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In Vitro Cultures for the Production of Secondary Metabolites

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Abstract

Plants' secondary metabolism is an important source of medicinal and industrial products. Even though natural ecosystems are still the most important font of this kind of substance, excessive harvesting of spontaneous flora can act as a direct cause of biodiversity loss. Different technologies are used for *in vitro* production which, in addition to being useful for safeguarding biodiversity, make available to industry substances that are difficult to produce *in vivo*. Moreover, the growing demand for secondary metabolites encourages the use of new biotechnology tools to create new, more productive *in vitro* transgenic plant cultures.

Keywords: medicinal plants, metabolites, vitro, secondary metabolism, medicinal plants, elicitor, cell multiplication

1. Introduction

Several problems might arise when producing secondary metabolites using both spontaneous and cultivated plants or parts of plants. If the material for extraction is collected by spontaneous plants, the major risk is related to the impoverishment of resources and biodiversity, consequently. Although natural ecosystems are usually rich in officinal plants that can be used by humans, an excessive collection of spontaneous flora can act as a direct cause of biodiversity loss [1, 2]. Currently, it is estimated that at least 50,000 plant species are used, which in the majority of cases grow spontaneously, however, sometimes products come from specific cultivation. Based on what was reported by the 2020 edition of the State of the World's Plants and Fungi [3], climate change is threatening two-fifths of the plants currently known; this value is doubled compared to what observed in 2016 and, among these, species are included many medicinal plants used both as a natural remedy and for drug production. According to such data collection that involved 210 scientists and 42 countries, over 140,000 plants should be classified as under extinction threat, including 730 medicinal plants. Among known species, 5500 medicinal plants can be found and approximately 13% of these are under extinction threat [4]. Concerning the most vulnerable plants, we can mention *Brugmansia sanguinea* (Ruiz & Pav.) D. Don, used in medicine to treat cardiovascular disorders, which can only be found as a cultivated plant. Fate similar to

Nepenthes khasiana Hook. f., mostly used to treat skin problems as well as *Warburgia salutaris* (G. Bertol.) Chiov., indicated when respiratory problems occur [3, 5].

1.1 Historical notes

A large number of species belonging to the plant kingdom have always coexisted on Earth, over the years they have created a great heritage of biodiversity. Plants have always been a primary source of sustenance for herbivorous and omnivorous animals including the human species, the latter, however, over time, has realized the possibility of using plant biomass to also obtain substances to be utilized in various effective ways, for example as medication or food supplements.

Western medical culture can be traced back to the Sumerian Nippur tablets of 3000 BC on which the names of medicinal herbs are reported. The first known writing on the subject is a papyrus (1552 BC), dating back to an Egyptian dynasty. It features numerous herbal formulas and, between magic and medicine, even invocations to ward off disease and a catalog of plants, minerals, magical amulets, and useful spells. It is based on more than 500 plants, nearly a third of which are still found in today's Western pharmacopoeias.

The most famous Egyptian physician was Imhotep (Memphis around 2500 BC) whose "materia medica" included practices to reduce head and thoracic trauma, wound care, prevention, treatment of infections, and principles of hygiene.

The first Chinese manual of materia medica, Shennong Ben Cao Jing (Emperor Shennong's classic Materia medica), written in the first century, describes 365 medicines, 252 derived from herbs.

Ancient literature also provided the manuscript "Recipes for fifty-two foods," the longest medical text found in the Chinese tomb of Mawangdui, (168 BC), the Wushi'er Bingfang (9950 characters). It along with others shows the early development of Chinese medicine while subsequent generations have developed Yaoxing Lun, a "Treatise on the Nature of Medicinal Herbs."

Ayurveda is the traditional medicine in India that emphasizes plant-based treatments, hygiene, and the balance of the state of the body. The Indian Materia Medica included knowledge of plants, the place of its growth, the methods of conservation, and the duration of the collected materials; includes also directions for extracting juices, powders, cold infusions, and extracts.

Later in Greece, it was Hippocrates, a philosopher known as the father of medicine, who in 460 BC founded a school focused on the necessity to discover the causes of disease to combat them. His treatises, aphorisms, and prognostics, in addition to describing 265 drugs, supported the importance of diet for the treatment of diseases.

Theophrastus (390–280 BC), a disciple of Aristotle's, historically known as the "father of botany," wrote the treatise *Historia Plantarum*, the first attempt to classify plants and botanical morphology in Greece with details of medicinal herbs and concoctions based on them.

Later Galen, philosopher, physician, pharmacist, and prolific writer of medical matters, collected the medical knowledge of his time in an extensive report and wrote on the structure of organs, the impulse and its association with respiration, arteries, and blood circulation, and the uses of the "Theriac" "In treatises such as on Theriac to Piso, on Theriac to Pamphilus, and on Antidotes, Galen identified in the Teriaca a compound of 64 ingredients, which can be defined as a polypharmaceutical, suitable for treating every known disease." His work rediscovered in the fifteenth century became the authority on medicine and healing for the next two centuries.

The Greek physician Dioscorides treated medical questions in five volumes, entitled *Περὶ ὕλης ἰατρικῆς* in Greek and *De Materia Medica* in Latin; they include about 500 plants and direct observations of the plants and the effects that the various drugs have had on patients. *De Materia Medica* was the first extensive drug system comprising a 1000 natural drugs (products for most basic plants). The classification used by Dioscorides is of an elementary type even if he uses a botanical taxonomy. The books written by Dioscorides on medicinal herbs of history are considered the precursors of the modern pharmacopeia remaining in use until the 1600s.

2. Secondary metabolism

Active principles synthesized through secondary metabolites act as a defense strategy, playing an active role in plant ecophysiology against herbivores, attacks by pathogens but also as a response to abiotic stress, and competition with other plants; at the same time, they play a crucial role in attracting beneficial organisms, such as symbionts and pollinators. Recently, several studies on “secondary metabolism” highlighted additional features related to these molecules, which make them essential for the organism that produces them as they provide useful information on quality and on specific features of a range of raw materials, both of animal and vegetal origin as well as on food produced with them [6, 7]. As a matter of fact, the secondary metabolites pool is often influenced by specific environmental conditions, for instance, in the case of essential oil profile; for this reason, secondary metabolite products in essential oils may provide important support in acquiring valuable information on their origin.

Unlike primary metabolites that are stable in concentration and chemical structure, ensuring cell structural and functional integrity, secondary metabolites show a “high degree of freedom” as far as these aspects are concerned [8, 9].

Due to an enormous diversity in structure and intraspecific variability, biosynthesis in secondary metabolites is limited to definite groups of plants and thus they are not ubiquitous. Synthesis in secondary metabolites was selected when during evolution such compounds managed to respond to specific needs by vegetal organisms [10]. This is the case, for instance, of the variation of scents and colors in flowers to attract pollinators and promote and increase efficiency in pollination [11].

Secondary metabolism-derived molecules are released in the environment through different mechanisms, among others we can mention volatilization that leads to a dispersion of substances such as ethylene and sesquiterpenes that can be absorbed by surrounding plants directly through the soil or atmosphere; lisciviation, instead, promotes the release of substances, such as sugar, amino acids, alkaloids, fatty acids, terpenoids, and phenolic acids, from the aerial part of the plant through hydrosolubility caused by rain or fog. Other mechanisms promoting dispersion are exudation and decomposition.

The activity of substances released also depends on the physiological and nutritional status of plants and environmental abiotic factors, such as light, rain, and temperature [12].

During the nineteenth century, chemists showed interest in the study of secondary metabolism and metabolites, concerning especially drugs, poison, aromatizers, and industrial products, all representing as a whole the final products of metabolic pathways or networks of these; actually, more than 200,000 are known to date.

Recently, potential roles of secondary products at the cell level that have been identified are—plant growth regulation, gene expression modulation, and compounds involved in signal transduction [13, 14]. Hence, while for centuries secondary metabolites have been used in traditional medicine, nowadays, they act as valuable pharmaceutical, cosmetic, chemical compounds, and nutraceuticals in the recent past [15].

2.1 Secondary metabolites in natural environment

Active principles can be divided into three big molecule families based on the biosynthesis pathways from which they are originated—terpenoids and steroids, alkaloids and phenolics [15].

2.1.1 Terpenoids

These are the most recurrent compounds; lipid molecules synthesized starting from acetyl CoA or from glycolysis intermediates reaching a total of 35,000, abundant in essential oils, resins, rubber, volatile molecules, scented, colorless, soluble in oil or highly lipophilic solutions, and inflammable. They function as protectors for wood tissues, exert antibacterial effects, are responsible for insect attraction and repulsion, as well as represent the base material for vegetal hormones or pigments (chlorophyll and carotenoids) synthesis; they also take part in the mitochondrial electron transport and plastoquinone.

2.1.2 Alkaloids

These molecules, which accumulate nitrogen becoming an important source of it, are produced by approximately 20% of plants; more than 20,000 different alkaloids are known and are synthesized principally from amino acids.

They play an important role as an advanced chemical defense system of plants under predators' pressure (larvae, insects, herbivores, mammals). They work as antibiotics and pesticides with a deterrent action to prevent plants from being ingested.

Alkaloids used as drugs, poison, with stimulating and narcotizing effects were used even by Greek and Romans, such as atropine (*Atropa belladonna* L), cocaine (*Erythroxylon coca* Lam. leaves), morphine and opium (*Papaver somniferum* L. fruits), nicotine (*Nicotiana tabacum* L. leaves), and strychnine (*Strychnos nux-vomica* L. seeds).

2.1.3 Phenolic compounds

Secondary plant metabolites belonging to the big family of polyphenols [16], having mostly hydrosoluble characteristics. They represent one of the main classes of secondary metabolites that includes a wide range of highly heterogeneous substances having all in common an aromatic ring. They are formed through the biosynthesis pathway of shikimic or mevalonic acids; a total of 15,000 are known and represent a group of substances easily occurring in superior plants; the most common cinnamic acid derivatives are caffeic, p-Coumaric, ferulic, gallic, and synaptic acids.

Compounds of different colors accumulate especially in aerial plant organs (stems, leaves, flowers, and fruits) rather than in roots; such a preferred location is related to a light-induced effect on phenolic metabolism; besides, phenolic compounds play a protective role against UV that are successfully absorbed and accumulated into leaves epidermis to avoid damage caused to cell DNA [16]. They influence the color,

generally yellow, of flowers and fruits where they can be found as glycosides diluted in cell juice except for anthocyanidines and their glucosides (anthocyanins) that are red, purple, or blue depending on the pH of cell juice [17]. The flavonoids content in plants depends not only on the genotype but is also closely related to environmental conditions especially by light radiation such as UV; the latter, in fact, induces a significant increase of flavonoids in leaves [18, 19].

Flavonoids and phenolic acids are the most important antioxidants in the diet and can be found also in tea, wine, and beer [8].

They are considered pharmacologically active compounds having anti-inflammatory activity, active against liver injury due to hepatotoxicity, and acting as antitumoral, antimicrobials, antivirals, enzyme inhibitors, antioxidants, protect against capillary fragility, as well as playing a role as insect repellents and signaling in plant-organism interactions.

In the recent past, the most common use involving the antioxidant properties has been represented by the “scavenger” activity exerted by a series of enzymes, such as dismutase, superoxide, catalase, glutathione peroxidase; they play a role in halting the radical reaction cascade causing acceleration of cell senescence processes.

Among multiple biological activities exerted by these secondary metabolism molecules, we highlight the role of antioxidants against aging, such as in the case of cocoa (*Theobroma cacao* L.), coffee (*Coffea arabica* L.), tea (*Camellia sinensis* L.). The content of phenolic compounds in vegetal tissues varies based on the species, variety, specific organ considered, physiological status, and pedoclimatic conditions.

The high variety of phenolic structures shows the same amount of function diversification—they can play a role as low molecular weight flower pigments, antibiotics, and anti-UV screens.

Likewise, elicitation on a secondary metabolic pathway by a pathogen can lead to *ex novo* production and accumulation of phytoalexins in a plant. This event is exploited through some biotechnological applications in which elicitors are used to stimulate the production of secondary metabolites.

2.2 Applications in food

Antioxidants can be defined as any substance that is able to delay or significantly inhibit oxidation in a specific substrate even if it shows a really low concentration compared to the oxidable substrate [20]. Nutrition plays a crucial role in ensuring the efficacy of antioxidant enzyme defenses—many essential oligoelements, such as selenium, copper, manganese, and zinc, are involved in the molecular structure or in the catalytic activity of these enzymes. The main antioxidant compounds in food are—ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids, flavonoids.

Over the years, pharmaceutical companies have been focusing on antioxidant compounds from food to promote healthy properties of food as available data show that an increase in oxidant intake from natural sources, specifically from fruit and vegetables, may have a beneficial effect on disease prevention. Their production can be effectively achieved through *in vitro* cultures.

3. Production through *in vitro* culture

Secondary metabolites can be produced *in vivo* from spontaneous or cultivated plants or *in vitro*. Unlike primary metabolites, an accumulation can be detected in

vacuoles, they are not ubiquitous and synthesis depend on the development stage of the plant. Production conventional methods from vegetal tissues include different extracting methodologies through solvents, steam, or supercritical CO₂ [21]. *In vivo* culture refers to plants grown in the natural environment or cultivated in non-sterile conditions. Instead, the definition “*in vitro* culture” refers to the culture of explants, tissues, or isolated cells on the artificial substrate, under controlled conditions, in a sterile environment. In *in vitro* culture, the same metabolites that plants naturally produce can be accumulated through physiological stimulation, stress, or hormones. The development of methodologies such as vegetal tissue culture, enzyme production, and fermentation technologies gave a significant contribution to the production of this kind of molecule [21].

In vitro secondary metabolite production is based on a procedure in two separate phases—mass production and secondary metabolites synthesis. These phases are performed separately and are independent each other; at the same time, they have different requirements and can be optimized separately [22, 23].

Cultures of vegetal tissues (**Figure 1**) or isolated cells (**Figure 2**) are inoculated in sterile conditions starting from explants, such as leaves, stems, meristems, roots, buds, callus (**Figure 3**) both for multiplication and secondary metabolite production. Production can take place in more than one tissue.

3.1 Biomass production

Depending on the species, biomass production can be initiated from an undifferentiated callus or cell suspension. In other cases, sprouts, roots, and somatic embryos can be cultured. Using differentiated tissues or organs is crucial when the requested metabolite is produced in specific plant tissue or organ or also in specialized glands

Figure 1.
Culture of shoots on liquid substrate.

Figure 2.
Cell suspension culture.

Figure 3.
Isolated callus on solidified medium.

such as in the case of essential oils [24, 25]. Although different studies showed efficacy in secondary metabolite production through cultures of differentiated tissues and callus, the technique mostly used is cell suspension [22, 23, 26, 27]. The latter is a culture of cells isolated in a liquid medium that exploits cell totipotency for large-scale production. Each cell, in fact, keeps the biosynthesis ability of the plant and under the right conditions can produce metabolites identical to the ones produced by the mother plant. Furthermore, it can be noticed that cell cultures have greater and faster potential application to the market compared to other production methods [25, 28]. This technique ensures the continuous production of metabolites of interest while offering an elevated quality standard and product uniformity. In addition, it is possible through biotechnology applications to produce new metabolites not synthesized by the mother plant [29, 30]. Currently, different metabolites with an interesting market

value are produced using cell suspension culture, such as taxol [31, 32], resveratrol [33], artemisinin [34], ginsenosides [35], raubasine [36].

Among differentiated tissues, hairy roots should be highlighted as they enable the production of secondary metabolites from a considerable number of plants.

Hairy roots are formed in nature on plants following an infection caused by *Agrobacterium rhizogenes*. This bacterium carries genes that encode phenotypic mutation inducing the formation of hairy roots. After infection, a DNA (T-DNA) segment is transferred in the plant genome through the root-inducing (Ri) plasmid [37].

Agrobacterium can transfer genetic information to plants inducing transformations. Once the infection takes place, a plasmid fragment called T-DNA can be integrated into the plant nuclear DNA where genes are integrated. The composition and organization of T-DNA sequences vary considerably. As some cT-DNA genes show strong growth effects when expressed in other species, they can also influence the growth of natural transformants. However, there is still a need to fully identify the mechanisms through which these genes alter growth models and their regulation by promoters and plant transcription factors [38]. Among the advantages of such a technique, we can mention the high level of cell differentiation, rapid growth, relatively easy production, genetic, and biochemical stability. It should also be taken into consideration the potential accumulation of secondary metabolites in the aerial part of the plant. However, technical problems might arise in cultivation systems for the market [37].

3.2 Immobilization

The process can involve both cells and elicitors. They are bound inside a matrix through trapping, absorption, or covalent bonding. The system must be integrated with an adequate substrate as in the case of cultures of suspended cells, as well as regulating chemical and physical parameters, such as pH and temperature.

In a system of this kind, secondary metabolites must be released by cells in the culture media naturally or through induced secretion. One of the advantages of this methodology is the potential stabilization of a continuous production process through the adoption of a specific system of bioreactors.

4. Substrates

In vitro production includes cultural techniques on explants, tissues, or vegetal cells under controlled conditions supported by a substrate that plays a vital role for plants; in fact, it acts as the “place” where all elements needed for plant survival are located [39]. The explant sometimes represented by the cell alone has to be able to regenerate; thus, the substrate provides it with all substances needed, such as macro and microelements, vitamins, carbon sources, growth regulators, and in the case of solid substrates jellifying agents, usual agar in quantities that can vary between 0.7–0.8 g/L, agarose, and starch.

Generally, the substrate contains mineral elements formed by macro and microelements and an organic component formed by vitamins, amino acids, and other nitrogen components as well as carbohydrates. There are different substrates that can play a specific role in achieving different objectives, as a consequence of the concentration of specific substances contained in them, such as those indicated, for example, in **Table 1**.

Macro and microelements	MS (mg/L)	WPM (mg/L)	B5 (mg/L)	NN (mg/L)
Ammonium nitrate	1650.000	400.000		720.000
Boric acid	6.200	6.200	3.000	10.000
Anhydrous calcium chloride	332.200	72.500	113.24	
Cobalt chloride hexahydrate 6H ₂ O	0.025		0.025	
Tripotassium phosphate	170.000	170.000	130.500	68.000
Potassium iodide	0.830		0.750	
Sodium molybdate 2H ₂ O	0.250	0.250	0.250	0.250
Calcium nitrate		386.000		
Potassium nitrate	1900.000		2500.000	950.000
Ammonium sulfate			134.000	
Iron sulfate·7H ₂ O	27.800	27.800	27.850	27.850
Anhydrous magnesium sulfate	180.700	180.700	122.09	90.340
Manganese sulfate·H ₂ O	16.900	22.300	10.000	18.940
Potassium sulfate		990.000		
Copper sulfate 5H ₂ O	0.025	0.250	0.025	0.025
Zinc sulfate 7H ₂ O	8.600	8.600	2.000	10.000
VITAMINS				
Folate				0.500
Nicotinic acid	0.500	0.500	1.000	5.000
Biotin				0.050
Myo-inositol	100.000	100.000	100.000	100.000
Pyridoxine·HCl	0.500	0.500	1.000	0.500
Thiamine·HCl	0.100	1.00	10.000	0.500
OTHER ADDITIVES				
Disodium EDTA (·2H ₂ O)	37.260	37.300	37.250	37.250
Glycine	2.000	2.000		2.000

MS = Murashige & Skoog [40]; WPM = woody plant medium [41]; B5 [42]; NN = Nitsch & Nitsch [42].

Table 1.
Composition of main substrates for in vitro culture.

The choice of an appropriate substrate should be based on the following [43]:

- the type of ions contained
- macroelements balance
- total ionic concentration of medium

Microelements are used in small quantities; lack of such elements causes specific symptoms as they intervene in plant metabolism; they are integrated into enzymes.

Some microelements can influence the production of secondary metabolites, acting as elicitors [44].

Hormones carry out an essential role as growth regulators in plants [45, 46]. The need to add growth regulators to substrates is based on the fact that normal tissues or small organs placed *in vitro* are not able to synthesize a sufficient quantity of them.

Among known hormones mostly utilized we can find:

1. Auxin: Natural auxin is Indole-3-acetic acid (IAA, 3-IAA); in substrates for *in vitro* culture mostly synthetic compounds are used with an auxin-like function such as:

- IBA (Indole-3-butyric acid), the most commonly used;
- NAA (1-Naphthaleneacetic acid);
- 2,4 D (2,4-Dichlorophenoxyacetic acid).

Natural auxins are added in variable quantities (0.01–10 mg/l) and the synthetic ones are added in quantities between 0.001 and 10 mg/l, determining—elongation and tissue distension, cell division, adventitious roots formation [47, 48].

2. Cytokines: Natural cytokinins are as follows:

- Kinetin (N6-Furfuryladenine, 6-Furfurylaminopurine)
- Zeatin [6-(4-Hydroxy-3-methylbut-2-enylamino)purine]
- 2Ip [N6-(2-Isopentenyl)adenine]

Cytokines are used in concentrations between 1 and 10 mg/l to stimulate cell division, stimulate adventitious buds production from tissues or from callus, and growth of somatic embryos, to induce the development of axillary buds. In addition, cytokines inhibit root development [49].

3. Gibberellins: Among gibberellins, the most used is GA3 (gibberellic acid) which promotes internode elongation, meristem, and bud development while inhibiting the formation of roots; thus, it is employed in subsequent phases after planting [50, 51].

pH: Another factor essential for a cultural substrate is pH as its value influences—salt solubility, elements absorption, and substrate solidification; for these reasons, the pH range is quite limited ranging from 5.2 to 5.8. As far as secondary metabolites are concerned, optimal ranges are established both for pH and temperature according to the cultured species.

5. Elicitors

To achieve secondary metabolite production, elicitation is one of the most important strategies and is used to increase productivity; it takes place through the addition

Figure 4.
Classification of elicitor based on different features.

of compounds called elicitors—they can be defined as stress-inducing compounds that induce or improve biosynthesis of specific compounds when a specific amount is applied to a living system [52, 53].

Elicitors can be biotic, such as jasmonic acid, hydrolyzed casein, cellulase, macerozyme, yeast extract, fungal extract, chitin; in addition, chemical compounds usually synthesized from pathogens; abiotic elicitors that include nonorganic substances and can be divided into physical, chemical, and hormonal factors (**Figure 4**) [53, 54].

6. Growth curve

In cell suspension, depending on environmental parameters and on bioreactor features, the development of cells cultured on the liquid substrate is based on specific phases illustrated in **Figure 5**. The graph shows time (horizontal axis) and cell number (vertical axis). At the beginning a slow-growth phase is shown, known as lag phase followed by a phase in which cell concentration grows based on a logarithmic scale,

Figure 5.
Growth curve of a cell suspension culture.

log phase; then a second slow-growth phase occurs followed by a phase in which the culture is numerically constant indicated as a plateau or steady state.

During the latency phase, reduced growth and an accumulation of substances useful for cell development occur, while during the exponential growth phase a considerable biomass increase can be observed. In a discontinuous culture, in the case that cells accumulate metabolites in vacuoles, the biomass is removed at the end of the exponential phase; during the stationary phase a balance occurs between new cells and dead cells, then secondary metabolites are excreted in culture media. In this case, the collection is carried out by replacing from time to time or continuously the culture media.

7. Production increase

In the production of high-value secondary metabolites, a good strategy is offered by the use of technologies that ensure elevated yield and stable over time. It should be underlined that the production of secondary metabolites from plants is genotype-dependent and this fact influences both metabolite type and quantity. Mother plants can be selected to a first selection to identify plants that ensure also *in vivo* a higher production of compound needed. Once the *in vitro* culture is stabilized, both from cells and organs, hyperproductive lines can be selected [23]. Selection is carried out through *in vitro* growth analysis on cell lines or organs, evaluating the multiplication degree but also assessing the quantity and quality of metabolite produced through chromatography and spectroscopic analysis [23].

The output can also be increased through conventional systems or metabolic engineering methodologies [22, 55].

7.1 Biosynthesis pathways

By using metabolic engineering, the biosynthesis pathways can be studied more efficiently [56, 57] through studying gene overexpression that alters pathways. The study design includes analysis of enzyme reactions and biosynthesis processes at genetic, transcriptomic, and proteomic levels; in addition, it is also studied the manipulation of genes that encode critical enzymes and those that regulate the speed in the biosynthesis pathways [58, 59]. However, to date this system is limited to experimental settings and no method has been identified yet for industrial transfer of such methodology. Currently, the study of the biosynthesis pathway in phenylpropanol seems to be one of the most promising given that this substance is involved in the biosynthesis of different secondary metabolites in plants [60, 61].

8. Conventional technologies

Culture parameters are among the factors that mostly influence secondary metabolite production—substrate composition both in terms of mineral and organic compounds; pH; characteristics of cell inoculation; physical parameters, such as temperature, light intensity, duration, shaking, and aeration [22, 23, 27]. The substrate should be selected based on the requirements of plant species. Each substrate parameter can be modified to better adjust to the species and to metabolites to be obtained by it—salt type and concentration, carbon source, growth regulators. In nature,

secondary metabolites production is in response to environmental stimuli, or for defensive purposes. This mechanism can be simulated in the laboratory through the modification of the culture parameters, for example, light, temperature, or through the use of substances called elicitors. To elicitors belong both organic and inorganic molecules, such as methyl jasmonate, salicylic acid, microbial cell wall extracts (e.g., yeast extract, chitosan), inorganic salts, heavy metals, physical agents (e.g., UV radiation) among others (**Tables 2 and 3**).

Aid to the production of new secondary metabolites, or increased production of those already known and used, can come from new technologies, such as transgenic

Elicitor	Plant species	Culture	Compound	References
Ozone (O ₃)	<i>Melissa officinalis</i>	Shoot	Rosmarinic acid	[62]
	<i>Hypericum perforatum</i>	Cell suspension	Hypericin	[63]
	<i>Pueraria thomsnii</i>	Cell suspension	Puerarin	[64]
pH	<i>Bacopa monnieri</i>	Shoot	Bacoside A	[65, 66]
	<i>Withania somnifera</i>	Hairy root	Withanolide A	[67]
	<i>Withania somnifera</i>	Cell suspension	Withanolide A	[68]
Sucrose	<i>Hypericum adenotrichum</i>	Seedling	Hypericin and pseudohypericin	[69]
	<i>Corylus avellana</i>	Cell suspension	Paclitaxel	[70]
	<i>Bacopa monnieri</i>	Shoot	Bacoside A	[65, 66]
	<i>Withania somnifera</i>	Cell suspension	Withanolide A	[68]
Ultraviolet C	<i>Vitis Vinifera</i>	Cell suspension	Stilbene	[71]
Proline	<i>Stevia rebaudiana</i>	Callus and suspension	Steviol glycoside	[72]
Polyethylene glycol	<i>Stevia rebaudiana</i>	Callus and suspension	Steviol glycoside	[72]
	<i>Hypericum adenotrichum</i>	Seedling	Hypericin and pseudohypericin	[69]
Jasmonic acid	<i>Bacopa monnieri</i>	Shoot	Bacoside A	[73]
	<i>Plumbago indica</i>	Hairy root	Plumbagin	[74]
	<i>Plumbago rosea</i>	Cell suspension	Plumbagin	[75]
Methyl jasmonate	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinone	[76]
	<i>Perovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[77]
	<i>Vitis vinifera</i>	Cell suspension	Stilbene	[71]
	<i>Bacopa monnieri</i>	Shoot	Bacoside	[73]
	<i>Salvia officinalis</i>	Shoot	Diterpenoid	[78]
	<i>Silybum marianum</i>	Cell suspension	Silymarin	[79]
	<i>Salvia castanea</i>	Hairy root	Tanshinone	[80]
	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[81]
	<i>Withania somnifera</i>	Hairy roots	Withanolide A, withanone, and withaferin A	[82]

Elicitor	Plant species	Culture	Compound	References
	<i>Andrographis paniculata</i>	Cell suspension	Andrographolide	[83]
	<i>Vitis vinifera</i>	Cell suspension	trans-Resveratrol	[84]
	<i>Taverniera cuneifolia</i>	Root	Glycyrrhizic acid	[85]
Gibberellic acid	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinones	[86]
	<i>Echinacea pupurea</i>	Hairy root	Caffeic acid derivatives	[87]
Salicylic acid	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinone	[76]
	<i>Vitis vinifera</i>	Cell suspension	Stilbene	[71]
	<i>Digitalis purpurea</i>	Shoot	Digitoxin	[88]
	<i>Hypericum hirsutum</i>	Shoot	Hypericin and pseudohypericin	[89]
	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[81]
	<i>Withania somnifera</i>	Hairy root	Withanolide A, withanone, and withaferin A	[82]
	<i>Datura metel</i>	Root	Hyoscyamine and scopolamine	[90]
	<i>Glycyrrhiza uralensis</i>	Adventitious root	Glycyrrhizic acid	[91]
Sodium salicylate	<i>Salvia officinalis</i>	Shoot	Carnosol	[92]
Sodium chloride	<i>Catharanthus roseus</i>	Embryogenic tissues	Vinblastine and vincristine	[93]
Sorbitol	<i>Perovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[77]
Silver (Ag)	<i>Perovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[77]
	<i>Vitis vinifera</i>	Cell suspension	Resveratrol	[94]
	<i>Salvia castanea</i>	Hairy root	Tanshinone	[80]
	<i>Datura metel</i>	Hairy root	Atropine	[95]
Cadmium (Cd)	<i>Vitis vinifera</i>	Cell suspension	Resveratrol	[94]
	<i>Datura stramonium</i>	Root	Sesquiterpenoid	[96]
Cobalt (Co)	<i>Vitis vinifera</i>	Cell suspension	Resveratrol	[94]
Copper (Cu)	<i>Ammi majus</i>	Shoot	Xanthotoxin	[97]
	<i>Bacopa monnieri</i>	Shoot	Bacoside	[73]
	<i>Datura stramonium</i>	Root	Sesquiterpenoid	[96]

Table 2.
Abiotic elicitors.

cultures. Several works have demonstrated the safety of these technologies, and their effectiveness, at low cost, for the production of secondary metabolites for medicine and industry [103].

Elicitor	Plant species	Culture	Compounds	References
Chitin	<i>Hypericum perforatum</i>	Shoot	Hypericin and pseudohypericin	[98]
	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[99]
	<i>Vitis vinifera</i>	Cell suspension	trans-Resveratrol and viniferins	[83]
Pectin	<i>Hypericum perforatum</i>	Shoot	Hypericin and pseudohypericin	[98]
Dextran	<i>Hypericum perforatum</i>	Shoot	Hypericin and pseudohypericin	[98]
Yeast extract	<i>Perovskia abrotanoides</i>	Adventitious roots	Cryptotanshinone and tanshinone IIA	[77]
	<i>Plumbago rosea</i>	Cell suspension	Plumbagin	[75]
	<i>Silybum marianum</i>	Cell suspension	Silymarin	[79]
<i>Trichoderma atroviride</i>	<i>Salvia miltiorrhiza</i>	Hairy root	Tanshinone	[100]
<i>Protomyces gravidus</i>	<i>Ambrosia artemisiifolia</i>	Hairy root	Thiarubrine A	[101]
<i>Claviceps purpurea</i>	<i>Azadirachta indica</i>	Hairy root	Azadirachtin	[102]
<i>Mucor hiemalis</i>	<i>Taverniera cuneifolia</i>	Root	Glycyrrhizic acid	[85]
<i>Fusarium oxysporum</i>	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[99]
<i>Phoma exigua</i>	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[99]
<i>Botrytis cinerea</i>	<i>Hypericum perforatum</i>	Cell suspension	Phenylpropanoid and naphthodianthrone	[99]
<i>Aspergillus niger</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[55]
<i>Saccharomyces cerevisiae</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[55]
<i>Agrobacterium rhizogenes</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[55]
<i>Bacillus subtilis</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[55]
<i>Escherichia coli</i>	<i>Gymnema sylvestre</i>	Cell suspension	Gymnemic acid	[55]
	<i>Datura metel</i>	Hairy root	Atropine	[95]
<i>Bacillus cereus</i>	<i>Datura metel</i>	Hairy root	Atropine	[95]
<i>Staphylococcus aureus</i>	<i>Datura metel</i>	Hairy root	Atropine	[95]
<i>Rhizobium leguminosarum</i>	<i>Taverniera cuneifolia</i>	Root	Gymnemic acid	[85]

Table 3.
Biotic elicitors.

9. Potential applications in agriculture

Focusing on biodiversity can be useful to strengthen food security and human nutrition aiming at promoting general sustainable development. Traditional crops represent an important biodiversity source and carry out a key role in preserving the identity of specific production areas as well the consumer behavior and transfer of cultural heritage to next generations. However, these cultures and foods require to be preserved from genetic erosion that can determine tragic effects on biodiversity, environmental sustainability, and rural economies.

As a matter of fact, this methodology based exclusively on a phenotypic evaluation does not allow to easily distinguish between genotype and effects on the environment. Recent methodologies based on gene markers enable us to identify species, cultivars, and autochthone varieties easily and rapidly.

Elevated costs and technical problems that might arise when the relationship between phenotype features and gene expression is studied, make the application of these methodologies often difficult. Recently, secondary metabolite analysis has been proposed as a crucial tool to identify a specific species; the metabolic profile, in fact, can lead to the identification of a huge quantity of local autochthone varieties, acting against globalization of agriculture production and being at the same time a tool to identify metabolites useful in traditional project characterization.

10. Importance of modern biotechnologies secondary metabolites production

The parts of plants to be used for therapy, nutrition, and other activities can be obtained from spontaneous or cultivated plants; the choice of production method is mostly determined by economic factors is affordable to collect spontaneous plants when abundant and costs are relatively low, however, in case of high collection costs and lack of spontaneous plants, cultivation can be less expensive [17]. Furthermore, a lot of spontaneous plants are collected without any control and are currently under extinction threat; just a small percentage is cultivated [104]—all these factors are of concern due to the decrease and loss of gene diversity and environmental degradation. Advantages of open field cultivations are related not only to the fact that they give a solution to a lack of vegetal material available in nature, but also to the fact that the wild plant often offers a highly heterogeneous which might be at the same time inadequate in terms of continuous supply and quality standards. Production of secondary metabolites from cell cultures is a valuable option for molecules that have elevated extraction costs and low output from plant material coming from cultivation [105, 106].

For these reasons and due to the current increased demand for natural food products and drugs of natural origin, the employment of biotechnological artificial culture systems might be a good alternative to conventional cultivations for *in vitro* production of secondary metabolites as well as a viable option to replace industrial biosynthesis products. These issues, together with the need to increase the production of plant materials with uniform quality standards, are encouraging pharmaceutical companies to innovate research aiming also at gene and cell technologies indicated as biotechnologies [107].

On one hand, *in vitro* cultivation systems give us the chance to exploit cells, tissues, organs, or organisms as a whole also through gene manipulation to obtain desired compounds [25]; on the other hand, they play a potential role in terms of large-scale productions, production from secondary metabolic pathways.

Plant tissue culture is based on the principle that the same substances found in nature inside an organ, a fruit, or other plant tissues can be induced to accumulate in undifferentiated cells while keeping gene information and the ability to produce that range of active principles detected in the mother plant [108].

Multiple factors influencing *in vitro* secondary metabolite synthesis can be found: type of raw material, environmental and climate conditions, culture media, the quantity of carbohydrates contained that influences biomass, type and quantity of hormones, light (optimal light quantity and intensity is a prerequisite for maximum expression of metabolites), temperature. Substrate composition strongly influences secondary metabolite production, especially for what concerns salt and growth regulators besides subsequent glucose addition that might increase accumulation if compared to cultures in which a fix concentration is used [109].

Plant cell cultures are defined also as “chemical factories for secondary metabolites” [25] and represent to date a viable alternative to the cultivation of pharmaceutical plants both from *in vitro* and non *in vitro* origin.

The most important reason for pharmaceutical companies to obtain valuable secondary metabolites in this way is due to the fact that conventional cultivations in fields of pharmaceutical plants of some species are time-consuming, expensive, and generate a reduced output.

Some large-scale protocols of productions for the market have been set up for extractions of berberine, shikonin, and *Ginseng* saponins [25, 109] by using bioreactors. Berberine is produced *in vitro* from two members of *Ranunculaceae* (*Thalictrum minus* and *Coptis japonica*); shikonin is produced *in vitro* from *Lithospermum erythrorhizon* in quantities 800 times higher than quantities obtained from plant roots; saponins are produced *in vitro* from *Panax ginseng*.

Further research was performed on other secondary metabolites such as flavoring agents (i.e., vanillin produced in bioreactors from calluses explanted from *Vanilla planifolia* by the company ESCAgenetic Corporation—San Carlos—CA, USA), food coloring (e.g., anthocyanins from *Euphorbia milii*), drugs (e.g., taxol), different essential oils and natural insect repellents [25].

11. Conclusions

Although for the production of food from plants there is an increasing tendency toward natural agriculture, in the production of substances intended for industry, in particular the medicinal industry, a cultivated or spontaneous plant cannot always guarantee a constant and high-quality product. Pollution problems, climate change, and the political unsafe of some harvesting and cultivation areas also make production uncertain. In this situation, the production of secondary metabolites *in vitro* ensures a safe and constant making of the substances of interest.

New technologies, always evolving, can give an even greater push toward *in vitro* culture, since they guarantee safe products, at lower costs, often difficult to obtain in nature.

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Conflicts of interest

The authors declare no conflict of interest.

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