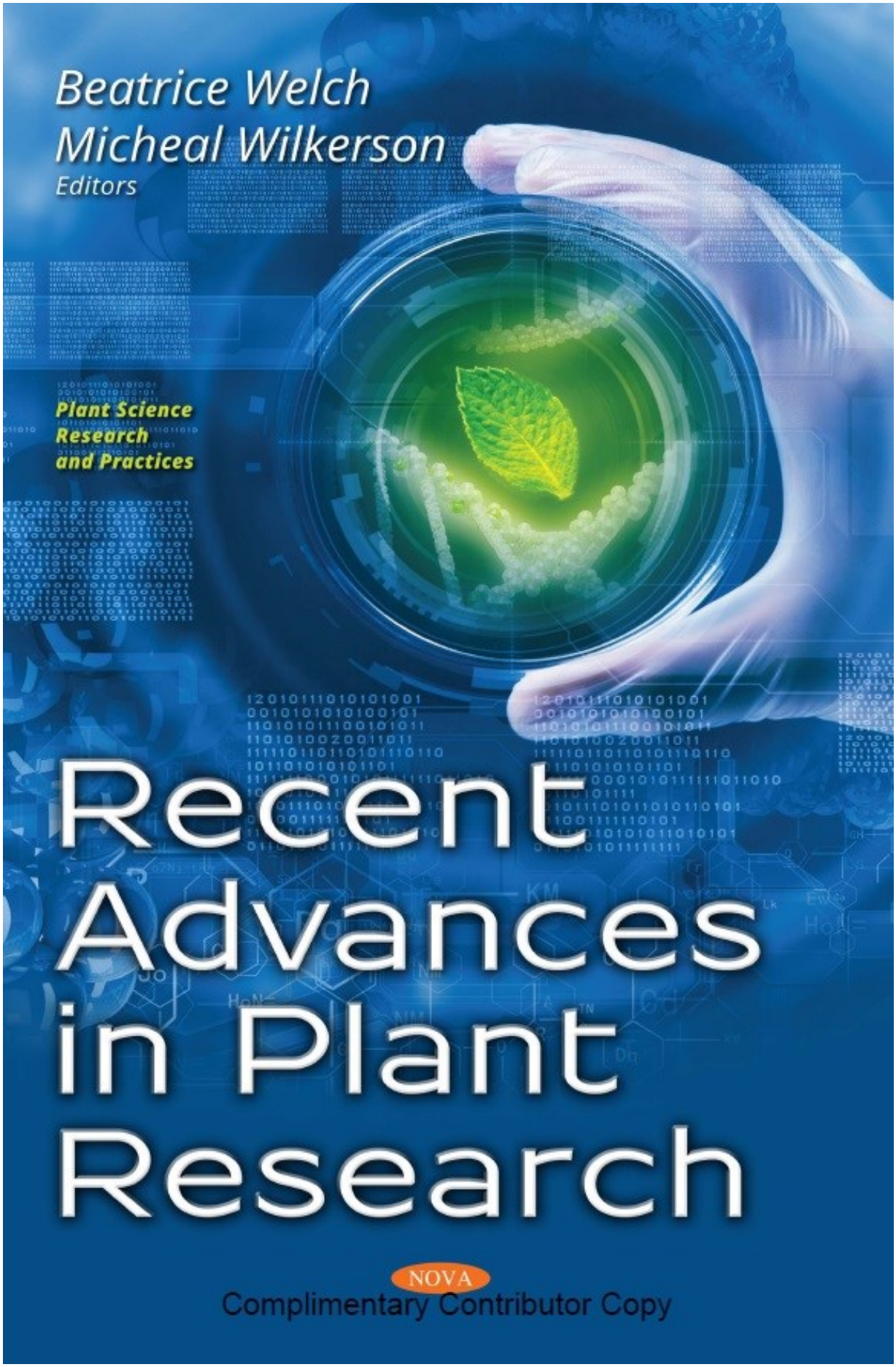
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Plant Science Research and Practices
Recent Advances in Plant Research
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RECENT ADVANCES IN PLANT RESEARCH

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RECENT ADVANCES IN PLANT RESEARCH

BEATRICE WELCH
AND
MICHEAL WILKERSON
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PREFACE

Recent Advances in Plant Research begins by providing a summary of work performed on the effects of environmental factors and growth treatments on secondary metabolite production in both conventionally and *in vitro* cultivated plant material, focusing on different classes of volatile and non-volatile secondary metabolites.

Following this, the authors set out to determine the diversity of *Fabaceae* in vegetational fragments of Cerrado in the state of Maranhão. Monthly expeditions were carried out in the period between September 2016 and June 2017 for the observation, collection, and identification of botanical material.

The great potential of the most important by-products of apples, grapes and berry fruits processing as a source of antioxidants is presented in one chapter. Some novel and practical aspects of extraction of natural antioxidants are also discussed.

Also discussed is gray mold rot caused by *Botrytis cinerea* Persoon: Fries [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel]. It is a serious disease causing severe damage to fruits and vegetables at both pre- and post-harvest periods. The authors aim to highlight try to highlight the disease distribution and hosts range, pathogenesis of the pathogen, symptoms of the disease, disease cycle and epidemiology of the pathogen as well as the current status of the disease in Malaysia.

Plants produce an array of toxins and defensive proteins through various metabolic pathways for their defence against insects. To cope with these defences, herbivores have developed counter-defences which the authors examine in this compilation. Furthermore, insects have employed a diverse array of strategies that enable them to bypass defensive barriers, or to metabolise these chemicals after ingestion.

Next, angiosperm flowers that have been found in mid-Cretaceous Myanmar (Burmese) amber are analyzed. Flowers in amber often show details of their reproductive parts that are not evident in blooms preserved in sedimentary deposits. Two new genera are described, *Chenocybus allodapus* gen et sp. nov. and *Diaphoranthus burmensis* gen et sp. nov.

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Japan, which extends north and south, has four clearly defined seasons that provide a variety of unique habitats for the growth of various aromatic and medicinal plants. Recent focus on these traditional plants has begun to revitalize local communities, providing diversification to alternative medicines and the development of other products, as well as suggesting increased and use of plants.

Aiming to better understand the phytodiversity of a vegetation area in the Piauí state, Brazil, in addition to aspects such as the geographical distribution of species and their uses, the concluding study was carried out in municipality of Bom Princípio, northern Piauí. The diverse flora was represented by trees, shrubs and herbaceous species, distributed in 54 families, 114 generas, and 146 species.

Chapter 1 - Secondary metabolites production, accumulation and translocation are dependent on the presence of highly specialized anatomical structures within the plant organism. Therefore secondary metabolites content is strongly affected by the developmental patterns and morphogenesis of the plant individual.

The present chapter summarizes work performed on the effects of environmental factors and growth treatments on secondary metabolite production in both conventionally and *in vitro* cultivated plant material. Different classes of volatile and non-volatile secondary metabolites have been concerned.

The author's own experience in the impact of tissue culture treatments on the developmental patterns and morphological features *in vitro* and the subsequent modification of secondary metabolite production of species of the genera *Hypericum*, *Sideritis* and *Artemisia* have been summarized and discussed.

Chapter 2 - *Fabaceae* is one of the largest families among the angiosperms, with representatives widely distributed throughout the globe, where 770 genera and 19.500 species are recognized and divided into six subfamilies (*Caesalpinioideae*, *Cercidoideae*, *Detarioideae*, Dialioideae, Duparquetioideae, and *Papilionoideae*). Floristic and taxonomic works indicate *Fabaceae* as the largest species family in Brazil and most representative in forest formations. The Cerrado has a significant representation of the family, where the state of Maranhão covers a broad area. In this way, the research had as goal to know the diversity of *Fabaceae* in vegetational fragments of Cerrado in the state of Maranhão. Monthly expeditions were carried out in the period between September 2016 and June 2017 for observation, collection, and identification of botanical material. The Cerrado area sampled belongs to the municipality of São João do Sóter, in the Maranhense´s East. After the collections, the specimens were herborized, analyzed with the help of taxonomic keys, specialized literature for identification, and determined by area expert. A total of 68 specimens, 31 genera, and 45 species were cataloged. Of all the specimens collected, the subfamilies *Papilionoideae* and *Caesalpinioideae* were the most representative of 21 species each. As for the Life form, was observed that the prevailing growth habit was the shrub type (20). Concerning to the physiognomies of the Cerrado, was observed the predominance of the species in the gallery forest environment (28).

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Were obtained three new records to the Maranhão, the species: *Desmodium subsecundum* Vogel. *Aeschynomene viscidula* Michx and *Vigna lasiocarpa* (Mart. ex Benth.) Verdc. being *V. lasiocarpa* a new record for the Northeast region of Brazil. Taxonomic keys, descriptions and photo plates were elaborated with all the studied species, composing a taxonomic treatment. In this way, it can be established that the Cerrado of Maranhão possesses a diversity of species for the *Fabaceae* family and that the research carried out has provided a basis for later studies, since these are few for Maranhão.

Chapter 3 - The awareness of consumers regarding the functional food led to an increasing demand for functional foods especially for foods rich in antioxidants, which have a wide range of activities such as antitumoral, antiviral, cardioprotective and antimutagenic activities. Phenolic compounds are an essential part of the human diet and are of considerable interest due to their antioxidant activities. Wastes from fruits represent a major disposal problem for the industry concerned. The by-products, which are results of the fruit processing, are rich in valuable compounds and represent a novel, natural and economic sources of flavorings, colorants, polyphenols and natural antioxidants, which can be used in the food industry as a source of natural food additives. This chapter is underlying the great potential of the most important by-products of apples, grapes and berry fruits processing as a source of antioxidants. Some novel and practical aspects of extraction of natural antioxidants will be discussed in this chapter.

Chapter 4 - The gray mold rot is caused by *Botrytis cinerea* Persoon: Fries [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel]. It is a serious disease causing severe damages on fruits and vegetables at both pre and post-harvest periods. *Botrytis cinerea* is an ascomycetous fungus as well as a necrotrophic plant pathogen that was ranked second in the top 10 most devastating fungal plant pathogens worldwide. Due to its broad host range, the fungus infects more than 200 plant species of agricultural importance such as cucumbers, strawberries, grapes, tomatoes, cut flowers and ornamental plants. The fungus occurs mostly where its host plants are cultivated, under climatic conditions ranging from cool temperate zones to subtropical regions. The fungus is highly concentrated in the temperate areas between latitudes 25 and 30. In Malaysia, the occurrence of this disease has been widespread in the greenhouses of the commercial farms in the Cameron Highland area of Pahang, Malaysia. This hindered the growing industries, hence detection, and characterization of the pathogen using molecular approaches provide an essential tool for establishing disease management programs. Since the pathogen is highly destructive, in this book chapter the authors will try to highlight the disease distribution and hosts range, pathogenesis of the pathogen (*B. cinerea*), symptoms of the disease, disease cycle and epidemiology of the pathogen (*B. cinerea*) as well as the current status of the disease (gray mold rot) in Malaysia.

Chapter 5 - Insect herbivores use different feeding strategies to obtain nutrients and energy from plants for their growth and reproduction. Plants produce an array of toxins and defensive proteins through various metabolic pathways for their defence against

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insects. To cope with these defences, herbivores have developed counter defences. These responses of insect herbivores are important in insect plant interactions. Among these interactions, host plant selection and feeding behaviour are important factors and play a significant role in habitat selection and discrimination mechanisms between host species. Furthermore, insects have employed a diverse array of strategies that enable them to bypass defensive barriers, or to metabolise these chemicals after ingestion.

Chapter 6 - Amberization is a unique type of fossilization that preserves both animal and plant parts for millions of years through a unique fixation-dehydration process. Flowers in amber often show details of their reproductive parts that are not evident in blooms preserved in sedimentary deposits. The present chapter discusses angiosperm flowers that have been found in mid-Cretaceous Myanmar (Burmese) amber. Two new genera are described, *Chenocybus allodapus* gen et sp. nov. and *Diaphoranthus burmensis* gen et sp. nov. These two new genera, along with other flowers described from this amber source, namely, *Antiquifloris latifibris*, *Cascolaurus burmitis*, *Endobeuthos paleosum, Eoepigynia burmensis*, *Jamesrosea burmensis*, *Lachnociona terriae, Micropetasos burmensis, Palaeoanthella huangii, Programinis burmitis, Programinis laminatus, Tropidogyne pikei* and *Tropidogyne pentaptera*, are considered extinct at the generic level. However, the level of extinction of the various lineages varies and they can be arranged in roughly three groups. Group one consists of flowers with no obvious affinities to any extant lineages. Group 2 are flowers with possible affinities to extant lineages above the family level. Group 3 are flowers that can be placed in extant families. Comments on possible pollinators in the Myanmar amber forest and extinction events regarding Myanmar amber flowers are included.

Chapter 7 - Japan, which extends north and south, has four clearly defined seasons that provide a variety of unique habitats for the growth of various aromatic and medicinal plants. Recent focus on these traditional plants has begun to revitalize local communities, providing diversification to alternative medicines and the development of other products. Aromatic plants are widely used in Japanese foods such as Washoku, the cuisine of Japan, which is recognized on the UNESCO Heritage list. The traditional Japanese "Ko-do" (an elegant incense ceremony) uses several aromatic plants that provide graceful aromas. Indigenous aromatic and medicinal plants continue the connection of plants with life and with spirit for the Japanese. Due to global warming, a change may limit plant growth and alter constituents, endangering both domestic and wild species. Thus, conservation of genetic resources is necessary to insure that the unique aromatic plant species of Japan, such as *Eutrema japonicum* (Wasabi) and *Cryptomeria japonica* (Sugi), are maintained. While universities, companies, institutes, and homes own a number of gardens and conservatories, the most important concerns are the use, conservation, and return of natural resources in a daily life. Various locations in Japan practice a circulation-type forestry. This method creates new jobs that eventually lead to regional revitalization. Recently, the introduction of profiles and advantages of domestic Japanese

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medicinal and aromatic plants have developed markets with remarkable growth in Japan, suggesting increased interest and use of plants for better health, education, and environment in Japan.

Chapter 8 - Aiming to better understand the phytodiversity of a vegetation area in the Piauí state, Brazil, in addition to aspects such as the geographical distribution of species and their uses, this study was carried out in municipality of Bom Princípio, northern Piauí. The diverse flora was represented by trees, shrubs and herbaceous species, distributed in 54 families, 114 generas, and 146 species. The families of richest generas were *Fabaceae* (22 generas), followed by Malvaceae and Rubiaceae (seven generas) and Poaceae (five generas). Approximately 64,81% (35) of families were represented by a single genre at this location. It was found, according to the literature, that 29 species belonging to 16 families have economic potential. Most species have medicinal potential, with 22 species; followed by 15 timbers species, with 14 honey producer species; 12 ornamental species, food with eight species, forage nine and use for fuel, eight species. Of the species recorded in area 97 (66.4%) are not endemic in Brazil and 47 (32,2%) are endemic in this country. As consultations held in the collections of several Virtual Herbarium and Herbarium "HDelta," checked that 17 species did not have occurrence records in Piauí state. Analyzing the geographical distribution of species recorded in the study area and comparing with other surveys, it was found that 13 species found in survey's "cerrado" vegetation and 18 species in "caatinga" vegetation areas. The floristic survey in this semideciduous vegetation area in northern Piauí showed that in the area there are a number of species belonging to the plant formations of "cerrado" and "caatinga" vegetation, according to data of floristic lists made these vegetational formations, thus suggesting, treat itself to a transition area between these two types of vegetation.

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Chapter 1

ROLES OF DEVELOPMENTAL PATTERNS AND MORPHOGENESIS IN THE SECONDARY METABOLITE PRODUCTION OF CONVENTIONALLY AND BIOTECHNOLOGICALLY CULTIVATED MEDICINAL AND AROMATIC PLANTS

Kalina Danova
Institute of Organic Chemistry with Centre of Phytochemistry,
Bulgarian Academy of Sciences, Bulgaria

ABSTRACT

Secondary metabolites production, accumulation and translocation are dependent on the presence of highly specialized anatomical structures within the plant organism. Therefore secondary metabolites content is strongly affected by the developmental patterns and morphogenesis of the plant individual.

The present chapter summarizes work performed on the effects of environmental factors and growth treatments on secondary metabolite production in both conventionally and *in vitro* cultivated plant material. Different classes of volatile and non-volatile secondary metabolites have been concerned.

The author's own experience in the impact of tissue culture treatments on the developmental patterns and morphological features *in vitro* and the subsequent modification of secondary metabolite production of species of the genera *Hypericum*, *Sideritis* and *Artemisia* have been summarized and discussed.

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1. Introduction

Growth and development are determined by the complex interrelations of the plant with its surrounding environment. Thus, the combined effects of climatic and geographic factors, as well as the plant phenology have been shown to strongly affect secondary metabolites productivity of medicinal and aromatic plants in nature. In addition, agro-technique has been shown to be non-less decisive, than the genotype for the productivity of these plants when being transferred from the natural habitats to the field.

The controlled environment of plant cell tissue and organ culture breeding makes plant biotechnology a flexible approach for targeting valuable phytopharmaceuticals through modification of growth conditions. The extent of differentiation/de-differentiation has been shown to play vital role in secondary metabolite levels in the different types of *in vitro* cultured cells and tissues. This phenomenon being determined by plant growth regulators treatments makes them one of the most widely utilized tools in modification of developmental patterns, morphogenesis and hence secondary metabolite production *in vitro*. The comprehension of the role of developmental and morphogenetic features for the biogenesis of plant secondary metabolites is essential for optimization of secondary metabolite productivity *in vitro*. In addition, identification of key factors responsible for the productivity of certain secondary metabolites in plant cell tissue and organ culture could be useful to also develop suitable agro-technique approaches for the enhanced productivity of these metabolites also on the farm.

2. Metabolite Production in the Plant Organism

2.1. Primary Plant Metabolites

Primary metabolites (such as carbohydrates, amino acids, fatty acids and organic acids, nucleotides, sugars and lipids) are widely distributed in the plant kingdom. They are produced as a result of primary cell metabolism and are vital for the plant survival (Figure 1). They are involved in processes such as growth and development, respiration and photosynthesis, as well as protein and hormone synthesis. Humankind has been using plant primary metabolites as food and raw materials since ancient times (Balandrin & Kloecke 1988; Hounsome et al. 2008).

2.2. Secondary Plant Metabolites

During its growth and development, the plant organism exists in a dynamic equilibrium with the numerous stimuli of its surrounding environment. Each ecosystem is

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characterized by numerous factors such as bacteria, viruses, fungi, nematodes, insects and herbivores. In addition, each habitat is characterized by its specific climatic factors and seasonal fluctuations, soil and water parameters, etc. Given their attached way of life, plants cannot directly avoid unfavorable impacts by simply changing their location at the moment of the unfavorable interaction. Although plants are capable of migrating, this process is complicated and time consuming, and occurs on the population level taking several seasons to be realized. In addition this process depends strongly on the physiological adaptability capacity of the individual plants constituting the population. Therefore the intermediate survival of the plant individual is bound to other mechanisms encompassing a number of complex biochemical adaptations which have evolved during evolution. Plants produce a wide array of chemical compounds which seemingly are not directly related to their growth and development (Figure 1). These compounds, designated secondary metabolites (natural compounds, biologically active compounds), do not have a direct widely accepted role in the processes of photosynthesis, respiration, mineral assimilation and transportation, differentiation, protein and lipids biosynthesis (Taiz and Zeiger 2006). A distinctive feature for secondary metabolites is their limited distribution in the plant kingdom. Often certain secondary metabolites are found only in certain species, genus or family, being a chemotaxonomic feature of this respective taxon. A vital parameter for secondary metabolite production and especially translocation and accumulation is the presence of the respective structural prerequisites, related to the morphological development of the plant.

2.2.1. Classification of Secondary Metabolites According to Their Functional Role for the Plant Organism

Secondary metabolites could functionally be classified as (Taiz & Zeiger 2006):

- *Attractants* – their function is to attract pollinating insects or animals whose biological role is to disperse plant diaspore.
- *Factors of chemical adaptation* towards the unfavorable environmental conditions – compounds with protective, repelling and warning functions.
- *Growth regulators and signaling molecules* – having in mind their vital role for synchronizing all physiological processes within the integral plant organism their functions are close to the ones of primary metabolites.

It is worth mentioning that unlike primary metabolites, plant secondary metabolites are "expensive to produce" for plant cellular energetics. Therefore in order to avoid the unnecessary waste of resources, plants have evolved complicated early signal systems, enabling them to distinguish between a purely mechanical damage and the attack of an insect/herbivore (Fürstenberg-Hägg et al. 2013). Thus, for example plants are able to

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evaluate the extent of damage, caused by herbivore feeding guilds, insect oral secretions, oviposition fluids, etc. These events lead to a succession of activities, leading to the production of certain metabolites: metabolic changes, followed by gene activation, jasmonic acid (JA) changes, kinase cascades, hydrogen peroxide production, cytosolic calcium ion fluxes, as well as membrane potential changes (Fürstenberg-Hägg et al. 2013 and references cited within).

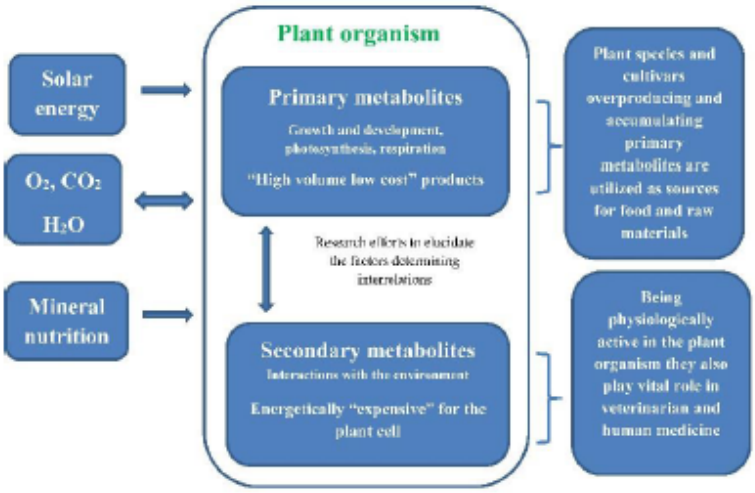

Figure 1. Schematic overview of primary and secondary plant metabolites.

Many of the models of biotic/abiotic stress response of the plants in their indigenous environment underline the experimental set-up of secondary metabolite elicitation of plant cell tissue and organ culture. The good knowledge and understanding of the physiological role of given secondary metabolite for the plant organism of interest is the key to its successful production by any breeding technique (i.e., by different types of cultivation techniques in soil, hydroponics, etc. as well as by the tools of plant biotechnology).

2.2.2. Main Biosynthetic Pathways and Regulation of Secondary Metabolism in the Plant Organism

The building blocks of secondary metabolites are derived from the primary metabolism (Figure 2). With the advancement of plant sciences, more light has been shed on the transcriptional regulation of secondary metabolites biosynthesis. It is now clear that the expression of activators and repressors comes as a complex response to phytohormones and different environmental signals, resulting in a dynamic regulatory network which determines the timing, amplitude and tissue specific expression of

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pathway genes and the subsequent accumulation of secondary metabolites (Patra et al. 2013). Transcription factor families such as MYC, MYB, WRKY and AP2/ERF have been identified to be responsible for secondary metabolite regulation and Jasmonic acid (JA) has been found to be an inducer in these processes (Afrin et al. 2015).

2.2.3. Main Classes of Secondary Metabolites and Their Production from the Cellular (Different Plant Organelles) to the "Supra-Cellular" (Secretory Glands, Trichromes, etc.) Level. Structural Prerequisites, Responsible for Their Production, Translocation and Accumulation

Secondary metabolites can be categorized into three major groups: *(i)* terpenes (Figure 3) *(ii)* phenolic compounds (Figure 4) and *(iii)* nitrogen-containing. The physiological roles of the respective compounds for the survival of the plant organism in its indigenous environment define also the pharmacological role which these substances play in the mammalian organism and hence their applicability in the veterinarian and humanitarian medicinal practices. Data from literature sources on the two first classes of compounds are summarized in Figures 3 and 4 (Gersbach 2002; Taiz and Zeiger 2006; Hassan and Mathesius 2012; Li et al. 2012; Gupta and Chakrabarty 2013; Agati et al. 2013; Singh and Sharma 2015).

The biogenesis, translocation, secretion and accumulation of the different secondary metabolites are bound to specific cellular organelles and require strictly the presence of the respective anatomic structures.

Plants respond by secondary metabolite production in dependence of the following factors:

- *Environmental impact*: water, nutritional elements, altitude, light, temperature, pathogens and herbivore pressure, etc. affect secondary metabolite production through affecting plant growth and development, morphology and primary metabolism (photosynthetic efficiency, carbon fixation and sugar accumulation) (Table 1).

Thus, herbivore attack signal is being perceived by undamaged tissues and low molecular messenger compounds are being released, followed by a cascade of defense reactions at the wounding site such as alteration of calcium ion fluxes, phosphorylation and systematic and jasmonate signaling. The bioactive compounds released as a result function to repel and intoxicate insects. The volatiles released repel herbivores, attract predators or serve for signaling between the different plant leaves and induce defense responses. Other morphological features as waxes, trichomes and lattices also serve to deter insects and herbivores (Fürstenberg-Hägg et al. 2013).

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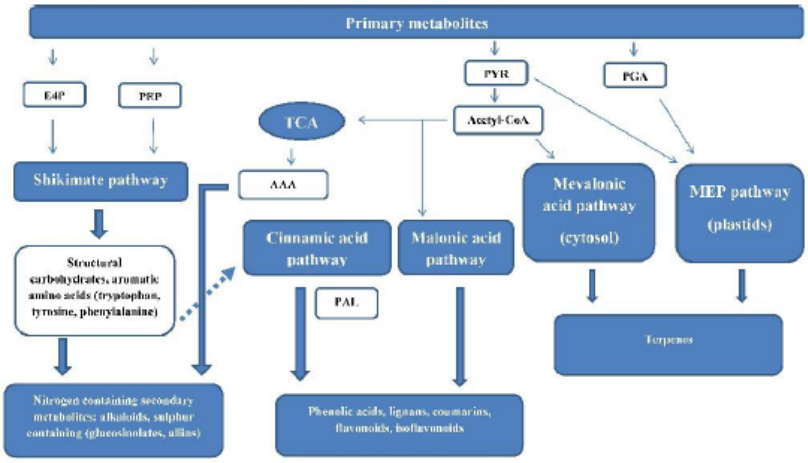

Figure 2. Schematic representation of the secondary metabolites biosynthetic pathways (Bajaj et al. 1998; Bouvier et al. 2000; Kasahara et al. 2004; Taiz and Zeiger 2006; Kougan et al. 2013). *PAL – Phenylalanine ammonia lyase (EC 4.3.1.24); E4P – erythrose-4-phosphate; PEP –phosphoenolpyruvate; PYR – pyruvate; PGA – 3-phospho-glycerate; Acetyl-CoA – acetyl coenzyme a; TCA – tricarboxylic acid cycle; AAA – aliphatic amino acids; MEP – 2-C-methyl-D-erythritol 4-phosphate.*

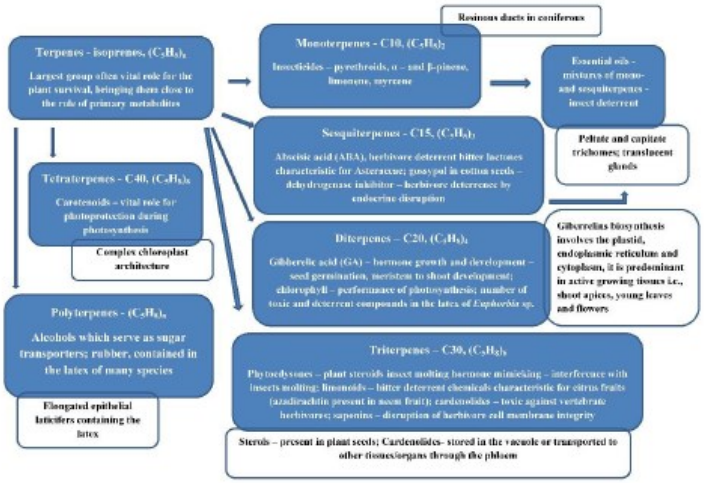

Figure 3. Schematic representation of some of the main roles of terpenes for the adaptive response and survival of the plant organism in its environment. Examples of anatomical structures for their biogenesis, accumulation secretion are denoted.

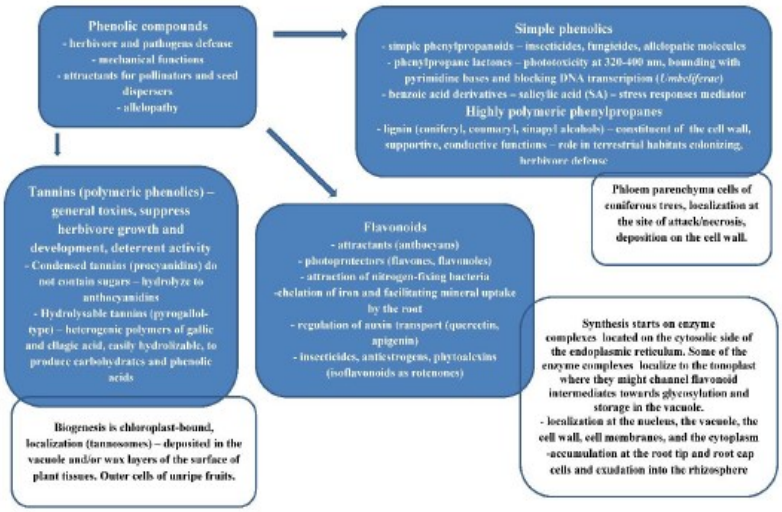

Figure 4. Schematic representation of some of the main roles of phenolics for the adaptive response and survival of the plant organism in its environment. Examples of anatomical structures for their biogenesis, accumulation and secretion are denoted.

Table 1. Effect of diverse environmental factors on the secondary metabolite levels in different species

Plant species	Secondary metabolites produced	Effect of environmental factors	Reference
Nitrogen containing compounds			
Papaver somniferum	Alkaloid levels	Morphine reaches maximum value of 1.1% in terminal and lateral capsules six weeks after fool bloom, then levels drop with 10% by the stage of dry harvest; in leaves the maximal 0.1% is at the same stage but drops by 50% at harvest.	Laughlin 1980
Pinus ponderosa *Pinus contorta*	2,6-disubstituted Piperidine alkaloids	Geographical dependence of alkaloid levels	Gerson and Kelsey 1998
Coffea arabica L. *C. canephora* P.	Caffeine and trigonelline of wild accessions (*C. arabica* - 38 genotypes originating from Ethiopia and Kenya and *C. canephora* - 38 genotypes originating from Côte d'Ivoire, Guinea, Congo, Cameroon and Central African Republic)	Higher trigonelline content for *C. arabica*, higher caffeine content and genetic diversity for *C. canephora* Both alkaloids content varied in *C. arabica* between the accessions	Ky et al. 2001
Conium maculatum	Piperidine alkaloids	Comparison of three locations with different infestation levels. Higher alkaloid production and less herbivore damage by the caterpillar *Agonopterix alstroemeriana* in locations with higher overall infestation levels.	Castells et al. 2005
Survey on the distribution of alkaloid-rich plant species in shortgrass steppe vegetation	Identification of alkaloid containing species	All alkaloid-positive taxa occurred in "water enriched" habitats, which composed less than 20% of the study area	Hazlett and Sawyer 2008
Catharanthus roseus	Vindoline, catharanthine and vinblastine	Highest vindoline and catharanthine in leaves; no vindoline in roots; highest vinblastine in blossoms; vindoline and catharanthine reach maximum between August and September; vinblastine reaches maximum after September; highest biomass in the beginning of September	Yu et al. 2010
Duboisia myoporoides	Alkaloid levels	69% higher in leaves harvested in February as compared with July or October	Ram et al. 2010
Camelia sinensis	L-theanine and caffeine	Effect of leaf age, bud and first two leaves, and shade levels: the content decreased as leaf age increased moving from bud to first and then second leaf	Song et al. 2012
Camellia sinensis	Theanine and theobromine	Highest levels in March, as compared with May and September	Fang et al. 2017
Thalictrum foliolosum	Berberine in root samples	Levels dropped with increasing the altitude	Pandey et al. 2017
Phenolic compounds			
Review on different plant species	Flavonoid compounds	Solar radiation and specifically UV-B wavelengths (280 – 320 nm) increase flavonoid levels	Caldwell et al. 1983 and references cited within

Table 1. (Continued)

Plant species	Secondary metabolites produced	Effect of environmental factors	Reference
Coffea arabica L. C. canephora P.	Chlorogenic acids of wild accessions (*C. arabica* - 38 genotypes originating from Ethiopia and Kenya and *C. canephora* - 38 genotypes originating from Côte d'Ivoire, Guinea, Congo, Cameroon and Central African Republic)	Higher chlorogenic acids levels and genetic diversity for *C. canephora*. Levels varied significantly between accessions, being dependent on the country of origin	Ky et al. 2001
Lycopersicon esculentum Mill.	Total phenolics and phenolic acids in the fruit	Comparison of effect of blocking UV solar radiation (290-400 nm). The studied metabolites were 20% higher in irradiated plants, as compared with plants which were protected from wavelengths < 380 nm.	Luthria et al. 2006
Pteridium arachnoideum	Phenolics and condensed tannins	High altitude related to higher secondary metabolite levels, attributed to sunlight exposure, elevation, and water restriction	Alonso-Amelot et al. 2007
Camellia sinensis	(-)-epigallocatechin gallate, (-)-epigallocatechin, (-)-epicatechin, and (-)-epicatechin gallate	Effect of leaf age, bud and first two leaves, and shade levels: the content increased from the bud to first and second leaves	Song et al. 2012
Camellia sinensis (L.) O. Kuntze	Proanthocyanins and O-glycosylated flavonols, phenolic acids, lignin	Shaded leaves: proanthocyanins and O-glycosylated flavonols decreased 53.37% and 43.26%, respectively; marked increase in phenolic acids and increase of lignin accumulation	Wang et al. 2012
Vitis vinifera L.	Flavonols, flavanols, phenolic acids,	Effect of UV-B radiation on specific signalling pathway in the skin of fruits leading to elevated flavonols, stilbenes, protocatechuic and p-coumaric acids; phenolic acids from the methanol-insoluble fraction, flavanols, and anthocyanins did not show any significant variation	Carbonell-Bejerano et al. 2014
Camellia sinensis	Phenolic acids and flavonoid content	Sampling in March, May and September. Higher afzelechin gallate, theogallin, kaempferol coumaroyl hexoside, abundance of kaempferol derivatives and lower epicatechin and catechin in March	Fang et al. 2017
Thalictrum foliolosum	Total phenolic and flavonoid contents of root samples	Levels increased at higher altitudes	Pandey et al. 2017
Camellia sinensis	Flavonoids and tannins	Sampling at different growth stages (from April to August). Flavonols and theaflavins increased gradually; gallotannins and flavan-3-ols decreased continuously depending on the growth stage	Ryu et al. 2017

Plant species	Secondary metabolites produced	Effect of environmental factors	Reference
Terpenes			
Artemisia alba Turra	*Artemisia alba* Turra	*Artemisia alba* Turra	*Artemisia alba* Turra
Artemisia alba Turra (*A. lobelia* All.)	*Artemisia alba* Turra (*A. lobelia* All.)	*Artemisia alba* Turra (*A. lobelia* All.)	*Artemisia alba* Turra (*A. lobelia* All.)
Artemisia alba Turra	*Artemisia alba* Turra	*Artemisia alba* Turra	*Artemisia alba* Turra
Artemisia alba Turra	Essential oil (EO) terpenoid profile	Sesquiterpenoid domination in the oils of samples from Montenegro, Ljevište	Jović et al. 2001
Artemisia alba Turra	Essential oil (EO) terpenoid profile	Monoterpenoid domination - generalization of data on majority of studied populations in different parts of Europe and grown in different natural environments	Radulović and Blagojević 2010 and ref. cited within
Artemisia alba Turra	Essential oil (EO) terpenoid profile	Monoterpenoid domination – samples from calcareous soils; sesquiterpenoid domination - Serpentinite (water and Ca deficient, supraoptimal Al, Fe, Mg, Ni, Co, Cr, low plant nutrition elements, pH basic to ultrabasic) – 4 times lower oil yields	Radulović and Blagojević 2010
Artemisia alba Turra	Essential oil (EO) terpenoid profile	Four Italian populations compared – in one of them – over 60% of the oil – sesquiterpenoid domination; in the other three – comparable levels of the mono- and sesquiterpenoids	Maggio et al. 2012
Lavandula angustifolia cv. Etherio	Essential oil (EO) profile	The EO content positively regulated by temperature and flowering stage development and negatively - by rainfall during flowering period. Rainfalls remarkably decreased linalool production, which was restored within 10 days period	Hassiotis et al. 2014
Origanum compactum Benth.	Essential oil profile	Two major and one intermediate type of populations according to their chemical composition: *(i)* high levels of thymol, *α*-terpineol, linalool, and carvacryl methyl oxide of samples growing in regions with humid climate, clayey, sandy, and alkaline soils; *(ii)* high levels of carvacrol, α-thujene, α-terpinene, and myrcene content in plants from semi-arid climate, and growing at high altitudes and silty soils; *(iii)* intermediate type populations exposed to sub-humid climate, appeared less homogeneous and belong mainly either to the first or second group	Aboukhalid et al. 2017

Table 2. Effect of diverse agro technique treatments on the secondary metabolite levels

Plant species	Secondary metabolites produced	Effect of agro technique treatments	Reference
Nitrogen containing compounds			
Papaver somniferum L.	Alkaloid production	Experiment of light and temperature – optimal growth at 1.6 x 10^4 lux light intensity and "low temperature" (daily rhythm of 12.5/7.5°C increasing to 18.5/11.5°C during vegetation); maximal dry weight at "low temperature" and higher lux intensity of 3.2 x 10^4 lux; "low temperature" hindered development by 10-15 days and modified the ratio of organs: proportion of leaves increased, and that of the stems diminished; highest alkaloid content at "high temperatures" (daily rhythm of 12.5/7.5°C reaching to 26.0/16.0°C) and at 3.2 x 10^4 lux illumination	Bernáth and Tétányi 1981
Papaver somniferum L.	Morphine	Morphine content in the capsules increased with the increasing supply of N from 0.85 to 1.01%	Lošák and Richter 2004
Lolium perenne L.	Pyrrolopyrazine alkaloid peramine, ergot alkaloid ergovaline and mycotoxin lolitrem B.	Endophyte-free plants did not produce peramine, ergovaline or lolitrem B. Both alkaloids declined with increasing N at ambient CO_2 but remained roughly constant across N levels at elevated CO_2	Hunt et al. 2005
Camellia sinensis	Caffeine	Nitrogen, potassium and micronutrients (Zinc and Copper) stimulated caffeine production	Sedaghathoor et al. 2009
Erythroxylum novogranatense var. *novogranatense*	Truxillines	Increased alkaloid levels with increased UV exposure	Lydon et al. 2009
Duboisia myoporoides	Alkaloid levels	1 m x 1 m spacing optimal for maximizing biomass harvest and alkaloid content	Ram et al. 2010
Papaver somniferum L.	Alkaloid content	Pre-anthesis water deficit caused a reduction in the biomass production without apparent reduction in capsule yield, which implies an increased harvest index for plots under water deficit	Mahdavi-Damghani et al. 2010
Spilanthes oleracea cv. Jambuarana	spilanthol	No significant differences of spilanthol when comparing organic and mineral (urea) fertilization	Borges et al. 2012
Withania somnifera Dunal.	Alkaloid levels	Highest alkaloid yield at 3000 ppm CCC (chlormequat chloride – GA biosynthesis inhibitor and growth retardant), at 30 x 20 cm planting distance (as compared with IAA and GA treatments and 30 x 30 cm plot).	Shukla and Shukla 2012
Colonization of *Claviceps purpurea* on winter rye (*Secale cereale* L.)	Ergot alkaloids	Application of gametocidal agents - maleic hydrazide and 2-chloroethylphosphonic acid to enhance *C. purpurea* infestation on unfertilized ovaries	Hanosová et al. 2015

Plant species	Secondary metabolites produced	Effect of agro technique treatments	Reference
Lupinus angustifolius	Sum of angustifoline, isolupanine, lupanine, and 13-hydroxylupanine	Alkaloid content was significantly influenced by the growing system, year and genotype. Higher N-content in the soil was related to higher concentrations of alkaloids; thus organically grown crop displayed lower productivity than the conventionally grown one	Jansen et al. 2015
Piper longum Linn.	Assay of total alkaloid content	"Protected cultivation system" experiments. Highest alkaloid production was recorded by planting in hanging pots and fertilization through mist	Jayanth et al. 2015
Ephedra sinica	Total alkaloids, ephedrine and pseudoephedrine	Achieved stabilization of high alkaloid levels by selective breeding and stolon propagation; selection criteria after 3 years of cultivation – total alkaloid content, high ephedrine/pseudoephedrine ratio, minimal variation between lowest and highest total alkaloid values, stolon formation capacity of the individuals	Hiyama et al. 2017
Phenolic compounds			
Geranium thunbergii Sieb. Et Zucc.	*Geranium thunbergii* Sieb. Et Zucc.	*Geranium thunbergii* Sieb. Et Zucc.	*Geranium thunbergii* Sieb. Et Zucc.
Camellia sinensis	*Camellia sinensis*	*Camellia sinensis*	*Camellia sinensis*
Vanilla planifolia A.	*Vanilla planifolia* A.	*Vanilla planifolia* A.	*Vanilla planifolia* A.
Brassica campestris L. ssp. *chinensis* var. *communis*	Flavonoids and hydroxycinnamic acids	Pre-harvest UV-B irradiation resulted in elevated hydroxycinnamic acid derivatives at low temperature (9°C) and of flavonoids at ambient temperature (22°C)	Harbaum-Piayda et al. 2010
Camellia sinensis	Optimal biomass formation and meeting market specifications of "Light leaf" and "Dark leaf" tea.	Production of hybride, biclonal and polyclonal seeds; spacing, pruning, mineral nutrition, shading, drainage, irrigation, pest management, soil rehabilitation (growing Guatemala grass, *Mimosa invisa*, Pusa Hybrid Napier for 18-24 months).	Barbora 2013
Lactuca sativa *(red leaf lettice)*	Cyanidin -3-O-(6"-O-malonyl)-glucoside and caffeoylmalic acid	Cultivation in greenhouse at low temperature stimulates anthocyanin biosynthesis	Becker et al. 2014
Calendula officinalis L.	Flavonoids	Blossom-mass and flavonoid levels related to the developmental stage - highest yield of flavonoids was obtained three days after anthesis	Honório et al. 2016
Lactuca sativa L. var. *crispa* L. cv. *Eventai* RZ and L. *sativa* L. var. *crispa* L., cv. *Satine* (Red Oak Leaf and Red Lollo lettuce)	Phenolic acids, flavonol and flavone glycosides, anthocyanidin glycosides	CO_2 enrichment resulted in high yields of red leaf lettuce rich in phenolic compounds (most flavonoid glycosides and only some caffeic acid derivatives)	Becker and Kläring, 2016

Table 2. (Continued)

Plant species	Secondary metabolites produced	Effect of agro technique treatments	Reference
Morus sp.	Tannins, phenolics	Elevated CO_2 increased biomass formation, primary metabolites (sugars, carbohydrates) and phenolic compounds	Lavanya et al. 2017
Lavandula angustifolia Mill.	Total phenolic and flavonoid content	Established optimal K fertilization for enhanced phenolic and flavonoid content	Chrysargyris et al. 2017
Terpenes			
Spilanthes oleracea cv. Jambuarana	*Spilanthes oleracea* cv. Jambuarana	*Spilanthes oleracea* cv. Jambuarana	*Spilanthes oleracea* cv. Jambuarana
Satureja hortensis L.	*Satureja hortensis* L.	*Satureja hortensis* L.	*Satureja hortensis* L.
Melissa officinalis	Citronellal, β-caryophyllene, neral, geranial and geranyl acetate	Vermicompost, biophosphate and chemical fertilizers treatments significantly increased the dominant component content	Mafakheri et al. 2016
Rosmarinus officinalis	Essential oil profile comparison based on 32 components	Vermicompost treatment stimulated phenolic compounds in the essential oil	Ganjali and Kaykhaii 2016
Lavandula angustifolia Mill.	Essential oil profile with main constituents 1,8-cineole, borneol, camphor, α-terpineol, myrtenal	Established optimal K fertilization levels for stimulated root growth and stimulated essential oil yield	Chrysargyris et al. 2017

Figure 5. Schematic representation of some biological activities and plant sources of the main groups of terpenes.

- *Phenology state* and the role of growth and development to fulfill the different roles in the plant's life cycle. The phonological stage is decisive on the secondary metabolites produced and has been studied for numerous species of economic importance (Çirak 2007 and references cited within);
- *Genotype and heredity* are also crucial for the production of secondary metabolites. Together with the selection of proper agrotechniques the selection of highly producing genotypes plays a central role for selection starting material for field cultivation (Nejadhabibvash et al. 2012; Kitzberger et al. 2013; Danova 2015; Fang et al. 2017);
- *The role of agrotechnique* for the production of secondary metabolites on the field. Domestication and introducing plant species from their wild habitats to field conditions is related to alteration of many factors which would have been responsible for the production of certain secondary metabolites in their indigenous environments (climatic challenges, soil chemistry, pressure of

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specific concrete insects and herbivores). Thus the correct agrotechnique measures are vital for the proper productivity of the crop (Table 2).

3. Application of Plant Derived Secondary Metabolites in Pharmaceutical Practice

Many biologically active compounds of plant origin find medical application as therapeutic molecules either in their indigenous form or as models for the total synthesis and also as matrices for the semi-synthesis of drugs. Examples for terpenes (Figure 5) and phenolics (Figure 6) are illustrative to the wide array of pharmacological activities of secondary metabolites for the human organism (Taiz and Zeiger 2006; Evans 2009).

According to the World Health Organization, about 80% of the World population still largely depends on medicinal plants as a traditional therapeutic method. In addition, up to now, 25% of the prescribed medicines and 50% of the "Over the counter drugs" in industrialized countries are of plant origin (Bajaj et al. 1988; Tripathi and Tripathi, 2003). In addition, about 52% of the anti-inflammatory and 51% of the anticancer drugs are also of plant origin (Arora 2010 and references cited within).

The wide application of secondary metabolites requires the possibility for their abundant delivery for the needs of the practice. Many compounds are currently synthetically produced (for example caffeine, theophylline, theobromine, ephedrine, pseudoephedrine, emetine, papaverine, L-dopa, salicylic acid). Nevertheless, due to their structural complexity, still many therapeutic molecules are being derived from the raw plant material (Bajaj et al. 1988).

Thus, obtaining raw plant material with desirable quality (proper biomass formation and stable and sufficient concentrations of the secondary metabolites) still holds a vital role for the supply of therapeutic molecules, vital for human health and survival.

These qualitative and quantitative characteristics are strongly dependent on the origin of the plant material. Often cultivation in the field of the target crop leads to poor results as compared to the characteristics of the material obtained from the wild habitat, the reason being the dependence of secondary metabolism on the interrelations of the plant organism with its surrounding environment, as discussed above. With its many applications and options, plant cell tissue and organ culture comes as an indispensable supplementary tool to conventional breeding techniques for meeting the needs of medicinal and aromatic plants raw material supply.

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Figure 6. Schematic representation of some biological activities and plant sources of the main groups of phenolics.

4. Principles of Plant Cell Tissue and Organ Culture Breeding

Plant biotechnology techniques are based on the possibility to cultivate separate cells, tissues, organs or integral plants in culture medium and sterile conditions out of the indigenous natural environments of the plants. This is possible due to the property "totipotency" of the plant cell and its capability to regenerate up to a whole integral organism.

The contemporary stage of development of plant cell tissue and organ cultures techniques has turned the method into a standard procedure technique for modern biotechnology with the following main areas of application (Loyola-Vargas and Vázquez-Flota 2006; Dias et al. 2016):

- Large-scale propagation of plant materials with superior (elite) quality;
- Generation of genetically modified fertile individuals;
- Model system for fundamental plant cell physiology aspects;

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- Preservation of endangered species,
- Metabolic engineering of fine chemicals.

4.1. The Plant Cell

The cell is the main building unit of the living plant organism, maintaining the integrity between structure and function (Teiz and Zeiger, 2006). There are several features distinguishing the plant from the animal cell:

- Green plants are the primary producers on the planet, due to their capability to assimilate solar energy and include it into macroergic compounds, during photosynthesis. Thus solar energy is being transformed into chemical energy. Hence are the structural and functional elements unique of the plant cell:
  - Presence of chloroplasts (the chlorophyll containing organelles where photosynthesis occurs);
  - Nucleus of the plant cell (responsible for encoding of enzymes and proteins, realizing the coordination of all processes taking place within the cell);
  - The plant cell membrane (allowing gas and water to pass in and out of the cell while controlling the passage of other molecules);
  - The vacuole (containing cell sap to keep the cell turgid, serving as storage cell compartment where many water soluble polar secondary metabolites are deposited);
  - Cell wall (lignin and pectin incorporated structure which serves for cell strengthening);
  - The cytoplasm (the spatial localization of the enzymes and other proteins used in photosynthesis, primary and secondary metabolites production);
- As already discussed, plants have an attached way of life. Therefore they have evolved complex mechanisms to direct their growth during their whole individual development towards vital resources as light, water and mineral components;
- Terrestrial plant species develop specific supporting structures, in order to accomplish biomass formation towards the sunlight and against the forces of gravity;
- The latter plants are also characterized by the constant loss of water through evaporation, and have therefore established specific mechanisms for prevention of drying;
- Plants are also capable of moving water and minerals from the soil towards the sites where photosynthesis and growth are accomplished, as well of transporting the photosynthetic products towards the non-photosynthesizing tissues and organs;

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- The plant cell membrane is surrounded and enforced by a hard cell wall, as neighboring cells are cemented by a pectin middle lamella. As a consequence of this, the development of the plant organism depends entirely on factors as cellular division and growth;
- Plant cell division is realized at specific spatially defined sites called "meristematic centers". They constitute the cell apparatus, in which the processes of cell division, growth and differentiation determine the size, form and structure of the whole plant organism. Neighboring to the sites of cell division are the sites of elongation in which the cells enlarge in width and length. After elongation, the cells undergo differentiation into a specific cell type. Except differentiated cells, however, during its whole life, the plant also produces non-differentiated meristematic cells, which are capable of the initiation of any type of cell, tissue or organ necessary for the plant's survival and development at any time of its individual development. This autonomous feature of the plant cell to store and express at any moment the whole genetic information of the whole organism is called "totipotency".

Plant cell tissue and organ culture is based on cell's totipotency and the possibility to regenerate the whole plant organism from a single cell.

4.2. *In Vitro* Culture Initiation

Sterile working conditions require the sterilization also of starting plant germplasm, which is used for the *in vitro* culture induction. Irrespectively of the starting material origin (collected from the wild or from controlled field/pot cultivation, seeds, above- or underground parts explants) it is vital to provide the maximal possible extent of environmental pathogens elimination without excessive harm being imposed on the vitality of the explants. This is usually achieved by surface sterilization. Initially the explant might be rinsed in up to 70% ethanol which provides on one hand an initial pre-sterilization, but also provides wetting of the explant, due to dissolving of the waxy layer. Then disinfection is performed by the aid of sodium hypochlorite, calcium hypochlorite, mercuric chloride, silver nitrate, hydrogen peroxide, benzalkonium chloride, etc. A detergent, for example Tween 20 or 80 could be added in order to increase the wetting of the explant by the sterilization agent. The choice of the sterilization agent, timing of sterilization, as well as the thorough rinsing with sterile distilled water of its traces are crucial for providing the vitality of the future development and normal morphogenesis of *in vitro* bred plants. After sterilization the explant is placed in medium suitable for either direct morphogenesis of differentiated organs (from existing meristematic centers) or callusogenesis or indirect morphogenesis of differentiated organs occurring through the

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initially induced callus tissue. The obtained cells or tissues are further on transferred to maintenance medium and the cultivation of the respectively obtained lines takes place.

4.3. Fundamental Aspects of Plant Biotechnology and Requirements for the Maintenance of Plant Cell Tissue and Organ Cultures

The plant cell and tissue cultures are a prospective additional tool and even sometimes an alternative in plant breeding and also in the yield of secondary metabolites in standardized conditions. Due to the long years of fundamental development and achievements, plant biotechnology offers a number of advantages as compared with the conventional plant breeding in the field (Petersen and Alfermann 1993; Murch et al. 2000; Alfermann et al. 2003; Arora 2010):

- Micropropagation and reintroduction into nature of rare, valuable or endangered species. The approach allows for the vegetative reproduction of plants which do not bear seeds, or their germination is difficult or slow;
- Due to the aseptic way of work the obtained plant material is pathogen-free, which makes it possible to quickly reproduce large quantities of clean planting material;
- The limitations on mass propagation of plant material, such as disease, pathogen invasion and unfavorable climatic conditions are being avoided;
- The rapid introduction and testing of new cultivars, selected by the methods of genetic engineering is made possible;
- There is a constant access to cultivated plant material, irrespectively of the fluctuations of the economic, climatic and geographic conditions, characteristic for the natural habitats of the plant species. The losses due to crop destruction by pathogens or herbivores are excluded;
- The standardization of the breeding conditions is related to standardization of the content of the active ingredients, and the yield of toxic/narcotic compounds is accomplished in a strictly controlled environment;
- The yield of secondary metabolites of rare or endangered species is conducted at laboratory conditions, without harming their natural habitats;
- The biotechnological breeding is flexible, and is easily adapted to the market requirements;
- In addition the technique is compact and allows for large scale breeding material preparation at the expense of comparatively small working space requirements as compared with the conventional plant nursery facilities;
- All the above factors contribute for the *in vitro* culture system of medicinal and aromatic plants to become a source of novel natural compounds;

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- The *in vitro* plant system is also a source of the isolation of a broad array of enzymes, which can be utilized in the conduction of positional- and stereo-specific chemical reactions, which would otherwise be complicated to be conducted;
- Plant *in vitro* systems are a valuable fundamental model for the elucidation of the biosynthesis of secondary metabolites. They are able to provide plant genetic material unified in its characteristics and in almost unlimited quantities. In addition, the metabolic activity in these conditions is high and for several weeks it is possible to achieve secondary metabolite biosynthesis, which in the natural conditions would require months or even more.

Generally plant cell tissue and organ culture is the cultivation of living plant tissue (with different differentiation degree), at constant environmental conditions and culture medium in which the culture is being developed. *In vitro* growth and development are in complex dependence of numerous factors such as:

4.3.1. Genotypic Prerequisites of the Plant

It is important whether the species is mono- or dicotyledonous, whether it is a gymnosperm or angiosperm, whether it is herbaceous or arboreal, seed setting or not. In medicinal plants cultivation, the susceptibility of the tissues towards the different techniques is also strongly dependent on the type of metabolites produced.

4.3.2. Factors of the Culture Medium. Approaches for Enhancement of Secondary Metabolite Production through Medium Optimization

- *Water constitutes* up to 95% of the culture medium, therefore its quality is of crucial importance. Plant tissue culture technique requires the utilization of distilled water, and protoplast and meristem tissue culture – bi-distilled water;
- *Solidification of the culture medium* is most often performed with agar (linear polygalactoside prepared from red algae (most often of the genera *Gelidium* and *Gracilaria*). Depending on the purposes agar concentration could vary from 0.6 – 0.8%. Agar concentration influences the availability and extent of assimilation of the medium components by the explant. It has been established that agar stimulates shikonin production in *Lithospermum erythror-hizon* suspensions (Fukui et al. 1983). It was further on established that agar interacts with the mineral salts in the medium, thus affecting their availability and up-take capacity by the plant cells (Gribble et al. 2002);
- *Sugar* is exogenously supplemented in the culture medium due to the lower effectiveness and sometimes complete lack of photosynthesis and carbon fixation in *in vitro* conditions. However, *in vitro* photosynthesis has been established for

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certain species (Pospišilová et al. 1997). The degree of plant heterotrophy (full dependence on exogenously supplied sugar), mixotrophy (the capability of partial carbon fixation) and even autotrophy (effective *in vitro* photosynthesis and "autonomous nutrition") do not only depend on the photosynthetic capacity of the concrete species but also on the culture medium composition, volume and aperture of the cultivation vessels and on their material and closure (Fujiwara et al. 1989; Kozai 1991; Kozai & Smith 1995; Kozai et al. 2002). According to Lucchesini et al. (2009) at optimal "mixotrophic conditions", *in vitro* shoots could perform photosynthesis simultaneously with the assimilation of exogenously applied sucrose in the culture medium. Therefore the modernization and scale-up of micropropagation techniques often utilize the methods of autotrophic culture in which the plant independently performs photosynthesis. Sugar concentration can vary significantly (1 – 6%), as the modification of this factor is widely utilized in plant biotechnology. For sugars supplementation most often sucrose is used, but some monosaccharides have also been applied (for example glucose and mannitol). Noteworthy, the quantity of the given monosaccharide, equivalent to the respective disaccharide, reduces in double its osmotic potential and hampers water utilization by the explant;

- *The main macro-elements* which are mandatory in the formulation of the culture medium are most often nitrogen, phosphorus, potassium, calcium, magnesium and sulphur;
- *Micro-elements* being supplied are iron, zinc, boron, manganese, copper, cobalt, nickel, aluminum, molybdenum, iodine. The macro- and micro-elements are being supplied in the form of soluble salts, and iron is usually chelated with EDTA in order to avoid its precipitation;
- *Growth regulators (plant hormones)* are vital endogenous factors of growth and development of the plant organism. The best studied groups of hormones are the auxins, gibberellins, cytokinins, ethylene and abscisic acid. The last years led to widening the knowledge on the steroid plant hormones brassinosteroids, which regulate diverse processes of differentiation in the plant organism. A number of other molecules are also related to the herbivore and pathogen resistance, such as jasmonic and salicylic acids (Taiz & Zeiger 2006). With the elaboration of molecular analytical methods in plant physiology, the number of hormones and hormone-like signal molecules is increasing. The exogenous supplementation of plant growth regulators is crucial for targeting, and maintenance of the degree of de-differentiation, micropropagation and rooting, as well as influence of *in vitro* secondary metabolites production. Cytokinins and auxins are usually applied at concentrations 0.01 - 0.5 - 5.0 mg/l. The effect of different plant growth regulators (PGR) on secondary metabolite production has been widely explored in plant cell, tissue and organ culture. A number of literature sources discuss on

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the inhibitory activity of 2,4-D on secondary metabolite production in non-differentiated cell cultures such as berberine (Nakagawa et al. 1986), antraquinones (Zenk et al. 1975) and indole alkaloids (Zenk et al. 1977a). In *Morinda citrifolia* cell cultures, low auxin concentrations led to high anthraquinone levels and low growth rate, high auxin concentrations have the reverse effect; 2,4-D was active at approximately a hundred-fold lower concentration than NAA (Hagendoorn et al. 1997). This pattern of inhibitory activity of 2,4-D on several types of secondary metabolites in cell cultures of diverse species, might be attributed to the larger degree of de-differentiation brought by this hormone. Therefore, auxins utilized for callusogenesis are either applied at low concentrations or are being removed from the medium during optimization of secondary metabolites production *in vitro* (Misawa 1994). The alteration of two-stage cultivation has been often applied with the purpose of firstly obtaining intensive biomass formation and then – production of secondary metabolites by reducing the growth rate of the cultures. Examples are the production of shikonin in cell cultures of *Lithospermum erythrorhizon* (Tabata & Fujita 1985), rosmarinic acid by cell culture of *Colleus blumei* (Ulbrich et al. 1985), berberine from cell cultures of *Coptis japonica* (Fujiata & Tabata 1987). The latter examples are illustrative of the stimulation of de-differentiation and inhibitory effect of auxins on secondary metabolites production by non-differentiated cultures. The opposite effect of the same plant growth regulator however has been observed in the differentiated genetically modified "hairy roots" of a number of medicinal plant species. Thus, auxin supplementation (IBA, IAA and NAA) in the culture medium of *Rubia akane* stimulated both growth and alizarin and purpurine production (Park & Lee 2009). Similar results were obtained in lobeline production in *Lobelia inflata* (Yonemitsu et al. 1990), valepotriates in *Centranthus rubber* (Granicher et al. 1995), rutin in *Fagopyrum esculentum* (Lee et al. 2007) and withanolide A in *Withania somnifera* (Murthy et al. 2008). In addition to this finding, in hairy root cultures of *Cichorium intybus* L. cv. Lucknow the combination of even low concentrations of cutokinin (kinetin) in combination with auxin led to de-diffreniation, callus formation and subsequent drop of coumarin content in the cultures (Bais et al. 2001).

- *Amino acids and inositol* – organic compounds such as aminoacids (for example glycine) and the polyol inositol are also exogenously applied to the culture medium;
- *The pH value* of the medium is an important factor for the proper conduction of the physiological processes of the *in vitro* grown plant. Usually pH values vary within the interval 5.5 – 6.0;
- *Other components of the culture medium* – activated charcoal being finely porous carbon material finds wide array of applications in tissue culture. Its effects may

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be attributed to establishing a darkened environment for the root; adsorption of undesirable/inhibitory substances; adsorption of growth regulators and other organic compounds, or the slow release of growth promoting substances present in or adsorbed by activated charcoal (Pan and van Staden 1998);

The most broadly used culture medium formulations in plant biotechnology are the ones of Murashige and Skoog (1962), White (1963), Linsmaier and Skoog (1965) and Gamborg (1968).

When discussing the issue of media optimization of secondary metabolite production it is vital to consider that growth and biomass formation are often not related and in many cases have different requirements. As discussed above, secondary metabolites production is related to the interactions of the plant organism with its environment (Harborne 1996). Therefore, the successful application of the methods of plant biotechnology for stimulation of secondary metabolism, the knowledge of the physiological role of the concrete secondary compounds for the plant physiology is of major importance. Thus, in literature there are examples of enhancement of secondary metabolite production achieved by limitation of nutritive resources in the medium (most often decreasing the content of nitrates, potassium, ammonium and phosphates salts results in secondary metabolites stimulation *in vitro*) (Petersen and Alfermann 1993). In this way by providing all other optimal breeding conditions and limiting growth resources, it is possible to stimulate secondary metabolism at the expense of primary metabolism.

4.3.3. Factors of Culture Conditions

- *Sterility* is a major principle for all *in vitro* culture manipulations. This is due to the application in the culture medium of a wide array of components which stimulate biogenic growth and development not only of the plant organism but also of different pathogens like bacteria, fungi and mould. Therefore plant biotechnology is a method for the aseptic cultivation of plant material, which is accomplished by the sterilization of culture media, vessels and instruments. All manipulations are carried out in sterile environment. This is most often achieved by autoclaving at 121°C (1 atm) of consumables, instruments and culture media and work under a constant flow of sterile air in the laminar cabinet. Thermo-degradable components are supplemented to the media by sterile filtration (pore size 0.45µm);
- *Cultivation space* – it is needed to provide a controllable temperature regime. Usually the temperature values are set between 24 - 26°C but this parameter might vary considerably according to the purposes of the cultivation. Thus cold treatment might be applied for the purpose of hardening of explants and higher temperature – for Heat Shock Proteins production and inducing adaptation

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reaction towards diverse types of stress. Modification of the temperature regime is a factor in biosynthesis of both primary and secondary metabolites. Thus, the total content of fatty acids is being increased in reducing temperatures in *Catharanthus roseus* suspensions (due to the rise of unsaturated C18 fatty acids), as this effect does not influence indole alkaloids content (Toivonen et al. 1992). Increasing temperatures up to 28°C and 30°C in *Lavandula vera* cell suspensions, has shown the achievement of maximal biomass and rosmarinic acid, respectively (Georgiev et al. 2004). According to the research of the same working group, aeration and agitation in bioreactor conditions stimulate rosmarinic acid production as well (Pavlov et al. 2005);

- *Illumination* is provided by luminescent tubes, whose spectral characteristics, intensity and photoperiod are selected according to the plant species requirements, but also can be a valuable experimental tool according to the culture type and results which are targeted;
- *Humidity, oxygen and carbon dioxide content* depend largely on the microclimate inside of the culture vessels (related to their volume and shape, the aperture diameter and closure type). It is important to also notice that the oxygen which is dissolved in a liquid medium is to a higher extent available for the explant, as compared to the one in agar solidified medium. In addition, gaseous supply is largely controllable in bioreactor systems and is a widely used variable in targeting secondary metabolite production *in vitro*.

4.4. Role of Differentiation and Morphogenesis in Plant Biotechnology. Main Types of *In Vitro* Cultures

The plant organism consists of different organs, which are constituted of different tissues, build up by cells. The vegetative body of vascular plants consists of three main organs: leaves (with its primary vital function of performing photosynthesis), stem (having supportive and translocation functions) and roots (having supportive and assimilation functions). According to the degree of organization of cultured cells *in vitro* cultures can be classified into differentiated and non-differentiated types (De Fossard 1977; Pierik 1987; Razdan 2003).

However, secondary metabolites production most often requires a certain differentiation level of plant tissues and is often lacking in non-differentiated cultures. Therefore secondary metabolite production in non-differentiated cultures often requires additional measures for optimization the production of secondary metabolites which are often empirical. Examples for such approaches will be discussed below.

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4.5. Differentiated Cultures. Role of Differentiation in Successful *In Vitro* Yield of Secondary Metabolites

Differentiated cultures are characterized by a diverse degree of organization and communication between the cells, tissues and organs. These are:

- *Cultures of integral sterile plant.* Such cultures can be obtained by directly germinating seeds in sterile conditions, or through morphological differentiation of organs from non-differentiated culture types. They have the highest level of morphological development and communication between the three organ types. In these cultures it is mostly achievable to obtain the anatomical structures needed for production, translocation and accumulation of secondary metabolites;
- *Embryo cultures* are integral whole plants which are obtained by discarding the seed covering, excising of the embryo and growth of the integral plant from it. This approach is applied in cases when for diverse reasons rescue of the fertilized material is required, for example in the case of seeds which have hard covering which hampers the germination process;
- *Organ cultures* are separate plant organs grown in culture medium such as shoot cultures (aerial parts), root cultures, etc. The "hairy roots" are a specific genetically modified root culture type, where genetic material has been transferred to the plant genome from the Ri plasmid of *Agrobacterium rhizogenes* through inoculation of a wounded segment on the plant body and the subsequent infection of the plant with the bacterium. The inserted genes encode proteins which participate in the biosynthesis of hormones stimulating the excessive cell multiplication at the site of the infection. The obtained cell mass produces opines, which serve as nutritive substrate for the plants. This phenomenon is a creation of a biological niche by the bacterium which enables it to develop and reproduce. After the integration and expression of the genetic information, the "hairy root" phenotype can be observed – which is manifested as increased branching and negative gravitropism of the roots. Over 450 species have been reported to be susceptible to the Gr (-) *Agrobacterium rhizogenes* infection. The experimental application of "hairy root" cultures dates from 1980. The main advantage of these cultures is that they are "hormonally autonomous" and can maintain the state of root morphogenesis without formation of aerial parts and exogenous supplementation of growth regulators to the medium (Pistelli et al. 2010).

There are many examples of species being cultivated *in vitro* as differentiated cultures, such as conventional (Becker and Chavadej 1988; Maurmann et al. 2006) and transformed (Gränicher et al. 1992; Gränicher et al. 1995) roots of *Valeriana officinalis*; differentiated shoots of *Hypericum perforatum* (Kirakosyan et al. 2004).

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Noteworthy, the development of transformed "hairy roots" often allows for the production of secondary metabolites characteristic for the aerial parts of the respective plants. Thus, artemisinin, isolated of the aerial parts of the field grown *Artemisia annua*, has been successfully yielded from genetically transformed roots of the plant (Weathers et al. 1994; Jaziri et al. 1995; Liu et al. 1999). Another example is the production of the 2-OH-1,4-naphtoquinone lawsone by genetically transformed roots of *Lawsonia inermis* (Bakkali et al., 1997). In addition, important advantages of "hairy roots" are their genetic stability, hormonal autonomy and the relatively constant levels of secondary metabolites produced.

Sometimes, however the successful scale-up of differentiated cultures to bioreactor scale might be difficult due to problems arising from the necessity to supply unified amounts of aeration and nutritive compounds, as well as illumination to a non-homogenous biomass.

4.6. Non-Differentiated Cultures. Main Principles and Approaches to Enhance Secondary Metabolite Production

In this culture type the plant cells are not organized into tissues and organs.

4.6.1. Protoplast Cultures are Obtained by the Enzymatic Degrading of the Cell Wall and the Subsequent Maintenance of Plant Cells Which are Only Surrounded by the Cytoplasmic Membrane

The fusion of protoplasts of different species is a widely utilized approach for obtaining interspecies hybrids.

4.6.2. Callus Cultures are Aggregates of Non-Differentiated Plant Cells Cultivated on Solid Medium

They are obtained through inducing de-differentiation of differentiated plant tissue by means of plant growth regulators treatment of diverse explant types. The balance between cytokinins and auxins is decisive for maintenance of the plant culture in the non-differentiated state. As discussed above, the degree of de-differentiation and "multiplication speed" of the culture are usually inversely related to the secondary metabolites produced by it.

4.6.3. Suspension Cultures are Cells or Actually Cell Aggregates with Different Extent of Aggregation, Cultivated in Liquid Culture Medium

In order to provide a unified nutritive compounds and oxygen supply to the whole cultivated biomass, agitation of culture vessels, usually on an orbital shaker, at 30 – 150

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rpm is applied. Depending on the cultivation method plant suspensions can be classified into:

- *Batch cultures* are maintained through a regular sub-culture of a small aliquot part of the generated biomass into fresh culture medium after a defined period of time. Biomass formation has the following sequence: *lag phase* immediately after transfer into the fresh medium – no new growth is occurring yet; *exponential phase* after the adaptation and overcoming the stress of sub-culture manipulations, a period of intensive cell growth is observed; *stationary phase* after 3 – 4 consecutive cell divisions, the nutritional resources are being exhausted and toxic by-products of cellular metabolism are being accumulated, growth slows down and cellular density remains constant. If no sub-culture occurs at this stage, the cell number gradually starts decreasing and the suspension line cannot continue its existence. These suspensions are usually maintained in 100 – 250 ml Erlenmeyer flasks containing 20 – 75 ml of liquid culture medium;
- *Continuous cultures* - this technique provides for the long-term cultivation through the constant supply of fresh medium into the system. Therefore cultures are grown in special vessels, which would allow for the sterile supply of fresh medium and discarding of the old one. These cultures might be of closed type when only the exhausted medium is discarded from the system, but the cells remain and increase their density with a point when saturation is achieved. The open type continuous cultures are characterized with drawing out equal volumes of nutrition medium and cells. The latter line is capable of prolonged maintenance.

4.6.4. Main Practical Problems in Utilization of Non-Differentiated Cultures for Secondary Metabolites Production

Although callus and suspension cultures are characterized by a faster reproduction and biomass formation, as compared with differentiated cultures, practical problems arise which are being solved in dependence of the genotypic peculiarities of each concrete species, being developed.

- *Lack of synchronization of growth and development* - growth and cell division of the many cells/cellular aggregates in the vessel are usually not synchronous in these types of cultures. Therefore a biomass of cell units with different age, instability of enzymatic processes and hence instability of secondary metabolites production is observed. This problem is solved by measures for synchronization through different physical and chemical methods.

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- *Genetic instability* – this is another major problem due to the intensively ongoing cell division. Therefore, plant organs or whole plants differentiated and regenerated of non-differentiated plant tissues (indirect morphogenesis) are mostly likely to differ from the parent plants. Desirable or non-desirable traits could be obtained in this way. Selection, identification, isolation and clonal propagation of cultivars with desirable qualities are often applied.
- *Lack of specialized anatomic and morphologic structures* needed for secondary metabolite production, translocation and accumulation. In spite of the many advantages of non-differentiated *in vitro* cultures (such as the rapid growth, the numerous possibilities for optimization of secondary metabolite production), there still remains the main drawback, namely the lack of differentiation. Although the plant cell is characterized by its totipotency, the biosynthesis of many secondary metabolites still requires a certain level of differentiation of the plant tissue. The development of differentiated cultures is a solution when the production of secondary metabolites cannot be realized in the non-differentiated tissues. Thus, it has been established that although the non-differentiated culture displayed a lack of certain secondary metabolites, the induction of somatic embryogenesis, root or aerial parts led to the regaining of the biosynthetic activity of the plant tissues (Charlwood et al. 1990). Another example is the finding that although alkaloids were only present in traces in the suspension of *Leucojum aestivum*, differentiation and subsequent aerial parts induction led to a significant increase of alkaloid content (Pavlov et al. 2007). The lines selected in the latter work produced over 200µg/gDW in liquid medium and displayed stability regarding growth and secondary metabolites production in the liquid system.

Thus plant cell tissue and organ culture systems represent a prospective complementary tool to conventional breeding techniques for the delivery of desirable secondary metabolites. In order to successfully utilize the potential of this valuable technique, however it is necessary to be able to interpret the physiological behavior of the plant as a result of the different optimization measures and relate it to the resulting increased or decreased levels of metabolites produced. There are many examples of the successful delivery of plant secondary metabolites through plant cell tissue and organ cultures, where the productivity has surpassed the levels obtained for the source "mother" plant (Table 3).

Analysis of the plant response to biotic and abiotic stress is an important prerequisite in the development of optimization strategies for secondary metabolites production *in vitro* (Broeckling et al. 2005). Elicitation for example is one of the main approaches as far as phytoalexines production is targeted. Thus, medium optimization, screening and

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selection could lead to 20-30 times higher levels of the secondary metabolites produced *in vitro* (Verpoorte et al. 2002).

- *Selection of highly productive lines* could be performed even by the naked eye when pigmented compounds are targeted such as the examples with anthocyanins, shikonins or berberine. In other cases the application of analytical methods to monitor the produced raw plant material is necessary. The utilization of different selective methods has led to the achievement of highly productive lines with significant improvement of secondary metabolite production (Table 4).
- *Impact of stress factors* – according to Brodelius (1988) stress factors exogenously applied on the plant cell cultures could be classified in the following categories:
  - *Culture medium parameters* – such factors include carbon, nitrogen, phosphate sources and plant growth regulators;
  - *Physical stress* – extreme light treatment, ultraviolet light, aeration, medium osmolarity and pH. Thus, the application of electric impulses has led to the intercellular accumulation of taxuyunnanine C (Tc) in *Taxus chinensis* suspension (Hong et al. 2004). The phenomenon has been explained by the induction of defense response and possible alteration of the dielectric properties of the cell membrane;
  - *Chemical stress* – heavy metals, abiotic elicitors, etc. Cultivation at Fe^{2+} stress has been reported to induce oxidative stress, lipid peroxidation and cell death related to the increased levels of ethylene and β-thujaplicin production in *Cupressus lusitanica* suspension (Zhao et al. 2005);
  - *Biotic stress* – infection with pathogens/biotic elicitors. In order to be protected from pathogenic microorganisms and herbivores, plants have evolved a complicated inducible chemical defense system. Plants are capable, as a response of external pathogenic stimuli to react by the *de novo* synthesis of certain compounds designated as phytoalexins. The process is called elicitation. Being protective compounds against exogenously arising stimuli, phytoalexins can also be artificially induced by exogenous treatment of *in vitro* tissues by the controlled application of elicitation stress factors. The rapidness of the response can vary from several hours, to several days and often even excretion of the compounds into the medium can be observed (Petersen and Alfermann 1993). Numerous plant species, grown as cell suspensions have shown positive results regarding secondary metabolites elicitation by the exogenous application of methyl jasmonate. Its application initiates the *de novo* transcription of genes, encoding the expression of phenylalanine ammonia lyase, related to the chemical defense protective mechanisms of the plant cell (Gundlach et al. 1992). In literature there are

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numerous reports concerning the successful secondary metabolites stimulation *in vitro* as a result of exogenous methyl jasmonate treatments. Such examples are the production of rosmarinic acid in *Lavandula vera* suspension (Georgiev et al. 2007);

- *Application of biosynthetic precursors and biotransformation* – irrespectively of the level of enzyme expression at a given moment, the plant cell possesses the whole genetic information for the full range of all functioning enzymatic systems. Therefore, it is possible to feed cheaper lower molecular precursors of valuable target metabolites, and enhance their yields *in vitro*. One of the first such experiments have been performed by the working group of Zenk who fed tryptophan to *Catharanthus roseus* suspensions (Zenk et al. 1977a) and phenylalanine to *Coleus blumei* suspensions (Zenk et al. 1977 b). The rosmarinic acid production was increased as a result. Plant cell cultures are a valuable source of enzymes, which are readily available in them and much easier to access as compared of fresh plant tissues of different species derived from the field. Extracted enzymes can further on included into various biochemical reactions outside of the plant organism. This makes it possible to complete perform biosynthetic experiments and biotransformation in laboratory conditions (Kutney 1997).

Table 3. Comparative yield of some secondary metabolites of *in vitro* cultures with field samples of the respective plant species

Compound	Plant species/ *In vitro* system	Yield (% of the dry weight)		*In vitro*/ Field content ratio	Reference
		In vitro	Field samples		
Non-differentiated cultures					
Nitrogen containing compounds					
Bisoclaurine	*Stephania cepharantha* Suspension	2.3	0.8	2.9	Akasu et al. 1976
Ajmalicine	*Catharanthus roseus* Suspension	1.0	0.3	3.3	Misawa 1994
Benzylisoquinoline alkaloids	*Coptis japonica* Suspension	11	5 - 10	2.2 - 1.1	Misawa 1994
Berberine	*Thalictrum minor* Suspension	10	0.01	1000	Misawa 1994
Berberine	*Coptis japonica* Suspension	10	2 - 4	5 - 2.5	Misawa 1994
Nicotine	*Nicotiana tabacum* Suspension	3.4	2.0	1.7	Misawa 1994
Caffeine	*Coffea arabica* Suspension	1.6	1.6	1	Govil et al. 2017
Serotonin	*Peganum harmala* Suspension	2	2	1	Govil et al. 2017
Trigonelline	*Trigonella foenum-graecum* Suspension	5	0.4	12.5	Govil et al. 2017
Trigonelline	*Trigonella wilfordii* Suspension	0.2	0.01	20	Govil et al. 2017

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Table 3. (Continued)

Compound	Plant species/ *In vitro* system	Yield (% of the dry weight)		*In vitro*/ Field content ratio	Reference
		In vitro	Field samples		
Serpentine	*Catharanthus roseus* suspension	2	0.26	7.7	Govil et al. 2017
Vomilenien	*Rauwolfia serpentina* Suspension	0.214	0.004	53.5	Govil et al. 2017
Phenolics					
Shikonin	*Lithospermum erythrorhizon* Suspension	20	1.5	13.3	Misawa 1994
Antraquinones	*Morinda citrifolia Suspension*	18	0.3	60	Misawa 1994
Antraquinones	*Galium verum* Suspension	5.4	1.2	4.5	Misawa 1994
Antraquinones	*Galium aparine* Suspension	3.8	0.2	19	Misawa 1994
Rosmarinic acid	*Coleus blumeii* Suspension	15	3	5	Misawa 1994
Ubiquinone - 10	*Nicotiana tabacum* Suspension	0.036	0.003	12	Misawa 1994
Anthocyanins	*Vitis* sp. Cell culture	16	10	1.6	Zhong 2001
Anthocyanins	*Euphorbia milli* Cell culture	4	0.3	13.3	Zhong 2001
Anthocyanins	*Perilla frutescens* Cell culture	24	1.5	16	Zhong 2001
Rosmarinic acid	*Coleus blumei* Cell culture	15	3	5	Misawa 1994
Isoflavonoids daidzein, retuzin, genistein and formononetin and the pterocarpans maakiain and medicarpin	*Maackia amurensis* Callus	2.08	0.52	4	Fedoreyev et al. 2004
Shikimic acid	*Galium mollugo* Suspension	10	2.3	4.3	Govil et al. 2017
Terpenoids					
Ginsenosides	*Panax ginseng* Callus	27	4.5	6	Misawa 1994
Triptolide	*Tripteryguium wilfordii* Suspension	0.05	0.001	50	Misawa 1994
Diosgenin	*Dioscorea deltoides* Suspension	7.8	2.4	3.3	Govil et al. 2017
Differentiated cultures					
Camptothecin	*Camptotheca acuminate* roots/hairy roots of *Ophiorrhiza pumila*	0.1	0.02 – 0.5	5 - 0.2	Khani et al. 2012
Podophyllotoxin	*Podophyllum peltatum* Callus derived adventitious roots, transferred onto PGR-free MS medium	1.6	0.2	8	Sakata et al. 1990
Hypericin (Hyp), pseudohypericin (psHyp)	*Hypericum rumeliacum* Shoot cultures	Hyp – 0.03 psHyp – 0.12	Hyp – 0.01 psHyp – 0.01	Hyp – 3 psHyp – 12	Danova et al. 2012a

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5. Research Evidence on the Successful Bioreactor Scale-Up of Secondary Metabolites

As discussed above the successful biotechnological yield of bioactive secondary metabolites requires the good knowledge and understanding of the biochemical and physiological aspects of plant growth and development related to secondary metabolism.

Scale-up of secondary metabolites production to bioreactor presumes the stabilization of the laboratory-scale processes and the gradual reaching larger, up to industrial-scale volumes similarly to microbial cultures. Usually scale-up begins in a 250 – 300 ml Erlenmeyer flask in laboratory conditions, goes through volume of up to 1 liter and afterwards the cultured line can to be adapted to 30 – 150 – 1000 l bioreactor. Unlike bacterial cell lines, the plant cells have certain specificities like for example their slower growth, larger cell size, tendency to form cell aggregates, as well as their sensitivity towards agitation and stirring. Therefore special adaptation of microorganism bioreactor processes is needed, as well as search for novel ideas in the tailoring of bioreactor for plant cells. The plant bioreactor processes could be compared to the above described "continued cultures" of suspension lines (Razdan 2003).

5.1. Scale-Up of Non-Differentiated Cell Cultures to Bioreactor

The non-differentiated cell/suspension cultures are considered as the most prospective source for the scaled-up yield of secondary metabolites (Georgiev et al. 2009). One of their biggest advantages is the fast growth and rapid biomass accumulation as compared with the differentiated cultures. As discussed above, the non-differentiated tissue is easier to scale-up to larger volumes and to bioreactor conditions, due to the possibility to provide uniform aeration and nutrition to the cell aggregates than to tissue mass of different differentiation types. As extensively reviewed by Bajaj et al. (1988) great success has been already achieved in the scale-up of secondary metabolite production of non-differentiated plant cell cultures in bioreactors (serpentine production by *Catharanthus roseus* in 85 l; rosmarinic acid – by *Coleus blumei* in 450 l; shikonin derivatives by *Lithospermun erythrorhizon* in 750 l; plant biomass by *Nicotiana tabacum* in 20 000 l; saponins by *Panax ginseng* in 2 000 l; polysaccharides by *Echinacea purpurea* and *Echinacea angustifolia* in 75 000 l).

5.2. Scale-Up of Differentiated Cell Cultures to Bioreactor

The differentiated state of the tissues in these systems is related to the better cell-to-cell communication, presence of secretory and translocation anatomic structures.

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Table 4. Selection strategies of highly productive lines based on modification of morphogenesis and growth and development patterns of the *in vitro* cultured plants

Plant species	Type of *in vitro* culture	Optimization measures/ Selection agents	Obtained results	References
Selection of explant type as starting material for *in vitro* lines selection				
Rauvolfia tetraphylla L.	Callus	Utilization of different explant types for callus induction	Leaf explants – 90% callus induction response, cotyledon – 64%, hypocotyl – 60%, node and internode – 50%, petiole – 46%, root – 33%; leaf derived callus expressed highest levels of reserpine production	Anitha and Kumari 2013
Utilization of the coloration of the target compounds as selection criteria				
Euphorbia milli	Cell culture	Clonal selection (cell aggregates cloning) and 24-times consecutive subcultures of cells with intensive coloration	Selected line with over seven fold increase of anthocyanins content	Yamamoto et al. 1982
Muscadinia ssp. (muscadine grape), *Vitis aestivalis* var. *Cynthiana*	Cell culture	Choice of variety, selection of appropriate explant, reduction of browning by activated charcoal or Amberlite XAD4, repeated selection and sub-culture of colored clusters	Anthocyanins production, mainly anthocyanidin-3,5-O-diglucosides	Ananga et al. 2013
Induction of morphogenesis and establishment of higher levels of differentiation of the cell lines				
Coptis japonica	Cell culture	Continuous selection of cell aggregates	Lines with rapid growth and enhanced berberine production; 14 l bioreactor scale-up obtained; stability of metabolite levels for over 27 generations	Yamada & Sato 1981
Hypericum perforatum	Suspension	Selection of globular berries-like structured cell aggregates	Hypericin production exceeding the one of the differentiated shoots and callus of the plant	Vardapetyan et al. 2000
Application of chemical selection factors				
Lavandula vera	Suspension	Pimelic acid supplementation, gamma ray irradiation	Biotin production	Watanabe et al. 1982
Catharanthus roseus	Suspension	Selection of compact globular aggregates	Alkaloid stimulation	Verpoorte 1996
Rhodiola sachalinensis	Suspension	Selection of compact callus aggregates; higher cytokinin/auxin ratio and sucrose; optimization of inoculum volume	Enhanced salidroside production	Xu et al. 1999

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All these factors are prerequisites for the production of higher molecular compounds by the plant organism as its morphological development is closer to the field cultivated plant, as compared with the non-differentiated cultures. Examples are the production of ginsenosides by adventitious roots of *Panax ginseng* C.A. Meyer in a 1 – 10 t two stage bioreactor system (Hahn et al. 2003); saponins by conventional roots of *Panax ginseng* in 5 and 20 l bioreactor (Paek et al. 2009) and ginsenosides (levels of 500 mg/l/day) by conventional roots of *Panax ginseng* in 20 t bioreactor (Charlowood and Charlwood 1991).

6. Commercial Utilization of Plant Biotechnology Tools in Mass Propagation of Planting Material for the Needs of Agriculture. Application of Plant Cell Tissue and Organ Culture Techniques as a Complementary Tool to Conventional Methods of Plant Breeding

The rapid development of plant biotechnology within the last years has led to its mass application for the production of planting material for agricultural needs.

According to the USDA in 2004 "biotechnological crops" constituted up to 46% of corn, 76% of cotton and 85% of soybean plantations, about a decade later these numbers rose up to 90, 90 and about 93%, respectively (Fernandez-Cornejo et al. 2014). Presently in the USA 60 - 70% of the food products on the market contain certain ingredient or oils which are biotechnologically obtained (Biotechnology Innovation Organization 2018).

The biotechnological selection and market realization of new cultivars however require the strict observation and critical application of a number of ethical considerations included into the legislation of the respective countries.

7. Commercial Utilization of Plant Biotechnology Tools for Industrial-Scale Secondary Metabolites Production

7.1. Patented Processes for the Biotechnological Delivery of Secondary Metabolites

The commercial yield of plant secondary metabolites by plant cell tissue and organ cultures is still limited as compared to the wide market application of plant biotechnology in agriculture and ornamental plants breeding. Nevertheless, numerous successful examples show the prospective for the development of this field.

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First attempts for the production of secondary metabolites by *in vitro* cultivated plant tissues were performed by the Charles Pfizer Co Company in 1950 (Lombardino 2000; Dias et al. 2016). Production, experimented on *in vitro* cultured water-melon tissue turned unsuccessful. Further on, the first patent obtained in this area was by Routian and Nickell as assignors to Chas. Pizer & Co., Inc., New York, N. Y., a corporation of Delaware (Routian and Nickell 1956). The invention consisted of "a method for cultivating plant tissue under submerged, aerated conditions in liquid culture" and was "also concerned with the production of useful materials by this method". Tissues of several plant species were included, designated as "tissues of those plants classified above the Thallophytes in systematic botany" (including *Rumex acetosa* L., Sweet clover, *Agave toumeyana*, Sunflower stem and petiole, Periwinkle, Tobacco, *Opuntia* cactus, *Datura* and avocado). The delivery of "vitamins, steroids, alkaloids of various types, antimicrobial agents, sugars, enzymes, organic acids, aromatic materials, and so forth" was defined.

Further development after 1978 in the field led to the feasible biotechnological production of secondary metabolites in Germany and Japan (Loyola-Vargas and Vázquez-Flota 2006; Dias et al. 2016). Thus, now-a-days the number of patents, involving plant cell cultures has risen to he impressive number of 28 000, with companies, applying plant cell cultures or hairy roots for the production of ingredient for cosmetics, food and pharmaceutical industry intensively expanding (Ochoa-Villarreal et al. 2016). Some examples of such processes patented by different companies for the industrial and semi-industrial scale delivery of plant secondary metabolites are summarized in Table 5.

The large-scale commercial production of secondary compounds for pharmaceutical industry from *in vitro* culture is recognized for shikonin, ginsenosides, berberine and taxol (Malik et al. 2016). Thus, industrial-scale production of ginsenosides developed by Nitto Denko Corporation, Japan in 1985 is still used till today. The yielded material is produced without the application of genetic engineering and utilizes the indigenous biosynthetic capacity of the wild genotype with only application of optimization methods. The company inserts the material into different drinks.

Initially berberine production by root cultures of *Coptis japonica* and *Thalictrum minus* was introduced by Mitsui Petrochemical Industries. *C. japonica* cell cultures production was firstly developed by Furuya et al. in Kitasato University (1972) and then Yamamoto and Shoyakugaku in Nippon Paint (1981) selected a highly productive line which was adopted by the company.

Table 5. Patented processes for secondary metabolites semi-commercial and commercial production in plant cultures *in vitro* systems

Manufacturer	Biotechnologically produced secondary metabolite	References
A. Nattermann & Cie. Gmbh, Cologne, Germany	*Coleus blumei* – rosmarinic acid - anti-inflammatory	Ochoa-Villarreal et al. 2016
Boehringer (Germany)	*Digitalis lanata* – (cell cultures in 300 l airlift system) digoxin for medical application	Malik et al. 2016
Mitsui petrochemical Ind. (Japan)	*Geranium sp.* – geraniol for flavor and fragrance industries	Misawa 1994; Eibl and Eibl 2002; Petersen and Amstutz 2008; Wilson and Roberts 2012; Malik et al. 2016; Ochoa-Villarreal et al. 2016
	Catharanthus roseus (cell culture) – arbutin for cosmetics – skin lightening and antiseptic agent	
	Carthamus tinctorius – carthamine - natural dye for food and pharmaceutical industries	
	Lythospermum erythrorizon (batch cell cultures 750 l bioreactor) – shikonin – pigment in cosmetics	
	Coptis japonica and *Thalictrum minus* (cell cultures in batch and continuous flow; impeller driven 4000 l bioreactor) – berberins – as anticancer, anti-inflammatory and antibiotic in medicine	
	Rubia akane (cell culture) – purpurin – pigment for dyes production	
	Lithospermum erythrorhizon – shikonin – red pigment for food industries	
Kibun (Japan)	*Carthamus tinctorius* (cell culture) carthamine - natural dye for food and pharmaceutical industries	Malik et al. 2016; Ochoa-Villarreal et al. 2016
Sumitomo Chem. Ind. (Japan)	*Duboisia* sp. (cell cultures) – scopolamine – active ingredient pharmaceutical industry – anticholinergic, anti-muscarinic, used in treatment of motion sickness, nausea and intestinal cramping	Petersen and Amstutz 2008; Malik et al. 2016 Ochoa-Villarreal et al. 2016
Nippon Oil Company (Japan)	*Podophyllum* sp. – podophyllotoxin (cell culture, differentiated organs) – anticancer active ingredient pharmaceutical industry	Petersen and Amstutz 2008; Malik et al. 2016; Ochoa-Villarreal et al. 2016
	Taxus sp. – paclitaxel - active ingredient pharmaceutical industry	
Nippon Paint Company (Japan)	*Euphorbia millii* and *Aralia cordata* (cell cultures in rotary culture system) - dyes for the textile and color for food industries, respectively	Eibl and Eibl 2002; Malik et al. 2016
Osaka (Japan)	*Aralia cordata* – anthocyanins as food coloring agents	Ochoa-Villarreal et al. 2016
ESCA genetics (USA)	*Taxus* sp. – paclitaxel - active ingredient pharmaceutical industry	Petersen and Amstutz 2008; Malik et al. 2016
	Vanilla planifolia (cell cultures in 72 l impeller driven reactor) – vanillin - flavoring agent for food and beverage industry, as well as pharmaceutics	
Phyton Catalytic (USA/Germany)	*Taxus* sp. – paclitaxel - active ingredient pharmaceutical industry; Anticancer World's largest cGMP plant cell culture facility with bioreactors specifically designed to meet the needs of plant cell culture	Petersen and Amstutz 2008; Malik et al. 2016; Ochoa-Villarreal et al. 2016
Nattermann (Germany)	*Coleus blumei* - rosmarinic acid - medicine, food industry as preservative	Petersen and Amstutz 2008
Diversa (Germany)	*Echinacea purpurea* and *E. angustifolia* - polysaccharide complex - active ingredient pharmaceutical industry	Petersen and Amstutz 2008; Malik et al. 2016
Nippon Shinyaki (Japan)	*Beta vulgaris* (cell culture) – betacyanins - natural dye for food and pharmaceutical industries	Petersen and Amstutz 2008; Malik et al. 2016

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Table 5. (Continued)

Manufacturer	Biotechnologically produced secondary metabolite	References
Nippon Paint (Japan)	*Euphorbia milli* and *Aralia cordata* – anthocyanins – natural dyes	Petersen and Amstutz 2008
Nitto Denko Corp. (Japan)	*Panax ginseng* (cell and root cultures, 20 000 l) - ginsenosiedes – dietary supplements	Malik et al. 2016; Ochoa-Villarreal et al. 2016
	Rubia akane (cell culture) – purpurin – pigment for dyes production	
CBN Biotech (Republic of Korea)	*Panax ginseng*- ginsenosiedes – pharmaceutical industry	Malik et al. 2016
ROOTec bioactives GmbH (Switzerland)	*Camptotheca acuminata* (hairy roots in mist bioreactor systems) – camptothecin - DNA topoisomerases, Type I inhibitor – pharmaceutical industry	Lehmann et al. 2014; Malik et al. 2016; Ochoa-Villarreal et al. 2016; ROOTee factsheets
	Podophyllum sp. – podophyllotoxin (hairy roots in mist bioreactor systems) - active ingredient pharmaceutical industry	
	Atropa belladonna (hairy roots in mist bioreactor systems) – atropine	
	Carlina acaulis (hairy roots in mist bioreactor systems) flavonoids with	
	atibacterial and diuretic effects, UV protective effects for cosmetics	
	Nicotiana glauca (hairy roots in mist bioreactor systems) – vitamin D3 derivative and alkaloids (anabasine, nicotine,	
	Panax ginseng (hairy roots in mist bioreactor systems) – ginsenosides – food supplements, cosmetics	
Nattermann (Germany)	*Coleus blumei* (cell cultures) – rosmarinic acid – medical application	Malik et al. 2016
Vipont Research Labs (USA)	*Papaver somniferum* (cell cultures in 300 l airlift system) – sanguinarine – cell toxin	Malik et al. 2016
Samyang, Genex Co. Ltd. (Korea)	*Taxus sp.* – paclitaxel - active ingredient pharmaceutical industry	Malik et al. 2016; Ochoa-Villarreal et al. 2016
Bristol-Myers Squibb Co. (Germany)	*Taxus sp.* – paclitaxel - active ingredient pharmaceutical industry	Malik et al. 2016
Unhwa Biotech Corp., (Jeonbuk, South Korea)	*Panax ginseng* (cultivated cambial meristematic cells "stem cell Ttobyeo") - ginsenosiedes – dietary supplements, cosmetic and medical products	Ochoa-Villarreal et al. 2016
Sederma, Le Perray-en-Yvelines (France)	Cell suspension cultures and hairy roots cultures, different species - about fifteen active ingredients for cosmetic use	Ochoa-Villarreal et al. 2016
Lumene Oy (Finland)	*Rubus chamaemorus* (callus cultures) – cosmetic products application - day creams, foundation creams, lipsticks, skin serums, mascaras, products for hair and/or scalp care, washing products for skin or hair, or as products for skin hygiene	Nohynek et al. 2014; EP2817070 (A4) — 2015-12-23
Mibelle (Switzerland)	*Malus domestica Solar vitis* (wave-type bioreactor cultivated suspension) – the whole cells decomposed in high pressure with a lipid fraction, which encapsulated all the cell constituents in liquid nanoparticles to be used in cosmetic products	Schürch et al. 2008; Nohynek et al. 2014; Ochoa-Villarreal et al. 2016
	Argania spinosa (cell suspension) – cosmetic application.	

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Purpurin production was firstly developed in cell cultures of *Rubia akane* by Mitsui Petrochemical Ind. Ltd. Mitsui Petrochemical Ind. Ltd. initially developed shikonin production in *Lythospermum erythrorhizon* suspension. CBN Biotech Co, Korea developed ginsenosides production by conventional root cultures of *Panax ginseng* in a research group at the Research Center for the Development of the Advanced Horticultural Technology (HortTech), Chungbuk National University with the financial support of the Ministry of Science and Technology, Engineering Foundation and Agricultural Development institution in Korea. Phyton Biotech, Germany is the international leader for large-scale paclitaxel production by cell cultures of *Taxus* ssp.

8. Short Outline of the Author's Own Experience in the Developmental Patterns and Morphological Features *In Vitro* and the Subsequent Modification of Secondary Metabolite Production of Species of the Genera *Hypericum*, *Sideritis* and *Artemisia*

8.1. *Hypericum* Species *In Vitro* Secondary Metabolites Productivity

Hypericum perforatum L. is a plant species with long years of medicinal application by humankind. It has been used since the 1st century A.D. It is native to Europe, North Africa and Asia, but also naturalized to North America. It possesses a broad spectrum of biological activities, determined by its rich pharmacological spectrum. Its most important biologically active constituents are the polyphenolic compounds, flavonoids, naphthodianthrones and phloroglucinols, terpenes, which have been shown to possess antidepressant, anticancer, antiviral and antibiotic activity.

Throughout the years the species has been an incessant object of scientific interest in terms of secondary metabolites production in plant cell tissue and organ culture (Danova 2015 and references cited within). In our laboratory shoot cultures as a model of elucidation of biotechnological productivity of different *Hypericum* species have been studied.

In a comparative work of the content of hypericin and pseudohypericin, it was established that the Balkan endemic *H. rumeliacum*, representative of the evolutionary more developed *Drosocarpium* section produced both *in situ* and *in vitro* higher hypericin as compared with *H. tetrapterum* (representative of section *Hypericum*, which is evolutionary older as compared with *Drosocarpium*) (Danova et al. 2012a). Interestingly, pseudohypericin level was higher in *H. tetrapterum*, as compared with *H. rumeliacum*, however the total hypericins of *H. rumeliacum* still remained higher. These findings corroborate with the ones of other authors that representatives of the evolutionary more

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developed *Hypericum* species have higher hypericins producing capacity (Kitanov 2001; Kirakosyan et al. 2003, 2004). Then an *in vitro* experiment on tissue culture development of these species showed that the higher hypericin productivity of *H. rumeliacum* was preserved (Danova et al. 2012a). Moreover, in an experiment of modification of vitamin supplementation of the latter two species, as well as of hypericin non-producing *H. calycinum* showed that the production of hypericins and phenolic and flavonoid compounds can be differentially targeted *in vitro*. It was established that Gamborg vitamins supplementation which increased the index of compactness (number of leaf couples per cm of stem length) and average leaf area also stimulated the polyphenolics levels in all three species, as compared with media with Murashige and Skoog vitamins (Danova 2010; Danova et al. 2012a). When studying the levels of hypericins in this experimental design it was established that they were higher in the medium with lower flavonoid and phenolics levels (Murashige and Skoog vitamins). The morphologic study showed that the number of hypericin dark glands per leaf was a comparatively conservative species-related feature and did not vary significantly within one and the same species, irrespectively of the vitamins modification (when comparing one and the same segment of the stem – basal, medium and apical) (Danova et al. 2012a). However, the morphological differences of obtaining a higher leaf portion within the total biomass in Gamborg treated plants led to a lower dark glands density per mm^2 of leaf area in the latter medium. Thus in Murashige and Skoog medium the total plant biomass contained a larger ratio of dark glands per unit of dry biomass which led to higher hypericins levels per gram dry material. Thus, this simple and reproducible experiment illustrated the importance of growth and development for the biosynthetic capacity of *Hypericum in vitro*.

8.2. *Sideritis scardica* Griesb Productivity *In Vitro*

The Balkan endemic *Sideritis scardica* Griesb. is traditionally utilized as a pulmonary treatment, as well as anti-flu and wound healing remedy (Ivancheva and Stantcheva 2000). It has been established that presently there are less than 2 500 mature individuals in Bulgaria and over 250 in Serbia (IUCN 2016). The plant has been determined with a near threatened status, with a decreasing current population trend (Khela 2013). The low germination rate and collection pressure imposes significant risk on its natural populations. Thus, as a part of wider program for medicinal and aromatic plants germplasm conservation, the species was introduced *in vitro* and experiments on the polyphenolic productivity under different organic and inorganic treatments was experimented. An array of plant growth regulators and activated charcoal (AC) treatments was applied in order to study their effects on the developmental patterns and polyphenolics productivity of the species *in vitro*. Low (0.02 and 0.05 g/l) as well as high (0.2 and 0.5 g/l) concentrations of activated charcoal were applied. The higher AC

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concentrations (0.2 and 0.5 g/l) led to a profound stimulation of axillary shoot formation, enlargement of leaf area and stimulation of polyphenolic production. In addition, photosystem II quantum yield revealed that the control (untreated) and all carbon-treated variants were physiologically fit. However, a slower apparent electron transport rate for 0.2 and 0.5 g/l treated plants was established, as compared with the control and 0.02 and 0.05 g/l treated plants. Thus, it was hypothesized that the higher leaf mass and leaf area in 0.2 and 0.5 g/l treated *S. scardica* might be a compensatory reaction/mechanism that allows for more efficient light utilization. Thus, the AC efficiency in affecting the biosynthetic capacity and physiological status of the plant, without the addition of organic supplements such as plant growth regulators, seems to be a prospective approach in tissue culture optimization of this specis *in vitro* (Koleva et al. 2016). In order to compare with AC treatments, plant growth regulators (PGR) were also applied to shoot cultures of the plant. The following combinations were experimented: 0.2 mg/l BA + 0.02 mg/l NAA; 0.2 mg/l BA + 0.5 mg/l NAA; 0.2 mg/l BA + 1.0 mg/l NAA; 0.5 mg/l BA + 0.5 mg/l NAA and 0.5 mg/l BA + 1.0 mg/l NAA treatments were compared with the plant growth regulators free control (Danova et al. 2017). The comparison showed that the PGR treatment led to the formation of elongated elyptical leaves with larger areas of the leaf blades, longer petiols and well expressed toothing of the leaf margins. Leaves of AC treated plants were with ovate shape, smaller area of the leaf blade, shorter petiols and less expressed toothing of the leaf margins. The addition of activated charcoal in the medium prolonged the sub-culture period, as compared with the control plants to at least 2 months without significant browning and necrosis of the plantlets. On the contrary, maintenance of *S, scardica* in PGR-free and PRG supplemented media was related to intensive polyphenolics leakage in the medium and required a shorter sub-culture period of maximum 6 weeks. Both treatments increased the polyphenolic levels *in vitro*.

8.3. *Artemisia* Productivity *In Vitro*

The medicinal plant *Artemisia alba* Turra is utilized for various purposes – decoction of the aerial parts is applied as a digestive in traditional medicine; a study on the ethanol extract revealed its anti-inflammatory activity and the essential oils were established to exert strong spasmolytic and antimicrobial properties. However, as summarized above (Table 1) great variability of the essential oil profiles has been reported in literature by many authors. This effect is attributed to the factors of the environment or to the genotype. Shoot cultures of the plant were initiated and supplementations of indole-3-butyric (IBA) acid and benzyl adenine (BA) were experimented: PGR-lacking control, 0.5 mg/l IBA; 1.0 mg/l IBA; 0.2 mg/ BA + 0.5 mg/l IBA and 0.2 mg/l BA + 1.0 mg/l IBA. Two main morphotypes were achieved as a result – the PGR-free control and IBA supplemented plants displayed shoot and root development, and the plants where BA and IBA were combined, displayed lack of root formation and callusogenesis at the explant

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base (Danova et al. 2012b). Comparison of the essential oils of the aerial parts of the two morphotypes revealed the presence of two main terpenoid profiles – oils with higher monoterpenoid/ sesquiterpenoid ratio (plants with root development, irrespectively of the PGR concentrations) and oils with sesquiterpenoid domination (the group with inhibited rooting). Given the decisive role of roots for cytokinin biogenesis in the plant organism, further research was completed in order to assess the levels of endogenous cytokinins, as well as the chloroplast architecture in the samples of the different treatments. Inhibited rooting also resulted in a significant drop of endogenous isoprenoid CK bioactive-free bases and ribosides as well as CK *N*-glycoconjugates and in decreased *trans*-zeatin (*trans*Z):*cis*-zeatin (*cis*Z) ratio in the aerials. Marked impairment of the structural organization of the photosynthetic apparatus and chloroplast architecture were also observed in samples with suppressed rooting. It is well known that in the plant cell monoterpenoid and *trans*Z type CKs biogenesis are spatially bound to plastids, while sesquiterpenoid and *cis*Z production are compartmented in the cytosol. The observed dependencies suggest an interplay between the biosynthesis of terpenoids and CK bioactive free bases and ribosides in *A. alba in vitro*. Possible moderation of the two terpenoid biosynthetic pathways because of the alteration of chloroplast structure in the experimental model has been hypothesized (Danova et al. 2018). In addition, the dependencies obtained by this experimental design provide clues of the possibilities to target terpenoid production by means affecting morphogenesis in this plant species. In conclusion, secondary metabolism is strongly dependent on growth and development and morphogenetic patterns of the plant organism. Therefore, the knowledge and understanding of these dependencies makes it possible to optimize culture conditions and target secondary metabolite production in both conventionally and biotechnologically cultivated plants and utilize the indigenous biosynthetic capacity of the wild genotype without performing genetic modifications.

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