2. CULTURE MEDIA

Characteristics of some of the more Common tissue Culture media

The type of tissue culture medium selected depends upon the species to be cultured. Some species are sensitive to high salts or have different requirements for PGRs. The age of the plant also has an effect. For example, juvenile tissues generally regenerate roots more readily than adult tissues. The type of organ cultured is important; for example, roots require thiamine. Each desired cultural effect has its own unique requirements such as auxin for induction of adventitious roots and altering the cytokinin:auxin ratio for initiation and development of adventitious shoots (Fig. 6).

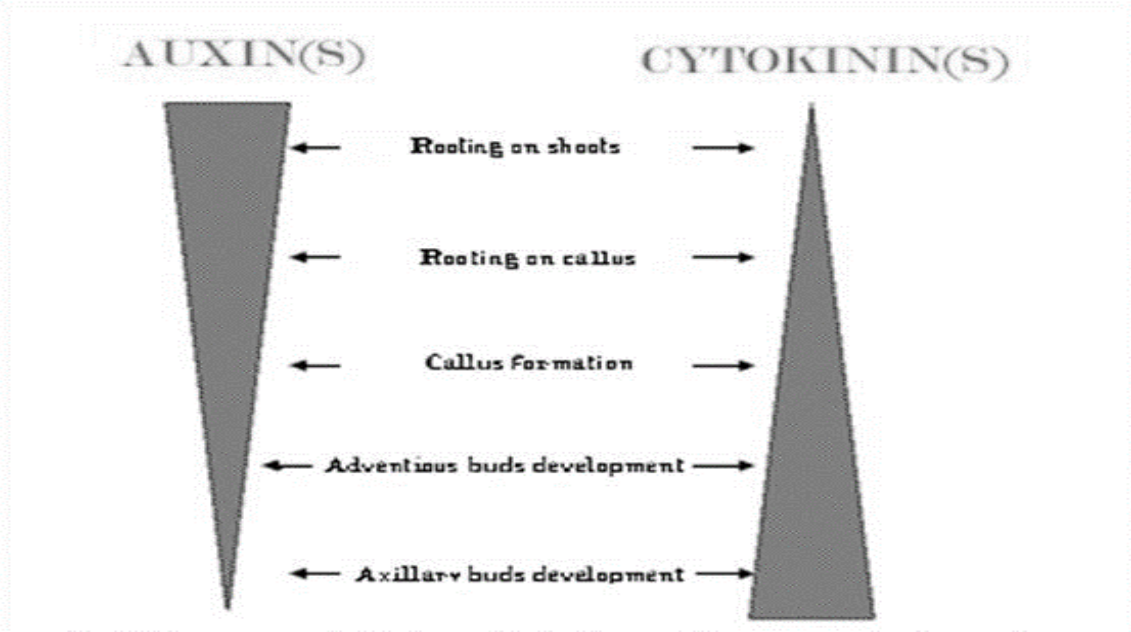

Figure 6. The relative concentrations of auxin and cytokinin were added to the nutrient medium to achieve the indicated purpose.

Development of culture medium formulations was a result of systematic trial and experimentation. The composition of several of the most commonly used plant tissue culture media with respect to their components in mg/L and molar units is presented in Table 2.1. Murashige and Skoog medium (MS) (Table 4) is suitable for many applications and the most commonly used basic tissue culture medium for plant regeneration from tissues and callus. It was developed for tobaccobased primarily upon the mineral analysis of tobacco tissue. This is a “high salt” medium due to its content of potassium and nitrogen salts. Linsmaier and Skoog medium (LS) is basically MS medium with respect to its inorganic portion, but only inositol

and thiamine HCl are retained among the organic components. To counteract salt sensitivity of some woody species, Lloyd and McCown developed the woody plant medium (WPM).

Gamborg's medium (B5) was devised for soybean callus culture and has lesser amounts of nitrate and, particularly, ammonium salts than does the MS medium. Although B5 was originally developed for the purpose of obtaining callus or for use with suspension culture, it also works well as a basal medium for whole plant regeneration. Schenk and Hildebrandt (SH) developed SH medium for the callus culture of monocots and dicots. White's medium, which was designed for the tissue culture of tomato roots, has a lower concentration of salts than MS medium. Nitsch's medium was developed for anther culture and contains a salt concentration intermediate to that of MS and White's media.

Many companies sell packaged mixtures of the better known media recipes. These are easy to make because they merely involve dissolving the packaged mix in a specified volume of water. These can be purchased as the salts, the vitamins, or the entire mix with or without PGRs, agar, and sucrose. These are convenient, less prone to individual error, and make keeping stock solutions unnecessary. However, they are more expensive than making media from scratch.

Components of the tissue Culture medium

Growth and development of an explant in vitro is a product of its genetics, surrounding environment, and components of the tissue culture medium, the last of which is easiest to manipulate to our own ends. Tissue culture medium consists of 95% water, macro- and micronutrients, PGRs, vitamins, sugars (because plants in vitro are often not photosynthetically-competent), and sometimes various other simple-to-complex organic materials. All in all, about 20 different components are usually needed for tissue culture medium.

Table 4. Composition of five commonly used tissue culture media in milligrams per liter and molar concentrations with inorganic components.

Compounds	Murashige and Skoog	Gamborg B-5	WPM	Nitsch and Nitsch	Schenk and Hildebrandt	White
			Macronutrients in mg/L (mM)			
NH_4NO_3	1650 (20.6)	—	400 (5.0)	720 (9.0)	—	—
$NH_4H_2PO_4$	—	—	—	—	300 (2.6)	—
NH_4SO_4	—	134 (1.0)	—	—	—	—
$CaCl_2 \cdot 2H_2O$	440 (3.0)	150 (1.0)	96 (0.7)	166 (1.1)	151 (1.0)	—
$Ca(NO_3)_2 \cdot 4H_2O$	—	—	556 (2.4)	—	—	288 (1.2)
$MgSO_4 \cdot 7H_2O$	370 (1.5)	246 (1.0)	370 (1.5)	185 (0.75)	400 (1.6)	737 (3.0)
KCl	—	—	—	—	—	65 (0.9)
KNO_3	1900 (18.8)	2528 (25)	—	950 (9.4)	2500 (24.8)	80 (0.8)
K_2SO_4	—	—	990	—	—	—
KH_2PO_4	170 (1.25)	—	170 (1.3)	68 (0.5)	—	—
$NaH_2PO_4 \cdot H_2O$	—	150 (1.1)	—	—	—	19 (0.14)
Na_2SO_4	—	—	—	—	—	200 (1.4)
			Micronutrients in mg/L (μM)			
H_3BO_3	6.2 (100)	3.0 (49)	6.2 (100)	10 (162)	5 (80)	1.5 (25)
$CoCl_2 \cdot 6H_2O$	0.025 (0.1)	0.025 (0.1)	—	—	0.1 (0.4)	—
$CuSO_4 \cdot 5H_2O$	0.025 (0.1)	0.025 (0.1)	0.25 (1)	0.025 (0.1)	0.2 (0.08)	0.01 (0.04)
$Na_2EDTA \cdot 2H_2O$	37.2 (100)	37.2 (100)	37.2 (100)	37.2 (100)	20.1 (54)	—
$Fe_2(SO_4)_3$	—	—	—	—	—	2.5 (6.2)
$FeSO_4 \cdot 7H_2O$	27.8 (100)	27.8 (100)	27.8 (100)	27.8 (100)	15 (54)	—
$MnSO_4 \cdot H_2O$	—	10.0 (59)	—	—	10.0 (59)	5.04 (30)
$MnSO_4 \cdot 4H_2O$	22.3 (100)	—	22.3 (100)	25.0(112)	—	—
KI	0.83 (5)	0.75 (5)	—	—	0.1 (0.6)	0.75 (5)
$NaMoO_3$	—	—	—	—	—	0.001 (0.001)
$Na_2MoO_4 \cdot 2H_2O$	0.25 (1)	0.25 (1)	0.25 (1)	0.25 (1)	0.1 (0.4)	—
$ZnSO_4 \cdot 7H_2O$	8.6 (30)	2.0 (7.0)	8.6 (30)	10 (35)	1 (3)	2.67 (9)
			Organics in mg/L (μM)			
Myo-inositol	100 (550)	100 (550)	100 (550)	100 (550)	1000 (5500	—
Glycine	2.0 (26.6)	—	2.0 (26.6)	2.0 (26.6)	—	3.0 (40)
Nicotinic acid	0.5 (4.1)	1.0 (8.2)	0.5 (4.1)	5 (40.6)	5.0 (41)	0.5 (4.1)
Pyridoxine HCl	0.5 (2.4)	0.1 (0.45)	0.5 (2.4)	0.5 (2.4)	0.5 (2.4)	0.1 (0.45)
Thiamine HCl	0.1 (0.3)	10.0 (30)	1.0 (3.0)	0.5 (1.5)	5.0 (14.8)	0.1 (0.3)
Biotin	—	—	—	0.2 (0.05)	—	—

Inorganic mineral elements

Just as a plant growing in vivo requires many different elements from either soil or fertilizers, the plant tissue growing in vitro requires a combination of macro- and micronutrients. The choice of macro- and microsalts and their concentrations is species dependent. MS medium is very popular because most plants react to it favorably; however, it may not necessary result in the optimum growth and development for every species since the salt content is so high.

The macronutrients are required in millimolar (mM) quantities in most plant basal media. Nitrogen (N) is usually supplied in the form of ammonium (NH^+_4) and nitrate (NO^-_3) ions, although sometimes more complex organic sources such as urea, individual amino acids such as glutamine, or complex mixtures of amino

acids found in casein hydrolysate are used, too. Although most plants prefer NO3- to NH^{+}_{4}, the right balance of the two ions for optimum in vitro growth and development for the selected species may differ considerably.

In addition to nitrogen, potassium, magnesium, calcium, phosphorus, and sulfur are provided in the medium as different components referred to as the macrosalts. $MgSO_4 \bullet 7H_2O$ provides both magnesium and sulfur; NH_4 H_2PO_4, KH_2PO_4, or NaH2PO4•H2O provide phosphorus; $CaCl_2 \bullet 2H_2O$ or $Ca(NO_3)_2 \bullet 4H_2O$ provide calcium; and KCl, KNO_3, or KH_2PO_4 provide potassium. Chloride is provided by KCl and/or $CaCl_2 \bullet 2H_2O$.

Microsalts typically include boron (H_3BO_3), cobalt ($CoCl_2 \bullet 6H_2O$), iron (complex of $FeSO_4 \bullet 7H_2O$ and Na 2EDTA•2H2O or rarely as $Fe_2(SO_4)_3$), manganese ($MnSO_4$•H2O or $MnSO_4 \bullet 4H_2O$), molybdenum ($NaMoO_3 \bullet 2H_2O$), copper ($CuSO_4 \bullet 5H_2O$), and zinc ($ZnSO_4 \bullet 7H_2O$). Microsalts are needed in much lower (micromolar; μM) concentrations than the macronutrients. Some media may contain very small amounts of iodide (KI), but sufficient quantities of many of the trace elements inadvertently may be provided because reagent-grade chemicals contain many inorganic contaminants.

organic compounds

Sugar (most commonly sucrose) is a very important part of any nutrient medium, and its addition is essential for in vitro growth and development of the culture. Most plant cultures are unable to photosynthesize effectively for a variety of reasons, including insufficiently organized cellular and tissue development, lack of chlorophyll, limited gas exchange and CO_2 in the tissue culture vessels, and less than optimum environmental conditions such as low light. A concentration of 20–60 g/L sucrose (a disaccharide made up of d-glucose and d-fructose) is the most often used carbon and energy source, since this sugar is also synthesized and transported naturally by the plant. Other mono- or disaccharides and sugar alcohols such as glucose, fructose, sorbitol, and maltose may be used. The sugar concentration chosen is dependent on the type and age of the explant in culture. For example, very young embryos require a relatively high sugar concentration (>3%). Fructose was better suited for proliferation of mulberry buds in vitro than sucrose, glucose, maltose, raffinose, or lactose. For apple, sorbitol and sucrose

supported callus initiation and growth equally as well, but sorbitol was better for peach after the fourth subculture.

Sugar (sucrose) that is bought from the supermarket is usually adequate, but be careful to get pure cane sugar as corn sugar is primarily fructose. Raw cane sugar is purified and according to the manufacturers analysis consists of 99.94% sucrose, 0.02% water, and 0.04% other materials (inorganic elements and also raffinose, fructose, and glucose). Nutrient salts contribute approximately 20%–50% to the osmotic potential of the medium, and sucrose is responsible for the remainder. The contribution of sucrose to the osmotic potential increases as it is hydrolyzed into glucose and fructose during autoclaving. This may be an important consideration when performing osmotic sensitive procedures such as protoplast isolation and culture.

Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions of the vitamins, only thiamine (vitamin B1 at 0.1–5.0 mg/L) is essential in culture as it is involved in carbohydrate metabolism and the biosynthesis of some amino acids. It is usually added to tissue culture media as thiamine hydrochloride. Nicotinic acid, also known as *niacin*, *vitamin B*3, or *vitamin PP*, forms part of a respiratory coenzyme and is used at concentrations between 0.1 to 5 mg/L. MS medium contains thiamine HCl as well as two other vitamins, nicotinic acid and pyridoxine (vitamin B6), in the HCl form. Pyridoxine is an important coenzyme in many metabolic reactions and is used in media at concentrations of 0.1 to 1.0 mg/L. Biotin (vitamin H) is commonly added to tissue culture media at 0.01–1.0 mg/L. Other vitamins that are sometimes used are folic acid (vitamin M; 0.1–0.5 mg/L), riboflavin (vitamin B2; 0.1–10 mg/L), ascorbic acid (vitamin C; 1–100 mg/L), pantothenic acid (vitamin B5; 0.5–2.5 mg/L), tocopherol (vitamin E; 1–50 mg/L), and *para*-aminobenzoic acid (0.5–1.0 mg/L).

Sometimes characterized as one of the B complex vitamin group, myo-inositol is really a sugar alcohol involved in the synthesis of phospholipids, cell wall pectins, and membrane systems in cell cytoplasm. It is added to tissue culture media at a concentration of about 0.1–1.0 g/L and has been demonstrated to be necessary for some monocots, dicots, and gymnosperms.

In addition, other amino acids are sometimes used in tissue culture media. These include l-glutamine, l-asparagine, l-serine, and l-proline, which are used as sources

of reduced organic nitrogen, especially for inducing and maintaining somatic or nonzygotic embryogenesis. l-Glycine, the simplest amino acid, is a common additive since it is essential in purine synthesis and is a part of the porphyrin ring structure of chlorophyll.

Complex organic compounds are a group of undefined supplements such as casein hydrolysate, coconut milk (the liquid endosperm of the coconut), orange juice, tomato juice, grape juice, pineapple juice, sap from birch, banana puree, etc. These compounds are often used when no other combination of known defined components produces the desired growth or development. However, the composition of these supplements is basically unknown and may also vary from lot-to-lot causing variable responses. For example, the composition of coconut milk (used at a dilution of 50–150 mL/L), a natural source of the PGR, zeatin, not only differs between young and old coconuts, but also among coconuts of the same age.

Some complex organic compounds are used as organic sources of nitrogen such as casein hydrolysate, a mixture of about 20 different amino acids and ammonium (0.1–1.0 g/L), peptone (0.25–3.0 g/L), tryptone (0.25–2.0 g/L), and malt extract (0.5–1.0 g/L). These mixtures are very complex and contain vitamins as well as amino acids. Yeast extract (0.25–2.0 g/L) is used because of the high concentration and quality of B vitamins.

Polyamines, particularly putrescine and spermidine, are sometimes beneficial for somatic embryogenesis. Polyamines are also cofactors for adventitious root formation. Putrescine is capable of synchronizing the embryogenic process of carrot and enhances somatic embryogenesis in cotton.

Activated charcoal is useful for absorption of the brown or black pigments and oxidized and polymerized phenolic compounds (melanins). It is incorporated into the medium at concentrations of 0.2–3.0% (w/v). It is also useful for absorbing other organic compounds including PGRs, such as auxins and cytokinins, vitamins, iron, and zinc chelates. Explants transferred to media containing no PGRs sometimes continue to exhibit growth and development typical of the medium they were on previously that contained PGRs. These carryover effects of PGRs are minimized by adding activated charcoal when transferring explants to media without PGRs. Another feature of using activated charcoal is that it changes the light environment by darkening the medium, so it can help with root formation

and growth. It may also promote nonzygotic embryogenesis and enhance growth and organogenesis of woody species.

Leached pigments and oxidized polyphenolic compounds and tannins can greatly inhibit growth and development. These are formed by some explants as a result of wounding. If charcoal does not reduce the inhibitory effects of polyphenols, addition of polyvinylpyrrolidone (PVP, 250–1000 mg/L), or antioxidants, such as citric acid, ascorbic acid, or thiourea, can be tested.

Plant Growth Regulators (PGRs)

PGRs exert dramatic effects at low concentrations (0.001–10 μM). They control and modulate the initiation and development of shoots and roots on explants and embryos on semisolid or in liquid medium cultures. They also stimulate cell division and expansion. Sometimes, a tissue or an explant is autotrophic and can produce its own supply of PGRs. Usually PGRs must be supplied in the medium for growth and development of the tissues or cells in culture.

The most important classes of the PGRs used in tissue culture are the auxins and cytokinins. The relative effects of auxin and cytokinin ratio on morphogenesis of cultured tobacco tissues were demonstrated by Skoog and Miller and still serve as the basis for plant tissue culture manipulations today. Some of the PGRs used are hormones (naturally synthesized by higher plants), and others are synthetic (man-made) compounds. PGRs exert dramatic effects depending on the concentration used, the target tissue, and their inherent activity even though they are used in very low concentrations in the media (from 0.1 to 100 μM). The concentrations of PGRs are typically reported in mg/L or in μM units of concentration. Comparisons of PGRs based on their molar concentrations are more useful because the molar concentration is a reflection of the actual number of molecules of the PGR per unit volume.

Auxins play a role in many developmental processes, including cell elongation and swelling of tissue, apical dominance, adventitious root formation, and somatic embryogenesis. Generally, when the concentration of auxin is low, root initiation is favored and, when the concentration is high, callus formation occurs. The most common synthetic auxins used are 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram). Naturally occurring indoleacetic acid (IAA) and

indolebutyric acid (IBA) are also used. IBA was once considered synthetic, but has also been found to occur naturally in many plants including olive and tobacco (Epstein et al., 1989). Both IBA and IAA are photosensitive so that stock solutions must be stored in the dark. IAA is also easily broken down by enzymes (peroxidases and IAA oxidase). IAA is the weakest auxin and is typically used at concentrations between 0.01 to 10 mg/L. More active auxins such as IBA, NAA, 2,4-D, and picloram are used at concentrations ranging from 0.001 to 10 mg/L. Picloram and 2,4-D are examples of auxins used primarily to induce and regulate somatic embryogenesis.

Cytokinins promote cell division and shoot initiation and growth in vitro. The cytokinins most commonly used are zeatin, dihydrozeatin, kinetin, benzyladenine, thidiazuron, and 2iP. In higher concentrations (1–10 μM), they induce adventitious shoot formation but inhibit root formation. They promote axillary shoot formation by opposing apical dominance regulated by auxins. Benzyladenine has significantly stronger cytokinin activity than the naturally occurring zeatin. However, a concentration of 0.05–0.1 μM thidiazuron, a diphenyl substituted urea, is more active than 4–10 μM BA, but thidiazuron may inhibit root formation, causing difficulties in plant regeneration.

Gibberellins (GA) are less commonly used in plant tissue culture. GA_3 is the most often used, but it is very heat sensitive (after autoclaving 90% of the biological activity is lost). Typically, it is filter sterilized and added to autoclaved medium after it has cooled. Gibberellins help to stimulate elongation of internodes and have proved to be necessary for meristem growth for some species. Abscisic acid (ABA) is not normally considered an important PGR for tissue culture except for somatic embryogenesis and in the culture of some woody plants. For example, it promotes maturation and germination of somatic embryos of caraway, citrus, and spruce.

Organ and callus cultures are able to produce ethylene, a gaseous PGR. Since culture vessels are almost entirely closed, ethylene can sometimes accumulate. Many plastic containers also contribute to ethylene content in the vessel. There are contrasting reports in the literature concerning the role played by ethylene in vitro. It appears to influence embryogenesis and organ formation in some gymnosperms. Sometimes, in vitro growth can be promoted by ethylene. At other times, addition of ethylene inhibitors results in better initiation or growth. For

example, the ethylene inhibitors, particularly silver nitrate, are used to enhance embryogenic culture initiation in corn. High levels of 2,4-D can induce ethylene formation.

Agar and alternative culture support systems

Agar is used to solidify tissue culture media into a gel. It enables the explant to be placed in intimate contact with the medium (e.g., on the surface or embedded) but remain aerated. Agar is a high molecular weight polysaccharide that can bind water and is derived from seaweed. It is added to the medium in concentrations ranging from 0.5 to 1.0% (w/v). High concentrations of agar result in a harder medium. If a lower concentration of agar is used (0.4%) or if the pH is low, the medium will be too soft and will not gel properly. The consistency of the agar can also influence the growth. If it is too hard, plant growth is reduced. If it is too soft, plants that have a translucent water-soaked (hyperhydric) appearance may be the result. To gel properly, a medium with 0.6% agar must have a pH above 4.8. Sometimes, activated charcoal in the medium will interfere with gelling. Typical tissue culture agar melts easily at ~65°C and solidifies at ~45°C.

Agar also contains organic and inorganic contaminants, the amount of which varies between brands. Organic acids, phenolic compounds, and long-chain fatty acids are common contaminants. A manufacturer's analysis shows that Difco Bacto agar also contains (amounts in ppm) 0.0–0.5 cadmium, 0.0–0.1 chromium, 0.5–1.5 copper, 1.5–5.0 iron, 0.0–0.5 lead, 210.0–430.0 magnesium, 0.1–0.5 manganese, and 5.0–10.0 zinc. Generally, relatively pure, plant tissue culture-tested types of agar should be used, such as Phytagar. Poor quality agar can interfere with or inhibit the growth of cultures.

Agarose is often used when culturing protoplasts or single cells. Agarose is a purified extract of agar that leaves behind agaropectin and sulfate groups. Since its gel strength is greater, less is used to create a suitable support or suspending medium.

Gellan gums such as Gelrite and Phytagel are alternative gelling agents. They are made from a polysaccharide produced by a bacterium. Rather than being translucent (like agar), they are clear, so it is much easier to detect contamination, but they cannot be heated and gelled again, and the concentration of divalent

cations such as calcium and magnesium must be within a restricted range or gelling will not occur.

Mechanical supports such as filter paper bridges or polyethylene rafts do not rely on a gelling agent. They can be used with liquid media, which then circulates better, but keeps the explant at the medium surface so that it remains oxygenated. The types of support systems that have been used are as varied as the imagination and include rock wool, cheesecloth, pieces of foam, and glass beads.

Phases of the nutrient media

1. Solid media

It is a medium that contains agar, gelatin, or Biogles to give it a gelatinous consistency. It is preferable to use pure agar. Agar is added at 1.0 - 0.6% (w/v) or gelatin at 1% (w/v).

2. Liquid media

It is a medium that does not contain agar and is preferred by many researchers in tissue culture instead of a solid medium.

3. Double phase media

Solid and liquid media are used separately in laboratory cultivation. It may cause Verification. Therefore, we use a double-profile medium. It is prepared by adding the medium to the agar at the bottom of the planting pot. The explant is grown, and then a liquid medium is added to it that contains the same components as the solid medium but without the soil. The plant material is between a solid and a liquid medium containing the same nutritional components. Accordingly, changing the liquid from the medium is easy whenever required .Comparison between solid and liquid media

1. Some plant species grow well in liquid media, such as plants in the Bromeliaceae family, while others grow well in solid media.
2. The explant grown in liquid media need a good supply of air. Therefore, the planted vessels are placed on an electric vibrator to shake them so they do not become damaged.
3. The low rate at which the explant or callus benefits from the components of solid media, as only the surface of the explant in contact with the solid medium is the beneficiary. In contrast, all surfaces of the explant immersed in the liquid medium can absorb nutrients and growth regulators due to direct contact with

the medium.

4. Exudates explant in one place on the solid medium, causing damage to new growth. While these secretions spread in the liquid medium by shaking, they have a less harmful effect on growth.
5. The explant immersed under the surface of the solid medium does not benefit from the exchange of gases. In contrast, the continuous shaking of the liquid media helps exchange gases, and the explant is immersed in it to benefit from the components of the nutrient medium.
6. Extracting the roots from the solid medium intact and without damage is difficult, while removing them from the liquid medium is easy.
7. Recording the growth parameters and respiratory rate of growths grown on a liquid medium is easier.
8. Polarization resulting from the effect of gravity is evident on callus grown on a solid medium. At the same time, it does not have an apparent impact on callus grown in a liquid medium.
9. Irregularity in the spread of light between plants growing on a solid medium affects their growth, while the spread of light is more significant in the case of a liquid medium.
10. Cell division, unfolding, and growth are faster in liquid media.