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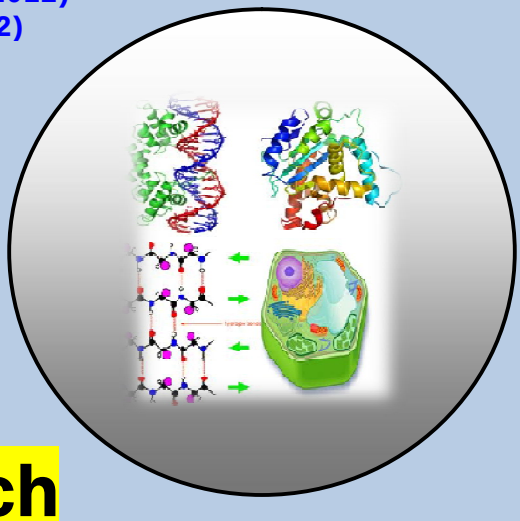

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REVIEW ARTICLE

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Study of Plant Tissue Culture Technology

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ABSTRACT

Development of efficient plant regeneration protocol from either single cell or organized tissue is a important of many commercially important crops like wheat. Because wheat, among the food crops, a common source of energy and proteins for the world population. It is well known that the frequencies of callus induction and plant regeneration in wheat tissue culture completely depend on medium composition and explant sources. For explant source; immature embryos have some disadvantages as explants such as their availability is limited in a period of growing time. In wheat media composition, amino acids are important supplement and widely used in tissue culture systems. Amino acids prove an organic form of nitrogen (reduced state), which are readily metabolized by plant cells, several species to enhance somatic embryogenesis and regeneration. Therefore, the additional amino acids appear to have the potential to enhance to some extent the roles of suitable nitrogen source. These results of thid paper indicate that plant genotype and medium had significant effects on regeneration capacity of cultivars. In this study, genotype and medium had significant effects on callus induction and regeneration capacity of plants. In fin we showed that 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

Key words: Plant Regeneration Protocol, Tissue Culture and Media Composition.

INTRODUCTION

Tissue culture is a method of plant propagation done in a laboratory. There are many methods of plant propagation including using seeds, cuttings and division. Plant tissue culture involves growing plants in an artificial medium under sterile conditions in a jar, flask or test tube. In theory, this process can go on indefinitely, producing many plants over time. Some plant tissue cultures are started from very small sections of plant tissue. The plants produced this way are exact copies, or clones, of one another.

While cloning is important for producing multiples (hundreds, thousands, even millions) of an individual, tissue culture offers other ways to propagate plants that are not "clones." Some plant tissue cultures are started from seeds. When seeds are used for tissue culture, each new plant is genetically different from its parent plant. Many of the orchids grown in the lab are started from seed. Tissue culture is commonly called "cloning" and "micropropagation."

Why Tissue Culture?

The plant tissue culture lab allows Atlanta Botanical Garden staff to research new methods of reproducing rare plants and using this information to further plant conservation efforts. Many plant species are threatened because people illegally take plants out of the wild to sell. Commercial laboratories can use the results of our research to produce thousands of plants for retail, thereby lightening the pressure on natural, wild plant populations. With tissue culture, a large number of plants can be produced in a short amount of time compared with traditional methods of planting seeds, cuttings or divisions. Many of everyday plants ware produced from tissue culture, especially tropical houseplants. Tissue cultures started from seeds are sometimes easier and faster than conventional cloning and offer the additional benefit of increased genetic diversity (Fennell et al. 1996) .

How to Do Tissue Culture

Flasks of nutrient medium are first sterilized under high heat and pressure to kill any fungal or bacterial contaminants. The medium in the containers is made of sugars, inorganic salts, plant hormones and a gelling agent (like agar). The plant material used to start a culture is called an explant and must be disinfected before being placed on a sterile medium. To maintain the sterility of the plant cultures, the containers are opened only in a laminar flow hood, which contains high-efficiency particle absorption (HEPA) filter. This creates an environment free of airborne organisms that could contaminate the tissue culture. The container of nutrient medium is first sterilized under high heat and pressure to kill any fungal or bacterial contaminants. After the culture has been started, it is stored under lights and carefully monitored for contamination. After a period of days or up to several months, the explants will show signs of growth. Leaves, roots and other organs will begin to develop. In time, under the proper conditions, the cultures will grow and multiply. They can be divided or subcultured into new flasks as the nutrients in the medium eventually run out. This process can go on indefinitely, producing many plants over time. Plants grown in the laboratory can be transferred to soil conditions carefully by selecting the correct soil mix and gradually lowering the humidity level.

How Does Tissue Culture Benefit Conservation?

The Atlanta Botanical Garden is working to find new ways of propagating native plants like native orchids and trillium. As our research develops, we share our results with the nursery industry. When nurseries are able to get large numbers of native plants from commercial tissue culture labs, they are less likely to sell plants that have been taken out of the wild.

Some of the rare plants that are grown in the tissue culture lab will be replanted in their native habitats. Others are made available to institutions like nurseries, universities and other botanical gardens for research. Plants grown in this laboratory will be used in endangered species recovery work. One of the main focuses in this lab is the germination, growth and multiplication of native terrestrial orchid species such as the monkey face orchid (Platanthera integrilabia) and the Kentucky ladyslipper orchid (Cypripedium kentuckiense). Tropical orchid species are also grown in the lab for use in Atlanta Botanical Garden displays, for exchange with other institutions, and for recovery work in countries such as Ecuador. Wheat plantlets regenerated from tissue culture and genetic management are normally very weak and need to be strengthened and propagated so that they can be transplanted. If the regenerants do not survive after transplant, the genetic management means uselessness in wheat improvement. Zhu et al. reported that using N6 base medium supplemented with IAA 10 mg/L and sugar 80 g/L to propagate unpollinated-ovary-derived haploid plantlets did not induce clonal variations (Chu et. al., 1975). Li et al. reported that for Triticum aestivum x Haynaldia villosa hybrid plantlets, the above medium was very efficient for rooting and strengthening. When we used this medium to propagate the anther-derived haploid plantlets of wheat for 3-4 subcultures, the growth was slowed down and the rooting was inhibited. Medium is a key factor in plantlets propagation, strengthening and rooting, while research concerning the medium for wheat plantlet propagation is very poor.

N6 medium

N6 medium was originally designed for another culture of rice, and it does not contain Cu and Mo. If the plantlets propagated on the medium for a long time, Cu and Mo deficiency symptoms appeare. Increasing CaCl2_H2O concentration did not relieve the symptoms, but caused Cl- poisoning showing withered leaf tip. We can use 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mM Ca(NO3)2_4H2O instead of CaCl2_H2O, and added 40 mg/L (approx. 0.5 mM) of KCl to supply Cl, reduced KNO3 content to adjust the nitrogen composition to the level of N6 medium to test the effects of Ca(NO3)2 on relieving the calcium deficiency symptoms. The optimal rooting was achieved with 2.0 and 3.0 mM Ca (NO3)2, So 2.5 mM (590 mg/L) of Ca(NO3)2_4H2O was chosen as the best concentration table 1, Components of M10 medium for Triticum aestivum.

NUTRIENT MEDIA

The intact plants can make their own food but the in vitro culture of plant parts or cells requires a variety of nutrients and suitable physical conditions for their growth. The composition of plant tissue culture medium depends upon the type of plant tissues or cells that are used for culture. No single medium can be used for all types of plants and organs, so the composition of the culture medium for each plant material has to be worked out.

A typical nutrient medium consists of the following components:

a) Inorganic nutrients (both micro- and macro-elements - C, H, O, N, P, S, Ca, K, Mg, Fe, Mn, Cu, Zn, B, Mb), the six elements namely nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are the essential macronutrients for tissue culture. The ideal concentration of nitrogen, and potassium is around 25 mmol l-1 while for calcium, phosphorus, sulfur and magnesium, it is in the range of 1-3 mmol l-1.

Among the micronutrients, iron requirement is very critical. Chelated forma of iron and copper are commonly used in culture media.

b) A carbon source and energy source (usually sucrose) Plant cells and tissues in the culture mediumare heterotrophic and therefore depend on the external carbon for energy. Among the various energy sources, sucrose is the most preferred. During the sterilization of the medium, sucrose gets hydrolysed to glucose and fructose and the plant cells utilize first the glucose and then the fructose. The other carbohydrates such as lactose, maltose, galactose etc have been used in culture media but with limited success.

c) Organic supplements vitamins (e.g. nicotinic acid, thiamine, pyridoxine and myo-inositol), amino acids (e.g. arginine) The plant cells in culture are able to synthesize vitamins just like natural plants, but in suboptimal quantities which does not support proper growth of cells in culture. Therefore the medium is supplemented with vitamins to achieve good growth of cells. Similarly amino acids are added to the cell cultures to stimulate the cell growth and estabilish the cell lines. Organic acids especially the intermediates of krebs cycle e.g. citrate, malate, succinate, pyruvate also enhances the growth of plant cells. Sometimes antibiotics (e.g. streptomycin, kanamycin) are also added to the medium to prevent the growth of the microorganisms.

d) Growth regulators (e.g. auxins, cytokinins and gibberellins Plant hormones play an important role in growth and differentiation of cultured cells and tissues. The growth hormones included in culture media involve: auxins, cytokinins, and gibberellins. The auxins facilitate the cell division and root differentiation. The cytokinins induce cell division and differentiation and the gibberellins is mainly used to induce plantlet formation from adventive embryos formed in culture.

Auxins induce cell division, cell elongation, and formation of callus in cultures. 2,4-dichlorophenoxy acetic acid is one of the most commonly added auxins in plant cell cultures. Cytokinins, promotes RNA synthesis and stimulate protein and enzyme activities in tissues. Kinetin and benzyl-aminopurine are the most frequently used cytokinins in plant cell cultures.

The ratio of auxins and cytokinins play an important role in the morphogenesis of culture systems. When the ratio of auxins to cytokinins is high, embryogenesis, callus initiation, and root initiation occur. For axillary and shoot proliferation, the ratio of auxins to cytokinins is kept low.

Among the gibberellins, gibberellin A3 (GA3) is the most commonly used for tissue culture. GA3 enhances callus growth and induces dwarf plantlets to elongate.

e) Solidifying agents like agar. Generally a gelling agent agar (a polysaccharide obtained from red algae, Gelidium amansil) is added to the liquid medium for its solidification. . The agar obtained from seaweeds provides solid surface for the growth of cells because in the liquid medium, the tissue will be submerged and die due to lack of oxygen. Cells are grown in suspension medium without agar but such cultures are aerated regularly either by bubbling sterile air or by gentle agitation. Some other less frequently used solidifying agents are biogel (polyacrlyamide pellets), phytagel, gelrite, and purified agarose Table 2 - Chemical Components of Tissue Cultured Panax ginseng.

f) Other compounds like casein hydrolysate, coconut milk, malt extract, yeast extract, tomato juice, etc. may be added for specific purposes.
g) pH - An optimum pH (usually 5.7) is also very important. At pH higher than 7.0 and lower than 4.5, the plant cells stop growing in cultures.
The most extensively used nutrient medium is MS medium (developed by Murashige and Skoog in 1962).

RESULTS AND DISCUSSION

MAJOR TYPES OF MEDIA

White's medium - is one of the earliest plant tissue culture media
MS medium - formulated by Murashige and Skoog (MS) is most widely used for many types of culture systems
B5 medium - developed by Gamborg for cell suspension and callus cultures and at present its modified form used for protoplast culture
N6 medium - formulated by Chu and used for cereal anther culture
Nitsch's medium developed by Nitsch and Nitsch and used for anther culture

PREPARATION OF MEDIA

The methodology for media preparation involves preparation of stock solutions (in the range of 10 x to 100x concentrations) of highly purified chemicals and demineralized water. The stock solutions are stored in glass or plastic containers and frozen till further requirement. Now a day, plant tissue culture media are commercially prepared, and are available in the market as dry powders. The culture media is usually sterilized in an autoclave at 1210C and 15 psi for 20 minutes. Hormones and other heat sensitive organic compounds is filter sterilized and added to the autoclaved medium.

MAINTENANCE OF ASEPTIC ENVIRONMENT

It is very important to maintain aseptic environment during the in vitro culture of plant cells and tissues. Following are some of the methods adopted for sterilization:
(a) Sterilization of Glassware- The glassware can be sterilized in a hot air oven at 160-1800C for 2-4 hours.
(b) Sterilization of instruments- The metallic instruments are incinerated by dipping them in 75% ethanol followed by flaming and cooling.
(c) Sterilization of nutrient media- The culture media are transferred into glass container, plugged with cotton or sealed with plastic closures and sterilized by autoclaving at 15 psi for 30 min. The autoclaving denatures the vitamins, plant extracts, amino acids and hormones therefore the solution of these compounds are sterilized by using Millipore filter paper with pore size of 0.2 micrometer diameter.
(d) Sterilization of plant materials- The surface of the plant material is made sterile by using disinfectants e.g. sodium hypochlorite, hydrogen peroxide, mercuric chloride, or ethanol. The transfer of sterile plant material on to the nutrient medium is done under the cabinet of laminar airflow.

(e) Sterilization of Culture room and transfer area- the floor and walls of the culture room should be washed with detergent followed by 2% sodium hypochlorite or 95% ethanol. The sterilization can also be done by exposure to UV light. The cabinet of laminar air flow is sterilized by exposing to UV light for 30 min. and 95% ethanol 15 minutes before starting the work (Chu et al. 1975).

Fig 1. Tissue culture from its parent plant.

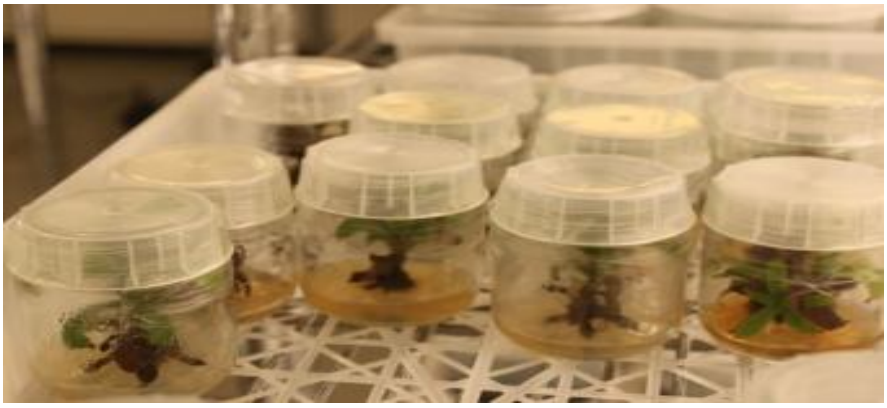

Fig 2. Sample tissue culture methods of plant propagation.

Fig 3. Microscopy study of tissue culture and parent plan.

Table 1. Components of M10 medium for *Triticum aestivum.*

Component	Content (mg/L)	Component	Content (mg/L)
KNO_3	2323	H_3BO_3	3.2
NH_4NO_3	560	KI	0.2
KC1	40	$Na_2MoO_4 \cdot 2H_2O$	0.25
$MgSO_4 \cdot 7H_2O$	246	Cu-EDTA	0.28 (0.8 μM)
KH_2PO_4	204	$CoCl_2 \cdot 6H_2O$	0.025
$Ca(NO_3)_2 \cdot 4H_2O$	590	Thiamine	0.5
$FeSO_4 \cdot 7H_2O$	27.8	IAA	10
Na_2-EDTA$\cdot 2H_2O$	37.3	Sugar	80 x 10^3
$MnSO_4 \cdot 4H_2O$	8.8	Agar	6 x 10^3
$ZnSO_4 \cdot 7H_2O$	3.0	pH	5.8-6.0

Table 2.Chemical Components of Tissue Cultured *Panax ginseng*

Item	Unit	*Panax ginseng* C.A. Meyer (6 years old)			Tissue Cultured *Panax ginseng*
		Planted ginseng A	Planted ginseng B	Planted ginseng C	Dry Powder
Water	%	8.3	9.4	10.4	3.5
Protein	%	13.4	16.7	9.0	15.0
Fat	%	1.0	0.8	0.7	1.3
Fiber	%	6.4	3.6	2.9	9.7
Ash	%	4.1	3.7	2.7	14.1
Sugar	%	66.8	65.8	74.3	56.4
Phosphorus (P)	mg/100g	207	356	280	243
Iron (Fe)	mg/100g	6.74	5.46	2.77	11.9
Calcium (Ca)	mg/100g	545	237	264	378
Sodium (Na)	mg/100g	12.4	62.8	11.0	151
Potassium (K)	mg/100g	1,200	1,240	908	6,790
Magnesium (Mg)	mg/100g	145	121	117	212
Chlorine (Cl)	mg/100g	67.0	79.1	56.7	286
Food fiber	%	21.3	13.1	11.4	27.2
Water-soluble indigestible polysaccharides	%	2.01	2.08	1.91	2.26
Hemicellulose	%	8.84	5.50	5.15	10.4
Cellulose	%	8.98	5.11	3.94	8.76
Lignin	%	1.47	0.39	0.42	5.81
Vitamin B_1 (VB_1)	mg/100g	0.17	0.31	0.27	33.8
Vitamin B_2 (VB_2)	mg/100g	0.18	0.11	0.15	2.40
Vitamin B_6 (VB_6)	mg/100g	2.03	1.36	0.98	4.26
Vitamin B_{12} (VB_{12})	mg/100g	0.15	0.13	0.07	ND
Total vitamin C (VC)	mg/100g	ND	ND	ND	117
Vitamine E (VE)	mg/100g	0.8	0.6	0.4	1.4
α-tocopherol (α)	mg/100g	0.8	0.6	0.4	1.4
β-tocopherol (β)	mg/100g	ND	ND	ND	ND
γ-tocopherol (γ)	mg/100g	ND	ND	ND	ND
δ-tocopherol (δ)	mg/100g	ND	ND	ND	ND
Choline	%	0.08	0.06	0.05	0.13
Niacin	mg/100g	3.90	7.04	5.06	36.4
Folic acid	μg/100g	44	60	30	260
Pantothenic acid	mg/100g	0.86	1.32	0.97	1.83
Biotin	μg/100g	9.0	6.5	4.2	24.0
Inositol	mg/100g	318	354	267	306
Arsenic (as As_2O_3)	ppm	0.5	0.4	ND	ND
Lead (Pb)	ppm	0.09	0.41	ND	ND
Cadmium (Cd)	ppm	0.07	0.05	0.02	ND
Total mercury (Hg)	ppm	ND	ND	ND	ND
Copper (Cu)	ppm	7.00	13.8	8.53	0.55
Zinc (Zn)	ppm	11.6	15.2	10.5	26.3
Manganese (Mn)	ppm	37.0	22.6	15.1	134
Cobalt (Co)	ppm	ND	0.11	ND	0.12
Selenium (Se)	ppm	ND	0.27	ND	ND
Germanium (Ge)	ppm	ND	ND	ND	ND

The prepration Plant materials for Triticum sp.

Callus culture was initiated from both mature seeds of two Triticum durum Desf. "Kunduru-1149" and "Ç-1252" and two Triticum aestium "İkizce-96" and "Bolal" genotypes. Mature seeds immersed in 70% ethanol for 10-15 min and rinsed with sterile distilled water. Then the grains were placed in 30% Clorox solution (1.5% v/v sodium hypochlorite) having a few drops of Tween-80 for 30 min with continuous shaking. After that, grains were rinsed 5- 6 times with sterile distilled water.in order to remove excess of the chemical. After sterilization, seeds were imbibed in sterile water for 90 min at 33°C for easy seperations of embryo from endosperma following the procedure suggested by Özgen et al. Embryos were then isolated aseptically by cutting from the top of the grain with a sharp scalpel blade and placed in MS callus induction medium.

Callus induction and regeneration

Stocks were prepared and stored in refrigerator. Vitamin stock was also prepared in which thiamine, nicotinic acid, pyridoxine and glycine were added. Stocks of 2,4-D and Kinetin were also prepared. These stocks were used for fresh medium preparation. 30 g/L of sucrose as carbon source and 7 g/L of agar as gelling agent were added to the medium and its pH was adjusted at 5.8. The mature embryos were aseptically removed from the imbibed seeds and inoculated in MS medium which containing 1mg/l 2,4-D + 1,5 mg/lKin. Inoculated culture vessels were incubated at 25±1°C in continuous darkness for 3 weeks. Induced calli were transferred to hormone free MS regeneration medium containing of L-Serine, L-Glutamine, L-Alanine and L-Serine (0.5 mM). The cultures were maintained at 25±1°C under a 16/8-h (light/dark) photoperiod (Benson 2000) .

CONCLUSION

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 macro elements and those required in concentrations less than 0.5 mM.l-1.

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