SECONDARY METABOLITES PRODUCTION

Plant cell produces two types of metabolites: primary metabolites involved directly in growth and metabolism, viz., carbohydrates, lipids, and proteins, and secondary metabolites considered as end products of primary metabolism and are in general not involved in metabolic activity, viz., alkaloids, phenolics, and terpenes. Primary metabolites are produced as a result of photosynthesis, and these products are further involved in the cell component synthesis and stored as reserve food. Primary metabolites obtained from higher plants for commercial use are high volume low value bulk chemicals. They are primarily used as industrial raw materials, foods, or food additives, for example, vegetable oils, carbohydrates (sucrose, starch, pectin, and cellulose), and proteins (Fig. 16).

Secondary metabolites are compounds biosynthetically derived from the primary metabolites but are of limited occurrence in the plant kingdom, may be restricted to aparticular taxonomic group (genus, species or family) and not required for the growth of plant. Medicinal plants are rich in secondary metabolites that is why these plants are termed as “medicinal” or “officinal” plants. Still 15% of flora has been explored phytochemically, and thus the scope of work is enormous. The required carbon skeleton is derived from carbohydrates synthesized from photosynthesis. The other major primary products involved in various classes of secondary metabolites are amino acids. Acetyl-CoA and mevalonic acid play a key role in the synthesis of various terpenoids, while skikimic acid pathway is involved in the synthesis of lignins and indole alkaloids. These secondary metabolites are synthesized by coordinated expression of several genes producing enzymes required for step by step biosynthesis. Genes responsible for synthesis of such secondary metabolites are highly expressive in some organs where these compounds are accumulated. However, there are examples such as nicotine (pyrrolidine alkaloid of Nicotiana species), which is mostly biosynthesized in roots but transported and accumulated in leaves. Therefore, specific mechanisms that evolved for such functions are also energy expensive involving ATP binding transporters. Out of several thousand genes present in plants, it is not clear how some of these genes are expressed in a coordinated manner. Interest in medicinal plants as a source of bioactive natural molecules has gained again in spite of intrinsic difficulties as combinatorial chemistry [comprising chemical synthetic methods that make it possible to prepare a large number (tens to thousands or even millions) of compounds in a single process] failed to deliver sufficient number of new drugs (only 36% out of 1073 molecules) in the recent past. From 1981 to 2014, 136 drugs were recorded effective against cancer in which 17% were synthetic while 83% were of natural origin. Hence medicinal plants are a source of several important medicines, and interest in discovery of new molecules is ever lasting. Some of the plants derived compounds approved by FDA (Food and Drug Administration, USA) in the last three decades as drug, and others which are under clinical trials are presented in Table 5. It is evident that these drugs have

complex structures and are used for a wide range of diseases. Complexity of these secondary metabolites prevents their chemical synthesis, and nonavailability of uniform material for extraction is difficult in developing technology toward drug approval. Plant cell and tissue cultures provide an excellent source of continuous and uniform supply of raw material throughout the year without seasonal or geopolitical interferences. Though secondary metabolites are considered as waste products, their production requires energy; these metabolites are known to involve in various biological processes including plant defense. Therefore, their production is influenced by external abiotic and biotic factors. In brief, following are important functions of secondary metabolites:

1. As plant defense molecule against microbes, fungi and insects
2. Role in plant and also insect reproduction
3. Provide protection to insects against parasites
4. As nitrogen storage molecules
5. Mitotic inhibitors and modulation of microtubule structures
6. Inhibition of DNA and protein synthesis
7. As germination inhibitor (as allelochemical)
8. Involve in plant immunity and programmed cell death
9. Signaling molecules in angiosperm parasites

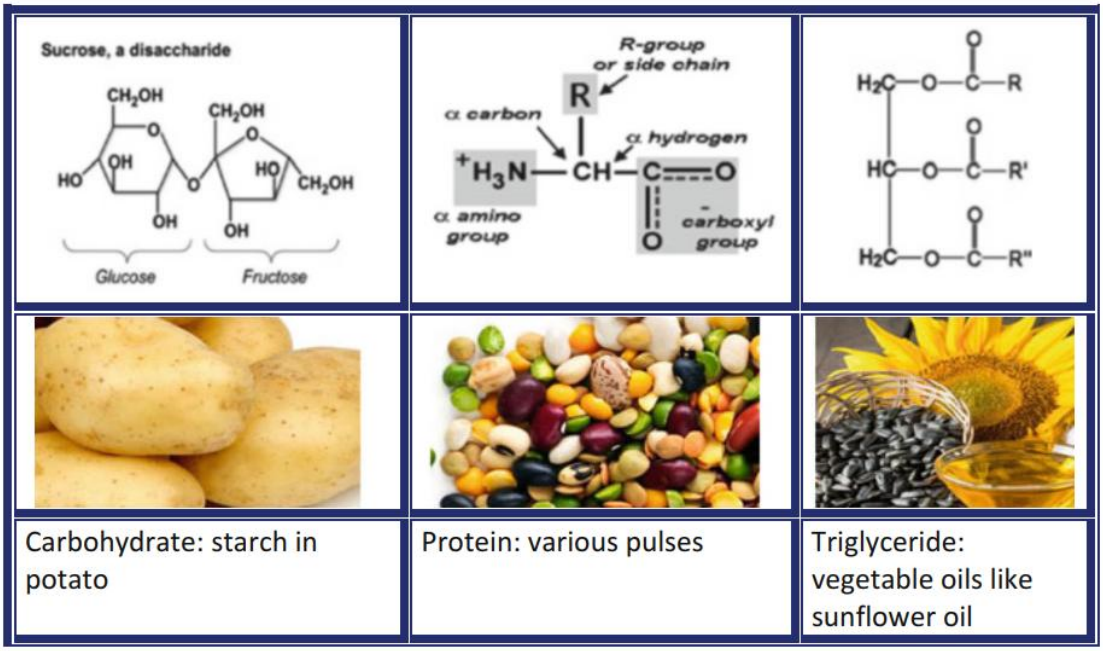
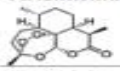
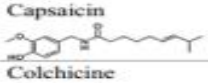
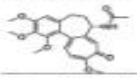
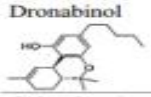
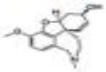
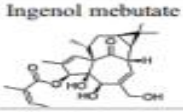
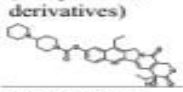
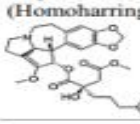
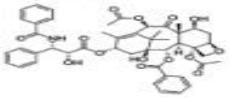
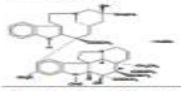
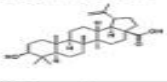
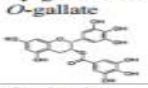
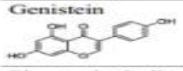
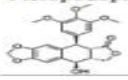


Figure 16. Three primary metabolites commonly present in storage organs of plants.

Table 5. Selected examples of plant derived natural products entered in drug market as approved therapeutic or under clinical trials in last three decades.

Generic name and chemical structure	Plant species	Trade name (year of introduction)	Disease (mechanism of action)
A. Approved therapeutic			
Artemisinin 	*Artemisia annua*	Artemisin (1987)	Malaria treatment (radical formation)
Capsaicin 	*Capsicum annum*	Qutenza (2010)	Postherpetic neuralgia (TRPV1 activator)
Colchicine 	*Colchicum* spp.	Colcrys (2009)	Gout (tubulin binding)
Dronabinol/ Cannabidol Dronabinol 	*Cannabis sativa*	Sativex (2005)	Chronic neuropathic pain (CB1 and CB2 receptor activation)
Galanthamine 	*Galanthus caucasicus*	Razadyne (2001)	Dementia associated with Alzheimer's disease (ligand of human nicotinic acetylcholine receptors (nAChRs))
Ingenol mebutate 	*Euphorbia peplus*	Picato (2012)	Actinic keratosis (inducer of cell death)
Irinotecan (structure), Topotecan (camptothecin derivatives) 	*Camptotheca acuminata*	Camptosar (2004), Hycamtin (2007)	Solid tumors of colon, lung, and ovarian cancers (topoisomerase I inhibitors)
Omacetaxine mepesuccinate (Homoharringtonine) 	*Cephalotaxus harringtonia*	Synribo (2012)	Oncology (protein translation inhibitor)
Generic name and chemical structure	Plant species	Trade name (year of introduction)	Disease (mechanism of action)
Paclitaxel 	*Taxus brevifolia*	Taxol (Taxotere® 1993), Abraxanec (2005), Nanoxelc (2007) Cabazitaxel (Jevtana®) (2010)	Cancer chemotherapy (mitotic inhibitor)
Sphingomyelin/ cholesterol liposomal vincristine 	*Catharanthus roseus*	(Marqibo®) 2012	Relapsed acute lymphoblastic leukemia (cell-cycle-specific)
B. Under clinical trials			
Betulinic acid 	*Betula* spp., *Diospyros* spp., *Syzygium* spp., *Ziziphus* spp., *Paeonia* spp., *Sarracenia flava*, *Anemone raddeana* *Lycopodium cernuum*	Various cancers, e.g., colorectal lung, colon, breast, prostate, hepatocellular, bladder, head and neck, stomach, pancreatic, ovarian and cervical carcinoma, glioblastoma, chronic myeloid leukemia cells (induction of apoptosis in mitochondria)	
Curcumin 	*Curcuma longa* (Turmeric)	26 different trials: cognitive impairment, different types of cancers, cardiovascular disease etc. (NF-κB inhibition) etc.	
Epigallocatechin-3-*O*-gallate 	*Camellia sinensis* (Green tea)	14 different trials: different types of cancer, Epstein-Barr virus reactivation, Alzheimer's disease, cystic fibrosis, diabetic nephropathy, obesity, influenza infection etc. (cell growth arrest and apoptosis induction)	
Genistein 	*Genista tinctoria*	5 different trials: various intestinal and lung cancers, Alzheimer's disease, osteopenia, osteoporosis (protein-tyrosine kinase inhibitor, antioxidant)	
Picropodophyllotoxin 	*Podophyllum hexandrum* (syn. *Sinopodophyllum hexandrum*)	Trial for various cancer cells (tubulin binding/IGF-1R Inhibitor)	

Advantages of production in plant biotechnology

Obtaining by-products from plant tissue cultures has many benefits, including:

1. The quantity and quality of the product are more stable in the case of cells grown outside the body because they are not affected by environmental conditions (temperature, humidity, soil type).
2. The possibility of scheduling production due to the ease of control and control of laboratory conditions.
3. The possibility of increasing the production of these materials in vitro cultivation by adding in the nutrient medium with initiators to form these materials or by genetic treatment of the cultivated cells.
4. In vitro cultivation technologies allow large-scale production of these by-products from beneficial plants.
5. The problem of product contamination is non-existent because the implants are created in completely sterile conditions.

Disadvantages of in vitro production

Despite the advantages mentioned above of tissue culture in producing these materials, there are some disadvantages and undesirable aspects, including:

1. In tissue culture, not all cultured cells produce these secondary materials, and this can be attributed to the cessation of genetic activity to create these materials at the cellular level due to a change in the physiological and morphological state of the cells cultivated.
2. During in vitro cultivation, the tissues remain in an undifferentiated meristematic state, whether they are callus tissues or cell suspensions. This condition will negatively affect the production of secondary materials because these materials usually accumulate in highly differentiated tissues such as mint glandular tissues and celery oil ducts. and the bases of swollen leaves (onions.)
3. The readiness of the initiators needed to form these secondary products in the food environment is sometimes low, especially at the cellular level, which leads to a sharp decline in the production of secondary materials.
4. Some incubation conditions, such as lighting, temperature, and sometimes the accumulation of certain growth regulators in the nutrient medium, negatively affect the by-products.

Stages of production of secondary metabolites in plant biotechnology

The production of secondary materials through secondary metabolic pathways outside the living body is gaining significant importance in biotechnology, and this importance is increasing daily. To establish a program to produce these materials, the following steps are followed:

- Selecting cell lines to produce large quantities of these secondary materials. Two methods can be used in this field, which are the following:

a. Single-cell cloning

Theoretically, cloning a single cell is the best way to isolate cells that produce large quantities of secondary materials. Still, many cloned cells resulting from a single cell are heterogeneous or heterogeneous in their ability to produce secondary materials, and cases of polyploidy occur.

B- Cell aggregate cloning

This method is more accessible than the previous method and includes the following steps:

1. Induction of callus formation.
2. Selection of groups of cells specialized in producing specific compounds.
3. Cultivation of these selected cell groups.
4. Divide each cell group into two halves: the first for subculturing and the second for quantitatively estimating the product in question.
5. Select the cell group that produces the most significant byproduct.
6. Ensure the stability of the production of secondary materials in the selected groups by repeated replanting.
7. Producing callus from selected groups, cultivating it, and propagating it to produce these metabolic materials, using the method of developing callus in liquid or solid media, using suspended cell cultures, or using Bioreactors.

Cell and Tissue Differentiation and Production of Secondary Metabolites

Plant cell and tissue cultures are extensively used to study cellular and tissue differentiation and also production of associated secondary metabolites. Most anthocyanins are glycosylated, accumulated in the vacuoles, and the most studied phenolic compound because they impart flower color. Plant cell culture is an excellent alternative for the production of anthocyanins of uniform quality as compared to in vivo materials like grape skins from wine industry, sweet potato, and red cabbage. Isolated single cells in liquid medium are specifically interesting systems to study secondary wall formation by lignin synthesis using Zinnia elegans and a few other cultures, which is not possible using in vivo plant model. Lignin precursor monolignols are synthesized from phenylalanine through shikimate pathway involving several steps. At low phenylalanine levels, this amino acid is used for protein synthesis. Cytokinin and IAA are key plant growth hormones that regulate root development, its vascular differentiation, and root gravitropism; these two hormones, together with ethylene, regulate lateral root initiation. Pathway for biosynthesis of some compounds (consequently many enzymes or genes) leading to cellular differentiation is same and expressed sequentially in a coordinated manner like a harmonium; keys are same but produce different rhythm when (ex)pressed in different sequences.

Secondary metabolites are present in most of the cells of plant in its cytoplasm and vacuole, sometimes in higher amounts in specialized cells called glands, glandular cells, and/or trichomes. The structures of these secretory tissues may vary considerably from single cells (e.g., idioblasts or laticifer cells) to many cells and complex structures (trichomes, colleters, nectaries, osmophores, secretory

cavities, and ducts). A laticifer is a single cell or a row of specialized cells that contain latex. Latex, which is cytoplasm of the cell, contains several compounds, minerals, proteins, and specialized compounds such as alkaloids or rubber (isoprene). As compared to laticifers, resin ducts are more complex organized tissues, having epithelium lining and lumen containing resin. This is a phylogenetic conserved trait to have laticifers or resin ducts. Plant secondary metabolites are generally accumulated at high levels in specific tissues or cell types of plants of a genus (e.g., Urticaria) or family (e.g., Rutaceae, Burseraceae). Economically important examples of tissuespecific metabolites include alkaloids (e.g., morphine and codeine) and latex in laticifer cells in poppy, papaya (latex contain papain), and rubber trees (isoprene in latex), terpenes and saponins in epidermal cells of many plant families, and resins in *Pinus trees*. Special metabolites are synthesized and accumulated in laticifers, resin canals, and specialized tissues. Details of these special structures are not presented here, but this state of tissue differentiation is a prerequisite for the production of compounds present in these structures. Therefore, this is a major hurdle in producing such tissue specific secondary metabolites in cell and callus cultures, and production of such compounds remains low, irrespective of various permutation and combinations of the medium or effectors, e.g., in *Commiphora wightii*. Various types of cellular and tissue differentiations obtained in cell and tissue culture are presented in Fig. 17.

Lignin is an important structural and defensive component of plant secondary cell walls, and the second most abundant biopolymer on earth. Lignin is composed primarily of three hydroxycinnamyl alcohol monomers, referred to as monolignols. These monolignols are variedly polymerized to form complex structure of lignin. These monolignols are also involved in the formation of suberin with lipids, bark, and cork formation. Lignin formation is an integral part of the process of differentiation, and tracheid-like structures are formed in callus and cell cultures, which ultimately form meristemoids and shoot buds. This complex process involves several precursor substrates and enzymes depending upon the cell type. It is well established that carbon source in the medium has a profound effect on vascularization (secondary wall formation) as basic carbon skeleton is provided by sugars.

Terpenoids are the most structurally divergent secondary metabolites. About 25,000 compounds have been isolated, and their structures are elucidated. Accumulation of terpenes is known in many plants, e.g., monoterpenoids in Lamiaceae plants are biosynthesized in secretory cells and accumulate in the epicuticular cavity of glandular trichomes, osmophores, conical-papillate cells, ducts, and cavities, while terpenoids of woody plants are secreted into the resin duct. For volatile mono- and sesquiterpenenoids, their emission from flowers of several plants (*Caesalpinia pulcherrima, Parkia pendula, Bauhinia rufa, Mucuna urens, Rosa x hybrid*) and leaves of conifer and other woody plants is well known. Terpenoids act as defense molecules when herbivores attack the plants and

defense mechanism is activated to produce volatiles. It is evident that this mechanism is activated in response to insect attack by gene expression.

Morphine, a major isoquinoline alkaloid in the latex of opium poppy, is accumulated in the large membranous vesicles of such latex. Immunofluorescence analyses using antibodies specific for five enzymes of alkaloid formation in opium poppy. Weid and coworkers showed that two O-methyltransferases and an O-acetyltransferase were found predominantly in parenchyma cells within the vascular bundle of capsule and stem, while codeinone reductase was localized to laticifers. Another group reported that three of those biosynthetic enzymes of morphine were localized in sieve elements of this plant. Therefore, it is evident that the transport of the intermediate from specific cell-type of vascular tissue to laticifer was involved with ABC transporters (located on cell membranes).

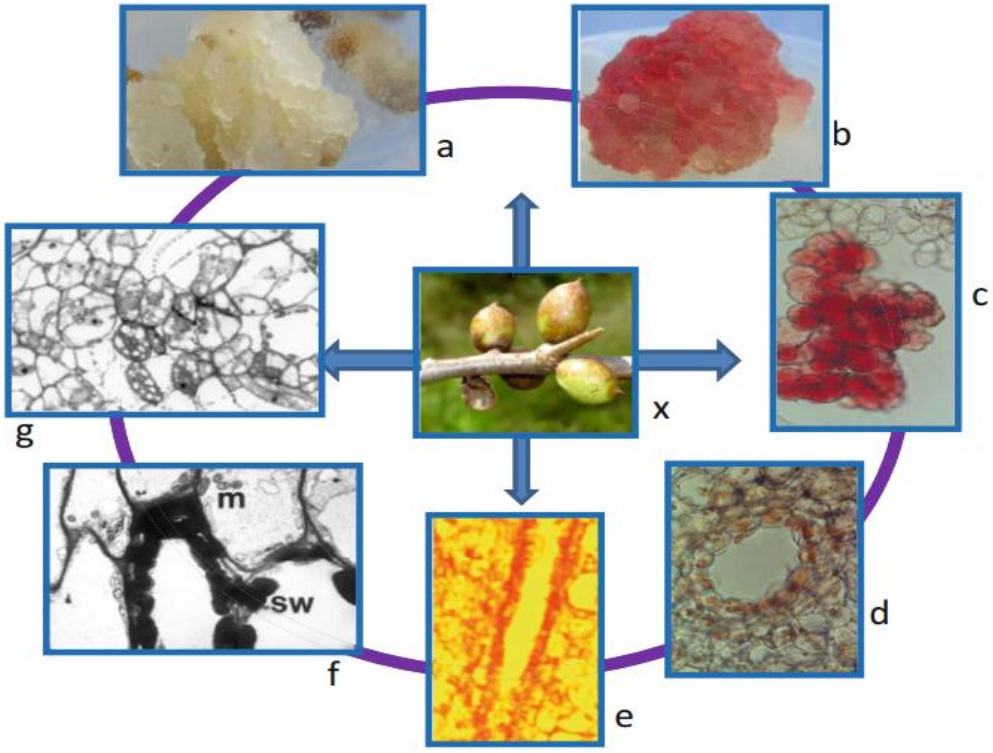


Figure 17. Various cellular and tissue differentiation in callus cultures initiated from explant (x). Symbolic photos represent: (a) Unorganized callus; (b) red callus due to presence of anthocyanin; (c) anthocyanin containing cells under light microscope; (d) resin canal showing epithelium lining; (e) L.S. resin canal; (f) secondary wall (sw) formation by lignification under TEM; (g) meristemoid formation as seen under light microscope. (All photos by the author)

Bioreactors

Bioreactors are vessels for cultivation outside the living body. They generally have a large capacity and are equipped with aeration mixing. Circulation systems to obtain a cellular mixture and techniques to control contamination and replenish the nutrient medium or the cultured cells. Bioreactors of various

designs and capacity have been extensively used for growing cell cultures of different species but growth of organized cultures though attempted in many species albeit in a smaller capacity bioreactor. It is necessary to optimize the growth of the organized cultures in shake flasks before the use of any type of bioreactor. Both roots and shoot cultures have been grown in static and liquid cultures. Adventitious shoot culture and micropropagation have become commercial level technology. Biomass of several medicinal plants is generated through tissue culture for downstream processing. Hairy roots are developed and grown in many more plant species as production system for secondary metabolites, e.g., Artemisia annua, Atropa belladonna, Azadirachta indica, Beta vulgaris, Catharanthus roseus, Echinacea purpurea, Glycyrrhiza glabra, Hyoscyamus muticus, Lithospermum erythrorhizon, Panax ginseng, Picrorhiza kurroa, and Tagetes patula and references therein). Shoot cultures grow well on static medium but in liquid medium (submerged, temporary immersion, bioreactor) pose problems of aeration, hyperhydracity (vitrification), and sometimes low biomass production. The major problem with developing scale-up technology for organized cultures is distribution of nutrients and aeration of cultures. The other issues related to the growth of shoots/root cultures in large bioreactor are high shear pressure, providing light to the cultures, and sealing and stability of shaft. However, in spite of these difficulties, shoot cultures have been attempted in Ananas comosus (10 L airlift bioreactor), Hypericum perforatum (2 L stirred tank bioreactor) Lavandula officinalis (5 L bubble column bioreactor), and several others. These problems of large-scale organized/unorganized cultures are discussed in detail elsewhere.

Factors affecting the production of secondary metabolites in plant biotechnology

1. Components of the medium

Cell growth occurs in tissue cultures when the necessities for division and development are available, such as nutrients, growth regulators, and other additives, all of which affect the metabolic activities within the cells. To achieve optimal productivity of secondary metabolic compounds, it is preferable to produce the cells in an optimal medium for increasing biomass, after which the cells are transferred to the production medium that achieves the highest production of the desired compound. Note that the callus induction or maintenance medium is not necessarily ideal for producing secondary compounds. Thus, many growth regulators and other additives are tried to obtain the production medium. The components of the medium, in general, such as sources of carbon and nitrogen, phosphates, growth regulators, starters, catalysts, vitamins, additives, and others, also affect the fluctuation in the production of secondary compounds.

a. Carbon source

Carbon source generally affects the production of secondary compounds. For example, increasing sucrose in the production medium to 4-10% led to an

increase in the production of alkaloids in tissue cultures of *Catharanthus roseus*. It was also found that adding sucrose as a carbon source was better than fructose and galactose when producing diosgenin from tissue cultures of *Dioscorea deltoidea* and *Dalanites aegyptiaca*. An increase in the compound 10-Ubiquinone was also recorded from tobacco tissue cultures when low levels of sucrose were added to the growing medium. The effect of adding different concentrations of sucrose on the concentration of flavonoids was studied in the presence or absence of light in tissue cultures of the plant (*Hypericum perforatum*). It was found that there was a significant increase in the proanthocyanidin compound in the dark and the presence of 40 g/L of sucrose, while the compound kaempferol accumulated in the exposed crops for continuous light and in very high quantities after adding 50 g/L of sucrose.

b. Effect of nitrogen source

It is usual to add a mixture of ammonia and nitrite to the nutrient medium as a nitrogen source. Most plant cells tolerate high ammonia levels, as the cells invest nitrogen in manufacturing amino acids and proteins, including enzymes and nucleic acids. It is known that nitrogen contains primary compounds that directly affect the formation of secondary compounds. In general, high concentrations of ammonia inhibit the synthesis of secondary compounds, and reducing it increases cell productivity. Inhibition of the production of anthocyanins by 90% and alkaloids by 80% was recorded when the nutrient medium was prepared with potassium nitrate and ammonium nitrate.

C. Effect of phosphates

Inorganic phosphates are essential in photosynthesis and respiration. Many secondary compounds are produced through Phosphorylated intermediates, which release phosphate. For example, phenylpropanoid compounds, terpenes and terpenoids are released. It is generally observed that high levels of phosphate encourage cells to divide, grow, manufacture primary compounds, and produce energy. At the same time, low phosphate levels are very useful in producing secondary compounds, although the situation cannot be generalized. Many researchers have recorded contradictory results regarding the levels of concentrations of phosphate added to the food media. They may increase, decrease, or not affect secondary metabolic compound production.

2. The Effect of plant growth regulators

Plant growth regulators, such as auxins and cytokinins, affect cell division, various metabolic processes, and the emergence of plants from their tissue cultures. Many scientific studies have reported that the type of growth regulator and its concentration impact the productivity of tissue cultures from secondary metabolites. It is clear that there is a wide diversity of plant growth regulators, and from time to time, new types of them are discovered that need to be studied and investigated in terms of their impact on the production of primary and secondary compounds (Table 6).

Table 6. Increasing the production of secondary compounds for a group of plant species by adding different combinations of plant growth regulators.

Compounds	Media+PGRs	Plants
Isoflavones	MS+TDZ+BA	*Psoralea cordifolia*
Resveratrol	MS+IAA+GA3+UV	*Vitis vinifera*
Azadirachtin	MS+2,4-D	*Azadirachta indica*
Catharathine	MS+2,4-D+UV-B	*Catharanthus roseus*
Serpentine	MS+BAP+IAA	*Rauwolfia serpentina*
Reserpine	MS+IAA+Cu+2	*Rauwolfia serpentina*
Stevioside	MS+BA+NAA	*Stevia rebaudiana*
Capsaicin	MS+2,4-D+Kin	*Capsicum annum*
Rosmarininc acid	MS+IAA+Kinetin	*Zataria multiflora*
Anthocyanin	MS+BAP+NAA	*Vitis vinifera*
Gymnemic acid	MS+2,4-D+IAA	*Gymnema sylvestre*

3. Precursors

Precursors are the base molecules that can be incorporated into secondary metabolism compounds through the nutrient medium and increase or accelerate the production of desired compounds. Adding starters generally accelerates the production of secondary metabolites, although they inhibit the growth of crops in many cases. For example, the synthesis of alkaloids increased in Datura tissue cultures, but this was offset by an inhibition in the crops' growth after adding ornithine, phenylalanine, tyrosine, or sodium phenylpyruvate. Ajmalcin production increased in C farms. roses after supplementing the nutrient medium with the initiator's tryptamine or occulogenin. There was a significant increase in rosmarinic acid accumulation in Coleus blumei callus cultures when the callus maintenance medium included 50 g/L of sucrose compared to lower concentrations. Rosmarinic acid also accumulated in high quantities in parts of the carpet plant stems when the liquid MS medium contained 10 and 20 mg/L of proline.

4. Elicitations

Elicitations are compounds that are either Biotic elicitors or Abiotic elicitors. Endogenous bioactives, such as pectin, pectic acid, cellulose, and other polysaccharides, may be used. In the case of manifestations produced by microorganisms, they are called Exogenous. Examples of chitin, chitosan, and glucan. As for non-biotic expressions include Physical, such as exposing crops to cold, heat, ultraviolet radiation, and osmotic pressure.

The manifestations are abiotic, including chemical agents such as treatment with ethylene, fungicides, antibiotics, heavy metal salts, or drought stress (Table 7). Sodium chloride salt was used in many tissue cultures as a cheap abiotic agent (Table 8), and it increased the productivity of secondary compounds added to the tissue cultures. Some supplements were added to the nutrient medium of the

rosehip callus farms to increase the production of phenolic compounds and terpenes, and increases were achieved in most of these compounds.

Table 7. The effect of moisture stress on increasing the productivity of tissue cultures from secondary metabolites.

Plant	**Secondary metabolites**
Scrophularia ningpoensis	Glycosides
Papaver somniferum	Morphine alkaloids
Glycine max	Trigonelline
Brassica napus	Glucosinolates
Lupinus angustifolius	Chinolizidin alkaloids
Camellia sinensis	Epicatechins
Hypericum Brasiliense	Betulinic acid
Hypericum Brasiliense	Rutine
Prisms sativum	Flavonoids
Prisms sativum	Anthocyanins

Table 8. Increase in metabolic compounds in a group of plant species after adding sodium chloride to the media of their tissue cultures.

Compound	**plant**
Lycopersicon esculentum	*Sorbitol*
Sesamum indicum L	*GABA*
Hordeum vulgare	*Flavonoids*
Lycopersicon esculentum	*Jasmonic acid*
Cakile maritime	*Polyphenol*
Datura innoxia	*Tropane alkaloids*

5. Environmental and physical factors

Physical factors affect, directly or indirectly, the productivity of plant cells from secondary compounds, the most important of which are the following:

a. Light: Light directly affects, as it is a source of carbon fixation, the process of photosynthesis in plants grown in the field in the field. Given that there is no need to fix carbon and that the need for it is limited in tissue plant cultures to prepare it as medium components, light therefore has no role in producing plant cells from primary compounds. The matter is different in terms of its effect on the production of secondary compounds in cells and directly affects their release because it is the mediator in enzymatic reactions. Exposing tissue cultures to blue and white colors has encouraged the production of anthocyanin pigments and essential oils of some plants.

b. Temperature: The growth of the cultivated cells increases with increasing incubation temperature until the ideal temperature is reached (30-25ºC), but in general, when wanting to produce secondary compounds, the cultures need to lower the temperature. For example, the accumulation of indole alkaloids increased twofold when *C. roseus* cultures were incubated at a temperature of

16°C instead of 27°C. A decrease in caffeine production was recorded in coffee tissue cultures, as well as nicotine in tobacco tissue cultures.

c. pH of the nutrient medium: It is known that the pH of the medium suitable for the growth of plant cells is within the range of 5-6, but the matter differs when producing secondary compounds. For example, tissue carrot cultures had lower amounts of anthocyanins when the pH of the medium was 5.5, while it increased when it was reduced to 4.5. This may be due to increased damage to anthocyanin pigments with increasing medium pH.

d. Aeration of cultures: Plant cells need lower oxygen levels than bacterial cultures due to the low rates of respiration of plant cells. If the latter breathes at a rate of 0.2 mmol/g/hour, it must be grown in a liquid medium at a rate of 10 g/L, taking into account not allowing the dissolved oxygen to drop below 20%. For experimental purposes, when flasks are used to grow cell suspensions, it is required that the medium with the suspended cells occupy a volume not exceeding one-third of the flask's volume to allow for gaseous exchange and to ensure appropriate levels of oxygen.

e. Treatment with radiation: Radiant energy sources have been widely invested in producing genetic mutations and stimulating plant tissues to produce secondary compounds such as γ, X, β, neutrons and protons.

- **Gamma rays:** For a long time, they have been the most widely used ionizing rays in producing mutations in plants, and their ability to increase the production of commercial compounds has been shown. When treating plant tissue with atoms and molecules, gamma rays interfere with the formation of free radicals inside plant cells, resulting in the modification of essential cell components. Studies have shown that free radicals affect plants' morphological, anatomical, biochemical and physiological appearance depending on radiation doses. Treatment with gamma rays increased phenolic compounds (as antioxidants) in many plant species within amounts ranging from 50 to 150 Cr. Therefore, radiation is an essential abiotic stress in stimulating plant tissues to increase their productivity of secondary compounds.

- Ultraviolet rays (UV): Ultraviolet rays are divided into three types: UV-C, with high energy with a wavelength of 200-280 nm, which is entirely absorbed by atmospheric gases, and UV-B, with high energy with a wavelength of 280-320 nm, which is partially absorbed by The ozone layer, whose damage has increased in recent years as a result of various pollutants, especially chlorofluorocarbons, which has led to an increase in the levels of UV-B coming from the sun to the surface of the Earth and may lead to major damage to the biological system on the planet. The latter, UV-A, has an energy wavelength of 400-315 nm, which is difficult to be absorbed by the ozone layer. Although UV-B inhibits plant growth, reduces biomass, and has many environmental effects, an increase in the accumulation of secondary metabolic compounds has been recorded when absorbing this type of rays. Plants generally have synthetic and induced protection mechanisms or may activate some corrective responses to resist UVB

stresses. One of these responses may be for the plant to accumulate secondary metabolites to absorb radiation at this wavelength.

- **Ultrasound:** The method of using ultrasound waves is unique among the strategies used because it is simple, cheap, and multi-functional, as it is typical when used in tissue cultures without physical contact. For example, treatment with sound waves allows for the reversible dissolution of various cellular membranes, making the method very useful in biotechnology. Technology requires cooperation between tissue culture specialists, engineers, and physicists to obtain suitable solutions. Systems based on fermentation are used with conductors that produce different frequencies and energy to separate molecules and sift and release secondary compounds. The technology of ultrasound waves will certainly open broad areas for producing secondary metabolites, especially for the industrial sector.

Conclusion

During the last five decades, plant tissue and cell cultures have become technology from a mere technique of growing tissues aseptically, and various branches developed to a commercially viable industrial level production system such as micropropagation, production of useful metabolites, and a platform to produce new proteins. Several high value primary and secondary metabolites are produced through cell culture technology in spite of unresolved constraints. There is no triggering mechanism for the production of secondary metabolites from cells and their release into the medium so that production can be achieved without sacrifice of the productive cultures. With the development and availability of new tools and techniques, focus has been shifted to study metabolomics and genomics. However, efforts are continuous to unravel the biosynthetic pathways, isolating and transferring the genes involved in biosynthesis and knowing the gene expression by metabolomics and genomics. The ground has become set for combining different approaches to unravel the mystery surrounded around triggering and development of high level of production system.