



Review

Evolution and current status of ecological phytochemistry

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Affectionately dedicated to Prof. Jeffrey B. Harborne

Abstract

Phytochemical studies have experienced a great deal of change during the last century, not only regarding the number of compounds described, but also in the concept of phytochemistry itself. This change has mainly been related to two key points: the methodologies used in phytochemical studies and the questions regarding ‘why secondary metabolites appeared in plants and in other living organisms?’ and ‘what is their role?’. This transformation in the field has led to new questions concerning such different subjects as evolution, paleobotany, biochemistry, plant physiology and ethnography. However, the main issue is to clarify the role that secondary metabolites play in the plant (and other organisms) and whether the resources invested in their production (C and N allocation, genes encoding their biogenetic pathways, specific enzymes, energy-rich molecules such as ATP and NADPH) have or lack a reasonable reward in terms of advantages for survival. Consequently, in this review the main emphasis will be placed on two subjects related to the evolution of phytochemical studies. The first aim is to describe briefly the influence that the development of the methodologies needed for compound isolation and structure elucidation have had on the field of phytochemistry. The second area to be covered concerns the new theories addressing the role of secondary metabolites from an ecological point of view: co-evolution of plants and their potential enemies (phytophagous insects, microbes, herbivores and other plants), chemical plant defence, adaptative strategies of phytophagues to plant toxins (among them sequestration will be briefly mentioned), and models and theories for carbon and nitrogen allocation. Some final remarks are made to summarize our opinion about the immediate future of phytochemical ecology and phytochemical studies.

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1. Introduction

Natural products have been used by man since antiquity for a number of applications, such as drugs, pigments and flavours. For example, the Tyrian purple pigment obtained from the mollusc *Murex brandaris* was used as a colorant. However, mankind was not aware of the specific importance and distinct chemical nature of such compounds until the end of the 19th century, and it has only been during the last century that natural products have experienced a great surge as a strong branch of organic chemistry. Many factors have led to this change, including the development of new and more powerful spectroscopic and chromatographic techniques and the change in the perception of society about chemicals.

While plants have been used for medicinal purposes since the time before historical records, it is also true that many of the poisons and venoms used by man also come from natural sources and a significant number of them originate from plants. The names *Nux vomica*, hemlock and others are linked to historical characters such as Lucrecia Borgia and Socrates. Many other compounds were used for their hallucinogenic properties in such diverse cultures as witches in the middle ages of European culture, shamans in the indigenous cultures of Central and South America, or opium in South and East Asia. Consequently, it is clear that plants are a rich source of bioactive compounds that mankind has learned to use (or misuse) throughout history, regardless of the knowledge of the particular chemicals and processes underlying their effects.

This picture changed during the 20th century with the development of studies in Natural Products. However, as always, the answer itself gave rise to many other questions. Phytochemical studies and, subsequently, Natural Products studies led to the discovery of an enormous number of compounds with a variety of chemical structures (many of them bioactive), all of which are present in the chemical composition of living organisms (from unicellular to higher organisms, mankind included). The two questions that immediately arose were why do these compounds appear in plants and other organisms? and what is their role? Undoubtedly, the biosynthesis of such compounds requires the use of energy (ATP, NADPH, NADH) as well as carbon and nitrogen allocation. These resources must be taken from the primary metabolism and there must be some kind

of advantage for them to be preserved through the evolutionary process.

The story of how life appeared on Earth is long and is yet to be fully understood. It is generally accepted that Earth was formed some 4600 million years (MY) ago. However, the first living organisms – bacteria and primitive unicellular prokaryotic cells – did not appear until 800 MY later, during the Hadean period, followed by the first autotrophic photosynthetic cyanobacteria in the Archean period, ca. 3100 MY ago. The production of oxygen by these organisms and its eventual accumulation in the atmosphere finally caused the first massive extinction of anaerobic organisms (ca. 2000 MY ago). Terrestrial bryophytes date back to around 550 MY ago, followed by terrestrial vascular plants at 440 MY ago during the Devonian period. Other species like fungi and lichens are believed to have appeared early, not long before the first bryophytes. The first forests, formed mainly by primitive trees and brackens, appeared during this period (Stein et al., 2007). It is important to highlight that insects appeared only 40 MY later, ca. 400 MY ago, and since then both kingdoms have been living together.

The purpose of this overview is to point out that while plants are among the oldest living complex organisms, they have been sharing the planet with insects, bacteria and fungi almost from the very beginning. While plants tried to defend themselves from attack by phytophagous insects and microbe-caused diseases, these organisms have tried conversely to overcome plant defence mechanisms. One of the hypotheses is that this action–reaction process caused the co-evolution of plants and their enemies and led to the great biodiversity that is found today. Such biodiversity can be observed not only in the enormous number of species present in Nature, but also in the vast number of secondary metabolites in their chemical composition.

2. An historical glance

The development of phytochemical studies in particular, and of Natural Products Chemistry in a wider sense, would never have been possible without knowledge of the chemical structures of these compounds. Two key issues continuously challenge this goal: (i) to obtain sufficient amounts of pure compounds and (ii) the use of the

appropriate tools to elucidate their chemical structures. As isolation, purification and structural elucidation methods improved, the procedures for extraction were adapted to the needs and the changes in the viewpoint: from massive extracts of many grams or kilos of starting material with boiling water and, later, organic solvents, to micro-, supercritical fluid, and high-pressure extractions among others. In this review we will discuss briefly structural elucidation and isolation.

Initially, before spectroscopic techniques were developed, the structural elucidation of organic molecules relied on destructive chemical derivatization and the ability and imagination of chemists to solve the puzzle from all the pieces. This approach, in many cases, led to remarkable results but in most cases it was not applicable due to the amounts of compound needed to perform the reactions. However, the development of spectroscopic techniques during the 1950s and 1960s, and their widespread application during the 1970s and the following decades, gave rise to a great expansion in Natural Products chemistry. Two of these techniques warrant particular attention because of their importance and contribution to the elucidation of new compounds: X-ray diffraction and NMR analysis. Nevertheless, we cannot overlook the importance that Combustion Analysis and Mass Spectrometry (MS) have had in the elucidation of chemical structure by providing the molecular masses of compounds. The different MS techniques (e.g., chemical ionization, electron impact, FAB, MalDI-tof) also provide important structural information. However, in the interest of brevity we will focus only on those techniques that, in our opinion, have contributed most to structure elucidation: NMR spectroscopy and X-ray diffraction.

2.1. Developing the tools: X-ray diffraction analysis

The first spectroscopic technique that led to a great step forward in the elucidation of organic and inorganic

chemical structures was X-ray diffraction analysis. X-rays were first discovered in 1895 by W.C. Röntgen. However, they were not used with organic molecules until some thirty years later. The first publications were devoted to the study of diffraction patterns of liquid organic molecules such as benzene (Eastman, 1924) and several fatty acids (Wyckoff et al., 1925) but the use of X-ray diffraction analysis for the structural elucidation of organic compounds was not successful until some 20 years later when it was used to determine the structure of penicillin (Hodgkin, 1949), purpurogallin (Hanson et al., 1953), longifolene (Moffett and Rogers, 1953) and vitamin B12 a few years later (Brink et al., 1954; Hodgkin, 1958) (Fig. 1). Nevertheless, the most important contribution was the elucidation of the double helical structure of the DNA molecule (Franklin and Gosling, 1953).

As the methods for calculation were improved and refined, it was possible to achieve the elucidation of structurally more complex natural products such as gibberellic acid (as *p*-bromobenzoate methyl ester; McCapra et al., 1962; Hartsuck and Lipscomb, 1963) (Fig. 1). Soon afterwards, during the 1960s, the use of computers allowed chemists to run more complex and accurate calculations with the immediate consequence of widening the scope of structures that could be calculated and elucidated. Nowadays, X-ray diffraction analysis is a routine technique for structural elucidation of crystalline natural products and it is clear that this advance has contributed significantly to Natural Products Chemistry and Phytochemistry to a point where it is comparable only with NMR analysis. However, in our opinion X-ray diffraction suffers from three major disadvantages.

The first limitation is that this technique can only be applied to crystalline compounds, although the requirements in terms of size and quality of the crystals are not as restrictive as they were decades ago.

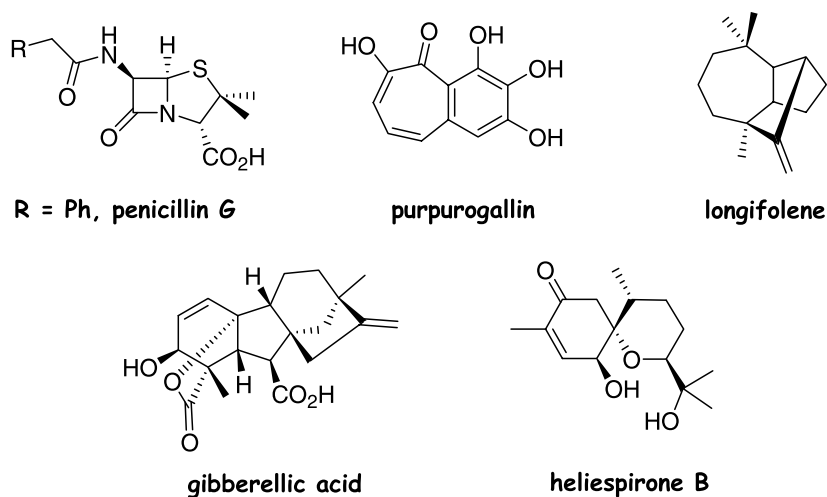


Fig. 1. The first natural products with structures elucidated using X-ray diffraction analysis. Heliespirone B is the first example of the elucidation of absolute stereochemistry in an oxygen-containing compound.

A second, albeit minor, disadvantage concerns the determination of the absolute stereochemistry. In this case the presence of a heavy atom is needed, usually bromide. However, a method for the calculation of the absolute configuration of oxygen-containing molecules has been developed and used with heliespirone B (Macías and Fronczek, 2007). This new improvement will provide scientists with the absolute configurations of more than half of the crystalline natural products.

The third drawback has arisen more recently as studies into the mode of action of natural products have gained importance. The relative and absolute stereochemistries obtained by diffraction analysis give the spatial configuration of solid crystals. The problem is that most of the reactions in the cell occur in an aqueous medium, where the configuration may vary significantly. This information may be crucial to develop models and, e.g., docking studies in QSAR. For such studies it is of great interest to have a technique that allows the study of the configuration in liquid systems.

2.2. Developing the tools: NMR analysis

Along with X-ray diffraction techniques, the structural elucidation of organic compounds relies heavily on NMR analysis. The first experiments were carried out in 1957, when the magnetic moments of *p,p'*-dialkoxyazoxybenzenes (Lippmann and Weber, 1957) were used to confirm the results of reactions. Initially, the resonance frequency of the N atom was the most commonly used signal. The first reports of H resonance frequencies for isobutyl and tert-butyl groups were reported for organoboranes to confirm their chemical structures (Davies et al., 1960).

However, it was not until the 1960s that NMR spectroscopy began to provide reliable results for the chemical elucidation of natural products. The first total assignment of a proton spectrum in a natural product was α -thujaplicinol (Zavarin et al., 1961), soon followed by the spectrum of

the first isolated phytoalexin, pisatin (Perrin and Bottomley, 1961; Perrin and Perrin, 1962) (Fig. 2), the structure of which had previously been elucidated by X-ray analysis. This particular result was of special importance since the isolation and structural elucidation of pisatin confirmed the phytoalexin theory that was proposed by Mueller and Börger some twenty years before (1940).

The stereochemical aspects of molecules were first addressed by NMR techniques in 1963 when long range spin–spin coupling and decoupling proton experiments were carried out to propose the relative stereochemistries of the methyl groups in betulin (Lhen and Ourisson, 1962; Lehn and Vystreil, 1963). Soon afterwards this technique allowed the correction of the structure of morellin proposed by X-ray diffraction analysis (Kartha et al., 1963) (Fig. 2).

From that starting point the technique of NMR spectroscopy has improved markedly in terms of accuracy, skills and sensitivity. As the increasing power of the magnets boosted the super-conductivity boundary, the increase in the magnetic field allowed higher resolution spectra to be obtained with smaller amounts of compound. This improvement widened the scope of this technique to encompass minor metabolites, which are usually present in small quantities and as non-crystalline forms. The development of new pulse sequences led to new techniques that combined mono- (double resonance, nOe difference experiments, TOCSY-1D, etc.) and bidimensional (COSY, TOCSY, ROESY, NOESY, INADEQUATE, DOSY) spectra. The combination of more than one nucleus gave rise to heteronuclear correlation experiments (HETCOR, HSQC, HMBC, etc.). Inverse detection, soft pulse and gradient pulse are other steps forward that have increased the utility of NMR for structure elucidation. Other recent advances have enabled the absolute configurations of allylic alcohols to be established under certain conditions, with this approach based on the differential shifts of MTPA ester derivatives (Takahashi et al., 1999).

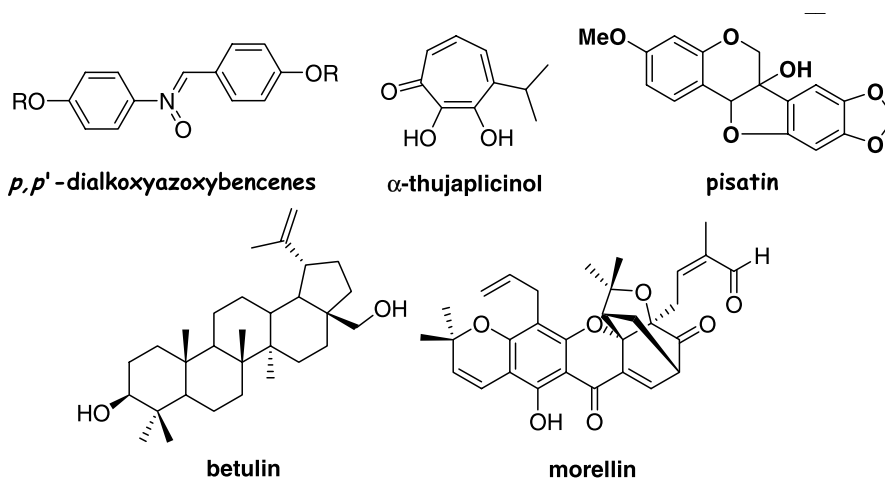


Fig. 2. The first natural products with structures elucidated using ^1H NMR spectroscopy. The stereochemistry of the methyl groups in betulin was proposed based on long-range spin–spin coupling constants.

All of these advances, which are far beyond the scope of this review, culminated in such spectacular results as the elucidation of the complete structure (relative stereochemistry included) of marine polyethers that are causative agents leading to the poisonous effects of the red tide maitotoxin, brevetoxin, okadaic acid, etc. (e.g., maitotoxin; Fig. 3; Murata et al., 1994; Nonomura et al., 1996; Sasaki et al., 1996; for a complete review see Murata and Yasumoto, 2000; Yasumoto, 2005). Such endeavour was accomplished mainly through NMR analysis (plus chemical correlation and other spectroscopic techniques) of the whole molecule and some of its derivatives. This advance represents a huge effort that has broadened the scope of the technique beyond any boundary reached before.

Other recent advances include hyphenated techniques, where LC–MS–UV–NMR tandem detection can be carried out together to accomplish HPLC sample purification and spectroscopic data collection in a single step. Moreover, the aforementioned DOSY technique (Morris and Johnson, 1993; Morris et al., 1994) can now be exploited as a routine analytical tool for the study of mixtures (Politi et al., 2006) in which the components have a minimum of only 5% difference in their respective molecular weights. The resolution and unequivocal structure elucidation of complex mixtures can also be achieved by TOCSY-1D techniques, as described recently for the resolution of a complex mixture of saponins (Macías et al., 2007b) (Fig. 4).

It is difficult to summarize in such a short space the relevance and power of the NMR techniques developed during the last 30 years. However, there is no doubt that advances in NMR techniques have allowed the elucidation of the structures of thousands of secondary metabolites. Without this tool and other spectroscopic techniques the chemistry of Natural Products would be very different today.

2.3. Developing the tools: chromatographic methods

As mentioned above, two of the most challenging factors affecting phytochemical studies are the amount of

compound isolated and its purity. Both of these factors greatly affect the possibilities for structural elucidation and the use of a compound in bioassays – something that has become increasingly important in recent decades.

Chromatography has developed in a similar way to spectroscopic techniques from early beginnings to the present. Indeed, there is a gap of several orders of magnitude between early analytical tools such as Thin Layer Chromatography (TLC) on paper and silica gel to current chromatographic equipment. Advances such as GC, HPLC, chiral, affinity, exclusion, ion exchange and counter-current (CCC) chromatography have widened the possibilities and lowered the quantities of compound required for analysis (e.g., Schreier et al., 1998; Hostettmann et al., 1997).

Besides the development of instrumental chromatographic techniques, the implementation of hyphenated tandem techniques that combine chromatographic separation with spectroscopic data collection and detection (first UV and MS, and later NMR) has again extended the possibilities for compound isolation, purification and structural elucidation (e.g., Zhang et al., 2006; Wolfender et al., 2006). In addition, the implementation of fast and reliable methodologies for analysis based on GC–MS, LC–UV–MS, etc., made possible the identification and quantification of known compounds in plants and other organisms (Fig. 5).

The techniques described above are of particular importance since biosynthetic pathways could not be proposed without knowledge of the structures of minor secondary metabolites. Also, the study of the role of secondary metabolites could not be properly addressed without techniques that allow the isolation of sufficient amounts of compounds with a high degree of purity to run the corresponding bioassays. In this sense, the technical advances achieved in chromatographic and spectroscopic techniques have been a great influence in changing the direction of phytochemical ecology from a descriptive approach of the structures to a global vision of their role within the plant or in relation to environmental (biotic and abiotic) factors.

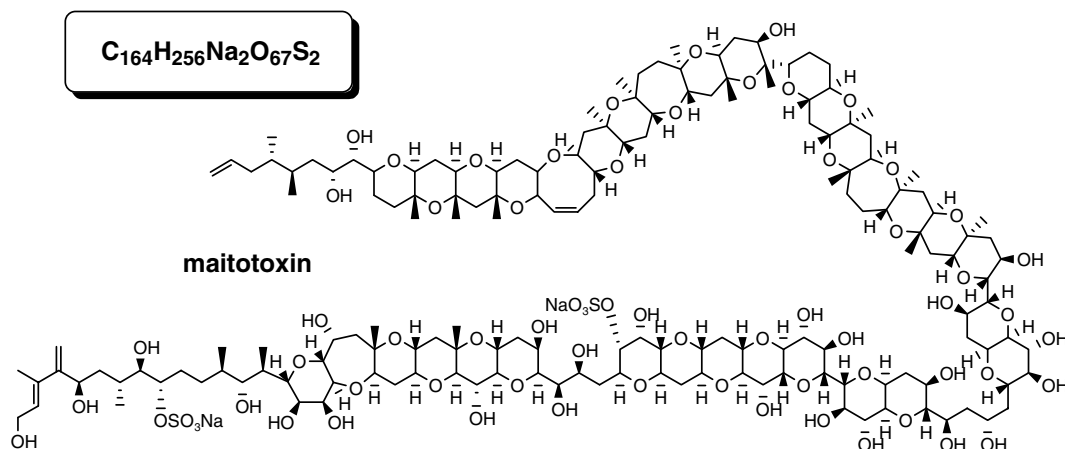


Fig. 3. Maitotoxin with a structure mainly elucidated by extensive NMR studies (Murata et al., 1994; Nonomura et al., 1996; Sasaki et al., 1996).

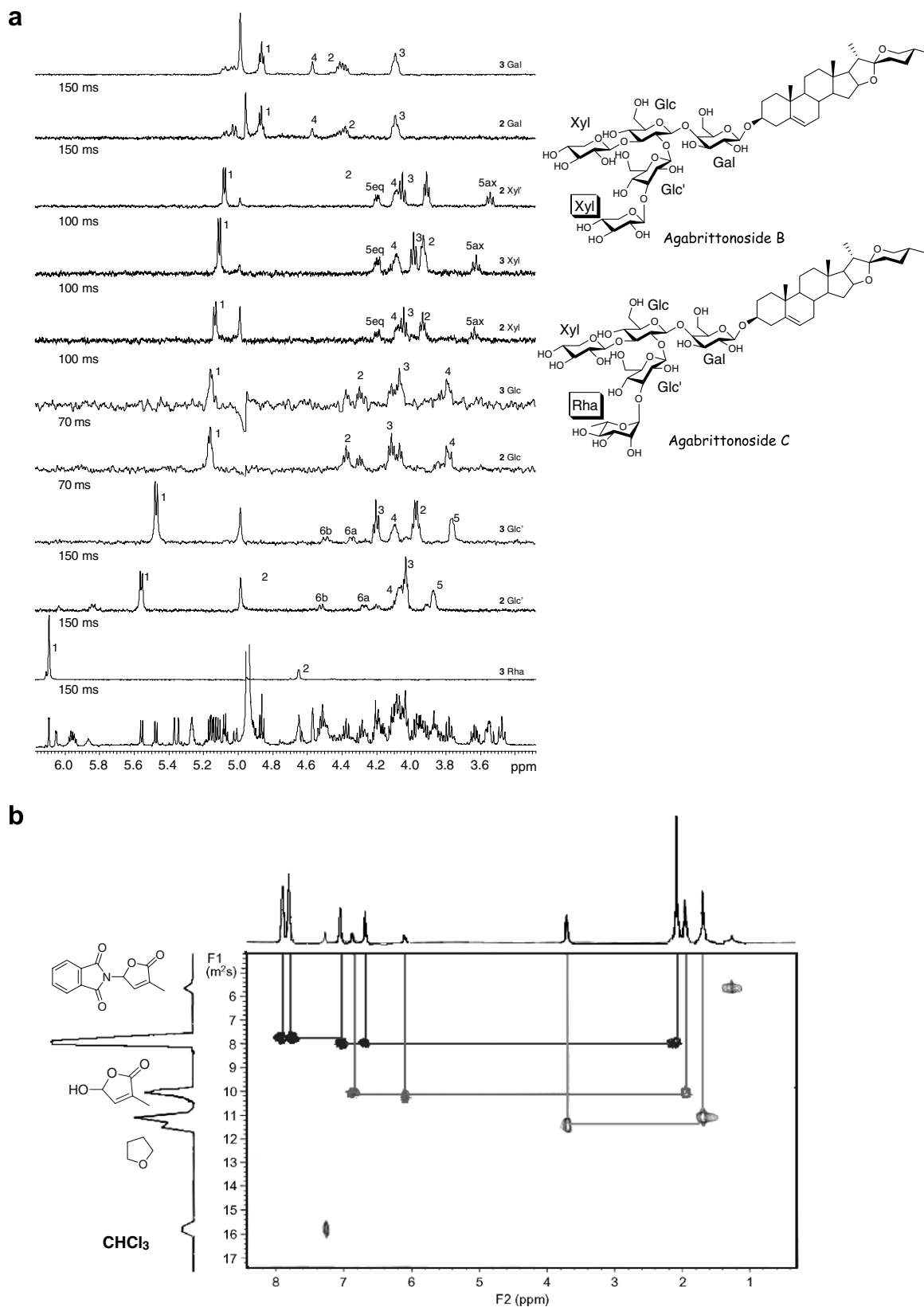


Fig. 4. (a) Elucidation of the structures of agabrittonosides B y C in a mixture using TOCSY-1D and ROESY-1D techniques (Macías et al., 2007b) (© by Journal of Magnetic Resonance, reproduced with permission). (b) Elucidation of a mixture of three compounds in a reaction mixture in the synthesis of sorgolactone-like compounds (Macías et al., unpublished results).

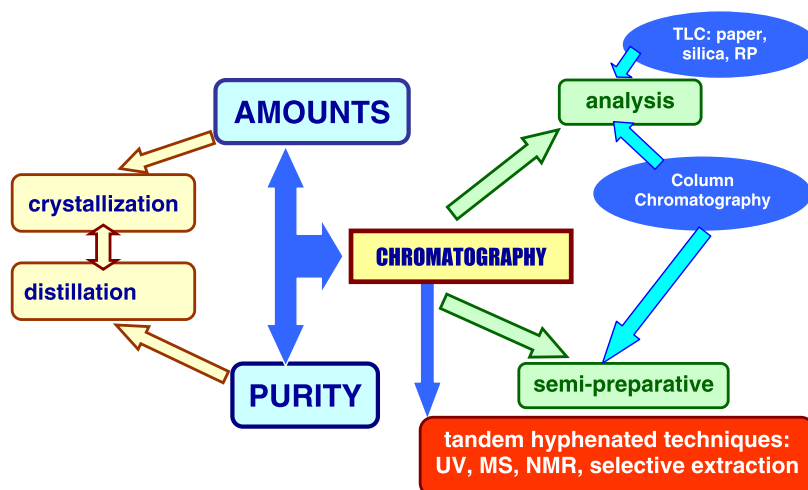


Fig. 5. Evolution of separation techniques in connection with demands of natural products studies.

2.4. From chemical hunting to phytochemical ecology

Phytochemical studies have undergone a marked change from their humble beginnings to the present day. The first studies mirrored the naturalist's point of view at the end of the 18th century and these can be categorized as "chemical hunting": plants were analyzed by searching for their major compounds, mainly for descriptive purposes and/or bioactive properties. Clearly, the isolation, chemical characterization and identification of chemicals were greatly challenged by the methodologies available at a given time. During the first half of the last century, massive amounts of extracts obtained from large quantities of plant material were needed to obtain sufficient crude extract to separate compounds by successive and tedious crystallizations and re-crystallizations, liquid–liquid differential extraction, distillation and other separation techniques. The advances in chromatography and structural elucidation techniques allowed an increase in the number of compounds isolated and characterized and expanded the study of their ecological and physiological roles: the everlasting questions of 'why?' and 'for what?'

The massive expansion of phytochemical studies began during the 1960s and 1970s, when thousands of natural products were isolated from plants all over the world and then structurally characterized. At the same time, these studies were extended to many other organisms, such as microorganisms, insects, lichens, algae and, during the 1980s, marine organisms. Of course, this does not mean that any real advances in describing phytochemicals were not made prior to this time. It simply means that new techniques were available to speed up the process and to lower the detection levels to boundaries that were impossible to reach during the first half of the century. Many authors contributed greatly to this expansion, and names such as Sir Robert Robinson, Ferdinand Bohlmann, Nikolaus H. Fischer and Antonio G. González should be mentioned. At the same time, a large number of scientists of outstand-

ing professionalism built up a solid body of knowledge by describing hundreds of thousands of plant natural products from different plant sources from different ecosystems. A large number of minor compounds were described and these results have been used to propose primary metabolic pathways and to explain the biogenesis of many important natural products. The work of these scientists has enabled many other studies and advances in phytochemistry and has also provided new points of view. Three of these areas deserve to be highlighted: chemotaxonomy, ethnobotanical studies and phytochemical ecology.

Aspects of the chemotaxonomic approach are of interest in the discussion of natural products as tools for phylogenetic discussion and classification of species and taxa. Once the chemical compositions of many plants had been described, scientists realized that the plant's chemical profile could be used as a clue for plant classification (Herout, 1966). Indeed, many controversies initiated by chemotaxonomy and the potential changes in plant classification that arose at that time were later confirmed by modern DNA studies. One example was the different stereochemistry shown by sesquiterpene lactones from Umbelliferae and Compositae, a fact explained by different metabolic pathways arising from different phylogenetic branches (Holub et al., 1987).

Even though chemical studies are still used for chemotaxonomy (Benedek et al., 2007; Ott et al., 2007; Nikolova and Ivancheva, 2006) and have incorporated the use of advanced informatics (Da Costa et al., 2005), this approach has been surpassed by DNA phylogenetic and taxonomic studies. The utility of these chemical techniques is challenged by the fact that secondary metabolite profiles are subject to change under environmental conditions. However, they have been and continue to be of use as a complementary approach to DNA studies (Komatsu, 2001; Zidorn, 2006) or for the study of the basis of chemo-differentiation in connection with molecular studies (Tetenyi, 2002). Such research is related to the study of plant

evolution, as the increasing complexity of natural products can be traced through almost all plant families from less evolved systems to more modern ones.

At the same time as “pure” phytochemical studies were performed and chemotaxonomic approaches applied as a new tool based on these results, two other important applications of these results were developed: the ethnobotanical and ecological approaches. With regard to the ethnobotanical approach, humankind has used plants from prehistoric times for a number of purposes: drugs, venoms, hallucinogens, etc. The discovery of their uses was based upon the close contact of man with Nature and the observation of animal behaviour. This observation expanded to experimentation through the collection of ancient knowledge and oral traditions. Thus, in 1896 the botanist William Harshberger coined the term ethnobotany to describe the study of plant use by humans. This knowledge has been transmitted down through the ages and is now an integral part of most traditions (Talhouk et al., 2007; Koorbanally et al., 2006). This area has given rise to the ethnopharmacy concept, which encompasses all aspects (including phytochemical) of drug searches based on an ethnobotanical approach. Ethnographic studies can, and in fact do, give clues about plants with bioactive properties. These plants then become targets of chemical studies in the search for bioactive compounds. Such an approach has been used extensively in the discovery of new bioactive compounds with applications, mainly as pharmaceuticals, to address all kinds of human afflictions as anti-inflammatory agents, to treat digestive disorders and as painkillers, but also anticancer and antiviral compounds.

There are many interesting examples of successful ethnobotanical studies. The alkaloid camptothecin was isolated in 1966 from *Camptotheca acuminata* (Wall et al., 1966) and was finally approved as an anti-cancer agent by the FDA in 1996 in the form of two pro-drug derivatives named irinotecan and topotecan (Heinrich and Bremner, 2006). This is a representative and famous example (Fig. 6).

Many of the most important pharmaceutical companies have ongoing research programmes to discover new drugs based upon the enormous biodiversity in jungles and forests and the intimate botanical knowledge of the people living there. The ethnobotanical approach to phytochemical studies is of such importance that questions concerning the rights of the owners of the original knowledge and the rights of the countries where the compounds are discovered have arisen. The Convention of Biological Diversity (“The Rio Convention”) addresses these matters and encourages the sharing of the potential benefits and profits of any discoveries made between the companies, the countries and the indigenous tribes that are the source of the original knowledge.

Sustainability is another important point of discussion that often arises, as the synthesis of the natural compound may not be economically viable and the compound has to be harvested directly from its source, thus endangering the survival of the species. Taxol (Fig. 6) was isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) (Wani et al., 1971) and was originally approved as an anticancer drug for the treatment of ovarian (1992) and mammary (1994) carcinomas. Demands on this compound endangered the species until a closely related analogue named bacattin III was obtained from a more available source (*Taxus baccata*) and used as a starting material in its hemisynthesis. Alternative methods for drug production include cultivation of the target species, synthesis and biotech production (e.g., callus cultures, biotech methods, genetically modified microorganisms). These approaches closely follow the original phytochemical study in order to assess the quality and yield of the production and the purity of the final product. To be economical, these methods must also include fast analytical techniques.

As chemicals from different plants were being isolated and characterized, many scientists were becoming aware of connections between the chemical content of plants and important ecological facts and observations related

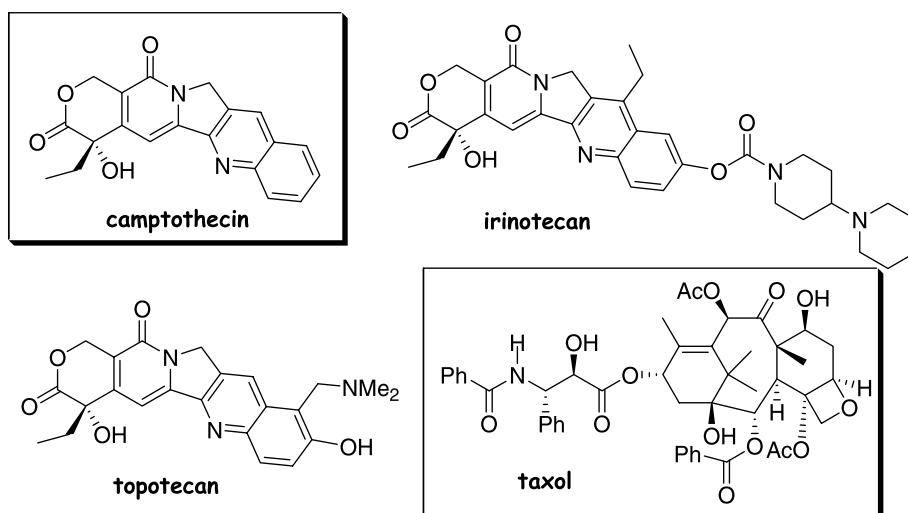


Fig. 6. Anticancer agent camptothecin and the derivatives irinotecan and topotecan developed through ethnobotany studies.

to the relationships between plants and other living beings in their ecosystems. How and when this link was identified is difficult to say. However, it was clearly acknowledged that secondary metabolites produced by the plant must have an important role in both plant self-regulation and plant interaction with the ecosystem. The pioneering work carried out by Professor J.B. Harborne stands out in the area of phytochemical ecology, without dismissing the contributions of many other important scientists. Besides the scientific achievements of Professor Harborne in the area of phytochemical ecology, his work also gave birth to a comprehensive treatise entitled “Ecological Biochemistry” (Harborne, 1977, 1993). This opus initiated and gave form to what we now know as phytochemical ecology as an important part of ecological biochemistry. In the following section we will try to briefly summarize some of the most important milestones in the field of Phytochemical Ecology and, by extension, Ecological Biochemistry.

All of these approaches illustrate the history and the changes that have occurred in phytochemical studies during the last century and represent a reasonable picture of the current situation. Among these areas, phytochemical ecology warrants a more detailed analysis and this is provided by the following section.

3. Phytochemical ecology: some important milestones

It is now accepted that the synthesis of toxic secondary metabolites by plants for defence purposes may be constitutive or inducible. However, the real situation is more complex. The same compounds may also be sequestered by phytophagues, involved in symbiotic relationships, or play a role as chemical signals for other purposes (ovoposition, feed stimulants, etc.) (Harborne, 1999). In any case, it is impossible to separate the evolution of phytochemical ecology from the development of ecological biochemistry. As will be shown in the following sections, despite the uses that mankind may have for natural products, they are compounds that have two main purposes in the organism that produces them: self-regulation (hormones and protectors against abiotic stress) and communication with individuals within the same species (pheromones) or belonging to another species (allomones). In a broad sense, all of them should be addressed as semiochemicals, “message carriers”, even though the nomenclature itself is not yet commonly agreed upon by the scientific community. Starting with the discovery of phytohormones in plants as a defence against insects (Takemoto et al., 1967; Imai et al., 1967), through the discovery of gibberellins (Yabuta and Hayasi, 1939) and other natural phytohormones (Teale et al., 2005) that control plant metabolism and development, and ending with allelopathy as the last discipline added to the chemical ecological study of plants, we will discuss in this section why natural products are described as a result of evolutionary and co-evolutionary forces. In this scheme they play a role in self regulation (Li et al., 2007; Wu

et al., 2006; Covarrubias et al., 2006), plant defence (Harborne, 1993, 1999) and communication with the environment (Harborne, 1993; Silva et al., 2006; Kaufman et al., 2005; Szabo et al., 2003). In addition, the encapsulation of potential autotoxic products (not only secondary metabolites, but also defence toxic proteins, e.g., Liu et al., 2006) in vacuoles (Iglesias and Meins, 2005), trichomes (Antonious et al., 2005; Lahtinen et al., 2004; Wagner, 1991) and other organs (e.g., polymethoxyflavones in orange peel, Ortuno et al., 2006) will be discussed as examples of defence strategies. As an example of direct co-evolution, specialized herbivores often take advantage of defence compounds synthesized by plants and sequester them (e.g., butterflies, Engler-Chauat and Gilbert, 2007; Nishida, 2002) for their own protection.

3.1. The discovery of phytohormones

One of the discoveries that have probably been the most helpful in changing the viewpoint about natural products and their role in organisms was that of compounds that regulate the biochemical processes within individuals.

The discovery of moulting hormones (MH) in insects dates back to 1954, when ecdysone was isolated from half a ton of silkworm pupae (Btenandt and Karlson, 1954). The structure of this compound (Fig. 7) was not elucidated by X-ray diffraction until ten years later (Karlson et al., 1965) for the reasons pointed out in Section 2.1. However, the most striking aspect was not only the fact that this compound was present in such small quantities, but that it was able to govern the entire moulting process of the silkworm. This showed that secondary metabolites were of great importance in self-regulation in insect development. More startling was the fact that soon afterwards, 20-hydroxyecdysone (also isolated as a minor compound along with ecdysone from silkworm pupae, Fig. 7) was present in the chemical composition of several plants in massive amounts in comparison with those obtained from the insect. The same amount obtained from the pupae (25 mg) was obtained from only 25 g of leaves from the common yew (*T. baccata*) (Takemoto et al., 1967) and other yew species (*Taxus cuspidata*) (Imai et al., 1967). Moreover, an equivalent amount could be isolated from just 2.5 g of rhizomes of sweet fern (*Polypodium vulgare*) (Jizba et al., 1967) and bracken fern (*Pteridium aquilinum*) (Kaplanis et al., 1967; Takemoto et al., 1968). Several other plants were also found to contain large amounts of these and other structurally related compounds (ponasterones, cyasterone, etc.; Fig. 7) (Imai et al., 1967).

These compounds evidently play a protective role in the plant, as no other benefits or functions could be found for their presence or their biosynthesis in terms of energy (ATP, NADPH) or carbon allocation. The evolution of genes that encode the enzymes for the whole pathway would be an incredible waste of resources if benefits were not derived from it. Other compounds with similar structures and even higher activities than ecdysones were later

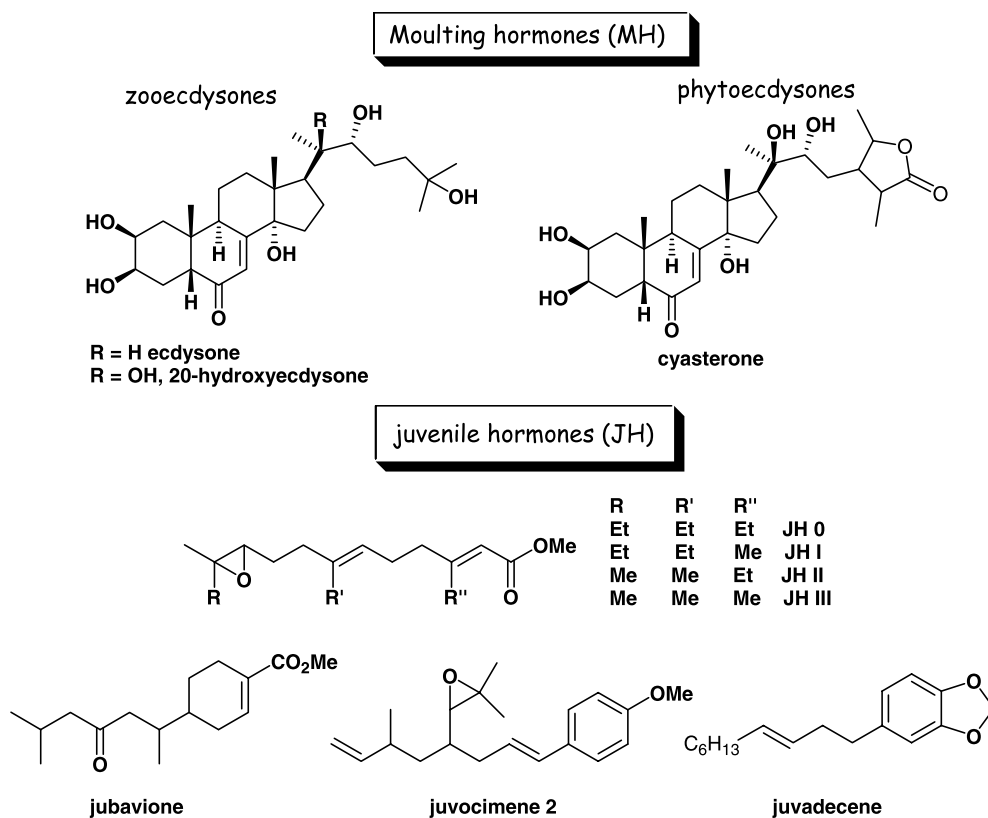


Fig. 7. Juvenile and moulting hormones in insects and compounds with moulting and juvenile hormonal activity found in plants.

isolated from different plants and ferns, giving rise to a complete class named phytoecdysteroids comprising more than 200 different compounds (Dinan, 2001). The story goes far beyond this point, but an important note to this discussion is the fact that phytoecdysones are mainly present in plants that are ancient in terms of evolution: almost 30 different ferns and eight gymnosperm species, with very few angiosperms. This finding has been explained in terms of an early mechanism of defence against insects that has been improved upon through evolution by more sophisticated methods in angiosperms.

The discovery of MHs was soon followed by that of the juvenile hormone analogues in plants – jubavione (Bowers et al., 1966), jувocimenes I and II (Bowers and Nishida, 1980) and jувadecene (Nishida et al., 1983), a class of compounds with a very limited number of examples (Fig. 7). However, these compounds are effective in regulating the development of certain insects, maintaining them in an immature and never-ending larval state. Despite their relatively low number, the usefulness of these compounds as pest controllers has caught the attention of many scientists (e.g., Henrich and Weinberger, 2005).

3.2. The theory of phytoalexins

The phytoalexin theory was another major breakthrough in the development of phytochemical ecology. The phytoalexin theory was proposed by Mueller and Bör-

ger (1940; Mueller, 1966) as a kind of immunologic system of defence against microorganisms. Phytoalexins were defined as plant-derived antibiotics that were synthesized *de novo* in living plant tissue as a response to pathogenic attack. The phytoalexin response is extremely localized and is subjected to healthy tissue surrounding the place of infection. Also, the phytoalexins are not a constitutive part of the plant's chemical composition, but are induced upon tissue damage. This aspect was not fully proven until the first phytoalexin was isolated (Cruickshank and Perrin, 1960) and structurally characterized (Perrin and Bottomley, 1961) from *Pisum sativum* plants infected with *Sclerotinia fructicola* some twenty years later. Interestingly, the isolation and chemical characterization both occurred at almost the same time as plant phytohormones were described, again showing the importance of advances in chromatographic separations and spectroscopic techniques in the characterization of secondary metabolites. These facts contributed to the gradual change in the vision of phytochemistry and, subsequently, of chemical ecology.

After the initial discovery of the first phytoalexin, many other compounds were identified as belonging to several skeletal types: isoflavones in Leguminosae (Barz and Welle, 1992; Dixon et al., 1993), sesquiterpenoids in many Solanaceae (Kuch, 1982; Dixon et al., 1993), diterpenoids in rice and other Gramineae (oryzalexins and momilactones) (West et al., 1990; Peters, 2006), tryptophan-derived sulphur-containing phytoalexins in Brassicaceae (Pedras

et al., 2003), stilbenes like resveratrol (Jeandet et al., 2002) and many others (Strange et al., 1994), along with numerous other examples (Coxon, 1982) (Fig. 8). These findings could be considered as indicative of separate evolutionary paths through which each family of plants (Solanaceae, Leguminosae, etc.) has ended up with different skeletal types for phytoalexin production (even though there are examples of plants belonging to different families that may produce phytoalexins from the same biosynthetic pathways, e.g., phenolics).

The fact that the *de novo* production of phytoalexins under pathogen stress takes place is now generally acknowledged. It is also widely accepted that the synthesis of protective compounds against diseases provides an advantage in those plants in which this process occurs. In fact, disease sensitive and resistant plant genotypes may be characterized, among other traits, by their response time: those plants that are able to synthesize phytoalexins faster will be able to control the disease at the early stages and will become resistant. This is a trait that can be

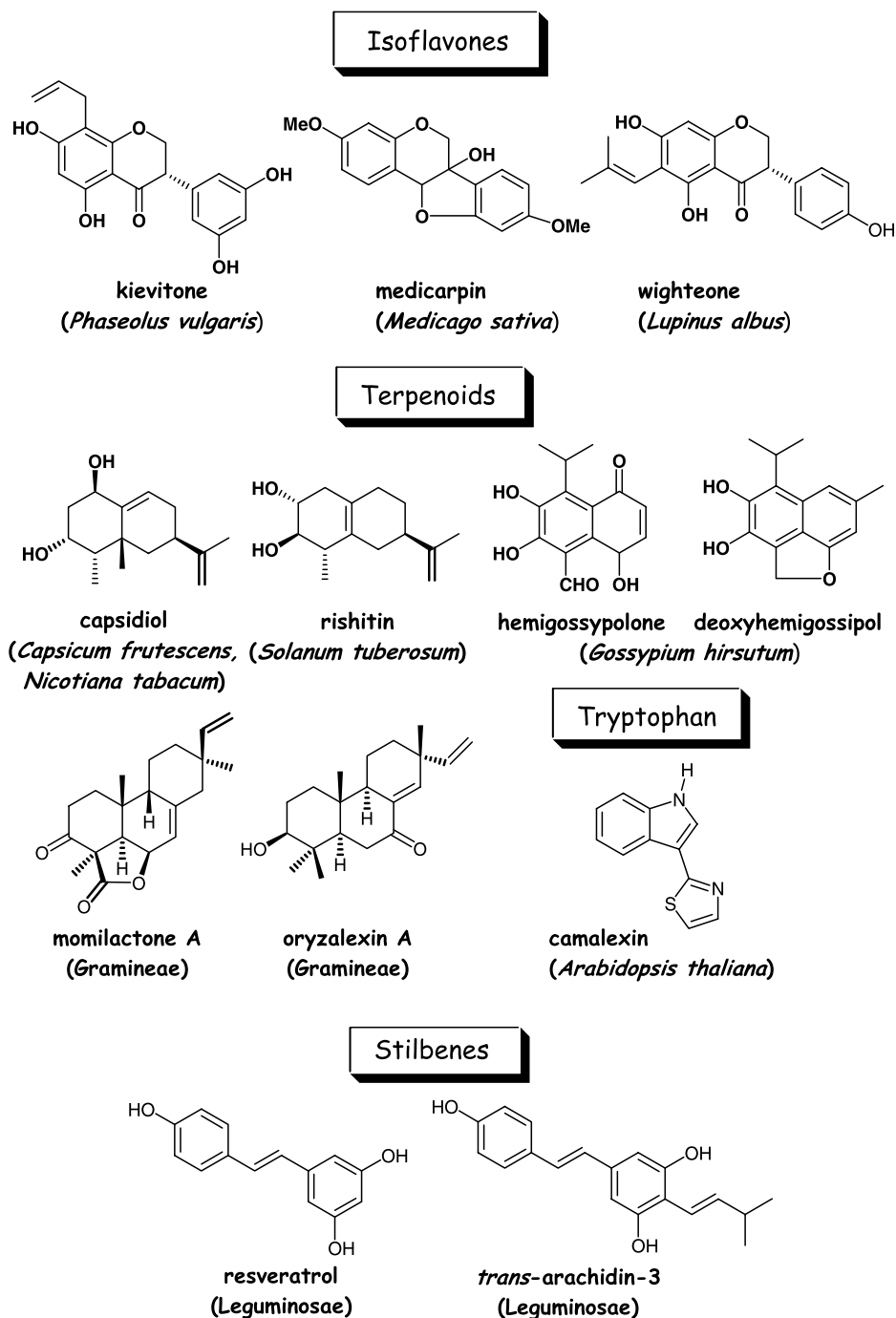


Fig. 8. Main families of phytoalexins.

enhanced or lost through breeding and thus provides indirect proof that plants probably developed the ability through evolution.

Moreover, plant chemical defences against diseases are not only limited to phytoalexins as inducible defence mechanisms. Many plants present a systemic defence mechanism based on constitutive pre-infectious fungicidal metabolites (prohibitins) or systems that may be enhanced upon infection (inhibitins). Furthermore, many plants synthesize and store anti-fungal compounds as non-active precursors that are released upon tissue injury (post-inhibitins). Once again, it is clear that all of these defence mechanisms have a high metabolic cost that would eventually disappear during plant evolution if advantages were not obtained from this investment.

3.3. Allelopathy

Allelopathy, a term coined by *Molisch (1937)*, is another word of Greek origin; “allelo” (respective) and “pathos” (damage). The concept was developed at around the same time as the phytoalexin concept, but the theory could not be demonstrated until the isolation of corresponding causative allelopathic agents. The first allelopathic agents to be characterized were simple phenolic and volatile substances from desert plants (e.g., 3-acetyl-6-methoxybenzaldehyde, camphor, pinenes, cineoles) (*Friedman, 1987*). Since then, secondary metabolites belonging to many skeletal types (e.g., simple phenolics, flavonoids, terpenoids, alkaloids, fatty acids, polyacetylenes, alkaloids, sulphur compounds, oligopeptides) belonging to terrestrial (*Rice, 1984; Macías et al., 2007c*) and aquatic (*Macías et al., 2007a*) ecosystems have been acknowledged as allelopathic agents (*Fig. 9*).

Although the three different concepts about secondary metabolites involved in plant defences (insects, microorganisms, and plants) arose in phytochemical studies at approximately the same time, between 1940 and 1960 their development has been certainly very different. The phytoalexin theory, and the fact that plants synthesize insect hormones or their analogues for self-defence, has been enthusiastically accepted by the scientific community.

However, the acceptance of the allelopathy phenomenon has been, and still it is, controversial. Phytotoxicity of secondary metabolites is relatively easy to check. However, for a compound to be named as a causative allelopathic agent it has to be released into the environment and taken up by the target plants. The main concern of most scientists is the difficulty in showing the causal-effect correspondence between the reputed allelopathic agents and any ecological effect related with plant distribution and/or predominance. In this way, the plant invasive species has been claimed as a paradigm for allelopathy based on the “novel weapon hypothesis”. Release of allelochemicals by a foreign plant into the environment may result in its predominance if native plants/microorganisms lack the proper detoxification/defence mechanisms developed for the other toxins within their ecosystem through co-evolution (*Hierro and Callaway, 2003*). In fact, this hypothesis was supported by recent work on spotted knapweed, where data obtained supported the flavonoid (–)-catechin as the causative allelopathic agent of its invasive behaviour (*Bais et al., 2003*). There is still some controversy in this area as a recent publication (*Blair et al., 2006*) reported the impossibility of reproducing the results previously published for spotted knapweed with regard to the levels in the soil and subsequent phytotoxic effects. This research reinforces the belief that allelopathy is especially difficult to demonstrate in the field – more than in the previous two cases of plant–environment interactions (microorganisms and insects). We have been able to demonstrate and quantify to a reasonable extent under controlled laboratory conditions that allelochemicals are exuded from Gramineae and taken up by the target plant in a dose high enough to induce a deleterious effect (*Macías et al., manuscript in preparation*). However, it is difficult to extend these experiments to field conditions. In any case, indirect evidence of the allelopathic phenomenon has been provided in a number of cases (*Macías et al., 2007c*).

The case of momilactone B is worthy of further comment. Momilactone B was considered to be a phytoalexin, as the presence of similar compounds in its family was detected and enhanced in rice plant tissue under attack

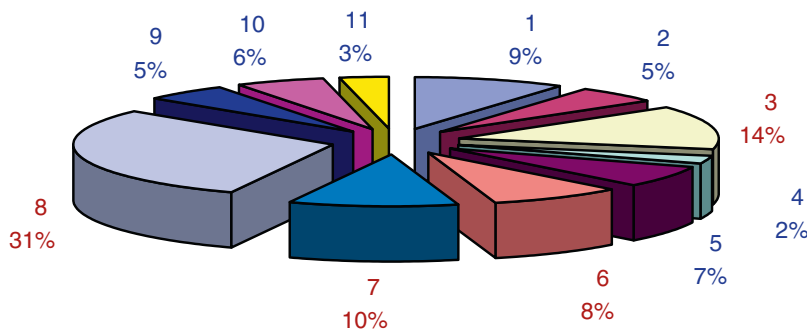


Fig. 9. Classification of allelopathic agents and relative abundance according to results published until year 2001. Key: 1: simple aliphatic compounds; 2: fatty acids and polyacetylenes; 3: benzoic and cinnamic acids; 4: quinines; 5: coumarins; 6: flavonoids; 7: monoterpenes; 8: sesquiterpenes; 9: other terpenoids; 10: nitrogen-containing compounds; 11: sulphur-containing compounds. Classes number 3, 6, 7, and 8 account for 63% of total number of compounds, being the most widespread and cited allelopathic agents.

by *Pyricularia oryzae* (Cartwright et al., 1977), UV irradiation (Kodama et al., 1988) and exogenous treatment with *N*-acetylchitoooligosaccharides (Yamada et al., 1993). However, it was not until recently that their possible role as allelopathic agents has been studied in depth. A single compound cannot have a role as a phytoalexin and allelopathic agent at the same time, as allelopathic agents are constitutive defence compounds while phytoalexins need to be expressed and synthesized *de novo*. However, Wilderman et al. (2004) showed that the mRNA levels encoding a specific syn-copalyl diphosphate 9 β -pimara-7,15-diene synthase were up-regulated in leaves subjected to conditions where phytoalexin production was stimulated, but it was constitutively expressed in roots, where momilactones are constantly synthesized and released into the soil. The differential expression of genes is not uncommon, but this case constitutes a good example to show how genomics can help to explain two apparently contradictory facts: how an allelopathic agent can be, at the same time and in the same plant, a phytoalexin, thus making a point for the “economy of resources” principle that could be operating in the metabolism of diterpenoids in rice.

3.4. Sequestration

Sequestration of toxic compounds by other living organisms is another interesting phenomenon that occurs in Nature and supports the idea of co-evolution. The sequestration and/or use of toxic compounds present in the diet of herbivores (e.g., insects, slugs and nudibranchs) or predators (toads) for defence purposes or as building blocks for their own venoms or pheromones (e.g., butterflies) is a widespread behaviour in Nature. Cardenolides, iridoid glycosides and pyrrolizidine alkaloids are good examples of this phenomenon.

Cardenolides are plant defence compounds that provide reasonable protection from many grazers. However, the monarch butterfly (*Danaus plexippus*) and moths' larvae, both of which feed on leaves of *Asclepias curassavica* (Asclepiadaceae), have become able to store cardenolides for their own defence purposes and the adults become protected against predation by birds (Trigo, 2000; Frick and Wink, 1995). These are classical examples of sequestration. Other common examples of sequestration by butterflies (Danainae and Ithomiinae) and moths (e.g., *Tyria jacobaea*, *Nyctemera annulata* and *Utetheisa ornatrix*) involve pyrrolizidine alkaloids (PA) contained in plants of the genus *Senecio* and Boraginaceae and in the tribe Eupatorieae (Seigler, 1998). Even though it is difficult to prove the acquired resistance through toxin sequestration in more than two trophic levels, it has been proven to a reasonable degree that under controlled laboratory conditions plant-acquired PA may provide effective protection for phytophagous against their predators (Narberhaus et al., 2005).

Defence may not be the only purpose or advantage for plant-toxin sequestration. Butterflies of the families Danaidae and Ithomiinae (Edgar, 1982; Schulz et al., 2004) are

able to detoxify the pyrrolizidine alkaloids present in the plant composition on which they feed to serve as building blocks for the biosynthesis of their own male sex pheromones danaidal, hydroxydanaidal and danaidone, among others. These compounds are usually stored in the insect hair pencils. Plant-acquired open chain pyrrolizidine alkaloids have also been reported to serve as precursors for other insect-specific compounds that might be maintained or changed through the different insect developmental stages (Hartmann et al., 2005). Other examples can be observed in many ecosystems and these are not only terrestrial. Thus, it is common to find clues about sequestration in nudibranchs (Andersen et al., 2006; Cronin et al., 1995), terrestrial snails (Hesbacher et al., 1995), frogs (Saporito et al., 2004), Lepidoptera (Nishida, 2002) and bugs (Aliabadi et al., 2002) (Fig. 10).

In most cases, sequestration leads to a close specific plant–predator relationship. In these situations most of the vital cycle of the herbivore takes place in the same plant(s). For example, this process is common in many insects that feed on Cruciferae, where the main defence chemicals are glucosinolates (Mueller and Sieling, 2006), but also in other families like Ranunculaceae (Prieto et al., 2007).

Many other cases in which the sequestration of plant toxins takes place have been reported. These examples are interpreted in terms of a dynamic process of insect adaptation to the plant mechanisms of defence (Mello and Silva-Filho, 2002; Dobler, 2001). Co-evolution again plays an important role in explaining secondary metabolite diversity, not only in plants but also in other organisms.

3.5. Co-evolution and co-adaptation

In a broad sense, plants have to defend themselves from attack by phytophagous (mainly insects, but also animals and birds) and microbes, but they also have to face competition with other plants for the soil, nutrients and other resources (Fig. 11). This is a situation that has been taking place from the very beginning of plant history, as all of these organisms have been sharing the different ecosystems since plants appeared on earth. Through millions of years of evolution, environmental pressure has ensured that each plant species ends up with its particular solution to the survival problem, thus creating enormous biodiversity. Several morphological and biochemical adaptations have arisen through evolution to solve this problem and ensure plant survival, with a kind of chemical warfare based on toxic secondary metabolite production, storage and, eventually, release into the environment being one important strategy. However, the role that plant secondary metabolites may play in evolution has not been proposed until recently. Ehrlich and Raven (1964) postulated that “the evolution of secondary plant substances and the stepwise evolutionary responses to these by phytophagous organisms have been clearly the dominant factor in the evolution of butterflies and other phytophagous groups” and they were pioneers

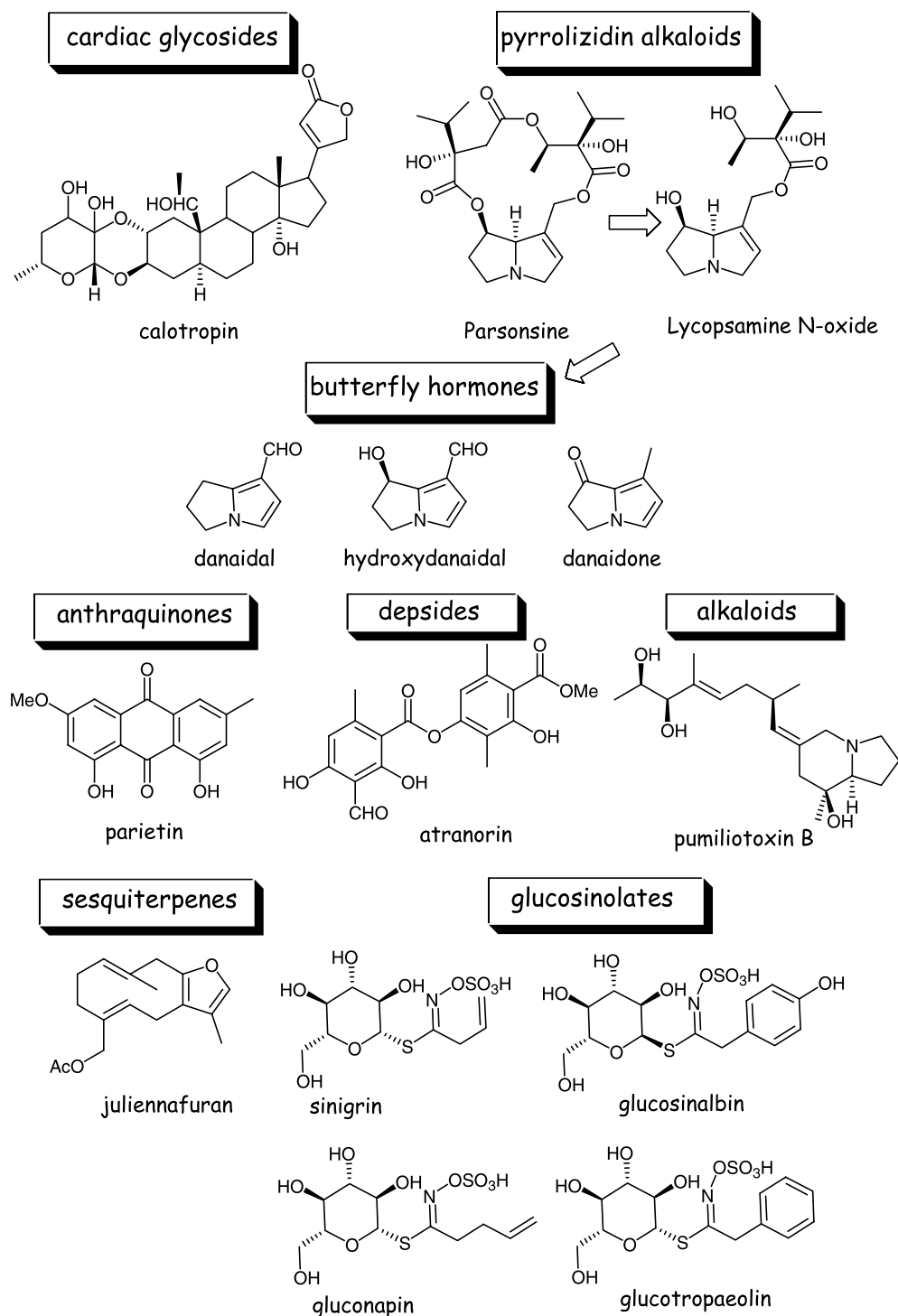


Fig. 10. Examples of secondary metabolites sequestered by several organisms. Cardiac glycosides and pyrrolizidine alkaloids (PA) are sequestered by many families of butterflies (Nishida, 2002) and/or stored and used as building blocks for hormone biosynthesis. Anthraquinones and depsides are sequestered by snails feeding on lichens (Hesbacher et al., 1995). Pumiliotoxin is sequestered by frogs from arthropods (Saporito et al., 2004). Juliennafuran is sequestered by the nudibranch *Tritonia hammerorum* feeding on the sea fan *Gorgonia ventalina* (Cronin et al., 1995). Glucosinolates are common examples of sequestration by the specialized harlequin bug *Murgantia histrionica* feeding on Brassicaceae (Aliabadi et al., 2002).

in envisioning an adaptative point of view to justify the presence and diversity of secondary metabolites: “plants and phytophagous insects have evolved in part in response to one another”. According to this concept, the synthesis and accumulation of plant toxins occurred first as a

response to insect grazing pressure producing avoidance in all species. Co-evolution of plants and insects then forced adaptation by a few insect species, with the deterrent becoming a feeding attractant; as more species become adapted, an action–reaction scheme involving the two play-

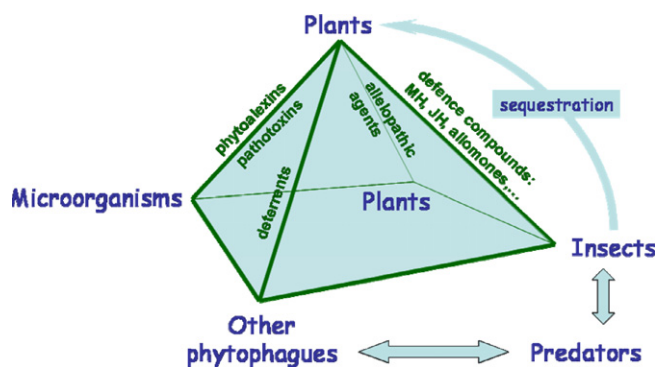


Fig. 11. Schematic representation of "chemical warfare" relationships among plants and competitors/enemies/phytophagues.

ers in this scenario takes place where more species become adapted and new toxins are synthesized by the plant, thus leading to a highly specific relationship between some insects and their feeding sources.

Secondary metabolism is not a static process. Rather, it changes in response to numerous factors. The synthesis of chemicals for defence implies a flow of precursors (carbon and nitrogen compounds), enzymes and energy-rich molecules (NADH, ATP) from the primary metabolism to the biosynthetic pathways. In addition, the plant response to phytophagous grazing pressure is greatly influenced by the environmental availability of resources (Silva et al., 2006), especially of nitrogen as carbon can be obtained through photosynthesis. This factor should be of particular importance in those plants with nitrogen-based toxins due to the higher metabolic cost of nitrogen-based compounds. However, it has been demonstrated that nutrient availability also influences allocation in carbon-based toxins. Consequently, deviation of resources in one or other direction is directly related with defence or growth/development costs (Herms, 1999). This situation represents a dilemma that the plant needs to solve at each point, depending on environmental pressures, in order to ensure survival – thus producing a dynamic response. Several hypotheses have been proposed concerning the way in which plants address this situation and modify their secondary metabolite content and the function of their biosynthetic pathways in order to better optimize their resources.

Most of the theories that address changes in carbon allocation focus on phenolics, probably because they are the most widespread and also the most studied type of secondary metabolites as they are present in all terrestrial higher plants (Harborne, 1997). In comparison, little has been published on the changes in, for example, total terpenoid (Gershenson, 1994) or alkaloid content (Frischknecht et al., 2001). This fact is also due to the lack of appropriate analytical methods to measure total terpenoid or alkaloid contents, a situation in contrast to the methods that exist for phenolics (e.g., Nitao et al., 2001; Julkunen-Tiito and Sorsa, 2001).

To date, the three main hypotheses that address the dynamic response of plant metabolism to environmental

factors are the Carbon-Nutrient Balance (CBN), the Growth-Development Balance (GDB) and the Protein Competition Model (PCM).

The CBN hypothesis (Bryant et al., 1983) proposes that changes in secondary metabolism are a result of imbalances between requirements (C and N) for growth and availability in the environment. Carbon-based metabolites positively correlate with the carbon-nutrient balance: when this balance is positive there is more carbon available than nutrients and, in this case, phenolic allocation is predicted to occur. Consequently, the theory predicts that under enhanced CO₂ conditions (e.g., a scenario of climatic change with high levels of greenhouse gases) in which nutrient availability does not change but more carbon is available, toxin (phenolic) production will be enhanced because of nutrient limitation. This will result in lower food quality for herbivores and may change the relationships between plants and insects and will challenge feeding behaviours for many insects and other phytophagues.

The growth-differentiation balance (GDB) (Herms and Mattson, 1992) addresses these matters by suggesting the classification of plants into two broad categories: (i) fast-growing plants with a dominance of growth over systemic chemical defence system, which may be highly inducible, and (ii) slow-growing plants with high basal levels of toxin in their tissues that provides them with a perennial and constitutive defence that, in turn, is poorly induced to higher levels. The Systemic Induced Resistance (SIR) hypothesis proposes that previously uninfected parts of the plant may develop systemic resistance mediated by (or associated with) the accumulation of certain compounds after hypersensitive cell death caused by a pathogen. In this way sensitization should work as a kind of immunologic system (Bonello and Blodgett, 2003; Van Loon, 2000). After the induction event (tissue injury caused either by a microbe or an insect) the SIR hypothesis predicts that SIR will be sustained for an extended period of time by enhancing the chemical machinery involved in toxin production (e.g., phenolics in coniferous trees) as long as carbon/nutrient assimilation can maintain this process. However, since more resources are being allocated in defence, continuous induction may result in breakdown of the plant's defence system and collapse of the plant resistance. In this case it is predicted that Systemic Induced Susceptibility (SIS) is expressed. As long as SIR is expressed, carbon flow to secondary metabolite production will slow plant growth (Bonello et al., 2006), a situation in good agreement with the GDB hypothesis. In fact, this is the expected situation in a healthy plant living in an ecosystem: a continuous state of "alert" due to insect wounding and pathogen attack. However, if the level of induction is too high or the nutrient resources are cut, this situation may finally end with the death of the plant. Coniferous trees living in temperate-to-cold ecosystems are good examples of this situation as they are slow-growing species with a strong constitutive defence system based on phenolic compounds.

SIR has been demonstrated to occur in several pine tree species (Bonello et al., 2006).

The PCM (Jones and Hartley, 1999) hypothesis focuses specifically on phenolic allocation. Phenylalanine ammonia-lyase (PAL) enzyme is a crossover point between primary and secondary metabolism as it is involved both in phenolic and protein synthesis. The hypothesis proposes that phenolic allocation depends on protein demand and the activity of PAL. Protein and phenolic syntheses compete for PAL and high rates of both processes cannot occur at the same time.

All of these hypotheses represent a vision of a dynamic defence response modulated by environmental factors including phytophagous/pathogen attacks and nutrient availability. Since dynamic defence responds to environmental pressure, it is likely that it will force co-evolution, thus explaining, at least in part, the great diversity of toxic secondary metabolites, their use as one plant defence mechanism and their great diversity due to the wide variety of situations that have been operating and prompting plants to find solutions to predatory and disease pressures over millions of years.

4. Final remarks

Phytochemical studies have experienced great change over the last century. The point of view has evolved from a descriptive approach to a global understanding of the role that secondary metabolites play in plants and living beings. This has led to a situation of increasing complexity where phytochemical studies interact with many other disciplines, including plant physiology, pharmacy and drug design, medicine, palaeontology, ecology, and biochemistry. The results obtained from these studies can be applied in many fields such as pharmaceuticals, agrochemicals, evolutionary studies, paleobotany, ecosystem management and conservation and basic science.

Given this scenario, the future of phytochemical ecology and the trends that will unfold is a very open question. Attempts to make such predictions would be like taking a glance into a crystal ball. However, in our opinion there are some facts that cannot be denied and we will try to indicate the fields in which the next advances are likely to occur. That plant toxins are a form of chemical defence towards attack by herbivores and microbes can be considered as demonstrated to a reasonable extent. Also, plant interaction and competition through chemicals is currently accepted by many scientists. Even though new chemicals are being isolated and characterized every day, a deeper understanding of the role that they play in each ecosystem and their influence on the architecture of the biocenosis is still required. Studies into the degradation pathways of secreted allelochemicals, their half-life periods, biotransformation by herbivores, and biological activities should also be common areas of work in years to come. Besides, the expression of secondary metabolism pathways in response

to environmental factors and their connection with genomics, proteomics and metabolomics, aided by advanced informatics and neural framework systems, should focus scientists' attention and will provide answers to such important questions as 'what is the energy cost of toxin production?' and 'how are these traits preserved and transmitted through evolution?'

Furthermore, the localization of genes that encode the biosynthetic pathways of toxins, in conjunction with molecular biology techniques, will allow the growth of transgenic plants with enhanced resistance to weeds and pests and the development of high-yielding plants for mass pharmaceutical production, which can be cultured and harvested like any other crop.

Advances in the isolation and structural elucidation of secondary metabolites will extend the scope of a range of studies. Hyphenated techniques will be more commonly used than they are today, particularly those that combine selective extraction (e.g., supercritical fluids, high-pressure extraction, etc.) techniques with chromatography. Tandem UV/NMR/MS spectroscopy will allow the resolution of complex mixtures in shorter times and with lower detection limits than now. These techniques will allow the detection of the small amounts of compounds that are exchanged between different trophic levels.

It is our opinion that ecological phytochemistry is yet to render its best results: i.e., a deeper understanding of the architecture of the ecosystems and the roles that secondary metabolites play in plants. As a result of this knowledge, new and useful applications for mankind in such diverse areas as pharmacy, environment management and conservation, global climate change, and green agriculture practices can be achieved based on the chemical relationships between plants and other organisms. It is expected that many other applications are still waiting to be discovered in the future.

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