Chapter 2

Plant Tissue Culture Media

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Additional information is available at the end of the chapter

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1. Introduction

Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Moreover, tissues from different parts of plants may also have different requirements for satisfactory growth [1]. Tissue culture media were first developed from nutrient solutions used for culturing whole plants e.g. root culture medium of White and callus culture medium of Gautheret. White's medium was based on Uspenski and Uspenska's medium for algae, Gautheret's medium was based on Knop's salt solution [2]. Basic media that are frequently used include Murashige and Skoog (MS) medium [1], Linsmaier and Skoog (LS) medium [3], Gamborg (B_5) medium [4] and Nitsch and Nitsch (NN) medium [5].

2. Media composition

Plant tissue culture media should generally contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, undefined organic supplements, growth regulators and solidifying agents. According to the International Association for Plant Physiology, the elements in concentrations greater than 0.5 $mM.l^{-1}$ are defined as macroelements and those required in concentrations less than 0.5 $mM.l^{-1}$ as microelements [6]. It should be considered that the optimum concentration of each nutrient for achieving maximum growth rates varies among species.

2.1. Macronutrients

The essential elements in plant cell or tissue culture media include, besides C, H and O, macroelements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) for satisfactory growth and morphogenesis. Culture media should contain at least 25-60 mM of inorganic nitrogen for satisfactory plant cell growth. Potassium is required for cell growth of most plant species. Most media contain K in the form of nitrate chloride salts

at concentrations ranging between 20 and 30 mM. The optimum concentrations of P, Mg, S and Ca range from 1-3 mM if other requirements for cell growth are provided [2].

2.2. Micronutrients

The essential micronutrients (minor elements) for plant cell and tissue growth include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). Iron is usually the most critical of all the micronutrients. The element is used as either citrate or tartarate salts in culture media, however, there exist some problems with these compounds for their difficulty to dissolve and precipitate after media preparation. There has been trials to solve this problem by using ethylene diaminetetraacetic acid (EDTA)-iron chelate (FeEDTA) [1]. A procedure for preparing an iron chelate solution that does not precipitate have been also developed [7]. Cobalt (Co) and iodine (I) may be added to certain media, but their requirements for cell growth has not been precisely established. Sodium (Na) and chlorine (Cl) are also used in some media, in spite of reports that they are not essential for growth. Copper and cobalt are added to culture media at concentrations of 0.1μM, iron and molybdenum at 1μM, iodine at 5μM, zinc at 5-30 μM, manganese at 20-90 μM and boron at 25-100 μM [2].

2.3. Carbon and energy sources

In plant cell culture media, besides the sucrose, frequently used as carbon source at a concentration of 2-5%, other carbohydrates are also used. These include lactose, galactose, maltose and starch and they were reported to be less effective than either sucrose or glucose, the latter was similarly more effective than fructose considering that glucose is utilized by the cells in the beginning, followed by fructose. It was frequently demonstrated that autoclaved sucrose was better for growth than filter sterilized sucrose. Autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars such as fructose. Sucrose was reported to act as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots [8].

It was found that supplements of sugar cane molasses, banana extract and coconut water to basal media can be a good alternative for reducing medium costs. These substrates in addition to sugars, they are sources of vitamins and inorganic ions required growth [9, 10].

2.4. Vitamins and myo-inositol

Some plants are able to synthesize the essential requirements of vitamins for their growth. Some vitamins are required for normal growth and development of plants, they are required by plants as catalysts in various metabolic processes. They may act as limiting factors for cell growth and differentiation when plant cells and tissues are grown *In vitro* [2]. The vitamins most used in the cell and tissue culture media include: thiamin (B_1), nicotinic acid and pyridoxine (B_6). Thiamin is necessarily required by all cells for growth [11]. Thiamin is used at concentrations ranging from 0.1 to 10 $mg.l^{-1}$. Nicotinic acid and pyridoxine, however not essential for cell growth of many species, they are often added to

culture media [12]. Nicotinic acid is used at a concentration range 0.1-5 $mg.l^{-1}$ and pyridoxine is used at 0.1-10 $mg.l^{-1}$. Other vitamins such as biotin, folic acid, ascorbic acid, pantothenic acid, tocopherol (vitamin E), riboflavin, p-amino-benzoic acid are used in some cell culture media however, they are not growth limiting factors. It was recommended that vitamins should be added to culture media only when the concentration of thiamin is below the desired level or when the cells are required to be grown at low population densities [14]. Although it is not a vitamin but a carbohydrate, myo-inositol is added in small quantities to stimulate cell growth of most plant species [13]. Myo-inositol is believed to play a role in cell division because of its breakdown to ascorbic acid and pectin and incorporation into phosphoinositides and phosphatidyl-inositol. It is generally used in plant cell and tissue culture media at concentrations of 50-5000 $mg.l^{-1}$.

2.5. Amino acids

The required amino acids for optimal growth are usually synthesized by most plants, however, the addition of certain amino acids or amino acid mixtures is particularly important for establishing cultures of cells and protoplasts. Amino acids provide plant cells with a source of nitrogen that is easily assimilated by tissues and cells faster than inorganic nitrogen sources. Amino acid mixtures such as casein hydrolysate, L-glutamine, L-asparagine and adenine are frequently used as sources of organic nitrogen in culture media. Casein hydrolysate is generally used at concentrations between 0.25-1 $g.l^{-1}$. Amino acids used for enhancement of cell growth in culture media included; glycine at 2 $mg.l^{-1}$, glutamine up to 8 mM, asparagine at 100$mg.l^{-1}$, L-arginine and cysteine at 10 $mg.l^{-1}$ and L-tyrosine at 100$mg.l^{-1}$ [2].

2.6. Undefined organic supplements

Some media were supplemented with natural substances or extracts such as protein hydrolysates, coconut milk, yeast extract, malt extract, ground banana, orange juice and tomato juice, to test their effect on growth enhancement. A wide variety of organic extracts are now commonly added to culture media. The addition of activated charcoal is sometimes added to culture media where it may have either a beneficial or deleterious effect. Growth and differentiations were stimulated in orchids [15], onions and carrots [16, 17], tomatoes [18]. On the other hand, an inhibition of cell growth was noticed on addition of activated charcoal to culture medium of soybean [17]. Explanation of the mode of action of activated charcoal was based on adsorption of inhibitory compounds from the medium, adsorption of growth regulators from the culture medium or darkening of the medium [19]. The presence of 1% activated charcoal in the medium was demonstrated to largely increase hydrolysis of sucrose during autoclaving which cause acidification of the culture medium [20].

2.7. Solidifying agents

Hardness of the culture medium greatly influences the growth of cultured tissues (Figure 1). There are a number of gelling agents such as agar, agarose and gellan gum [21].

Figure 1. Agar-solidified medium supporting plant growth.

Agar, a polysaccharide obtained from seaweeds, is of universal use as a gelling agent for preparing semi-solid and solid plant tissue culture media. Agar has several advantages over other gelling agents; mixed with water, it easily melts in a temperature range 60-100^{o}C and solidifies at approximately 45^{o}C and it forms a gel stable at all feasible incubation temperatures. Agar gels do not react with media constituents and are not digested by plant enzymes. It is commonly used in media at concentrations ranging between 0.8-1.0%. Pure agar preparations are of great importance especially in experiments dealing with tissue metabolism. Agar contains Ca, Mg and trace elements on comparing different agar brands [22]; Bacto, Noble and purified agar, in concern with contaminants. The author, for example reported Bacto agar to contain 0.13, 0.01, 0.19, 0.43, 2.54, 0.17% of Ca, Ba, Si, Cl, SO_4^-, N, respectively. Impurities also included 11.0, 285.0 and 5.0 mg.l^{1-} for iron, magnesium and copper as contaminants, respectively. Amounts of some contaminants were higher in purified agar than in Bacto agar of which Mg that accounted for 695.0 mg.l^{1-} and Cu for 20.0 mg.l^{1-}.

Reduction of culture media costs is continually targeted in large-scale cultures and search for cheap alternatives provided that white flower, potato starch, rice powder were as good gelling agents as agar. It was also experienced that combination of laundry starch, potato starch and semolina in a ratio of 2:1:1 reduced costs of gelling agents by more than 70% [23].

2.8. Growth regulators

Plant growth regulators are important in plant tissue culture since they play vital roles in stem elongation, tropism, and apical dominance. They are generally classified into the following groups; auxins, cytokinins, gibberellins and abscisic acid. Moreover, proportion of auxins to cytokinins determines the type and extent of organogenesis in plant cell cultures [24].

2.8.1. Auxins

The common auxins used in plant tissue culture media include: indole-3- acetic acid (IAA), indole-3- butric acide (IBA), 2,4-dichlorophenoxy-acetic acid (2,4-D) and naphthalene- acetic acid (NAA). IAA is the only natural auxin occurring in plant tissues There are other synthetic auxins used in culture media such as 4-chlorophenoxy acetic acid or p-chloro-phenoxy acetic acid (4-CPA, pCPA), 2,4,5-trichloro-phenoxy acetic acid (2,4,5 T), 3,6-dichloro-2-methoxy- benzoic acid (dicamba) and 4- amino-3,5,6-trichloro-picolinic acid (picloram) [2].

Auxins differ in their physiological activity and in the extent to which they translocate through tissue and are metabolized. Based on stem curvature assays, eight to twelve times higher activity was reported on using 2,4-D than IAA, four times higher activity of 2,4,5 T in comparison with IAA and NAA has as doubled activity as IAA [25]. In tissue cultures, auxins are usually used to stimulate callus production and cell growth, to initiate shoots and rooting, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture. The auxin NAA and 2,4-D are considered to be stable and can be stored at 4°C for several months [26]. The solutions of NAA and 2,4-D can also be stored for several months in a refrigerator or at -20°C if storage has to last for longer periods. It is best to prepare fresh IAA solutions each time during medium preparation, however IAA solutions can be stored in an amber bottle at 4°C for no longer than a week. Generally IAA and 2,4-D are dissolved in a small volume of 95% ethyl alcohol. NAA, 2,4-D and IAA can be dissolved in a small amount of 1N NaOH. Chemical structures of some of the frequently used auxins are given in Figure (2).

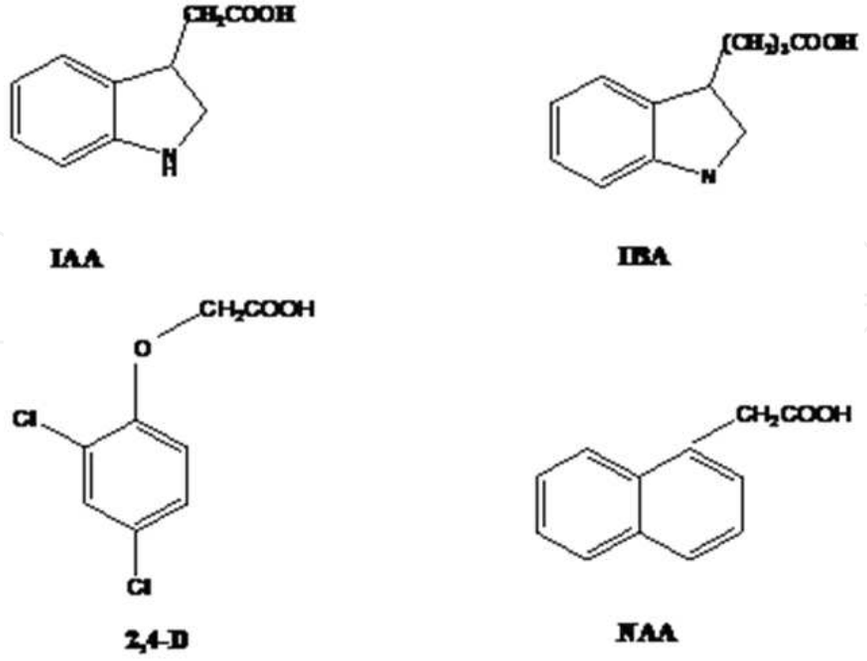

Figure 2. Chemical structure of commonly used auxins. IAA indole acetic acid, IBA Indole butyric acid, 2,4-D dichlorophenoxyacetic acid and NAA naphthalene acetic acid.

There are also some auxinlike compounds (Figure 3) that vary in their activity and are rarely used in culture media.

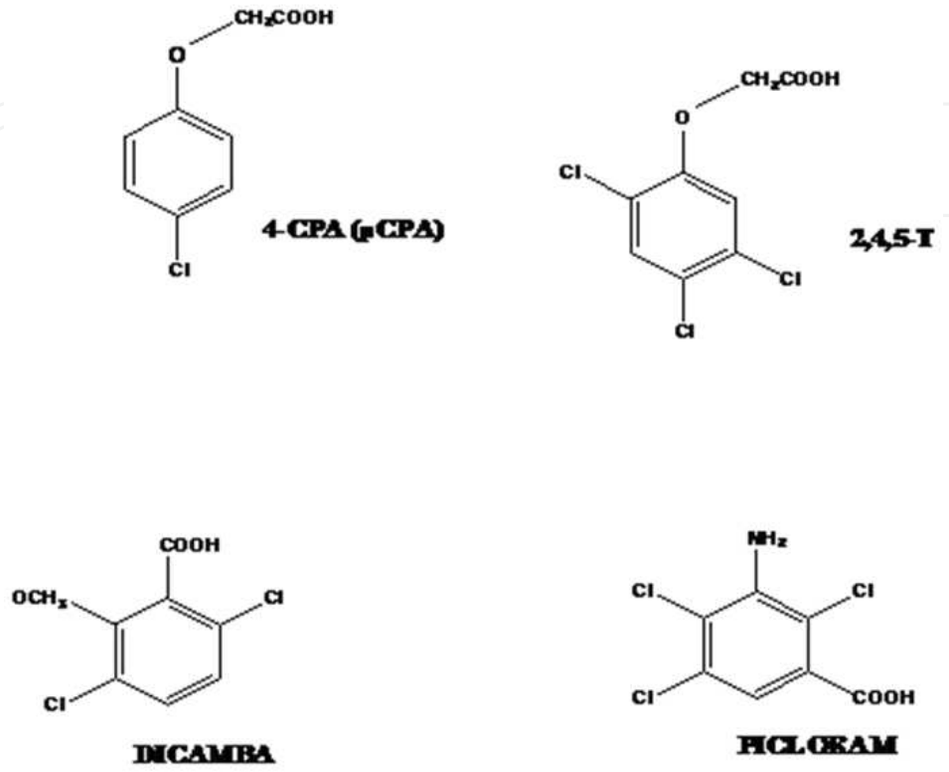

Figure 3. Some auxinlike compounds. pCPA, p-chloro-phenoxy acetic acid and 2,4,5T, 2,4,5-trichloro-phenoxy acetic acid.

2.8.2. Cytokinins

Cytokinins commonly used in culture media include BAP (6-benzyloaminopurine), 2iP (6-dimethylaminopurine), kinetin (N-2-furanylmethyl-1H-purine-6-amine), Zeatin (6-4-hydroxy-3-methyl-trans-2-butenylaminopurine) and TDZ (thiazuron-N-phenyl-N-1,2,3 thiadiazol-5ylurea). Zeatin and 2iP are naturally occurring cytokinins and zeatin is more effective. In culture media, cytokinins proved to stimulate cell division, induce shoot formation and axillary shoot proliferation and to retard root formation. The cytokinins are relatively stable compounds in culture media and can be stored desiccated at -20^{o}C. Cytokinins are frequently reported to be difficult to dissolve and sometimes addition of few drops of 1N HCl or 1N NaOH facilitate their dissolution. Cytokinins can be dissolved in small amounts of dimethylsulfoxide (DMSO) without injury to the plant tissue [27]. DMSO has an additional advantage because it acts as a sterilizing agent; thus stock solutions containing DMSO can be added directly to the sterile culture medium. Chemical structure of the frequently used in plant tissue culture media is given in Figure (4).

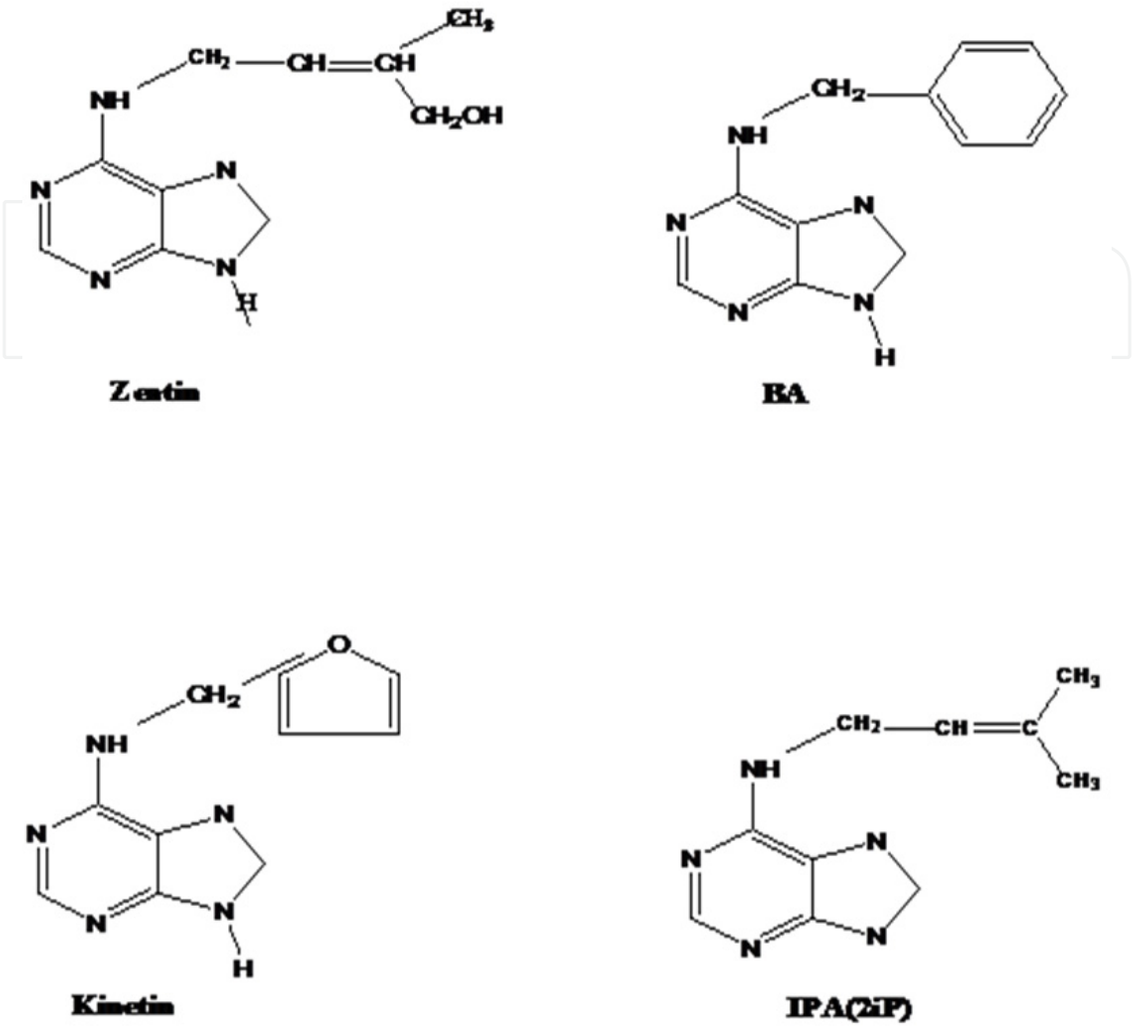

Figure 4. Chemical structure of some cytokinins, BA, benzyladenine, IPA dimethylallylamino purine.

2.8.3. Gibberellins

Gibberellins comprise more than twenty compounds, of which GA_3 is the most frequently used gibberellin. These compounds enhance growth of callus [13] and help elongation of dwarf plantlets [2].

Other growth regulators are sometimes added to plant tissue culture media as abscisic acid, a compound that is usually supplemented to inhibit or stimulate callus growth, depending upon the species. It enhances shoot proliferation and inhibits later stages of embryo development [18]. Although growth regulators are the most expensive medium ingredients, they have little effect on the medium cost because they are required in very small concentrations [21].

A comparison of the chemical composition of the frequently used plant tissue culture media appears in Table (1) which was given in the appendix of the proceedings of the Technical meeting of the International Atomic Energy Agency (IAEA) [28].

Medium Components (mg.l^{1-})	MS	G_5	W	LM	VW	Km	M	NN
Macronutrients								
$Ca_3(PO4)_2$					200.0			
NH_4NO_3	1650.0			400.0				720.0
KNO_3	1900.0	2500.0	80.0		525.0	180.0	180.0	950.0
$CaCl_2.2H_2O$	440.0	150.0		96.0				166.0
$MgSO_4.7H_2O$	370.0	250.0	720.0	370.0	250.0	250.0	250.0	185.0
KH_2PO_4	170.0			170.0	250.0	150.0	150.0	68.0
$(NH4)_2SO4$		134.0			500.0	100.0	100.0	
$NaH_2PO_4.H_2O$		150.0	16.5					
$CaNO_3.4H_2O$			300.0	556.0		200.0	200.0	
Na_2SO_4			200.0					
KCl			65.0					
K_2SO_4				990.0				
Micronutrients								
KI	0.83	0.75	0.75			80.0	0.03	
H_3BO_3	6.20	3.0	1.5	6.2		6.2	0.6	10.0
$MnSO_4.4H_2O$	22.30		7.0		0.75	0.075		25.0
$MnSO_4.H_2O$		10.0		29.43				
$ZnSO_4.7H_2O$	8.6	2.0	2.6	8.6			0.05	10.0
$Na_2MoO_4.2H_2O$	0.25	0.25		0.25		0.25	0.05	0.25
$CuSO_4.5H_2O$	0.025	0.025		0.25		0.025		0.025
$CoCl_2.6H_2O$	0.025	0.025				0.025		
$Co(NO_3)_2.6H_2O$							0.05	
Na_2EDTA	37.3	37.3		37.3		74.6	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8		27.8		25.0	27.8	27.8
$MnCl_2$						3.9	0.4	
$Fe(C_4H_4O_6)_3.2H_2O$					28.0			
Vitamins and other supplements								
Inositol	100.0	100.0		100.0				100.0
Glycine	2.0	2.0	3.0	2.0				2.0
Thiamine HCl	0.1	10.0	0.1	1.0		0.3	0.3	0.5
Pyridoxine HCl	0.5		0.1	0.5		0.3	0.3	0.5
Nicotinic acid	0.5		0.5	0.5			1.25	5.0
Ca-panthothenate			1.0					
Cysteine HCl			1.0					
Riboflavin						0.3	0.05	
Biotin							0.05	0.05
Folic acid							0.3	0.5

MS Murashige and Skoog, G_5= Gamborg *et al*., W= White, LM= Lloyd and McCown, VW= Vacin and Went, Km= Kudson modified, M= Mitra *et al*. and NN= Nitsch and Nitsch media.

Table 1. Composition of media most frequently used.

3. Media preparation

Preparation of culture media is preferred to be performed in an equipped for this purpose compartment (Figure 5). This compartment should be constructed so as to maintain ease in cleaning and reducing possibility of contamination. Supplies of both tap and distilled water and gas should be provided. Appropriate systems for water sterilization or deionization are also important [29]. Certain devices are required for better performance such as a refrigerator, freezer, hot plate, stirrer, pH meter, electric balances with different weighing ranges, heater, Bunsen burner in addition to glassware and chemicals [30]. It is well known now that mistakes which occur in tissue culture process most frequently originate from inaccurate media preparation that is why clean glassware, high quality water, pure chemicals and careful measurement of media components should be facilitated.

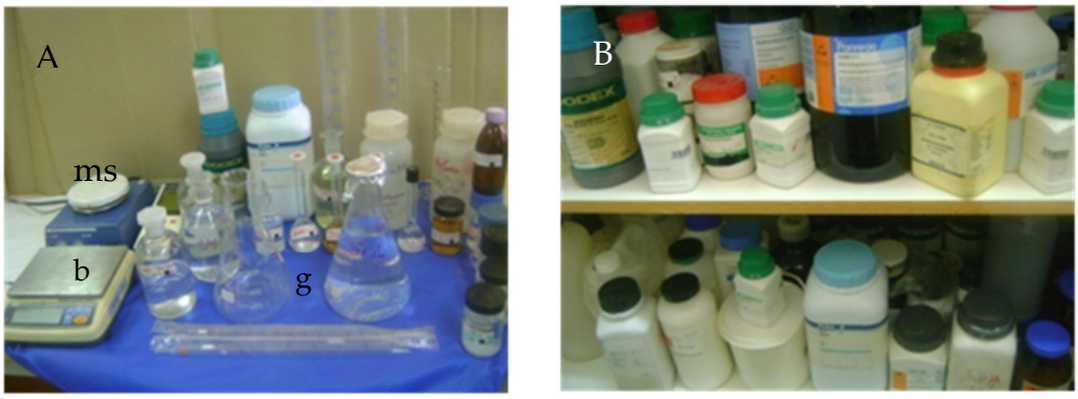

Figure 5. Some of components of the preparation room. A, some equipments used; ms magnetic stirrer hot plate, b electric balance, g glassware. B shelves for keeping chemicals.

A convenient method for preparation of culture media is to make concentrated stock solutions which can be immediately diluted to preferred concentration before use. Solutions of macronutrients are better to be prepared as stock solutions of 10 times the strength of the final operative medium. Stock solutions can be stored in a refrigerator at 2- 4^{o}C. Micronutrients stock solutions are made up at 100 times of the final concentration of the working medium. The micronutrients stock solution can also be stored in a refrigerator or a freezer until needed. Iron stock solution should be 100 times concentrated than the final working medium and stored in a refrigerator. Vitamins are prepared as either 100 or 1000 times concentrated stock solutions and stored in a freezer (-20^{o}C) until used if it is desired to keep them for long otherwise they can be stored in a refrigerator for 2-3 months and should be discarded thereafter [31]. Stock solutions of growth regulators are usually prepared at 100-1000 times the final desired concentration.

Concentrations of inorganic and organic components of media are generally expressed in mass values (mg.l^{-1}, mg/l and p.p.m.) in tissue culture literature. The International Association 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 weights of the atoms in the chemical formula of the substance. One liter of solution containing 1mole of a substance is 1 molar (1M) or 1 $mol.l^{-1}$ solution of the substance (1 $mol.l^{-1}$= 10^3 $mmol.l^{-1}$= 10^6 μmol/l). It is routinely now to accepted to express concentrations of macronutrients and organic nutrients in the culture medium as mmol/l values, and μmol/l values for micronutrients, hormones, vitamins and other organic constituents. This was explained on the basis that mole values for all compounds have constant number of molecules per mole [32].

4. Media selection

For the establishment of a new protocol for a specific purpose in tissue culture, a suitable medium is better formulated by testing the individual addition of a series of concentrations of a given compound to a universal basal medium such as MS, LS or B_5. The most effective variables in plant tissue culture media are growth regulators, especially auxins and cytokinins. Full strength of salts in media proved good for several species, but in some species the reduction of salts level to ½ or ¼ the full concentration gave better results in *in vitro* growth.

Sucrose is often assumed to be the best source of carbon for in vitro culture, the levels used are from 2 to 6% and the level has to be defined for each species.

5. Media sterilization

Prevention of contamination of tissue culture media is important for the whole process of plant propagation and helps to decrease the spread of plant parasites. Contamination of media could be controlled by adding antimicrobial agents, acidification or by filtration through microporous filters [33]. To reduce possibilities of contamination, it is recommended that sterilization rooms should have the least number of openings. Media preparation and sterilization are preferred to be performed in separate compartments. Sterilization area should also have walls and floor that withstand moisture, heat and steam [29].

Sterilization of media is routinely achieved by autoclaving at the temperature ranging from 115^o – 135^o C. Advantages of autoclaving are: the method is quick and simple, whereas disadvantages are the media pH changes and some components may decompose and so to loose their effectiveness. As example autoclaving mixtures of fructose, glucose and sucrose resulted in a drop in the agar gelling capacity and affecting pH of the culture medium through the formation of furfural derivatives due to sucrose hydrolysis[2].

Filtration through microporus filters (0.22- 0.45) is also used for thermolabile organic constituents such as vitamins, growth regulators and amino acids [2]. Additives of antimicrobial agents are less commonly applied in plant tissue culture media. Limitation for their use was reported and attributed to harm imposed on plants as well [34].

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