Tissue Culture Media-Composition

MEDIA COMPONENTS

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. The basic nutrient requirements of cultured plant cells are very similar to those of whole plants.

Plant tissue and cell culture media are generally made up of some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other undefined organic supplements, solidifying agents or support systems, and growth regulators. Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations include those described by White, Murashige and Skoog, Gamborg et. al., Schenk and Hilderbrandt, Nitsch and Nitsch, and Lloyd and McCown. Murashige and Skoog's MS medium, Schenk and Hildebrand's SH medium, and Gamborg's B-5 medium are all high in macronutrients, while the other media formulations contain considerably less of the macronutrients.

Macronutrients

The macronutrients provide the six major elements-nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S)-required for plant cell or tissue growth. The optimum concentration of each nutrient for achieving maximum growth rates varies considerably among species.

Culture media should contain at least 25-60 mM of inorganic nitrogen for adequate plant cell growth. Plant cells may grow on nitrates alone, but considerably better results are obtained when the medium contains both a nitrate and ammonium nitrogen source. Certain species require ammonium or another source of reduced nitrogen for cell growth to occur. Nitrates are usually supplied in the range of 25-20 mM; typical ammonium concentrations range between 2 and 20 mM. However, ammonium concentrations in excess of 8 mM may be deleterious to cell growth of certain species. Cells can grow on a culture medium containing ammonium as the sole nitrogen source if one or more of the TCA cycle acids (e.g., citrate, succinate, or malate) are also included in the culture medium at concentrations of approximately 10 mM. When nitrate and ammonium sources of nitrogen are utilized together in the culture medium, the ammonium ions will be utilized together in the culture medium, the ammonium ions will be utilized more rapidly and before the nitrate ions.

Potassium is required for cell growth of most plant species. Most media contain K, in the nitrate or chloride form, at concentrations of 20-30 mM. The optimum concentrations of P, Mg, S, and Ca range from 1-3 mM when all other requirements for cell growth are satisfied. Higher concentrations of these nutrients may be required if deficiencies in other nutrients exist.

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Micronutrients

The essential micronutrients for plant cell and tissue growth include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). Chelated forms of iron and zinc are commonly used in preparing culture media. Iron may be the most critical of all the micronutrients. Iron citrate and tartrate may be used in culture media, but these compounds are difficult to dissolve and frequently precipitate after media are prepared. Murashige and Skoog used an ethylene diaminetetraacetic acid (EDTA)-iron chelate to bypass this problem.

Cobalt (Co) and iodine (I) may also be added to certain media, but strict cell growth requirements for these elements have not been established. Sodium (Na) and chlorine (Cl) are also used in some media but are not essential for cell growth. Copper and Cobalt are normally added to culture media at concentrations of 0.1 μM, Fe and Mo at 1 μM, I at 5 μM, Zn at 5-30 μM, Mn at 20-90 μM, and B at 25-100 μM.

Carbon and Energy Source

The preferred carbohydrate in plant cell culture media is sucrose. Glucose and fructose may be substituted in some cases, glucose being as effective as sucrose and fructose being somewhat less effective. Other carbohydrates that have been tested include lactose, galactose, rafinose, maltose, and starch. Sucrose concentrations of culture media normally range between 2 and 3 percent. Use of autoclaved fructose can be detrimental to cell growth.

Carbohydrates must be supplied to the culture medium because few plant cell lines have been isolated that are fully autotropic, e.g., capable of supplying their own carbohydrate needs by CO_2 assimilation during photosynthesis.

Vitamins

Normal plants synthesize the vitamins required for their growth and development. Vitamins are required by plants as catalysts in various metabolic processes. When plant cells and tissues are grown in vitro, some vitamins may become limiting factors for cell growth. The vitamins most frequently used in cell and tissue culture media include thiamin (B_1), nicotinic acid, pyridoxine (B_6), and *myo*-inositol. Thiamin is the one vitamin that is basically required by all cells for growth. Thiamin is normally used at concentrations ranging from 0.1 to 10.0 mg/liter. Nicotinic acid and pyridoxine are often added to culture media but are not essential for cell growth in many species. Nicotinic acid is normally used at concentrations of 0.1-5.0 mg/liter; pyridoxine is used at 0.1-10.0 mg/liter.

Myo-inositol is commonly included in many vitamin stock solutions. Although it is a carbohydrate not a vitamin, it has been shown to stimulate growth in certain cell cultures. Its presence in the culture medium is not essential, but in small quantities *myo*-inositol stimulates cell growth in most species. *Myo*-inositol is generally used in plant cell and tissue culture media at concentrations of 50-5000 mg/liter.

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Other vitamins such as biotin, folic acid, ascorbic acid, pantothenic acid, vitamin E (tocopherol), riboflavin, and p-aminobenzoic acid have been included in some cell culture media. The requirement for these vitamins by plant cell cultures is generally negligible, and they are not considered growth-limiting factors. These vitamins are generally added to the culture medium only when the concentration of thiamin is below the desired level or when it is desirable to grow cells at very low population densities.

Amino Acids or Other Nitrogen Supplements

Although cultured cells are normally capable of synthesizing all of the required amino acids, the addition of certain amino acids or amino acid mixtures may be used to further stimulate cell growth. The use of amino acids is particularly important for establishing cell cultures and protoplast cultures. Amino acids provide plant cells with an immediately available source of nitrogen, which generally can be taken up by the cells more rapidly than inorganic nitrogen. The most common sources of organic nitrogen used in culture media are amino acid mixtures (e.g., casein hydrolysate), L-glutamine, L-asparagine, and adenine. Casein hydrolysate is generally used at concentrations between 0.05 and 0.1 percent. When amino acids are added alone, care must be taken, as they can be inhibitory to cell growth. Examples of amino acids included in culture media to enhance cell growth are glycine at 2 mg/liter, glutamine up to 8 mM, asparagine at 100 mg/liter, L-arginine and cysteine at 10 mg/liter, and L-tyrosine at 100 mg/liter. Tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used in an agar medium. Supplementation of the culture medium with adenine sulfate can stimulate cell growth and greatly enhance shoot formation.

Undefined Organic Supplements

Addition of a wide variety of organic extracts to culture media often results in favorable tissue responses. Supplements that have been tested include protein hydrolysates, coconut milk, yeast extracts, malt extracts, ground banana, orange juice, and tomato juice. However, undefined organic supplements should only be used as a last resort, and only coconut milk and protein hydrolysates are used to any extent today. Protein (casein) hydrolysates are generally added to culture media at a concentration of 0.05-0.1%, while coconut milk is commonly used at 5-20% (v/v).

The addition of activated charcoal (AC) to culture media may have a beneficial effect. The effect of AC is generally attributed to one of three factors: absorption of inhibitory compounds, absorption of growth regulators from the culture medium, or darkening of the medium. The inhibition of growth in the presence of AC is generally attributed to the absorption of phytohormones to AC. 1-Napthaleneacetic acid (NAA), kinetin, 6-benzylaminopurine (BA), indole-3-acetic acid (IAA), and 6-γ-γ-dimethylallylaminopurine (2iP) all bind to AC, with the latter two growth regulators binding quite rapidly. The stimulation of cell growth by AC is generally attributed to its ability to bind to toxic phenolic compounds produced during culture. Activated

charcoal is generally acid-washed prior to addition to the culture medium at a concentration of 0.5-3.0 percent.

Solidifying Agents or Support Systems

Agar is the most commonly used gelling agent for preparing semisolid and solid plant tissue culture media. Agar has several advantages over other gelling agents. First, when agar is mixed with water, it forms a gel that melts at approximately 60°-100° C and solidifies at approximately 45°C; thus, agar gels are stable at all feasible incubation temperatures. Additionally, agar gels do not react with media constituents and are not digested by plant enzymes. The firmness of an agar gel is controlled by the concentration and brand of agar used in the culture medium and the pH of the medium. The agar concentrations commonly used in plant cell culture media range between 0.5 and 1.0%; these concentrations give a firm gel at the pH's typical of plant cell culture media.

Another gelling agent commonly used for commercial as well as research purposes is Gelrite. This product is synthetic and should be used at 1.25-2.5 g/liter, resulting in a clear gel which aids in detecting contamination.

Alternative methods of support have included use of perforated cellophane, filter paper bridges, filter paper wicks, polyurethane foam, and polyester fleece. Whether explants grow best on agar or on other supporting agents varies from one species of plant to the next.

Growth Regulators

Four broad classes of growth regulators are important in plant tissue culture; the auxins, cytokinins, gibberellins, and abscisic acid. Skoog and Miller were the first to report that the ration of auxin to cytokinin determined the type and extent of organogenesis in plant cell cultures. Both an auxin and cytokinin are usually added to culture media in order to obtain morphogenesis, although the ratio of hormones required for root and shoot induction is not universally the same. Considerable variability exists among genera, species, and even cultivars in the type and amount of auxin and cytokinin required for induction of morphogenesis.

The auxins commonly used in plant tissue culture media are 1H-indole-3-acetic acid (IAA), 1H-indole-3-butyric acid (IBA), (2,4-dichlorophenoxy) acetic acid (2,4-D), and 1-napthaleneacetic acid (NAA). The only naturally occurring auxin found in plant tissues is IAA. Other synthetic auxins that have been used in plant cell culture include 4-chlorophenoxyacetic acid or p-chlorophenoxyacetic acid (4-CPA, PCPA), (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), 3,6-dichloro-2-methoxybenzoic acid (Dicamba), and 4-amino-3,5,6-trichloropicolinic acid (Picloram).

The various auxins differ in their physiological activity and in the extent to which they move through tissue, are bound to the cells, or metabolized. Naturally occurring IAA has been shown to have less physiological activity than synthetic auxins. Based on stem curvature assays, 2,4-D has eight to twelve times the activity, 2,4,5-T has four times the activity, PCPA and Picloram

have two to four times the activity, and NAA has two times the activity of IAA. Although 2,4-D, 2,4,5-T, PCPA, and Picloram are often used to induce rapid cell proliferation, exposure to high levels or prolonged exposure to these auxins, particularly 2,4-D, results in suppressed morphogeneic activity. Auxins are generally included in a culture medium to stimulate callus production and cell growth, to initiate shoots, particularly roots, and to induce somatic embryogenesis and stimulate growth from shoot apices and shoot tip cultures.

The cytokinins commonly used in the culture media include 6-benzylaminopurine or 6-benzyladenine (BAP, BA), 6-γ-γ-dimethylaminopurine (2iP), N-(2-furanylmethyl)-1H-puring-6-amine (kinetin), and 6-(4-hydroxy-3-mehty-trans-2-butenylamino)purine (zeatin). Zeatin and 2iP are considered to be naturally occurring cytokinins, while BA and kinetin are synthetically derived cytokinins. Adenine, another naturally occurring compound, has a base structure similar to that of the cytokinins and has shown cytokinin-like activity in some cases. Many plant tissues have an absolute requirement for a specific cytokinin for morphogenesis to occur, whereas some tissue are considered to be cytokinin independent, i.e., no cytokinin or a specific cytokinin may be required for organogenesis.

The cytokinins are generally added to a culture medium to stimulate cell division, to induce shoot formation and axillary shoot proliferation, and to inhibit root formation. The type of morphogenesis that occurs in a plant tissue culture largely depends upon the ratio and concentrations of auxins and cytokinins present in the medium. Root initiation of plantlets, embryogenesis, and callus initiation all generally occur when the ration of auxin to cytokinin is high, whereas adventitious and axillary shoot proliferation occur when the ration is low. The concentrations of auxins and cytokinins are equally as important as their ratio.

Gibberellins (GA_3) and abscisic acid (ABA) are two other growth regulators occasionally used in culture media. Plant tissue cultures can usually be induced to grow without either GA_3 or ABA, although, certain species may require these hormones for enhanced growth. Generally, GA_3 is added to culture media to promote the growth of low-density cell cultures, to enhance callus growth, and to elongate dwarfed or stunted plantlets. Abscisic acid is generally added to culture media to either inhibit or stimulate callus growth (depending upon the species), to enhance, inhibit, or stimulate callus growth (depending upon the species), to enhance shoot or bud proliferation, and to inhibit latter stages of embryo development.

Stock Solutions

The use of stock solutions reduces the number of repetitive operations involved in media preparation and, hence, the chance of human or experimental error. Moreover direct weighing of media components (e.g., micronutrients and hormones) that are required only in milligram or microgram quantities in the final formulation cannot be performed with sufficient accuracy for tissue culture work. For these components, preparation of concentrated stock solutions and subsequent dilution into the final media is standard procedure. In addition, concentrated

solutions of some materials are more stable and can be stored for longer periods than more dilute solutions.

To prepare a stock solution, weigh out the required amount of the compound and place it in a clean flask. It is common practice to make a stock solution 10x or 100x, depending upon the solubility of the compound. Once the chemical is in the flask, dissolved it in a small amount of water, ethyl alcohol, 1 N NaOH, or 1 N HCL. Next, slowly add double-distilled water to the flask, while agitating. Continue this until the proper volume is reached. Label the flask with the name of the solution, preparation and expiration dates, and the name of the person who prepared the solution. Certain items, e.g., IAA, must be prepared and stored in amber bottles to prevent photodecomposition. The volumes of stock solutions prepared at various concentrations that must be used to achieve various final concentrations are presented in tabular form in the Plant Growth Regulator Section.

Macronutrients

Stock solutions of macronutrients can be prepared at 10 times the concentration of the final medium. A separate stock solution for calcium salts may be required to prevent precipitation. Stock solution of macronutrients can be stored safely for several weeks in a refrigerator at 2°-4°C.

Micronutrients

Micronutrient stock solutions are generally made up at 100 times their final strength. It is recommended that micronutrient stocks be stored in either a refrigerator or freezer until needed. Micronutrient stock solutions could be stored in a refrigerator for up to 1 year without appreciable deterioration. Iron stock solutions should be prepared and stored separately from other micronutrients in an amber storage bottle. Formulations for preparing stock solutions of iron are presented later.

Vitamins

Vitamins are prepared as 100X or 1000X stock solutions and stored in a freezer (-20°C) until used. Vitamin stock solutions should be made up each time media is prepared if a refrigerator or freezer is not available. Vitamin stock solutions can be stored safely in a refrigerator for 2-3 months but should be discarded after that time.

Growth Regulators

The auxins NAA and 2,4-D are considered to be stable and can be stored at 4°C for several months; IAA should be stored at -20°C. Auxin stock solutions are generally prepared at 100-1000 times the final desired concentrations. Solution of NAA and 2,4-D can be stored for several months in a refrigerator or indefinitely at -20°C. Generally IAA and 2,4-D are dissolved in a small volume of 95% ethyl alcohol or KOH and then brought to volume with double-distilled

water; NAA can be dissolved in a small amount of 1 N NaOH or KOH, which also can be used to dissolve 2,4-D and IAA.

The cytokinins are considered to be stable and can be stored at -20°C. Cytokinin stock solutions are generally prepared at 100X to 1000X concentrations. Many of the cytokinins are difficult to dissolve, and a few drops of either 1 N HCL, 1 N NaOH, in KOH or DMSO, are required to bring them into solution.

Storage of Stock Solutions

Storage conditions for most stock solutions have already been pointed out; however, some additional points can be made. For convenience, many labs prepare stock solutions and then divide them into aliquots sufficient to prepare from 1 to 10 liter of medium; these aliquots are stored in small vials or plastic bags in a freezer. This procedure removes the inconvenience of having to un-thaw a large volume of frozen stock each time medium is prepared. Some have found that heating in a microwave oven is a satisfactory and quick method of thawing concentrated medium.

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