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Secondary Metabolites

Sources and Applications

Edited by Ramasamy Vijayakumar and Suresh S.S. Raja

SECONDARY METABOLITES - SOURCES AND APPLICATIONS

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Meet the editors

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Contents

Preface

Metabolites are the intermediate products of metabolism. Metabolites have various functions, including fuel, structure, signaling, stimulatory and inhibitory effects on enzymes, catalytic activity of their own, defense, and interactions with other organisms. A primary metabolite is directly involved in normal growth, development, and reproduction. A secondary metabolite is not directly involved in those processes but usually has an important ecological function. They are found in microorganisms, plants, and animals. Herbal plants, animals, and microorganisms such as bacteria, actinobacteria, cyanobacteria, fungi, and algae have attracted more attention in research that has led to the discovery of secondary metabolites. The exploration of secondary metabolites from various resources subsequently led to the development of drugs for the treatment of human diseases of microbial origin. Routine screening of natural resources will introduce novel secondary metabolic products with a high pharmaceutical value. Thus, this book is very apt and needed.

This book consists of an introductory overview of secondary metabolites, which are classified into four main sections: microbial secondary metabolites, plant secondary metabolites, secondary metabolites through tissue culture technique, and regulation of secondary metabolite production. This book provides a comprehensive account on the secondary metabolites of microorganisms, plants, and the production of secondary metabolites through biotechnological approach like the plant tissue culture method. The regulatory mechanisms of secondary metabolite production in plants and the pharmaceutical and other applications of various secondary metabolites are also highlighted. The 8 chapters have been contributed by the authors around the world, including Austria, Portugal, Malaysia, the United Kingdom, India, Sri Lanka, and the United States.

This book is considered as necessary reading for microbiologists, biotechnologists, biochemists, pharmacologists, and botanists who are doing research in secondary metabolites. It should also be useful to MSc students, MPhil and PhD scholars, scientists, and faculty members of various science disciplines. We are thankful to all the contributors for their valuable work. We offer our special thanks and appreciation to Ms. Ivana Glavic, Publishing Process Manager/Author Service Manager, for her encouragement and help in bringing out the book in the present form.

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Section 1

Microbial Secondary Metabolites

Chapter 1

An Introductory Chapter: Secondary Metabolites

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Additional information is available at the end of the chapter

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1. Introduction

The metabolism can be defined as the sum of all the biochemical reactions carried out by an organism. Metabolites are the intermediates and products of metabolism and are usually restricted to small molecules. The term "secondary" introduced by A. Kossel in 1891 implies that while primary metabolites are present in every living cell capable of dividing, the secondary metabolites are present only incidentally and are not of paramount significance for organism's life. Though secondary metabolites are derived from primary metabolism, they do not make up basic molecular skeleton of the organism. Its absence does not immediately curtail the life of an organism, a feature contrary to primary metabolite, but survival of the organism is impaired to a larger extent. Its presence and synthesis are observed in ecologically disadvantaged species within a phylogenetic group [1].

The difference between primary and secondary metabolite is ambiguous since many of the intermediates in primary metabolism is overlapping with the intermediates of secondary metabolites [2]. Amino acids though considered a product of primary metabolite are definitely secondary metabolite too. Contrary to the observation that sterols are secondary metabolites that are indispensable part of many structural framework of a cell. The mosaic nature of an intermediate indicates common biochemical pathway being shared by primary and secondary metabolism [3]. The secondary metabolites serve as a buffering zone into which excess C and N can be shunted into to form inactive part of primary metabolism. The stored C and N can revert back to primary metabolite by the metabolic disintegration of secondary metabolite when on demand. There is dynamism and a delicate balance between the activities of the primary and secondary metabolism (**Figure 1**) being influenced by growth, tissue differentiation and development of the cell or body, and also external pressures [4].

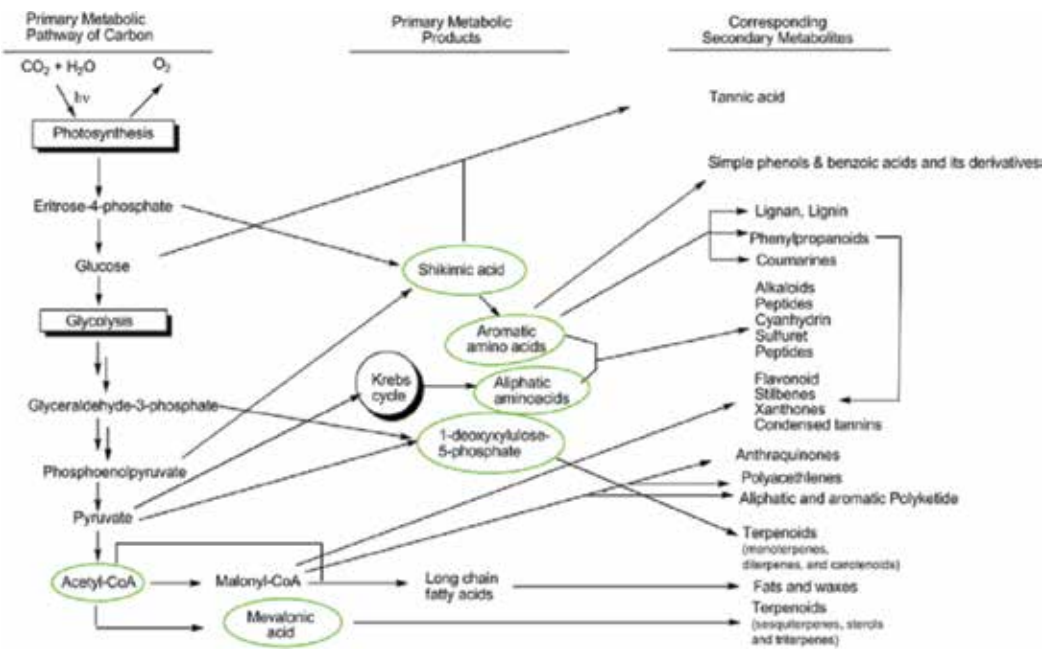

Figure 1. Schematic diagram representing integration of primary and secondary metabolism.

Hence, secondary metabolites or natural products can be defined as a heterogeneous group of natural metabolic products that are not essential for vegetative growth of the producing organisms, but they are considered differentiation compounds conferring adaptive roles, for example, by functioning as defense compounds or signaling molecules in ecological interactions, symbiosis, metal transport, competition, and so on [5]. The multitude of secondary metabolite secretions is harvested by human kind to improve their health (antibiotics, enzyme inhibitors, immunomodulators, antitumor agents, and growth promoters of animals and plants), widen the pyramid of healthy nutrition (pigments and nutraceuticals), enhancing agricultural productivity (pesticides, insecticides, effectors of ecological competition and symbiosis and pheromones), and hence impacting economics our society in a certain positive way. They are a source of antibiotics.

2. Classification of secondary metabolites

Over 2,140,000 secondary metabolites are known and are commonly classified according to their vast diversity in structure, function, and biosynthesis. There are five main classes of secondary metabolites such as terpenoids and steroids, fatty acid-derived substances and polyketides, alkaloids, nonribosomal polypeptides, and enzyme cofactors [6].

2.1. Terpenoids and steroids

They are major group of substances derived biosynthetically from isopentenyl diphosphate. Currently, over 35,000 known terpenoid and steroid compounds are identified. Terpenoids

have different variety of unrelated structures, while steroids have a common tetracyclic carbon skeleton and are modified terpenoids that are biosynthesized from the triterpene lanosterol.

2.2. Alkaloids

There are over 12,000 known compounds of alkaloids, and their basic structures consist of basic amine group and are derived biosynthetically from amino acids.

2.3. Fatty acid-derived substances and polyketides

Around 10,000 compounds are identified and are biosynthesized from simple acyl precursors such as propionyl CoA, acetyl CoA, and methylmalonyl CoA.

2.4. Nonribosomal polypeptides

These amino acids derived compounds are biologically synthesized by a multifunctional enzyme complex without direct RNA transcription.

2.5. Enzyme cofactors

Enzyme cofactors are nonprotein, low-molecular enzyme component [6].

3. Functions of secondary metabolites

The major functions of the secondary metabolites including antibiotics are:

(i) competitive weapons against other livings such as animals, plants, insects, and microorganisms

(ii) metal transporting agents

(iii) agents for symbiotic relation with other organisms

(iv) reproductive agent and

(v) differentiation effectors

(vi) agents of communication between organisms

The other functions include interference in spore formation (not obligatory) and germination [5]. Predominantly, the secondary metabolites are used for variety of biological activities like antimicrobial and antiparasitic agents, enzyme inhibitors and antitumor agent, immunosuppressive agents, etc. [7].

4. Sources of secondary metabolites

The major sources of secondary metabolites are plants (80% of secondary metabolite), bacteria, fungi, and many marine organisms (sponges, tunicates, corals, and snails) (**Table 1**) [8].

Source	All known compounds	Bioactives	Antibiotics
Natural products	Over one million	200,000–250,000	25,000–30,000
Plant kingdom	600,000–700,000	150,000–200,000	~25,000
Microbes	Over 50,000	22,000–23,000	~17,000
Algae, lichens	3000–5000	1500–2000	~1000
Higher plants	500,000–600,000	~100,000	10,000–12,000
Animal kingdom	300,000–400,000	50,000–100,000	~5000
Protozoa	Several hundreds	100–200	~50
Invertebrates	~100,000	NA	~500
Marine animals	20,000–25,000	7000–8000	3000–4000
Insects/ worms/ *etc.*	8000–10,000	800–1000	150–200
Vertebrates (mammals, fishes, amphibians, *etc.*)	200,000–250,000	50,000–70,000	~1000

NA – Data Not Available.
Source: Bérdy [8].

Table 1. Approximate number of known natural metabolites.

4.1. Secondary metabolites of plants

Plant secondary metabolites represent highly economically valuable products. These are used as high value chemicals such as drugs, flavors, fragrances, insecticides, dyes, etc. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found to have *in vitro* antimicrobial properties. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives [9]. About 25,000 terpenoids are known as secondary compounds and are derived from the five-carbon precursor isopentenyl diphosphate (IPP). In total, around 12,000 known alkaloids are identified, and they possess one or more nitrogen atoms which are biosynthesized from amino acids. The 8000 known phenolic compounds are synthesized either through the shikimic acid pathway or through the malonate/acetate pathway [10].

Many alkaloids are used in medicine, usually in the form of salts. Some examples include vinblastine which has antitumor properties [11]; quinine which has antipyretics and antimalarial properties [12]; and reserpine which can be used to treat high blood pressure. Alkaloids are regarded as reserve materials for protein synthesis, as protective substances discouraging animal or insect attacks, and as plant stimulants or regulators or simply as detoxification products. Alkaloids currently in clinical use include the analgesics morphine and codeine, the anticancer agent vinblastine, the gout suppressant colchicine, the muscle relaxant tubocurarine, the antiarrhythmic ajmalicine, the antibiotic sanguinarine, and the sedative scopolamine.

In vitro studies have shown that natural phenols have antimicrobial [13], antiviral [14], anti-inflammatory [15], and vasodilatory actions [16]. It protects the plant against adverse factors

which threaten its survival in an unfavorable environment, such as drought, physical damage or infections. Resistance of plants to UV radiations is due to the phenolic compounds especially the phenylpropanoids present in them [17]. Phenolic compounds act as antioxidants protecting cells from oxidative stress scavenging of free radicals by hydrogen atom donation. The action of phenolic as neuroprotective [18], fungicidal [19], bactericidal [20] compounds and their anti-atherosclerosis [21] effects, and anticancer [22] activity is well documented.

Terpenoids are commercially important fragrance and flavoring agents [23]. Prenol and α-bisabolol are used in fragrance due to fruity odor and sweet floral aroma, respectively. Mono and sesqui terpenes are basis of natural perfumes and also of spices and flavorings in the food industry. The roles of terpenoids as pharmaceutical agents with activities such as antibacterial and antineoplastic are still under investigation. There are examples of diterpenes that exhibited *in vitro* cytotoxic, antitumor, and antimicrobial activities. Terpenes are vital for life in most organisms exerting metabolic control and mediating inter and intra species interactions, for example, manufacture compounds in response to herbivory or stress factors, and it has also been shown that flowers can emit terpenoids to attract pollinating insects and even attract beneficial mites, which feed on herbivorous insects. Cheng et al. [24] have reported that terpenes may act as chemical messengers influencing the expression of genes involved in plant defensive functions or influence gene expression of neighboring plants. Other secondary metabolite of plant origin and their functions is given in **Table 2** [25].

4.2. Production of secondary metabolites from plants

4.2.1. Conventional

The conventional method of secondary metabolite production relies on extraction of metabolite, not production, from the tissues of plant by different phytochemical procedures like solvent, steam, and supercritical extraction. The recent developments in biotechnological methods like plant tissue culture, enzyme and fermentation technology have facilitated *in vitro* synthesis and production of plant secondary metabolites. The major processes include:

4.2.2. Immobilization

Cell or biocatalysts are confined within a matrix by entrapment, adsorption or covalent linkage. On addition of suitable substrate and provision on optimum physico chemical parameters, the desired secondary metabolites are synthesized. Immobilization with suitable bioreactor system provides several advantages, such as continuous process operation, but for the development of an immobilized plant cell culture process, natural or artificially induced secretion of the accumulated product into the surrounding medium is necessary.

4.2.3. In vitro tissue, organ, and cell culture

Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots, meristems, etc., both for multiplication and extraction of secondary metabolites. Shoot, root, callus, cell suspension, and hairy root culture are used to synthesize metabolite of interest. Metabolites which are localized in multiple tissues

S. No.	Secondary metabolites	Biological activity
1.	Pyrethrins	Insecticidal
2.	Nicotine	Insecticidal
3.	Rotenoids	Insecticidal
4.	Azadirachtin	Insecticidal
5.	Phytoecdysones	Insecticidal
6.	Baccharine	Antineoplastic
7.	Bruceantine	Antineoplastic
8.	Gsaline	Antineoplastic
9.	3-Doxycolchicine	Antineoplastic
10.	Ellipticine	Antineoplastic
11.	9-methoxyellipticine	Antineoplastic
12.	Fagaronive	Antineoplastic
13.	Tlarringtovinl	Antineoplastic
14.	Jandicine N-oxide	Antineoplastic
15.	Maytansive	Antineoplastic
16.	Podophyllotoxin	Antineoplastic
17.	Taxol	Antineoplastic
18.	Thalicarpine	Antineoplastic
19.	Tripdiolide	Antineoplastic
20.	Vinblastin	Antineoplastic
21.	Quinine	Antimalarial
22.	Digoxin	Cardiac tonic
23.	Diosgunin	Antifertility
24.	Morphine	Analgesic
25.	Thebaine	Source of codeine
26.	Suolpolanine	Antihypertension
27.	Alropine	Muscle relaxant
28.	Codeine	Analgesic
29.	Shikonin	Dye, pharmaceutical
30.	Anthroquinones	Dye, laxative
31.	Rosamarinic acid	Spice, antioxidant, perfume
32.	Jasmini	Sweetner
33.	Stevioside	Saffron
34.	Croun	Chili

S. No.	Secondary metabolites	Biological activity
35.	Capsacin	Vanilla
36.	Vanillin	Rubber
37.	Gutla percha	Essential oils
38.	Terpendids	Spasmolytic
39.	Papaverive	Hypertensive
40.	Ajmalicive	Stimulant
41.	Caffeine	Antispasmadic
42.	Birberine	NA

NA – Not Assessed.
Source: Ramawat and Merillon [25].

Table 2. Biological activities of some secondary metabolites of plants.

can be synthesized through unorganized callus or suspension cultures. But when the metabolite of interest is restricted to specialized part or glands in host plant, differentiated microplant or organ culture is the method of choice. Saponins from ginseng are produced in its roots, and hence *in vitro* root culture is preferred for saponin synthesis. Similarly, antidepressant hypericin and hyperforin are localized in foliar glands of *Hypericum perforatum*, which have not been synthesized from undifferentiated cells [26].

The quantum of secondary metabolite production in cell cultures can be enhanced by treating plant cells with biotic and/or abiotic elicitors. Methyl jasmonate, fungal carbohydrates, and yeast extract are the commonly used elicitors. Methyl jasmonate is an established and effective elicitor used in the production of taxol from *Taxus chinensis* [27] and ginsenoside from *Panax ginseng* [28–32]. The most recently evolved and designed metabolic engineering can be employed to improve the productivity.

The production of metabolites through hairy root system based on inoculation with *Agrobacterium rhizogenes* has garnered much attention of late. The quality and quantity of secondary metabolite by hairy root systems is same or even better than the synthesis by intact host plant root [33]. In addition, stable genetic make up, instant growth in plant tissue culture media san phytohormones provides additional scope for biochemical studies. Root tips infected with *A. rhizogenes* are grown on tissue culture media [Murashige and Skoog's (MS) Gamborg's B5 or SH media] lacking phytohormones. Srivastava and Srivastava [34] have recently summarized the attempts to adapt bioreactor design to hairy root cultures; stirred tank, airlift, bubble columns, connective flow, turbine blade, rotating drum, as well as different gas phase reactors have all been used successfully. Genetic manipulation in hairy root culture for secondary metabolite production is being tried out. The established roots are screened for higher growth and production of metabolites. Transgenic hairy roots generated though *Agrobacterium rhizogenes* have not only paved way for plantlet generation but also for synthesis of desired product through transgenic hairy root cultures.

4.3. Secondary metabolites of microorganisms

Microbial secondary metabolites are low molecular mass products with unusual structures. The structurally diverse metabolites show a variety of biological activities like antimicrobial agents, inhibitors of enzymes and antitumors, immune-suppressives and antiparasitic agents [7], plant growth stimulators, herbicides, insecticides, antihelmintics, etc. They are produced during the late growth phase of the microorganisms. The secondary metabolite production is controlled by special regulatory mechanisms in microorganisms, as their production is generally repressed in logarithmic phase and depressed in stationary growth phases. The microbial secondary metabolites have distinctive molecular skeleton which is not found in the chemical libraries and about 40% of the microbial metabolites cannot be chemically synthesized [35].

4.3.1. Features of microbial secondary metabolites

- The principle and process of natural fermentation product synthesis can be successfully scaled up and employed to maximize its application in the field of medicine, agriculture, food, and environment.
- The metabolite can serve as a starting material for deriving a product of interest, extended further through chemical or biological transformation.
- New analog or templates in which secondary metabolite serve as lead compounds will lead discovery and design of new drugs.

4.4. Applications of microbial secondary metabolites

4.4.1. Antibiotics

The discovery of penicillin initiated the researchers for the exploitation of microorganisms for secondary metabolite production, which revolutionized the field of microbiology [5]. With the advent of new screening and isolation techniques, a variety of β-lactam-containing molecules [36] and other types of antibiotics have been identified. About 6000 antibiotics have been described, 4000 from actinobacteria (**Table 3**). In the prokaryotic group, unicellular bacteria *Bacillus* (**Table 3**) and *Pseudomonas* (**Table 3**) species are the most recurrent antibiotic producers. Likewise in eukaryotes, fungi are dominant antibiotic producers next to plants (**Table 3**). In the recent years, myxobacteria and cyanobacteria species have joined these distinguished organisms as productive species.

The pharmaceutical product, especially anti-infective derivatives comprise 62% antibacterials, 13% sera, immunoglobulins, and vaccines, 12% anti-HIV antivirals, 7% antifungals, and 6% nonHIV antivirals. There are over 160 antibiotics. *Streptomyces hygroscopicus* with over 200 antibiotics, *Streptomyces griseus* with 40 antibiotics, and *Bacillus subtilis* with over 60 compounds are the major contributors to the antibiotic market [7].

4.4.2. Antitumor agents

Natural product and its derivatives account for more than 60% of anticancer formulations. Actinobacteria derived antineoplastic molecules currently in use are actinomycin D,

Name of secondary metabolites	Source of secondary metabolites	Biological activities	References
Secondary metabolites of Actinobacteria			
Resistomycin	*S. corchorusii*	HIV-1 protease inhibitor	Shiono et al. [39]
Himalomycins A and B	*Streptomyces* sp. B6921	Antimicrobial	Maskey et al. [40]
Bonactin	*Streptomyces* sp. BD21–2	Antibacterial	Schumacher et al. [41]
Trioxacarcins	*S. ochraceus* and *S. bottropensis*	Antitumor and antimalarial	Maskey et al. [42]
Chinikomycins A and B	*Streptomyces* sp.	Antitumor and antiviral	Li et al. [43]
Daryamides	*Streptomyces* sp. CNQ-085	Cytotoxic polyketides	Asolkar et al. [44]
Resistoflavine	*S. chibaensis*	Antibacterial	Gorajana et al. [45]
Chalcomycin A and terpenes	*Streptomyces* sp. M491	Antibacterial	Wu et al. [46]
Napyradiomycin (C-16 stereoisomers)	*S. antimycoticus*	Antibacterial	Motohashi et al. [47]
Oxohexaene and Cephalaxine	*Streptomyces* sp. RM17; *Streptomyces* sp. RM42	Antibacterial	Remya and Vijayakumar [48]
Citreamicin θ A, Citreamicin θ B, and Citreaglycon A	*S. caelestis*	Antibacterial	Liu et al. [49]
Spiramycin	*Streptomyces* sp. RMS6	Antibacterial	Vijayakumar and Malathi [50]
N-isopentyltridecanamide	*Streptomyces labedae* ECR 77	Antibacterial	Thirumurugan et al. [51]
Staurosporine	*Streptomyces champavatii* KV2	Antimicrobial	Cholarajan and Vijayakumar [52]
Secondary metabolites of *Bacillus* spp.			
Coagulin	*B. coagulans*	Bactericidal, Bacteriolytic	Le Marrec et al. [53]
Bacthurucin f4	*B. thuringenesis* sp.	Fungicidal sub sp., *kurstaki* BUPM4	Kamoun et al. [54]
Cerein	*B. cereus*	Bactericidal, bacteriolytic	Bizani et al. [55]
Megacin	*B. megaterium*	,	Lisboa et al. [56]
Thuricin S	*B. thuringenesis*	,	Chehimi et al. [57]
Thuricin CD 19	*B. thuringenesis DPC6431*	,	Rea et al. [58]
	B. anthracis		
Halobacillin 5b	*B. licheniformis*	Hemolytic, cytotoxic	Kalinovskaya et al. [59]
Bacillomycin	*B. amyloliquefacins* FZB42,	Antifungal hemolytic	Ramarathnam et al. [60]
	B. subtilis		
Bacilysocin	*B. subtilis*	Fungicidal, antibacterial	Tamehiro et al. [61]
Bacilysin 1	*B. subtilis* 168, *B. pumilus*	Antifungal, antibacterial	Steinborn et al. [62]
	B. amyloliquefaciens GSB272		

Name of secondary metabolites	Source of secondary metabolites	Biological activities	References
Secondary metabolites of *Pseudomonas* spp.			
Pseudomonine	*P. stutzeri* KC	Competitive inhibition of phytopathogens	Lewis et al. [63]
Hydrogen cyanide	*P. pseudoalcaligenes* P4	Antifungal	Ayyadurai et al. [64]
Secondary metabolites of Fungi			
Lovastatin	*Monascus ruber;* *Aspergillus terreus*	Enzyme inhibitor	Dewick [65]
Limonene and guaiol	*Trichoderma viride*	Antimicrobial	Awad et al. [66]
Tubercullariols	*Tubercularia* sp. TF5	Anticancer	Xu et al. [67]
Oxaline	*Penicillium raistricki*	Anti-cell proliferation	Sumarah et al. [68]
Benzomalvin C	*Penicillium raistrickii*, *Penicillium* sp. SC67	Antimalarial	Stierle et al. [69]
Roquefortine C	*P. roqueforti; P. crustosum*	Neurotoxin	Kim et al. [70]; Xu et al. [67]
Pravastatin	*Penicillium citrinum*	Anticholesterolemics	Gonzalez et al. [71]

Table 3. Secondary metabolites produced by microorganisms.

anthracyclines (daunorubicin, doxorubicin, epirubicin, pirarubicin, and valrubicin), bleomycin, mitosanes (mitomycin C), anthracenones (mithramycin, streptozotocin, and pentostatin), enediynes (calicheamicin), taxol, and epothilones [37].

Taxol is the nonactinobacterial molecule derived from plant *Taxus brevifolia* and endophytic fungi *Taxomyces andreanae* and *Nodulisporium sylviforme.* It interferes with microtubule breakdown, an essential event leading to cell division, thereby inhibiting rapidly dividing cancer cells. It is effective against breast and advanced form Kaposi's sarcoma. It is also found to exhibit antifungal activity against *Pythium*, *Phytophthora,* and *Aphanomyces*.

4.4.3. Pharmacological and nutraceutical agents

One huge success was the discovery of the fungal statins, including compactin, lovastatin, pravastatin, and others which act as cholesterol-lowering agents. Lovastatin is produced by *A. terreus.* Of great importance in human medicine are the immunosuppressants such as cyclosporin A, sirolimus (rapamycin), tacrolimus, and mycophenolate mofetil. They are used for heart, liver, and kidney transplants and were responsible for the establishment of the organ transplant field. Cyclosporin A is made by the fungus *Tolypocladium niveum*. Mycophenolate mofetil is a semisynthetic product of the oldest known antibiotic, mycophenolic acid, and is also made by a fungus. Sirolimus and tacrolimus are products of streptomycetes [7]. Metabolites of probiotic bacteria are considered as a remedy for controlling weight gain, preventing obesity, increasing satiety, prolonging satiation, reducing food intake, reducing fat deposition, improving energy metabolism, treating and enhancing insulin sensitivity, and

treating obesity. *Firmicutes* and *Bacteroidetes* are the dominant beneficial bacteria present in the normal human gastrointestinal tract, and the latter was reported in lower numbers in constipation-predominant irritable bowel syndrome patients [38]. Carotenoids of microbial origin are used as food colorant, fish feeds, nutraceuticals, cosmetics, and antioxidants. Food colorant widely used is carotene derived from *Blakeslea trispora, Dunaliella salina* and lycopene from *B. trispora* and *Streptomyces chrestomyceticus, subsp. rubescens.* Astaxanthin produced from *Xanthophyllomyces dendrorhous* is an approved fish feed. Astaxanthin, lutein, β-carotene, zeaxanthin, and canthaxanthin are used as nutraceuticals due to their excellent antioxidant property. Docosahexaenoic acid (DHA) used in infant formula as nutritional supplements is derived from microalgae *Schizochytrium* spp. [7].

4.4.4. Enzymes and enzyme inhibitors

Enzymes produced from microorganism have annual sales of US $ 2.3 billion enzymes that find application in detergents (34%), foods (27%), agriculture and feeds (16%), textiles (10%), and leather, chemicals, and pulp and paper (10%). The protease subtilisin used in detergents has an annual sale of $ 200 million. The other major enzymes include glucose isomerase (100,000 tons) and penicillin amidase (60,000 tons). Nitrilase (30,000 tons) and phytase are amounting for $135 million worth of production. *Streptomyces* glucose isomerase is used to isomerize D-glucose to D-fructose, to make 15 million tons per year of high fructose corn syrup, valued at $1 billion [7].

The most important enzyme inhibitors are clavulanic acid, synthesized by *Streptomyces clavuligerus*, the inhibitor of β-lactamases. Some of the common targets for other inhibitors are glucosidases, amylases, lipases, proteases, and xanthine oxidase. Amylase inhibitors prevent absorption of dietary starches into the body, and hence can be used for weight loss [38].

4.4.5. Agricultural and animal health products

Secondary metabolites find wide applications in the field of agriculture and animal health: kasugamycin and polyoxins are used as biopesticides; *Bacillus thuringiensis* crystals, nikkomycin, and spinosyns are used as bioinsecticides; bioherbicides (bialaphos) find application as bioherbicides; ivermectin and doramectin as antihelmintics and endectocides against worms, lice, ticks, and mites; ruminant growth promoters in the form of coccidiostats; plant hormones like gibberellins as growth regulators are the most common application [7].

4.5. Production of secondary metabolites from microorganisms

Secondary metabolites branch out from the pathways of primary metabolism. Commercially, important secondary and primary metabolic pathways are given in **Table 4**.

4.5.1. Liquid fermentation

Batch or fed-batch culture in submerged fermentation is employed for production of secondary metabolites. Inoculum is developed after careful strain improvement of producing organism. Initially, shake flasks culture is employed, and the culture which are in active growth

S. No.	Intermediates from primary metabolic pathway	Secondary metabolites derived
1.	Shikimic acid	Ergot alkaloids, antibiotics: candicidin and chloramphenicol
2.	Amino acids	Antibiotics: penicillin, cephalosporins and cephamycins, and gramicidin, immunosuppressive cyclosporine
3.	Acetyl-CoA and other Kreb's cycle intermediates	Antibiotics: erythromycin, antiparasitic avermectin antitumor doxorubicin, taxol
4.	Sugars	Antibiotics: streptomycin and kanamycin.

Table 4. Intermediate from primary metabolism and their secondary metabolite derivatives.

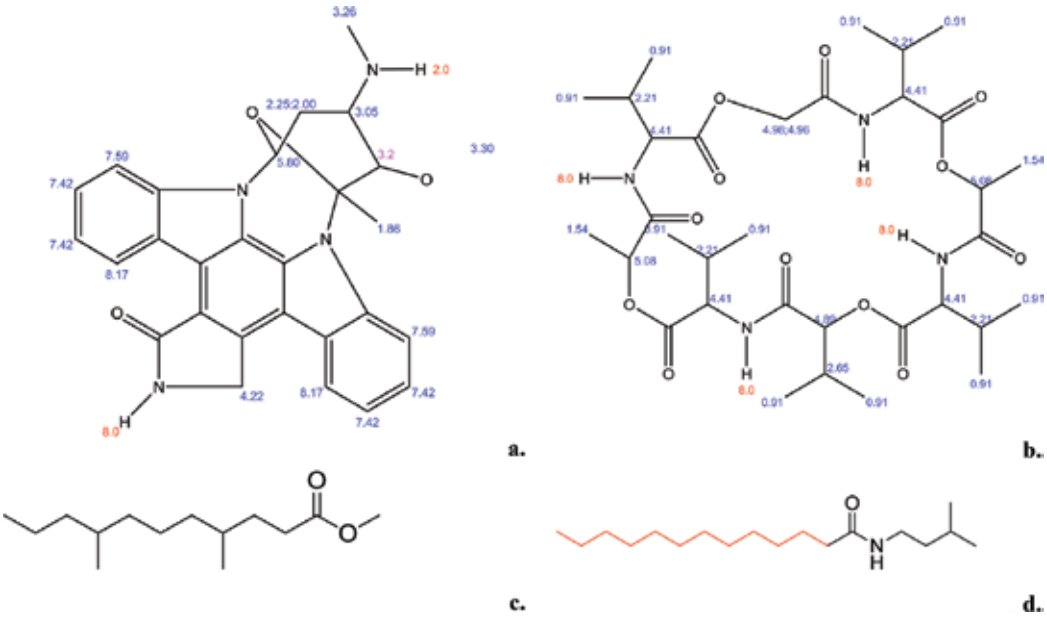

Figure 2. Chemical structures of actinobacterial secondary metabolites. (a) Staurosporine, (b) octa-valinomycin, (c) methyl-4,8-dimethylundecanate, and (d) N-isopentyltridecanamide from actinobacteria. Source: Cholarajan and Vijayakumar [52]; Cholarajan [72]; Thirumurugan et al. [73].

phase are transferred to a small fermenter and later into a larger fermenter with production medium. Several parameters, like medium composition, pH, temperature, and agitation and aeration rate, are controlled. An inducer such as methionine is added to cephalosporin fermentations, phosphate is restricted in chlortetracycline fermentation, and glucose is avoided in penicillin or erythromycin fermentation.

4.5.2. Solid-state fermentation

Solid-state fermentation, defined as a microbial culture that develops on the surface and at the interior of a solid matrix and in the absence of free water, holds an important potential for the production of secondary metabolites. Two types of SSF can be distinguished, depending on

the nature of solid phase used [7]: (a) solid culture of one support-substrate phase solid phase and (b) solid culture of two substrate-support phase solid phase. The advantages of solid-state fermentation in relation with submerged fermentation include: energy requirements of the process are relatively low, since oxygen is transferred directly to the microorganism. Secondary metabolites are often produced in much higher yields, often in shorter times, and often sterile conditions are not required [7].

It is important here to note our own experience of deriving actinobacterial secondary metabolite. Actinobacteria from terrestrial and marine habitats were screened for their antimicrobial activity. The bioactive metabolites were extracted and purified by thin layer and column chromatography, and the structure of the metabolite was elucidated by UV-spectrometry, FT-IR, mass spectrum analysis, and NMR. The derived metabolites staurosporine, octa-valinomycin, methyl-4,8-dimethylundecanate, and N-isopentyltridecanamide are known for their biological activity (**Figure 2**).

5. Conclusion

This review emphasizes the importance of secondary metabolites from various sources like plants, microorganisms including bacteria, actinobacteria, and fungi and its classification, production and applications in various fields. Since there is a constant and crucial requirement for new pharmaceutical agents to fight cancers, cardiac disorders, pests, cytotoxic, mosquitoes, infectious diseases, and autoimmune disorders of both animals and plants as climate changes provide conditions favorable to repeated outbreaks of these events. The battle against any disease is a vibrant symmetry between advances in chemotherapy and natural selection on infectious or invasive agents. If the scientific community is to put constant importance in this never ending effort, then new sources of bioactive secondary metabolites with novel activities must be found. Secondary metabolites are one of their essential means of growth and defense, and these metabolites are readily available for discovery. Secondary metabolites with noteworthy biological activity are considered as an alternative to most of the synthetic drugs and other commercially valuable compounds.

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Chapter 2

Secondary Metabolites in Cyanobacteria

Bethan Kultschar and Carole Llewellyn

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75648

Abstract

Cyanobacteria are a diverse group of photosynthetic bacteria found in marine, freshwater and terrestrial habitats. Secondary metabolites are produced by cyanobacteria enabling them to survive in a wide range of environments including those which are extreme. Often production of secondary metabolites is enhanced in response to abiotic or biotic stress factors. The structural diversity of secondary metabolites in cyanobacteria ranges from low molecular weight, for example, with the photoprotective mycosporine-like amino acids to more complex molecular structures found, for example, with cyanotoxins. Here a short overview on the main groups of secondary metabolites according to chemical structure and according to functionality. Secondary metabolites are introduced covering non-ribosomal peptides, polyketides, ribosomal peptides, alkaloids and isoprenoids. Functionality covers production of cyanotoxins, photoprotection and antioxidant activity. We conclude with a short introduction on how secondary metabolites from cyanobacteria are increasingly being sought by industry including their value for the pharmaceutical and cosmetics industries.

Keywords: cyanobacteria, secondary metabolites, nonribosomal peptides, polyketides, alkaloids, isoprenoids, cyanotoxins, mycosporine-like amino acids, scytonemin, phycobiliproteins, biotechnology, pharmaceuticals, cosmetics

1. Introduction

1.1. Cyanobacteria

Cyanobacteria are a diverse group of gram-negative photosynthetic prokaryotes. They are thought to be one of the oldest photosynthetic organisms creating the conditions that resulted in the evolution of aerobic metabolism and eukaryotic photosynthesis [1, 2]. They

are primarily photoautotrophic performing oxygenic photosynthesis using photosystems I and II to produce energy, requiring water, carbon dioxide, inorganic compounds and light to grow. They are also able to grow under heterotrophic conditions without light using an organic carbon substrate to obtain energy [3].

Morphologically cyanobacteria can be unicellular or filamentous and have spherical, rod and spiral shapes [4, 5]. Taxonomically they are divided broadly into five major sub-sections using morphological and physiological characteristics as described in [5]: Subsection I (order: Chroococcales), II (order: Pleurocapsales), III (order: Oscillatoriales), IV (order: Nostocales) and V (order: Stigonematales). Subsections I and II are unicellular as single cells or aggregates that reproduce by binary fission or budding (I) and multiple fission or both binary and multiple fission (II). Subsections III-V are filamentous, which are composed of trichomes (chain of cells), these reproduce by trichome breakages to produce short motile fragments known as hormogonia. Subsection III cyanobacteria divide in one plane only and are composed of vegetative cells only whereas subsections IV and V are capable of cell differentiation. An example includes the production of heterocysts in the absence of a nitrogen source, which is used for nitrogen fixation [5]. The classification of cyanobacteria is constantly evolving with newer systems based on phylogenetic analyses [6].

Cyanobacteria live in a wide range of habitats encompassing freshwater, marine and terrestrial ecosystems (**Table 1**). A key feature of cyanobacteria is their ability to thrive under extreme conditions and their ability to adapt and evolve to cope with abiotic stress factors such as high light, UV and extreme temperatures. As extremophiles cyanobacteria can exist as thermophiles (high temperature tolerant) e.g. *Synechococcus* found in hot springs and geotherms, psychrophiles (cold tolerant), acidophiles (low pH tolerant), alkaliphiles (high pH tolerant) and halophiles (salt tolerant) [7].

Species of cyanobacteria	**Order**	**Habitat**
Unicellular		
Microcystis sp.	Chroococcales	Freshwater
Synechococcus sp.	Chroococcales	Marine
Synechocystis sp.	Chroococcales	Freshwater
Hyella caespitosa	Pleurocapsales	Marine
Filamentous		
Lyngbya majuscula	Oscillatoriales	Marine (tropical)
Oscillatoria sp.	Oscillatoriales	Freshwater
Anabaena sp.	Nostocales	Freshwater
Nostoc sp.	Nostocales	Terrestrial
Fischerella muscicola	Stigonematales	Freshwater

Table 1. Cyanobacterial species by morphology, order and habitat.

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1.2. Cyanobacterial secondary metabolites

Secondary metabolites, also described as natural products, are usually described as compounds that are not directly required for an organism's primary metabolism. These secondary metabolites are usually unique to specific organisms and are not present during all environmental conditions.

Although most metabolites can be categorised as primary or secondary there is some overlap between the two. Some are essential for primary metabolism but are only synthesised by specific species and are therefore also secondary metabolites.

Secondary metabolites are often produced by cyanobacteria in response to biotic or abiotic stress in the surrounding environment by providing protection and aiding in survival giving an advantage over other species [2, 8]. Because of their ability to survive under a diversity of environments, cyanobacteria are a rich source of secondary metabolites. Different suites

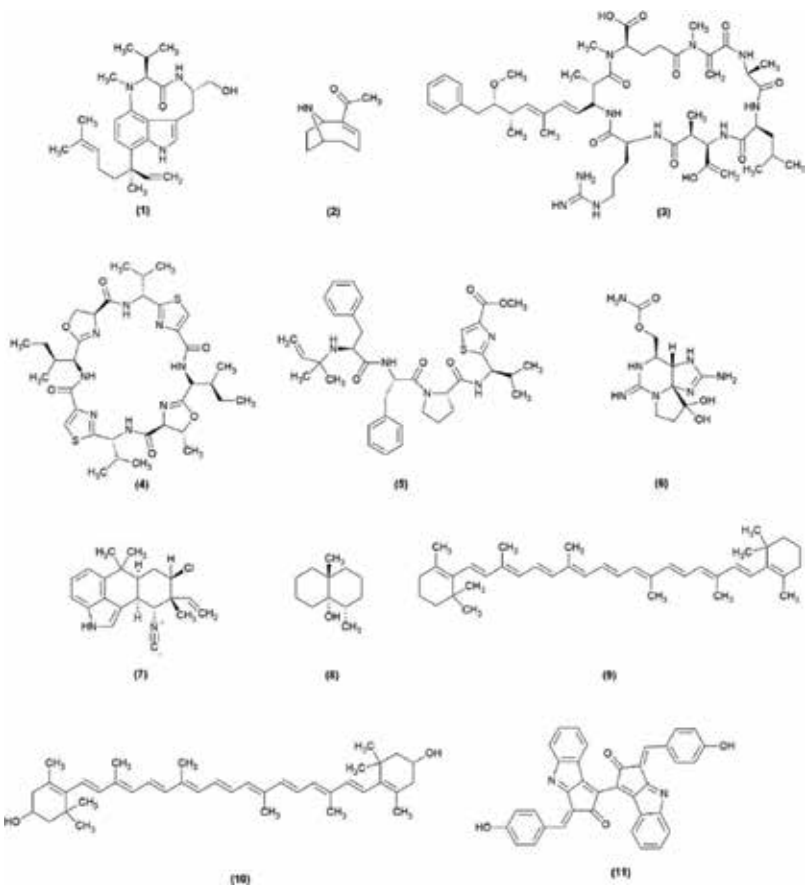


Figure 1. Chemical structures of a variety of secondary metabolites; lyngbyatoxin-a (1), anatoxin-a (2), microcystin-LR (3), patellamide-a (4), aeruginosamide (5), saxitoxin (6), hapalindole-a (7), geosmin (8), β-carotene (9), zeaxanthin (10) and scytonemin (11).

of secondary metabolites can be produced according to the stress environment with a high degree of structural variation across the different compound classes. These suites of metabolites include; peptides, polyketides, alkaloids, terpenoids and UV-absorbing (**Figure 1**). Accordingly they possess a wide variety of functions to protect the cells such as; defence against predators and grazers, chemosensory, photoprotection and antioxidant roles. These properties can be utilized in industrial biotechnology as nutraceuticals, cosmeceuticals and pharmaceuticals.

2. Cyanobacterial secondary metabolites by chemical structure and biosynthesis

2.1. Nonribosomal peptides and polyketides

Commonly occurring as secondary metabolites in cyanobacteria are nonribosomal peptides NRPs. These are produced using specialised nonribosomal peptide synthases (NRPS). NRPS contains modules, which are responsible for integrating specific amino acids into peptide chains. These modules consist of an adenylation domain, peptidyl carrier domain and a condensation domain, which incorporates proteinogenic and nonproteinogenic amino acids. Other domains can also be present for further modifications such as N-methylation, epimerization and cyclisation of the amino acid backbone, which gives rise to the intricate chemical structures produced [9]. Lyngbyatoxins, such as lyngbyatoxin-a (**Figure 1 (1)**), are biosynthesised *via* NRPS pathway in *Lyngbya majuscule* and comprise of an indolactam ring composed of L-valine, L-tryptophan and methionine [10]. Lyngbyatoxin-a is a dermatoxin with potent tumour promoting activity by activation of protein kinase C (PKC) [11].

Another large class of secondary metabolites found in cyanobacteria are the polyketides, which are biosynthesised from acetyl-CoA using polyketide synthases (PKS). Similarly to NRPS, PKS modules consist of a acyltransferase domain, acyl carrier protein domain and ketosynthase domain as well as additional domains for further modification [12]. The neurotoxin anatoxin-a (**Figure 1 (2)**) from *Anabaena* sp. Binds irreversibly to nicotinic acetylcholine receptors and is biosynthesised from L-proline using three PKS modules [9].

Hybrid metabolites are primarily derived from the attachment of polyketide or fatty acids using PKS to nonribosomal peptides in a natural combinatorial biosynthetic pathway to produce an array of chemical structures with specific roles and bioactivity. Microcystin-LR (**Figure 1 (3)**) is biosynthesised using multi-enzymes of NRPS and PKS modules and has potential as a lead compound for the treatment of cancer due to its cytotoxicity [13].

2.2. Ribosomal peptides

Ribosomal peptides (RPs) are synthesised on the ribosome and only use proteinogenic amino acid. They are similar to NRPs due to their posttranslational modifications. A prevalent group of ribosomal peptides found in cyanobacteria are the cyanobactin. These are cyclic and less commonly linear peptides formed through the post-ribosomal peptide synthesis (PRPS) pathway, which then undergoes post modifications to form their final complex structures [14],

formally known as ribosomally synthesised and posttranslationally modified peptides (RiPP). Examples include the cyclic peptides patellamides, such as patellamide A (**Figure 1 (4)**) and the linear peptide aeruginosamide (**Figure 1 (5)**) [9].

2.3. Alkaloids

Alkaloids are nitrogen containing natural compounds, which usually have toxic properties, an example includes the saxitoxins also known as paralytic shellfish poisons (**Figure 1 (6)**), which are neurotoxins found in a number of cyanobacteria [15].

Indole alkaloids are a class of alkaloids containing an indole moiety such as the hapalindoles (hapalindole-A, **Figure 1 (7)**), hapalindolinones, ambiguines, fischambiguines, fisherindoles, and welwitindolinones, which are only found in cyanobacteria of subsection V. Their structural diversity is due to the cyclisation, methylation, oxygenation and chlorination of terpene precursors [16]. Hapalindole isolated from *Fischerella* sp. has been found to possess antibacterial activity against gram negative and gram positive bacteria such as; *Escherichia coli* ATCC25992 and *Staphylococcus aureus* ATCC25923 [13].

2.4. Isoprenoids

A wide range of isoprenoids (also known as terpenoids) are produced by cyanobacteria, which have a common pathway utilising isoprene diphosphate (IDP) and dimethylallyl triphosphate (DMADP) precursors. These have many possible configurations resulting in high structural diversity due to modification by cyclisation, rearrangements and oxidation [17]. They are biosynthesized through the methylerythritol-phosphate (MEP) pathway. Using glyceraldehyde-3-phosphate and pyruvate produced from photosynthesis, the five carbon building blocks IPD and DMADP are formed [17].

The smallest group of isoprenoids is the hemiterpenes, which are formed from a single isoprene unit composed of five carbons. Monoterpenes have 10 carbons and are formed from IDP and DMADP or two molecules of DMADP monomers to form geranyl diphosphate (GDP). An example includes 2-methylisabomeol, which gives taste and odour to water. Geosmin (**Figure 1 (8)**) in an odorous sesquiterpene found in *Nostoc punctiforme* PCC 73102, which gives rise to its earthy smell and is synthesised from the condensation of an IDP molecule to the monoterpene GDP to form farnesyl diphosphate (FDP) [17, 18].

An abundant group of isoprenoids found in cyanobacteria are the carotenoids. These are tetraterpenes formed from the head to head condensation of two geranyl geranyl diphosphate (GGDP) molecules [17]. Located within cell membranes due to their hydrophobic nature, this group of metabolites can be divided into two classes; carotenes, hydrocarbon carotenoids such as β-carotene (**Figure 1 (9)**) and xanthophylls, which are oxygenated derivatives of hydrocarbon carotenoids such as zeaxanthin (**Figure 1 (10)**). Other carotenoids commonly found within cyanobacteria are echinenone, canthaxanthin and myxoxanthophyll. In many cases individual carotenoids could be considered as primary rather than secondary metabolites because of their role in photosynthesis however, other carotenoids are more specifically involved in photoprotection and in antioxidant protection and therefore fall into the secondary metabolites category [19].

3. Cyanobacterial secondary metabolites by function

3.1. Toxic metabolites

A wide variety of toxic metabolites (**Table 2**) are produced by cyanobacteria that have a negative effect on target species in their surrounding areas and are referred to as cyanotoxins [2]. These toxins are found during cyanobacterial blooms on stagnant surface water bodies. Cyanobacteria that bloom include the unicellular *Microcystis* and the filamentous *Anabaena*, and *Nostoc* [20].

Cyanotoxins have a diverse range of chemical structures including ribosomal peptides and NRPs, polyketides alkaloids and lipopolysaccharides. These toxins can be classified according to their biological effect; neurotoxins targeting the nervous system, hepatotoxins targeting the liver, cytotoxins targeting cells, dermatoxins targeting the skin or endotoxins, which are irritants [15]. The most prevalent and potent hepatotoxins are the cyclic peptides microcystins, which are produced through NRPS in *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* [15].

An example of a non-protein amino acid neurotoxin is β-N-methylamino-L-alanine, which can be produced by a variety of cyanobacteria [21]. It was originally isolated from cycad seeds in Guam and many investigations have implicated this neurotoxin in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Parkinsonism dementia complex (PDC) [22]. Other neurotoxins include saxitoxin (paralytic shellfish poisons) and the anatoxins [15].

Cyanotoxin	Biological effects	Cyanobacteria
Microcystin	Hepatotoxin, inhibits eukaryotic protein phosphatases (types 1 and 2A)	*Microcystis, Anabaenopsis, Nostoc*
Nodularin	Hepatotoxin, inhibits eukaryotic protein phosphatases (types 1 and 2A)	*Nodularia*
Saxitoxin	Neurotoxin, binds to voltage-gated Na^+ channels, causing neuronal communication blockage	*Aphanizomenon*, *Anabaena*, *Lyngbya*
Anatoxin-a	Neurotoxin, binds to nicotinic acetylcholine receptors irreversibly	*Cylindrospermum, Planktothrix, Oscillatoria*
β-*N*-methylamino-L-alanine	Neurotoxin, damages motor neurons	Many species including; *Anabaena, Nostoc,* [24]
Lyngbyatoxin	Cytotoxin, binds to protein kinase C, tumour promoting	*Lyngbya majuscula*
Aplysiatoxins	Cytotoxin, binds to protein kinase C, tumour promoting	*Lyngbya majuscula*
Lipopolysaccharides	Endotoxin, irritant	*Microcystis, Anabaena, Spirulina, Oscillatoria*

Table 2. Cyanotoxins and their biological effect.

Although dangerous to animals, fish and humans, these toxins have potential uses as biocides (algaecides, fungicides, herbicides and insecticides) and pharmaceuticals (antimicrobial, anti-cancer, antiviral and immunosuppressant) [15, 23].

3.2. Photoprotective metabolites: Mycosporine-like amino acids (MAAs) and scytonemin

Mycosporine-like amino acids (MAAs) are a group of about 30 colourless, water soluble, low molecular weight molecules found primarily within the cytosol of cells and sometimes found glycosylated on the outer cell membrane such as in *Nostoc commune* [25]. MAAs have strong absorption in the UV region between 310 and 365 nm [2] with high molar extinction coefficients (ε = 28,100–50,000 $l{\cdot}mol^{-1}{\cdot}cm^{-1}$) providing photoprotection with the ability to disperse energy without producing reactive oxygen species (ROS) [26].

They consist of cyclohexenone or cyclohexenimine chromophores conjugated to nitrogen substituents from amino acids or imino alcohols. The variety in absorption is due to the differing nitrogen substituents and side groups [27, 28] (**Table 3**).

There are two biosynthetic routes involved in the production of MAAs. The first is the shikimate pathway (biosynthesis of aromatic acids) [29], by first forming deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) from phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) using DAHP synthase. DAHP is then converted to 3-dehydroquinate and subsequent transformation into 4-deoxygadusol (4-DG). The primary MAA mycosporine-glycine is then formed from the reaction of 4-DG with glycine, which can then be converted into a secondary MAA by addition of other amino acids such as serine (to produce shinorine) and threonine (to produce porphyra-334) [25]. The other pathway involved is the pentose-phosphate pathway, which also produces the intermediate 4-DG from sedoheptulose-7-phosphate *via* 2-*epi*-5-*epi*-violiolone [19].

Another photoprotective metabolite produced by cyanobacteria alone is scytonemin (**Figure 1 (11)**), this is located in the extracellular polysaccharide sheath of cyanobacteria [19]. With a molecular weight of 544 Da it is a hydrophobic alkaloid comprising of idolic and phenolic substituents usually linked by a carbon–carbon double bond. It has an absorption maximum at 380 nm [2, 26]. Scytonemin has an extinction coefficient of 136,000 $l{\cdot}mol^{-1}$ cm^{-1} at 384 nm, which makes it an excellent photo-protective compound. It is biosynthesized in response to UV-A and has two major forms, an oxidised state (brown) and reduced state (red).

3.3. Antioxidants

Unavoidably ROS are produced by cyanobacteria during photosynthesis and respiration. Abiotic factors that produce these species include UVR, osmotic perturbations, desiccation and heat. Hydrogen peroxides (H_2O_2), superoxides ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$) which damage biomolecules within cells are all examples of ROS [30].

Cyanobacteria require multiple approaches to prevent inhibitory effects of stressful environments. They can prevent the production of ROS by energy dissipation in the photosynthetic

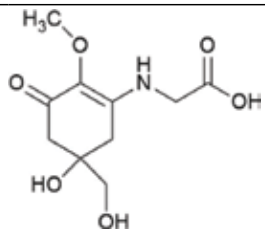
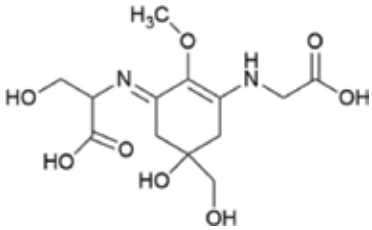
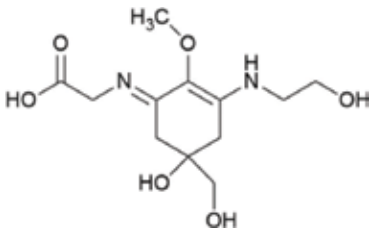
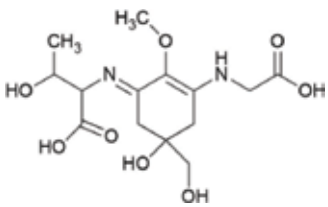
MAA	Molecular structure	λmax (nm), ε ($l \cdot mol^{-1} \cdot cm^{-1}$)	Species of cyanobacteria
Mycosporine-glycine		310, 28,800	*Nostoc commune*
Shinorine		334, 44,668	*Anabaena* sp.
Asterina-330		330, 43,800	*Gloeocapsa* sp.
Porphyra-334		334, 42,300	*Nodularina baltica*

Table 3. Example of MAAs [27, 28].

apparatus. One mechanism is the non-photochemical quenching (NPQ) of excitation energy *via* photosystem II using the carotenoid zeaxanthin. They also produce enzymatic antioxidants such as; superoxide dismutases (SOD), catalases and peroxidases) as well as non-enzymatic antioxidants such as; carotenoids, phycobiliproteins, tocopherols and ascorbic acid [31].

Carotenoids absorbs light in the region of 400–500 nm and have several roles including sun-screening, singlet oxygen quenching, releasing excessive light as heat through the xanthophyll cycle and radical scavenging [30].

Another group of antioxidants are the phycobiliproteins (PBP). These are present only in cyanobacteria and are primarily used as major light harvesting antennae but also have antioxidant

roles within the cells [32]. They are water soluble proteins that are brightly coloured due to the covalently attached linear tetrapyrrole prosthetic groups called bilins, which gives rise to cyanobacteria prominent colour. They, along with linker protein are able to form giant supramolecular structures known as phycobilisomes [33].

4. Potential of cyanobacterial secondary metabolites in industrial biotechnology

Sustainability in industry is increasingly important due to global warming and the depletion of fossil fuels. A considerable amount of research has been conducted to find new sources of industrially important compounds to reduce the carbon footprint and increase sustainability.

Cyanobacteria has received much interest in becoming a promising alternative due to their diversity, simple growth needs and simple genetic background, which are easily manipulated to form cell factories [34].

Some strains of cyanobacteria are already being used in industry, examples include the edible *Arthrospira* (*Spirulina*) and *Nostoc*, which have been used as a food source for thousands of years [35].

Spirulina has been well researched for its application within industry. It is used as a health food due to its extensive source of proteins, polyunsaturated fatty acids (γ-linoleic acid, GLA), antioxidants (phycocyanin and carotenoids) and vitamins [36].

A challenge remains in assessing and understanding the ability of cyanobacteria to produce target metabolites in sufficient quantities to be of use under standard and repeatable conditions. This will be easier moving into the future as 'omic' studies enable improved understanding on metabolite pathways using a whole systems approach.

4.1. Pharmaceuticals and cosmetics

Natural products have been used to treat disease for thousands of years and are a useful source of bioactive compounds used in the pharmaceutical industry as leading compounds in drug discovery. They can be used as templates for synthesis of new drugs to treat complex diseases. Cyanobacteria have been widely researched for their applications in this field. They have found to possess a wide range of potential antimicrobial, anticancer, antiviral and anti-inflammatory activities [37]. Some known bioactives are listed below (**Table 4**) [11].

Chemotherapies currently used in the treatment of cancer cause serious side effects; naturally derived alternatives give opportunities for synthesising new highly potent drugs with fewer side effects [15, 42]. Cytotoxic metabolites produced by cyanobacteria usually target tubulin or actin filaments in eukaryotic cells, which make them promising anticancer agents. Dolastatins found within *Leptolyngbya* and *Simploca* sp. are synthesised by NRPS-PKS enzymes and are able to disrupt microtubule formation. Other cyanobacterial metabolites act as proteases inhibitors such as the lyngbyastatins, which are cyclic depsipeptide derivatives, which are

Species of cyanobacteria	Bioactive compound	Biological activity	References
Spirulina platensis	Spirulan	Antiviral	[38]
	γ-linolenic acid	Precursor to prostaglandins	[39]
	Phycocyanin	Cosmetic colourants	[1]
Lyngbya majuscule	Apratoxins	Anticancer	[23]
Nostoc commune	Nostodione	Antifungal	[38]
	Carotenoids	Antioxidant	[36]
	MAAs	Sunscreen	[40]
Anabaena circinalis	Anatoxin-a	Anti-Inflammatory	[11, 38]
Fischerella muscicola	Fischerellin	Antifungal	[11]
	Scytonemin	Anti-inflammatory, Anti-proliferation	[41]

Table 4. Potential applications of cyanobacterial natural products in pharmaceutical and cosmetics industry.

thought to be elastase inhibitors. Apratoxins such as Apratoxin-a from *Lyngbya majuscule* is another metabolites biosynthesized from a hybrid NRPS-PKS pathway. It is cytotoxic due its ability to induce G1-phase cell cycle arrest and apoptosis [42].

Antibacterial metabolites produced by cyanobacteria are effective against gram negative and gram positive bacteria. In the age of antibacterial resistance, new drugs are essential to combat bacterial infections. The hapalindole-type class of indole alkaloids has been found to possess antimicrobial (bacteria, fungi) and antialgal activity [16].

Secondary metabolites can be used as natural ingredients in the cosmetics industry. Uses include the photoprotective MAAs in sunscreens to protect the skin from harmful UVR. Pigments such as carotenoids and phycobiliproteins could be used as natural colourants but also as antioxidants to protect the skin from damage caused by UV exposure [11].

Other potential uses for cyanobacterial secondary metabolites include their use in the nutraceutical and agricultural industry [11, 43].

5. Conclusion

Cyanobacteria have a long evolutionary history and have adapted to deal with natural and anthropogenic stress. The morphological, biochemical and physiological diversity of cyanobacteria gives rise to the vast amount of secondary metabolites produced all with their own specific functions that aid in the organism's survival. These secondary metabolites can also be utilised in drug discovery as lead compounds due to their complex structures and varied bioactivities. New natural products can be identified through biosynthetic pathway analysis using genomic data with around 208 cyanobacterial genomes sequences publically available [12]. Although extensive research has been conducted on cyanobacterial secondary metabolites

there is still a large selection of species, which have yet to be sequenced and investigated with many potentially important secondary metabolites yet to be discovered.

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Conflict of interest

No conflict of interest.

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Chapter 3

Secondary Metabolites of Mycoparasitic Fungi

Verena Speckbacher and Susanne Zeilinger

Additional information is available at the end of the chapter

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Abstract

Mycoparasitic fungi, fungi preying on other fungal species, are prolific producers of volatile and non-volatile secondary metabolites. Several secondary metabolites are produced during mycoparasitism to weaken the host and support attack and parasitism. Further, evidence accumulated that some secondary metabolites also act as communication molecules. Besides their antagonistic activity, several fungal mycoparasites exhibit beneficial effects on plants and some of their secondary metabolites have plant growth-promoting and defense stimulating activities. As many secondary metabolism-associated gene clusters remain silent under standard laboratory conditions, the full variety as well as the underlying biosynthetic pathways employed by fungal mycoparasites for secondary metabolite production still await clarification. Nonetheless, the variety of currently known secondary metabolites and their range of activities is impressive already and they exhibit a great potential for agriculture, pharmacology and other industrial applications.

Keywords: secondary metabolites, volatile organic compounds (VOCs), peptaibiotics, mycoparasitism, biocontrol

1. Introduction

Mycoparasitic relationships, where a predatory fungal species gains nutrients on the expense of a host fungus, are widespread within the fungal kingdom. By the modalities of this non-mutual relationship, biotrophic and necrotrophic mycoparasitic fungi with different gradations within this classification (contact, invasive or intracellular necrotrophic; haustorial or fusion biotrophic) can be distinguished [1]. Biotrophic mycoparasites co-exist and nourish on their living host in a balanced way and are specifically adapted to one or few host species. In contrast, necrotrophic fungi destructively invade and kill a broad range of hosts to gain

nutrients from the remains of their prey [2]. Mycoparasitic fungi are prolific producers of a plethora of volatile and non-volatile secondary metabolites, favoring their ecological fitness and survival under certain environmental conditions. For example, the excretion of siderophores – affecting high affinity iron chelation – is strongly up-regulated under iron-limiting conditions [3] and several antimicrobial metabolites empower the successful perseverance within the ecological niche [4]. The mycoparasitic lifestyle obviously substantiates the overrepresentation of secondary metabolism-associated genes and the extensive excretion of a variety of secondary metabolites [2] enabling the fungus´ successful access to its prey as well as its thriving persistence in or assassination of the host. Furthermore, selected fungal secondary metabolites are known to exhibit beneficial effects on plants: They may promote vitality and growth of roots and shoots, enhance the resilience against abiotic stress factors and prime the plants immune system (induced systemic resistance; ISR) thereby enhancing its resistance and survival in case of prospective infections with pathogens [5]. In recent times, evidence accumulated that some secondary metabolites also act as communication molecules over species boundaries [6, 7].

A great diversity of mycoparasitic species exists in the fungal kingdom, especially within the order *Hypocreales* [8]. In this aspect *Trichoderma* (teleomorph *Hypocrea*), a worldwide abundant, diverse fungal genus, is one of the best-studied examples [2]. *Trichoderma* comprises necrotrophic mycoparasitic species like *Trichoderma atroviride* or *Trichoderma virens*, which are successfully applied in agriculture as biocontrol agents against plant pathogenic fungi of crop plants. Further they are reported to promote plant growth, vitality and systemic resistance via priming the plants' immune system. The genomes of several *Trichoderma* species have been sequenced and analyzed revealing the ancestral mycoparasitic lifestyle of these fungi [9, 10]. The second largest lineage of mycorparasites within the *Hypocreales* is the genus *Tolypocladium*. *Tolypocladium* comprises, besides of some entomopathogenic species like *Tolypocladium inflatum,* mostly mycoparasitic species like the widespread on northern hemisphere *Tolypocladium ophioglossoides* which mycoparasitizes with a narrow host range on truffles of the genus *Elaphomyces* [11, 12]. In contrast to *Trichoderma*, the genus *Tolypocladium* exhibits an ancestral entomopathogenic lifestyle and developed to a mycoparasite by host jumping. The genomes of *Tolypocladium* species are rich in secondary metabolite gene clusters of which some, like the clusters for the production of peptaibiotics, seem to be exclusive to mycoparisitic lineages [12]. A further well-investigated mycoparasitic fungus is *Escovopsis weberi*. *E. weberi* is a contact necrotrophic mycoparasite on *Leucoagaricus* sp. in leaf-cutting ant agriculture [13]. As foraging for leaves causes considerable economic damages in neotropic agriculture by defoliation of a wide variety of crop plants, *E. weberi* would be a suitable biocontrol agent as it causes a breakdown of the fungal feeding structures, thereby starving out the ant colony leading to a collapse of the whole system [14]. Like other very specialized mycoparasites, the *E. weberi* genome exhibits a reduced size and content, but very unique secondary metabolite clusters for host attack, facilitating the excretion of fungicidal substances which can break down the host mycelia even without contact [15, 16]. Further examples of secondary metabolite analysis on mycoparasitic species include *Stachybotrys elegans,* a potential biocontrol agent against plant

pathogenic *Rhizoctonia solani* [17] as well as *Coniothyrium minitans* and *Microsphaeropsis ochracea* co-culture antagonizing the plant pathogenic *Sclerotinia sclerotiorum* [18].

A characteristic trait of filamentous fungi is that their secondary metabolism-associated genes are mostly situated within subtelomeric regions of the chromosomes in large biosynthetic gene clusters present in the genomes in significantly greater numbers than secondary metabolites currently identified [19]. The unique and often uncommon biosynthetic pathways are mostly characterized by signature enzymes, often also transcription factors and transporters present in the respective gene clusters, which enable the secondary metabolite synthesis starting from simple precursors gained from primary metabolism like amino acids and acetyl-CoA [20]. Most common core enzymes are non-ribosomal peptide synthases (NRPSs), polyketide synthases (PKSs) and terpene-synthases or -cyclases [4]. In necrotrophic mycoparasitic species like *T. atroviride* and *T. virens*, genes for the biosynthesis of secondary metabolites are enriched compared to the only weakly mycoparasitic relative *Trichoderma reesei*. Within the two mentioned strong mycoparasites nearly half of all secondary metabolism-associated genes are positioned on non-syntenic regions in the genome and do not exhibit orthologs in the respective other species [21]. Depending on the species, environmental conditions, and even the strain, a plethora of different compounds is derived by the genus *Trichoderma* [2, 4], awakening hope on the detection of new substances. Since resistance development to the currently applied substances constitutes an increasing problem in agriculture and medicine, the need for environmentally sustainable biological control of pathogens and the discovery of novel antagonistic substances is essential. Secondary metabolites of mycoparasitic fungi could contribute to the solution.

2. Non-ribosomal peptides

Non-ribosomal peptides (NRPs) are synthesized by NRPSs, enzymes that characteristically consist of multiple domains synthesizing the peptide in one by one steps. Characteristic for NRPSs are the core domains for adenylation, thiolation and condensation. The generated NRPs are very diverse: they mostly comprise of proteinogenic and non-proteinogenic amino acids, can be linear or branched to cyclic with a varying length. After their synthesis outside of the ribosome, they frequently pass extensive secondary modifications. Many fungal NRPs have high economic and/or ecologic value like β-lactam antibiotics, the immunosuppressant cyclosporine A but also mycotoxins like gliotoxin.

The occurrence of NRPS genes is enriched within the genome of mycoparasitic *T. atroviride* for 60% and in *T. virens* for 180% to the wood-degrading *T. reesei* [21]. Further, the functional involvement of NRPS and PKS in the mycoparasitic interaction was supported by deletion experiments of the *T. virens* 4-phosphopanteteinyl transferase-encoding gene (*ppt*) – which is essential for NRPs and PKSs activation – resulting in mutant strains defective in mycoparasitism and induction of systemic resistance (ISR) in plants [22]. The main NRPSs derived metabolites in *Trichoderma* species are peptaibiotics, epipolythiodioxypiperazines and siderophores.

2.1. Peptaibiotics

Peptaibiotics are mostly linear to rarely cyclic polypeptides, with a size of 0.5–2.1 kDa consisting of 4–21 residues. Characteristic for peptaibiotics is the inclusion of the non-proteinogenic amino acid α-aminobutyric acid (Aib). By module-skipping one NRPS is frequently capable of synthesizing a whole set of peptaibiotics [23, 24]. According to their sequence alignment and structure, peptaibiotics can be divided into several sub-clades: peptaibols, lipopeptaibols, lipoaminopeptides, cyclic peptaibiotics and two very special, small categories [25]. Because of their unusual synthesis and appearance, they are not included in regular protein databases, but in the "Comprehensive Peptaibiotics Database" [25].

Peptaibols are solely described for filamentous fungi exhibiting a mycoparasitic lifestyle, with a high abundance of over 80% of all known substances being derived from *Trichoderma* species [25]. Peptaibols are linear peptides, which besides of Aib include a characteristic acetylation of the N-terminus and a 1.2-amino-alcohol at the C-terminus. The first peptaibols, suzukacillin and alamethicin, have been described in the 1960s [26]. As demonstrated for alamethicin, the amphipathic character of peptaibols allows the voltage-dependent formation of helical structures acting as ion channels, thus spanning and permeabilizing the cell membrane and leading to cytoplasmic leakage and cellular breakdown [27].

Whereas all *Trichoderma* strains produce peptaibols, some substances are synthesized in a species- or even strain-specific manner [25]. For instance, in five different biocontrol agents containing species from the *Trichoderma harzianum* complex, peptaibols were the dominant secondary metabolites including three new and recurrent major groups present in all formulations [28]. Peptaibols play an important role in the mycoparasitic interactions as well as in induction of ISR in plants via up-regulation of the jasmonic acid and salicylic acid synthesis [29, 30]. In *T. harzianum*, peptaibols synergistically act together with hydrolytic, cell wall degrading enzymes on the cell wall destruction of the host fungus [31, 32]. Other mycoparasites such as *T. ophioglossoides* and *E. weberi* also comprise peptaibiotics-associated gene clusters, which are absent in plant- and entomopathogenic lineages of *Hypocreales,* suggesting the restriction of these genes to mycoparasitic species, further indicating their importance in the mycoparasitic interaction [11, 15]. The antifungal activity of the peptaibol trichokonin from *Trichoderma pseudokongii* caused extensive apoptosis by loss of the mitochondrial transmembrane potential resulting in apoptotic cell death in *Fusarium oxysporum* [33]. Similar evidences suggest a major involvement of peptaibiotics in mycoparasitism, substantiated by reports of antifungal action of peptaibols secreted by *Clonostachys rosea* against *S. sclerotiorum* [34], or *Sepedonium tulasneanum* against *Botrytis cinerea* and *Phytophthora infestans* [35].

2.2. Epipolythiodioxypiperazines

Epipolythiodioxypiperazines (ETPs) are characterized by the presence of an inter- or intramolecular disulfide bridge and a diketopiperazine core. The toxicity of ETPs lies in the disulfide bridging which is facilitating the inactivation of proteins by conjugation and the elicitation of reactive oxygen species (ROS) [36]. The best known substance of this class is gliotoxin derived

from *T. virens* Q-strains [37]. *T. virens* P-strains antagonize *Pythium ultimum* and do not produce gliotoxin but the terpenoid gliovirin, whereas Q-strains affect *R. solani* but not vice versa [38]. *C. rosea* also produces ETPs like verticillin A and gliocladines involved in the antagonism on nematodes [39] and glioperazine exhibiting antibacterial properties [40]. Whereas the role of gliotoxin (cluster comprising of 12 genes) as a virulence factor in human *Aspergillus fumigatus* infections and the self-protection via the *gliT* gene product of the biosynthetic gliotoxin cluster is well investigated, there is little and partially adverse information on the role of ETPs in biocontrol [36].

The weak mycoparasitic *T. reesei* exhibits an incomplete gliotoxin cluster whose genes were not expressed during confrontation with *R. solani* [4], whereas highly mycoparasitic *T. atroviride* does not contain a gliotoxin cluster [2]. The gliotoxin gene cluster of *T. virens* Q-strains consists of eight genes encoding the core enzyme *gliP* – an NRPS dioxypiperazine synthase – whose expression was induced during confrontation with *R. solani* [4]. Deletion of *gliP* resulted in gliotoxin production-deficient mutants, going hand in hand with a significantly reduced induction of ISR in cotton seedlings and antagonistic action against *P. ultimum* and *S. sclerotiorum.* Adversely, the mutants' antifungal activity against *R. solani* remained unaltered [41]. The involvement of ETPs in mycoparasitic interactions stays unresolved and seems to depend on the combination of several – largely unknown – factors like synergistic interactions with other metabolites or enzymes [42], environmental conditions, the species, strain and even the host organism.

2.3. Siderophores

Siderophores of fungal origin are high affinity iron chelating, linear to cylic oligomeric secondary metabolites mostly characterized by a N5-acyl-N5-hydroxyornithine basic unit [3]. Several siderophores are derived by one NRPS and post-synthetic subsequent modification [43]. As bio-available iron is rare in natural habitats, but an essential trace element to most organisms, efficient chelation, uptake and storage mechanisms for iron play an important role in competition and perseverance, especially within dense microbial communities like in soil [44]. Siderophores are important metabolites in the response against oxidative stress in several fungi like *Aspergillus nidulans, A. fumigatus, Cochliobolus heterostrophus, Gibberella zeae* and *T. virens;* furthermore, they play a role in conidial germination and sexual development [4, 45–47].

Evidences accumulate that siderophores act in biocontrol as virulence factors against other microbes during iron competition. Further, they promote plant growth by the reduction of oxidative stress: in biocontrol of *Fusarium* wilt disease by *Trichoderma asperellum* strain T34, the tomato plants exhibited reduced numbers of infected roots and a decrease in iron-associated abiotic stress [48]. The over-representation of siderophores in *Trichoderma hamatum* strain GD12 was reported to beneficially influence the biocontrol of *S. sclerotiorum* and plant growth promotion in lettuce [49]. More direct evidence for an involvement of siderophores in mycoparasitic interactions exists in *C. minitans:* the expression of *CmSIT1,* a gene-mediating siderophore-iron transport, not only enhanced antifungal abilities but also reduced growth [50].

3. Polyketides (PKs)

Polyketides (PKs) are derived from simple building blocks like acetyl-CoA or malonyl-CoA via consecutive PKS-mediated decarboxylative condensation and subsequent post-synthetic modification. Fungal PKSs are complex multi-modular enzymes, which obligatory include a characteristic ketoacyl-CoA-synthase (KS), an acyltransferase (AT) and an acyl-carrier (ACP) domain [20]. The structurally diverse PKs are the main class of secondary metabolites derived from fungi. The spectrum of substances ranges from spore pigments over antibiotics to toxins [2].

The *T. virens* and *T. atroviride* genomes are enriched for about 60% in PKS genes compared to *T. reesei* [21]. The *C. rosea* genome even exceeds this number with a total of 31 PKS genes [51], whereas the *T. ophioglossoides* genome comprises 15 PKSs [11]. The TMC-151 type PKs derived from *C. rosea* exhibits antibacterial properties [52], whereas *T. ophioglossoides* produces two antifungal and antibacterial substances: the polyketide ophiocordin and the NRPS-PKS hybrid enzyme-derived ophiosetin [11, 53, 54]. The deletion of *pks4* – encoding an orthologue of the aurofusarin and bikerfusarin forming PKSs of *Fusarium* spp. – in *T. reesei* caused extensive changes in morphology as well as physiology and metabolism. In Δ*pks4* mutants, the pigmentation of conidia and the generation of teleomorph structures were inhibited, and the stability of the conidial cell wall was reduced. *Pks4* deletion decreased *T. reesei*'s antagonistic abilities in confrontation assays, lowered its antifungal effect mediated by water soluble and volatile metabolites and altered the expression pattern of other PKSs [55]. It seems that also within this metabolite class, the effects are more diverse and global, than hitherto expected.

4. Terpenoids

Terpenoids are synthetized from the acetyl CoA-derived C-5-isopentenyl-diphosphate intermediates isopentenyl- and dimethylallyl-diphosphate. The C-5 units are subsequently processed via head-to-tail condensation by prenyl synthases and are post-synthetically modified by various enzymes resulting in different terpenoids originating from very few C-5 precursors [56]. Terpenoid biosynthetic clusters are characterized by the presence of a terpene cyclase gene [4]. Terpenoids are volatile to non-volatile substances constituting the highest abundant natural products on earth [37]. Terpenoids of fungal origin comprise phytohormones, mycotoxins as well as antibiotics and antitumor substances.

The *C. rosea* genome contains eight terpene synthase genes [51] and *E. weberi* comprises an expansion of six genes for terpene synthases in its genome, of which three lie within unique biosynthetic secondary metabolite clusters [15]. The majority of secondary metabolites of *S. elegans* secreted during mycoparasitizing *R. solani* are trichothezenes and atranones belong to the terpenoid class of secondary metabolites [17]. The *T. virens* genome comprises an enrichment of terpene cyclase genes compared to *T. atroviride* and *T. reesei* [57]. The production of several terpenoids was proven for *Trichoderma* species [58, 59], whereas their biosynthetic pathways mostly still remain obscure. The putative terpene cyclase *vir4* is well-researched

and only present in *T. virens,* but not in *T. reesei* or *T. atroviride*. Analysis of a mutant which exhibited defects in antibiotic production, a lack of viridin and viridiol synthesis and an under-expression of most of the genes of the *vir4* cluster evidenced that the cluster is involved in viridin biosynthesis [60]. Generation of a *vir4* deletion mutant and metabolic screening validated its involvement in terpene biosynthesis; the terpene cyclase gene *vir4*, however, turned out not to be involved in viridin or viridiol biosynthesis but in the synthesis of more than 20 volatile sesquiterpenes [61]. The involvement of terpenes in mycoparasitism relies unresolved, but there are hints: it is probable that genes underlying the mevalonate pathway also influence terpene synthesis. The *hmgR* gene codes for the glycoprotein 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which processes HMG-CoA to mevalonic acid. Accordingly *hmgR*-silenced mutants of *T. harzianum* exhibited decreased antifungal abilities [62]. Deletion of the trichodiene synthase genes *tri4* and *tri5* in *Trichoderma arundinaceum* resulted in a loss of harzianum A production, a reduced antagonism against host fungi and a decreased ISR in tomato plants [63, 64]. Expression of the *T. arundinaceum tri4* and/or *tri5* genes in *T. harzianum* mainly influenced plant wealth and defense by induced production of trichodiene and 12, 13-epoxytrichothec-9-ene (EPT) [65, 66], whereas *tri5* overexpression in *Trichoderma brevicompactum* boosted the excretion of antifungal trichodermin [67, 68].

5. Ecology and regulation of secondary metabolism in mycoparasitic fungi

Ancestral and recent lifestyles fundamentally influence the existence as well as the expression of secondary metabolite genes and clusters up to the species or even strain level. The transcriptional responses of *T. reesei, T. atroviride* and *T. virens* – which all share an ancestral mycoparasitic lifestyle – to the confrontation with *R. solani* illustrates this fact very well. All three species exhibit few common metabolic responses but autonomous and specific strategies in defeating their opponent. Both potent mycoparasites, *T. atroviride* and *T. virens*, attack their hosts in the stage before physical contact, but with distinct strategies of antibiosis. *T. virens* offends mostly via the NRP biosynthetic pathway for gliotoxin synthesis, whereas *T. atroviride* combats mainly via the PKS biosynthetic pathway, as well as the excretion of 6-pentyl-α-pyrone (6-PP) – a volatile organic compound (VOC) with antifungal and plant growth-promoting properties [2]. Conversely, the transcriptional response of the only slightly mycoparasitic *T. reesei* is more defense-related and targeted on the excretion of cellulases, hemicellulases and ribosomal proteins before hyphal contact [69]. The long-term specialized lifestyle and co-evolution of *E. weberi* with its host in its relatively demarcated ecological niche facilitated the loss of a manifold of genes leading to a more or less obligatory mycoparasitism with limited growth and viability without the presence of the host. Hence, the *E. weberi* genome demonstrates a high degree of specialization, with a unique secretome containing an unusually high content of over 50% of proteins with unknown function. Further, the genome contains only 20% homologs with the closely related *T. atroviride, T. virens* and *T. reesei* and only 12% of the 1066 unique genes exhibit homology with proteins in the whole subdivision of *Pezizomycotina* [15].

Several environmental cues like temperature, light, carbon, nitrogen, pH and competing or synergistic organisms are known to influence the transcriptional regulation of secondary metabolism-associated gene clusters (**Figure 1**). Suboptimal environmental conditions thereby often facilitate and promote transcriptional activation or transcriptional reprogramming events [70]. In media containing chitin or *B. cinerea* cell walls, the predicted cutinase transcription factor 1 encoding gene of *T. harzianum* (*Thctf1*) was up-regulated. *Thctf1* deletion mutants exhibited reduced antagonistic and antifungal ability, and the mutant strain did not synthesize two 6-PP derivatives, indicating a role of *Thctf1* in secondary metabolism of *T. harzianum* [71]. Furthermore, the overexpression of the gene encoding multiprotein bridging factor 1 (*Thmbf1*) of *T. harzianum* – a transcriptional co-activator of *Thctf1* – negatively regulated the antifungal abilities, as well as the expression of VOCs [72].

Like known for the production of mycotoxins in non-mycoparasitic species [73], secondary metabolite production in mycoparasitic fungi is governed by heterotrimeric G protein signaling and the associated cAMP-pathway, as well as mitogen-activated protein kinase (MAPK) cascades [74, 75]. *T. atroviride* mutants, lacking the MAPK-encoding gene *tmk1* showed an enhanced production of peptaibols and of 6-PP [74]. First evidence for a positive regulation of the secondary metabolism by cAMP signaling came from *T. virens* Δ*tac1* mutants bearing a deletion of the adenylate cyclase-encoding gene. The mutants were unable to offend *Sclerotium rolfsii* and *R. solani*, but showed a clear inhibition zone in direct confrontation with *Pythium* sp., pointing to a host-dependent expression of secondary metabolism-associated

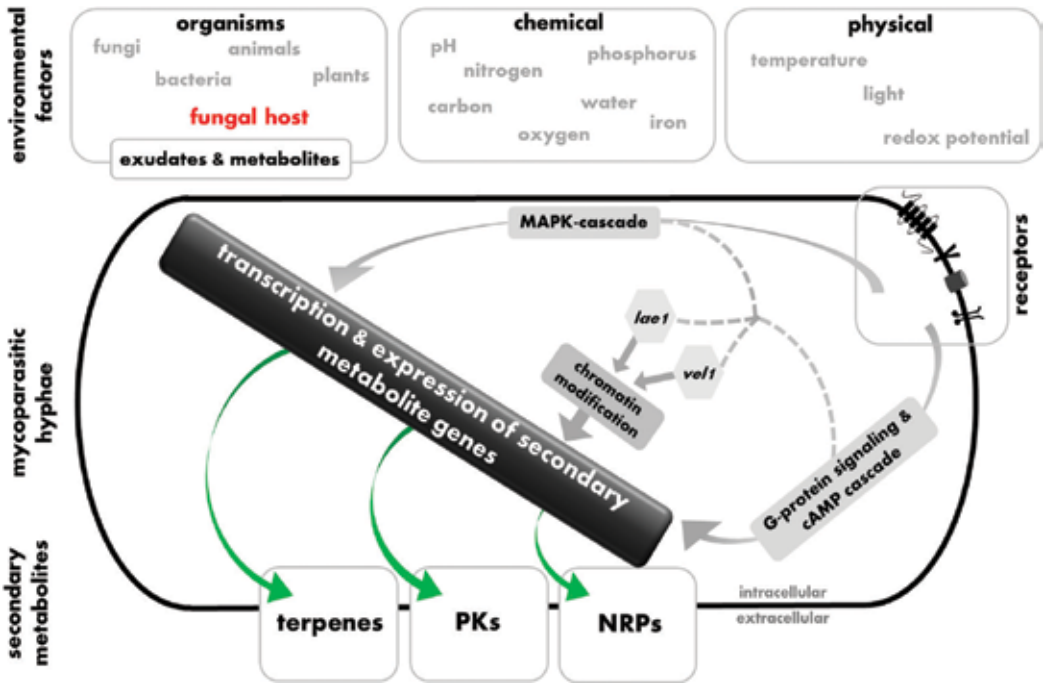

Figure 1. Overview on mycoparasitism-influencing factors and pathways in secondary metabolite biosynthesis of mycoparasitic fungi.

genes. Further, the mutant exhibited a diminished production of secondary metabolites like viridiol and a reduced expression of secondary metabolism-associated genes [75]. Prior to that, similar results were obtained for *T. atroviride* Δ*tga1* mutants missing the subfamily I Gα protein-encoding gene. Deletion of *tga1* led to a complete loss of overgrowth and mycoparasitism of different preys during direct confrontation and a decrease of 6-PP and sesquiterpene production as well as chitinase gene transcription. Despite the reduction in chitinase and 6-PP accumulation, the Δ*tga1* mutant caused a strong growth inhibition of prey fungi in the interaction zone mediated by yet unidentified low molecular weight antifungal metabolites, thereby evidencing opposite roles of *tga1* in regulating the biosynthesis of different antifungal substances in *T. atroviride* [76]. Similar to Δ*tga1* mutants, transformants bearing a deletion of the subfamily III Gα protein-encoding gene *tga3* were unable to overgrow and lyse prey fungi. However, absence of the adenylyl cyclase-stimulating Tga3 protein led to significantly reduced antifungal activity [77]. The global regulation of secondary metabolism and morphogenesis by the heterotrimeric VELVET protein complex, consisting of the S-adenosylmethionine-dependent methyltransferase LaeA and the velvet proteins VeA and VelB, was first described in *A. nidulans* [78]. Deletion of the *laeA* orthologue *lae1* in *T. atroviride* led to a loss of mycoparasitic abilities in direct confrontation and a major reduction in the synthesis of 6-PP and water-soluble secondary metabolites. Further, the expression of eight mycoparasitism-related genes was decreased in the mutant. The deletion of *vel1* – the *veA* orthologue – in *T. virens* caused defects in overgrowth and offense against the host in direct confrontation as well as in bioprotective plant interaction, accompanied by a decrease in the expression of several secondary metabolism-associated genes [79, 80].

6. Cross-talk by and response to secondary metabolites in mycoparasitic interactions

In bacteria, it has been shown that at sub-inhibitory concentrations antibiotics serve as mediators of microbial communication and interaction with one of the outcomes being the production of cryptic metabolites [81]. Accordingly, the interaction with other fungi may shape the secondary metabolite profile of a specific fungus, making co-cultures a valuable tool for eliciting the activation of silent secondary metabolism-associated gene clusters.

Studies on the mutual effects of secondary metabolites produced during mycoparasitic interactions are rare however. *Trichoderma*-derived 6-PP was shown to suppress the synthesis of the *Fusarium* mycotoxins fusaric acid and deoxynivalenol (DON) [82–85], suggesting that 6-PP acts as communication molecule that elicits biological responses in the interaction partners. On the other hand, fusaric acid and DON modulate 6-PP production as well as chitinase gene expression in *T. atroviride* and recent studies provided evidence that *Fusarium* mycotoxins induce defense mechanisms in mycoparasites such as *T. atroviride* and *C. rosea* which results in mycotoxin detoxification [59, 86]. *C. rosea* was shown to open the ring structure of zearalenone (ZEN), while *Trichoderma* spp. seem to convert ZEN into its reduced and sulfated forms and metabolize DON to deoxynivalenol-3-glucoside, a detoxification product of DON previously identified in plants [87, 88]. In the interaction of the mycoparasite *T. arundinaceum*

with *B. cinerea*, *Botrytis*-derived mycotoxins botrydial and botcinins attenuated trichothecene biosynthesis gene expression in *Trichoderma* while botrydial production was repressed by *Trichoderma*-derived harzianum A and aspinolide [89–91].

Co-culturing of mycoparasites with prey fungi simulates the conditions occurring during the mycoparasitic interaction in natural or agricultural systems and could hence encourage the production of secondary metabolites via communication and signaling molecules. Accordingly, pairwise interactions of *Aspergillus niger*, *Fusarium verticilliodes* and *C. rosea* led to metabolites that occurred in single cultures but were suppressed in dual cultures, and many new metabolites not present in single cultures were found in dual cultures [92]. Similar results were obtained in co-culturing experiments of *T. harzianum* and *Talaromyces pinophilus* with the accumulation of siderophores being induced in both interaction partners and the production of *Talaromyces*-derived 3-*O*-methylfunicone and herquline B being reduced. In addition, the novel substance harziaphilic acid was detected in the co-culture only [93].

Based on these studies, it is evident that secondary metabolites contribute to mycoparasitic interactions in various ways including inhibition of the activity or synthesis of mycoparasitism-relevant enzymes and other substances, by eliciting defense and detoxification responses or by triggering the production of cryptic metabolites. In most cases, however, information on the spatial distribution of the secreted substances is lacking and it is hence difficult to assign novel secondary metabolites specifically induced during the co-cultivation to its actual producer. Recently, mass spectrometry-based imaging (MSI) turned out as a valuable tool for in situ visualizing the dynamics and localization of small molecules released during microbial interactions [94]; however, reports on its application to mycoparasitic fungus-fungus interactions are still rare. By applying matrix-assisted laser desorption/ionization (MALDI)-based MSI for visualization and identification of secondary metabolites being exchanged during the mycoparasitic interaction of *T. atroviride* with *R. solani* [95], the diffusion of *Trichoderma*-derived peptaibols toward the prey and the accumulation of *Rhizoctonia*-derived substances at the borders of fungal interaction was tracked. Monitoring of the *T. harzianum* interaction with the fungal phytopathogen of cacao plants *Moniliophthora roreri* by MSI lead to the detection of T39 butenolide, harzianolide, sorbicillinol and an unknown substance specifically produced in the co-culture with a spatial localization in the interaction and overgrowth zones [96].

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Section 2

Plant Secondary Metabolites

Chapter 4

Comprehensive Insight into the Elderflowers and Elderberries (*Sambucus nigra* L.) Mono and Sesquiterpenic Metabolites: Factors that Modulate Their Composition

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Additional information is available at the end of the chapter

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Abstract

Plant secondary metabolites are synthesized for their protection and regulation purposes. Quite often, due to their properties, these metabolites have relevant organoleptic and biological properties and can play important roles in human health and general well-being. A relevant case study in this context is berries and flowers from *Sambucus nigra* L., which have been used for generations in folk medicine. Although those effects are mainly linked to phenolic compounds, mono and sesquiterpenic secondary metabolites may also play a key role. Despite their potential, *S. nigra* mono and sesquiterpenic compounds are yet largely unexplored. Complex and dynamic external and internal plant-related phenomena deeply affect terpenes profile, as metabolism, abiotic and biotic stresses, and understanding these phenomena is the first step for *S. nigra* berries and flowers' valuation. This chapter will cover aspects linked to elder plant uses, mono and sesquiterpenic composition, and the influence of preharvest and postharvest effects over these metabolites. This knowledge is crucial for scientists and industries to understand and improve the quality of *S. nigra*-based products.

Keywords: elderberry, elderflower, *Sambucus nigra* L., secondary metabolites, mono and sesquiterpenic compounds

1. Introduction

A growing interest in the exploitation of natural products as sources of bioactive compounds with potential health benefits has been observed, particularly in the consumption of plant-based products that are able to prevent, ameliorate, or even treat chronic diseases with increasing incidence in the advent of the twenty-first century [1]. The elderberry plant (*S. nigra* L.) may be reported to illustrate this trend, especially due to its potential impact on the local economies as a raw material used to produce, specially, food and beverages, but also nutraceutical and cosmetic products derived from berries and flowers.

S. nigra flowers and berries have been widely used in folk medicine for numerous applications that include antimicrobial, antiviral, antioxidant, anti-inflammatory, anticancer, antidiabetic applications, among others [2–6]. The detailed knowledge of the chemical composition of *S. nigra* is not only extremely important to understand its biological effects but also to improve its value and applicability. Elderberries and elderflowers received increased attention due to the presence of phytochemicals with many reported health benefits, comprising, among others, vitamins, terpenic, and phenolic compounds [5]. Volatile and semi-volatile terpenic components (mono and sesquiterpenics), in particular, are plant secondary metabolites that play key roles in their protection and communication processes. They are often reported as toxic to some microorganisms involved in plant-insect interactions [7, 8]. Additionally, they confer plant protection against oxidative stress, namely as thermo-tolerance mediators [9], playing important roles in their adaptation to biotic and abiotic stresses [10]. On the other hand, the transcriptional regulatory network to different biotic and abiotic stresses is reflected on the plant metabolism, as well as phenological processes, such as on ripening [11], which ultimately will impact the plant secondary metabolites' profile. Nonetheless, these effects are still poorly explored and understood, particularly in the case of *S. nigra.*

Understanding those effects could be of extreme importance, given that mono and sesquiterpenic-based extracts are commercially important, namely for pharmaceutical/nutraceutical, agronomic, food, sanitary, and cosmetic industries. For instance, limonene and linalool, two of the most used monoterpenic compounds, are often employed in perfumes, creams, soaps, as flavor additives for food, as fragrances for household cleaning products and as industrial solvents [8]. In the particular case of potential health benefits-related applications, these compounds have been reported as exhibiting hepatoprotection [12], anti-inflammatory [13], analgesic [14], and antioxidant [15] activities, among others [16]. These effects are strongly dose-dependent, which reinforce the need to study in detail the terpenic composition of *S. nigra* berries and flowers, in-depth, and the variables that have an impact on their composition.

The challenge to understand the impact of pre- and postharvest processes over the mono and sesquiterpenic compounds is the first step to further establish approaches that can control the variables that have a significant effect over these processes. Hence, the present chapter is devoted to a detailed discussion of *S. nigra* mono and sesquiterpenic composition, and of the parameters, such as pre- and postharvest conditions, that modulate their composition. Also, a general perspective of *S. nigra* L. berries and flowers relevance and uses is reported.

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2. Elderberry and elderflower applications

Flowers and berries from *S. nigra* species have long been used in folk medicine for prophylactic and therapeutic purposes, being considered the medicinal "chest" from the days of Hippocrates [17]. The relief of early symptoms of common cold using elderflowers fulfills the requirement of medicinal use according to the Committee on Herbal Medicinal Products [3]. Elderflowers were also used to alleviate bronchial and pulmonary diseases, tumors, and ulcers [18]. Laxative [19], treatment of asthma [20], toothache, colic, cold, and rheumatic conditions [21, 22] have been reported, as well as bronchitis, whooping cough, hemorrhoids, expectorant [20], insect bites, and fever [21]. Elderberry preparations are traditionally used as laxative, diaphoretic [2], for fever reduction [19], anti-rheumatic, and to treat colic in infants [23]. Their juice has also been used to treat sciatica, headache, dental pain, heart pain, nerve pain, while the syrup was recommended to treat cough and cold [2].

This species has gained attention due to its diverse uses, assimilating in markets from food and herbal industries. In 2010, *S. nigra* (flowers and berries) was the most harvested medicinal plant intended for export trade and for infusions and phytopharmaceutical production in Bulgaria and Romania [24]. Additionally, elderberry was ranked on the Top 20 best-selling herbal dietary supplements in the medicinal, food, and mass market in the USA in 2011–2014, with increasing sales of 64% in 2014 [24, 25].

One of the main uses of elderberries is the production of natural food colorants, juices, and concentrates, due to their high content in phenolic compounds [26]. Those are also exploited for the formulations of decoctions [2, 19], infusions [23], juice and syrup/concentrate [2], extracts, supplements, pies, ice creams, jellies, juices, beverages, beers, wines, liqueurs, and fruit bars [27–29]. In addition to color, flavor (taste and aroma) is also an important parameter in the consumer perception and product acceptance [30]. Due to the pleasant and characteristic floral aroma, elderflowers are often used as flavoring agents [31] for the preparation of infusions, decoctions [19–22], pastry products [23], nonalcoholic cordials, and fermented beverages [18, 23]. Elderflowers are characterized by an intense, pleasant, and characteristic aroma, currently named as elderflower aroma [32, 33]. Despite the role of esters, alcohols, and aldehydes, monoterpenes, as limonene, terpinolene, and terpinene, present a relevant contribution for the elderflowers fruitiness aroma [34], and more exotic notes, such as woody and spicy, have been attributed to some mono and sesquiterpenic compounds [32, 34].

3. *S. nigra* mono and sesquiterpenic metabolites composition

Terpenic compounds form a large and structurally diverse family of secondary metabolites derived from C_5 isoprene units, with over 35,000 known structures [35]. The volatile and semi-volatile ones, that is, mono and sesquiterpenic compounds, result from two main biosynthetic routes, starting from the mevalonate and the methylerythritol phosphate pathways (**Figure 1**). These are produced through the activity of a large family of enzymes, the mono and sesquiterpene synthases and cyclases, but others are formed through transformation of the initial

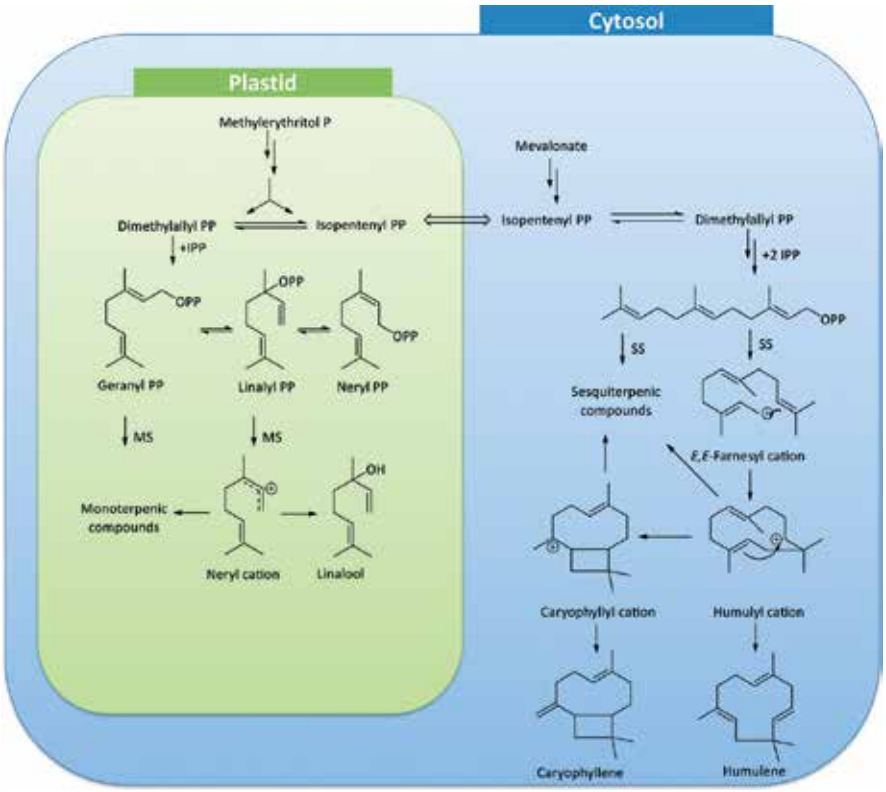

Figure 1. Simplified mono and sesquiterpenic compounds biosynthetic pathways, illustrated with the routes for linalool, caryophyllene and humulene found in *S. nigra*. MS, monoterpene synthases; SS, sesquiterpene synthases; PP, pyrophosphate.

products by acylation, dehydrogenation, oxidation, and other reaction types, such as acetylation [35, 36]. For instance, in **Figure 1**, illustrates the biosynthesis of linalool, caryophyllene, and humulene, three compounds present in *S. nigra* plant. The biosynthesis occurs from their respective linear precursors, geranyl pyrophosphate and farnesyl pyrophosphate, originating. Both linear and cyclic structures [35].

Information about mono and sesquiterpenic compounds from elderflowers and elderberries is still scarce and disperse. Thus, to systematize this data, the information related with ripe berries and fresh flowers and minimally processed products, such as infusions, syrups, and juices, is presented in **Table 1**. The reported studies are mainly focused on analytes' identification rather than on their quantification, however, when available, quantitative data is also provided.

So far, 89 mono and sesquiterpenic compounds are reported in elderflowers (64) and elderberries (61). Recent studies using an advanced gas chromatographic methodology (comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry detection—GC × GC-ToFMS) have contributed to substantially increase the knowledge about *S. nigra* berries and flowers volatile terpenic profile by reporting dozens

Metabolites	Elderflowers[a]	Elderberries[a]	References
Monoterpenic compounds			
Hydrocarbon type			
Camphene	—	✓	[37]
3-Carene	✓	✓	[31, 38, 39]
Cosmene	✓	—	[31, 40]
o-Cymene	—	✓	[41]
p-Cymene	✓	—	[31, 32, 37]
2,6-Dimethyl-2,6-octadiene	✓	—	[31]
D-Limonene[b]	✓	2.24–9.92	[31, 32, 37, 38, 41–43]
1,3,8-p-Menthatriene	—	✓	[37]
Myrcene	✓	✓	[31, 37, 38, 42]
Ocimene[b]	✓	1.55–9.32	[31, 32, 34, 41]
α-Phellandrene	✓	✓	[32, 34, 37, 41]
α-Pinene	✓	✓	[31, 37]
β-Pinene	✓	✓	[31, 37, 38]
α-Terpinene	✓	✓	[32, 34, 41]
γ-Terpinene	✓	✓	[31, 32, 34, 41]
Terpinolene	✓	✓	[31, 32, 37, 38]
Verbenene	—	✓	[37]
Oxygen-containing type			
Artemisia alcohol	—	✓	[37]
Borneol	—	✓	[37, 41]
Camphor	✓	✓	[37, 41, 42]
3-Caren-2-ol	—	✓	[37]
Carvacrol	✓	—	[42]
Carvone	✓	✓	[37, 42]
1,8-Cineole	✓	✓	[31, 34, 41, 42]
Citral	✓	✓	[31, 37, 41]
Citronellal	✓	—	[31, 37]
Citronellol	✓	✓	[34, 37, 42, 44]
Citronellyl formate	✓	—	[31]
p-Cymen-8-ol	—	✓	[37]
Dehydroxylinalool oxide	✓		[31]
Dihydromyrcenol	—	✓	[37]

Metabolites	Elderflowers[a]	Elderberries[a]	References
Fenchol	—	✓	[37]
Fenchone	✓	—	[31]
Geranial	✓	✓	[31, 41]
Geraniol[b]	✓	1.05–7.21	[31, 32, 37, 41]
Geranyl acetate	—	✓	[37]
Hydroxylinalool	✓	—	[32, 34]
Hotrienol[b]	✓	2.56–8.08	[31, 34, 37, 40–44]
Hydroxycitronellol	—	✓	[41]
Lilac aldehyde	✓	—	[31]
Lilac alcohol	✓	—	[31]
Limonene oxide	✓	—	[31]
Linalool[b]	✓	1.18–128.89	[31, 37, 40–43, 45]
E-Linalool oxide (furanic form)	✓	✓	[31, 37, 40, 42, 44]
Z-Linalool oxide (furanic form)	✓	✓	[31, 37, 40, 42, 44]
E-Linalool oxide (pyranic form)	✓	—	[31, 32, 34, 40, 42, 44]
Z-Linalool oxide (pyranic form)	✓	—	[31, 32, 34, 40, 42, 44]
Linalool methyl ether	✓	—	[31]
Menthol	✓	✓	[37, 41, 42]
Methyl citronellate	✓	—	[31]
Methyl geranate	✓	—	[31]
Myrcenol	✓	—	[31]
Myternol	✓	—	[31]
Nerol	✓	✓	[31, 32, 37, 41]
Nerolidol	✓	—	[42]
Nerol oxide[b]	✓	1.02–7.80	[31, 34, 41, 42, 45]
Pinocarvone	—	✓	[37]
E-Rose oxide	✓	✓	[31, 34, 37, 40–42, 45]
Z-Rose oxide[b]	✓	1.68–8.34	[31, 34, 37, 40–42, 45]
Tagetone	✓	—	[31]
α-Terpineol[b]	✓	70.85–2699.56	[31, 37, 41, 42, 45]
Terpinen-4-ol	✓	✓	[31, 32, 37, 41]
β-Terpinyl acetate	—	✓	[37]
α-Thujone	✓	—	[31, 42]
β-Thujone	✓	—	[31, 42]
Thymol	✓	—	[42]

Metabolites	Elderflowers[a]	Elderberries[a]	References
Verbenone	✓	—	[31]
Sesquiterpenic compounds			
Hydrocarbon type			
Aromadendrene	✓	✓	[31, 37]
α-Bergamotene	✓	—	[31]
β-Bourbonene	✓	✓	[31, 37]
Cadinene	✓	✓	[31, 37]
α-Calacorene	—	✓	[37]
Calamenene	✓	✓	[31, 37]
Calarene	—	✓	[43]
β-Caryophyllene	✓	✓	[31, 32, 34, 37]
α-Copaene	✓	✓	[31, 37, 42]
Cubebene	✓	✓	[31, 37]
β-Elemene	✓	✓	[31, 37]
α-Farnesene	✓	—	[31]
D-Germacrene	✓	—	[31]
α-Humulene	—	✓	[31, 38]
Longifolene	—	✓	[37]
α-Muurolene	—	✓	[37]
Oxygen-containing type			
β-Bourbonen-13-ol	—	✓	[37]
t-Cadinol	—	✓	[37]
Caryophyllene oxide	—	✓	[37]
Cubenol	—	✓	[37]
Globulol	—	✓	[37]
Epiglobulol	—	✓	[37]

[a]When available, quantitative information was reported;

[b]μg/kg of fresh berries; Marks "✓" correspond to nonquantified compounds or quantified but not expressed as berry or flower weight basis.

Table 1. Mono and sesquiterpenic compounds reported in *S. nigra* L. berries and flowers and related products, such as infusions, syrups, or juices.

of monoterpenic and sesquiterpenic compounds for the first time in these matrices [31, 37]. Representative total ion GC × GC chromatogram contour plots from fresh elderflowers and ripe elderberries are illustrated in **Figure 2**, highlighting the complexity of the natural matrices.

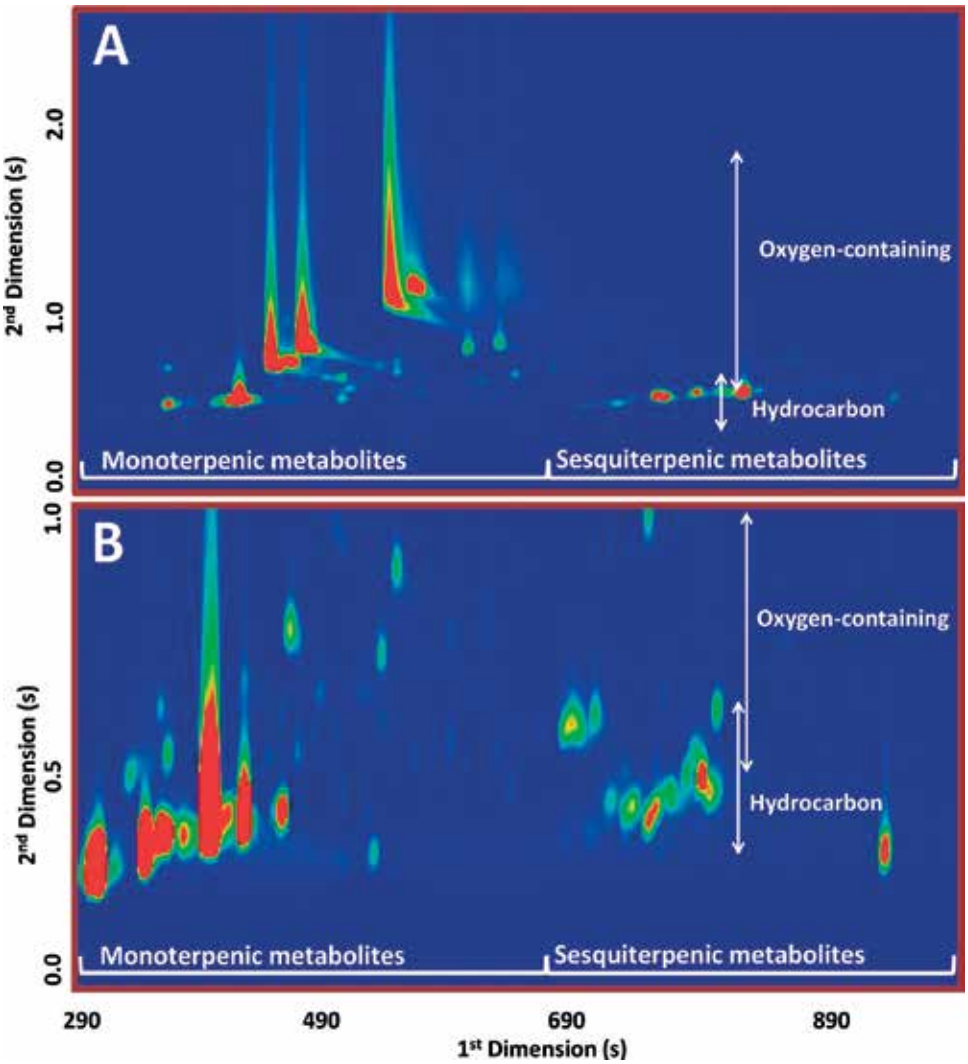

Figure 2. GC × GC–ToFMS chromatogram contour plots from fresh elderflowers (A) and fresh ripe elderberries (B). The chromatographic spaces corresponding to monoterpenic and sesquiterpenic compounds are highlighted.

As evidenced in **Table 1**, of the 64 volatile terpenic compounds reported from elderflowers, 40 are oxygen-containing structures. As shown in **Figure 2**, the peak intensities of the monoterpenic metabolites predominate, representing up to 99 and 77% of the overall elderflowers and elderberries terpenic content, respectively [31, 37]. Linalool oxide (in the pyranoid form) is a major component from fresh elderflowers, accounting for up to 87% (relative to the overall GC peak area) [31]. Other authors reported that hotrienol (14%, w/w), rose oxide (5%, w/w), linalool (4%, w/w), and linalool oxide (furanic forms, 3%, w/w) were the major monoterpenic metabolites from dried elderflowers [42] (chemical structures illustrated in **Figure 3**).

Regarding ripe elderberries, limonene and *p*-cymene are reported as the major monoterpenic components (**Figure 3**). Along with limonene (2.2–9.9 μg/kg of fresh berries), other authors reported as major components in fresh elderberries the monoterpenic compounds

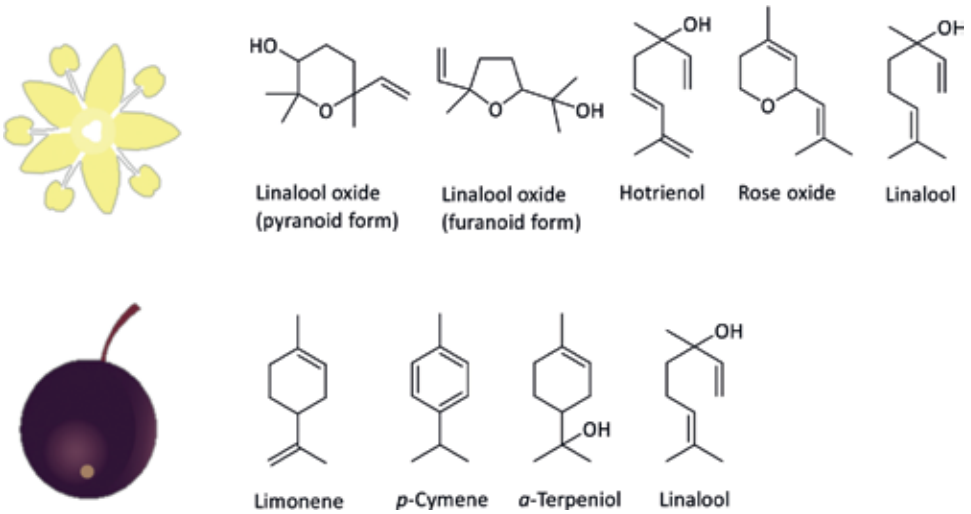

Figure 3. Main monoterpenic components from *S. nigra* flowers and berries.

linalool (1.2–128.9 μg/kg of fresh berries) and α-terpineol (70.8–2699.5 μg/kg of fresh berries) (**Figure 3**) [41]. Monoterpenic compounds also prevailed in its juice, ranging from 8.9 to 77.2 ng/mL, limonene and linalool being the main monoterpenic components (**Figure 3**) [38]. Sesquiterpenic compounds are present in lower amounts, when compared to the monoterpenic ones, both in elderflowers and in elderberries. They represent up to 0.6% in elderflowers, with β-caryophyllene and α-farnesene as major sesquiterpenic components, while in elderberries, they account for up to 13% of the terpenic content, being β-caryophyllene and aromadendrene the major ones [31, 37]. No quantitative data for the resquiterpenic composition of fresh flowers and berries is available in literature.

4. Factors that modulate mono and sesquiterpenic profile

4.1. Preharvest impact

Crop quality could be defined as a set of agronomic/commercial, organoleptic, and nutritional qualities that are variable among (1) distinct species but also among different cultivars within the same species (genetic factors); (2) different climatic conditions, such as water availability and light exposition; and (3) different agronomic conditions, such as cultivation systems, fertilization, and harvesting date [46]. Altogether, these preharvest factors may have an impact on the final quality of the elderberry fruits and flowers; however, the information about these effects is scarce. The impact of preharvest factors is often focused on parameters with direct agronomic and commercial relevance, as plant yield, fruit size, sugar content and acidity (e.g., reviews on *S. nigra* plant [18, 23]), from which some nutritional quality parameters can be inferred. However, the comprehensive impact of these parameters on the chemical composition, specially in what concerns the target molecules with determining biological properties, still remains unknown. As relevant examples in the present appraisal, the impact of preharvest factors on *S. nigra* mono and sesquiterpenic compounds is still in the beginning and the

available literature is mainly focused on ripening and cultivar effects. However, considering that these components, as plant secondary metabolites, play an important role in plant growth and development, in the interaction with surrounding environment, such as temperature, water, radiation, chemicals, mechanical (as wind or soil movement), pathogen attacks, and nutrient deficiencies [10, 47, 48], as well as in their potential health benefits, the interest in the detailed understanding of the impact preharvest parameters on their profile is of obvious interest.

The production of terpenic metabolites depends on the physiological and developmental stage of the plant [10, 37]. Fruit ripening, in particular, is a crucial phenomenon that affects different physiological and biochemical processes, which are determinant to the development of nutritional and organoleptic characteristics [30]. The fruit organoleptic characteristics such as taste, color, and aroma are important quality and consumer acceptance-determining features [30, 49].

During ripening, major events occur, including cell expansion and softening, dismantling of the photosynthetic apparatus, and degradation of chlorophyll [11]. Elderberry ripening takes place from the 1 to 2-month period, starting with a green appearance and they ripen over a period of 6–8 weeks from July to September (depending on the geographic location). When elderberries become ripe, they have a characteristic deep purple color [23]. The accumulation of sugars (expressed as total soluble solids [TSS]) and decrease in acidity (pH and titratable acidity [TA]) have been routinely used by growers as a decision-making parameter to establish the harvesting moment and even the commercial price of the berries [18, 23, 37]. The ripe elderberries' pH ranges from 3.8 to 4.8; TA ranges from 0.48 to 1.43 g citric acid/100 g FW berries, while TSS ranges from 10.1 to 17.5°Brix [37, 38, 50, 51]. **Figure 4** illustrates the impact of ripening in those tree parameters on elderberries harvested in a Portuguese location (Tarouca, Távora and Varosa Valley), in the harvest season of 2013.

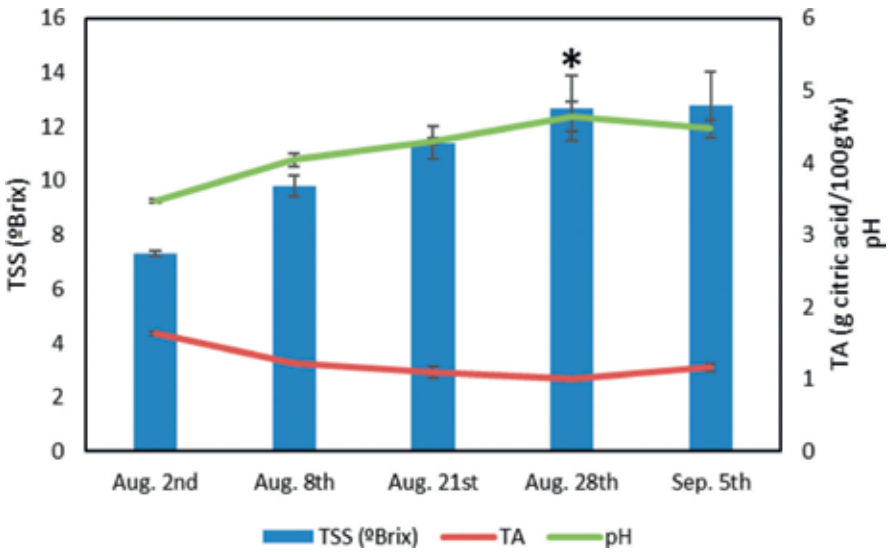

Figure 4. Total soluble solids (TSS), titratable acidity (TA), and pH from elderberries at five ripening stages [37], harvested in a Portuguese plantation (Tarouca, Távora and Varosa Valley), in the harvest season of 2013. The harvesting date assigned with * represents the ripe stage.

During the ripening process, several other phenomena occur, namely biosynthesis and degradation of a wide range of secondary metabolites that may have direct relevance in elderberry sensorial characteristics. A recent metabolomics-based study that exploited the effects of the developmental stages of different cultivars on the volatile terpenic components [37] demonstrated that the variability of monoterpenic compounds (β-pinene, 1,3,8-*p*-menthatriene, terpinolene, dihydromyrcenol, fenchol, α-terpineol, and citral) and of the sesquiterpene β-elemene was linked to elderberries ripening. Overall, monoterpenic and sesquiterpenic content exhibited a similar trend of variation through ripening, that is, gradually decreased over the ripening stages, which was mainly ruled by the major components, namely limonene, *p*-cymene, β-caryophyllene, and aromadendrene. These components were proposed as quality markers to follow-up the ripening process [37].

Plant cultivars generally differ in yield, organoleptic, and nutritional characteristics [23, 46], and their genetic background is a factor that influences quality traits [46]. In the particular case of elderberries, cultivars are classified based on their morphological characteristics and yield [52], as no definitive taxonomic DNA-based studies have been conducted in this species. Although, efforts have been made for their classification with molecular data. For instance, Portuguese *S. nigra* clones explored from local growers using different molecular characterization tools [52], and a genebank has been created for different *Sambucus* species and dozens of cultivars [53].

It is reported that elderberry yield ranges anywhere from 1 to over 30 kg per bush, depending on cultivar [54, 55]. This aspect, together with the fact that several cultivars are nowadays explored for the formulation of various products, where formula standardization is required, implying the comparison of cultivars' composition, can play a significant role in their application (e.g., [56]) and then become important decision tool for producers. The fact that mono and sesquiterpenic synthesis is encoded by a variety or cultivar-related genes implies that their levels can be cultivar-dependent, which, on the one hand, might be used to trace its varietal origin [57] and, on the other hand, can be used to better manage their final product and to maximize the commercial value of the crop. An exploratory study, suggested a possible cultivar effect over the mono and sesquiterpenic compounds profile from fresh elderflowers [31]; however, more consolidated data is still required to sustain the stated remarks, namely in what concerns the number of analyzed samples and different harvesting years.

The specific cultivar metabolite profile may imply differences at the sensorial level in *S. nigra*-based products, as shown for elderflower- and elderberry-based products obtained from different cultivars [32, 34, 56]. In a study that merges the results from the sensory evaluation and information on the aroma of the individual volatile compounds, the results highlighted that different elderberry cultivars had specific sensory characteristics (as fresh-fruity-sweet aroma) and, hence, volatile composition [38]. Differences in linalool and α-terpineol (ranging from 2.8 to 21.7 and from 213.6 to 2699.6 μg/kg, respectively) were reported for thawed ripe elderberries from different cultivars [41]. Likewise, the terpenic alcohols and oxides in elderflowers from different cultivars ranged from 0.8 to 3870 ng/mL for hotrienol; from 1.2 to 2320 ng/mL for *cis*-rose oxide; from 2.3 to 1840 ng/mL for linalool; and from 1.3 to 1100 ng/mL for linalool oxide (furanic form) [32, 34].

Despite the studies reported earlier, a more comprehensive understanding of the influence of preharvest parameters will require their analysis in an integrated approach, including,

among others, climate, agricultural practices, soil, and harvesting year to fully understand how these affect the biochemical mechanisms involved in the formation of mono and sesquiterpenic metabolites from elderflowers and berries and also to improve its valorization potential, particularly when related with health benefits and relevant sensorial characteristics. Also, the influence of climate change on the *S. nigra* plant response should be a noteworthy issue.

4.2. Postharvest impact

Postharvest management includes a set of postproduction practices comprising, among others, cleaning to eliminate undesirable elements and improve product appearance, sorting, cooling, control of variables such as temperature and relative humidity, and packing, ensuring that the product complies with the established quality standards for fresh and processed products [58, 59]. Postharvest practices may deeply affect the quality of a product in many aspects such as chemical and sensorial characteristics but also their potential health benefits, and ultimately, it may affect product's acceptability and marketability [30]. Therefore, reliable and objective quality-control tools to measure the impact of postharvest practices (ideally integrated with preharvesting conditions) over product quality and in the present appraisal on sensory quality are essential.

Elderflowers and elderberries go through different postharvest handling and storage conditions that precede processing, to prepare stable formulations for commercialization. **Figure 5** illustrates the main steps from harvesting for the storage of elderberries and elderflowers and the main chemical changes that may occur throughout these processes [31, 33, 42, 60–63].

S. nigra flowers or berries are typically collected during the morning and transported to processing facilities in specific plastic crates, avoiding damage caused by their own weight [5]. Flowers are often frozen or air-dried and then the stems are removed, while elderberries are sun dried or refrigerated, and the stems are removed and stored in silos at subzero

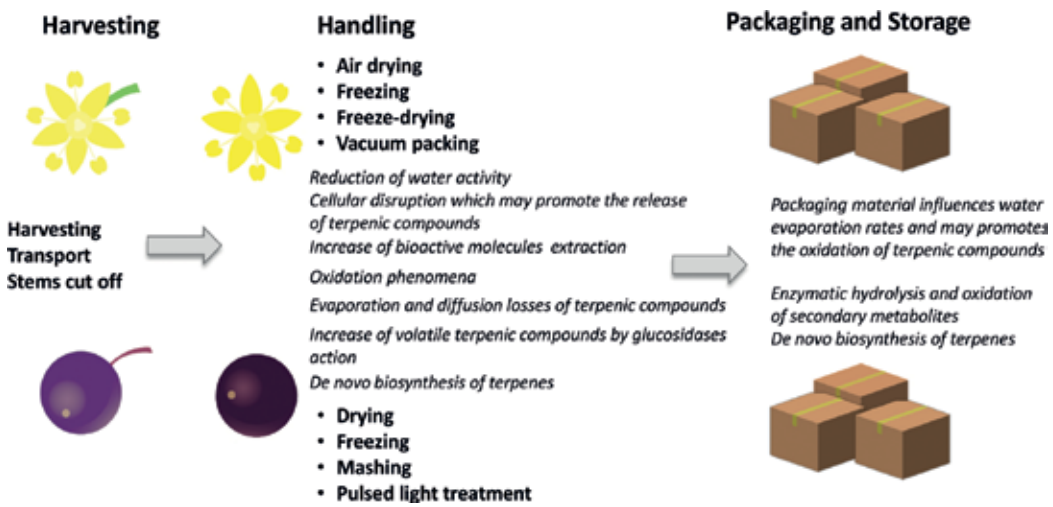

Figure 5. From harvesting to elderberry and elderflower storage. The main chemical changes that may occur through different steps are included [31, 33, 42, 60–63].

temperatures [5]. Elderberries can also be pulse-light treated and further crushed and mashed to produce concentrate juices [64–67]. These later steps promote the degradation of berry cell walls, contributing to the alteration of metabolites' profile, namely increasing the anthocyanin content of juices [63]. All these handling and storage processes may have an impact on the chemical composition of these matrices, as discussed as follows.

The knowledge of the impact of handling and storage conditions on the terpenic metabolites of *S. nigra* is still scarce for both, berries and flowers. However, more information is available regarding postharvest effects over the elderflowers' matrix. For instance, the impact of freezing, freeze-drying, air-drying, and vacuum packing over the volatile terpenic compounds was monitored for up to 1 year (**Figure 6**) [31].

After 1 year of storage, a decrease of the total terpenic content up to 47% for frozen elderflowers; up to 67 and 71% when vacuum packed and kept under light exposure and without light exposure, respectively; up to 82% for air-dryed elderflowers; and up to 85% for freeze-dried elderflowers (**Figure 6**) [31]. Under vacuum packing, there was no significant impact from light exposure. Linalool oxides were suggested as markers of the impact of the studied postharvest conditions over the volatile terpenic metabolites of elderflowers [31].

Drying methodologies, as air-drying or freeze-drying, often fail to completely preserve volatile aroma compounds [68], as reported in dried elderflowers, mainly due to diffusion and evaporation losses [31, 33, 69, 70]. Drying of elderberries or their products promotes a water activity reduction, contributing to the preservation of the samples against microbial contamination and also decreases the degradation of anthocyanins [60], by increasing their stability

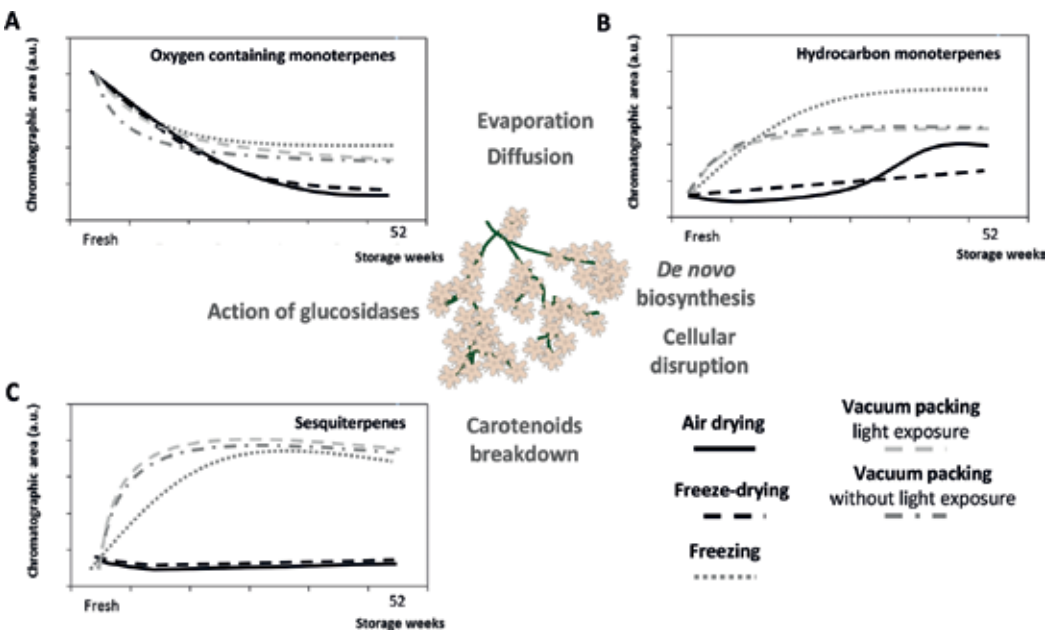

Figure 6. Variation trends of the abundance of oxygen-containing monoterpenes (A), hydrocarbon monoterpenes (B) and sesquiterpenes (C) toward the different handling and storage conditions for up to 1 year, based on the corresponding GC peak areas (au: Arbitrary units). Adapted with permission [31].

[60]. Other strategies have been used to preserve the elderberries' bioactive components or to enhance their nutritional value, as for instance, their processing with pulsed ultraviolet light to enhance the phenolic content [61]. However, no studies were performed so far on mono and sesquiterpenic fractions of elderberries.

Storage time also plays an important role in the mono and sesquiterpenic composition illustrated by the fact that 15 compounds, including rose oxides, hotrienol, linalool, α-terpineol, hydroxylinalool, and limonene, partially or completely vanished during storage of dried elderflowers [33]. Cellular disruption might explain the release of volatile compounds in certain postharvest conditions, namely freezing storage [31, 71, 72]. Likewise, the packaging material might affect their profile, being reported that elderflower's tea bags made of aluminum had the highest average concentrations of rose oxide, linalool oxide, nerol oxide, and hotrienol, when compared to paper and plastic bags [33].

Some components, such as hotrienol, were observed to increase during storage of elderflowers, which could be associated with the action of enzymes, such as glucosidases, that unbound the volatile components from glycosides present in the matrix [33]. Non-oxygen-containing structures, that is, monoterpenes and sesquiterpenes, also increased under certain postharvest conditions (**Figure 6**), again assuming that *de novo* biosynthesis of terpenes may play a key role in this phenomenon [31, 33].

The modifications in *S. nigra* terpenic profile upon different postharvest conditions have a significant impact on sensorial characteristics of its products, being linked to a dynamic and complex network of enzymatic and physicochemical phenomena. Understanding the postharvest impact is a step forward to manage and control the production of elderflower and elderberry formulations [31].

5. Concluding remarks

Plant secondary metabolites play key role in the plants' protection and communication processes. Beyond that, these components, and particularly, mono and sesquiterpenic compounds, are nowadays explored in industrial sectors due to their pleasant aroma characteristics and potential on the prevention and management of human diseases. The exploitation of *S. nigra* L. plant has gained increasing attention in the last decade. Literature highlights that this plant has been used, both, for the formulation of food products and in folk medicine. More recently, herbal supplements and nutraceuticals are also available. Given the diversity of biological activities reported for mono and sesquiterpenic components, as hepatoprotection [12], anti-inflammatory [13], analgesic [14], and antioxidant [15], among others [16], it allows to infer strong potential to foster its economic value. Actually, to go further in that direction, several challenges have to be overcome, namely (1) to in-depth know the mono and sesquiterpenic composition, (2) to study the impact of preharvest factors, and (3) to select the appropriate postharvest procedures to preserve bioactive molecules, as elderflower and elderberries seasonality requires handling and storage steps. Quality of crops, specifically elderberries and elderflowers, is a complex concept which could be defined by the yield efficiency, the organoleptic and the nutritional quality [46]. If,

for several years, the agronomic and organoleptic qualities were the main market drivers, nowadays, the importance of the nutritional value is strongly increasing, thanks to increased consumer awareness on the dietary health effects of plant consumption [46]. It is for this reason that understanding how *S. nigra* mono and sesquiterpenic metabolites respond to exposures of different biotic and abiotic stresses is of major importance. Understanding and managing the effects of preharvest and postharvest factors are critical for further economical exploitation of these natural products, namely to (1) provide the growers of robust decision-making tools, (2) fulfill the current standardization requirements for production of plant-based extracts and products, and (3) contribute to assure the high-quality *S. nigra*-based products.

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Section 3

Secondary Metabolites Throuht Tissue Culture Technique

Chapter 5

Production of Plant Secondary Metabolites by Using Biotechnological Tools

Sandra Gonçalves and Anabela Romano

Additional information is available at the end of the chapter

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Abstract

Plants are a remarkable source of high-value secondary metabolites with applications in various domains. Plant cell and tissue culture techniques appear as environmentally friendly alternatives for the production of secondary metabolites when natural supply is limited or chemical synthesis is unviable. In this chapter, the main advantages of using plant cell and tissue culture techniques for the production of plant secondary metabolites are presented as well as the different biotechnological approaches available to improve their production. In addition, the production of anticancer compounds (camptothecin, podophyllotoxin, taxol, vinblastibe, and vincristine) and metabolites from *Lamiaceae* spp. (phenolics as rosmarinic acid) were selected as examples to be highlighted. The study reviewed shows that undifferentiated cells are the preferred culture system used for the production of high-value secondary metabolites *in vitro* although there are many examples reporting the production in differentiated tissues particularly in hairy roots. Efforts have been made to scale up the production, and several strategies have been successfully applied to increase the production yields at the laboratorial scale. Nevertheless, there are only few examples of plant secondary metabolites production at commercial level, and further in-depth studies are still required.

Keywords: alkaloids, anticancer compounds, cell suspension cultures, elicitation, *Lamiaceae*, metabolic engineering, phenolics

1. Introduction

Plant kingdom, comprising about 250,000 species, is a repository of probably hundreds of thousands of low-molecular-weight structurally complex chemical compounds known as secondary metabolites [1]. These high-value metabolites are biosynthesized through

phenylpropanoid, mevalonate, 2-C-methyl-D-erythritol-4-phosphate, amino acid, glucose, acetate-malonate pathway, or combined pathways. Secondary metabolites have an important role in the interaction between plants and their environment (e.g., defense against herbivores and pathogens, protection against ultraviolet light, etc.) and, thus, are vital for their existence and subsistence. They are accumulated in specific tissues and structures (e.g., vacuoles, specialized glands, trichomes, etc.), and their production is affected by several factors, like genotype, plant physiology, climate, environmental conditions, and pathogens; in some cases, they are only produced during certain developmental stages [2, 3].

Over the past decades, efforts have been directed at the extraction, structure elucidation, and evaluation of biological activity of many plant secondary metabolites. Plants continue to be the main source for many important bioactive molecules/pharmacophores [4, 5]. About 25–28% of modern medicines are derived from higher plants [6], and over 60% of anticancer drugs are directly or indirectly derived from plants [7]. According to a recent report of the British Broadcasting Corporation (BBC), plant-derived drugs will grow from $29.3 billion in 2017 to around $39.2 billion by 2022 with an annual growth rate of 5.9% [8].

In the last decades, considerable progress has been made concerning the production of secondary metabolites by using plant tissue culture techniques owing to the advantages of this platform over other production systems as discussed in the next section of this chapter. The most studied classes of plant secondary metabolites using plant cell and tissue culture production systems are alkaloids and the landmark example is the anticancer-registered drug Taxol® [3]. Plant tissue culture techniques were even endorsed by Food and Agriculture Organization as safe for the production of compounds for food application [9]. This chapter aims to discuss the main advantages of using plant cell and tissue culture techniques for the production of plant secondary metabolites as well as the different biotechnological approaches available to improve their production. Important and representative examples produced through these methods, as is the case of plant anticancer compounds and metabolites from *Lamiaceae* spp., are addressed.

2. Advantages of plant tissue culture techniques for the production of secondary metabolites

In a context where consumers demand for safe natural products increases, because synthetic chemicals are perceived as potentially toxic, the interest in plant secondary metabolites from research and industry also increases [10]. Few important plant products with simple chemical structures can be produced via chemosynthesis; however, many compounds like alkaloids are difficult to be synthesized or the cost of their synthesis outweighs their commercial availability [11, 12]. Some compounds can be obtained from naturally grown plants, but sometimes there are regional and environmental restrictions, which can limit the commercial production [13]. Also, traditional cultivation of some plant species is difficult or takes several years. In this context, plant cell and tissue culture techniques appear as environmentally friendly alternative methods for the production of secondary metabolites when natural supply is limited

and traditional methods are unfeasible. The mass propagation of plants in aseptic and environmental controlled conditions, and the large-scale production of secondary metabolites in a year-round system without seasonal constraints, are some of the advantages of plant tissue culture techniques [3]. Moreover, cultures can be established in any part of the world independently of the plant growth requisites and are free of microbes and insects avoiding the use of pesticides and herbicides [14, 15]. Plant tissue culture techniques provide a reliable and predictable method for isolating the secondary metabolites at a high efficiency within a short time when compared to the extraction from wild plant populations [16]. Also, the simplicity in the extraction of the metabolites from *in vitro*-produced tissues makes the method appellative for commercial application [17].

Apart from the abovementioned advantages, there are metabolites that are generally not found in the intact plant but can be produced by *in vitro* cultures [18]. Biotechnology opens the opportunity to apply traditional or metabolic engineering strategies to promote the accumulation of desired compounds by *in vitro* cultures. Products from *in vitro* cultures can still be used as models of whole plants, and cell cultures can be radiolabeled so that secondary products can be traced metabolically [19].

The massive and indiscriminate collection of plant material from plants producing important bioactive compounds has threatened the survival of some species. *In vitro* propagation through plant tissue culture techniques allows the large-scale multiplication of true-to-type plants within a short span of time and without a negative impact on the natural resources [16]. This method is particularly valuable for plants difficult to propagate by conventional techniques or with slow propagation rates. In this context, in the last years, there has been an increased interest on the use of these methodologies for the propagation and conservation of medicinal plants.

3. Culture systems

The production of secondary metabolites by *in vitro* cultures usually occurs in a two-step process, biomass accumulation and secondary metabolites synthesis, in which both steps need to be optimized independently [3, 14]. Production could be accomplished by using undifferentiated calli, cell suspension cultures, or organized structures like shoots, roots, or somatic embryos. In some cases, a certain degree of differentiation may be needed for the biosynthesis to occur [20]. The use of differentiated organ cultures is required, for instance, when the target metabolite is only produced in specialized plant tissues or glands as is the case of essential oils [20, 21].

Among differentiated tissues, hairy roots culture offers new opportunities for the *in vitro* production of plant-valuable compounds [22]. Hairy roots are induced by the infection of plants with *Agrobacterium rhizogenes*, a Gram-negative soil bacterium. During the infection, a DNA segment (T-DNA) from the large root-inducing (Ri) plasmid of the bacterium is transferred into the genome of the infected plant. The higher level of cellular differentiation, rapid growth, genetic and biochemical stability, and maintenance facility are some of the

advantages of hairy roots [22]. Also, they can accumulate metabolites in the aerial parts of the plant. However, the difficulties in cultivating hairy roots in an industrial system limit their commercial use to produce valuable plant secondary metabolites.

Although there are many studies reporting the production of secondary metabolites using callus cultures and differentiated tissues [3, 14, 23], in most cases, undifferentiated cells are the preferred culture system [13]. Cell suspension culture is a simple and cost-effective method that has been extensively used to overcome the problems of large-scale production. Plant cell is biosynthetically totipotent, which means that under suitable conditions, each cell has theoretically the capacity to produce compounds identical to those present in the parent plant [13]. Plant cell cultures have more immediate potential for commercial application than tissue or organ cultures [21, 24]. They are considered as a stable system for the continuous production of secondary metabolites of uniform quality and yield. Another great advantage of plant cell cultures is the possibility to synthesize novel products not usually produced by the native plant [25, 26]. This is the preferable biotechnological platform to produce high-value secondary metabolites, as taxol [27, 28], resveratrol [29], artemisinin [30], ginsenosides [31], and ajmalicine [32].

4. Strategies to improve the production of secondary metabolites

In the commercial exploitation of plant cell cultures for the production of high-value secondary metabolites, it is fundamental to achieve high yields and consistent productions. The production of secondary metabolites in plants is genotype-dependent; thus, the first step to initiate cell or organ cultures is the choice of the parent plant containing higher contents of the secondary product of interest for callus or organ induction, and the selection of high-producing cell/organ lines [14]. The selection is made by analyzing cell/organ growth and then by quantifying the desired product by chromatographic and spectroscopic techniques [14]. Nevertheless, even selecting a highly productive line, the production yields are not always adequate, and after long periods of cultivation they lose their production efficiency. Thus, many alternative strategies can be used to stimulate the production of secondary metabolites and obtain efficient yields including traditional and metabolic engineering strategies [3, 19].

4.1. Traditional strategies

There are several factors that can be optimized to improve the growth and metabolites productivity of the *in vitro* cultures. Among them, the following can be appointed: the culture medium composition, the medium pH, the inoculum density, the culture medium environment (e.g., temperature, light density and quality, etc.), the agitation and aeration, etc. [3, 14, 15]. The culture medium strongly affects the biomass and metabolites productivity, and thus the selection of the suitable culture medium formulation is an imperative step [3]. It must be selected according to the physiological requirements of the plant species, and there are several parameters that can be optimized, namely nutrients composition, salt strength, nitrate

and phosphate levels, plant growth regulators type and concentration, carbon source, etc. For instance, carbon source plays significant roles in the signal transduction systems through regulating gene expression and developmental processes [3].

Secondary metabolites are produced by plant cells in response to environmental stimuli or as defensive mechanisms against invading pathogens. In this sense, the strategy available to improve the productivity of secondary metabolites, elicitation, aims to misguide the cells or tissues for a possible biotic/abiotic attack by using agents that trigger the defense response [33]. Elicitors have the ability to control an array of cellular activities at the biochemical and molecular level since they induce the upregulation of genes [33]. The elicitors can be biotic or abiotic and may comprise signaling molecules like 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 [1, 34]. Methyl jasmonate and its related signal molecules, and salicylic acid are probably the most extensively used elicitors [5]. The combination of some elicitors with physical factors (e.g., UV light, temperature regime, and pulsed electric field) yielded good results for secondary metabolite production [35]. As reviewed by Giri and Zaheer [5], cell suspension culture is the most used culture system for elicitation treatment and secondary metabolites production. Due to its inherent characteristics of hormone autotrophy, uncontrolled growth, biosynthetic, and genetic stability distinctiveness, hairy root cultures have proved to be also a valuable culture system for elicitation experiments. In addition, there are some secondary metabolites that are synthesized only in the roots [14, 36, 37]. Multiple shoots culture is a less used culture system for elicitation treatments for the production of secondary metabolites which is particularly useful in the case of metabolites present in the leaves [5]. The elicitors can change the secondary metabolites production quantitatively and also qualitatively [5]. For extra information, consult the recent reviews on this subject [1, 5].

Nutrient and precursor feeding are also used to improve the yields of secondary metabolites production. Nutrient feeding involves the replenishment of nutrient medium, and in precursor feeding, plant cell cultures are used to convert precursors into products by utilizing preexisting enzyme systems [14]. Immobilization of plant cells is another strategy used to overcome problems of low shear resistance and cell aggregation. This procedure can be done by several methods, and the most widely used are surface immobilization or gel entrapment. In this technique, the cells are entrapped in a specific gel or a combination of gels. Examples of matrices used are calcium alginate (the most used), agarose, gelatin, carrageenan, or polyacrylamide [14]. This strategy has several advantages, such as the extension of cells' viability in the stationary stage, the simplification of downstream processing, the high-cell density within small bioreactors reducing the costs and risk of contamination, an increased product accumulation, the minimization of fluid viscosity, among others [38].

The permeabilization of plant cell membranes with chemicals, the use of electric field stress, and ultrasound techniques are strategies used to facilitate the removal of secondary metabolites from vacuoles and membrane systems of the plant cell, facilitating the secretion of products into the culture medium and thus simplifying the purification process [14, 17].

The cultivated cells have the capacity for biotransformation of supplied compounds, which are not necessarily natural intermediaries of plant metabolism, into high-value compounds.

This can occur through different reactions as hydroxylation, oxidation of hydroxyl group, reduction of carbonyl group, hydrogenation of carbon-carbon double bond, glycosyl conjugation, and hydrolysis, catalyzed by plant enzymes [14]. This is probably one of the most commercially realistic approaches; however, in some cases, the costly precursors may limit the economic viability [38].

4.2. Metabolic engineering

Metabolic engineering offers a new perspective to understand the expression of genes involved in the biosynthesis of secondary metabolites through overexpression studies allowing the alteration of biosynthetic pathways [39, 40]. This involves the study of enzymatic reactions and biosynthetic processes at gene, transcriptomic, and proteomic levels, and the manipulation of the genes encoding the critical and rate-limiting enzymes in the biosynthetic pathways [41, 42]. Theoretically, the secondary metabolites productivity of plant cell cultures can be improved through the overexpression of genes encoding regulatory enzymes involved in their biosynthetic pathways [16]. However, the overexpression of certain genes may not always improve production [16].

Metabolic engineering approach also uses the inhibition of competitive pathways to increase metabolic flux of targeted biosynthetic pathway intermediates for a higher production through a variety of approaches. Certain steps in the biosynthetic pathway could be inhibited to induce the accumulation of preceding intermediates. The understanding of phenylpropanoyl biosynthetic pathway that is involved in the biosynthesis of several plant secondary metabolites is the most successful and recent application [43, 44].

The in-depth understanding of the biosynthetic pathways is still a barrier to the practical use of this strategy to enhance production [45, 46]. For the large-scale production of important secondary metabolites to meet industry demand, more studies are needed to identify rate-limiting steps and regulation along with bottlenecks on the lack of clarity of their biosynthetic pathways.

5. Scale-up production

Owing to the importance of some plant secondary metabolites, efforts have been made to study the feasibility of their production at the industrial scale. This is not always a simple process because plant cells have a relatively unstable productivity, a high shear sensitivity, a slow growth rate, and low oxygen requirements [14]. The scale-up involves the use of bioreactors of varying sizes and features, and cell suspension culture is the better culture system having several advantages in comparison with the other. The simplicity, predictability, and high efficiency at which the metabolites can be isolated from biomass or cultivation media are some of these advantages. Nevertheless, there are some examples of the use of differentiated tissues like shoots and somatic embryos [47].

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Some important milestones in the production of secondary metabolites by plant cell cultures are the production of shikonin [48] and ginseng [49], and the most successful example of the scale-up process is probably the production of taxol by Phyton Biotech Company (Germany) to supply part of the demands of Bristol-Meyers Squibb Company during the year 2002 [50]. Phyton Biotech operates the largest cGMP plant cell culture facility in the world designed for large-scale production of Taxanes in 75,000 L-size bioreactors that run up to 880,000 L per year [51]. Berberine, ginsenosides, shikonin, scopolamine, and rosmarinic acid are also examples of plant secondary metabolites presently produced at the commercial scale (**Figure 1**) [3, 17].

Several factors should be considered in scaling up the production of secondary metabolites using bioreactors, namely the optimization of culture conditions, biomass production measurement (especially with tissue and organ cultures), and so on [52, 53]. Several bioreactor designs have been tested and used for plant cell cultures. Some of them as is the case of stirred tank reactors, bubble column reactors, airlift reactors, and ebb and flood reactors are merely extension of microbial culture. For plant cells with a high shear sensitivity, Wang and Zhong [54] develop the centrifugal impeller bioreactors that are based on the principles of a centrifugal pump. Mechanically driven "wave reactors," "slug bubble reactor," and "undertow reactor" are also adequate for high shear-stress-sensitive cells [14]. On the other hand, airlift bioreactors are suitable for not highly shear-sensitive cells and

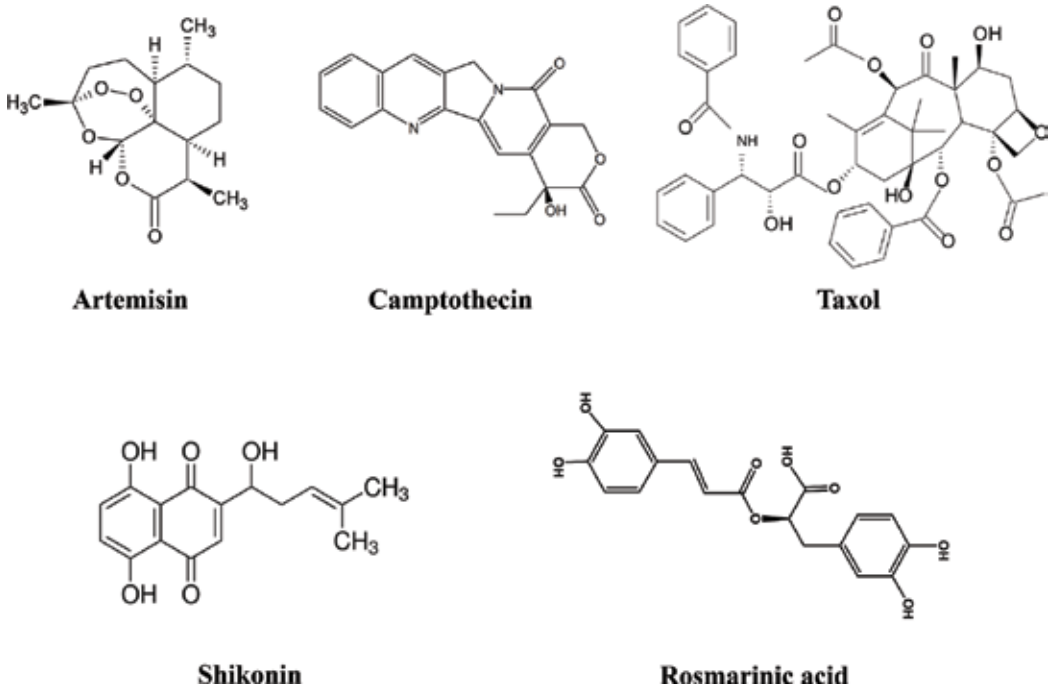

Figure 1. Structures of some relevant plant secondary metabolites produced on a commercial scale.

for hairy and adventitious root cultures [14]. The interested reader can find more important details about the scale-up process in the works by Murphy et al. [14], Yue et al. [13], and Isah et al. [3].

6. Selected examples

There are several plant secondary metabolites including among others alkaloids, terpenes, flavonoids, and glycosides, which can be produced by plant tissue culture techniques using different strategies [3, 13, 14]. Two examples were selected to be described in this chapter: the production of important anticancer compounds and the production of metabolites from *Lavandula* spp.

6.1. Anticancer compounds

As mentioned before in this chapter, over 60% of anticancer drugs are directly or indirectly derived from plants [7]. The search for anticancer compounds from plants started in the 1950s when the alkaloids vinblastine and vincristine from *Catharanthus roseus* (L.) G. Don and podophyllotoxin from *Podophyllum* spp. were discovered. The United States National Cancer Institute initiated an extensive program in 1960 that led to the discovery of many novel chemotypes with cytotoxic activities [55], taxanes and camptothecins being some of the examples [7]. Camptothecin, podophyllotoxin, taxol, vinblastine, or vincristine are the most important plant-derived anticancer compounds [19, 56]. Most compounds with anticancer properties are alkaloids, and some of them have a complex structure, with multiple rings and chiral centers, and therefore the chemical synthesis is prohibitively expensive [17]. Plant cell and tissue culture techniques appear as environmentally friendly alternative methods for the production of these secondary metabolites [17, 19].

Taxanes from *Taxus* spp., terpenoid indole alkaloids from *C. roseus*, camptothecin from *Camptotheca acuminata* Decne among other species, and podophyllotoxin from *Podophyllum* and *Linum* spp. are the main compounds produced by using biotechnological approaches (**Table 1**). For the production of taxanes, cell suspension cultures are definitively the most adequate culture system. However, some studies demonstrated that differentiated tissues are more adequate than undifferentiated cells to produce other anticancer compounds. For instance, intact plants of *C. acuminata* contain around 0.2–5 mg/g dry weight (DW) of camptothecin while callus and suspension cultures produced only 0.002–0.004 mg/g DW or lesser [57]. Hairy root cultures have also proven to be a good option for *in vitro* production of secondary metabolites as indole alkaloids due to their higher level of cellular differentiation and improved genetic or biochemical stability. The hairy roots of *Ophiorrhiza pumila* Champ. ex Benth showed a high capacity to produce camptothecin (0.1% DW), although the callus culture failed to produce this compound [58].

Several researchers have focused on studies aiming for the optimization of biomass growth conditions and on the application of biotechnological strategies to increase production yields of anticancer compounds. By manipulating empirical factors related to plant cell and organ

Compound(s)	Group	Source (plant species)	Culture system	Reference(s)
Camptothecin	Monoterpene indole alkaloid	*Camptotheca acuminata* Decne	HRC	[75]
		Camptotheca acuminata Decne	CSC	[76]
		Nothapodytes foetida (Wight) Sleumer	CC	[77]
		Nothapodytes nimmoniana (J. Grah.)	CSC	[78]
		Ophiorrhiza alata Craib	HRC	[79]
		Ophiorrhiza mungos Linn.	CSC	[80]
		Ophiorrhiza prostata D. Don	ARC	[81]
		Ophiorrhiza pumila Champ. ex Benth.	HRC	[58]
Podophyllotoxin	Aryltetralin lignan	*Linum* spp.	HRC	[82, 83]
		Linum album Kotschy ex Boiss.	CSC	[84]
Taxanes (taxol)	Diterpene alkaloids	*Taxus* spp.	CSC	[27, 28]
		Corylus avellana L.	CSC	[85]
Vinblastine and vincristine	Terpene indole alkaloids	*Catharanthus roseus* (L.) G. Don	HRC	[86, 87]

ARC: adventitious root culture; HRC: hairy root culture; CSC: cell suspension cultures; CC: callus culture.

Table 1. Some examples of studies reporting the production of plant anticancer compounds using biotechnological approaches.

cultures, it has been possible to enhance production yields. Several factors have been optimized, such as nutrients, carbon source, plant growth regulators, or culture environmental conditions, and several biotic and abiotic elicitors have been tested. Studies have also been focused on the elucidation and regulation of biosynthetic pathways and on aiming the increase of production yields of anticancer compounds as taxanes [41] and indole alkaloids [59] by using elicitors to activate genes involved in metabolic pathways. In spite of all the advantages of producing anticancer compounds by using plant cell and tissue culture techniques and the significant advancements in the last years, the examples of the production of plant anticancer compounds on an industrial level are scarce. As previously mentioned in this chapter, the best success example is the production of taxanes by the Germany company Phyton Biotech [51].

Plant cell and tissue culture techniques have also been applied for the propagation of several anticancer plants. *In vitro* propagation allows the rapid mass multiplication of true-to-type plants within a short span of time which is particularly important in the case of endangered species. Some recently selected examples comprise plants producing the important anticancer compounds camptothecin [60, 61] and podophyllotoxin [62, 63].

6.2. *Lamiaceae* spp. metabolites

The mint family (*Lamiaceae*) contains about 236 genera and more than 7000 species with cosmopolitan distribution [64]. Some of the most important genera are *Hyptis*, *Lavandula*, *Nepeta*, *Salvia*, *Scutellaria*, *Thymus*, and *Teucrium*. Species from the family inhabit different natural ecosystems, and many are already cultivated. Most of the species belonging to this family are aromatic (possess essential oils) and are widely used in traditional medicine to cure various disorders. They also have great economic value due to their use in culinary or as ornamentals, and for cosmetic, flavoring, fragrance, perfumery, pesticide, and pharmaceutical applications [65]. Many *Lamiaceae* contain high levels of phenolics, which are probably the most relevant group of secondary metabolites synthesized by plants due to their health promotion effects [64]. Among phenolic compounds, rosmarinic acid is present in the tissues of many of these species being used as a chemical marker of the family [64, 66, 67]. In some species, this compound is accumulated as the main phenolic compound at a concentration above 0.5% dry weight [64]. Several species in the *Lamiaceae* family can also accumulate high levels of other phenolic acids, flavonoids, or phenolic terpenes [64]. There are some phenolic compounds as carnosic and clerodendranoic acids that are exclusive from this family [68, 69]. The interested reader can find an excellent overview on the phytochemical characterization and biological effects of *Lamiaceae* species in Trivellini et al. [64].

Phenolic compounds are generally produced as a defense mechanism or as a response to stressful environment conditions [9]. The activation of these protective mechanisms by applying stress stimulus can be used as a strategy to increase the production of phenolic compounds in plant cell and organ cultures [70]. Recently, several attempts were made regarding the production of secondary metabolites by several *Lamiaceae* species (mainly phenolics) using plant tissue cultures particularly applying elicitation as a strategy to achieve higher production yields [64]. These studies involve mainly the use of chemical elicitors like jasmonic acid (or methyl jasmonate), or physical elicitors as UV-B and ozone (O_3), to increase the production of many compounds as essential oil constituents, phenylpropanoids, flavonoids, and phenolic acids. Overall, the results demonstrated that these elicitors had an immediate effect on enhancing the production of phenolics [64].

The revised study showed that a high number of studies reported an increase in the production of rosmarinic acid after elicitation of cultures of several *Lamiaceae*, such as *Coleus*, *Lavandula,* and *Salvia* genera [64, 66, 71]. Several studies reported the increase in rosmarinic acid production through the application of elicitors (**Table 2**). Elicitation with jasmonic acid induces a 4.6-fold increase of rosmarinic acid production in *L. officinalis* L. cell suspension cultures [72], and elicitation with methyl jasmonate induces a 3.4-fold increase in *C. forskohlii* (Willd.) Briq. hairy root cultures [73]. The production of this compound also increased (2.3-fold) in leaves of *Rosmarinus officinalis* L. after 14 days of UV-B exposure [74]. Recently, rosmarinic acid attracted the attention of the scientists due to its broad range of biological activities, such as anti-inflammatory, antioxidant, cognitive-enhancing, cancer chemoprotection effects, among others [71]. In the last years, there are many progresses in the

Plant species	Tissue	Elicitor	Reference
Coleus blumei Benth.	HRC	Methyl jasmonate	[88]
Coleus forshohlii (Willd.) Briq.	HRC	Methyl jasmonate	[73]
Lavandula officinalis Chaix	CSC	Jasmonic acid	[72]
Lavandula vera MM	CSC	Vanadyl sulphate	[89]
Lavandula vera MM	CSC	Methyl jasmonate	[90]
Mentha × *piperita*	CSC	Methyl jasmonate	[91]
Rosmarinus officinalis L.	L	UV-B	[74]

HRC: hairy root culture; CSC: cell suspension cultures; L: leaves.

Table 2. Selected examples of elicitation treatments applied in cultures of *Lamiaceae* species to increase the production of rosmarinic acid.

biotechnological production of this compound but its large-scale production still requires further optimization. The molecular understanding of its biosynthesis and the application of metabolic engineering tools are crucial to improve the biotechnological production.

7. Conclusions and prospects

Plant cell and tissue culture techniques are an attractive system for the cultivation of a broad range of secondary metabolites, including important alkaloids with anticancer properties and bioactive phenolics. This alternative provides a continuous, sustainable, economical, and viable production of secondary metabolites, independent of geographic and climatic conditions, which is particularly useful for the production of species at risk. Despite the great progresses in this area in the last decades, in some cases, production occurs at very low yields, and there are many difficulties in scaling up the production, and limited commercial success is achieved. Incomplete knowledge about the biosynthetic pathways of bioactive molecules limited the improvement of the production yields. Exploiting modern molecular biology techniques emerged as an alternative that needs to be harnessed to improve production efficiency by engineering biosynthetic pathway(s) of the molecules in plant cells. Also promising are new elicitors and permeabilizing agents such as coronatin or cyclodextrins. The production of bioactive molecules in endophytes also appears as an attractive alternative, although till date, there is no reported commercial exploitation.

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Chapter 6

Secondary Metabolite Research in Malaysia: Current Status and Future Prospects

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Abstract

Herbal medicine is gaining acceptance worldwide for their effective pharmacological effects and relative safety. Plants have metabolic pathways that lead to the production and accumulation of secondary metabolites such as alkaloids, glycosides, and terpenoids that exhibit various biological activities. Plant secondary metabolites have been the major foci of investigation for several years and have been successfully used against a number of communicable and noncommunicable diseases such as diabetes, cancer, and viral infections. This chapter will explore Malaysian plants, their secondary metabolites, and their biological/medicinal properties with a particular focus on some selected species under a national project. Other aspects such as plant tissue culture to produce secondary metabolites and a case study on the use of secondary metabolites in the prevention and treatment of dengue fever are also described. While a lot of effort has been put in, further research and development into plant secondary metabolites are needed including using the plant tissue culture approach toward reaching high-value herbal industry.

Keywords: biological activities, dengue, medicinal plants, secondary metabolites, tissue culture

1. Introduction

Secondary metabolites are defined as natural products that often have an ecological role in regulating the interactions between plants and their environment [1]. Plants execute several defense

mechanisms against pathogenic microbes, herbivores, and diseases. One of these defense mechanisms is the accumulation of secondary metabolites such as alkaloids, flavonoids, glycosides, terpenes, saponins, and tannins, which are important in health, food, and environmental fields [2]. For centuries, herbal extracts from a variety of plant species have been used as remedies for a wide spectrum of diseases. Majority of these extracts, the medicinal properties of which are attributable to the secondary metabolites present in the plants and serve as lead molecules in current drug design and development. It is interesting to note that a large number of drugs that have been approbated within the last 25 years are of natural origin and incorporate bioactive material with "drug-like properties" [3]. Even more pertinent is the fact that 12 of the world's 25 best-selling pharmaceutical agents were obtained from natural products [4].

Classic examples of plant secondary metabolites that later become lead for drug development include artemisinin from *Artemisia annua* (sweet wormwood, qinghao) for treatment of malaria, digoxin from *Digitalis lanata* (foxglove) for treatment of various heart conditions, and paclitaxel from *Taxus brevifolia* (Pacific yew) as chemotherapy medication.

This chapter will explore Malaysian plants, their secondary metabolites, and their medicinal properties with a particular focus on some selected species under a national project. Other aspects such as plant tissue culture to produce secondary metabolites and a case study on the use of secondary metabolites in the treatment of dengue fever are also described.

2. Secondary metabolites from Malaysian plants

A recent review by Buenz et al. described the ethnopharmacologic contribution to bioprospecting natural products [5]. Many databases of traditional medicine uses of natural products have been established, for instance, the PharmDB-K (covering traditional Korean medicine) [6], FERN Ethnomedical Plant Database (covering fern species) [7], AfroDb (covering African medicinal plants) [8], and Traditional Chinese Medicine Information Database (TCM-ID) [9]. In 2002, the Institute for Medical Research (IMR), Malaysia, was granted an approval to host a global electronic information resource on traditional and complementary medicine (TCM) known as Glob*in*Med (http://www.globinmed.com). The project was initially discussed during the 12th Commonwealth Health Ministers Meeting in Barbados in 1998 where the main idea was to establish a working group on TCM-related activities. Glob*in*Med also partners with ASEAN Task Force on Traditional Medicine (ATFTM) and several local institutions to enhance its service.

As one of the 17 megadiversity countries with 15, 000 estimated known plant species, Malaysia has a great potential for bioprospecting toward discovery of compounds with medicinal value. Together with ethnopharmacologic evidences from its rich traditional medicinal practice, several plant species have been selected for the agriculture NKEA-EPP1 (National Key Economic Area, Entry Point Project 1: High Value Herbal Products) [10], namely, tongkat ali (*Eurycoma longifolia* Jack), Misai Kucing (*Orthosiphon aristatus* (Blume) Miq.), Hempedu Bumi (*Andrographis paniculata* (Burm.f.) Nees), Dukung Anak (*Phyllanthus niruri* L.), Kacip Fatimah (*Marantodes pumilum* (Blume) Kuntze (*syn. Labisia pumila* (Blume) Mez)), Mengkudu (*Morinda citrifolia* L.), Roselle (*Hibiscus sabdariffa* L.), ginger (*Zingiber officinale*), Mas Cotek (*Ficus deltoidea* Jack), Belalai

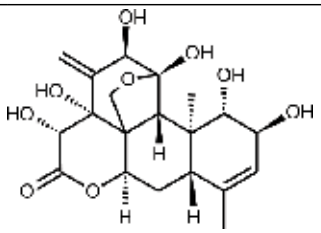
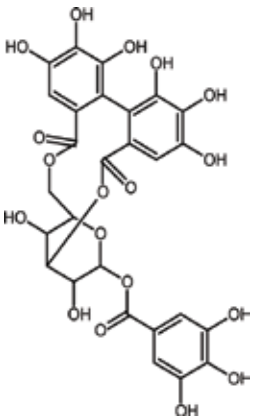
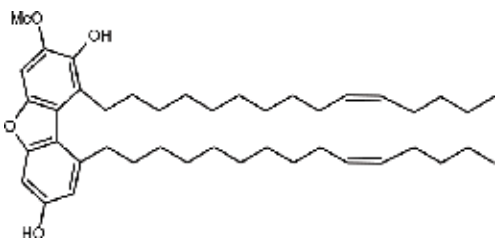
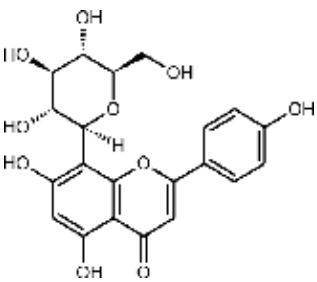
Plant	Secondary metabolites	Biological activity	Reference
***Eurycoma longifolia* Jack (Tongkat Ali)**	 Eurycomanol (class: quassinoids)	Antimalarial against *P. falciparum*	[12]
***Phyllanthus niruri L.* (Dukung Anak)**	 Corilagin (class: tannins)	Anti-hyperalgesic	[13]
***Marantodes pumilum (Blume) Kuntze (syn. Labisia pumila (Blume) Mez* (Kacip Fatimah)**	 Fatimahol (class: alkylphenols)	Not available	[14–16]
***Ficus deltoidea* Jack (Mas Cotek)**	 Vitexin (apigenin-8-*C*-glucoside)	Antidiabetic (α-glucosidase inhibitors)	[17]

Table 1. Selected secondary metabolites from plants under the Malaysia's agriculture NKEA-EPP1.

Gajah (*Clinacanthus nutans* (Burm.f.) Lindau), and Pegaga (*Centella asiatica* (L.) Urb) [10]. It is noteworthy that a dietary supplement combining *Labisia pumila* and *Eurycoma longifolia* has reached the clinical trials investigating the effects on menopausal women and their quality of life (trial registration number NCT02269891) [11].

Not discounting many other important local plants, **Table 1** listed some of the important secondary metabolites from the agriculture NKEA-EPP1-select plants to illustrate the myriad of secondary metabolites responsible for various biological effects. The secondary metabolites range from flavonoids, quassinoids, phytosterols, and terpenoids.

3. Secondary metabolites from plant tissue culture

Overharvesting of medicinal plants for their secondary metabolites may lead to their disappearance from the natural habitats. Due to this, researchers turn to alternative and innovative methods to meet the increased demand for these natural products. In particular, plant tissue culture has become a well-established and attractive alternative for mass production of secondary metabolites through callus, suspension, and organ culture [18, 19]. Tissue culture itself is defined as the technique of maintaining plant tissue in vitro in a synthetic medium under controlled conditions, and it is reported to be extremely useful for commercial production of therapeutically important compounds [20].

There are many advantages of using plant tissue culture for producing metabolites including the (i) ability to improve the production of certain compounds within the plant cell using elicitors and plant hormones to manipulate the cultured cells [21], (ii) the ability to continuously produce secondary metabolites through propagation in sterile bioreactors independently of growth conditions such as soil content and microclimate [22], (iii) the ability of in vitro plant tissue culture to achieve higher rates of metabolism than the in vivo differentiated intact cells [22], and (iv) the ability to bypass the structural complexity of the plant organism rendering it to be a convenient tool in research studies [23].

Plant species	Type of plant culture	Secondary metabolites	Biological activities	Reference
***Boesenbergia rotunda* (Kencur)**	Embryogenic and non-embryogenic callus	Flavonoid (panduratin, pinocembrin, pinostrobin, cardamonin, and alpinetin)	Antimicrobial, antiulcer, antiviral, and antitumor activities	[24]
***Centella asiatica (L.) Urb* (Pegaga)**	Cell suspension	Triterpenoids (asiatic acid, madecassic acid, asiaticoside, and madecassoside)	Antibacterial, antimalarial, antiproliferative, and wound-healing properties	[25]
	Cell suspension	Flavonoid (quercetin, kaempferol, luteolin, and rutin)	Antibacterial, antiviral, antiallergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant activities	[26]

Plant species	Type of plant culture	Secondary metabolites	Biological activities	Reference
***Eurycoma longifolia* Jack (Tongkat Ali)**	Hairy roots	Alkaloid (9-methoxycanthin-6-one)	Cytotoxicity activity against human breast cancer (MCF-7) and human lung cancer (A-549) cell lines	[27]
	Cell suspension culture	Alkaloid (9-hydroxycanthin-6-one and 9-methoxycanthin-6-one)		[28]
***Ficus deltoidea* Jack (Mas Cotek)**	Cell suspension	Flavonoids	Cardiovascular diseases and postpartum treatments, antidiabetic	[29]
***Justicia gendarussa* (Gandarusa)**	Callus, cell suspension	Phenolics	Antioxidant	[30]
***Morinda elliptica* (Mengkudu)**	Cell suspension	Phenolics (anthraquinones)	Antiviral, antimicrobial, cytotoxic, and antitumor-promoting and antioxidant activities	[31]
***Orthosiphon stamineus* (Misai kucing)**	Cell suspension	Phenolics	Antioxidant	[32]
***Pogostemon cablin* (Nilam)**	Cell suspension	Terpene (patchouli alcohol)	Nausea, diarrhea, and headache	[33]

Research conducted in various institutions in Malaysia.

Table 2. Different types of plant tissue culture and their secondary metabolites.

The current evaluation of secondary metabolite compounds in various culture types of medicinal plants available in Malaysia is summarized in **Table 2**. Most of these studies involved with the cell suspension culture compared to other types of plant culture that are also frequently used for secondary metabolite production such as hairy roots and shoot culture.

4. Case study: secondary metabolites in the prevention and treatment of dengue

4.1. Dengue fever

The dengue prevalence in Malaysia remains overwhelming, with increasing rate of incidence reported annually. According to the latest WHO report, in 2016, more than 375,000 suspected cases of dengue were recorded in the Western Pacific Region, 100,028 of which occurred in Malaysia [34]. The steady surge of cases over the years has prompted serious efforts from the government and the community, including intensive efforts in both vector control and elucidation of potential therapeutic agents. Although evaluation of several vaccines is currently in progress, the ambivalence of such treatment with regard to serotype

interference, incomplete protection, and dose sufficiency [35] makes the search for new antiviral agents imperative.

In Malaysia, the National Dengue Strategic Planning 2015–2020 (the Sixth Strategy: Dengue Research) highlights the importance of research aimed at enhancing the effectiveness, cost-effectiveness, sustainability, and the scale of existing interventions, as well as producing ideas and new methods, while promoting collaboration with relevant agencies. In this regard, it is anticipated that comprehensive research focusing on the local natural heritage, including the

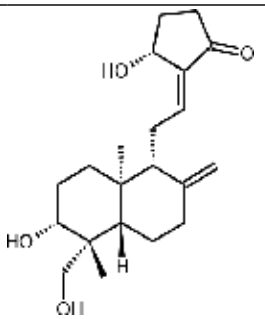
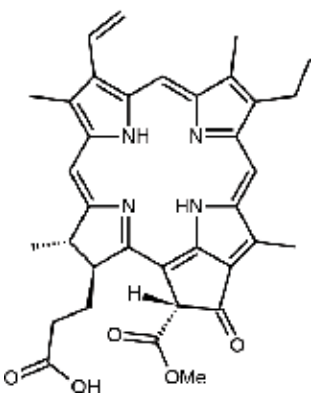
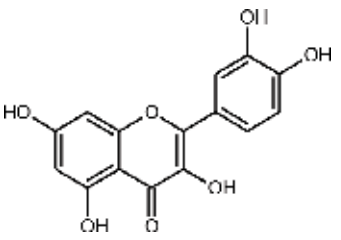
Plant	Secondary metabolites	Biological activity	Reference
Andrographis paniculata **(Hempedu Bumi)** leaves	 Andrographolide (class: terpenoid)	Anti-dengue activity against the primary dengue vector *Aedes aegypti*	[38]
Clinacanthus nutans (Burm.F) **(Belalai Gajah)** leaves	 Pheophorbide (class: chlorophyll)	Anti-DENV-2 activity by inhibiting the production of viral RNA and viral protein	[39]
Psidium guajava **(Jambu Batu)** leaves	 Quercetin (class: flavonoid)	Anti-dengue activity against different stages of DENV-2 infection and replication cycle	[40]

Table 3. Selected secondary metabolites from plants with effects against dengue or its vector.

abundance of medicinal plants, should be prioritized, as this is deemed the best strategy for the development of new dengue fever treatment regimens.

4.2. Secondary metabolites from Malaysian plant species used for the prevention and treatment of dengue

Malaysia, due to its prolific nature and wide diversity of plant sources, offers a wide range of pharmaceutical options, with high potential as anti-DENV agents. Most of these plant species have not been studied extensively, and the existing research tends to focus on folklore medications. It is important to emphasize that the characterization of secondary metabolites responsible for anti-DENV activities is still very limited. Consequently, none of these medicinal plants have reached the clinical stage in the drug design pipeline, thus necessitating further verification studies. To date, only several secondary metabolites from a number of local Malaysian plant species have been identified and their chemical structures elucidated (**Table 3**), while many more remain to be explored. For instance, the effects of local plant extracts on in vitro dengue replication were recently documented [36], whereby *Hydrocotyle sibthorpioides* Lam. extracts showed variable effects on dengue viral replication, depending on the treatment, cell lines, and solvent types. In an earlier study, extract from *Phyllanthus* spp. also exhibits antiviral activity against DENV-2, which was supported by differential regulation of various hosts and viral proteins [37].

A summary of plant secondary metabolites found useful in dengue treatment and prevention is depicted in **Table 3**.

5. Challenges and future prospects

Despite a more concerted efforts and strategic approach to add value to the country's herbal industry, the desired output has not reached its optimum. The main challenges including the lack of good research documentation, monographs, standardization in farming practices (good agricultural practices, GAP), good laboratory practice (GLP), and development and commercialization of products still persist. Further, the increased harvesting of medicinal herbs raises the concerns about the extinction of plant populations and degradation of natural plant habitat causing shortage in plant raw materials which may affect the effort to reach high mass production of secondary metabolites. To this end, biotechnological applications such as plant tissue cultures have been recognized an alternative tool for scaling-up the production of secondary metabolites. However, challenges inherent in plant tissue cultures must be overcome in order for it to contribute to significant cost-effective production of secondary metabolites. This includes the understanding of the secondary metabolites and their metabolic pathways, identification of the highest yielding populations, control of specific gene expression and regulatory enzymes, and the use of economic sterile bioreactors. Leveraging the country's wealth of flora species, a continuous effort from the government, academia, and industry to further nurture and uphold the herbal industry is indispensable.

6. Conclusion

Malaysian tropical rainforests comprise a wide range of medicinal plants that have been screened for their secondary metabolites and potential biological and therapeutic effects. Selected plant species from the agriculture NKEA-EPP1 demonstrated numerous pharmacological activities which were attributed to the presence of biologically active secondary metabolites, namely, flavonoids, quassinoids, phytosterols, and terpenoids. The use of local plants toward combating the country's high-prevalent disease such as dengue could be further explored. While a lot of effort has been put in, further research and development into plant secondary metabolites are needed including using the plant tissue culture approach toward reaching high-value herbal industry.

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Section 4

Regulation of Secondary Metabolites Production

Chapter 7

Microbial Biotransformation for the Production of Steroid Medicament

Rutuja Rokade, Selvan Ravindran, Pooja Singh and Jitendra K. Suthar

Additional information is available at the end of the chapter

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Abstract

Androstenedione (AD) is a steroid intermediate valuable for the production of steroid medicaments. Microbial biotransformation of phytosterol to produce AD is a well-researched area. However, low substrate solubility of phytosterol in aqueous media and nucleus degradation of AD to androstadienedione (ADD) or 9-hydroxy-AD are the major obstacles for AD production leading to detailed research for optimization of biotransformation process. In this review, microbial transformation of AD with respect to the existing methods of chemical or biochemical synthesis of AD are extensively discussed. This review examines the microbial biotransformation process and limitations for enhanced AD production. Factors affecting the effective biotransformation process to obtain AD are discussed and limitations are highlighted. The main content of this review focuses on the recent and futuristic biotechnological advances and strategies in techniques to enhance AD bioprocess.

Keywords: androstenedione, microbial biotransformation, phytosterol, steroids, nucleus degradation

1. Introduction

Steroids are terpenoid lipids of specific structure that contain the nucleus of four cycloalkane rings. Androstenedione (AD) is a natural steroid which belongs to the 17-ketosteroid family. It is produced by the adrenal cortex and gonads. In the body, cholesterol leads to the formation of steroidal hormones. AD is a compound specifically used as a precursor for the majority of pharmaceutically active steroids such as testosterone, estradiol, ethinylestradiol, testolactone,

progesterone, cortisone, cortisol, prednisone and prednisolone [1]. The steroid pharmaceuticals are of great importance for their role in the management of human fertility, osteoporosis, menopause and blood pressure regulation [2, 3]. Commercially, steroid production represents one of the largest sectors of medical products manufactured by the pharmaceutical industry [4]. Production of AD and androstadienedione (ADD) exceeds 1000 tons per year in the world. Therefore, production of AD on a large scale with cost effective process becomes an area of demanding research in biotechnology. Apart from the broadly used natural compounds, phytosterols gained an increasing importance as raw materials for the synthesis of steroidal drugs such as pregnenolone, boldenone, androstenedione and androstadienedione. The utilization of cholesterol and phytosterol as the sole carbon source by *Mycobacterium* sp. for growth and proliferation led to a serious development in microbial biotransformation processes for production of large number of steroidal compounds [1].

Phytosterols are thoroughly widespread in plants and are similar to cholesterol in terms of physiological functions and structure. Phytosterols differ from cholesterol by having a methyl or ethyl group at C-24 (**Figure 1**). Phytosterols participate in essential cellular processes since they modulate permeability and fluidity of membranes. In addition, they are precursors for the synthesis of steroid hormones and are involved in plant defense mechanisms. β-Sitosterol, campesterol, and stigmasterol are the main phytosterols found in plants (**Figure 1**). The most important phytosterols, β-sitosterol (C-29 carbon skeleton), campesterol (C-28), and stigmasterol (C-29), contribute up to 98% of all the phytosterols found in plants [5]. As cholesterol acts as a starting material for steroid production by adrenal cortex and gonads, phytosterol can act as a starting material for AD production by many microorganisms which are able to utilize phytosterols as carbon and energy source.

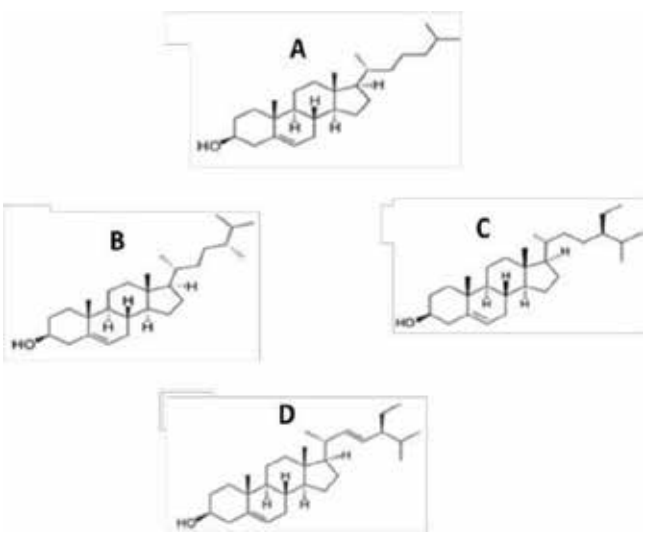

Figure 1. (A) Structure of cholesterol, (B) structure of campesterol, (C) structure of β-sitosterol, (D) structure of stigmasterol.

This property is a key element in the steroid precursor production and, therefore can be exploited to make AD production a commercially viable process.

Further, phytosterols being a polar substance, has very low solubility in aqueous media. Because of low substrate solubility of phytosterols, the process of production of AD gets hampered. Current review focuses on the mechanisms used for enhanced AD production, and includes current trends & future perspectives.

2. Synthesis of androstenedione from phytosterols

AD is synthesized in the body by adrenal cortex and gonads stimulating formation of testosterone or estradiol. Adrenocorticotropic hormone (ACTH) stimulates the steroid synthesis pathway from cholesterol leading to formation of AD. Phytosterol being similar to cholesterol is able to stimulate the formation of AD by its side-chain cleavage. (Elaboration of the pathway in **Figure 2**).

As shown in the **Figure 2**, CYP11A1 is an enzyme that is responsible for side chain cleavage of the sterol resulting in AD formation.

2.1. Chemical synthesis

Marsh demonstrated a chemical synthesis of AD (aromatase inhibitors) which involves multiple steps [6]. AD can be produced chemically by addition of thiol reagents to appropriate dienone intermediates. C19 steroids should retain the steroidal nucleus for their effective functions. However, the steroidal nucleus is sensitive to a temperature leading to its degradation [7]. Steroidogenic enzymes were also sensitive to endocrine-disrupting chemicals (EDCs) [8]. Apart from the above mentioned limitations, chemical synthesis leads to added costs and lower yield of AD with lengthy and sensitive procedures. Additionally, chemical synthesis of AD also requires use of toxic/harmful chemicals which affects the environment. Hence, there is a need to develop a process which is environment friendly and cost effective. Microbial biotransformation of phytosterol fulfills the need.

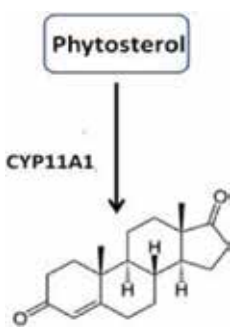

Figure 2. Side chain cleavage of sterol leading to AD formation.

2.2. Biotransformation and substrates used

Sterol rich substrates are used to obtain steroidal intermediates by biotransformation. There are a number of organisms known to transform phytosterols to AD such as *Aspergillus, Arthrobacter*, *Bacillus*, *Brevibacterium*, *Chryseobacterium*, *Fusarium*, *Gordonia*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Streptomyces* and *Mycobacterium* sp. [2, 3].

3. Factors affecting androstenedione production

3.1. Substrate solubility

Several researchers have reported their work in developing a microbial biotransformation process with high yield of AD with wide industrial applications [9–11]. Therefore, it was important to understand the factors affecting the actual biotransformation process. The basic and the most reported research is on low solubility of phytosterols in the aqueous media. This leads to low mass transfer rate and low substrate availability for conversion of phytosterol to AD. Oxygen transfer rate was also reported to be another critical parameter in the biotransformation of phytosterol [12]. Along with oxygen transfer rate (OTR) the effect of by-products such as 1,4-HBC formed during the bioprocess was found to affect the amount of the products produced leading to a loss in yield by around 16.3 percent [12].

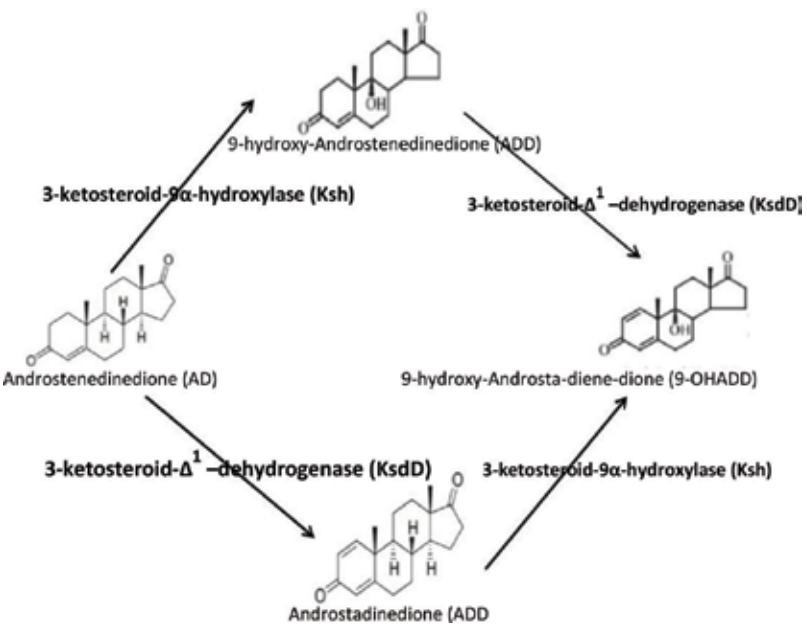

Figure 3. Enzymes involved in nucleus degradation of AD.

3.2. Nucleus degradation

The change in the steroidal nucleus of AD is one of the major obstacles reported in the microbial biotransformation of phytosterols. After formation of AD during microbial biotransformation, further degradation of AD takes place producing either ADD or 9-hydroxy androstenedione (9-OHAD) [7]. In a phenomenon which was coined as nucleus degradation by the researchers, two major enzymes have been implicated so far: 3-ketosteroid-1,2-dehydrogenase (KsdD) was found to be responsible for AD to ADD conversion and hence lead to low yield of AD. 3-ketosteroid-9-hydroxylase (Ksh), was observed to be responsible for AD to 9-OHAD conversion, leading to nucleus degradation and low yield of AD (**Figure 3**).

Xu et al. demonstrated that the enzymes involved in nucleus degradation of AD are temperature sensitive [7]. 3-ketosteroid-1,2-dehydrogenase (KsdD) and 3-ketosteroid-9-hydroxylase (Ksh) are enzymes sensitive at 37°. These enzymes break the B ring of steroidal nucleus which leads to degradation of the steroid. Hence it can be concluded that the microbial biotransformation of phytosterol is a temperature sensitive process.

4. Techniques to enhance production of androstenedione

As described earlier, to develop a process which is industrially applicable for high yields of AD, different techniques need to be incorporated. In the following section, we discuss the effective techniques to enhance AD production; thus making the process more economical.

4.1. Screening of micro-organisms

In the process of microbial biotransformation, selection of the microorganisms is a very crucial step. Microorganisms which are able to utilize sterols as their sole energy and carbon source are reported to have the ability to produce AD as an intermediate in the sterol degradation pathway [13]. Such microorganisms e.g., *Mycobacterium* sp., *Mycobacterium smegmatis, Rhodococcus* sp., *Deinococcus radiodurans, Pseudomonas* sp. have been documented to have an ability to produce AD or ADD. **Table 1** illustrates the organisms which are able to utilize sterols as their sole carbon and energy source to produce steroidal intermediates, AD, ADD, 9-hydroxy AD or 9-hydroxy ADD and also the various strategies implemented for enhancing the growth of the microorganisms.

4.2. Genome modifications

As mentioned in the earlier sections, researchers have demonstrated the pathways followed by microorganisms for biotransformation of phytosterol, the enzymes responsible for AD production and its nucleus degradation. Various studies have been performed on genome sequencing and proteomics to determine the sequence of the genes involved in the side chain cleavage of phytosterol. CYP11A1 in *Mycobacterium neoaurum* was reported to be involved in side chain cleavage of phytosterol leading to formation of AD [14]. Further, as described in Section 3.2, 3-ketosteroid-1,2-dehydrogenase (KsdD) and 3-ketosteroid-9-hydroxylase (Ksh) are the

Organism	Product	Strategy	Reference
Mycobacterium neoaurum	AD, ADD	Three-stage fermentation	[13]
Pseudomonas aeruginosa	AD,ADD, B	Selective media containing 8- hydroxyquinoline	[10]
Nocardioides simplex	AD, ADD, T	Selective media containing Hp-β- CD	[15]
Mycobacterium smegmatis	AD, ADD	Selective media containing Hp-β- CD	[16]
Mycobacterium tuberculosis	AD, ADD	Selective media containing Hp-β- CD	[17]
Moraxella ovis	AD, ADD	Natural medium containing rice bran oil	[11]
Deinococcus radiodurans,	AD, ADD	Natural medium containing rice bran oil	[11]
Corynebacterium equi	AD, ADD	Natural medium containing rice bran oil	[11]
Corynebacterium urealyticum	AD, ADD	Natural medium containing rice bran oil	[11]
Mycobacterium smegmatis	AD, ADD	Selective media	[18]

AD: Androstenedione, ADD: androstadienedione, T: Testosterone, B: Boldenone.

Table 1. Microorganisms reported to have the ability to transform sterols to steroidal intermediates.

enzymes responsible for nucleus degradation of AD. To avoid the nucleus degradation genome modification or mutation studies can be performed. Wei et al. has demonstrated a study to increase the yield of AD or ADD by mutations in Ksh or KsdD genes [14]. Ksh enzyme has two subunits namely A and B known as monooxygenase and reductase respectively. As described by Wei et al., the whole genome sequencing of *Mycobacterium neoaurum* explains six distantly placed gene clusters encoding KsdD enzyme and two gene clusters encoding KshA and KshB each. It was found that NwIB-02 *Mycobacterium neoaurum* with mutation in KsdD gene accumulated AD as the main product. Besides that, double mutated *mycobacterium neoaurum* i.e. mutations in both KsdD and Ksh genes accumulated AD as the main product of microbial biotransformation of phytosterol. Cloning of any one of the genes encoding these enzymes into a more economically cultivable strain may lead to enhanced production of desired products.

4.3. Liquid polymer-based systems

As described earlier, low substrate solubility is one of the major obstacles in aqueous bioconversion systems involving hydrophobic compounds. Researchers derived an alternative of using organic solvents in the aqueous media to increase the substrate solubility and mass transfer. On the other hand, organic solvents are not environment friendly. An alternative to organic solvents are the use of supercritical fluids, liquid polymers, ionic liquids and natural oils [19, 20]. Such strategies also negate the drawbacks caused by organic solvents i.e. damaging effects on microbial cells and its hazardous nature. Carvalho et al. demonstrated the use of poly (methylphenylsiloxane) oil (Silicone B oil) for AD extraction. It provides a suitable media for sitosterol side chain cleavage. AD yields close to 10 mM were obtained in almost 4–5 days of incubation, for an initial substrate concentration of 12 mM (referred to the polymer/organic solvent phase), with a biocatalyst concentration of 5 mg dry cell weight/ml. The researchers concluded the use of silicone B oil as non-volatile and non-toxic for providing a sustainable environment for the microbial side chain cleavage of sitosterol, both in single liquid phase system and in oil: aqueous two liquid phase systems.

To enhance the production of AD from phytosterol ionic liquid-aqueous biphasic systems were established by Yuan et al. [19]. Ionic liquids (ILs) with different cations and anions provided distinct but favorable substrate solubilization and product distribution for two phase conversion. The results of this study showed that AD production reached 2.23 g L^{-1} after 5 days of biotransformation with substrate concentration of 5 g L^{-1}. Further, ionic liquids which are easy to recycle produce negligible vapor pressure which indicates the industrial application of ILs in biphasic transformation process. Hence this strategy can be built up further for a more scaled up production.

4.4. Three-stage fermentation system

A recent strategy employed for enhanced AD production is the use of three-stage fermentation system. The well-known two-stage fermentation system uses a complex sugar (fructose in this case) as an initial carbon source followed by additional supplementation of simple sugar (glucose) which leads to elimination of the lag phase of the microorganism as well as an increase in biomass [21]. Shao et al. showed the effect of different carbon and energy sources on *Mycobacterium neoaurum* for enhancement of the desired steroid intermediate (AD/ADD) [13]. The researchers found that fructose acted as an optimal initial carbon source. Subsequent feeding by glucose maintained the metabolism of *mycobacterium neoaurum*. The researchers proposed three-stage fermentation by addition of phytosterol as third source and found enhanced ADD production (18.6 g/ l) which is reported to be the highest using *mycobacterium neoaurum*.

4.5. Use of PHB granules

Gerber et al. developed a whole–cell system based on recombinant *Bacillus megaterium* which encodes CYP11A1, the enzyme responsible for side chain cleavage of sterols [4]. The microorganism's PHB granules, aggregates of bioplastic coated with a protein/phospholipid monolayer act as substrate storage entities. As described earlier, substrate solubility of sterols in microbial biotransformation process is one of the major obstacles; PHB granules increase the conversion rate by serving as substrate storage entity. This phenomenon leads to increase in the mass transfer thereby increasing the desired product. Microorganisms which code for PHB production or the organisms having the ability to produce PHB granules can be screened for the biotransformation of sterols. These organisms may help in substrate availability thereby enhancing the efficiency of biotransformation process.

5. Conclusions

As per the available literature, there are number of strategies to increase the product yield. However, this area of research is still a hot topic as new strategies need to be developed which can lead to high yields of AD from cheaper substrates. Techniques involving industrially appropriate strains or addition of bioavailable catalysts to enhance the conversion or analysis of molecular pathway for generation of by-products. This may lead to development of a commercially cost effective process with high yields of the desired product. AD being one of the most important steroids which has high demand in pharmaceuticals as it is the precursor for

widely marketed drug category known as steroids. Therefore, the industrial production of this compound will greatly benefit the biomedical sector.

6. Future perspectives

The industrial production of androstenedione is challenging and economically important.

Therefore upcoming research in this field will be focused on:

a. Bioprospecting for finding organisms with enhanced production of androstenedione

b. Process optimization for economically viable production

c. Developing industrially applicable techniques to enhance the yield of AD.

Although there are a number of strains reported to have the biotransformation ability, yet the efficacy of these strains does not match the industrial applicability. Hence the need of the hour is to look for organisms having the capacity of transforming phytosterols to AD with high efficiency. Strategies can be applied for screening microorganisms having the ability to produce natural compounds like biosurfactants which increase the availability of complex substrates. Such organisms might play a role in substrate solubility of phytosterol thus minimizing the need of additional compounds.

Process optimization involves various parameters such as substrate inhibition, product inhibition, activities of biocatalysts, etc. To date, to the best of our knowledge, these parameters have not been addressed in the available literature. These parameters may result in the low yield of AD. Once addressed, these parameters might open a vast area of research. The mechanism of phytosterol biotransformation by various organisms has been reported but the enlisted parameters have not been paid attention to. All the aspects of the process need to be considered so as to develop an industrially applicable process for biotransformation of phytosterols.

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Conflict of interest

None declared.

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Chapter 8

Physiology and Pathology of Mitochondrial Dehydrogenases

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Additional information is available at the end of the chapter

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Abstract

Effective mitochondria bioenergetics requires the proper functioning of various intra-organelle dehydrogenases. By providing pyridine and flavin adenine dinucleotides to the electron chain, or Acyl-CoA for the reactions of the tris-carboxylic acid cycle, or the acylation of fatty acids to undergo β-oxidation, these dehydrogenases preside to the organelle production of ATP, required for a variety of cellular functions under physiological conditions. The operation of the various dehydrogenases is mainly regulated by hormones through changes in intra-mitochondrial cation levels and ratios, namely Ca^{2+} and Mg^{2+}. Dysregulation of specific dehydrogenases under pathological conditions results in marked changes in the energetic level of the organelle and ultimately the cell. The present review will attempt to highlight the role of the main mitochondrial dehydrogenases and their regulation, and provide a general assessment of their dysfunction and associated consequences under some of the most common human pathologies.

Keywords: mitochondria, dehydrogenases, calcium, magnesium, diabetes, obesity, cancer

1. Introduction

The oldest reports of intracellular structures that most likely represented mitochondria date back to 1840s [1], whereas the association of these organelles with specific biological functions essential for the hosting cells can be attributed to Altman [2]. The first utilization of the term 'mitochondrion' is attributed to Benda, in 1895 [3]. Regaud, in the early twentieth century, suggested that the organelle was constituted of protein and lipids [4], but it is not until the 1960s through the seminal work by Palade [5] and the use of electron microscopy that the structure and morphology of the organelle as it is currently known was defined. Alongside

these studies, the utilization of various experimental approaches resulted in the identification of a variety of enzymes and proteins localized either within the mitochondrial matrix or associated with the inner mitochondrial membrane or the inter-membraneous space, as well as the presence of mitochondrial DNA [for an historical overview see [6] and the refs. therein]. Because the reduplication of mitochondrial DNA occurs following the reduplication of cellular DNA, and mDNA only accounts for less than half of all the mitochondrial enzymes and proteins, sophisticated biological mechanisms are in place to coordinate the import and proper allocation of mitochondrial enzymatic components within the organelle following their cellular synthesis. Furthermore, mitochondria are not static organelles but undergo constant fusion and division (fission) to form ever changing tubular networks in most eukaryotic cells. These changes are important for the normal physiology of the cell, and may dramatically affect cell behavior under pathological conditions.

From the functional stand-point, mitochondria are regarded as the source of energy for the cell, in that they produce a steady flow of ATP that is utilized in a variety of cellular functions and signaling events. In addition to producing ATP, mitochondria play a major role in signaling, cell differentiation and growth, cell cycle, cell death (apoptosis), and in the production of reactive oxygen species (ROS) [7].

While certain mitochondrial functions are present almost exclusively in specific cells (e.g. ammonia detoxification in hepatic mitochondria), ATP production represents the predominant and ubiquitous function of the organelle in all eukaryotic cells. The chemiosmotic coupling of proton movement out of specific sites of the electron transport chain to their re-entry through the F0-F1 ATPase, with associated ATP production in a $3H^+$: 1ATP ratio, as proposed by Mitchell [8] in 1966, is now generally accepted, although some details of the process need further refinement. The generated ATP is then extruded in a $1ADP_{in}$: $1ATP_{out}$ ratio into the cytoplasm through the adenine nucleotide translocase (AdNT), one of the most abundant proteins present in the mitochondrial membrane.

Despite our current understanding of the mechanisms responsible for ATP synthesis within the mitochondria and its extrusion across the organelle membrane, the specific role of various mitochondrial dehydrogenases and their regulation in modulating ATP synthesis to maintain a stable and viable flow of energetic 'currency' for the cell based on the available substrate is still not completely elucidated.

The present review does not have the pretense of being comprehensive in addressing the role and regulation of all mitochondrial dehydrogenases. Rather, it will focus on the regulation of some of them, and the dysregulation occurring under specific pathological conditions, which ultimately impacts the proper functioning of specific organs or tissues.

2. Key mitochondrial dehydrogenases and their regulation

For the purpose of this review, we will focus on the following dehydrogenases:

Glutamate dehydrogenase (EC 1.4.1.4): The presence of this enzyme in eukaryotes is essential for urea synthesis in the urea cycle, in that it converts glutamate to α-ketoglutarate, and

vice versa. However, in addition to having a very low Km for ammonia (~1 mM), at equilibrium the reaction catalyzed by this enzyme favors ammonia and α-ketoglutarate production. Conversion of α-ketoglutarate to glutamate does occur in brain mitochondria as a result of local NAD^+:$NADH/H^+$ ratio [9]. In humans, the activity of the enzyme is regulated through ADP-ribosylation, but also by caloric restriction or hypoglycemia, as either of the latter two conditions increase glutamate dehydrogenase activity to increase the amount of α-ketoglutarate produced. In turn, α-ketoglutarate is used to provide energy thought the citric acid cycle, ultimately generating ATP. The activity of the enzyme does not appear to be regulated by an increase in the levels of Ca^{2+} and Mg^{2+}, which can accumulate to a significant extent within mitochondria under physiological conditions. On the other hand, Zn^{2+} has been reported to act as an allosteric regulator of the enzyme, together with ATP and possibly GTP.

α-ketoglutarate dehydrogenase (1.2.4.2): Also known as oxoglutarate dehydrogenase complex (OGDC), this enzymatic complex is known for its role in the citric acid cycle. Three different forms of this complex can be identified based on the specific substrate of interaction. One form is specific for pyruvate, a second one is specific for 2-oxoglutarate, and a third one is specific for branched-chain α-keto-acids. Because the same dehydrogenase subunit is utilized, the three forms of the complex utilized the same coenzymes: i.e. TTP, CoA, lipoate, FAD and NAD. Functionally, this dehydrogenase is involved in lysine degradation and tryptophan metabolism in addition to playing a key control point in the TCA cycle. In this contest, the dehydrogenase controls the level of reducing equivalents, such as NADH, generated in the cycle which, in turn increase the electrons flux through the mitochondrial electron transport chain, enhancing oxidative phosphorylation and ultimately ATP synthesis [10]. In a classic enzymatic inhibitory feedback, the activity of α-ketoglutarate dehydrogenase is inhibited by the reaction by-products succinyl-CoA and NADH, as well as by a high energy status (elevated ATP and NADH levels) within the cell, and by CoA-SH. Conversely, an increase in ADP level acts as an allosteric activator of the dehydrogenase. Calcium [10] and magnesium [11] ions also act as allosteric activators of the enzyme. The effects of Ca^{2+} and Mg^{2+} on the enzyme activity appear to be additive when the concentrations of free Mg^{2+} in the matrix is <1 mM. The presence of the divalent cations markedly decrease the K_m for α-ketoglutarate from ~4–5 mM in the absence of Ca^{2+} and Mg^{2+}, to 2.2 mM in the presence of Ca^{2+} alone, to 0.3 mM in the presence of both cations [11]. The effect of Mg^{2+} is only observed in the presence of thiamine pyrophosphate (TPP), suggesting that the enzyme requires both TPP and Mg^{2+} for maximal activity. The presence of both cations also decreases the affinity of the dehydrogenase for NAD^+ [11]. The modulatory effect of both Ca^{2+} and Mg^{2+} are observed at concentrations that are well within the range reported to occur in mitochondria for these cations under stimulatory conditions, e.g. by catecholamine or other hormones, thus supporting the likelihood that these regulatory effects can occur under in vivo conditions.

A side product of a-ketoglutarate activity is the generation of free radicals, which can lead to oxidative stress if accumulating in high levels due to increased production and/or reduced detoxification. Due to its ability to generate free radicals, this dehydrogenase is considered de facto a mitochondrial redox sensor [12] in that it can modulate the rate of mitochondrial functioning and consequently the level of oxidative stress, thereby limiting the associated damage [12]. Under conditions in which the level of free radicals becomes extremely elevated, the enzyme can undergo a fully reversible oxidative inhibition [13]. This temporary inhibition

appears to occur through the reversible glutathionylation of the E2-lipoic acid domain of the dehydrogenase [14], thereby protecting the E2 domain, and the catalytic site of the dehydrogenase, from damaging oxidative stress. As the flux of electrons through the electron chain decreases, so does the production of free radicals, optimizing the conditions for the mitochondrial detox systems to scavenger these toxic agents.

Isocitrate Dehydrogenase (1.1.1.42 and 1.1.1.41): This enzyme catalyzes the oxidative decarboxylation of isocitrate to generate a-ketoglutarate and CO_2 in the citric acid cycle. The reaction is a two-step process, supported by the conversion of NAD^+ to NADH. The mitochondrial isoform of this enzyme, IDH3, is a heterotetramer composed by two alpha, one beta, and one gamma subunits ($2\alpha1\beta1\gamma$) Two other isoforms (IDH1 and IDH2) of the enzyme are known in humans. They catalyze the same reaction in the cytosol and in peroxisomes converting $NADP^+$ to NADPH in the process.

Owing to the large negative free energy change involved in the reaction, the step catalyzed by this dehydrogenase represents one of the irreversible reactions in the citric acid cycle. Hence, tight regulation is required to avoid unnecessary depletion of isocitrate. The reaction is promoted by substrate availability and the presence of cofactors such as NAD^+ and Mg^{2+} (or Mn^{2+}), which both bind specific active sites on the IDH structure, and inhibited by ATP levels [15]

Succinate dehydrogenase (1.3.5.1): This enzyme participates in two key cycles within the mitochondrion. It is part of the respiratory complex II (whereby it is also defined as succinate-coenzyme Q reductase) and as such is key in controlling the electron flux through the electron transport chain [16]. In addition, it participates in the citric acid cycle, in which it catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol.

Structurally, the succinate dehydrogenase is composed of 2 hydrophilic and 2 hydrophobic subunits. The two hydrophilic subunits possess a covalently attached flavin adenine dinucleotide (FAD) cofactor and the succinate binding site (subunit SdhA) and three iron–sulfur clusters (SdhB). The subunit SdhA provides the binding site for the oxidation of succinate. The two hydrophobic subunits SdhC and SdhD act as membrane anchors. Human mitochondria contain 2 distinct SdhA isoforms (FpI and FpII), which form a membrane bound cytochrome b complex with 1 heme b group and 1 ubiquinone binding site. More specifically, the ubiquinone binding site is located in an enclosure formed by SdhB, and the two hydrophobic subunits SdhC and SdhD. These two subunits also present one cardiolipin and one phosphatidylethanolamine molecule attached [17].

Functionally, the succinate-binding site in SdhA and the ubiquinone-binding site in the pocket formed by the three other subunits are connected by a chain of redox centers that include FAD and the three iron–sulfur clusters [17]. Due to its localization, the succinate dehydrogenase participates to both the citric acid cycle and the respiratory chain, as indicated previously. Electron removed from succinate during its oxidation to fumarate are transferred through the iron–sulfur clusters present in SdhB to the ubiquinone molecule, and ultimately to the two hydrophobic subunits anchoring the complex to the mitochondrial membrane. The acceptance of the electrons by the ubiquinone occurs in a two-step process whereby, after the first electron transfer, the ubiquinone is converted to a semiquinone radical species. The transfer of the second electron fully converts this intermediate to ubiquinol [18]. Hence, SdhA acts as an intermediate in the enzymatic activity of the succinate dehydrogenase complex in that:

1) it converts succinate to fumarate as part of the citric acid cycle while converting FAD to $FADH_2$; 2) electrons from FADH2 are transferred to the iron–sulfur clusters of the SdhB subunits as part of the respiratory chain function, and 3) the electrons are ultimately transferred to ubiquinone Q via the hydrophobic subunits o the complex. Inhibition of the succinate-ubiquinone activity results in the transfer of the electrons flowing through the SdhB subunit to O_2, thereby generating reactive oxygen species (e.g. superoxide). Accumulation of reactive oxygen species stabilizes the production of HIF-1α, and facilitating its interaction with HIF-1b to form a critical heterodimeric complex that induces the expression of anti-apoptotic genes but also tumor-causing genes (see section 3 for further details). Under conditions in which succinate dehydrogenase activity is inhibited, succinate accumulates within the mitochondria and then diffuses into the cytoplasm where it inhibits the physiological hydroxylation of HIF-1α in the cytosol by prolyl-hydroxylase (PHD). Inhibition of HIF-1a hydroxylation works in conjunction with the accumulation of reactive oxygen species occurring through the succinate dehydrogenase complex to stabilize HIF-1a and promote the formation of a stable and active HIF complex that promotes the expression of tumor-inducing genes [19]. Because PHD activity requires oxygen, α-ketoglutarate as substrates, and ferrous iron and ascorbate as co-factors, increasing α-ketoglutarate levels could represents a viable therapeutic approach to limit tumor development and growth under SDH deficiency.

Pyruvate dehydrogenase (1.2.4.1): This enzyme is the first component of the pyruvate dehydrogenase complex (PDC), which is responsible for transforming pyruvate to acetyl-CoA via pyruvate decarboxylation. The Acetyl-CoA generated by the reaction then enters the citric acid cycle, contributing to cell respiration. As a result, pyruvate dehydrogenase links glycolysis to the citric acid cycle and the release of energy via NADH. The complex is constituted by the pyruvate dehydrogenase (E1) component, a dihydrolipoamide acetyltransferase (E2) component, a pyruvate dehydrogenase kinase (PDK) and a pyruvate dehydrogenase phosphatase. The reaction operated by E1 uses thiamine pyrophosphate (TPP) as a required cofactor, and it is considered to be the rate-limiting step for the whole pyruvate dehydrogenase complex (PDHc) activity. Phosphorylation of E1 by PDK inactivates E1 and consequently the whole complex. Pyruvate is a natural inhibitor of PDK, thereby resulting in a higher level of active, unphosphorylated PDH [20]. Alternatively, the phosphorylation state of E1 is reversed by the activity of pyruvate dehydrogenase phosphatase, which is stimulated by insulin, phospho-enol-pyruvate, and AMP, and competitively inhibited by ATP, NADH, and Acetyl-CoA. Physiological fluctuations in mitochondrial Ca^{2+} and Mg^{2+} levels, as observed following insulin stimulation, also stimulate the phosphatase activity, maintaining E1 in the active state [20].

3. Mitochondrial dehydrogenases and cancer

Cancer cells are characterized by increased glycolytic ATP production as a result of decreased mitochondria effectiveness. Inhibition (or decreased activity) of the citric acid cycle is considered to be one of the main causes forcing the cells to generate ATP through anaerobic glycolysis [21]. This reprogramming results from oncogene activation or inhibition of tumor suppressors [21]. Consistent with this observation, inhibition of glycolysis by dichloroacetate,

a pyruvate inhibitor, shifts cell metabolism back to oxidative phosphorylation, at least to a certain extent [22]. This metabolic reprogramming is considered to be necessary to meet the needs of the rapid proliferative rate exhibited by cancer cells. Two key bioenergetics parameters resulting from the mentioned metabolic reprogramming are anaerobic glycolysis, with associated extracellular acidification, and mitochondrial respiration.

Altered functioning of any of the mentioned dehydrogenases has been observed in different cancer cells, and specific correlations have been observed and documented. For example, glutamate dehydrogenase can be considered as biomarkers for cancer cell growth [23]. Similarly, defects in isocitrate dehydrogenase [24], a-ketoglutarate dehydrogenase [25] succinate dehydrogenase [26], and pyruvate dehydrogenase [27] have all been reported in a variety of cancer cells, and associated with the metabolic reprogramming these cells undergo. The picture that emerges is that many cancer cells are hypoxic and therefore metabolize lactate to pyruvate to generate ATP [28]. The occurrence of hypoxia results in the activation of hypoxia-inducible factor 1 (HIF-1) heterodimeric DNA-binding complex and pro-neoplastic genes in tumor cells. HIF-1α is continuously synthesized and degraded, with a half-life of ~6 min under normoxic conditions. Under hypoxia conditions, however, the rate of HIF-1α degradation decreases significantly as a result of prolyl hydroxylation and proteosomal degradation suppression by accumulating succinate and increasing ROS formation and enzyme modification. As a result, HIF-1 heterodimer accumulates, and translocated to the nucleus of the cells where it activates genes responsible for increased glucose uptake (mostly GLUT1) and lactate production. At the same time, the increase in succinate levels further depresses mitochondrial respiration, with increased routing of O_2 towards ROS formation. In addition to upregulating the glucose transporter, HIF-1 promotes the expression of various glycolytic enzymes as well as PDK-1, the kinase that phosphorylates and inactivates the pyruvate dehydrogenase subunit E1. Inactivation of this dehydrogenase prevents pyruvate entry into the TCA cycle, thus down-regulating mitochondria respiration [29].

Another pathway involved in modulating mitochondria activity and responsiveness in cancer cells is the one tapping onto peroxisome proliferator-activated receptor gamma co-activator 1alpha (PGC1α). This pathway has been observed to be overexpressed in some subsets of melanomas in which it activates mitochondrial oxidative phosphorylation and TCA-cycle specific genes for the metabolic needs of the cancer cells [30]. In addition, PGC1α promotes the expression of genes involved in *de novo* lipogenesis and in the pentose phosphate pathway, increasing NADPH production for fatty acid synthesis purposes [30]. While genes involved in oxidative phosphorylation and TCA cycle are located in mitochondria, those involved in the pentose phosphate pathway and fatty acid synthesis are located in the cytoplasm of the cell. How exactly Acyl-CoA generated within the mitochondrion through aerobic glycolysis (pyruvate dehydrogenase) is utilized to enhance fatty acid synthesis is not completely understood. Experimental evidence would suggest that PGC1α may do so by inducing the expression of genes responsible for converting citrate back to oxaloacetate and Acyl-CoA [30]. Irrespective of the precise mechanisms involved in the metabolic reprogramming of cancer cells, PGC1α-positive cancer cells are particular sensitive to pharmacological (e.g. metformin) or chemical (e.g. rotenone or FCCP) inhibitors of oxidative phosphorylation, in stark contrast to PGC1α-negative cancer cells, which are more sensitive to anti-glycolytic agents (e.g. 2-deoxyglucose, or 2-DG).

Mitochondrial ROS and Cancer: Reactive oxygen species (ROS) are by-products of mitochondrial electron transport chain, generated by the incomplete reduction of oxygen as electrons flow from one complex to the next. Under physiological conditions, 1–2% of the molecular oxygen utilized by the mitochondria is converted to ROS [31]. Reactive oxygen species are highly reactive molecules, which act as oxidants removing electrons from DNA, proteins, and lipids. While ROS can be generated at the level of the plasma membrane (NADPH oxidase) and the endoplasmic reticulum of cell abundant in cytochrome activity (e.g. hepatocytes), the main intracellular source of ROS in most cells is indeed represented by mitochondria. In particular, O_2^- is the main byproduct of oxidative phosphorylation, and acts as precursor of other ROS products such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). Due to its negative charge, O_2^- cannot diffuse across biological membrane. However, evidence suggests that it can cross the mitochondrial membrane and diffuse into the cytoplasm through VDAC and other not-yet identified mitochondrial channels [32]. Either in the mitochondria or in the cytoplasm superoxide dismutases convert O_2^- to H_2O_2, which can now diffuse freely across membranes. H_2O_2 is highly reactive towards cysteine residues, and this reactivity is further enhanced in the presence of O_2^- and iron or other transition metals (Fenton reaction or Haber-Weiss reaction), generating the more reactive but short-lived OH^-, which interacts with any surrounding macromolecule, including proteins, lipids, nucleic acids, and carbohydrates [33]. Interaction of ROS with surrounding macromolecules may result in damage of biological membrane, enzyme inactivation, and genotoxicity. High levels of ROS can induce apoptosis and cell death whereas low levels of ROS can act as signaling molecules, and either path can results in tumor initiation and progression.

Cellular DNA and mitochondrial DNA are both highly susceptible to ROS-induced damage. This susceptibility is more pronounced for mitochondrial DNA, due to the close proximity to the electron transport chain where ROS are generated, the absence of protective histones, and the limited DNA repair capability. As a result, the mutation rate of mitochondrial DNA is two orders of magnitude higher than that of nuclear DNA. Consistent with this notion, mutations in mitochondrial DNA - in particular mutations of oxidative phosphorylation enzymes - have been observed in many neoplastic cells (see previous section), in which they result in oxidative phosphorylation dysfunction, increased ROS formation, and energetic shift towards anaerobic glycolysis.

The role of ROs as signaling molecules in cell proliferation, differentiation, migration, metastatic colonization, and gene transcription is now fairly well recognized. The ability of ROS to act as signaling molecules depends on the presence of redox-sensitive proteins that operate as 'ROS sensors'. In most cases, the sensing ability lies in the reversible oxidation of sulfhydryl groups in specific cysteine residues by H_2O_2 or other ROS, resulting in the inhibition of that particular protein. For the most part, this inhibition is transient in that the cell possesses scavenging mechanisms (e.g. glutathione) to reduce the sulfhydryl groups back to the original state, thus restoring the protein's activity or signaling properties [34].

The levels of ROS produced by tumor cells are usually higher than those present in normal cells, and they results in DNA damage but also in increased tumorigenesis and metastasis via direct activation of signaling pathways [35]. Two signaling pathways activated by ROS are MAP Kinase and phosphoinositide 3-kinase, which both control cell survival and proliferation. The

upregulation of these pathways increases the expression of oncogenes and proteins involved in metastasis (e.g. matrix metalloproteinases) and in epithelial to mesenchymal transition [36]. Of note, oncogene activation has been reported to enhance mitochondrial ROS production, and ROS generation appears to be required for oncogene-mediated cell transformation [37].

4. Mitochondrial dehydrogenases under diabetic conditions

A vast body of literature covers the functional modifications of mitochondria under diabetic conditions. Because cardiovascular insults remain the leading cause of death for diabetic patients [38], most of the attention has been focused on the modifications occurring in cardiac mitochondria to provide a better rationale for the morphological and functional modifications observed in diabetic hearts. Further, the incidence and severity of cardiovascular complications are markedly increased in both male and female diabetic patients, with the latter exhibiting a greater incidence than the diabetic male counterparts [39].

In humans, our understanding of the disease is complicated by the presence of two pathological conditions: diabetes type 1, in which insulin production is lacking, and diabetes type 2, in which insulin is still produced, sometimes to a greater extent than under normal conditions, and yet it is not functionally efficient in controlling the glycemic state of the patient. An additional confounding parameter is that to a large extent type 2 diabetic patients are overweight or obese, with an altered lipid status both in serum and within tissues (lipotoxicity).

This variety of conditions is also reflected in the heterogeneity of animal experimental models utilized to investigate the disease. Also here, models of type 1 diabetes are conceptually simpler in that animals are injected with agents that damage pancreatic beta-cells and abolish insulin secretion, fully mimicking the human condition. Models for type 2 diabetes are more heterogeneous, spanning from genetically conditioned animals (KO animals for specific proteins, receptors, or signaling molecules) to genetically inbred animals (e.g. Koletsky rats), to animals that develop diabetes spontaneously, to animals fed assorted high fat diets with or without sucrose, and injected with subliminal doses of beta-cell damaging agents that limit but not completely abolish the endogenous production of insulin, or treated with various oral antidiabetic agents [40]. Because of this plethora of models, it is not simple to determine to which extent modifications of mitochondrial dehydrogenase are a primary or secondary insult in the pathogenesis of the diabetic condition and its complications.

The development of diabetic cardiomyopathy has been etiologically attributed to several factors including metabolic [41], biochemical [42] and ultra-structural [43] modifications within the cardiac myocytes. Scrutiny of mitochondrial function in cardiac mitochondria from streptozotocin-treated rats has shown a decline in respiration and oxygen consumption, more pronounced for state 3 than state 4, which translates into a decrease in ATP production [44]. This energy deficiency has been considered a key factor in the development of diabetes-related cardiac dysfunctions, although changes in cardiac microvasculature [45], metabolic and hormonal disturbances [46] and concurring hyperglycemia [47] can certainly act as co-factors. Combining defective mitochondrial respiration with increased ROS formation, studies from our laboratory have indicated that succinate dehydrogenase [48] and pyruvate dehydrogenase [49] are highly sensitive to ROS-induced damage, forming stable, non-functional adducts

within the mitochondrion of streptozotocin-treated rats. As mentioned in the previous section, the consequences of this dysfunction are three-fold: 1) the inability of the electron transport chain to operate properly is compromised, resulting in decreased ATP production; 2) the production of ROS is enhanced, further damaging macromolecules within the mitochondrion and its surroundings, and 3) forcing the cardiac myocytes to depend on glycolysis to produce ATP. Interestingly, these changes were observed predominantly in cardiac and liver tissue but not in the kidney of the diabetic animals [49], rising the intriguing question of which protective mechanism(s) may operate in the latter organ under diabetic conditions.

5. Mitochondrial dysfunction in obesity

The term obesity refers to a condition in which the amount of fat tissue in the body is increased to an excessive degree (i.e. more than 25% in body weight in men and more than 30% in women). The incidence of obesity or just overweight has been on the rising for the last several decades, and currently affects anywhere between 35 and 45% of the population, in developed and developing countries [50]. Obesity is the sixth most important risk factor, and the number of affected individuals has reached 2.1 billion worldwide, including 10% of all children. The main complications include particular forms of cancer (e.g. colon and breast cancer, in particular), type 2 diabetes mellitus, cardiovascular pathologies including stroke, and musculoskeletal dysfunctions, with a prohibitive price tag in terms of healthcare costs, morbidity and mortality [51]. Several reviews have addressed the changes in mitochondria functioning in obesity, and we refer to them for an in-depth understanding of the changes occurring in the organelle [52–53]. The emerging picture indicates that cardiac cells, adipocytes, skeletal muscle cells, beta-cells, liver cells and others are affected to a varying degree by lipotoxicity [54], which also affects mitochondria operation. Whether the dysfunction strictly depends on the abnormal presence of fatty acid in the cells and the biological membrane of the organelle, or other not-yet identified factors remains undefined. The most common mitochondrial dysfunctions associated with obesity have been identified in an abnormally low number of mitochondria with altered morphology, decreased expression of the F1-ATPase subunit, with consequent low ATP generation [53], higher expression of the uncoupling protein 2 (UCP2) [52] with associated increased production of O_2^-, ROS in general, and apoptosis [52]. At the same time, the abnormal presence of fatty acids and ceramide within the cell activate specific signaling that further impair the tissue response to insulin [53]. Due to the decline in number and the altered morphology of mitochondria, an unanswered question remains as to whether these modifications are secondary to intrinsic defects in mitochondrial dehydrogenases that link electron transport chain to TCA cycle and/or to proper ATP synthesis, or whether defects in mitochondria enzymes are consequence of defective synthesis of key components within the cell nucleus and defective import into already altered organelles.

6. Conclusions

The picture we have attempted to draw in the present review moves from the important role specific mitochondrial dehydrogenases play in cell metabolism. Under conditions in which metabolic reprogramming occurs (i.e. cancer, diabetes, obesity), cells move away from efficient

mitochondrial bioenergetics and come to rely on glycolysis for ATP generation purposes. This metabolic shift has the unintended consequence of increasing reactive oxygen species production, which further disrupt cell metabolism and activate genes and oncogenes through specific signaling pathways and nuclear transcription factors (HIF-1α, MAPKs, PI3K). Current and future lines of research aim at better understanding the signaling and metabolic routes connecting mitochondria to cell functions in the attempt to possibly recondition metabolism and energy production of target cell and mitochondria.

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