Chapter 3

Tissue Culture Media

3.1. INTRODUCTION

Nutritional requirements for optimal growth of a tissue in vitro may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige and Skoog, 1962). As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. When starting with a new system, it is essential to work out a medium that will fulfil the specific requirements of that tissue. During the past 25 years, the need to culture diverse tissues and organs has led to the development of several recipes (Table 3.1).

Some of the earliest plant tissue culture media, e.g. root culture medium of White (1943) and callus culture medium of Gautheret (1939), were developed from nutrient solutions previously used for whole plant culture. White evolved the medium from Uspenski and Uspenskaia's medium (1925) for algae, and Gautheret's medium is based on Knop's (1865) salt solution. All subsequent media formulations are based on White's and Gautheret's media.

While some calli (carrot tissue, blackberry tissue, most tumour tissues) may grow on simple media containing only inorganic salts and a utilizable sugar, for most others it is essential to supplement the medium with vitamins, amino acids and growth substances in different qualitative and quantitative combinations. Often, complex nutritive mixtures have been added to plant tissue culture media. A medium containing only 'chemically-defined' compounds is referred to as a 'synthetic medium'[1].

In tissue culture literature the concentrations of inorganic and organic constituents of the medium are generally expressed in mass values (mg l^{-1} and ppm are synonymous but only mg l^{-1} is now acceptable). This has been followed in Table 3.1. However, the International Association

[1]Even in a synthetic medium one knows only what has been added. Breakdown of certain compounds (sucrose, vitamins) during autoclaving and interaction between various ingredients may occur during preparation, thus changing the final composition of the medium.

TABLE 3.1

Composition of some plant tissue culture media[a]

Constituents	Media (amount in mg l^{-1})						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B_5[g]	Nitsch's[h]	NT[i]
Inorganic							
NH_4NO_3	–	–	1650	1200	–	720	825
KNO_3	80	–	1900	1900	2527.5	950	950
$CaCl_2 \cdot 2H_2O$	–	75	440	440	150	–	220
$CaCl_2$	–	–	–	–	–	166	–
$MgSO_4 \cdot 7H_2O$	750	250	370	370	246.5	185	1233
KH_2PO_4	–	–	170	340	–	68	680
$(NH_4)_2SO_4$	–	–	–	–	134	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	300	–	–	–	–	–	–
$NaNO_3$	–	600	–	–	–	–	–
Na_2SO_4	200	–	–	–	–	–	–
$NaH_2PO_2 \cdot H_2O$	19	125	–	–	150	–	–
KCl	65	750	–	–	–	–	–
KI	0.75	0.01	0.83	–	0.75	–	0.83
H_3BO_3	1.5	1	6.2	0.63	3	10	6.2
$MnSO_4 \cdot 4H_2O$	5	0.1	22.3	2.23	–	25	22.3
$MnSO_4 \cdot H_2O$	–	–	–	–	10	–	–
$ZnSO_4 \cdot 7H_2O$	3	1	8.6	–	2	10	–
$ZnSO_4 \cdot 4H_2O$	–	–	–	–	–	–	8.6
$ZnNa_2$·EDTA	–	–	–	15	–	–	–
$Na_2MoO_4 \cdot 2H_2O$	–	–	0.25	0.025	0.25	0.25	0.25
MoO_3	0.001	–	–	–	–	–	–
$CuSO_4 \cdot 5H_2O$	0.01	0.03	0.025	0.0025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	–	–	0.025	0.0025	0.025	–	–
$CoSO_4 \cdot 7H_2O$	–	–	–	–	–	–	0.03
$AlCl_3$	–	0.03	–	–	–	–	–
$NiCl_2 \cdot 6H_2O$	–	0.03	–	–	–	–	–
$FeCl_3 \cdot 6H_2O$	–	1	–	–	–	–	–
$Fe_2(SO_4)_3$	2.5	–	–	–	–	–	–
$FeSO_4 \cdot 7H_2O$	–	–	27.8	27.8	–	27.8	27.8
$Na_2EDTA \cdot 2H_2O$	–	–	37.3	37.3	–	37.3	37.3
Sequestrene 330Fe	–	–	–	–	28	–	–
Organic							
Inositol	–	–	100	–	100	100	100
Nicotinic acid	0.05	–	0.5	0.5	1	5	–
Pyridoxine·HCl	0.01	–	0.5	0.5	1	0.5	–
Thiamine·HCl	0.01	–	0.1	0.5	10	0.5	1
Glycine	3	–	2	2	–	2	–
Folic acid	–	–	–	–	–	0.5	–
Biotin	–	–	–	–	–	0.05	–

TABLE 3.1 (*continued*)

Constituents	Media (amount in mg l^{-1})						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B_5[g]	Nitsch's[h]	NT[i]
Sucrose	2%	–	3%	4%	2%	2%	1%
D-Mannitol	–	–	–	–	–	–	12.7%

[a]Growth regulators and complex nutrient mixtures described by various authors are not included here. The compositions of several media recommended for specific tissue and organ are given in relevant chapters.
[b]Concentrations of mannitol and sucrose are expressed in percentage.
[c]White (1963).
[d]Heller (1953).
[e]Murashige and Skoog (1962).
[f]Eriksson (1965).
[g]Gamborg et al. (1968).
[h]Nitsch (1969).
[i]Nagata and Takebe (1971).

for Plant Physiology has recommended the use of mole values. Mole is an abbreviation for gram molecular weight which is the formula weight of a substance in grams. The formula weight of a substance is equal to the sum of the weights of the atoms in the formula of a substance. One litre of solution containing 1 mole of a substance is said to be 1 Molar (1 M) or a 1 mol l^{-1} solution of the substance (1 mol l^{-1} = 1000 or 10^3 mmol l^{-1} = 1 000 000 or 10^6 μmol l^{-1}). According to the recommendations of the International Association for Plant Physiology, mmol l^{-1} should be used for expressing the concentration of macronutrients and organic nutrients and μmol l^{-1} for micronutrients, hormones, vitamins and other organic constituents in the plant tissue culture medium. One of the reasons for using mole values is that the number of molecules per mole is constant for all compounds.

When preparing medium according to a published recipe the original mole values can be used irrespective of the number of water molecules in the sample of the salt. This cannot be done when the concentrations are expressed in mass values.

3.2. MEDIA CONSTITUENTS

3.2.1. Inorganic nutrients

Mineral elements are very important in the life of a plant. For example, magnesium is a part of chlorophyll molecules, calcium is a constitu-

ent of the cell wall, and nitrogen is an important part of amino acids, vitamins, proteins and nucleic acids. Similarly, iron, zinc and molybdenum are parts of certain enzymes. Besides C, H, and O, 12 elements are known to be essential for plant growth: nitrogen, phosphorus, sulphur, calcium, potassium, magnesium, iron, manganese, copper, zinc, boron and molybdenum. Of these, the first six elements are required in comparatively large quantities and are, therefore, termed macro- or major elements. The other six elements are necessary in only small amounts and are called micro- or minor elements. According to the recommendations of the International Association for Plant Physiology the elements required by plants in concentrations greater than 0.5 mmol l^{-1} are referred to as macroelements and those in concentrations less than 0.5 mmol l^{-1} are microelements (De Fossard, 1976). Essentially, the 15 elements found important for whole plant growth have also proved necessary for tissue cultures. A survey of Tables 3.1 and 3.2 shows that the chief difference in the composition of various commonly used tissue culture media lies in the quantity of various salts and ions, respectively. Qualitatively, the inorganic nutrients required for various plant tissues appear to be fairly constant.

When mineral salts are dissolved in water they undergo dissociation and ionization. The active factor in the medium is the ions of different types rather than the compounds. One type of ion may be contributed by more than one salt. For example, in Murashige and Skoog's (1962) medium (MS) NO_3^- ions are contributed by NH_4NO_3 as well as KNO_3, and K^+ ions are contributed by KNO_3 and KH_2PO_4. Therefore, a useful comparison between the two media can be made by looking into total concentrations of different types of ions in them. 'Balance sheets' of ions for the seven media given in Table 3.1 are presented in Table 3.2.

White's medium, one of the earliest plant tissue culture media, includes all the necessary nutrients and is widely used for root culture. Experience of various investigators has, however, revealed that quantitatively the inorganic nutrients in this medium are inadequate for good callus growth (Murashige and Skoog, 1962). This deficiency was overcome by enriching the medium with complex mixtures like yeast extract, casein hydrolysate, coconut milk, amino acids, etc. (Reinert and White, 1956; Risser and White, 1964). With the objective of evolving suitable synthetic media, later investigators have effectively replaced the nutritive mixtures by increasing the concentrations of the various inorganic nutrients, particularly potassium and nitrogen. Most plant tissue culture media that are now being widely used (Tables 3.1 and 3.2) are richer in mineral salts (ions) compared to White's medium. Aluminium and nickel used by Heller (1953) could not be demonstrated as essential and, there-

TABLE 3.2

Balance sheet of ions for the media included in Table 3.1

Ions	Units	Media[a]						
		White's	Heller's	MS	ER	B_5	Nitsch's	NT
NO_3	mM	3.33	7.05	39.41	33.79	25.00	18.40	19.69
NH_4	mM	–	–	20.62	15.00	2.00	9.00	10.30
Total N	mM	3.33	7.05	60.03	48.79	27.03	27.40	29.99
P	mM	0.138	0.90	1.25	2.50	1.08	0.50	5.00
K	mM	1.66	10.05	20.05	21.29	25.00	9.90	14.39
Ca	mM	1.27	0.51	2.99	2.99	1.02	1.49	1.50
Mg	mM	3.04	1.01	1.50	1.50	1.00	0.75	5.00
Cl	μM	0.87	11.08	5.98	5.98	2.04	2.99	3.00
Fe	μM	12.50	3.70	100.00	100.00	50.10	100.00	100.00
S	μM	4502.00	1013.50	1730.00	1610.00	2079.90	996.80	5236.50
Na	μM	2958.00	7966.00	202.00	237.20	1089.00	202.00	202.00
B	μM	24.20	16.00	100.00	10.00	48.50	161.80	100.00
Mn	μM	22.40	0.40	100.00	10.00	59.20	112.00	100.00
Zn	μM	10.40	3.40	30.00	37.30	7.00	34.70	36.83
Cu	μM	0.04	0.10	0.10	0.01	0.10	0.10	0.10
Mo	μM	0.007	–	1.00	0.1	1.00	1.00	1.00
Co	μM	–	–	0.10	0.01	0.10	–	0.10
I	μM	4.50	0.06	5.00	–	4.50	–	5.00
Al	μM	–	0.20	–	–	–	–	–
Ni	μM	–	0.10	–	–	–	–	–

[a]For references, see Table 3.1.

fore, were dropped by subsequent workers. The indispensability of sodium, chloride and iodide has also not been established.

A detailed study of inorganic nutrients of plant tissue cultures was made by Heller (1953). He gave special emphasis to iron and nitrogen. In the original White's medium (1943), iron was added in the form of $Fe_2(SO_4)_3$, but Street and co-workers replaced it with $FeCl_2$ for root cultures because the former contained Mn and some other metallic ions as impurities (Street and Henshaw, 1966). However, $FeCl_2$ also did not prove to be an entirely satisfactory source of iron. In this form iron is available to the tissues at and around a pH of 5.2. It is known that in root cultures, within a week of inoculation, the pH of the medium drifts from the initial value of 4.9–5.0 to 5.8–6.0, and roots start showing iron-deficiency symptoms. To overcome this problem, in most media, iron is now used as Fe·EDTA. In this form iron remains available up to a pH of 7.6–8.0. Incidentally, unlike roots, callus cultures can utilize $FeCl_2$ up to a pH of 6.0 by secreting natural chelates which bind with iron (Heller,

1953). Fe·EDTA may be prepared by using $FeSO_4 \cdot 7H_2O$ and Na_2·EDTA as described in Table 3.6, or it may be possible to buy NaFe·EDTA.

(i) *Macroelements*. Nitrogen is one of the main elements contributing to the growth of plants in vitro and in vivo. It is a constituent of the amino acids, proteins, certain hormones and chlorophyll. The source of nitrogen in vitro could be either organic or inorganic. An indirect effect of nitrogen on tissue growth is through its influence on the pH of the medium (Dougall, 1980; Congard et al., 1986). The form of nitrogen, as NH_4^+ or NO_3^-, has a dramatic influence on the morphogenic response of plant tissues in vitro (see Section 6.3.4). Development of anthocyanin in vitro has been attributed to deficiency of NO_3^- ions (Heller, 1965).

Phosphorus is vital for cell division as well as in storage and transfer of energy in plants. Its role in photosynthesis is also important. Kozai et al. (1991) reported that in autotrophic cultures of strawberry, uptake of PO_4^{3-} is much greater than that of other minerals. Too little phosphorus causes plants to be abnormal and sickly.

Potassium is necessary for normal cell division, for synthesis of proteins, chlorophyll, and for nitrate reduction. The level of K^+ in vitro is rarely a problem but certain species are sensitive to high levels. Anderson (1975) showed that *Rhododendron* shoots grew better, without browning, when K^+ level was reduced.

Sulphur is present in some proteins. It is quite often present as an impurity in agar (Pochet et al., 1991).

Calcium as calcium pectate is an integral part of the walls of plant cells and helps maintain integrity of the membrane. High levels of calcium have been shown to promote callose deposition thereby inhibiting cell extension (Eklund and Eliasson, 1990). Atkinson (1991) found that stomata were more open in plants grown in the presence of high Ca^{2+}. Cytoplasmic Ca^{2+} is also involved in the regulation of hormone responses and mediates in responses to environmental factors such as temperature and light (Williams, 1995). Calcium could be having a pre-emptive role in morphogenesis (Hush et al., 1991). Calcium is not very mobile in plants. As a result, it is the new growth that suffers when there is a calcium deficiency either absolutely or because of poor mobility. The leaf tips and growing points tend to die back under such conditions.

Magnesium is a component of chlorophyll and a co-factor for many enzyme reactions. Magnesium uptake is not usually limited, except at low pH.

(ii) *Microelements*. The majority of the micro-elements are required in trace quantities and quite often may get carried into the medium as im-

purities in other ingredients. They may also get carried-over with the explant or tissues and support growth for several weeks without showing any deficiency symptoms. This and the interaction amongst the micro-elements makes the study of individual elements slightly complicated. The microelements are essential as catalysts for many biochemical reactions. Microelement deficiency symptoms include reduced lignification (Cu, Fe), rosetting (Zn, Mn), leaf chlorosis (Fe, Zn, Mn) and shoot tip necrosis (B). Certain elements, such as Co and Ni, can inhibit ethylene synthesis.

The availability of ions becomes critical sometimes because of the solubility problems. Dalton et al. (1983) suggested that an imbalance between Fe and EDTA can cause precipitation and make 45% of Fe, 20% of Zn and 13% of original PO_4^{3-} in MS medium unavailable within 2 days of media preparation. Interpretation of Fe status is complicated by the interactions between Fe and Mn or Zn. Excess Mn can lead to Fe deficiency while excess Fe or EDTA can reduce Zn uptake (Williams, 1995).

3.2.2. Organic nutrients

Most cultured plant cells are capable of synthesizing all essential vitamins but, apparently, in sub-optimal quantities (Czosnowski, 1952; Paris, 1955, 1958). To achieve the best growth of the tissue it is often essential to supplement the medium with one or more vitamins and amino acids. Various standard media show wide differences in their composition with respect to vitamins and amino acids (see Table 3.1).

(i) *Vitamins.* Animals require minor quantities of vitamins as necessary ancillary food factors which they get from extraneous sources. Plants, on the other hand, can produce their requirements of vitamins. However, plant cell cultures need to be supplemented with certain vitamins. The most widely used vitamins are thiamine (vitamin B_1), niacin (vitamin B_3), pyridoxine (vitamin B_6), and myo-inositol (a member of the vitamin B complex). Certain other vitamins which find specific uses in cell cultures are pantothenic acid, vitamin C, vitamin D and vitamin E.

The widely used Murashige and Skoog's (1962) medium lists four vitamins as necessary for tobacco callus growth. However, in a subsequent study, Linsmaier and Skoog (1965) removed niacin and pyridoxine but retained myo-inositol and increased the quantity of thiamine to 4 mg l^{-1}. Several later modifications of MS medium use only myo-inositol and thiamine.

Myo-inositol or meso-inositol is a natural constituent of plants and as phosphatidyl-inositol could be a crucial factor in the functioning of mem-

branes (Jung et al., 1972; Harran and Dickinson, 1978). In plants inositol as inositol phosphate may be acting as a second messenger to the primary action of auxins. It probably has a role as a carrier and in storage of IAA as IAA-myo-inositol ester. In plant tissue cultures myo-inositol could be a crucial precursor in the biosynthetic pathways leading to the formation of pectin and hemicelluloses needed in the cell wall synthesis (Loewus et al., 1962; Verma and Dougall, 1979) and may have a role in the uptake and utilization of ions (Wood and Braun, 1961).

Thiamine is involved in the direct biosynthesis of certain amino acids and is an essential co-factor in carbohydrate metabolism. Certain plant cultures appear to be self-sufficient for thiamine but most cultures do benefit by minute quantities of it, with the requirement increasing with consecutive passages. Thiamine could be having a synergistic interaction with cytokinins (Digby and Skoog, 1966).

Vitamin E is used as an anti-oxidant while vitamin C is useful to prevent blackening during explant isolation. Vitamin D has a growth regulatory effect on plant tissue cultures. Riboflavin has been found to inhibit callus formation and improve growth and quality of shoots (Drew and Smith, 1986).

(ii) *Amino acids.* There is little substantive evidence for the necessity or role of amino acids in plant tissue cultures. Even the often used glycine has little benefit in the sustained growth of tobacco callus (Linsmaier and Skoog, 1965) and may even be inhibitory at higher levels. Amino acids may be directly utilized by the plant cells or may serve as a nitrogen source. However, an organic source of nitrogen is preferred only when an inorganic source is lacking or exhausted (Williams, 1995). Cysteine has been included in media as an antioxidant to control the oxidation of phenolics and prevent blackening of tissue. The in vitro produced shoots of dwarf apple rootstocks formed more roots in the presence of arginine (Orlikowska, 1992).

(iii) *Undefined supplements.* Numerous complex nutritive mixtures of undefined composition, like casein hydrolysate (CH), coconut milk (CM), corn milk, malt extract (ME), tomato juice (TJ), and yeast extract (YE), have also been used to promote the growth of certain calli and organs. However, the use of these natural extracts should be avoided as far as possible. Different samples of these substances, especially the fruit extracts, may affect the reproducibility of results because the quality and quantity of the growth-promoting constituents in these extracts often vary with the age of the tissue and the variety of the donor organism. Moreover, it should be possible to effectively replace these substances by

a single amino acid. For example, for maize endosperm callus Straus (1960) could substitute yeast extract and tomato juice by L-asparagine alone. Similarly, Risser and White (1964) demonstrated that L-glutamine could replace a mixture of 18 amino acids earlier used by Reinert and White (1956) for tissue cultures of *Picea glauca*.

(iv) *Carbon source.* Haberlandt (1902) attempted to culture green mesophyll cells probably with the idea that green cells would have simpler nutritive requirements but this did not prove to be true. We now know that, as a rule, tissues which are initially green gradually lose their green pigments in cultures and depend on an external source of carbon. Even those tissues which acquire pigments through sudden changes or under special conditions during culture period are not autotrophs for carbon. Fully organized, green shoots in cultures also show better growth and proliferation with the addition of a suitable carbon source in the medium. Thus, it is essential to add a utilizable source of carbon to the culture medium.

The most commonly used carbon source is sucrose, at a concentration of 2–5%. Glucose and fructose are also known to support good growth of some tissues. Ball (1953, 1955) observed that autoclaved sucrose was better than filter-sterilized sucrose for the growth of *Sequoia* callus. Autoclaving seems to bring about hydrolysis of sucrose into more efficiently utilizable sugars, such as fructose and glucose. Bretzloff (1954) found that in a fungal medium sucrose breakdown during autoclaving was dependent on pH, with no hydrolysis occurring on setting the pH to 6.0.

In general, excised dicotyledonous roots grow best with sucrose whereas those of monocots do best with dextrose (glucose). Tissue cultures of *Malus pumila* (var. McIntosh) grow as well with sorbitol as with sucrose or glucose (Chong and Taper, 1972). Some other forms of carbon that plant tissues are known to utilize include maltose, galactose, mannose, and lactose (Gautheret, 1959). Tissue cultures of *Sequoia* (Ball, 1955) and maize endosperm (Straus and LaRue, 1954) can even metabolize starch as the sole carbon source.

Kozai (1991b) suggested that sucrose could either be reduced or completely eliminated from medium if autotrophic conditions of high CO_2 and light intensity could be maintained. However, despite such autotrophic conditions sucrose might become a limiting factor in the growth of certain cultures (Debergh et al., 1992a).

Sucrose in the medium is necessary for various metabolic activities. It is required for differentiation of xylem and phloem elements in cultured cells (Aloni, 1980). Sugars also represent the major osmotic component of the medium. Brown et al. (1979) replaced a third of sucrose in tobacco

callus medium with mannitol without effecting callus growth. Mannitol is an osmotic agent and is not taken into the plant cells or metabolized because of its molecular size.

3.2.3. Growth hormones

In addition to the nutrients, it is generally necessary to add one or more growth substances, such as auxins, cytokinins, and gibberellins, to support good growth of tissues and organs. However, the requirement for these substances varies considerably with the tissue, and it is believed that it depends on their endogenous levels.

The growth regulators are required in very minute quantities (μmol l^{-1} values). There are many synthetic substances having growth regulatory activity, with differences in activity and species specificity. It often requires testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new plant species.

(i) *Auxins*. In nature, the hormones of this group are involved with elongation of stem and internodes, tropism, apical dominance, abscission, rooting, etc. In tissue cultures auxins have been used for cell division and root differentiation. The auxins commonly used in tissue culture are: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), naphthoxyacetic acid (NOA), *para*-chlorophenoxyacetic acid (*p*-CPA), dichlorophenoxyacetic acid (2,4-D), and trichlorophenoxyacetic acid (2,4,5-T). Of these, IBA and IAA are widely used for rooting and, in interaction with a cytokinin, for shoot proliferation. 2,4-D and 2,4,5-T are very effective for the induction and growth of callus. 2,4-D is also an important factor for the induction of somatic embryogenesis. Auxins are usually dissolved in either ethanol or dilute NaOH.

(ii) *Cytokinins*. These hormones are concerned with cell division, modification of apical dominance, shoot differentiation, etc. In tissue culture media, cytokinins are incorporated mainly for cell division and differentiation of adventitious shoots from callus and organs. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance. More commonly used cytokinins are: benzylamino purine (BAP), isopentenyl-adenine (2-ip), furfurylamino purine (kinetin), thidiazuron (TDZ) and zeatin. Compared to the other cytokinins, thidiazuron is generally used at very low concentrations (0.1–5 μg l^{-1}). Cytokinins are generally dissolved in dilute HCl or NaOH. For thidiazuron, DMSO may be used as the solvent.

(iii) *Gibberellins*. There are over 20 known gibberellins. Of these, generally, GA_3 is used. Compared to auxins and cytokinins, gibberellins are used very rarely. They are reported to stimulate normal development of plantlets from in vitro formed adventive embryos. GA_3 is readily soluble in cold water up to 1000 mg l^{-1}.

(iv) *Ethylene*. All kinds of plant tissue cultures produce ethylene, and the rate of production increases under stress conditions. In cultures, ethylene is also produced abiologically when the organic constituents of the medium are subjected to heat, oxidation, sunlight or ionizing radiation (Matthys et al., 1995).

Pure ethylene or chemical compounds which release ethylene during their decomposition, such as 2-chloroethylphosphonic acid (marketed under the trade names Ethrel, Ethaphon, Floridimex, Camposan), can be applied to study the effect of this gaseous growth regulator on plant tissue cultures. Ethylene exerts various morphogenic influences on cultured tissues but its effects are not clear cut (Matthys et al., 1995). It may be promotory or inhibitory for the same process in different systems. For example, it promoted somatic embryogenesis in maize (Vain et al., 1989a,b) but the same process was inhibited in *Hevea brasiliensis* (Auboiron et al., 1990).

(v) *Others*. Abscisic acid is most often required for normal growth and development of somatic embryos and only in its presence do they closely resemble zygotic embryos (Ammirato, 1988). It is also known to promote morphogenesis in *Begonia* cultures.

More recently, there has been some interest in the application of growth retardants, such as paclobutrazol, during the acclimatization stage of micropropagation to reduce hyperhydricity and regulate leaf growth and function in relation to control of water stress (Smith and Krikorian, 1990a; Ziv, 1992). Ancymidol has been used to inhibit leaf formation and promote shoot formation in gladiolus (Ziv, 1989; Ziv and Ariel, 1991).

3.2.4. Gelling agents

In static cultures if liquid medium is used the tissue would get submerged and die due to lack of oxygen. A gelling agent is generally used to circumvent this problem. The most desirable property of a gelling agent is that it should withstand sterilization by autoclaving, and the medium should be liquid when hot but form a semisolid gel when cool. Plant tissues have been shown to do better when cultured on a semi-solid support

TABLE 3.3

The concentration of minerals ($\mu g\ g^{-1}$) in a range of gelling agents[a]

Minerals	Gelling agents					
	Merck agar[1,b]	Bacto-agar[2]	Phyta-agar[3]	TC-agar[4]	BiTek agar[5]	Gelrite gellan[6]
Na	1200	7194	1244	596	10949	6800
S	5900	–	–	–	7120	220
K	2000	317	86	24	885	28000
Ca	110	1997	2097	2542	90	4900
P	1300	42	331	51	1005	2100
Mg	62	1002	635	478	110	1530
Fe	31	8.3	226	25	26	280
Al	7.7	6.2	75	16	–	185
B	23	109	57	80	34	1.4
Mn	0.6	0.3	46	2.2	0.5	5.3
Zn	1.5	6.6	4.5	5.7	2.2	19
Cu	0.3	0.8	0.8	0.2	0.1	2.9

[a]Source of the gelling agent: 1, Merck, Germany; 2, Difco Laboratories, USA; 3, GIBCO, USA; 4, K.C. Biologicals, USA; 5, ?; 6, Kelco, USA.
[b]1,6, after Scherer et al. (1988); 2–4, after Singha et al. (1985); 5, after Williams (1993).

than otherwise. Most of the gelling agents (agar, agarose, gelrite) used in plant tissue culture media are biological products. Being natural products and subjected to varying degrees of processing and purification the composition of these gelling agents varies with the brand and the batch, particularly their mineral composition (Table 3.3).

(i) *Agar*. This most commonly used gelling agent is obtained from red algae, especially *Gelidium amansii* It is a complex mixture of related polysaccharides built up from the sugar, galactose. These include the neutral polymer fraction, agarose, which gives strength to the gel and the highly charged anionic polysaccharides agaropectins which give agar its viscosity (Araki and Arai, 1967). The agar quality and purity vary from batch to batch as it depends a lot on the culture conditions of the algae and the varying degrees of processing and purification. The proportion of agarose to total polysaccharides, which can vary from 50 to 90%, influences the gel strength (Adrian and Assoumani, 1983). Firmness of the gel produced by a given concentration of agar varies according to the brand and the pH during autoclaving. Agar is partly hydrolyzed if it is autoclaved in an acidic medium. Agar is used at varying concentrations from 0.8 to 1%.

(ii) *Agarose*. Agarose consists of β-D(1–3) galactopyranose and 3,6-anhydro-α-L(1–4) galatopyranose linked into polymer chains of 20–160 monosaccharide units. Agarose is obtained by purifying agar to remove agaropectins with its sulphate side groups. As the process is tedious, the cost of agarose is much higher than agar. It is only used where high gel strength is required, such as in single cell or protoplast cultures. Agarose is adequate at 0.4%.

(iii) *Gelrite*. Gelrite (Kelco Division, Merck & Co.) or Phytagel (Sigma Chemical Co.), a gellan gum, is a linear polysaccharide produced by the bacterium *Pseudomonas elodea*. It comprises of linked K-glucuronate, rhamnose and cellobiose molecules (Kang et al., 1982). The commercial product contains significant quantities of K, Na, Ca and Mg (Scherer et al., 1988) but it is said to be free of organic impurities found in agar. Gelrite requires a minimum level of cations in the solution for gelling. Unlike agar, which requires heating, gelrite can be readily prepared in cold solution. To prevent clumping it should be added to rapidly stirring culture medium at room temperature.

Gelrite is a good alternative to agar not only because of its low cost per litre of medium (0.1–0.2% is sufficient) but also for the many advantages it offers. Gelrite sets as a clear gel which assists easy observation of cultures and their possible contamination. Unlike agar, the gel strength of gelrite is unaffected over a wide range of pH (Bonga and Von Aderkas, 1992). Various plant species have shown as good results on gelrite as on agar, and sometimes gelrite proved to be better. However, certain plants show hyperhydricity on gelrite, apparently due to more freely available water (Debergh, 1983). This problem could be rectified by mixing small quantities of agar with gelrite (Pasqualetto et al., 1986). Kyte (1987) has recommended the use of a mixture of gelrite and agar in a ratio of 3:1.

3.2.5. pH

The pH of the medium is usually adjusted between 5.0 and 6.0 before sterilization. However, Straus and LaRue (1954) observed that the growth of maize endosperm callus on a fresh weight basis was best at pH 7.0 and on a dry weight basis pH 6.1 proved optimal. In general, a pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar.

The pH of the medium changes at various stages of preparation and culture. pH of the medium set after the addition of the gelling agent shows a remarkable drop on autoclaving. The pH of the medium further

changes once plant tissue is placed on it. The plant tissue and the medium interact to adjust the pH to an equilibrium irrespective of the initial pH adjusted (Skirvin et al., 1986; Williams et al., 1990). The ratio of NH_4^+ and NO_3^- ions in the medium also influences the pH. When NH_4^+ is predominantly taken up the medium gets acidified due to liberation of H^+ ions, while uptake of NO_3^- ions increases pH due to liberation of OH^- ions (Dougall, 1980; Congard et al., 1986). Such pH changes then influence the availability of various mineral ions in the medium and their uptake by the plant tissue.

3.3. MEDIA SELECTION

There is no one ideal approach to formulate a suitable medium for a new system. A convenient approach could be to select three media from the available recipes, that represent high, medium and low salt media and combine them factorially with different levels of plant growth regulators suitable for the desired response. For shoot proliferation or adventitious shoot bud differentiation a commonly used auxin (NAA) and cytokinin (BAP) may be used, each at five concentrations (0, 0.5, 2.5, 5, 10 μmol l^{-1}). All possible combinations of the five concentrations of the two substances would lead to an experiment with 25 treatments (Table 3.4) with each basal medium. Select the best of the 75 treatments and test some of the available auxins and cytokinins at that concentration. While varying cytokinins, keep the auxin constant and vice versa. Test a range of sucrose concentrations (2–6%) to decide its optimal level. However, there are limitless opportunities to further improve the selected medium by manipulating its nutrient salts and plant growth regulators.

De Fossard et al. (1974) have described a 'broad spectrum experiment' to select a suitable medium for an untested system. In this approach all the components of the medium are divided into four broad categories: (a) minerals, (b) auxins, (c) cytokinins, and (d) organic nutrients (sucrose, amino acids, inositol, etc.). For each group of substances three concentrations are chosen: low (L), medium (M), and high (H) (see Table 3.5). Trying various combinations of the four categories of substances at three different concentrations leads to an experiment with 81 treatments. The best of the 81 treatments is denoted by a four-letter code. For example, the treatment with medium salts, low auxin, medium cytokinin, and high organic nutrients would be represented as MLMH. Having reached this stage it would be desirable to test different auxins and cytokinins to find the best types.

TABLE 3.4

NAA (μM)	BAP (μM)				
	0	0.5	2.5	5	10
0	1	2	3	4	5
0.5	6	7	8	9	10
2.5	11	12	13	14	15
5	16	17	18	19	20
10	21	22	23	24	25

3.4. MEDIA PREPARATION

The most simple method of preparing media today is to use commercially available dry powdered media, containing inorganic salts, vitamins, and amino acids. The powder is dissolved in purified water (10% less than the final volume of the medium), and after adding sugar, agar, and other desired supplements, the final volume is made up with purified water. The pH is adjusted, and the medium autoclaved. Several standard plant tissue culture media and a range of complete mixes (together with agar) for micropropagation of specific plant species are now available as dry powder. The powdered media may be useful for routine purposes, such as micropropagation of plant species for which composition of the medium required is well established. In such cases the use of a powdered medium should save time. However, in experimental work where it is necessary to make major qualitative and quantitative changes in the organic and/or inorganic constituents of the medium, or where powdered media are either not available or considered expensive, there are two possible ways of preparing medium. One method is to weigh and dissolve the required quantities of the ingredients separately and mix them before the preparation of the medium. A more convenient and popular method, however, is to prepare a series of concentrated stock solutions. For example, to prepare Murashige and Skoog's basal medium, four different stock solutions may be prepared (Table 3.6): (a) major salts (20× concentrated); (b) minor salts (200× concentrated); (c) iron (200× concentrated), and (d) organic nutrients except sucrose (200× concentrated). For the preparation of stock solutions (a)–(d) each component should be separately dissolved to the last particle and then mixed with the others. Separate stock solutions are prepared for each growth regulator by dissolving it in a minimal quantity of the appropriate solvent (if insoluble in water; see Section 3.2.3) and making up the final volume with purified

TABLE 3.5

Constituents and concentrations of minerals, auxin, cytokinin, and organic nutrients of the broad spectrum experiment of De Fossard et al. (1974)

Constituents	Concentration range (mM)		
	Low	Medium	High
Minerals			
NH_4NO_3	5	10	20
KNO_3	–	10	20
KH_2PO_4	0.1	–	–
NaH_2PO_4	–	1	2
KCl	1.9	–	–
$CaCl_2$	1	2	3
$MgSO_4$	0.5	1.5	3
H_3BO_3	0.01	0.05	0.15
$MnSO_4$	0.01	0.05	0.1
$ZnSO_4$	0.001	0.02	0.04
$CuSO_4$	0.00001	0.0001	0.0015
Na_2MoO_4	0.00001	0.0001	0.001
$CoCl_2$	0.0001	0.0005	0.001
KI	0.0005	0.0025	0.005
$FeSO_4$	0.01	0.05	0.1
Na_2·EDTA	0.01	0.05	0.1
Auxin	0.0001	0.001	0.01
Cytokinin	0.0001	0.001	0.01
Organic nutrients			
Inositol	0.1	0.3	0.6
Nicotinic acid	0.004	0.02	0.04
Pyridoxine·HCl	0.0006	0.003	0.006
Thiamine·HCl	0.0001	0.002	0.04
Biotin	0.00004	0.0002	0.001
Folic acid	0.0005	0.001	0.002
D-Ca-pantothenate	0.0002	0.001	0.005
Riboflavin	0.0001	0.001	0.01
Ascorbic acid	0.0001	0.001	0.01
Choline chloride	0.0001	0.001	0.01
L-Cysteine·HCl	0.01	0.06	0.12
Glycine	0.0005	0.005	0.05
Sucrose	6	60	120

water. Depending on the levels of growth regulators used, their stock solutions may be prepared at the strength of 1 mmol l^{-1} or 10 mmol l^{-1}. All the stock solutions are stored in proper plastic or glass bottles under refrigeration. The iron stock must be stored in an amber-coloured bottle.

TABLE 3.6

Stock solutions for Murashige and Skoog's medium (MS)[a]

Constituents	Amount (mg l^{-1})
Stock solution I	
NH_4NO_3	33000
KNO_3	38000
$CaCl_2 \cdot 2H_2O$	8800
$MgSO_4 \cdot 7H_2O$	7400
KH_2PO_4	3400
Stock solution II	
KI	166
H_3BO_3	1240
$MnSO_4 \cdot 4H_2O$	4460
$ZnSO_4 \cdot 7H_2O$	1720
$Na_2MoO_4 \cdot 2H_2O$	50
$CuSO_4 \cdot 5H_2O$	5
$CoCl_2 \cdot 6H_2O$	5
Stock solution III[b]	
$FeSO_4 \cdot 7H_2O$	5560
$Na_2EDTA \cdot 2H_2O$	7460
Stock solution IV	
Inositol	20000
Nicotinic acid	100
Pyridoxine·HCl	100
Thiamine·HCl	20
Glycine	400

[a]To prepare 1 l of medium, take 50 ml of stock I, 5 ml of stock II, 5 ml of stock III, and 5 ml of stock IV.
[b]Dissolve $FeSO_4 \cdot 7H_2O$ and $Na_2EDTA \cdot 2H_2O$ separately in 450 ml distilled water by heating and constant stirring. Mix the two solutions, adjust the pH to 5.5, and add distilled water to make up the final volume to 1 l.

For storing coconut milk (liquid endosperm) the water collected from fruits is boiled to deproteinize it, filtered, and stored in plastic bottles in a deep freeze at –20°C. As a rule, before using the stocks the bottles must be shaken gently and if any of the solutions show a suspension of a precipitate or a biological contaminant they should be immediately discarded.

For preparing stock solutions and media, glass, distilled or purified water and chemicals of high purity (AnalaR grade) should be used.

The sequence of steps involved in preparing a medium is as follows:

(a) Required quantities of agar and sucrose are weighed and dissolved in water, about 3/4 the final volume of the medium, by heating them in a water bath or an autoclave at low pressure. This step is not necessary for a liquid medium because sucrose would dissolve even in lukewarm water.

(b) Appropriate quantities of the various stock solutions, including growth regulators and other special supplements are added. Some workers feel that it is better to add vitamins and auxins after autoclaving. If there is a special reason to do so the substance may be sterilized by filtering their solutions (adjusted to the desired pH) through microfilters[2] of pore size 0.22–0.45 μm (see Section 2.3.2).

(c) The final volume of the medium is made up with purified water.

(d) After mixing well, the pH of the medium is adjusted using 0.1 N NaOH and 0.1 N HCl.

(e) The medium is poured into the desired culture vessels. About 15 ml of the medium is dispensed in a 25 × 150 mm culture tube, and about 50 ml in a 150-ml flask. If during steps (b)–(e) the medium starts to gel, the flask containing the medium should be heated in a water bath or microwave oven and poured only when it is in a uniformly liquid state.

(f) Mouth of the culture vessels are closed with non-absorbent cotton wrapped in cheese-cloth (such closures exclude microbial contaminants but allow free gas exchange), or any other suitable closure.

(g) The culture vessels containing medium are transferred to appropriate baskets, covered with aluminium foil to check wetting of plugs during autoclaving, and sterilized by autoclaving at 120°C (1.06 kg cm^{-2}) for 15 min. If pre-sterilized, unautoclavable, plastic culture vials (petri-plates or jars) are being used the medium may be autoclaved in 250 or 500 ml flasks with suitable closures (large flasks are inconvenient for pouring) or narrow-mouthed bottles. The medium is allowed to cool to around 60°C before pouring into the vials under aseptic conditions.

(h) The medium is allowed to cool at room temperature and is stored at 4°C. When preparing a solid medium in culture tubes it is desirable to make slants by keeping the tubes tilted during cooling. Such slants provide a larger surface area for tissue growth. It is also easier to photograph cultures grown on such slants.

[2]Filter assemblies of various sizes and filter membranes of various porosity are manufactured by Millipore Intertech Inc., P.O. Box 255, Bedford, MA 01730, USA.

MEDIA NO.	SUMMARY COMPOSITION	VOLUME	PURPOSE	DATE:
				PREPARED BY:

CONSTITUENTS		TYPE	STOCK CONC.	AMOUNT ADDED
Minerals	Macro		20 x	
	Micro		200 x	
	Iron		200 x	
Organics			200 x	
Sugar				
Auxin			1 mmol.l^{-1}	
			10 mmol.l^{-1}	
Cytokinin			1 mmol.l^{-1}	
			10 mmol.l^{-1}	
Other				
Agar				
Container		pH Required pH Original pH Adjusted		

Fig. 3.1. A reference sheet used for media preparation.

3.5. CONCLUDING REMARKS

Recent studies have shown that a correct nutrient balance in the culture medium may be a prerequisite for any response of explants to plant growth regulators, and optimization of inorganic nutrients may reduce or eliminate the use of plant growth regulators (Preece, 1995). The number of adventitious buds differentiated from leaf explants of *Juinperus oxycedrus* was doubled by using SH (Schenk and Hildebrandt, 1972) medium instead of MS medium (Gomez and Segura, 1994). Whereas on MS medium optimum response was obtained in the presence of BAP and NAA, on SH medium it did not require NAA. Likewise, on full strength MS

medium the number of olive petiole explants that formed adventitious shoots was twice as many as on half strength MS medium and the amount of thidiazuron required in full MS medium was one quarter of that required with 1/2 MS (Mencuccini and Rugini, 1993). The best in vitro rooting of *Leucopogon obtectus* microshoots occurred on a agar-water medium devoid of an auxin but on media with increasing concentration of MS salts a requirement for auxin also increased (Bunn et al., 1989). Thus, it may be possible to reduce the use of plant growth regulators which sometime cause undesirable effects, such as callusing, vitrification and poor quality roots, by optimization of nutrient salts in the medium.

Street (1977a) remarked 'More false trials have been laid by mistakes in media preparation than by any other fault of technique'. To minimize human error all the steps listed above should be followed very carefully. The ingredients of the medium should be listed on paper and, after adding a component, it should be cancelled on the sheet. A reference sheet routinely used in some laboratories is depicted in Fig. 3.1. All the tubes, jars, flasks and petri-plates containing the medium should be clearly marked in such a manner that they can be identified even after autoclaving and long storage under light.

APPENDIX 3.I

Molecular weights of the compounds commonly used in tissue culture media

Compound	Chemical formula	Molecular weight
Macronutrients		
Ammonium nitrate	NH_4NO_3	80.04
Ammonium sulphate	$(NH_4)_2SO_4$	132.15
Calcium chloride	$CaCl_2 \cdot 2 \cdot H_2O$	147.02
Calcium nitrate	$Ca(NO_3)_2 \cdot 4H_2O$	236.16
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	246.47
Potassium chloride	KCl	74.55
Potassium nitrate	KNO_3	101.11
Potassium dihydrogen *ortho*-phosphate	KH_2PO_4	136.09
Sodium dihydrogen *ortho*-phosphate	$NaH_2PO_4 \cdot 2H_2O$	156.01
Micronutrients		
Boric acid	H_3BO_3	61.83
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	237.93
Cupric sulphate	$CuSO_4 \cdot 5H_2O$	249.68
Manganese sulphate	$MnSO_4 \cdot 4H_2O$	223.01
Potassium iodide	KI	166.01
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	241.95
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	287.54
Sodium EDTA	$Na_2.EDTA \cdot 2H_2O$ ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$)	372.25
Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	278.03
Ferric-sodium EDTA	FeNa·EDTA ($C_{10}H_{12}FeN_2NaO_8$)	367.07
Sugars and sugar alcohols		
Fructose	$C_6H_{12}O_6$	180.15
Glucose	$C_6H_{12}O_6$	180.15
Mannitol	$C_6H_{14}O_6$	182.17
Sorbitol	$C_6H_{14}O_6$	182.17
Sucrose	$C_{12}H_{22}O_{11}$	342.31
Vitamins and amino acids		
Ascorbic acid (vitamin C)	$C_6H_8O_6$	176.12
Biotin (vitamin H)	$C_{10}H_{16}N_2O_3S$	244.31
Calcium pantothenate (Ca salt of vitamin B_5)	$(C_9H_{16}NO_5)_2Ca$	476.53
Cyanocobalamine (vitamin B_{12})	$C_{63}H_{90}CoN_{14}O_{14}P$	1357.64
L-Cysteine·HCl	$C_3H_7NO_2S \cdot HCl$	157.63
Folic acid (vitamin B_c, vitamin M)	$C_{19}H_{19}N_7O_6$	441.40
Inositol	$C_6H_{12}O_6$	180.16
Nicotinic acid or niacin (vitamin B_3)	$C_6H_5NO_2$	123.11

APPENDIX 3.I (*continued*)

Compound	Chemical formula	Molecular weight
Pyridoxine HCl (vitamin B_6)	$C_8H_{11}NO_3 \cdot HCl$	205.64
Thiamine HCl (vitamin B_1)	$C_{12}H_{17}ClN_4OS \cdot HCl$	337.29
Glycine	$C_2H_5NO_2$	75.07
L-Glutamine	$C_5H_{10}N_2O_3$	146.15
Glutathione	$C_{10}H_{17}N_3O_6S$	307.33
Hormones		
Auxins		
p-Chlorophenoxyacetic acid (p-CPA)	$C_8H_7O_3Cl$	186.59
3,6-Dichloro-*o*-anisic acid (Dicamba)	$C_8H_6Cl_2O_3$	221.04
2,4-Dichlorophenoxyacetic acid (2,4-D)	$C_8H_6O_3Cl_2$	221.04
Indole-3-acetic acid (IAA)	$C_{10}H_9NO_2$	175.18
3-Indolebutyric acid (IBA)	$C_{12}H_{13}NO_2$	203.23
2-Methyl-4-chlorophenoxyacetic acid (MCPA)	$C_9H_9ClO_3$	200.62
α-Naphthaleneacetic acid (NAA)	$C_{12}H_{10}O_2$	186.20
β-Naphthoxyacetic acid (NOA)	$C_{12}H_{10}O_3$	202.20
4-Amino-3,5,6-Trichloropicolinic acid (Picloram)	$C_6H_3Cl_3N_2O_2$	241.46
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	$C_8H_4Cl_3O_3$	255.49
Cytokinins		
Adenine (Ad)	$C_5H_5N_5.3H_2O$	189.13
Adenine sulphate ($AdSO_4$)	$(C_5H_5N_5)_2.H_2SO_4 \cdot 2H_2O$	404.37
6-Benzyladenine or 6-benzylamino purine (BA or BAP)	$C_{12}H_{11}N_5$	225.20
6-γ,γ-Dimethylallylamino purine or *N*-isopentenylamino purine (2-ip)	$C_{10}H_{13}N_5$	203.3
6-Furfurylamino purine (kinetin)	$C_{10}H_9N_5O$	215.21
6-(Benzylamino)-9-(2-tetrahydropyranyl)-9H-purine (SD8339)	$C_{17}H_{19}N_5O$	309.40
n-Phenyl-*N*-1,2,3- thiadiazol-5-urea (thidiazuron)	$C_9H_8N_4OS$	220.2
6-(4-Hydroxy-3methylbut-2-enylamino)-purine (zeatin)	$C_{10}H_{13}N_5O$	219.20
Gibberellins		
Gibberellic acid (GA_3)	$C_{19}H_{22}O_6$	346.37
Other compounds		
Abscisic acid	$C_{15}H_{20}O_4$	264.31
2′-Isopropyl-4′-(trimethylammonium chloride)-5′methylphenyl piperidine carboxylate (Amo 1618)		

APPENDIX 3.I (*continued*)

Compound	Chemical formula	Molecular weight
α-Cyclopropyl-α-4-methoxyphenyl (ancymidol)	$C_{15}H_{16}N_2O_2$	256.3
β-Chloroethyltrimethyl ammonium chloride (CCC)	$C_5H_{13}Cl_2N$	158.07
Colchicine	$C_{22}H_{25}NO_6$	399.43
N-Dimethylaminosuccinamic acid (paclobutrazol)	$C_{15}H_{20}ClN_3O$	293.80
Phloroglucinol	$C_6H_6O_3$	126.11
1,4-Diaminobutane; tetramethylene-diamine (putrescine dihydrochloride)	$C_4H_{12}N_2 \cdot 2HCl$	161.1
N-(3-Aminopropyl)-1,4-butanediamine (spermidine)	$C_7H_{19}N_3$	145.2
N,*N*′-(Bis 3-aminopropyl)-1,4-butane-diamine (spermine)	$C_{10}H_{26}N_4$	202.3
2,3,5-Tri-iodobenzoic acid (TIBA)	$C_7H_3I_3O_2$	499.81

APPENDIX 3.II

Atomic weights

Name	Symbol	Atomic weight
Aluminium	Al	26.98
Boron	B	10.82
Calcium	Ca	40.08
Carbon	C	12.011
Chlorine	Cl	35.457
Cobalt	Co	58.94
Copper	Cu	63.54
Hydrogen	H	1.008
Iodine	I	126.91
Iron	Fe	55.85
Magnesium	Mg	24.32
Manganese	Mn	54.94
Molybdenum	Mo	95.95
Nickel	Ni	58.71
Nitrogen	N	14.008
Oxygen	O	16.00
Phosphorus	P	30.975
Potassium	K	39.10
Sodium	Na	22.991
Sulphur	S	32.066
Zinc	Z	65.38