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PLANT TISSUE CULTURE MEDIA[1]

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INTRODUCTION

Plant tissue culture techniques have become vitally important for pursuing a wide range of fundamental and applied problems in research and development. The techniques encompass a variety of procedures used for specific purposes. The growing of masses of unorganized cells (callus) on agar or in liquid suspension is widely employed in biochemical and growth studies (1–5). The culture of segments of stems, roots, leaves or of callus provides systems to study differentiation, morphogenesis and plant regeneration (6, 7). Shoot apex culture methods leading to plant regeneration have been adopted for plant propagation and production of virus-free stock (8). The culture of anthers and pollen provides new approaches to haploid plant formation (9). Recently the technology has been extended to include the isolation and culture of plant protoplasts which are employed in fusion and somatic cell hybridization (10–13).

The development of the various types of tissue culture has been based on empirical approaches, and some of the observations recorded in the literature may not be typical for plant cells. Differences in medium, environment, age, cell origin, and growth rates may explain the behavior of a particular line and need not represent a general characteristic of plant cells in culture. More uniformity in conditions of culture would assist in making data and observations more comparable.

There are two factors in particular which govern the success of cell cultures: the explant origin and the culture medium. In general the tissue from shoots or roots of seedlings, from nondormant buds, or from shoot tips offers the best materials for producing a friable callus. In cereals and grasses the mesocotyl or root tissues may be the most suitable for callus and eventually for suspension cultures.

The composition of the medium is a determining factor for growth. In many of the earlier plant tissue culture experiments, the media designed by White (14) were employed. These media contained the nutrients normally required by plant cells and are widely used especially for root cultures. However, the amounts particularly of nitrogen and potassium have since been found to be inadequate to sustain maximum growth of callus and cell suspension cultures (15, 16). The need for richer mineral salt mixtures was compensated for by adding yeast extracts, protein hydrolysates, amino acids, coconut milk or other organic supplements (17, 18).

Other media have now been designed and are adequate for cell culture without addition of complex substances under most experimental conditions. The objective of this report is to provide some guidelines and to present information and suggestions on various aspects of plant tissue culture media.

Comments on media. The methodology of plant tissue culture has advanced to the stage where tissues from virtually any plant species can be cultured successfully. The technique is not without problems, but a vast amount of knowledge and experience has been recorded in numerous publications. Several excellent reviews summarize a variety of observations on cell nutrition, differentiation, growth, biochemistry, physiology and differentiation (1, 2, 5–7, 19–22).

Successful plant tissue culture depends on the choice of nutrient medium. The cells of most plant species can be grown on completely defined media. The wide use of the Murashige-Skoog (MS) (15) medium or modifications thereof is an appropriate illustration. Such media con-

[1] The Plant Division of the Tissue Culture Association appointed a Committee on Tissue Culture Media Standards. The Committee was asked to investigate and formulate suggestions which would be helpful to those who wish to employ cell culture techniques and to companies planning to market media.

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TABLE 1

MINERAL SALT MEDIA FOR PLANT TISSUE AND CELL CULTURE

Salts	MS (15) 1		ER (28) 2		B5 (29) 3		SH (26) 4		HE (25) 5	
Macronutrients	mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM
NH_4NO_3	1650	20.6	1200	15.0						
KNO_3	1900	18.8	1900	18.8	2500	25	2500	25		
$CaCl_2 \cdot 2H_2O$	440	3.0	440	3.0	150	1.0	200	1.4	75	0.5
$MgSO_4 \cdot 7H_2O$	370	1.5	370	1.5	250	1.0	400	1.6	250	1.0
KH_2PO_4	170	1.25	340	2.5						
$(NH_4)_2SO_4$					134	1.0				
$NH_4H_2PO_4$							300	2.6		
$NaNO_3$									600	7.0
$NaH_2PO_4 \cdot H_2O$					150	1.1			125	0.9
KCl									750	10.0
Micronutrients	mg/L	μM	mg/L	μM	mg/L	μM	mg/L	μM	mg/L	μM
KI	0.83	5.0			0.75	4.5	1.0	6.0	0.01	0.06
H_3BO_3	6.2	100	0.63	10	3.0	50	5.0	80	1.0	16.2
$MnSO_4 \cdot 4H_2O$	22.3	100	2.23	10					0.1	0.5
$MnSO_4 \cdot H_2O$					10	60	10	60		
$ZnSO_4 \cdot 7H_2O$	8.6	30			2.0	7.0	1.0	3.5	1.0	3.5
Zn. versenate			15	37						
$Na_2MoO_4 \cdot 2H_2O$	0.25	1.0	0.025	0.1	0.25	1.0	0.1	0.4		
$CuSO_4 \cdot 5H_2O$	0.025	0.1	0.0025	0.01	0.025	0.1	0.2	0.8	0.03	0.12
$CoCl_2 \cdot 6H_2O$	0.025	0.1	0.0025	0.01	0.025	0.1	0.1	0.4		
$ALCl_3$									0.03	0.22
$NiCl_2 \cdot 6H_2O$									0.03	0.13
$FeCl_3 \cdot 6H_2O$									1.0	3.7
$Na_2 \cdot EDTA$	37.3	100	37.3	100	37.3	100	20	55		
$FeSO_4 \cdot 7H_2O$	27.8	100	27.8	100	27.8	100	15	55		
Sucrose (g)	30		40		20		30			
pH	5.7		5.8		5.5		5.8			

sist of mineral salts, a carbon source (generally sucrose), vitamins and growth regulators. The MS medium designed for tobacco cells is being used very extensively and with obvious success for culturing callus on agar, for cell suspension cultures in liquid media, and for morphogenetic studies.

The medium contains the correct amounts and proportion of inorganic nutrients to satisfy the nutritional as well as the physiological needs of many plant cells in culture. The cells, therefore, generally have no essential requirement for organic supplements such as amino acids, casein hydrolysates, yeast extract or coconut milk. A distinguishing feature of the MS medium is its high content of nitrate, potassium, and ammonium relative to other nutrient media. The salt composition of the Linsmaier-Skoog (23) medium is identical to that of MS. There are numerous other media in use. The majority are modifications of earlier media (4, 14, 20, 24–27).

By various criteria, many media were reported adequate but optimum cell growth was often dependent upon the addition of an organic supplement. The positive response to these additions indicated a requirement by the cells for nitrogen and other nutrients. In many cases these requirements can be provided by increasing concentrations of inorganic salts, particularly nitrogen, as well as sucrose and vitamins (6, 28–30).

Selected media. A number of basal salt mixtures have been tested and appear to be adequate to satisfy the needs of a variety of plant cells in culture. The five media listed in Table 1 contain relatively high concentrations of potassium

and nitrate (Table 3). A medium in this discussion is identified only by its mineral salt composition. Vitamins, hormones and other supplements are optional and the requirements for these vary with the plant species and the intended use of the tissue culture (Table 2).

The MS and Eriksson (ER) (28) media are very similar, but the ER contains twice the amount of phosphate and much lower concentrations of micronutrients than the MS medium. The latter may be an advantage for some cells. The B5 medium has been tested with a wide range of callus and suspension cultures (29, 31). Callus and cell cultures of some species prefer MS while others grow better in the B5 or ER media. The B5 medium contains relatively low amounts of ammonium, a nutrient that may repress growth in batch cultures (32).

The Schenk and Hildebrandt (SH) (26) medium resembles B5. The amounts of mineral salts are slightly higher, and ammonium and phosphate are supplied as one compound.

The Heller (HE) (25) medium has been widely used in Europe. The salt content is relatively low and it contains some unnecessary compounds. Potassium and nitrate are added through separate compounds. The nickel and aluminum salts likely are unnecessary. A molybdenum salt is not included but should be present in any plant nutrient medium.

The selection of the media in Table 1 should not be interpreted as implying that other media are unsuitable. These media are suggested as the starting point. The specific requirement of hormones and organic supplements for individual cell lines can then be determined. For a more complete discussion of nutrition, several reviews are available (2, 16, 20, 21).

TABLE 2

SUGGESTED AMOUNTS AND KINDS OF VITAMINS, HORMONES AND SUPPLEMENTS USED WITH THE MINERAL SALT MEDIA

Compound	MS (15) mg/L	ER (28) mg/L	B5 (29) mg/L	SH (26) mg/L
Inositol	100		100	1000
Nicotinic Acid	0.5	0.5	1.0	5.0
Pyridoxine·HCl	0.5	0.5	1.0	0.5
Thiamine·HCl	0.1	0.5	10.0	5.0
Glycine	2.0	2.0		
IAA[a]	1–30			
NAA[b]		1.0		
Kinetin	0.04–10	0.02	0.1	
2,4-D[c]			0.1–1.0	0.5
p-CPA[d]				2.0

[a] Indoleacetic acid.
[b] Naphthaleneacetic acid.
[c] 2,4-Dichlorophenoxyacetic acid.
[d] p-chlorophenoxyacetic acid.

TABLE 3

CONCENTRATIONS OF INORGANIC NUTRIENTS IN FIVE MEDIA FOR PLANT TISSUE CULTURE

Macro-Nutrients (mM)	MS	ER	B5	SH	HE
K	20	21.3	25	25	10
$N(NO_3)$	40	33.8	25	25	7.0
$N(NH_4)$	20	15.0	2.0	2.6	
Mg	1.5	1.5	1.0	1.6	1.0
P	1.25	2.5	1.1	2.6	0.9
Ca	3.0	3.0	1.0	1.4	0.5
S	1.5	1.5	1.0	1.6	1.0
Na	—	—	1.1	—	7.9
Cl	6.0	6.0	2.0	2.8	.11
Micro-Nutrients (μM)					
I	5.0		4.5	6.0	0.06
B	100	10	50	80	16
Mn	100	10	60	60	0.5
Zn	30	37	7.0	3.5	3.5
Mo	1.0	0.1	1.0	0.4	
Cu	0.1	0.01	0.15	0.8	0.12
Co	0.1	0.01	0.1	0.4	
Al					0.22
Ni					0.13
Fe	100	100	100	55	3.7

NUTRIENT MEDIA COMPOSITION

The nutrient medium for most plant tissue cultures is comprised of five groups of ingredients: inorganic nutrients, carbon source, vitamins, growth regulators and organic supplements (Tables 1 and 2).

Inorganic nutrients. The inorganic nutrients consist of mineral salts which supply the requirements for macro- and micronutrients. For most purposes the nutrient medium should contain at least 25 mM each of nitrate and potassium. For regular callus and cell suspension culture, the concentrations of nitrate plus ammonium possibly should be increased to 60 mM. Ammonium may be essential for some cultures, although amounts in excess of 8 mM can be deleterious (32). However, it should always be remembered that transfer of cells to a new medium can result in slower growth rates for

one or two subcultures until the cells become adapted. An adequate assessment of the suitability of a medium can be made only after 2 to 3 subcultures. A concentration of 1–3 mM of calcium, sulfate, and magnesium generally is adequate. Any need for sodium and chloride can be provided by calcium salts, phosphates or micronutrients. The required micronutrients include I, B, Mn, Zn, Mo, Cu, Co and Fe, although iodide may not be essential (28).

Carbon and energy source. Sucrose at 2–4% is preferred by most cells. It can be replaced by glucose. Fructose also is utilized but other sugars are poor carbohydrate sources. m-Inositol may not be essential, but is routinely added since it has been shown to enhance callus growth.

Vitamins. Of the vitamins, only thiamine may be required. Nicotinic acid and pyridoxine may enhance growth.

Amino acids and organic supplements. Amino acids should not be necessary. If inorganic nutrients do not seem to be adequate, the best approach is to add 0.05–0.1% casein hydrolysate (enzymatic digest) or Casamino acids to establish the cell culture. These materials later may be replaced by L-glutamine (2–10 mM), or the organic nitrogen can be omitted completely (29). The cells will require at least a month and several transfers to adjust to the gradual elimination of organic nitrogen. The advantages of using only inorganic nitrogen are self-evident.

Hormones. The hormone supplement falls into two categories. Callus production in most cases is successful by using only 2,4-D (10^{-6}–10^{-5} M). Addition of a cytokinin (kinetin, zeatin, or benzyladenine at 10^{-6}–10^{-7} M) can be beneficial, but 2,4-D alone is adequate in most cases. If the tissue culture is for morphogenesis, NAA and benzyladenine, zeatin or isopentenyl adenine in combination would possibly be better. While 2,4-D induces cell division, the compound tends to repress morphogenesis. NAA can be replaced by IAA but the latter should be preferably filter-sterilized. IAA also may be degraded by enzymes from the cells. A compound which effectively induces callus formation in cereals is 2,4-5-trichlorophenoxyacetic acid (2,4-5-T).

Media Preparation

It is not the intention to provide a detailed outline for the preparation of media. The suggestions may provide an adequate guide. Further information is available in some of the references (1, 31).

Water and chemicals. Most water sources may require being demineralized, glass-distilled or both. The chemicals should be of the highest grade available. Growth regulators may require recrystallization before use. Protein hydrolysates are available as acid or enzyme hydrolysates. The latter may be preferable, since the amino acids are preserved in their natural form.

Stock solutions. A convenient approach to media preparation for most operations is to have a series of stock solutions (31). Salts of major nutrients (NO_3, NH_4, P, Mg, Ca, and SO_4) can be made up in 10× final concentration. Minor salts as well as vitamins can be prepared in 1000× final concentration and stored in a freezer. It may be advantageous to have separate stock solutions of the calcium salt and potassium iodide (31). Naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid and similar compounds are dissolved in a small amount of ethanol and made to volume with water to yield a concentration of 2–3 mM (31). The cytokinins dissolve in a small amount of 0.5 N HCl with slight heat; and then can be made to volume with water (1–2 mM). These compounds also may be dissolved in dimethylsulfoxide (34).

Autoclaving and storage. It may be convenient to prepare and store complete mineral salt-vitamin media at 10× final concentration. Concentrated media can be stored frozen in plastic bags (Nasco Whirl-Pak bags, supplied by Fisher Scientific Co., Ltd., Don Mills, Canada). Each bag contains the correct amount to make 500 or 1000 ml of medium, depending upon the size of the operation. The sucrose, hormones and other ingredients can then be added when the desired complete medium is prepared. The pH is adjusted to a specified value between 5.5 and 6.2, and the medium autoclaved at 120°C for 15 to 20 min. Most components in the media tolerate autoclaving, although some caution should be exercised (33). Exceptions may be indoleacetic acid and glutamine. There also may be some loss of thiamine. The media can be stored at room temperature, but 10°C is preferable.

Tissue Culture—Nutrition and Growth Studies

New advances in the understanding of plant cell nutrition and growth requirements neces-

sitate a systematic and comprehensive approach. The specific requirement of particular nutrient compounds as well as their combined effects must be clearly ascertained (30, 36). Investigations of this type would result in the development of simple (minimal) media adequate for initiating callus and establishing suspension cultures of different plant genera. In any investigation we suggest that the Murashige and Skoog mineral salt mixture be used as control (15). [NOTE: the Murashige and Skoog medium is sometimes referred to as Linsmaier and Skoog (23).] The recommended amounts of vitamins, sucrose and inositol in the MS medium also is suggested as a starting point. For callus and suspension cultures, 2,4 dichlorophenoxyacetic acid (2,4-D) possibly with some cytokinin is the most appropriate hormone combination for general purposes. The need for organic nitrogen such as casein hydrolysate or Casamino acids should be tested with a range of concentrations. Organic nitrogen sources rarely are essential, but may enhance the growth rate especially during callus initiation.

Any experiments on vitamin requirements should include a control consisting of thiamine alone, since this vitamin is known to be required by some plant cells.

The efficiency of a medium should be adequately tested to demonstrate reproducibility and also should be compared with existing media. A minimum of three subcultures with quantitative growth measurements against a standard medium is necessary to determine the merit of a "new" medium.

DILEMMAS RECONSIDERED

Before a plant tissue culture system can be applied for a particular purpose, the problems of nutrition and environment must be resolved. In some areas, such as the application of morphogenesis in plant propagation, the nutritional and environmental options are defined fairly well but precise conditions, particularly with respect to hormones and explant origin, must be ascertained for each plant species (6, 16, 35).

Frequently much time has been spent on developing procedures for growing callus of a given plant, but with limited success. It should be clearly understood that getting fast-growing cultures with a cell generation (doubling) time of, for example, 24 to 36 hr involves not only suitable media and growing conditions, but also careful timing for subculture. In addition, a process of cell selection is taking place. A failure to recognize these points easily can lead to a lengthy search for a "super" medium. The results have been an array of "new" media, which is an unfortunate development. It may be appropriate to draw attention to this point, since the continued appearance of new media, which often increase in complexity, has introduced an unnecessary variable in comparative plant cell research. A strict comparison of physiological and biochemical data based on cell culture experiments is frequently invalid because the cells even from the same plant species may have been grown under widely different milieus.

Another related point concerns the interpretation of plant cell behavior in tissue culture. Misleading and contradictory conclusions may appear in reviews because of inadequate consideration to culture practices. Data obtained with cells from slow growing callus probably are not comparable in biochemistry, structure or genetic stability to rapidly growing cells from suspension cultures. Similarly, cells grown in complex media with extracts of unknown composition should not be expected to be identical to those cultured on simple, defined media.

It is also critical that adequate control experiments are included to establish the validity of a particular behavior. A case in point is the original proposal that coconut milk and several hormones were required for obtaining embryos from carrot cell cultures. Simple control experiments later showed that these substances were unnecessary.

The interest in plant tissue culture methods is growing very rapidly because the procedures for some purposes are far superior to using whole plants or organs. We hope the information and discussions in the present report may be of assistance in the development of satisfactory plant tissue culture systems, and that it may stimulate efforts to standardize appropriate aspects of the technology and expand its use in research and industry.

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