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Current Approaches for Enhanced Expression of Secondary Metabolites as Bioactive Compounds in Plants for Agronomic and Human Health Purposes – a Review

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The study of secondary metabolism in plants is an important source for the discovery of bioactive compounds with a wide range of applications. Today these bioactive compounds derived from plants are important drugs such as antibiotics, and agrochemicals substitutes, they also have been economically important as flavors and fragrances, dyes and pigments, and food preservatives. Many of the drugs sold today are synthetic modifications of naturally obtained substances. There is no rigid scheme for classifying secondary metabolites, but they can be divided into different groups based on their chemical components, function and biosynthesis: terpenoids and steroids, fatty acid-derived substances and polyketides, alkaloids, phenolic compounds, non-ribosomal polypeptides and enzyme cofactors. The increasing commercial importance of these chemical compounds has resulted in a great interest in secondary metabolism, particularly the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology and metabolomics. In today's world the use of bioactive compounds derived from plants plays an important role in pharmaceutical applications. This review presents information about these metabolites and their applications as well as their importance in agronomy and bioactive effects on human health as nutraceuticals. This review includes also the new tendencies to produce these bioactive compounds under different stresses conditions such as biotic and abiotic stress that could be included in production systems.

INTRODUCTION

Metabolism is defined as the sum of all the biochemical reactions carried out by an organism. Primary metabolic pathways converge into few end products, while secondary metabolic pathways diverge into many products. Primary metabolism requires the cell to use nutrients in its surroundings, such as low-molecular weight compounds, for cellular activity. There are three potential pathways for primary metabolism: the Embden Meyerhof-Parnas pathway (EMP), the Entner-Dourdorof pathway, and the hexose monophosphate (HMP) pathway [Canter *et al*., 2005]. The EMP occurs most widely in animal, plant, fungal, yeast, and bacterial cells. During primary metabolism, hexoses such as glucose are converted to single cell proteins by yeasts and fungi. This is generally done by using a combination of EMP and HMP pathways, followed by tricarboxylic acid cycle (TCA) and respiration [Shilpa *et al*., 2010]. Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances traditionally referred to as secondary metabolites, are often differentially distributed among limited taxonomic groups within the plant kingdom. The primary metabolites, in contrast, such as phytosterols, acyl lipids, nucleotides, aminoacids, and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident [Hussain *et al*., 2012]. Although noted for the complexity of their chemical structures and biosynthetic pathways, secondary metabolites based natural products have been widely perceived as biologically insignificant and have historically received little attention from most plant biochemists [Hussain *et al*., 2012; Parsaeimehr *et al*., 2011]. Pharmaceutical organic chemists, however, have long been interested in these novel phytochemicals (bioactive compounds) and have investigated their chemical properties extensively since the 1850s. Interest in bioactive compounds was not purely academic but was rather prompted by their great utility as dyes, polymers, fibers, glues, oils, waxes, flavoring agents, perfumes, and drugs. Recognition of the biological properties of myriad natural products has supported the current focus of this field, the search for new drugs, antibiotics, insecticides, and herbicides [Grindberg *et al*., 2011].

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Terpenes, phenols, flavanoids, alkaloids, sterols, waxes, fats, tannins, sugars, gums, suberins, resin acids and carotenoids are among the many classes of compounds known as secondary [Gottlieb, 1990; Grindberg *et al*., 2007; Nunnery *et al*., 2010]. All terpenoids, including both primary metabolites and more than 25,000 secondary compounds, are derived from the five-carbon precursor isopentenyl diphosphate (IPP). The 12,000 known alkaloids, which contain one or more nitrogen atoms, are biosynthesized principally from aminoacids. The 8,000 phenolic compounds are formed by either the shikimic acid pathway or the malonate/acetate pathway [Genovese *et al*., 2009]. In this review, we provide an overview of recent trends on enhanced production of secondary metabolites under different stress condition such as biotic and abiotic stresses and their application for agroindustry and pharmaceutical purposes.

OCCURRENCE OF SECONDARY METABOLITES

Natural products have provided key guidelines for new drug discovery [Newman & Cragg, 2007]. The search for novel natural products with interesting bioactive properties is an ongoing discipline. Thousands of bioactive compounds have the potential or have already been established as pharmaceuticals, or nutraceuticals [Rea *et al*., 2010]. The development of crops for bioactive compounds production presents both research and agronomic management challenges and market--related considerations. The population susceptible to develop chronic degenerative diseases represents more than 30% of the worldwide population, so the demand for new alternatives for control of these diseases has increased considerably in recent decades. Increasing consumption of functional foods with a high content of phytochemicals (bioactive compounds) is an option to prevent or correct chronic degenerative diseases [Cameron *et al*., 2005].

Conventional medicine included phytochemicals in some medications, such as actually pharmaceuticals contain 50––60% of phytochemicals or are synthesized from them. Between 10% and 25% of prescribed drugs contain one or more natural bioactive compounds. The estimated use of medicinal plants suggests that more than 35,000 species of chemical compounds are used worldwide [Canter *et al*., 2005]. A large number of compounds from various natural sources such as crop, roots, leaves and some other plants have been reported. These metabolites include different types of economically important compounds, among which are the antibiotics, pigments, toxins, pheromones, enzyme inhibitors, immunomodulatory agents, receptor antagonists and agonists, pesticides, antitumor agents and elicitors in animals and plants [Evangelista & Moreno, 2007].

CLASSIFICATION OF SECONDARY METABOLITES

Plant secondary metabolites are categorized in three main groups based on their biosynthetic pathways: phenolic compounds, terpenes and nitrogen-containing compounds [Rea *et al*., 2010; Krzyzanowska *et al*., 2010]. Studies have demonstrated that terpenes are synthesized *via* the mevalonic pathway from precursor acetyl-CoA, while phenolic compounds are aromatic substances formed *via* the shikimic acid pathway or the mevalonic pathway. Nitrogen-containing secondary metabolites such as alkaloids are synthesized primarily from aliphatic amino acids derived from the tricarboxilic acid pathway or aromatic acids derived from the shikimic acid pathway, *via* acetyl-CoA, mevalonic acid. These three classes of metabolites have been widely used in pharmaceuticals [Parsaeimehr *et al*., 2011].

Phenolic compounds

Phenolic compounds are secondary metabolites that encompass several classes of structurally diverse products biogenetically arising from the shikimate-phenylpropanoids--flavonoids pathways [Krzyzanowska *et al*., 2010]. Plants need phenolic compounds for growth, reproduction, pigmentation, resistance to pathogens, resistance to influences of heavy metal-salts or in a general form to biotic and abiotic stresses [Ferrari, 2010]. The term "phenolic" or "polyphenolic" can be precisely defined chemically as a substance which possesses an aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl substituents, including functional derivatives (esters, methyl ethers, glycosides, *etc*.) and most of them show estrogenic activities [Lattanzio *et al*., 2006; Parsaeimehr *et al*., 2011]. Phenolics can be broadly divided into non-soluble compounds such as condensed tannins, lignins, and cell-wall bound hydroxycinammic acids, and soluble phenolics such as phenolic acids, flavonoids and quinones [Krzyzanowska *et al*., 2010].

On the other hand, the compounds such as furano-coumarins, lignin, flavonoids, isoflavonoids, and tannins are included in phenols. Some authors report that biotic and abiotic stresses can induce the biosynthesis of phenolic compounds. For example, the concentration of phenolic compounds can be increased significantly in blueberries (*Vaccinium corymbosum*) under UV treatments, also it has been reported that UV radiation induced the flavonoids biosynthesis that consequently leads to an accumulation of phenolics in the leaves of silver birch (*Betula pendula*). The elevated ozone environment increased the concentrations of phenolics and antioxidant in red clover (*Trifolium pratense*) leaves [Morales *et al*., 2010; Saviranta *et al*., 2010]. The activity of some pathogens has been limited when phenolic compounds are expressed in some coffee genotypes, showing higher resistance to the leaf miner *Leucoptera coffeella*, a serious coffee pest [Magalhães *et al*., 2008].

The presence of fungi leads to accumulation of lignin, a type of phenolic glycoside present in the plants, in the phloem of Austrian pine tree. There is sufficient evidence about different phenolic compounds such as: benzaldehyde, catechol, protocatechuic acid, salicylic acid, and other compounds are over synthesized and expressed by plants under fungal attacks [Lattanzio *et al*., 2006; Rea *et al*., 2010]. Adequate levels of chlorogenic acid account for the resistance of potato tubers against *Streptomyces scabies*, *Verticillium alboatrum* and *Phytophthora infestans*, while at low concentrations it stimulates the growth of *P. infestans* and *Fusarium solani* var. *coeruleum* [Krzyzanowska *et al*., 2010]. Small concentrations of benzaldehyde totally inhibited spore germination of *Botrytis cinerea* and germination of *Monilia fructicola.* Naringenin (flavanone), dihydroquercetin (dihydroflavonol), kaempferol, and quercetin (flavonols) have been studied for their biological activity against two fungal rice pathogens, *Pyricularia oryzae* and *Rhizoctonia solani* [Bassoli *et al*., 2008].

Other important phenolic compounds are flavonoids which comprise a large and diverse group of polyphenolic plant secondary metabolites. The most important flavonoids include flavonols (quercetin, kaempferol, and isorhamnetin) present in onions, leeks, endives, and broccoli; and flavones (apigenin, luteolin, and chrysoeriol) present in parsley, thyme, and celery [Yao *et al*., 2004; Hounsome *et al*., 2008].

Phenolic acids, such as gallic and caffeic acids, are found in lettuce and pac choi (*Brassica rapa* var. *rosularis*); vanillic and cinnamic acids in onions, parsley, and spinach; whilst coumaric acid in tomatoes, carrots, and garlic [Hounsome *et al*., 2008; Krzyzanowska *et al*., 2010].

Nitrogen-containing compounds

Alkaloids are a large group of bioactive compounds containing usually basic (in some cases neutral or quaternary) nitrogen derived from an amino acid (or a purine, pyrimidine or other source such as transamination in the case of pseudoalkaloids), in a heterocycle (or aliphatic in the case of protoalkaloids). Most alkaloids are classified chemically according to the nitrogen-containing ring system (pyrrolidine, piperidine *etc.*) and their biosynthetic origin, amino acids, amines, alcamides, cyanogenic glycosides, and glucosinolates [Winks & Schimmer, 1999; Paiva *et al*., 2010; Khadem & Marles, 2012]. Alkaloids have traditionally been of great interest because of their pronounced physiological and medicinal properties (for example, caffeine, nicotine, morphine, atropine, quinine). Alkaloids are usually classified by their common molecular precursors, such as pyridine (coniine derived from *Conium maculatum* which is extremely toxic and nicotine, an essential alkaloid from *Nicotiana tabacum* which is present in a number of other plants belonging to the families *Lycopodiaceae, Crassulaceae, Leguminosae, Chepopodiaceae* and *Compositeae*), tropane group (atropine, cocaine), isoquinoline (morphine, codeine), purine (caffeine), and steroids (solanine) [Angelova *et al*., 2010; Khadem & Marles, 2012]. Most alkaloids are very toxic and, therefore, have the potential function in the chemical defense against herbivores and microorganisms. It has also been suggested that these compounds serve as protectants against damage by UV radiation [Parsaeimehr *et al*., 2011].

Among alkaloids, coniine derived from *Conium maculatum* is extremely toxic and affects the nerve system or sanguinarine, an alkaloid derived from *Sanguinaria canadensis* has a toxic action on the Na+-K+-ATPase trans-membrane protein [Lee *et al.,* 1993; Khadem & Marles, 2012]. Nicotine had inhibitions on the tobacco hornworm, *Manduca sexta* and cigarette beetle, *Lassioderma serricorne*. It seems that the wide usage of nicotine as one of the first insecticides used to control pests in agriculture lays behind Schmeltz [1971] explanation about this fabulous alkaloid that the pyridine alkaloid nicotine has one of the best-studied putative plant resistance traits because it can interact with the acetylcholine receptors in the nervous systems of animals and therefore nicotine is extremely toxic to most herbivores [Parsaeimehr *et al*., 2011].

Among the alkaloids caffeine, a purine alkaloid, plays the role of a chemical defense compound against pathogens and also herbivores and is even recognized as an allelopathic compound among plants [Baumann & Gabriel, 1984; Ashihara *et al*., 2008]. There are also fabulous reports about the usage of alkaloids in pharmaceuticals, for instance: rauwolscine – an alkaloid derived from *Rauwolfia canescens* is reported as a central nervous stimulator or coralyne as DNA-binding agent and anti-cancer, antioxidant, anti-inflammatory agent [Paiva *et al*., 2010; Parsaeimehr *et al*., 2011].

Tropane alkaloids include cocaine and atropine, then nicotine is a pyridine alkaloid, whilst noradrenaline (or norepinephrine), and adrenaline (epinephrine), papaverine, curarines and morphine arise from tyrosine [Iriti & Faoro, 2009]. Melatonin and serotonin are indolamines, vindoline and catharantine are indole alkaloids, quinine and capthotecin are quinoline alkaloids and lysergic acid diethylamide (LSD) is an ergot alkaloid, all these arising from tryptophan [Paiva *et al*., 2010]. Purine alkaloids include theophylline, theobromine and caffeine, and they can be found in tea, cacao, and coffee, and some important alkaloids and their mechanism of action, as such as, caffeine is originally *Caffea arabica, Camellia sinensis, Theobroma cacao*, causing mechanism of action in mammals, for example, increased arousal and decreased sleep, increased cautiousness by influence on central nervous system (CNS), and inhibitory actions in synthesis of adenosine A1 and A2 receptors. Physostigmine (eserine) is originally *Physostigma venenosum* and its effect in mammals includes inhibition of neurotransmitter-degrading enzymes, acetylcholinesterase inhibitors, and actions on the neuromuscular junction [Aires *et al*., 2009].

Among the alkaloids there are glucosinolates, a functional group of sulfur, containing a group derived from glucose [Angelova *et al*., 2010]. The glucosinolates: progoitrin and sinigrin are found in white and red cabbage, Brussels sprouts, and cauliflower; glucoiberin and glucoraphenin in broccoli, red radish, and daikon (*Raphanus sativus*); and sinigrin and gluconasturtiin in mustard and horseradish. Substances formed from glucosinolates act as natural pesticides and as a defense against herbivores [Hounsome *et al*., 2008; Aires *et al*., 2009]. Upon tissue disruption, glucosinolates are rapidly hydrolysed by the enzyme myrosinase, which cleaves off the glucose unit. The remaining molecule quickly converts to thiocyanate, isothiocyanate, or nitrile, which acts as an allelochemical, protecting plants against herbivores, pests, and pathogens [Paiva *et al*., 2010].

Terpenes

Terpenes are lipid-soluble compounds and their structure includes 1 or more 5-carbon isoprene units, which are ubiquitously synthesized by all organisms through 2 potential pathways, the mevalonate and, more recently identified, deoxy-D-xylulose pathways [Rohmer, 1999]. Terpenoids are classified according to the number of isoprene units they contain; isoprene, which itself is synthesized and released by plants, comprises 1 unit and is classified as a hemiterpene; monoterpenes, sesquiterpenes, diterpenes, sesterpenes, triterpenes, and tetraterpenes [Paiva *et al*., 2010]. Terpenes and terpenoids are the base constituents of many types of plant essential oils. It is proven that the terpenes have great biological activities such as: anti-cancers, anti-microorganisms and anti-inflammatory [Liu *et al*., 2000]. Terpenes in plants are synthesised by mevalonic acid pathway and they play some functional roles in primary metabolism and considerably, the hormones such as gibberellins and abscisic acid and pigments such as phytol and carotenoids are a part of triterpenoids. Meanwhile, resins in plants are monoterpenes or pyrethum and azadirachtin from *Chrysanthemum spp.* and meliaceae family as natural insecticides are terpenes. Considerably, menthol as a terpene is reported to have anti-herbivores influences and its smell warns that the plant contains toxic compounds [Parsaeimehr *et al*., 2011].

EFFECT OF DIFFERENT FACTORS ON BIOSYNTHESIS OF SECONDARY METABOLITES

Several environmental factors such as nutrient supply, temperature, light conditions and atmospheric CO_2 concentrations can influence the levels of secondary metabolites in plants [Fine *et al*., 2006]. As it is commonly known that secondary metabolites are associated with primary metabolism by the rates at which substrates are redirected from primary pathways to the secondary biosynthetic routes. Several environmental factors affecting growth, photosynthesis and other parts of the primary metabolism will also influence secondary metabolism [Ibrahim *et al*., 2011].

Light intensity, photoperiod and temperature have been reported to influence the biosynthesis of many secondary metabolites in a number of plant species [Vogt, 2010]. The long days and cool night temperature in the northern latitudes appear, for example, to increase production of aromatic compounds compared with the same plant species in the south [Fucile *et al*., 2008].

The shikimate pathway acts at a critical point between primary and secondary metabolism by transporting carbon from glycolysis and the pentose phosphate pathway towards the synthesis of a broad range of physiologically important aromatic compounds [Fucile *et al*., 2008; Chen *et al*., 2009]. The shikimate pathway is defined by seven metabolic steps beginning with the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) and ending with the synthesis of chorismate [Vogt, 2010]. The shikimate pathway, the common route leading to production of the aromatic amino acids phenylalanine, tyrosine, and tryptophan, constitutes a part of metabolism that is found only in microorganisms and plants, never in animals. This pathway is therefore an important target for herbicides, antibiotics, and live vaccines production [Gientka & Duszkiewicz-Reinhard, 2009]. Plants have several metabolic pathways leading to secondary products capable of effectively responding to stress situations imposed by biotic and abiotic factors [Chen *et al*., 2009]. These pathways, often recruited from essential primary metabolism pathways upon initial gene duplication, are frequently restricted to specific taxonomic groups and play a major role in the plant and environment interaction, and the plants are using biotic and abiotic stresses to activate the metabolic pathways in important points and these effects are used as a defense against pathogens with a high production of bioactive compounds [Vogt, 2010; Mazid *et al*., 2011].

The shikimate pathway is also the entry to the biosynthesis of phenylpropanoids. Transcriptional control was shown for 3-deoxy-D-arabinose-heptulosonate (DHAP) synthase [Gientka & Duszkiewicz-Reinhard, 2009], whereas arogenate and prephenate dehydratase are inhibited by phenylalanine, one end-product of the pathway [Yamada *et al*., 2008]. The plastidal location of two elusive steps of the shikimate pathway, arogenate dehydratase and dehydrogenase, leading to phenylalanine and tyrosine biosynthesis has recently been proven [Rippert *et al*., 2009]. With respect to its important contribution to the carbon flow within the plant, the response of individual genes of the shikimate pathway to variations in light or nutrient content appears tightly regulated and more complex than the transcriptional responses of the entry genes of phenylpropanoid and flavonoid biosynthesis, encoding phenylalanine ammonia lyase (PAL) or chalcone synthase (CHS) [Vogt, 2010]. Usually in plants, the defense system is activated through signaling pathways mediated by endogenous signaling molecules such as salicylic acid (SA), ethylene, and jasmonic acid (JA). These elicitors target secondary signals in the cell nucleus where they initiate the signal transduction pathways that lead to transcriptional activation of numerous genes and, consequently, induce the synthesis of a variety of defense proteins and secondary metabolites [Siddiqui *et al*., 2012]. For example, rice is devoid of β-carotene. Therefore, genetic engineering was used to produce β-carotene in rice endosperm. Daffodil *(Narcissus pseudonarcissus*) *Psy* gene, which encodes phytoene synthase and produces the first carotenoid phytoene, a key precursor of β-carotene, was expressed in rice under a CaMV 35S (constitutive) or Gt1 (endosperm-specific) promoter [Yu *et al*., 2008]. Transformation of canola seed with bacterial phytoene synthase (*crtB*) gene using seed-specific napin promoter resulted in orange coloured seed with a 50-fold higher (1000-1500 μg/gFW) total carotenoid level than the wild type (33 μg/gFW) [Fujisawa *et al*., 2008]. Dehydroascorbate reductase (DHAR) regenerates ascorbic acid (AsA) from its oxidized form and has been used to increase the level of AsA. Upon 100-fold increase in the expression of wheat *dhar* cDNA under the control of ubiquitin (ub) or shrunken (Sh2) promoter in corn, 2 to 4-fold increase in ascorbic acid (AsA) was quantified in kernels [Mattoo *et al*., 2010]. Expression of rice *dhar* (under the control of barley D-hordein promoter) in corn resulted in 6-fold increase in AsA (ascorbate) [Mattoo *et al*., 2010; Siddiqui *et al*., 2012].

Accordingly, plants have several metabolic pathways leading to tens of thousands of secondary products capable of effectively responding to stress situations imposed by biotic and abiotic factors [Krzyzanowska *et al*., 2010]. These pathways, often recruited from essential primary metabolic pathways upon initial gene duplication, are frequently restricted to specific taxonomic groups and play a major role in the plant and environment interaction [Mazid *et al*., 2011].

Biotic and abiotic stresses

Biotic and abiotic stresses are well known as inductors of different responses in plants. Biotic stress is defined as any condition that occurs as a result of damage done to plants by other living organisms, such as bacteria, viruses, fungi, parasites, harmful insects and weeds. Abiotic stress is defined as any condition generated by environmental factors such as drought, salinity, temperature and others [Fujita *et al*., 2006]. Stress response is initiated when plants recognize a disorder at a cellular level, activating signal transduction pathways that transmit information within individual cell and throughout plant, leading to changes in expressing many gene networks [Gorovits & Czosnek, 2007].

One way to generate resistance in plants to stress conditions is the use of elicitors, because their application on plant surface activates multiple signaling pathways of intracellular defense [Bent & Mackey, 2007]. Elicitors are very stable molecules that induce an immune defense response in plants. Elicitor needs to be recognized on plant by a receptor (usually a protein), which activates the expression of defense genes. There are two groups of elicitors, the biogenic and abiogenic [Holopainen *et al*., 2009; Ferrari, 2010; Spoel & Dong, 2012]. Biogenic elicitors are divided into two groups, exogenous and endogenous. The exogenous elicitors are isolated from pathogens or culture medium, while endogenous elicitors are isolated from some plants [Mejía-Teniente *et al*., 2010]. On the other hand, the abiogenic elicitors are heavy metal ions, inhibitors of certain metabolic stages, UV radiation, some kinds of antibiotics and fungicides. Compared with biogenic, the abiogenic elicitors induce defense reactions of plant when given in relatively high doses. In turn, the biogenic elicitors are active at very low doses. When applied, they cause no symptoms of demand and stress accumulation of toxic compounds that sensitize the plant tissue and improve their resistance to subsequent infections [Boller & Felix, 2009; Mejía-Teniente *et al*., 2010; Spoel & Dong, 2012].

UV radiation is a natural elicitor of secondary metabolites production in higher plants. There are three types of UV light which are separated according to wave-length, UV-A has the longest wave-lengths ranging from 400 to 315 nm and the least amount of energy at 3.10-3.93 e.V. per photon. UV-B ranges from 315 to 380 nm (4.43-12.40 e.V. per photon) while UV-C is the shortest at 280-100 nm and has between 8.28–124 e.V. per photon [Li & Kubota, 2009; Brechner, 2008; Gu *et al*., 2010]. UV-C is generally filtered out by the ozone layer and is not a factor in plant development. UV-A is less harmful to plants due to the lower energy level per photon and is transmitted through glass, meaning that all plants grown in glass greenhouse are exposed to UV-A radiation [Kotilainen *et al*., 2008; Victório *et al*., 2011]. UV-C radiation which can be obtained through germicidal lamps will kill plants. Supplemental exposure to UV-B light has been shown to increase the concentration of secondary metabolites in maize, basil, peanut, and lettuce [Li & Kubota, 2009]. UV-B has also been associated with antifeedant properties in many higher plants, leading to enhanced secondary metabolites production in some plants [Brechner *et al*., 2011].

Another important elicitor is salicylic acid (SA), a phenolic endogenous growth regulator which participates in the regulation of physiological processes in plants, and it is also important in immunity to diseases [Ferrari, 2010]. It is involved in endogenous signaling and in the plant defense response against pathogens. It is involved in plant resistance to pathogens by inducing the synthesis of pathogenesis-related (PR) proteins [Spoel & Dong, 2012]. It also plays a role in systemic acquired resistance (SAR) [Mejía-Teniente *et al*., 2010]. For example, SA has been found to induce heat tolerance in mustard, chilling tolerance in maize and wheat, salinity tolerance in wheat, heavy metal stress tolerance in barley, and drought tolerance in wheat. SA has been shown to act as a signaling molecule, modulating plant responses to various external biotic and abiotic stimuli [Ashraf *et al*., 2010].

In addition to jasmonic acid (JA), salicylic acid (SA) is another important signaling molecule in plants. It has been well documented that SA is involved in plant defense responses such as the activation of distinct sets of defense-related genes and development of systemic acquired resistance (SAR) [Xu *et al*., 2009]. Jasmonic acid (JA) is an important signaling molecule in plant defense responses [Dombrecht *et al.,* 2007].

Although numerous reports show that SA and JA may act through distinct signalling pathways in plants, there is a growing body of literature showing that these pathways do not function entirely independently. Rather, they are involved in a complex signalling network that influences the magnitude or amplitude of various signals derived from these pathways [Kazan & Manners, 2008]. One of the major roles postulated for JA is its antagonistic action on SA-dependent signalling pathway and the expression of SA-responsive genes [Xu *et al*., 2009]. Similarly, various pharmacological and genetic experiments have shown that SA is a potent suppressor of the JA signalling pathways and JA-dependent defense gene expression [Spoel & Dong, 2012].

ALLELOPATHIC EFFECTS OF SECONDARY METABOLITES

The term "allelopathic", from the Greek word "*allelon*" meaning mutual and "*pathos*" meaning harm affection, by which one plant influences another by chemical means. Those compounds that affect in a positive or negative way other organisms and hence affect the structure of ecosystems are termed as allelochemicals. Among allelochemicals some compounds that have been shown to have bioactivity as antibiotic, antifungal and anti-predator activities are allelopathic compounds [Leflaive & Ten-Hage, 2007].

Allelopathy may be either the result of a direct selection of secondary metabolism, or a secondary process where the biosynthesis of molecules was originally selected for other purposes [Chen *et al*., 2009]. It may have developed when the emitter organism first released some compounds in order to avoid their autotoxicity or when mechanisms of self-resistance evolved, which could then have led to a secondary advantage. In the case of terrestrial plants, allelopathy may have served primarily to protect the plant against attack by fungi or microorganisms [John & Sarada, 2012]. Allelopathy is manifested in cases of abiotic stress, invasion by organisms, and synthesis of a new molecule by the emitter organism, delay in the target adaptation, or accumulation of allelopathic compounds in the environment. Stress can enhance both the production of allelopathic compounds and the susceptibility of the target [Li *et al*., 2010; Bialczyk *et al.,* 2011].

Various secondary metabolites produced by plants and microorganisms have been considered as potential allelochemicals and to play an important role in shaping interactions and communities due to their bioactivity [Latkowska *et al*., 2008]. For example, the phenolic compounds are distributed in plants and very common in vegetative decomposition products, and they are important precursors of humic substances in soils [John & Sarada, 2012]. In soil, ortho--substituted phenolics, such as salicylic and o-coumaric acids, and dihydro-substituted phenolics, such as protocatechuic and caffeic acids, are adsorbed by clay minerals by forming chelate complexes with metals [Li *et al*., 2010]. Phenolic allelochemicals can also inhibit plants from absorbing nutrients from surroundings and affect the normal growth of plants. Spraying cucumber (*Cucumis sativus*) with benzoic acid and cinnamic acid derivatives, resulted in a decline in phenols glycosylation and phenyl-β-glucosyltransferase (PGT) activity decrease which are linked with increases in membrane permeability [Bialczyk *et al*., 2011].

In a study of allelopathic effects of phenolic acids such as benzoic, hydrobenzoic, vanillic, and caffeic were reported to have antimicrobial and antifungal activity, probably due to enzyme inhibition. Hydroxycinnamic acid derivatives such as caffeic, chlorogenic, sinapic, ferulic, and *p*-coumaric acids possess strong antioxidant activity due to inhibition of lipid oxidation and scavenging reactive oxygen species [Hounsome *et al*., 2008]. Plants use a variety of mechanisms to release secondary compounds into their surrounding environment. Each of these processes may release chemicals that mediate allelopathic interactions between plants. Therefore these compounds are being important for the pharmaceutical industry [Chen *et al*., 2009].

BIOTECHNOLOGICAL APPROACHES FOR BIOACTIVE COMPOUNDS PRODUCTION

Many biotechnological strategies have been hypothesized and used to enhance the production of secondary metabolites in plants such as: high yielding cell line screening, optimization of cultivation media, biosynthesis pathways engineering, usage of elicitors, large scale cultivation in bioreactor system, root culture, plant cell immobilization, and biotransformation [Peterhansel *et al*., 2008]. Several strategies have been followed to improve yields of secondary metabolites in plant cell cultures. In the past years new approaches have been developed such as: the culturing of differentiated cells *i.e*., shoots, roots and hairy roots, and induction by elicitors and metabolic engineering [Parsaeimhr *et al*., 2011]. In most cases the cultures of differentiated cells have been able to get productions of the desired compounds in levels comparable to the mother plant. However the culture of such differentiated tissues on a large-scale in bioreactors is a major constraint, but for studies of the secondary metabolites biosynthesis such systems are very useful [Niraula *et al*., 2010]. The second approach is mentioned as the use of elicitors, this technique has been successfully reported in several cases. Plant cell cultures provide an excellent system to study biosynthesis of secondary metabolites for the large scale production of these compounds, but unfortunately in most cases production is too low for commercialization, therefore advances in biotechnology particularly in plant cell cultures methods, should provide new means for the commercial processing of even rare plants and the chemicals they provide [Du *et al*., 2010]. Plant cell culture technologies were introduced at the end of 1960s as a possible tool for both studying and producing plant secondary metabolites. Different strategies using cell cultures systems have been extensively studied with the objective of improving the production of bioactive secondary metabolites and cell culture systems could be used for the large scale culturing of plant cells from which secondary metabolites can be extracted. The advantage of this method is that it can provide a continuous, reliable source of natural products [Niraula *et al*., 2010].

Production of secondary metabolites with biological activity in other organisms

Cyanobacteria are an ancient group of photosynthetic prokaryotic organisms. The secondary metabolites produced in cyanobacteria play an important role in toxicity as iron chelators, protease inhibitors, growth inhibitors as well as growth promoting properties and have been well documented [Prasanna *et al*., 2010; Yadav *et al*., 2011]. Some metabolites such as microcystins, saxitoxins or anatoxins, are of global significance because of their toxicity while other display significant pharmaceutical potential [Sainis *et al*., 2010]. It has been found that the cyanotoxins, anatoxin-a, microcystins and cylindrospermopsin obtained from *Anabaena*, *Microcystis* and *Cylindrospermopsis* (respectively), show larvicidal activity with >50% mortality. The aeruginosins particularly inhibit serine proteases (trypsin, chymotrypsin, thrombin or elastase) and have been regarded as a promising drug candidate [Yadav *et al*., 2011].

Alternatively, flavonoids are produced in yeast by expressing phenylpropanoid pathway. Flavanone has been successfully produced in yeast by expressing phenyl ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate-CoA (4CL), and chalcone synthase (CHS) genes. Flavones have also been produced in flavanone-producing recombinant yeast by expressing flavone synthase I (FSI) and flavone synthase II (FSII) genes [Yan *et al*., 2005]. The biosynthesis of terpenes in higher plant cells shows two entirely separate enzymatic pathways: mevalonic acid pathway (MVA) and methylerythritol 4-phosphate pathway. In yeast, only MVA pathway is involved in the biosynthesis of ergosterol as the major end product [Hussain *et al*., 2012]. Oswald *et al*. [2007] engineered yeasts to produce monoterpenoids by expressing linalool synthase and geraniol synthase genes, and yeast strains successfully produced those monoterpenoid alcohols by using internal geranyl pyrophosphate. *Schizosaccharomyces pombe* yeast is not able to produce any carotenoids but it synthesizes ergosterol from farnesyl pyrophosphate (FPP) through the sterol biosynthetic pathway. Gunel *et al*. [2006] cloned a gene encoding geranyl pyrophosphate synthase from bell pepper (*C. annum*) in *S. pombe* and successfully redirected carbon flow from the terpenoid pathway leading to ergosterol formation toward the production of carotenoid through the heterologous expression of carotenoid biosynthetic gene in a noncarotenogenic yeast, *S. pombe*.

Metabolomics engineering of bioactive compounds

The principal advantage of recent technologies with genomics and proteomics is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. Metabolic engineering involves the targeted and purposeful alteration of metabolic pathways found in an organism to achieve better understanding and use of cellular pathways for chemical transformation, energy transduction, and supramolecular assembly [Lessard, 1996]. This technique applied to plants will permit endogenous biochemical pathways to be manipulated and results in the generation of transgenic crops in which the range, scope, or nature of a plant's existing natural products are modified to provide beneficial commercial, agronomic, and/or postharvest processing characteristics [Du *et al*., 2010]. Metabolomics, or metabolite (metabolic) profiling, offers tremendous potential to discover novel genes and assign function to those genes. Metabolic profiling determines the consequences of a targeted change in gene activity and has the potential to provide information on gene function and its effects on the complex biochemical network [Vogt, 2010]. Changes in gross phenotypes (*e.g*., branching, flowering time, growth rate) are the integrated result of changes in the pattern of many interacting genes. Stated explicitly, metabolomics is the unbiased, relative quantification of the broad array of cellular metabolites, and their fluxes [Tyagi *et al*., 2010]. Genetical metabolomics aims at identifying genomic loci that regulate the level of secondary metabolites of interest using comprehensive metabolomics approaches coupled to large-scale genetic marker analyses. Genetical metabolomics metabolite profiling combined with quantitative trait locus (QTL) analysis has been proposed as a new tool to identify loci that control metabolite abundance [Oldiges *et al*., 2007].

Specifically, metabolomics involves the rapid, high throughput characterization of the small molecule metabolites found in an organism. Since the metabolome is closely tied to the genotype of an organism, its physiology and its environment, metabolomics offers a unique opportunity to look at genotype-phenotype as well as genotype-envirotype relationships [Oldiges *et al*., 2007; Vogt, 2010]. Metabolomics is increasingly being used in a variety of health applications including pharmacology, pre-clinical drug trials, toxicology, transplant monitoring, newborn screening and clinical chemistry [Du *et al*., 2010]. One of the challenges of systems biology and functional genomics is to integrate proteomic, transcriptomic, and metabolomic information to give a more complete picture of living organisms. Metabolomics is the scientific study of chemical processes involving metabolites [Tyagi *et al*., 2010].

On the other hand, several transgenic approaches have been used to increase the levels of the endogenous flavonols, quercetin and kaempferol in the peel and flesh of tomato fruits. Ectopic expression of a single CHI gene from Petunia (*Petunia sp.*) resulted in a tissue-specific increase of total flavonols in the fruit peel. This was mainly due to an accumulation of the flavonols rutin (quercetin-3-rutinoside), quercitrin (quercetin-3-glucoside), and to smaller but still substantial increases in kaempferol glycosides. In these high flavonol transformants, naringenin chalcone levels were strongly reduced, suggesting that the natural naringenin chalcone pool was utilized by CHI to stimulate the flux through the endogenous flavonoid pathway [Verhoeyen *et al*., 2002].

Up-regulation of the flavonoid pathway in tomato fruit flesh, a tissue that normally does not produce flavonoids, was achieved by the introduction and coordinate expression of the maize regulatory genes Lc and C1 [Le Gall *et al*., 2003]. Alternatively, RNAi-mediated suppression of the tomato regulatory gene DET1 resulted in a 'high pigment' fruit phenotype, consisting of an up to 3.5-fold increase in flavonoid content in addition to enhanced carotenoid levels [Schijlen *et al*., 2006]. Using structural genes from several plant sources and combinations, we were able to produce transgenic tomatoes accumulating high levels of stilbenes, deoxychalcones or flavones. These fruits displayed altered antioxidant profiles and a three-fold increase in total antioxidant activity of the fruit peel [Schijlen *et al*., 2006].

SECONDARY METABOLITES IN ECONOMICALLY IMPORTANT CROPS

The plants are sources of a great number of metabolic products of commercial importance and are used in the pharmaceutical industry [Krzyzanowska *et al*., 2010]. The demand for natural products has increased in recent years because of limitations in the process of obtaining drugs based on chemical synthesis [Fucile *et al*., 2008; Vogt, 2010]. To this is added the chemical diversity of these compounds on synthetic and intense biological activity. For example, studies have been performed on the composition of common pepper (*C. annuum*), *C. frutescens* and *C. chinense* [Helmja *et al.*, 2007; Rodriguez-Burruezo *et al*., 2009]. These studies have shown that peppers rank among the vegetables with the highest contents of several relevant health-promoting compounds. Many carotenoids present in *chili peppers*, particularly capsanthin and β-carotene, have shown a strong antioxidant activity, and some others, like the yellow/orange β-cryptoxanthin, and β-carotene, are vitamin A precursors, the latter having the highest activity. Furthermore, the antioxidant properties of carotenoids appear to provide protection against several heart diseases and different types of cancer [Rodriguez-Burruezo *et al*., 2009].

On the other hand, bioactive compounds not only increased interest in soybean products but also led to the incorporation of soybean isoflavone extracts into a range of commercial functional foods and to the development of numerous non-prescription food supplements, both also known as nutraceuticals. Isoflavones have been demonstrated to have antiatherosclerotic, antioxidative, antitumorial, and antiestrogenic properties that can be used in the treatment of important diseases such as cancer, heart and kidney diseases [Cheng *et al*., 2010].

Red kidney beans (*Phaseolus vulgaris*) contain significant amounts of lectins which have both beneficial and detrimental biological properties. Lectins are carbohydrate-binding glycoproteins that can react specifically with human blood cells, preferentially agglutinate malignant cells, and undergo mitogenic stimulation of lymphocytes. Some lectins are resistant to heat and proteolytic enzymes and can enter the circulatory system intact [Zhang *et al*., 2009].

Grapefruits are a rich source of bioactive compounds, which may serve as cancer chemopreventive agents. The red-fleshed varieties (Rio Red) contain flavonoids, limonoids and their glucosides, vitamin C, folic acid, carotenoids (*e.g.* lycopene and β-carotene), coumarin-related compounds (*e.g.* auraptene), soluble fiber and potassium. Many of these compounds as quercetin, α-tocopherol and β-carotene, among others, are natural antioxidants. Antioxidants are secondary metabolites present in plants, particularly fruits. The consumption of antioxidants in foods is key to maintenance of human health because the body lacks adequate levels of biochemical compounds to provide sufficient protection against the constant and inevitable formation of reactive oxygen species, which are powerful oxidants [Vanamala *et al*., 2006].

Antioxidants are associated with a reduced rate of heart disease mortality and incidents of mouth, pharynx, esophagus, lung, stomach and colon cancers, and other degenerative diseases and aging [Bhattacharya *et al*., 2010]. The most important natural antioxidants present in green chilli are vitamin C, carotenoids, and phenols. Moreover, the metalloenzyme, superoxide dismutase, which is universally present in all plants and imparts defense against oxidative stress, converts superoxide radical anion into hydrogen peroxide [Matsufuji *et al*., 2007]. Chilli pepper (*Capsicum annuum*) is reported to contain moderate to high levels of neutral phenolics or flavonoids, phytochemicals that are important antioxidant components of a plant-based diet. Capsaicin, the phenolic pungent principle of chilli (*Capsicum annuum*), can directly scavenge various free radicals [Bhattacharya *et al*., 2010].

Isolation and extraction techniques of bioactive compounds

Domestication and cultivation of plant species disclose the opportunity to solve problems by using biotechnologies to produce bioactive compounds. The general interest for the traditional plant cultivation comes from the necessity to guarantee a constant supply of natural chemical compounds to be also employed as a precursor of organic synthesis; in fact, neither the collection of wild plants can satisfy the continuous demands from the industry, nor the spontaneous plants [Lucchesini & Mensuali-Sodi, 2010]. *In vitro* culture techniques are a useful tool to improve production and marketing of plant species which allows making a rapid clonal propagation of plants selected for their active principles. The synthesis of target compounds such as bioactive compounds depends on adaptations to fluctuating temperatures and light conditions, stress pathogen infections or herbivore attacks [Lucchesini *et al*., 2009]. Different methodologies of *in vitro* culture can be employed, other than cell cultures, to produce biomass of medicinal plant regardless the conservation of both phenotype and genotype of initial parent plants (adventitious regeneration of organs, organogenesis from callus, somatic embryogenesis, cultivation of genetic transformed plant material) whereas, to produce true-type plants, the micropropagation techniques are necessary to clone selected plants and are helpful to start field cultivation for a mass herbal production [George *et al*., 2007].

The somatic embryogenesis is a process through which groups of somatic plant cells or tissues leading to the formation of somatic embryos, which resemble the zygotic embryos of intact seeds, retaining the ability to develop into seedlings if placed in an appropriate culture environment [Mattoo *et al*., 2010]. So, the regeneration of whole plants through somatic embryogenesis can offer, in comparison with the classic micropropagation through organogenesis, remarkable advantages thanks to the greater multiplication rate and the smaller costs supported to produce one plant [Jeong *et al*., 2006]. Somatic embryos can be induced to proliferate directly on plant tissues cultured *in vitro* or in liquid cell suspension culture. The initiation of embryogenic calli needs the employment of a narrow range of plant growth regulators for the production of bioactive compounds. The somatic embryos mature in few weeks if transferred into suitable maturation media, obtaining, usually in few months, plants ready to the transfer in a greenhouse [Matoo *et al*., 2010].

The direct manipulation of DNA sequences to alter gene expression in nutraceutical plant research is a booming business for the production of bioactive compounds. For example some terpenes biosynthetic pathway of *Mentha spp*. were engineered to alter the essential oils in thricomes (cell organs specialized in producing and accumulating secondary metabolites) and increase resistance to fungal infections and abiotic stress [Lucchesini *et al*., 2006; Wan *et al.,* 2011]. Rather than insist on the creation of GM plants with productive and qualitative improvement, the role of genetic transformation seems to go towards the genetic improvement of cell culture and the establishment of hairy roots culture [Lucchesini *et al*., 2010]. The processing methods can be the direct transfer of DNA or indirect taking advantage of the use of vectors (bacteria, viruses). The use of *Agrobacterium tumefaciens* Ti plasmid DNA or *Agrobacterium rhizogenes* RI plasmid for induction of hairy roots is among the indirect methods [Jeong *et al*., 2006]. These bacteria have the ability to induce adventitious roots (literally "hairy roots") to a large number of host plants, once integrated the T-DNA from Ri plasmid of *Agrobacterium rhizogenes*; Rol genes (or locus root) inserted in the plant DNA increase the sensitivity to auxin rather than codify for its biosynthesis. It allows transformed cells to develop hairy roots which can be cultured in a bioreactor, in the absence of growth regulators and can release secondary metabolites into medium or can be used to regenerate entire transformed plants [Lucchesini *et al*., 2006].

Perspectives in human health applications

The use of natural compounds as inhibitory agents for virulence factor production is a new approach to overcome increased antimicrobial resistance in pathogenic bacteria [Niraula *et al*., 2010]. Medicinal plants are the most exclusive source of lifesaving drugs for majority of the world's population. The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over past decades. The secondary metabolites are known to play a major role in the adaptation of plants to their environment and also represent an important source of pharmaceuticals [Sun *et al*., 2007]. Increasing epidemiological evidence associates diets rich in fruits and vegetables with reduced risk of heart disease, cancer, and other chronic diseases. A major benefit from such a diet may be increased consumption of antioxidants, including carotenoids, ascorbate, tocopherols, and phenolics. One phenolic fraction, flavonoids, are potent antioxidants and include compounds such as flavones, isoflavones, flavonones, catechins, and the red, blue and purple pigments known as anthocyanins [Cote *et al*., 2010].

Dietary flavonoids (quercetin, kaempferol, and isorhamnetin) possess antiviral, anti-inflammatory, antihistamine, and antioxidant properties. They have been reported to inhibit lipid peroxidation, to scavenge free radicals, to chelate iron and copper ions (which can catalyze production of free radicals), and to modulate cell signaling pathways [Cote *et al*., 2010]. Production of peroxides and free radicals, which damage lipids, proteins, and DNA, has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation, and neurodegenerative diseases (Parkinson and Alzheimer). Flavonoids protect low-density lipoprotein cholesterol from being oxidized, preventing the formation of atherosclerotic plaques in the arterial wall. They stimulate enzymes involved in detoxification of cancerogenic substances and inhibit inflammation associated with local production of free radicals [Hounsome *et al*., 2008; Aires *et al*., 2009].

There are also reports about the usage of alkaloids in pharmaceuticals and about *Rauwolfia canescens* being a central nervous stimulator [Parsaeimehr *et al*., 2011]. Dietary saponins cause a reduction of blood cholesterol, inhibit growth of cancer cells, and stimulate the immune system. Some saponins, such as sapotoxin, can be toxic for humans, causing irritation of membranes of the respiratory and digestive tract, and increase the membrane permeability of red blood cells and urticaria (skin rash) [Satwadhar *et al*., 2011].

Plant glucoalkaloids solanine, tomatine, and chaconine are called saponins, these are found in peas, beans, tomatoes, spinach, asparagus, onions, garlic, and potatoes. However, certain glucosinolates (glucoraphanin, glucobrassin, glucotropaeolin) and their breakdown products have been linked to a reduction in the prevalence of certain types of cancer [Hounsome *et al*., 2008; Aires *et al*., 2009]. A considerable pool of data shows a relationship between the risk of cancer and dietary carotenoid intake. A case-control study (2.706 cases of cancer of the oral cavity, pharynx, esophagus, stomach, colon and rectum *vs.* 2.879 controls) indicated that a high intake of tomatoes and tomato-based food, both of which are rich sources of lycopene, was strongly associated with a reduced risk of digestive tract cancers, especially stomach, colon and rectum [Rea *et al*., 2010]. Also resveratrol protects the cardiovascular system by mechanisms that include defense against ischemic-reperfusion injury, promotion of vasorelaxation, protection and maintenance of intact endothelium, anti-atherosclerotic properties, inhibition of low-density lipoprotein oxidation, suppression of platelet aggregation, and estrogen-like actions [Krzyzanowska *et al*., 2010].

CONCLUSIONS

Despite the success of certain naturally-occurring substances as drugs, their limited accessibility and sometimes challenging synthesis often make them only leads and not candidate drugs for scientists and pharmaceutical companies in the quest for new therapeutic hits. The introduction of new techniques of molecular biology, such as to produce transgenic cultures and to effect the expression and regulation of biosynthetic pathways, is also a significant step toward making cell cultures more generally applicable to the commercial production of secondary metabolites. The commercial values of plant secondary metabolites have been the main impetus for the enormous research effort put into understanding and manipulating their biosynthesis using various chemical, physiological, and biotechnological pathways.

A key to the evaluation of strategies to improve productivity is to carry out that all the problems must be seen in a holistic context. At any rate, substantial progress in improving secondary metabolite production from plant cell cultures has been made within last few years. These new technologies will serve to extend and enhance the continued usefulness of higher plants as renewable sources of chemicals, especially medicinal compounds. We hope that a continuation and intensification efforts in this field will lead to controllable and successful biotechnological production of specific, valuable, and as yet unknown plant chemicals.

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