

ARTICLE

Natural substances in tissue culture media of higher plants

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ABSTRACT Plant cell and tissue cultures are characterized by the use of isolated parts of plant obtained from an intact plant body and kept on, or in a suitable nutrient medium. This nutrient medium functions as replacement for the cells, tissue, or conductive elements originally neighbouring the explant. The exact conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. The empirical approach has shown that three factors, namely explant choice, medium composition, and control of the physical environment are important in successful cultures. When the completely defined plant culture media did not give the desired results, employing natural substances have beneficial effects on *in vitro* plant cell and tissue cultures. The composition of different culture media and the effects of natural compounds, including the supernatant and freeze-dried biomass of well-growing algal strains of Mosonmagyaróvár Algal Culture Collection (MACC), are presented in this short review.

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KEY WORDS

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Plant tissue culture can be considered to involve three phases: first, the isolation of plant tissue from its usual environment; second, the use of aseptic techniques to obtain clean material free of the usual bacterial, fungal, viral, and even algal contaminants; and third, the culture and maintenance of this material *in vitro* in a strictly controlled physical and chemical environment (Hall 1999). An extra, fourth phase may also be considered where recovery of whole plants for rooting and transfer to soil is the ultimate goal. The *in vitro* cell and tissue cultures of higher plants are characterized by the use of isolated parts of plants obtained from an intact plant body and kept on, or in a suitable nutrient medium. This nutrient medium functions as replacement for the cells, tissue, or conductive elements originally neighbouring the explant. Such experimental systems are usually maintained under aseptic conditions (Neumann et al. 2009). Cultures of individual or groups of plant cells, and whole organs, contribute to understanding both fundamental and applied science. The culture can be sustained as a mass of undifferentiated cells for an extended period of time, or regenerated into whole plants. The physiological state of the plant does have an influence on its response to attempts to initiate tissue culture. Younger tissue contains a higher proportion of actively dividing cells and is more responsive to a callus initiation programme. The exact conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. The empirical approach that has been extensively used in studies on *in vitro* organo-

genesis has shown that success is largely dependent on three factors: explant choice, medium composition, and control of the physical environment.

An overview of cell and tissue cultures of higher plants is presented in this paper. Also, the composition of different culture media with special regard to natural compounds, including the supernatant – after centrifugation – and freeze-dried biomass of well-growing algal strains of Mosonmagyaróvár Algal Culture Collection (MACC), will be discussed in detail.

Practical aspects of plant cell and tissue cultures

Nowadays the term plant cell and tissue culture has become the general title for a very broad subject. It covers all aspects of the cultivation and maintenance of any plant material (single cell, tissue, organ) *in vitro*. However, more recently, this technology has been increasingly exploited in a more applied context. In terms of practical aspects, basically five areas can be distinguished: callus cultures, cell suspensions, protoplast cultures, anther cultures, and organ or meristem cultures (Neumann et al. 2009).

Callus cultures

Isolated pieces of a selected tissue (explants) are obtained aseptically from a plant organ and cultured on, or in a suitable nutrient medium. Primary callus culture derived from tissues with high contents of parenchyma or meristematic cells. In such explants, mostly only a limited number of cell types occur, and so a higher histological homogeneity exists

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than in the entire organ. However, growth of the explant in the nutrient medium usually results in an unorganized mass or clump of cells – a callus – consisting largely of cells different from those in the original explant.

Cell suspensions

Ideally all cells are isolated in a cell suspension. There is usually a high percentage of cells occurring as multicellular aggregates under practical conditions in these cell populations. A supplement of enzymes is able to break down the middle lamella connecting the cells in such clumps. Often, cell suspensions are produced by mechanical shearing of callus material in a stirred liquid medium. These cell suspensions generally consist of a great variety of cell types, and are less homogenous than callus cultures.

Protoplast cultures

Initially the cell wall of isolated cells is enzymatically removed, and the explants is transformed into a single-cell culture. This method has been used to study processes related to the regeneration of the cell wall, and to better understand its structure. The main aim in using this approach in the past, however, has been interspecies hybridizations. Nowadays, protoplasts are still essential in many protocols of gene technology. Plants can be regenerated through somatic embryogenesis to be used in breeding programs.

Anther or microspore cultures

Culturing anthers, or isolated microspores from anthers under suitable conditions, haploid plants can be obtained through somatic embryogenesis or organogenesis. It is possible to produce dihaploids, and within one year a fertile homozygous dihaploid plant can be produced from a heterozygous mother plant. This method is advantageous for hybrid breeding, by substantially reducing the time required to establish inbred lines. During culturing microspores initially a callus is produced, with separate formation of roots and shoots that subsequently join, and in due time haploid plants can be isolated. Another aim in using anther or microspore cultures is to provoke the expression of recessive genes in haploids to be selected for plant breeding.

Plant propagation, meristem culture, somatic embryogenesis

Mostly isolated primary or secondary shoot meristems (shoot apex, axillary buds) are induced to shoot under aseptic conditions. Generally, this occurs without an interfering callus phase, and after rooting, the plantlets can be isolated and transplanted into soil. Highly valuable single plants can be propagated by using this procedure. This technique has received a broad interest in horticulture for mass propagation of clones for the commercial market, and the production of

virus-free plants.

Culture medium requirement of plant cells and tissues

The underlying principles involved in plant tissue culture are simple. First, it is necessary to isolate a plant part (explant) from the intact plant. Second, it is necessary to provide the plant part with an appropriate environment in which it can express its intrinsic or induced potential. Third, the above mentioned must be carried out aseptically (Biondi and Thorpe 1981). Much of the equipment used in a plant tissue culture laboratory is aimed at careful control of all the components pertaining to the physical and physiological environment of the system (e.g., media components, atmosphere, light and temperature regimes, etc.). The composition of a medium, preferable to a certain plant species, is nearly the main task for the establishment of a successful plant cell and tissue culture technique.

There are three essential sources of nutrition for plants growing in nature. The mineral nutrients are obtained, along with water, from the soil. Atmospheric carbon dioxide is used in the process of photosynthesis to provide carbon as a source of basic energy. The meristematic regions and young organs such as leaves, using fixed carbon and minerals, synthesizes all of the vitamins and various plant growth substances that are critical and essential for normal growth and development (Oziakis-Akins and Vasil 1985). The requirements of plant cells and tissues grown *in vitro* are similar in general to those of intact plants growing in nature. The isolated cells, tissues and organs lack the capacity to synthesize their own supply of carbohydrates, most vitamins, and plant growth regulators. The earliest attempts to culture plant tissues and organs *in vitro* utilized the simple inorganic solutions of Knop (1865) and Hoagland and Snyder (1933). They were used widely for the growth of whole plants. The difficulties encountered in obtaining sustained growth of plant tissues *in vitro* can be attributed to inadequate nutrition and poor choice of plant material.

There is a small number of standard culture media that are widely used with or without additional organic and inorganic supplements (White 1963; Heller 1953; Chu 1978; Murashige and Skoog 1962; Gamborg et al. 1968; Kao and Michayluk 1975). A common feature of all these media was the greatly elevated levels of mineral salts. These high-salt media are excellent for supporting callus growth and morphogenesis. Nitsch and Nitsch (1969) medium, with lower inorganic compounds, is widely used for obtaining haploid tissues or embryos from cultured anthers. There is an almost unending list of media that have been reported to be appropriate for specific purposes.

Plant-culture media generally consist of essential and optional components (Gamborg and Shyluk 1981). The essential nutrients consist of inorganic salts, a carbon and

energy source, vitamins, and plant growth regulators (PGRs, phytohormones). In addition to these standard components, in case of specific needs of particular species or tissues, other components, including organic nitrogen compounds, organic acids, and a wide variety of complex natural extracts, can be important but are optional. When the completely defined media did not give the desired results, employing coconut water (milk), other fluid endosperms, casein hydrolysate, yeast extract, malt extract, tomato and orange juice have beneficial effects on *in vitro* plant cell and tissue cultures.

Organic supplements of plant-culture media from natural origin

Growth and regeneration of plants from *in vitro* tissue cultures can be improved by small amounts of some organic nutrients. Many of these amendments can be a source of amino acids, peptides, fatty acids, carbohydrates, vitamins and plant growth substances in different concentrations. The amount of these substances required for successful culture varies with the species and genotype (Thorpe et al. 2008).

Coconut water

Coconut water (CW) is the colorless liquid endosperm of green coconuts (*Cocos nucifera*). However, coconut milk is the extract of white, solid endosperm of matured coconut after grinding and squeezing. Both of them are used in tissue culture media, but the coconut water is the more complex combination of compounds. The liquid endosperm contains a number of amino acids, organic acids, nucleic acids, several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals, and other unidentified substances, none of which alone is totally responsible for growth promoting qualities. When added to a medium containing auxin, the liquid CW can induce plant cells to divide and grow rapidly. It can be applied in tissue culture media successfully if any other compositions are not able to induce explant development (Neumann et al. 2009). Coconut water is commonly used in orchid tissue cultures, too (Kyte and Kleyn 1996). Unlike other undefined supplements to culture media (such as yeast extract, malt extract and casein hydrolysate) coconut milk has proved harder to replace by fully defined media. The liquid has been found to be beneficial for inducing growth of both callus and suspension cultures and for the induction of morphogenesis. Many workers try to avoid having to use coconut milk in their protocols. However, adding coconut milk to media often provides a simple way to obtain satisfactory growth or morphogenesis without the need to work out a suitably defined formulation (Thorpe et al. 2008).

Yeast extract

Yeast extract (YE) is used less as an ingredient of plant media nowadays than in former times, when it was added

as a source of amino acids and vitamins, especially inositol and thiamine (Vitamin B₁) (Robbins and Bartley 1937). In a medium consisting only of macro- and micro-nutrients, the provision of yeast extract was often found to be essential for tissue growth (White 1934; Robbins and Bartley 1937). Amino acids such as glycine, lysine and arginine, and vitamins such as thiamine and nicotinic acid, could serve as replacements for YE. It normally only enhances growth in media containing relatively low concentrations of nitrogen, or where vitamins are lacking. Yeast extract has been shown to have some unusual properties which may relate to its amino acid content. It elicits phytoalexin accumulation in several plant species. Also, induced direct formation of adventitious embryos was found in some plant species' tissue cultures (Thorpe et al. 2008).

Malt extract

Although no longer commonly used, malt extract seems to play a specific role in cultures of *Citrus*. Malt extract, mainly a source of carbohydrates, was shown to initiate embryogenesis in nucellar explants (Rangan 1984). Malt extract also promoted germination of early cotyledonary stage embryos arising from the *in vitro* rescue of zygotic embryos of sour orange (Carimi et al. 1998). Some plant hormones, such as auxins and gibberellins have been identified in malt extract (Dix and Van Staden 1982).

Potato extract

The extract of potato tubers contains carbohydrates, amino acids, important vitamins (C, B₁, B₆) and mineral elements (potassium, iron, magnesium) (Storey 2007). There was a sharp increase in the number of pollen plants produced from wheat anthers when they were cultured on an agar solidified medium containing only an extract of boiled potatoes and growth regulators. Potato extract alone or combined with components of conventional culture media has since been found to provide a useful medium for the anther culture of wheat and some other cereal plants. Potato extract being added to media for orchid micropropagation media has beneficial effects on some species (*Phalaenopsis*, *Doritaenopsis*) (Thorpe et al. 2008).

Banana homogenate

Homogenised banana fruit (or simply banana slices) is sometimes added to media for the culture of orchids and is often reported to promote growth. The reason for its stimulatory effect has not been explained. One suggestion mentioned earlier is that it might help to stabilise the pH of the medium. Similar to other natural extracts, it contains numerous compounds, that evolve its favourable effect. Cytokinin compounds were manifested in banana homogenate (Van Staden et al. 1975).

Fluids which nourish embryos

The liquid which is present in the embryo sac of immature fruits of *Aesculus* and *Juglans* genera has been found to have a strong growth-promoting effect on some plant tissues cultured on simple media. Immature *Zea mays* grains (less than two weeks after pollination) can have a similar effect. It contains zeatin (cytokinin), that can encourage the development of explants (Kyte and Kleyn 1996). However, the most readily obtained fluid with this kind of activity is coconut water.

Casein hydrolysate

Although protein hydrolysates are a convenient source of substances which may promote plant growth, they are by nature relatively undefined supplements. The proportion of individual amino acids in different hydrolysates depends on the nature of the source protein and the method by which the product has been prepared. Casein hydrolysates (CH) can be a source of calcium, phosphate, several microelements, vitamins and, most importantly, a mixture of up to 18 amino acids (George and de Klerk 2008). Several casein hydrolysates are available commercially but their value for plant tissue culture can vary considerably. Acid hydrolysis can denature some amino acids and so products prepared by enzymatic hydrolysis are to be preferred. The best can be excellent sources of reduced nitrogen, as they can contain a relatively large amount of glutamine. Several investigators have concluded that casein hydrolysate itself is more effective for plant culture than the addition of the major amino acids which it provides. In prepared mixtures of amino acids resembling those in CH, competitive inhibition between some of the constituents is often observed.

Algal compounds in plant tissue culture media

Several results in microalgal biotechnology show that PGRs are present in macro and microalgae, (Jameson 1993; Stirk et al. 2002; Ördög et al. 2004). Various compounds of algae (microalgae and cyanobacteria, extra- and intracellular) could be useful sources to enhance or substitute the influence of synthetic PGRs on tissue cultures of higher plants. Strains of Mosonmagyaróvár Algal Culture Collection (MACC) have been tested as a source of growth regulators for *in vitro* cultures of pea (*Pisum sativum* L.), tobacco (*Nicotiana tabacum* L.) and beet (*Beta vulgaris* L.) (Molnár and Ördög, 2004, 2005b). The supernatant (after centrifugation) from cultured microalgae, as an 'unknown organic mixture', have improved the shoot multiplication rate on media supplemented with other plant hormones in the case of pea and tobacco shoot cultures. Brown and compact calli were developed on media supplemented with only the supernatant of microalgal strains in pea mesocotyl cultures, while green calli and small shoots were obtained with combination of synthetic PGRs.

The combination of extracellular compounds from microalgae and synthetic PGRs produced more fresh weight and regenerated shoot numbers than both controls in all cultures. In shoot cultures the medium with 2 g L⁻¹ freeze-dried (f.d.) biomass was found better than hormon-free B5 medium, thus it did not substitute the synthetic plant hormones.

Natural compounds from cyanobacterial biomass alone have produced lower rates of shoot regeneration and gained smaller fresh weights compared to the PGRs control in pea cultures. They are not like real substitutes of synthetic PGRs but as a supplement in culture media resulting more vigorous cultures and regenerated shoots (Molnár and Ördög 2005a).

In some orchid species, such as *Phalaenopsis* and *Paphiopedilum*, the nutrition media supplemented with the biomass of algal strains enhanced the development of plants, but different strains depending on orchid species proved to be efficient. E.g., in *Oncidium* cultures different concentrations of MACC-612 were applied as a supplementation of nutrition media. Results showed, that higher biomass concentrations (0.5 - 1 g L⁻¹) have beneficial effects on plants grown *in vitro* (Virág et al., unpublished results).

Complex nutritive mixtures have been added to plant tissue culture media in the past decades. Nowadays media containing only chemically-defined compounds are commonly used. The dilution of freeze-dried biomass from some microalgae and cyanobacteria could be useful for the improvement of *in vitro* culture media of economically important crops.

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