B- Organic compounds

1- Carbon source

Plant cells and tissues in the culture medium are heterotrophic and therefore, are dependent on the external carbon for energy. Moreover, culture conditions are usually not optimum for photosynthesis due to many reasons including insufficiently organized cellular and tissue development, limited gas exchange and less than optimal light intensity. Consequently, sugar is a very important part of nutrient medium; it is used as carbon and energy source.

Sucrose is the preferred carbon source since it is also synthesized and transported naturally by the plant. Based on type and age of explant and purpose of culturing, sucrose is added to medium at 20-60 g/L. During the course of sterilization (by autoclaving) of the medium, sucrose gets hydrolyzed to glucose and fructose. The plant cells in culture first utilize glucose and then fructose. In fact, glucose or fructose can be directly used in the culture media. It may be noted that for energy supply, glucose is as efficient as sucrose while fructose is less efficient. Besides sucrose and glucose, **other carbohydrates** (such as lactose, maltose, galactose, raffinose, trehalose and cellobiose) or **sugar alcohols** (as mannitol) have been used in culture media but with a very limited success. For example, fructose is better for mulberry buds. In addition for its use as carbon source, sucrose is used as osmoticum keeping osmotic potential of medium suitable for cultured tissues.

2- Vitamins

Plant cells and tissues in culture (like the natural plants) are capable of synthesizing vitamins but in suboptimal quantities, inadequate to support growth. Therefore the medium should be supplemented with vitamins to achieve good growth of cells. Vitamins are organic substances used as parts of enzymes or cofactors. The vitamins added to the media include thiamine, riboflavin, niacin, pyridoxine, folic acid, pantothenic acid, biotin, ascorbic acid and vitamin E. **Thiamine (Vitamin B_1)** is the essential vitamin used in culture medium because its role in carbohydrate metabolism and biosynthesis of some amino acids. **Nicotinic acid, pyridoxine and myo-inositol** are usually added to all culture media at different concentrations. Other vitamins are sometimes used for certain purposes. For example, pantothenic acid is used for meristem cultures.

3- Amino acids

Although the cultured plant cells can synthesize amino acids to a certain extent, media supplemented with amino acids stimulate cell growth and help in establishment of cells lines. Further, organic nitrogen (in the form of amino acids such as L-glutamine, L-asparagine, L-arginine, L-cysteine) is more readily taken up than inorganic nitrogen by the plant cells. Amino acids are sometimes used as a source of reduced organic nitrogen especially for inducing and maintaining somatic embryogenesis. The **sulphur-containing amino acids** (methionine and cysteine) become incorporated into proteins. **Glycine** is simplest amino acid and usually used in culture media. It is essential in purine synthesis and is a part of porphyrin ring (chlorophyll biosynthesis). Despite frequent use, it is difficult to find hard evidence that glycine is really essential for so many tissue cultures, but possibly it helps to protect cell membranes from osmotic and temperature stress. We may conclude therefore, that for many cultural purposes, amino acids are not essential media components; but their addition as identified pure compounds, or more cheaply through **casein hydrolysates**, can be an easy way of ensuring against medium deficiency, or of providing a source of nitrogen that is immediately available to cultured cells or tissues.

4- Complex organics of chemically undefined composition

This class includes compounds of natural sources like casein hydrolysate, coconut milk, juices, pulps and extracts from various fruits…etc. which are known to have certain desired effects in tissue cultures though their exact chemical composition is not known. It is however, preferable to avoid the use of natural extracts due to high variations in the quality and quantity of growth promoting factors in them. It is now known that these organic complexes are rich in growth regulators, organic nitrogen and vitamins.

5- Activated Charcoal

Supplementation of the medium with activated charcoal stimulates the growth and differentiation of certain plant cells (carrot, tomato, orchids). Some toxic/inhibitory compounds (e.g. phenols) produced by cultured plants are removed by adsorption on activated charcoal, and this facilitates efficient cell growth in cultures (eg Faba bean culture). Addition of activated charcoal to certain cultures (tobacco, soybean) is found to be inhibitory, probably due to adsorption of growth stimulants such as phytohormones.

6- Growth regulators

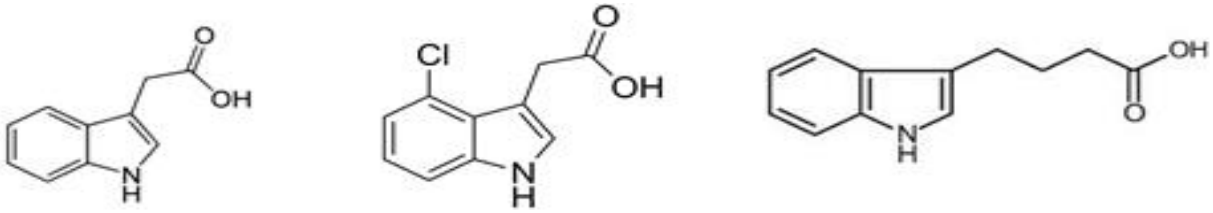
Plant hormones (phytohormones) are group of natural organic substances that synthesized in a discrete organ or tissue (at very low concentration) and transported to a specific target tissue where they exert a profound influence on physiological processes of plants as growth, development and differentiation. They are the most important components of *in vitro* culture media to promote growth, differentiation and organogenesis of plant tissues in a concentration dependent manner. Progress in the science of tissue culture is mainly attributed to discovery of their structure and mode of action. Growth regulators include naturally produced hormones and synthetic substances having the same effects. Their effects are rarely specific in their ultimate influences on growth and development as they can vary with cultural conditions, explant type and genotype. A combination between two or more growth regulators of different classes either applied simultaneously or sequentially, is usually required for different purposes.

In this section we will briefly present some information on each class of growth regulators and role played in tissue culture.

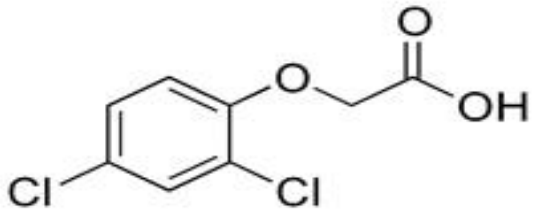
a- Auxins

The most common auxin found in plant is indole-3-acetic acid (IAA). However, depending on the species, age, season, and the conditions under which the plant grows, other natural auxins have been identified such as 4-chloroindole-3-acetic acid (4-Cl-IAA) and indole-3-butyric acid (IBA). In addition, substances like 2,4-dichlorophenoxy acetic acid (2,4-D) or 1-naphthaleneacetic acid (NAA) were found to have auxin activity.

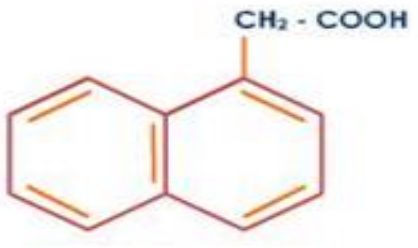
Auxins are characterized principally by their capacity to stimulate cell elongation in excised stem and coleoptile sections, but they exert a strong influence over processes such as regulation of apical dominance, formation of lateral and adventitious roots, floral bud development, delaying the onset of leaf abscission, promotion of fruit development and induction of vascular differentiation. **In tissue cultures**, auxins have strong effect on initiation of cell division and organization of meristems. Consequently they are used for callus induction and organogenesis (generally rooting). Auxins are also effective in induction of somatic embryogenesis.



IAA **4-chloroindole-3-acetic acid** **(IBA).**



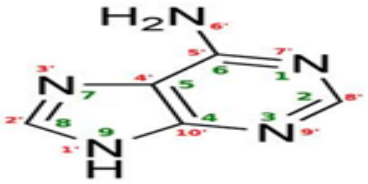
2,4-D



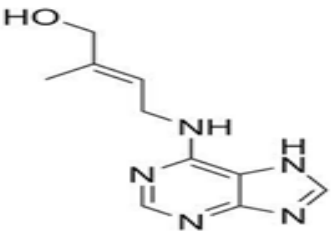
NAA

b- Cytokinins

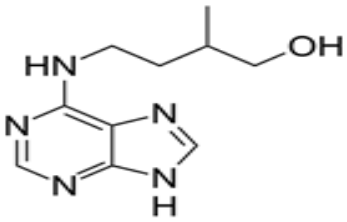
Cytokinins are N^6-substituted derivatives of the nitrogenous purine base adenine. The most widespread naturally occurring cytokinin in higher plants is zeatin. Other cytokinins including kinetin (KN), benzyl adenine (BA) or benzyl-amino purine (BAP), 2-isopentyl adenine (2iP), zeatin riboside (ZR) and dihydrozeatin (DHZ) were also identified. Among the cytokinins, kinetin and benzyl adenine are widely used in tissue cultures instead of the very expensive natural cytokinins. Cytokinins promote RNA synthesis and thus stimulate protein and enzyme activities in tissues. Physiological role of cytokinins also include regulation of cell division in shoot and root, modification of apical dominance, promotion of lateral bud growth, cell expansion in leaf and cotyledons via a mechanism different from that of auxin and delaying leaf senescence. **In tissue cultures**, cytokinins are used for stimulation of cell division (often together with auxin) and release of lateral bud dormancy. They also induce adventitious bud formation either in cutting or callus.



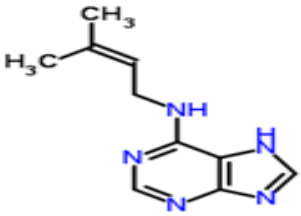
Adenine



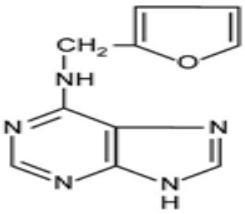
Zeatin



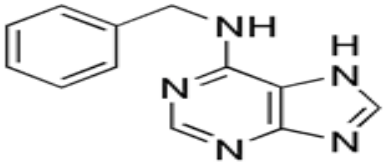
Dihydrozeatin



(2iP)



Kinetin



Benzyl adenine

Ratio of auxins and cytokinins:

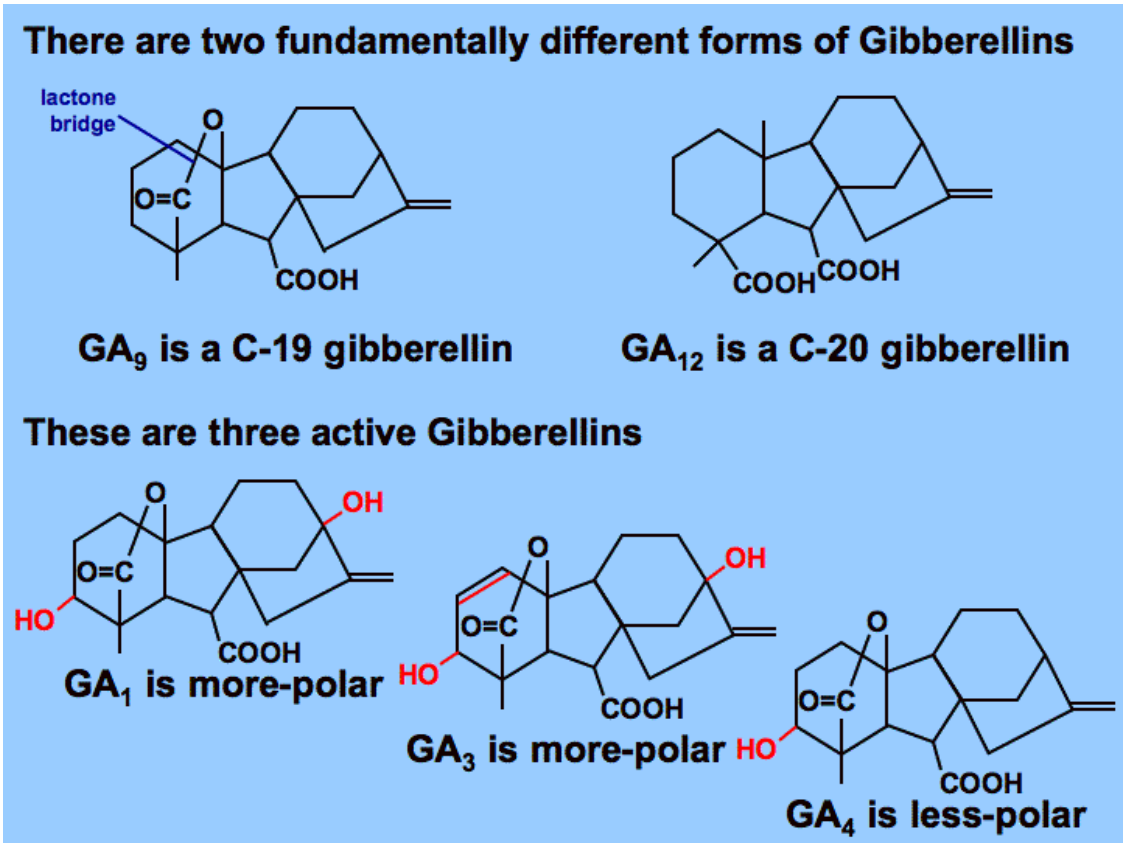
The relative concentrations of the growth factors namely auxins and cytokinins (auxin : cytokinin ratio) are crucial for the morphogenesis of culture systems. High ratio promotes root formation, low ratio promotes shoot formation and intermediate ratio makes the callus to remain growing as undifferentiated mass of cells.

The actual concentrations of the growth regulators used in culture media are variable depending on the type of tissue explant, the plant species, the internal auxin : cytokinin ratio as well as the purpose needed.

c- Gibberellins

Gibberellins are an extensive increasing family of more than 100 members. Chemically, they are diterpenes based on the 20-carbon ent-gibberellane structure. Some gibberellins retained the full complement of 20 carbon atoms and called C_{20}-gibberellins while others lost carbon atom number 20 and known as C_{19}-gibberellins. Chemically characterized gibberellins are assigned an "A" number that refers to order of discovery to become GA. Gibberellic acid (GA_3) is one of the first isolated and characterized gibberellins. It is isolated from fungal cultures of *Gibberella fujikuroi*. In tissue culture, **GA_3** is the most common and available gibberellin. Not all gibberellins are biologically active, however, mutants lacking gibberellins biosynthesis are dwarf and suffering male sterility and dormant seeds (if produced) unless treated with gibberellins. Gibberellins are found to stimulate cell division and elongation

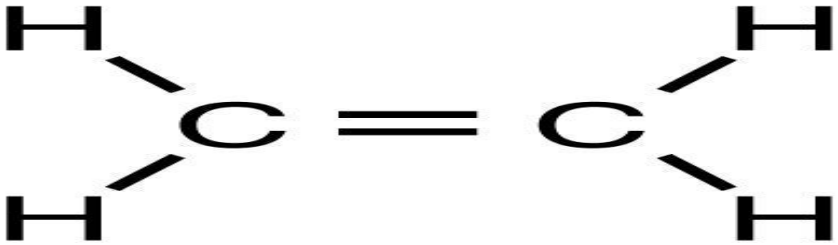
leading to abnormal stem elongation after spraying. GAs can promote flowering (particularly in species that require long days and/or cold). **In tissue culture**, gibberellins inhibit meristemoid initiation but they are required for assisting further growth and development of preformed organs. Generally, gibberellins are used for stem growth and elongation in meristem and shoot cultures.



d- Ethylene (Gas hormone)

It is a simple gaseous hydrocarbon produced by almost all parts of higher plants. The rate of production increased with exposure to stresses including wounding and physiological stresses like salinity and flooding. It is involved in many physiological processes including fruit ripening, breaking seed dormancy of some species, formation of adventitious roots and root hairs as well as promotes leaf senescence and abscission of the oldest leaves. **In tissue culture**, studies showed that ethylene can stimulates organogenesis and embryogenesis with optimum ethylene concentration, above which the gas becomes inhibitory. Ethylene is released easily from tissue, diffuses through the intercellular spaces to outside the tissue and accumulates in the closed culture vessel. Ethylene can be manipulated through enhancing its

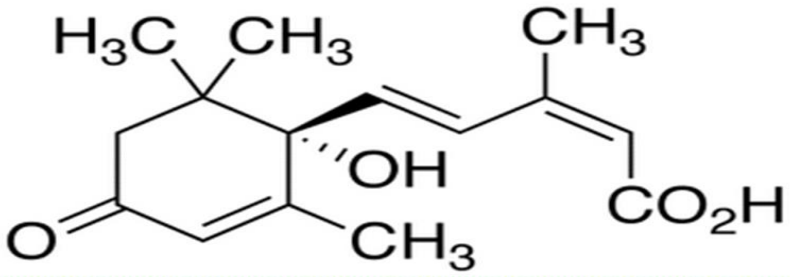
production for example by adding 2-chloroethylphosphonic acid (CEPA). On the other hand, aminoethoxyvinylglycine (AVG), α-aminoisobutyric acid (AIBA) and Co^{2+} inhibits specific step in ethylene biosynthesis. In addition, 2,5-Norbornadiene (NBD) and Ag^{+} (from silver thiosulfate or silver nitrate) effectively blocks ethylene action. Silver thiosulfate is preferred as it produces less toxic symptoms and is more mobile in the plant. Other inhibitors of ethylene action are chelating agents, such as i-hydroxyquinoline and diethyldithio-carbamic acid, and high concentrations of carbon dioxide by preventing the gas binding with its active site.



ethylene

e- Abscisic acid (ABA)

ABA is involved primarily in regulating seed maturation. The dormancy of seeds is controlled by the ratio of ABA and gibberellins. During water stress, ABA closes stomata (reduce transpiration) and control of water and ion uptake by roots. With other phytohormones, ABA promotes leaf abscission and senescence as well as root growth while inhibits shoot growth. ABA is often regarded as being an inhibitor, as it slows cell elongation and maintains bud and seed dormancy. **In tissue cultures**, exogenously applied ABA can affect callus growth, organogenesis and somatic embryogenesis (generally positively at low concentrations and negatively at high concentrations).



ABA

f- Substances with phytohormonal-like activity

They are a variety of compounds synthesized by plants and microorganisms, which show growth active properties. Brassinosteroides, polyamines, oligosaccharines, salicylates and jasmonates are examples of these substances. *Now Brasinosteroids are an independent class and the other substances are considered as hormones.*

- *Brassinosteroids* (BR)

They are steroidal hormones with chemical structure similar to the steroid hormones in animals. They play diverse roles in plant growth and development. Plants deficient in brassinosteroid (BR) biosynthesis or defective in signal transduction show many abnormal developmental phenotypes. Exogenous application of BRs regulate various aspects of plant development including cell expansion, cellulose biosynthesis, vascular differentiation, reproductive development, seed germination, flowering (anther and pollen development and formation) and fruit set in plants. They stimulate shoot elongation and inhibit root growth and development as well as vascular differentiation. Brassinolide is the most biologically active brassinosteroid and it is widely distributed through plant kingdom. Castasterone, analogue of brassinolide, is also intrinsically bioactive in some plant species, e.g. mung bean, although it serves as the immediate biosynthetic precursor of brassinolide in other species, such as *Catharanthus roseus*, tomato and *Arabidopsis*. **In tissue culture,** BR can be effectively used for regulating cell expansion and proliferation, shoot multiplication, regeneration and direct organogenesis.

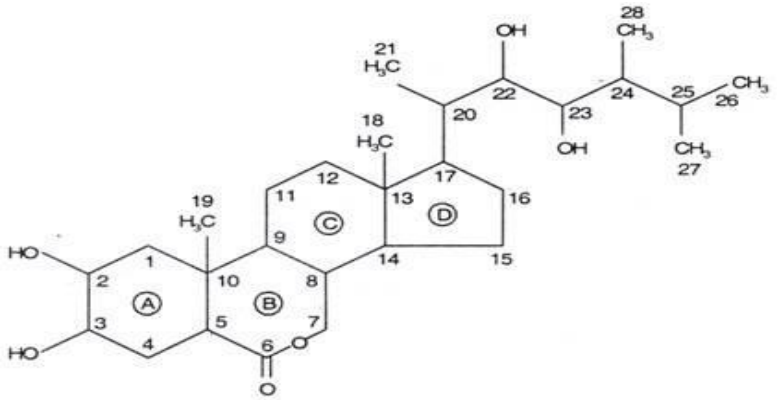


Fig. 17.33. Brassinolide.

- *Polyamines*

Polyamines are group of low molecular weight, highly charged polyvalent compounds containing two or more amine group as *putrescine* (diamine), *spermidine* (triamine) and *spermine* (tetramine). They are ubiquitously present in all living cells. They are involved in many cellular processes: participate in modulation of chromatin structure, gene transcription and translation, DNA stabilization, signal transduction, cell growth and proliferation, migration, membrane stability, functioning of ion channels and receptor-ligand interactions. Polyamines seem to exert their role through ionic interactions, owing to their unique structural feature of regularly spaced positive charges. Tissues deficient in polyamines are usually abnormal. **In tissue cultures**, polyamines involved in pollen maturation, flower development, adventitious vegetative shoot formation, adventitious rooting, and somatic embryogenesis.

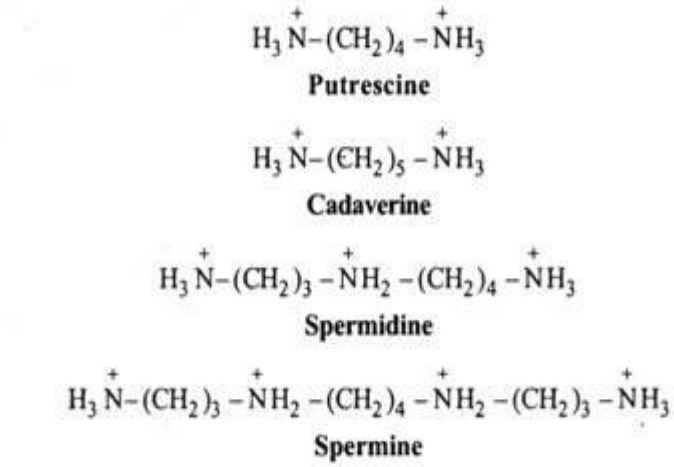
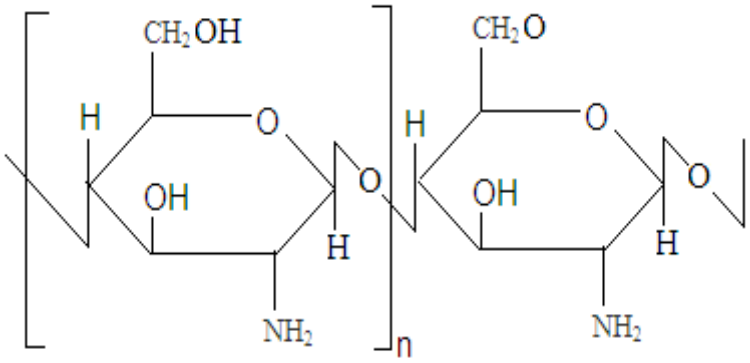


Fig. 17.35. Chemical structures of common polyamines in plants.

- *Oligosaccharins*

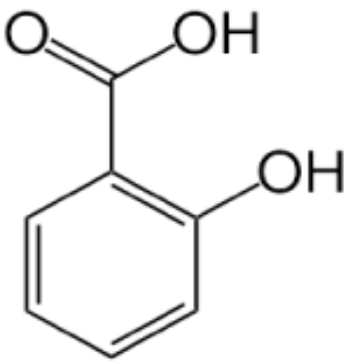
They are cell wall derived oligosaccharides that have a hormone-like function and can be released from the cell wall by hydrolytic enzymes secreted by pathogens signaling the hypersensitive response (HR). These bioactives, called oligosaccharins, are now relatively easy to produce and ready to face public acceptance because of their natural origin. They also act as carbon and energy sources. They exert, at low concentration, effects on development and morphogenesis. **In tissue cultures**, oligosaccharines promote callus

proliferation (with the aid of auxins), increase number of adventitious roots and restoration of the ability to form somatic embryos.



- *Salicylates*

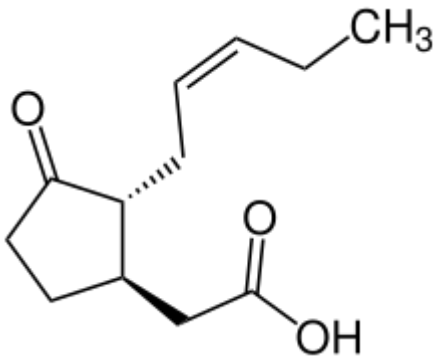
Salicylic acid (SA) and its derivatives belong to the large group of plant phenolic in higher plants. SA plays a role as an endogenous signal mediating local and systemic plant defense responses against biotic (against pathogens) and abiotic stresses such as drought, chilling, heavy metal toxicity, heat, and osmotic stress. It also regulates the following processes as seed germination, vegetative growth, photosynthesis, respiration, thermogenesis (heat production during flowering of thermogenic plants like *Arum lilies*), flower formation, seed production, senescence, and a type of cell death that is not associated with the hypersensitive response and inducer of certain pathogenesis-related (PR) proteins. **In tissue culture**, salicylic acid was found to be effective in the induction of tuberization *in vitro.*



Salicylic acid (salicylate)

- *Jasmonates*

Jasmonic acid (JA) and in particular its volatile methylester (MeJA) have been detected in many species. Wounding and pathogens can promote JA synthesis. Jasmonates are thus involved in the cellular transduction processes between external stress (herbivore, pathogen, desiccation, mechanical, or osmotic stresses) and macromolecular stress responses involving the expression of "defense genes" and production of JA- induced proteins (JIPS). Jasmonates display a multiplicity of effects in plants such as the promotion of leaf senescence, abscission, fruit ripening, tendril coiling, and tuber formation. Jasmonates also inhibit seed germination and stem elongation. **In tissue cultures**, JA stimulates bulb formation. Jasmonic acid has been shown to retard callus formation, inhibit rhizogenesis, and promote bud growth in cultured potato meristems. In cultured roots of tomato, low concentrations of JA promoted the frequency of lateral root initiation and elongation, whereas high concentrations inhibited root growth and reduced lateral root formation.



Interactions:

It should be mentioned that beside the direct effect of exogenously applied growth regulator on cellular mechanisms, the applied growth regulator may modify the synthesis, destruction, activation, transport or sensitivity to endogenous growth substance of the same class or other classes. For example, exogenous auxin stimulates GAs and ethylene biosynthesis. Cytokinins may block ethylene action, however, a synergism between auxin and cytokinins in ethylene production is observed. GAs may alter the availability of endogenous auxins. In addition, they antagonize the effect of ABA on promoting senescence. ABA can antagonize some of cytokinins' effect. Polyamines' synthesis is enhanced by auxins, cytokinins and gibberellins while they are inhibited by ethylene. Oligosaccharines inhibit growth induced by auxins and

gibberellins. Brassinosteroides may modulate auxin action and alter the sensitivity of cells to auxin they also promote ethylene synthesis. In some cases JA also promotes ethylene synthesis.

Note:

It is clear that manipulation of growth regulators in tissue culture medium in order to inducing certain effect is not an easy task. In addition to genotype, explant and effect of cultural conditions, the mutual effect of growth regulators on each other make a great challenge for any one aiming to induce any effect in tissue culture.

To avoid this labyrinth **adequate survey** for available related literature should be carried out to decide what is the growth regulator (or combination of growth regulators) that will be used for certain purpose. Otherwise, we should use **trials and errors**. The **previous facts** about the roles of growth regulators can be considered as **guide lines** in designing any experiment to induce certain effect in tissue cultures using growth regulators.

C- Gelling agent (solidifying agents)

Media for plant tissue culture can be used can be used either in liquid or solid (gelled) forms according to the tissue used. The use of liquid medium in tissue culture is often described as a means of reducing the cost and labor. In addition, availability of water and dissolved substances is higher in liquid medium. However, liquid medium may cause physiological disorder (vetrification) in which tissues become waterlogged and have glassy appearance.

For the preparation of semisolid or solid tissue culture media, solidifying or gelling agents are required. In fact, solidifying agents extend support to tissues growing in the static conditions. A number of solidifying agents is used including agar, gerlite, phytagel, and agarose.

Agar is the most frequently used solidifying agent, it has the desirable characteristic of high gel clarity, stability, being chemically inert and resistance to digestion by plant enzymes during use. The major disadvantages of agar are the presence of impurities and dependence, to some extent, of results on brand used. It is used for all types of cultures at a concentration of 0.5 to 1% (0.8% as average) in the medium can form a gel. **Agarose** is a purified extract of agar with higher gel strength. It is used for protoplast and single cell culture. **Gums**, such as gelan, produced by bacteria and commercialized under the name of gel-gro, gerlite and phyta-

gel. They are polysaccharides used as solidifying agents. They are used in lesser amount per liter than agar to obtain the same consistency and give more transparent media. The high cost of these products still limits their use in commercial cultures. **Gelatine** is used at a high concentration (10%) with a limited success. This is mainly because gelatin melts at low temperature (25°C), and consequently the gelling property is lost.

D- pH of medium

It is the measure of the concentration of hydrogen ions in a solution. It greatly affects the availability of ingredients and gelling efficiency of the solidifying agent. Although the pH of medium is altered during culture, an initial pH is selected before autoclaving. A pH of 5.7-5.9 (5.85 in average) is found to be suitable for availability of salts and solidification. The pH generally falls by 0.3-0.5 units after autoclaving. Before sterilization, pH can be adjusted to the required optimal level while preparing the medium using pH-meter and diluted KOH and HCl. It is usually not necessary to use buffers for the pH maintenance of culture media.

At a pH higher than 7.0 and lower than 4.5, the plant cells stop growing in cultures. If the pH falls during the plant tissue culture, then fresh medium should be prepared continuously. In general, pH above 6.0 gives the medium hard appearance, while pH below 5.0 does not allow gelling of the medium.

Note:

Plant tissue culture media are commercially prepared, and are available in the market as dry powders. The requisite medium can be prepared by dissolving the powder in a glass distilled or demineralized water. The general methodology for a medium preparation involves preparation of stock solutions (in the range of 10x to 100x concentrations) using high purity chemicals and demineralized water. The stock solutions can be stored (in glass or plastic containers) frozen and used as and when required. Sugar, organic supplements and agar (melted) are added, pH adjusted and the medium diluted to a final volume. Most of the growth regulators are not soluble in water. They have to be dissolved in NaOH or alcohol (details will be in lab).

III- Media sterilization

Tissue culture necessitates absolute sterility. The *in vitro* environment used is ideal for growth of microorganisms, so media should be autoclaved for 20 minutes at 121 ^{o}C and 1.21 kg cm^{-2} (15 psi). Autoclaving is a quick and simple method for sterilization while pH may change and some components may decompose and lose their effectiveness. Heat-labile substances are sterilized through 0.22 µm filters and aseptically added to autoclaved media just before solidification (at 40-45 ^{o}C).

IV- Storage of culture media

After sterilization, media should be stored in a dark cool place to minimize degradation of light-labile components eg: IAA. Storage in refrigerator prolongs time of storage (more than one month) but condensation encourages contamination. Media containing unstable ingredients must be used fresh and can't be stored.

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