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Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects

Vincenzo Lattanzio¹, Veronica M. T. Lattanzio² and Angela Cardinali²

¹Dipartimento di Scienze Agro-Ambientali, Chimica e Difesa Vegetale
Università degli Studi di Foggia, 71100-Foggia, Italy; ²Istituto di Scienze delle
Produzioni Alimentari – CNR, Via Amendola, 122/O - 70126 Bari, Italy

Abstract

Plant phenolics are secondary metabolites that encompass several classes structurally diverse of natural products biogenetically arising from the shikimate-phenylpropanoids-flavonoids pathways. Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. Therefore, they represent adaptive characters that have been subjected to natural

selection during evolution. Plants synthesize a greater array of secondary compounds than animals because they cannot rely on physical mobility to escape their predators and have therefore evolved a chemical defence against such predators.

This article, after a short review of plant phenols and polyphenols as UV sunscreens, signal compounds, pigments, internal physiological regulators or chemical messengers, examines some findings in chemical ecology concerning the role of phenolics in the resistance mechanisms of plants against fungal pathogens and phytophagous insects.

1. Introduction: Physiological and ecological significance of secondary metabolism

Phenolic compounds are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants. As stated by Harborne [1], the term "phenolic" or "polyphenol" 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.): as a general rule, the terms phenolics and polyphenols refer to all secondary natural metabolites arising biogenetically from the shikimate-phenylpropanoids-flavonoids pathways, producing monomeric and polymeric phenols and polyphenols. Phenol itself is a natural product but most phenolics have two or more hydroxyl groups. Unless they are completely esterified, etherified or glycosylated, plant phenolics are normally soluble in polar organic solvents. Most phenolic glycosides are water-soluble but the corresponding aglycones are usually less so. With a few exceptions, water solubility increases with the number of hydroxyl groups present. Some phenolics are solubilized in sodium hydroxide and sodium carbonate but in alkaline medium their oxidation is enhanced and therefore treatment with alkaline solvents should either be performed under N₂ or preferably avoided. Phenolics with only few hydroxyl groups are soluble in ether, chloroform, ethyl acetate, methanol, and ethanol [2]. Methanol, ethanol, water, and alcohol-water mixtures are most commonly used for dissolving phenolic compounds for analytical purposes. All phenolic compounds exhibit intense absorption in the UV region of the spectrum and those that are coloured absorb strongly in the visible region as well. Each class of phenolic compounds has distinctive absorption characteristics. For example, phenols and phenolic acids show spectral maxima in the range 250-290 nm; cinnamic acid derivatives have principal maxima in the range 290-330 nm; flavones and flavonols exhibit absorption bands of approximately the same intensity at about 250 and 350 nm; chalcones and aurones have an absorption peak of great intensity above 350 nm and a much less intense band at 250 nm; anthocyanins

and betacyanins show rather similar absorption in visible region (475-560 nm and 535-545 nm, respectively) and a subsidiary peak at about 270-275 nm [3, 4].

Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. These compounds form one of the main classes of secondary metabolites and several thousand (among them over 8,150 flavonoids) different compounds have been identified with a large range of structures: monomeric, dimeric and polymeric phenolics. Several classes of phenolics have been categorized on the basis of their basic skeleton: C_6 (simple phenol, benzoquinones), C_6-C_1 (phenolic acid), C_6-C_2 (acetophenone, phenylacetic acid), C_6-C_3 (hydroxycinnamic acids, coumarins, phenylpropanes, chromones), C_6-C_4 (naphthoquinones), $C_6-C_1-C_6$ (xanthenes), $C_6-C_2-C_6$ (stilbenes, anthraquinones), $C_6-C_3-C_6$ (flavonoids, isoflavonoids), $(C_6-C_3)_2$ (lignans, neolignans), $(C_6-C_3-C_6)_2$ (biflavonoids), $(C_6-C_3)_n$ (lignins), $(C_6)_n$ (catechol melanins), $(C_6-C_3-C_6)_n$ (condensed tannins) [5-10].

In contrast with basic metabolism that refers to the anabolic and catabolic processes required for cell maintenance and proliferation, secondary metabolism refers to compounds present in specialized cells that are not directly essential for basic photosynthetic or respiratory metabolism but are thought to be required for plants survival in the environment. Nowadays it is well established that the *raison d'être* of the so-called secondary metabolites could not be accommodated by the idea that they are simply waste products of primary metabolism, accumulating in the plant cell because of the absence of an efficient excretory system [9, 11-13]. Secondary metabolites apparently act as defence (against herbivores, microbes, viruses or competing plants) and signal compounds (to attract pollinating or seed dispersing animals), as well as protecting the plant from ultraviolet radiation and oxidants [14, 15]. Therefore, they represent adaptive characters that have been subjected to natural selection during evolution. This requirement for secondary metabolites to have highly diverse biological activities has led plants to accumulate a vast catalogue of compounds. Plant genomes are variously estimated to contain 20,000-60,000 genes, and perhaps 15-20% of these genes encode enzymes for secondary metabolism, while the genetic complement of the fruit fly (*Drosophila melanogaster*) is substantially lower (13,601 predicted genes). One explanation of this discrepancy in the relationship between biological and genetic complexity may lie in the differences between the ways that plants and animals protect themselves against predators, pests, diseases, and abiotic stress. Animals have developed nervous and immune systems that enable them to detect and respond to danger, and they are capable of avoiding perilous situations. By contrast, plants cannot escape from their biotic and abiotic stressors, being linked to the ground by means of their root system, and therefore they must stay and protect themselves. The production of chemicals

that deter or kill pests and pathogens represents one mean of self-protection. The pattern of secondary metabolites in a given plant is complex; it changes in a tissue- and organ specific way; regularly, differences can be seen between different developmental stages (e.g., organs important for survival and reproduction have the highest and most potent secondary metabolites), between individuals and populations. [16-22].

The defence hypothesis was not accepted by most botanists at that time because they were not convinced of evolution and adaptive explanations. Botanists preferred the simpler interpretation that secondary metabolites were waste products of primary metabolism and that structural diversity would only reflect a play of nature. The potential value of plant secondary metabolites to taxonomy has been recognised for nearly 200 years even if their practical application has been restricted to the 20th century, and predominantly to the last 40 years. The use of secondary compounds has clear advantages over the use of primary compounds in establishing phylogenetic relationships because differences in the complement of secondary compounds are qualitative differences whereas differences in the concentrations of primary compounds are quantitative differences, and these are subject to environmental as well as to genetic control. The existence of a common pattern of secondary compounds may indeed provide much clearer evidence of common ancestry than morphological similarities attributable either to common ancestry or to convergent evolution [22-25].

Phenolic compounds are found throughout the plant kingdom but the type of compound present varies considerably according to the phylum under consideration. Phenolics are uncommon in bacteria, fungi and algae and the classes of phenols recorded are few: flavonoids are almost completely absent. Bryophytes are regular producers of polyphenols including flavonoids, but it is in the vascular plants that the full range of polyphenols is found [1, 26]. Moreover, the existence of a characteristic phenolic pattern, which taxonomists use to separate species, can also have enough adaptive value for survival through natural selection. Phenolic compounds have been synthesized during the course of evolution by different plant species when the presence of a particular secondary metabolite has conferred a selectionary advantage on the plant containing it. More previously stated, plants synthesize a greater array of secondary compounds than animals because they cannot rely on physical mobility to escape to their predators and have therefore evolved a chemical defence against such predators [23]. Generally, the role of phenolic compounds in defence is related to their antibiotic, antinutritional or unpalatable properties. Besides their involvement in plant- animal and/or plant-microorganism relationships, plant phenolics have also key roles as the major red, blue and purple pigments, as antioxidants and metal chelators, as signalling agents both above and below ground between plant and other organisms, and as UV light

screens. This latter property has benefited very much some higher members of the Charophyceae, which are regarded as prototypes of amphibious plants that presumably preceded true land plants, when emerged from an aquatic environment onto the land. Thus the primary established roles of plant phenolics are clearly ecological in nature, some having dual or even multiple functions [27]. Furthermore, it must be stressed that some authors have pointed out that phenolics are often stored at strategically important sites where they play a signalling role, and often a direct role, in defence. Several studies have indicated a high degree of compartmentation of phenolic compounds and of the enzymes involved in their biosynthesis. Phenolics usually accumulate in the central vacuoles of guard cells and epidermal cells as well as subepidermal cells of leaves and shoots. Furthermore, some phenolics are found covalently linked to plant cell wall; others occur in waxes or on the external surfaces of plant organs. Some findings suggest also a deposition of flavonoids in nuclei of certain tree species: it has been suggested that a flavonoid-DNA complex provides a mutual protection against oxidative damage [27-34]. Plant phenolics may be divided in two classes: (i) preformed phenolics that are synthesized during the normal development of plant tissues and (ii) induced phenolics that are synthesized by plants in response to physical injury, infection or when stressed by suitable elicitors such as heavy metal-salts, UV-irradiation, temperature, etc. (phytoalexins). Induced phenolics may also be constitutively synthesized but, additionally, their synthesis is often enhanced under biotic or abiotic stress [35-42].

1.1. UV sunscreens

Plants in the field are exposed to ambient solar ultraviolet-B (UV-B) radiation (280–320 nm) that is an environmental challenge negatively affecting DNA, proteins and membranes, thus leading to altered metabolism through the generation of reactive oxygen species (ROS). Plants protect themselves from this harmful radiation by synthesizing phenolic compounds, which act as a screen inside the epidermal cell layer, and by adjusting the antioxidant systems at both cell and whole organism level [41, 43]. By means of this mechanism phenolics would prevent both mutagenesis or cellular death by dimerization of thymine units in the DNA, which shows an absorption maximum at 260 nm, and possible photodestruction of coenzymes NAD or NADP, which have a maximum at 260nm [26]. It has been proposed that flavonoids with their high absorptivity at 250-270 and 335-360 nm act as good UV screens.

It is noticeable that tropical and high altitude plants contain a higher proportion of flavonoids than temperate ones do. Several studies have demonstrated the change in flavonoid composition of plant leaves because of an excess of light or UV-radiation [44-47]. The activation of flavonoid

biosynthetic genes by UV-radiation has been shown in a number of studies [48].

The importance of flavonoids in UV protection has also been proved using mutants of *Arabidopsis* which have a block in flavonoid production and are, therefore, UV-hypersensitive phenotypes [49]. These studies suggest that other phenolic compounds may be at least as important as flavonoids in UV protection. Speculating about the role of phenylpropanoids as sunscreens to absorb UV-B irradiation in various phenylpropanoid mutants of *Arabidopsis thaliana*, Kliebenstein [13] observed that all phenylpropanoid-deficient mutants exposed to UV-B radiation were more UV-B sensitive in comparison with the wild type but there were dramatic UV-B sensitivity differences between these mutants. These results have suggested that both preformed hydroxycinnamic acid sinapoyl esters and induced flavonols act as UV-B protectant and that the importance of hydroxycinnamic acids and flavonols is directly related to their relative concentrations.

1.2. Phenolics as signal compounds

Several evidences suggest that phenolic compounds influence the pools and fluxes of inorganic and organic soil nutrients. Polyphenols enter the soil mainly as leachates from above- and belowground part plant and/or within above- and belowground plant litter. Phenolic compounds can directly affect the composition and activity of decomposer communities thus influencing the rates of decomposition and nutrient cycling. Different types of soluble phenolics, such as ferulic acid, gallic acid or flavonoids, have been found to either stimulate or inhibit spore germination and hyphal growth of saprophytic fungi. Mycorrhizal fungi might be even more sensitive to phenolic compounds, but again different types of polyphenols can have opposite effects. Plant mycorrhizal infection, nutrient uptake and plant growth can be impaired by specific phenolic released by competitors [7].

Allelopathy refers to the chemical inhibition of one species by another. Commonly this term is most used to describe the chemical interaction between two plants. In plants, allelochemicals can be present in leaves, bark, roots, root exudates, flowers, and fruits. The delivery of allelochemicals into the rhizosphere is often thought to occur through leaching from leaves and other aerial plant parts, through volatile emissions, by root exudation, and by the breakdown of bark and leaf litter. Some identified phenolic allelochemicals are: *p*-hydroxybenzoic acid and *p*-coumaric acid (present in leaves), quercetin, juglone and 2,4-dihydroxy-1,4(2H) benzoxazin-3-one (DIBOA) (present in leaves, bark and root exudates), (-)-catechin and sorgoleone (found in rhizosphere and root exudates) [50, 51]. Bais et al. [52] present evidence that *Centaurea maculosa*, an invasive species in western U.S.A., displaces native plant species by exuding the phytotoxin (-)-catechin from its roots. This

allelochemical triggers a wave of reactive oxygen species initiated at the root meristem, which leads to a Ca^{2+} signalling cascade triggering genome-wide changes in gene expression and, ultimately, death of the root system. In addition to affect the soil microorganisms responsible for nutrient mineralization, phenolic compounds can alter nitrogen availability by complexing proteins [53, 54]. Polyphenol-protein complexes originate either during senescence of plant tissues, when polyphenols stored in the vacuole come into contact with cytoplasmatic proteins [19, 20], or in the soil, when polyphenols complex proteins from litter or extracellular enzymes from microorganisms. These complexes cause the brown colouring of senescent leaves and are resistant to most decomposing organisms, except basidiomycetes with the appropriate polyphenol oxidase activity and earthworms that can directly use a large proportion of nitrogen contained in the complexes.

Plants depend on the ability of roots to communicate with microbes. The converse is also true: many bacteria and fungi are dependent on associations with plants that are often regulated by root exudates. Biological interactions that are driven by root exudates are more complex and include signal traffic between roots and soil microbes, and one-way signals that relate the nature of chemical and physical soil properties to the roots. Specific compounds identified in root exudates have been shown to play roles in these interactions. For example, isoflavonoids and flavonoids present in the root exudates of a variety of leguminous plants activate the *Rhizobium* genes responsible for the nodulation process and might be responsible for vesicular-arbuscular mycorrhiza colonization. Flavonoid profiles in root exudates differ considerably among legumes, and this specificity enables mutualists and beneficial bacteria such as rhizobia to distinguish their hosts from other legumes. [55]. Although rhizobia colonize roots in a way that is reminiscent of pathogenic microorganisms, no host plant defence reactions are triggered during successful symbioses: symbiotic interactions are beneficial to both partners. Nevertheless, the plants obviously control the invading bacteria; failures in effective nodule formation or infections with rhizobia defective in surface polysaccharides often result in pathogenic responses. Symbiosis between leguminous plants and rhizobia involves the *de novo* development of a specialized plant organ, the root nodule. In the nodules, rhizobia fix dinitrogen into ammonia, which is assimilated by the host plant, and, in turn, rhizobia are supplied with carbon compounds. The nodulation process in rhizobia–legume symbiosis requires a sequence of highly regulated and coordinated events, initiated by an exchange of specific signalling compounds between both partners [56]. The flavonoids in root exudates induce in conjunction with NodD protein, the product of the only nodulation (*nod*) gene constitutively expressed by rhizobia, the transcription of an important set of

Rhizobium *nod* genes. The *nod* genes are responsible for the synthesis of a sulphated acylated tetraglucosamine glycolipid (NodRm-1), which is secreted by induced rhizobia and initiates root hair curling and cortical cell division in the infectible zone of legume roots [6, 57-59]. Examples of flavonoids found to be active in the induction of nod gene expression are eriodictyol (3',4',5,7-tetrahydroxyflavanone) and apigenin-7-*O*-glucoside isolated from pea root exudate, active at a concentration lower than 50 nM, and luteolin and chrysoeriol (3'-methoxyluteolin) released from alfalfa seeds [60, 61]. Other flavonoid classes released naturally from legume plants to induce *nod*-gene expression in their appropriate microsymbionts are flavanones, such as naringenin and hesperetin, chalcones, and isoflavonoids, such as daidzein and genistein [62, 63].

1.3. Phenolics as pigments

An important role of flavonoids is to serve as visual signals for animals in attracting pollinators in flowers, and later for animals eating the fruits and thereby helping in seed dispersal, acting as pigments in fruits and flowers. Factors affecting fruit colour are primarily genetically determined. In addition, environmental factors such as nutrients, temperature and light conditions can have an effect on flavonoid composition and on the final hue of the fruit.

Concerning anthocyanins that are mainly responsible for the bluish to purple and reddish colours in plants, several different factors can affect the final colour of the fruit or flower. Delphinidin-derived anthocyanins are known to be responsible for the bluish colours, whereas cyanidin- and pelargonidin-derived anthocyanins are found from mauve and reddish tissues, respectively. Anthocyanins readily form complexes with so-called co-pigments that can intensify and modify the initial colour given by the pigment. Apparently, almost all polyphenols, as well as other molecules, such as purines, alkaloids and metallic cations, have the ability to function as co-pigments. In addition, the temperature and pH of the vacuolar solution may affect the final colour [64-66].

Chalcones and aurones are two classes of flavonoids that contribute to yellow flower colour in a number of plants: for example, the chalcone isosalipurposide is the sole yellow colouring matter of yellow carnation, while the aurone aureusidin, occurring as 6-glucoside aureusin, is the major yellow pigment in the snapdragon (*Antirrhinum majus*).

Variations in hydroxylation pattern of the five commonest flavones and flavonols (apigenin, luteolin, kaempferol, quercetin, and myricetin) produce structures that give white, yellow or ivory colours to the tissues in which they are located. For example, the insertion of a 2'-hydroxyl group into luteolin gives the flavone isoetin, which is a yellow flower pigment in several *Compositae* members. The introduction of a hydroxyl group at the 6- or

8-positions of quercetin causes a significant shift in colour and such resulting compounds (e.g. quercetagenin, found in flowers of *Coronilla*, *Lotus*, *Primula*, and *Rhododendron* species, and gossypetin, the pigment of *Gossypium hirsutum* flowers) are yellow instead of pale cream as in quercetin.

Finally, most of the naturally occurring phenolic pigments are quinones: benzoquinones, naphthoquinones, and anthraquinones. Benzoquinones are uncommon in plants, while they occur in fungi, mainly in the Hyphomycetes and Basidiomycetes. Primin (6-methoxy-2-*n*-pentylbenzoquinone) is a benzoquinone, which is present in the glandular hair of leaves of *Primula obconica*. On the contrary, most naphthoquinone pigments are of higher plant origin such as plumbagin, an orange pigment identified in *Plumbago capensis*, which is also present in bound form in members of Plumbaginaceae, Droseraceae, and Ebenaceae. Much the largest group of natural quinones is the tricyclic anthraquinones found especially in Leguminosae, Liliaceae, Polygonaceae, Rhamnaceae, Rubiaceae, and Scrophulariaceae. Some anthraquinone pigments such as alizarin (2,3-dihydroxyanthraquinone), anthragallol (2,3,4-trihydroxyanthraquinone) and purpurin (1,2,4-trihydroxyanthraquinone) have been used in the past for dyeing textiles [5].

1.4. Phenolics and plant growth

As far as the role of plant phenolics as internal physiological regulators or chemical messengers within the intact plant is concerned, some information is available. Hydroxycinnamic acids, particularly *p*-coumaric acid and ferulic acid, are found in the insoluble or cell wall fraction also as esters. These pools of wall-bound acids act as a reservoir of phenylpropanoid units for lignin biosynthesis or even that they represent the beginnings of lignification itself. In addition, by radical dimerization of ferulates, polysaccharide-polysaccharide cross-linking is effected: feruloylation occurs on the arabinose or galactose side chains of pectic polysaccharides. A possible role of feruloyl pectin may be in the regulation of cell expansion, possibly through coupling reactions leading to the production of diferulate [67-69]. Another proposed role for cell wall bound hydroxycinnamic acids, regarding phototropism in higher plants, concerns the chemical nature of the UV-A and the blue light photoreceptors and the mechanism of the transduction of light energy to physical changes in the properties of cell walls. It has been proposed that the *E* (*trans*)/*Z* (*cis*) reversible photoisomerism, in UV-A, of cell wall ferulate and diferulate-carbohydrate esters, concerning a large population of bound molecules, can be regarded as a mechanism for transduction of light energy leading to changes in wall structure and hence water flux, turgor pressure and growth. Unilateral light would cause phototropism [70-71].

Some intriguing effects of plant phenolics are the ones associated with the growth hormone auxin (indoleacetic acid, IAA). Monohydroxy B-ring

flavonoids are suggested as cofactors of peroxidase functioning as an IAA oxidase that destroys the hormone, whereas dihydroxy B-ring forms act as inhibitors of the IAA degrading activity [72-74]. Mono- and dihydroxy-flavonoids are also implicated as inhibitors of IAA transport across the plasma membrane by binding to a plasma membrane protein known as the naphthylphtalamic acid (NPA) receptor. NPA is a synthetic compound, which is believed to bind a regulatory protein that is associated with the transmembrane efflux of IAA anions mediated by carrier. Some flavonoids, such as quercetin, apigenin, and kaempferol, do not directly compete with IAA but act through their own receptor, the NPA receptor, in the plant cell plasma membrane, thus blocking the polar auxin transport [75-78].

In general, plants are rooted and unable to move from place to place by themselves. However, some plants are known to be able to move in certain ways. Especially, the circadian rhythmic leaf movement known as nyctinasty is widely observed in all leguminous plants. Nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvini, an organ located in the joint of the leaf. Nyctinastic movement has been believed to be controlled by Schildknecht's turgorins, which induce leaf-closing movement of the plants [79]. Schildknecht said that all leaf-movements (nyctinastic and seismonastic movements) are controlled by turgorins, a new class of phytohormones that regulates the turgor of the plants. Leaf-opening substances differed in certain aspects from indole-3-acetic acid (IAA) that has been believed to induce the leaf-opening of nyctinastic plants: i) the bioactivities of the leaf-opening substances ($10^{-5} - 10^{-7}$ M) were much stronger than that of IAA ($> 10^{-4}$ M), and ii) the bioactivity of the leaf-opening substances was specific to the genus of the plant while that of IAA was non-specific [80]. Some identified phenolic turgorins are: gallic acid 4-*O*-(β -D-glucopyranosyl-6'-sulfate) and gentisic acid 5-*O*- β -D-glucopyranoside that are pulvini-localized in *Mimosa pudica* L., *cis-p*-coumaric acid 4-*O*- β -D-glucopyranoside, found in *Cassia mimosoides* L., and *cis-p*-coumaroylagmatine, identified in *Albizzia julibrissin* Durazz [81-84].

Finally, there exist in plant material phenolics affecting seed germination and dormancy, and these substances, found in both seed coats and embryos, have been identified as phenolic acids, hydroxycinnamic acids, and coumarins. In this connection, non-germinating seeds of *Melilotus alba* were found to possess a large amount of free coumarin, while in rapidly germinating seeds coumarinic acid β -glucoside was more prevalent. Another naturally occurring phenolic compound inhibiting the germination of seeds of the same or of other species is ferulic acid: pure solution of ferulic acid gave strong inhibition of *Raphanus sativus* seed germination at concentration of 10^{-4} M. It has been suggested that phenolics may be active as germination inhibitors by inhibiting

the transport of amino acids and the formation of proteins in the seeds. [85, 86].

2. Phenolic compounds and plant defence

Plants encounter numerous pests and pathogens in the natural environment. An appropriate response to attack by such organisms can lead to tolerance or resistance mechanisms that enable the plant to survive. Resistance mechanisms refer to traits that inhibit or limit attack, while tolerance strategies do not limit attack but reduce or offset consequences on the plant fitness by adjusting its physiology to buffer the effects of herbivory or diseases. Resistance strategies include physical and/or chemical barriers, mechanisms that rapidly clear infection or herbivory (hypersensitive response), and processes that limit the spread of damage within the host (such as localized cell death). Tolerance often involves some degree of compensation for disease damage. Plants can tolerate infection or herbivory by increasing the chlorophyll concentration in leaves, increasing the size of new leaves or the number of new branches, advancing the timing of bud break, delaying the senescence of infected tissues, and increasing the nutrient uptake [87-90]. Most plants produce a broad range of secondary metabolites that are toxic to pathogens and herbivores, either as part of their normal program of growth and development or in response to biotic stress. Preformed antibiotic compounds that occur constitutively in healthy plants are likely to represent inbuilt chemical barriers to herbivorous and fungal enemies and may protect plants against attack by a wide range of potential pests and pathogens. In contrast, induced defence compounds are synthesized in response to biotic stress as part of the plant defence response and are restricted to the damaged tissue. Both tolerance and resistance traits require the reallocation of host resources, therefore defensive chemicals are considered to be costly for plants because of the resources consumed in their biosynthesis or the ecological consequences of their accumulation. One way for a plant to reduce these costs is to synthesize defence compounds only after an initial damage by a pathogen or insect: this strategy could be risky because the initial attack may be too rapid or too severe for an effective defence response. Therefore, plants that are likely to suffer frequent and/or serious damage may be better off investing mainly in constitutive defences, whereas plants that are attacked rarely may rely predominantly on induced defences. [91-94].

2.1. Fungal pathogens

It is estimated that there are about 250,000 species of higher plants, but six times as many (1.5 million) species of fungi. Fungi are, ultimately, all dependent on plants for their carbon and energy source, like most other

organisms that are not able to photosynthesize. Fortunately, for plants, the relationship between them and fungi is usually a mutually beneficial one (saprophytic fungi, mycorrhizae, and endophytes). A small minority of fungal species has developed further and broken the fine balance of mutual benefit to become plant pathogens [95]. Therefore, plants are continuously exposed to the biotic stress exerted by organisms with which plants have symbiotic or pathogenic interaction. Indeed, in interactions between plants and microbial pathogens, resistance is the rule and disease the exception. This phenomenon is known as non-host resistance or species-specific resistance¹ and is thought to explain why a pathogen can cause disease in particular plant species but not in others. Explanations why plants may be unsuitable as hosts are: (i) plants do not support the invading pathogen's lifestyle and therefore are not substrates for microbial growth; (ii) preformed physical or chemical barriers constitutively present on the plant surface (leaf hairs, wax layers, rigid cell walls, and antimicrobial secondary metabolites) prevent pathogen invasion and spread; (iii) the plant's recognition of pathogens induces its endogenous multicomponent defence system. There is considerable evidence that preformed defences are a major component of non-host resistance, particularly in non-domesticated plants. Plants contain preformed peptides, proteins and secondary metabolites (phenolics, sulphur compounds, saponins, cyanogenic glycosides, and glucosinolates). The multicomponent defence response induced after the pathogen attack requires a substantial commitment of cellular resources, including extensive genetic reprogramming, because the induced expression of a large number of defence related genes is essential for plants to counter pathogen attack. Many defence related genes encode proteins possessing antifungal and antimicrobial activities or enzymes that catalyse defence metabolites, known as phytoalexins. Thus, defence responses are kept under tight genetic control and are activated only when the plant detects a potential invader. Other responses of invaded cells result from allosteric enzyme activation initiating tissue reinforcement by oxidative crosslinking, apposition of callose and lignins. The initial signal perception also leads to the production of an endogenous systemically translocated signal that has the function to activate defence mechanisms in parts of the plant remotely located from the initial site of interaction, providing durable protection against challenge infection by a broad range of pathogens. Other defence genes encode these regulatory proteins, important for defence signal transduction, and this

¹ Non-host resistance in plants refers to a mechanism that provides resistance against a specific parasite or pathogen throughout all the members of a plant species. It is expressed by every plant towards the majority of potentially pathogenic microbes, whereas host resistance refers to resistance expressed by plant genotypes within an otherwise susceptible host species.

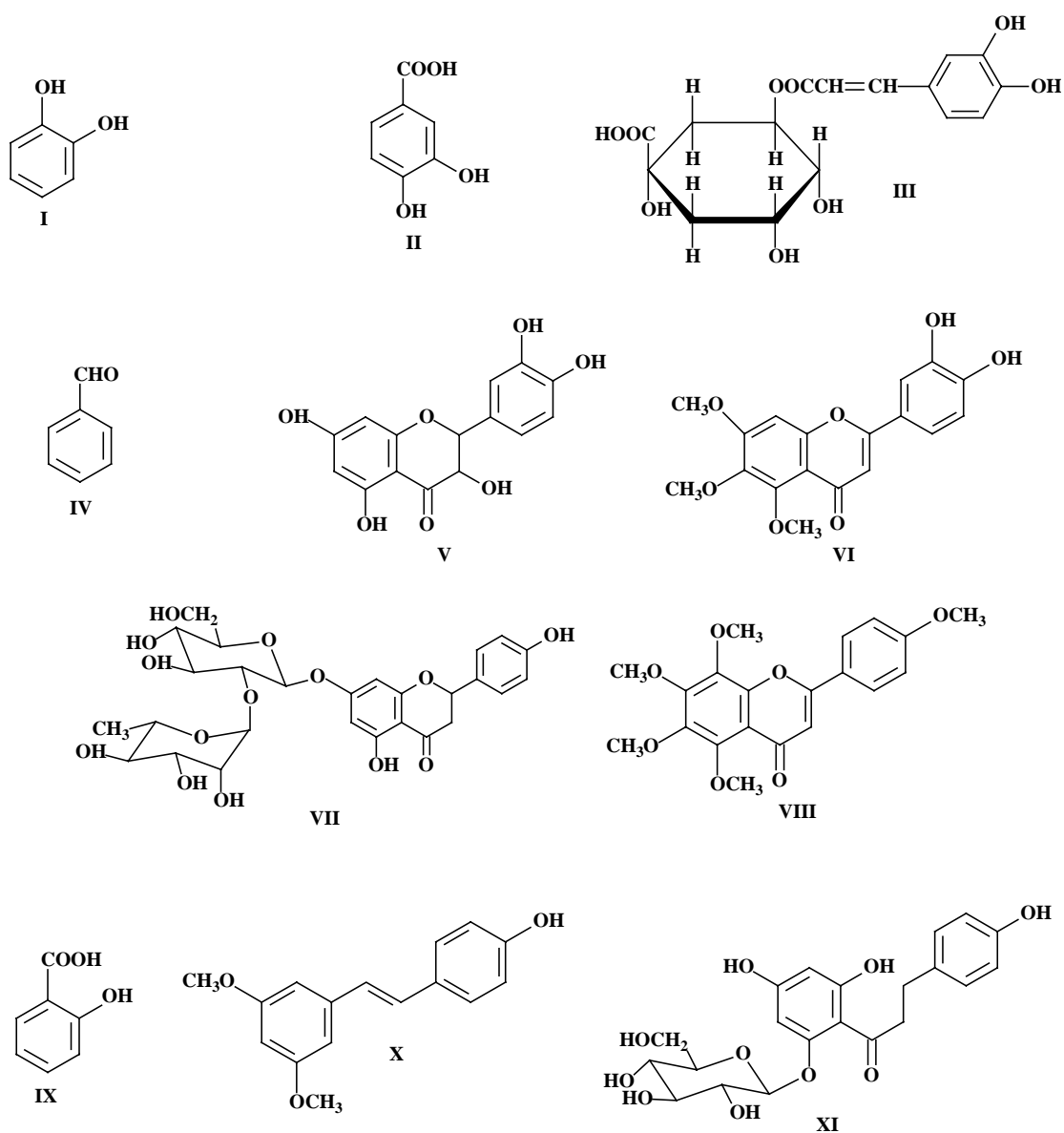
form of induced defence response is referred to as systemic acquired resistance (SAR) [96-101].

2.1. a Preformed antifungal phenolics

Pre-formed antibiotic compounds such as phenolic and polyphenolic compounds are ubiquitous in plants and play an important role in non-host resistance to filamentous fungi. The term “phytoanticipin” has been proposed to distinguish these preformed antifungal compounds from phytoalexins, which are synthesized from remote precursors in response to pathogen attack. Some antibiotic phenolics are stored in plant cells as inactive bound forms but are readily converted into biologically active antibiotics by plant hydrolysing enzymes (glycosidases) in response to pathogen attack. These compounds can also be considered as preformed antibiotics since the plant enzymes that activate them are already present but are separated from their substrates by compartmentalization, enabling rapid activation without a requirement for the transcription of new gene products [102]. In such cases, free phenolics are likely to be much more toxic to the invading organism than the bound forms. In addition, even if preformed antifungal phenolics are present in healthy plants at levels that are anticipated to be antimicrobial, their levels may increase further in response to challenge by pathogens.

The distribution of preformed antifungal phenolics within plants is often tissue specific and there is a tendency for many lipophilic compounds (*e.g.* flavone and flavonols methyl ethers) to be located at the plant surface (*e.g.* in leaf wax and bud exudates) or in the cytoplasmic fraction within the epidermal cells, suggesting that they may indeed act as deterrents to pathogens. In general, however, preformed antifungal phenolics are commonly sequestered in conjugated form, usually with glycosidic attachments, in vacuoles or organelles in healthy plants [27, 32, 35, 91, 102-104]. Biotrophs may avoid the release of preformed antibiotics by minimizing the damage to the host, whereas necrotrophs are likely to cause a substantial release of these compounds.

The first demonstrated example of phenolics providing disease resistance was the case of onion scales accumulating sufficient quantities of catechol (I) and protocatechuic acid (II) to prevent onion smudge disease, *Colletotrichum circinans*. The coloured outer onion scales of resistant onion varieties contain enough of these two phenols to reduce spore germination of *C. circinans* to below 2%, while susceptible varieties lack these compounds and the germination rate is over 90% [105-108]. Adequate levels of chlorogenic acid (III) account for the resistance of potato tubers against *Streptomyces scabies*, *Verticillium albo-atrum* and *Phytophthora infestans* [109-111], while at low concentrations it stimulates the growth of *P. infestans* and *Fusarium solani* var. *coeruleum*. Small concentrations of benzaldehyde (IV) totally inhibited spore germination of *Botrytis cinerea* and germination of *Monilia fructicola* [112].



Skadhauge *et al.* [113] found that a barley mutant (*ant* 18-159) showed extreme resistance to *Fusarium* attack: the hyphae were unable to penetrate the testa of this mutant. The testa layer of *ant* 18-159 accumulates, besides proanthocyanidins, small amounts of dihydroquercetin (V) as a result of nonsense mutation in the structural gene for dihydroflavonol reductase. *In vitro* bioassays showed that dihydroquercetin is a strong inhibitor of *Fusarium* growth and macrospore formation. The epicuticular wax of the leaves of *Arrabidaea brachypoda* contains four flavonoids, 3',4'-dihydroxy-5,6,7-trimethoxyflavone (VI), 3',4'-dihydroxy-6,7-dimethoxyflavone (cirsiol), 4'-hydroxy-6,7-dimethoxyflavone (cirsimaritin), and 4'-hydroxy-6-methoxyflavone (hispidulin), showing antifungal activity against *Cladosporium sphaerospermum* [114]. Two constitutive secondary metabolites of the bitter orange *Citrus aurantium*, the

flavanone, naringin (VII), and the polymethoxyflavone, tangeretin (VIII), showed antifungal activity against *Penicillium digitatum*, acting as first and second defence barriers, respectively, since tangeretin is mainly localised in the outermost tissue of the fruit, the flavedo, while naringin is located in the albedo which is immediately below the flavedo [115]. *In vitro* studies reveal that phenolic compounds extracted from olive plants (*Olea europaea* L.), tyrosol, catechin, and oleuropein, showed antifungal activity, thus affecting plant resistance against *Phytophthora* sp. [116]. Quercetin 3-methyl ether and its 4'-O-glucoside (6) and 7-O-glucoside, especially, completely inhibited conidial germination of the fungus *Neurospora crassa* [117]. Naringenin (flavanone), dihydroquercetin (dihydroflavonol), kaempferol, and quercetin (flavonols) have been tested for their biological activity against two fungal rice pathogens, *Pyricularia oryzae* and *Rhizoctonia solani* [118]. Naringenin and kaempferol showed a significant inhibition of spore germination of *P. oryzae* from 7 µg onwards. On the other hand, no such inhibition was found with *R. solani*. Finally, salicylic acid (IX) inhibited mycelial growth of *Eutypa lata* (Pers. Fr.) Tul., the causal agent of eutypa dieback, a severe disease of the grapevine and many other woody fruit plants, such as apricots. Salicylic acid (SA) acts in a concentration-dependent manner, the threshold value being 0.1 mM. In conditions mimicking the plant environment (in particular, a pH near the apoplastic value, i.e. 5.5), 1 mM SA showed only fungistatic properties. A fungicidal effect was obtained at 2 mM or higher concentrations and following this treatment, fungal filaments appeared empty [119].

In summary, preexisting antifungal phenolics are simple phenols, phenolic acids, flavonols and dihydrochalcones (Table 1). In addition, many flavones and flavanones have been shown to be active against fungal pathogens commonly found during the storage of fruits and vegetables, i.e. *Aspergillus* sp., *B. cinerea* and *F. oxysporum* [120]. The fungicidal activity of a number of stilbenes and related compounds has been tested against several fungi, including some pathogens, which infect grapes during storage. A linear relationship between bioactivity and hydrophobicity of the molecule has been found. When the activity of two natural stilbenes (resveratrol and 3,5-dimethoxy-4'-hydroxystilbene (X)), occurring in grapevines, was examined against different fungi, resveratrol was found less active than the dimethoxy stilbene [121, 122]. It is also believed that the stilbene structure may be modified *in vivo* by partial methylation to further enhance its activity. A similar structure/activity relationship was observed when the fungicidal properties of some flavonoids, especially flavones and isoflavones, were tested against *Fusarium* sp., *B. cinerea*, *Aspergillus* sp. and other storage fungi [120, 123-126]. Lipophilicity and/or the presence of at least one acidic hydroxyl group are, therefore, considered to be a structural feature essential for a good antifungal activity. Lipophilicity allows active phenols to penetrate biological

Table 1. Antifungal phenolics.

Compound	Fungus	References
Benzaldehyde, Ethyl benzoate	<i>Botrytis cinerea</i>	112
	<i>Monilinia fructicola</i>	112
3,4-Dihydroxybenzaldehyde	<i>Gloeosporium musarum</i>	156
Catechol, Protocatechuic acid	<i>Colletotrichum circinans</i>	108
2,5-Dimethoxybenzoic acid	<i>Botrytis cinerea</i>	274
	<i>Rhizopus stolonifer</i>	274
Salicylic acid	<i>Eutypa lata</i>	119
Vanillic acid, 4-Hydroxybenzoic acid	<i>Phytophthora infestans</i>	5
Chlorogenic acid	<i>Verticillium albo-atrum</i>	111
	<i>Phytophthora infestans</i>	110
	<i>Phlyctaena vagabunda</i>	103
Chlorogenic acid, Rutin	<i>Fusarium oxysporum</i>	275
<i>p</i> -Coumaric acid, Cyanidin	<i>Gloeosporium perennans</i>	276
3- and 7-Hydroxyflavone	<i>Penicillium glabrum</i>	277
	<i>Cladosporium herbarum</i>	277
	<i>Fusarium</i> spp.	113
Dihydroquercetin	<i>Pyricularia oryzae</i>	118
Naringenin, Kaempferol	<i>Penicillium digitatum</i>	115
Naringin, Tangeretin	<i>Venturia inaequalis</i>	278
Phloridzin, Phloretin	<i>Aspergillus</i> sp.	120
Flavone, Flavanone	<i>Cladosporium sphaerospermum</i>	114
Cirsiliol, Cirsimaritin, Hispidulin	<i>Botrytis cinerea</i>	279
7,4'-Dihydroxyflavan,	<i>Fusarium oxysporum</i>	120
5,8-Dihydroxy-6,7-dimethoxyflavan	<i>Helminthosporium oryzae</i>	120
	<i>Phytophthora</i> spp.	116
Oleuropein	<i>Phoma tracheiphila</i>	156
Nobiletin	<i>Monilinia fructicola</i>	280
Genistein, Biochanin	<i>Cercospora bieticola</i>	280
	<i>Helminthosporium sativum</i>	278

membranes while hydroxyl groups may act by uncoupling oxidative phosphorylation [117, 123, 127].

Toxicity of tannins, hydrolysable tannins and proanthocyanidins, usually estimated by the measurement of the reduction of the *in vitro* growth of mycelium, is well documented for several filamentous fungi, for example *B. cinerea*, *Aspergillus niger*, *Colletotrichum graminicola*, *Gloeophyllum trabeum*, *Trichoderma viride*, and *Penicillium* sp.. Tannins are quite potent antibiotics. In temperate trees, tannins and related phenolic compounds preserve heartwood from fungal decay and inhibit extracellular hydrolases from invading pathogens, thus preventing their rapid development in the plant [5, 14, 128]. It is possible that inhibition of extracellular fungal enzymes (cellulase, pectinase, laccase, xylanase, etc.), nutrient deprivation of substrates (metal complexation, protein insolubilization) and action on fungal membranes (inhibition of oxidative phosphorylation) are effectively involved in tannin toxicity. Finally, lignans, a phenolic class of dimeric phenylpropanoid units linked by the central carbons of their side chains, also play a role in plant-fungus interactions. Some of the fungistatic activity of lignans is attributable to their inhibition of the extracellular fungal enzymes, cellulase, polygalacturonase, glucosidase and laccase [129].

2.1. b Induced disease resistance

When a pathogen manages to overcome constitutive defence barriers it may become subject to recognition at the plasma membrane of plant cell. Activation of inducible plant defence responses is probably brought about by the recognition of invariant pathogen-associated molecular patterns (PAMP) that are characteristic of whole classes of microbial organisms: PAMP perception systems trigger signalling cascades whose recognition is very likely to activate defence responses in natural plant-pathogen encounters [101]. Plants respond to pathogens by activating broad-spectrum innate immune responses that can be expressed locally at the site of pathogen invasion as well as systemically in the uninfected tissue.

Locally induced defence responses, which restrict pathogen infections to the site of attempted ingress, are characterized by a hypersensitive response (HR), a complex, early defence response that causes necrosis and cell death in order to restrict the growth of a pathogen. A ring of tissue around the developing lesions became fully refractory to subsequent infection (localized acquired resistance). Host resistance (R) genes detect the pathogen and change the membrane potential and the ion permeability of the plasma membrane. In phase one of the response, the R genes trigger an increase in extra cellular pH and K^+ , while eliciting an influx of calcium and hydrogen ions into the cell. The outward K^+ and the inward Ca^{+2} and H^+ ion flux are dependent and trigger the HR, resulting in cell death and formation of local lesions, which contain antimicrobial compounds. In phase two, cells undergoing the HR produce reactive oxygen species (ROS; oxidative burst), including super oxide anions, hydrogen peroxide, and hydroxyl radicals. Several enzymes may be involved in generation of ROS: copper amine oxidase (which catalyzes the oxidative deamination of polyamines releasing hydrogen peroxide and ammonia), xanthine oxidase, NADPH oxidase, oxalate oxidase, and peroxidases. Lipid peroxidation and lipid damage may be partially responsible for some of these cell changes and probably affect membrane function. Phenolics and phytoalexins, and other compounds are synthesized in cells surrounding the lesion. Callose and lignin are deposited and pathogenesis-related proteins (PRs) are induced. These proteins include four families of chitinases, one β -1,3-glucanases, one proteinase inhibitors, and one specific peroxidase. Chitinases, together with glucanases, could be directed against fungal cell walls. Peroxidase is of the lignin-forming type and could be involved in the strengthening of the plant cell wall. Subsequently, the hypersensitive response triggers a general resistance mechanism rendering uninfected parts of the plant less sensitive to further attack by pathogens, a phenomenon called systemic acquired resistance (SAR), suggesting that as a result of the initial infection, a signal was generated, transported and propagated, that primed the plant to respond more effectively to subsequent infection [36, 130-135].

Thus, the potential of plants to react to an invader depends upon a signal released from the infected tissue and translocated to other parts of the plant where it induces defence reactions. Salicylic acid is considered one of the key signalling molecules that activate plant defence responses against invading pathogens. Evidence for the key role of salicylic acid came from the analysis of transgenic plants expressing the bacterial *nahG* gene, which encodes for the enzyme salicylate hydroxylase that inactivates salicylic acid by converting it to catechol. Transgenic NahG plants are unable to accumulate salicylic acid, and are also incapable of developing SAR, indicating that salicylic acid accumulation is required for the expression of SAR [136, 137]. Salicylic acid is synthesized from phenylalanine (phenylpropanoid metabolism), however this pathway cannot account for all of the salicylic acid in plant cells, suggesting the presence of an alternative biosynthetic pathway for salicylic acid. A pathway similar to that found in some bacteria synthesizes salicylic acid from chorismate via isochorismate. It has been shown that this simple phenolic metabolite is produced by plants locally, at the site of infection, but it has been also found in the phloem sap and in uninfected tissues. Salicylate acts as a secondary signal molecule, the level of which increases during the induced defence process and it is likely that this increase results from an increased expression of SA biosynthetic enzymes. Increased expression of such enzymes might not be induced by SA but by another earlier signal. This endogenous regulator, in turn, coordinately induces the full spectrum of SAR genes, encompassing all well-characterized PRs, in plants infected by pathogens. A mode of action for salicylic acid has been proposed by virtue of its ability to form a phenolic free radical upon inhibition of catalase and ascorbate peroxidase. It has been suggested that such phenolic radical is involved in lipid peroxidation, the product of which might activate defence gene expression. Other SA binding proteins have been identified that show a higher affinity for SA and related functional analogues than catalase [100, 134, 138-142]. However, recent advances in understanding plant defence signalling have revealed that plants employ a network of signal transduction pathways, some of which are independent from salicylic acid. Evidence is emerging that jasmonic acid and ethylene play key roles in these salicylic acid-independent pathways. Cross-talk between the salicylic acid-dependent and the salicylic acid-independent pathways provides a great regulatory potential for activating multiple resistance mechanisms in varying combinations [143-146].

If pre-existing antifungal phenolics are not sufficient to stop the development of the infectious process, plant cells usually respond by increasing the level of pre-existing antifungal phenols at the infection site, after an elicited increased activity of the key enzymes (PAL and chalcone synthase) of the biosynthetic pathway [147-155]. The increased level of phenolics provides an adequate substrate to oxidative reactions catalysed by PPO and/or

POD that, consuming oxygen and producing fungitoxic quinones, make the medium unfavourable to the further development of pathogens. Therefore, although many phenolic compounds present in plant tissues may not have any anti-microbial activity *per se*, there are several cases recorded where the oxidation products of pre-existing phenolics might have antimicrobial activity, which is often associated with an inhibition of the cell wall degradation by extracellular enzymes produced by pathogens. In addition, many simple low molecular phenolic compounds present in plants may be readily polymerized by oxidation to yield brown tannin-like substances (melanins) containing quinonoid groups. These can also precipitate protein and cross-link to other polymers. They are often formed in necrotic cells after the invasion by a pathogen, as shown by the browning, which takes place in and around the area [14, 156-158]. Thus, oxidized phenolics in resistant varieties of apple play an important role in the restricted lesion formation associated with the brown rot disease of fruits caused by *Sclerotinia fructigena* Aderh. [159] or with rotting of stored Golden Delicious apples caused by *Phlyctaena vagabunda* [103]. Raa and Overeem [160] have shown that phloridzin (XI) oxidation products may be involved in the defense mechanism of apple leaves against the scab fungus *Venturia inaequalis*. In addition, Lattanzio et al. [103] have shown that in cold stored Golden Delicious apples, when rot caused by *P. vagabunda* appears in infected tissues surrounding the rotten zone, a general increase in phenolic levels was observed, as compared to a healthy tissue of the same fruit. PPO activity also increased in these tissues, 2-3 times than in healthy tissues: these authors suggested the hypothesis that phloridzin oxidation in infected tissues, instead of preexisting phenols, plays an important role in host resistance and that the mechanism of resistance may be governed by the hypersensitive response linked to the host-pathogen interaction. In fact, changes in phenolic content and PPO activity may be considered as a part of this response of the host cells to pathogen that is useful arresting the development of fungus without causing further damage to the surrounding tissue. Postinfectious accumulation of preexisting phenolics, especially phloridzin and chlorogenic acid which are the best substrates of apple PPO ($K_M = 1.351$ and 1.139 mM, respectively), provides an adequate substrate to the increased PPO activity. Polyphenol oxidase, consuming oxygen and producing fungitoxic quinones, makes the medium unfavourable to further development of pathogens. In fact, *in vitro* bioassays showed that, when a crude extract of apple PPO was added to a spore suspension of *P. vagabunda* that contains 10^{-3} M of each apple phenolics, an inhibition of fungal spore germination was observed. These bioassays also showed a potential synergistic effect of phloridzin and chlorogenic acid, thus demonstrating that the simultaneous presence of chlorogenic acid and catechin in a model system increases the oxidation rate of phloridzin in the presence of polyphenol oxidase. This synergistic effect

should probably be considered in the overall defensive strategy of apple against fungal attack. Finally, when in long stored apples these primary responses (increased phenolic level and activation of apple PPO in infected tissues), which counteract the further development of the pathogen, fall during storage, a simultaneous increase in apple rot was observed.

Furthermore, antifungal activity of oxidized phenolics may also be related to the necrotic reaction, e.g. the oxidative polymerisation involving phenolic compounds, amino acids and proteins that yields brown melanins. This reaction results in the formation of an impermeable barrier to pathogenesis by plant parasites, and in a decrease of nutrients essential to the fungal development. The possibility that phenolic oxidation products could have an antifungal action by polymerizing and forming a protective seal on cell walls has been proposed by Beckman *et al.* [161] who showed that artificial membranes of calcium oxalate-pectin, that had been infused with the oxidation products of 3-hydroxy-tryptamine, were resistant to degradation by *Fusarium oxysporum* f. sp. *cubense*.

Secondary responses also include the release of toxic phenols that are normally stored as less toxic glycosides in the vacuoles of the plant cells, the formation of lignin, a biopolymer resistant to the degradation by most microorganisms, the accumulation of cell-wall appositions such as papillae, and, finally, the synthesis of specific antibiotics such as phytoalexins [35].

2.1. c Lignification

Lignification has been proposed as a general mechanism of resistance on the basis of experiments with cucumbers and the pathogen *Cladosporium cucumerinum* in which lignification was found after the inoculation of a resistant variety of the host plant but not of a susceptible one [162]. A close relationship between lignification and disease resistance has been demonstrated afterwards in a number of experiments which showed that resistant plants accumulated lignins more rapidly and/or exhibited enhanced lignin deposition as compared with susceptible plants (35, 131, 163-165). For example, Egea *et al.* [166] found that cell suspension cultures of three varieties of *Capsicum annuum* L., each with a different degree of sensitivity to *Phytophthora capsici*, a fungus causing blight in peppers, responded to elicitation by both lyophilized mycelium and fungus filtrate. The cells of all the three pepper varieties produced a hypersensitive reaction (rapid cell browning and collapse) when elicited by the fungus, which differed in the speed and the extent of necrosis (much faster in the resistant variety). They showed the synthesis or accumulation of PR-proteins with peroxidase activity and the accumulation of lignin-like polymer. After elicitation, the cell walls thickened through the accumulation of lignin, as can be observed by staining microscope preparations with methylene blue. Elicitation also reduced the

level of total peroxidase activity in the susceptible varieties, while such activity increased in resistant varieties, and was accompanied by *de novo* expression of acidic peroxidase isoenzymes in the extracellular and cell wall fractions. In addition, the ability of non-pathogenic isolates of *Fusarium oxysporum* (npFo) to induce systemic resistance and defence responses against subsequent challenge with a pathogenic strain of *F. oxysporum* f. sp. *asparagi* (Foa) has been examined in *Asparagus officinalis*. Roots inoculated with npFo exhibited a hypersensitive response and those subsequently inoculated with Foa displayed resistance. Induction of systemic resistance in npFo-treated plants led to significantly fewer necrotic lesions and reduced Foa disease severity compared with plants not treated with npFo. In hyphal-sandwich root inoculation experiments, the activities of peroxidase and phenylalanine ammonia-lyase and lignin content were higher in npFo-treated plants and increased more rapidly than in npFo-untreated plants after Foa inoculation [167]. Bishop [168] reports that when stressed plants exhibit symptoms of infection, syringyl lignin and ferulate dimers increase in the cell walls associated with the endophyte. Peroxidase activity and transcripts increase correspondingly in association with fungal infection. The author points out that peroxidase activity is widely related to the polymerization of phenolic compounds, the deposition of lignin, and the cross-linking of phenolics to cell wall proteins. A lack of penetration by the fungus *Colletotrichum orbiculare* has been also observed in cucumber plants in which the expression of induced disease resistance to leaf infecting pathogen has been associated with the rapid modification of the outer epidermal cell wall of the host at the point of attempted infection: histochemical analysis have detected lignin deposits under the appressoria that have been not successfully penetrated [169]. In addition, the use of specific chemical inhibitors of lignification led to the inhibition of the hypersensitive response in wheat plants challenged by *Puccinia graminis* Pers., rendering resistant plants susceptible to pathogen attack [131, 170]. Lignification is not restricted to incompatible interactions in gene-for-gene systems but is also observed in nonhost resistance and SAR [158]. However, the role of these wall modifications in disease resistance is often equivocal although their presence is commonly associated with penetration failure in situations where a fungus tries to enter a cell. In this connection, Kruger et al. [171] showed that inhibitors of phenylpropanoid biosynthesis reduced localized autofluorescence (caused by the presence of phenolics) of the plant cell wall and increase the penetration success of the barley powdery mildew fungus in a set of near-isogenic barley lines containing defined race-specific resistance genes. However, the authors concluded that the penetration resistance shown by these lines is not influenced by the presence of resistance genes, but comes from the background penetration resistance in the parent genotype.

Lignin is a three-dimensional phenolic structure resulting from the free-radical polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols within the plant cell wall, even if there are now many examples showing that other phenolics can be incorporated into lignins. As lignin polymerizes, it forms covalent crosslinks with carbohydrate and protein and renders cell walls highly resistant to mechanical and enzymatic disruption thus acting as a major line of defense against pathogens, insects, and other herbivores [164, 172-175]. Deposition of lignin has been hypothesized to interfere with the enzymatic hydrolysis and mechanical penetration of plant tissue by fungal pathogens and may also impair the movement of water and diffusible molecules between the plant and fungus and then help to starve a pathogen. Lignin precursors themselves might exert a toxic effect on pathogens or, by binding to fungal cell walls, make them more rigid and impermeable, thus hindering further growth or uptake of water and nutrients. Thus, *in vitro* coniferyl alcohol is toxic to fungi at low concentration. Moreover, it has been proposed that the polymerization of lignin precursors by free radicals in the intercellular space might also lead to a lignification of pathogen structures: each of these possible events provides a testable hypothesis, and some of these mechanisms have been demonstrated [35, 131, 158, 176-179]. Pectic fragments have been identified as elicitors for the accumulation of histochemically detected lignin-like materials in cucumber hypocotyls and suspension cultures of castor bean (*Ricinus communis* L.) [180, 181]. Other elicitors of lignin or lignin-like substances include chitosan, extracts from fungal cell walls, and fungal lipids [147, 182].

2.1. d Phytoalexins

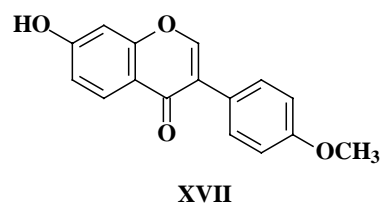
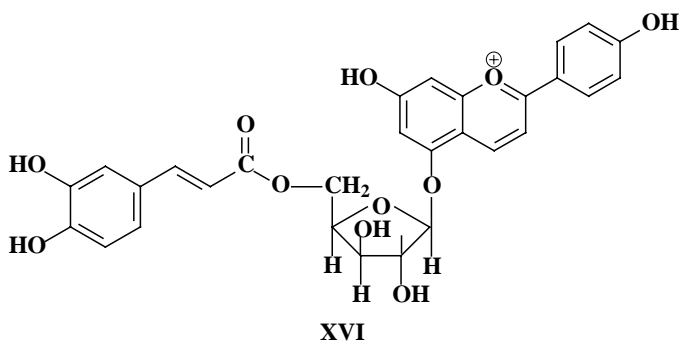
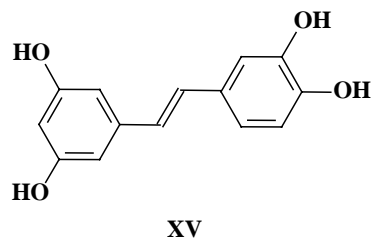
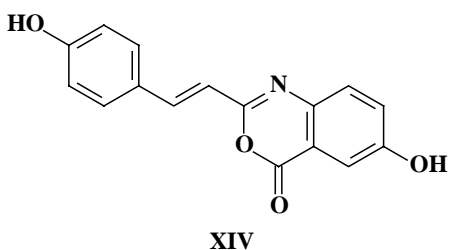
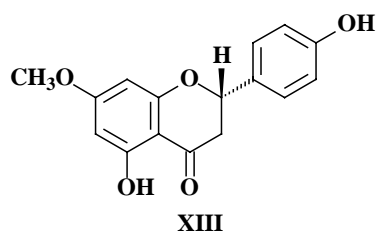
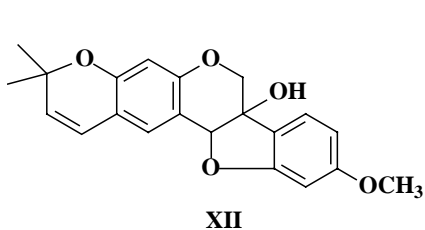
Phytoalexins are antimicrobial, low-molecular-weight secondary metabolites that are both synthesized by and accumulated in plant cells as a result of the interaction between the metabolic systems of the host and a fungal parasite, and that require *de novo* expression of the enzymes involved in their biosynthetic pathway [183, 184]. For example, the major phytoalexin in alfalfa is the pterocarpan medicarpin, synthesized from phenylalanine via the isoflavonoids pathway. The elicitation of medicarpin in alfalfa cell suspension cultures exposed to crude elicitor from cell walls of *Colletotrichum lindemuthianum* is preceded by increases in the activity of all 11 enzymes required for its biosynthesis and their transcriptional activation. The genes encoding L-phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chalcone reductase (CHR) were the most rapidly activated, within 10-20 min after the elicitation of the isoflavonoids biosynthetic pathway [185, 186].

Phytoalexins are substances not detectable before infection, and are considered to inhibit the further development of most attacking pathogens of the species under consideration. The molecules that signal plants to begin the

process of phytoalexin synthesis are called elicitors. Nowadays the term “elicitor” refers to both biotic elicitors (such as complex carbohydrates from fungal and plant cell walls, and microbial enzymes) and abiotic elicitors (such as heavy metals, autoclaved ribonuclease, chloroform, detergents, freezing, heating, exposure to UV light). There is some evidence that cell wall fragments, active as elicitors, are released by a plant enzyme that is activated by damaging plant cells. The main features that relate phytoalexin synthesis and metabolism are: (i) phytoalexins rapidly accumulate in response to infection to an antimicrobial level in resistant plants, while there is either lesser or slower accumulation in susceptible plants; (ii) when the accumulation of phytoalexins is either increased or decreased by manipulation of the experimental conditions, the plant becomes either more resistant or more susceptible; (iii) some pathogens can metabolize phytoalexins to less toxic products [36, 39, 158, 187-190]. In addition, some pathogens have developed mechanisms to suppress the defence response of the host plant, making the plant unable to perceive the pathogen entering it. This leads to a failure in the hypersensitive response that is often considered a prerequisite for induced disease resistance [191]. However, the role of phytoalexins in stopping pathogen development in host tissues is far from clear for many host-pathogen interactions [36]. Some very good information has been obtained from those systems where phytoalexin concentration at the site of infection has been quantified. Knowing when, where, and to what extent these antimicrobial compounds accumulate in response to infection is a critical step in determining if a phytoalexin is casually related to defence. In sorghum, taking advantage of the fact that the deoxyanthocyanidin phytoalexins produced in response to fungal infection are visible pigments, by utilizing adequate analytical techniques has been observed that the timing of phytoalexin accumulation and the quantity of phytoalexins at the infection site is more than sufficient to stop the development of fungal infection in the invaded tissues [192-193].

The phytoalexins of different plants are generally unique structures. However, closely related plants often have phytoalexins more closely chemically related than plants phylogenetically more distant. In addition, it must be emphasized that plants not only synthesize phytoalexins, plants catabolize phytoalexins: this is advantageous because phytoalexins are toxic to the plants that synthesize them. The maximum levels of phytoalexins accumulated in a plant upon challenge by a microbe or elicitor are determined by the rate of catabolism as well as the rate of synthesis of the phytoalexins [36, 37, 187, 194]. Clearly, not all plants produce phytoalexins, however phytoalexins have been found in both Gymnospermae and Angiospermae, and within the latter group in both Monocotyledoneae and Dicotyledoneae. The structures of phytoalexins are often unique at the family level: the majority of phytoalexins produced by the members of the family Leguminosae are

isoflavonoids (90 out of 113 identified structures), and the commonest isoflavonoid subclass are the pterocarpan such as medicarpin and glyceollin II (XII), whereas phytoalexins from Vitaceae seem to constitute a rather restricted group of molecules belonging to the stilbene family, the skeleton of which is based on the *trans*-resveratrol structure (3,5,4'-trihydroxystilbene) [37, 195-198]. Dicotyledonous species represent the majority of plants from which such compounds have been identified. Some monocotyledonous species producing phenolic phytoalexins are rice (sakuranetin (XIII)), oat (avenalumin I (XIV)), sugarcane (piceatannol (XV)), and sorghum (3- deoxyanthocyanidins) [193, 199-202].



Sorghum (*Sorghum bicolor*) synthesizes a complex mixture of 3-deoxyanthocyanidin phytoalexins in response to inoculation with the non-pathogenic fungus *Bipolaris maydis*. The sorghum phytoalexins have been identified as 3-deoxyanthocyanidins: luteolinidin, 5-methoxyluteolinidin, apigeninidin, caffeic acid ester of arabinosyl 5-*O*-apigeninidin (XVI), and 7-methoxyapigeninidin. These compounds accumulate in inclusion bodies in epidermal cells that migrate towards the site of fungal penetration. The inclusions eventually release their contents, killing both the fungus and the cell

that synthesized them [192, 203]. Fawe et al. [204], in their research on silicon-mediated accumulation of flavonoid phytoalexins in cucumber, found that silicon is involved in the increased resistance of cucumber to powdery mildew by enhancing the antifungal activity in infected leaves. This antifungal activity was attributable to the presence of low-molecular-weight metabolites. One of these metabolites, described here as a phytoalexin, was identified as a flavonol aglycone rhamnetin (3,5,3',4'-tetrahydroxy-7-*O*-methoxyflavone). This is the first report of a phytoalexin for this chemical group in the plant kingdom and of a flavonol phytoalexin in cucumber, a chemical defense long believed to be nonexistent in the family Cucurbitaceae.

Some evidence that phytoalexins are effective in providing plants with disease resistance has been obtained by transferring foreign phytoalexin expression from one plant to another, and such approach has been undertaken in the case of stilbenes and isoflavonoids [205, 206]. Stilbene phytoalexins, which include resveratrol, require only a single enzyme, stilbenes synthase, to link the two universally available precursors, malonyl-CoA and *p*-coumaroyl-CoA, for their synthesis. Pathogen resistance has thus been engineered into alfalfa by the transfer of a peanut cDNA encoding resveratrol synthase. Such plants constitutively accumulate a glucoside of the novel phytoalexin resveratrol (*trans*-resveratrol-3-*O*- β -D-glucopyranoside) and exhibit vastly reduced lesions following infection by the leaf spot pathogen *Phoma medicaginis* [207]. Similarly, the constitutive overexpression of isoflavone *O*-methyltransferase in transgenic alfalfa resulted in a more rapid and increased production of the phytoalexins formononetin (XVII) and medicarpin after infection by *P. medicaginis*, with a consequent amelioration of the symptoms [208]. However, it is not so easy to do the same for many other phytoalexins. For instance, for non-leguminous plants many new enzymes would be required to produce isoflavonoid phytoalexins, so that many genes would have to be transferred. Therefore, an alternative way of using the knowledge of phytoalexin production in plants is to stimulate the plants, even susceptible genotypes, to produce their own phytoalexins rapidly and in large quantity by inoculation with non-pathogenic microorganisms or with an incompatible strain of pathogen. For example, systemic resistance to *Pyricularia oryzae* in rice has been induced by inoculation with bacterium *Pseudomonas syringae* pv. *syringae*, which causes a hypersensitive response in rice [95, 209]. Likewise, phytoalexin (glyceollin I, II and III, and coumestrol) induction and accumulation in soybean cotyledon tissue was observed using four species of *Aspergillus*: *A. sojae*, *A. oryzae*, *A. niger*, and *A. flavus*. All the four *Aspergillus* species tested elicited phytoalexin accumulation in living soybean cotyledons. Results from a time course study indicated that maximum concentrations of the phytoalexin glyceollin, 955 $\mu\text{g/g}$ fresh weight (fw), occurred at day 3 in soybean cotyledon tissue inoculated with *A. sojae*. Other

Aspergillus species caused an accumulation of glyceollin at significantly lower levels. A maximum concentration of coumestrol of 27.2 $\mu\text{g/g}$ fw was obtained from soybean cotyledons inoculated with *A. niger*. [210].

2.1. e Antifungal compounds and fruit ripening

The ripe fruit is the one part of the plant, which is likely to be undefended chemically, since it is provided for animals in return for the widespread dispersal of the seed that lies within the fruit. By contrast, the seed and the seed coat usually possess some chemical toxins, although they are often well protected by physical structures. This is to ensure that the seed is not consumed along with the fruit. The unripe fruit will, however, differ from the ripe fruit in being protected to some degree by physico-chemical barriers from herbivory and fungal pathogens, since the seed within is not yet ready for distribution. Chemical changes during ripening will reduce or eliminate these barriers. In fact, it is well known that the resistance of unripe fruits to fungal decay has been associated with the presence of preformed antifungal compounds in the peel. Pathogens often infect unripe fruits but then they remain quiescent, with the onset of decay coinciding with decreases in concentrations of antifungal phenolics to subtoxic levels as the fruit ripens: the levels of these chemical barriers, as well as the ability of the harvested fruits to synthesize phytoalexins, decline during ripening more rapidly in disease-susceptible cultivars than in resistant ones [96, 97, 103, 211]. The principal phenols in the peach fruit epidermis and subtending cell layers include chlorogenic acid, catechin, and epicatechin. Fruits of certain genotypes such as the South American cv. Bolinha with a high level of resistance to the brown rot fungus, *M. fructicola*, have as much as three times the level of chlorogenic acid than susceptible ones at equivalent stages of maturity. The decline during fruit ripening in chlorogenic acid and other endogenous phenolics, together with the thinning of the cuticle, correspond to the transition to susceptibility that occurs in fruit of cv. Bolinha and of less resistant cultivars [212]. Similarly, after successful flower infection, *B. cinerea* remains quiescent in immature strawberry fruits with a high content of proanthocyanidins: an inverse relationship between the proanthocyanidin content of immature strawberry fruits of various cultivars and the colonization of *B. cinerea* has been observed. This negative correlation is attributable to an inhibitory effect of proanthocyanidins on the fungal polygalacturonase activity. When the inhibitory potential of proanthocyanidins in fruits decreases due to maturation, the hitherto quiescent fungus is no longer confined and can progress further into the fruit tissue [213].

Postharvest treatments such as irradiation, curing (heat treatment), high CO_2 concentration, superatmospheric oxygen levels and ultra low oxygen (ULO) concentration, affect postharvest quality of fruit and vegetables by inducing a relevant biosynthetic increase of endogenous phenolic compounds

that, in turn, enhances host resistance to postharvest pathogens [214-220]. UV-C irradiation alters the levels of the flavanone naringin and the polymethoxyflavone tangeretin in the peel of *Citrus aurantium* fruits and this, in turn, reduces the growth of *Penicillium digitatum* on irradiated fruits. It has been suggested that the two constitutive flavonoids, naringin and tangeretin, may act as fungitoxins in the resistance mechanism against fungal attack, acting as first and second defence barriers, respectively, since tangeretin is mainly localised in the outermost tissue of the fruit while naringin is located in the albedo which is immediately below the flavedo [115]. UV-C treatments are also used to elicit phytoalexin production in plant tissues. UV-C irradiation at 0.5 kJ m^{-2} reduces decay without causing damages or affecting the postharvest quality of grapefruit: phytoalexin accumulation and decay control are dependent on cultivar, treatment dose, and harvest date. After UV-C irradiation, the coumarin phytoalexins scoparone and scopoletin accumulate in flavedo tissues. Experimental evidence indicates that the effect of UV light in reducing the citrus *P. digitatum*, the most important postharvest rot of citrus fruit, is an induced resistance phenomenon (production of phytoalexins) rather than a germicidal effect of the UV-C treatment [217]. In grapevine berries UV light treatments induce the production of resveratrol, whose concentration has been found 2-30 folds higher in induced berries as compared to the non-induced ones, and this phenomenon has been particularly prominent in non-infected berries surrounding the infected site [190, 221]. Finally, curing (postharvest hot air treatment) of plant commodities before cold storage has been successfully utilized in order to improve the shelf life of fruit and vegetables. Curing, e.g. to cure wounds and injuries caused during postharvest handling, can have positive effects in reducing pathogen levels and disease development by direct effect of heat on fungal pathogens and by stimulating certain host-defence responses. These responses include the increase in constitutive antifungal phenolics that inhibit fungal development in the fruit tissues, the production of a lignin-like material that serves as a mechanical barrier against pathogen invasion, and the synthesis of phytoalexins. Studies on various citrus fruit cultivars have shown that curing of *Penicillium spp.*-inoculated fruits prevented the development of the pathogen and promoted the biosynthesis of the phytoalexin scoparone in cells adjacent to the wound [189, 222].

2.2. The role of phenolics in plant-insect interactions

The ecological relationship between plants and insects is a complex one with physical as well as chemical interactions. This relationship is also affected by plant factors, insect factors and by some insect-plant factors, including hypersensitive reaction and plant resistance to insect-borne diseases. Various environmental conditions can modify the expression of these factors by acting

primarily on the insect, the plant, or the insect-plant relationships. Each of the plant or insect mechanisms indicated may be the result of one or more genetic factors [223]. In this context, an interesting question concerns as to how insects select plants for both food and as sites for ovipositing. Insects possess a range of chemoreceptors (gustatory and olfactory chemoreceptive systems), mainly on their antennae and mouthparts, which enable them to discriminate a wide variety of chemical compounds at often unbelievably low concentrations and encode this information. Afterwards, this information is processed by the decoding command centers localized in the central nervous system [14, 224, 225]. A systematic evaluation of the kinds of plants fed upon by larvae of butterfly leads to the conclusion that secondary plant metabolites play a leading role in determining patterns of utilization. This seems true not only for butterflies but for all phytophagous insects. The biosynthesis in plant tissues of chemical compounds not directly related to their basic metabolic pathways but not inimical to normal growth and development serves to reduce or destroy the palatability of the plant in which they are produced [226-229]. Plant constituents that make unpalatable a host are secondary metabolites in sufficient concentration to exert an undesirable physiological effect. Such a plant protected from the attacks of phytophagous insects, would in a sense have entered a new adaptive zone. Therefore, plant secondary compounds have received much attention as proximate and ultimate determinants of host-plant range in phytophagous insects [230]. Phytophagous insects, however, can evolve in response to physiological obstacles. If a recombinant or mutation appeared in a population of insects that enabled individuals to feed on some previously protected plant group, selection could carry the line into a new adaptive zone. Changes in food plant choice would be especially favoured in situations where the supply of the preferred plant is sufficiently limited to be an important factor in the survival of the larvae. Therefore, Ehrlich and Raven [227] have proposed that through the process of co-evolution, insects are able to detoxify certain defensive substances that deter feeding so that, eventually, the same deterrent compounds become feeding attractants. Finally, it must be emphasized that insects are reported to utilise secondary metabolites to increase their fitness. It is well known the ability of certain insects to sequester plant toxins from their food plants in the larval stage. They then move these toxins into the adult imago and both larva and adult generally gain protection from bird predation. Besides clear and well-documented fitness benefits of sequestration in highly aposematic species, there are many cases, in which insects deposit chemicals in the cuticle yet are not warningly coloured, and others where they gain protection from predators as a result of the gut contents alone [231-234]. Butterflies (*Polyommatus icarus*) reared at larval stage on inflorescences of *Vicia villosa*, a plant species acceptable for larval development even if not used as food source in nature, showed some important

features of the uptake and sequestration of plant secondary metabolites by insects: (i) larvae incorporated only part of the flavonoids of their host plant (mainly quercetin and kaempferol, while other flavonoids were mostly excreted); (ii) the content of ingested flavonoids was correlated with the body mass; (iii) the flavonoids were metabolized and their conjugated forms were stored. These data also suggest that females are better adapted to sequester these compounds than males and that the accumulation of flavonoids in the smaller wings of the female is used in visual communication, as flavonoid-rich females are more attractive to males than flavonoid-free females [235-237].

Insects searching for an acceptable host plant must first locate and identify the appropriate plant species. We know that the speed of host-finding may be important. There may be time limits for various reasons, while other ecological circumstances commonly impose a need for speed, such as when resources are rare or scattered and predators make searching risky. The accuracy with which host taxa are selected, and individual plant quality assessed, are also important, especially for insects with narrow host ranges and specific nutritional requirements for larval development. The majority of insect species use a very restricted number of hosts that typically share characteristic phytochemicals, some volatile and some non-volatile. A subset of these compounds seems to be of great importance for the identification of the host, and in some extreme specialists, great sensitivity to one or few host-specific chemicals totally dominates in host selection [226, 233, 238-240].

Plant volatiles, visual and thigmotactic cues may be involved in an insect's recognition of, and migration to a host plant. After locomotion is arrested, probing occurs. The net result of individual feeding stimuli and deterrents determines whether the insect will remain and feed. Whether a plant is accepted or rejected as food by insects depends largely on its chemical composition in addition, of course, to physical factors such as toughness, thickness, hairiness, etc.. In addition, chemical inhibitors play an important role in the inhibition of oviposition on the host-plant and, in turn, in insect larval growth and survival of progeny [234, 241-243]. Studies on the role of inhibitors in host plant selection indicate that many different chemicals may be expected to have an inhibitory effect on feeding by different insects. Among plant constituents, it is now generally accepted that plant phenolics play a role in protecting plants from both insect and mammalian herbivory [244-248]. Traditionally, methods for demonstrating that constitutive phenolics are involved in plant defence have depended on measuring the total phenolic content of plant tissues. While such measurements might occasionally show some correlation, experience shows that it is an individual subclass of phenolic or an individual structure that is active against a particular herbivore. In addition, one of the most important advances in understanding plant-insect interactions has been the discovery of the induced defence: some plants

respond to insect feeding by increasing the synthesis of a particular phenolic toxin(s). Finally, the concentration of the toxic phenolic compound(s) in the plant is a key factor in deterrence and it is the accumulation of phenols in particular parts of the plant which represent a feeding barrier [228, 234].

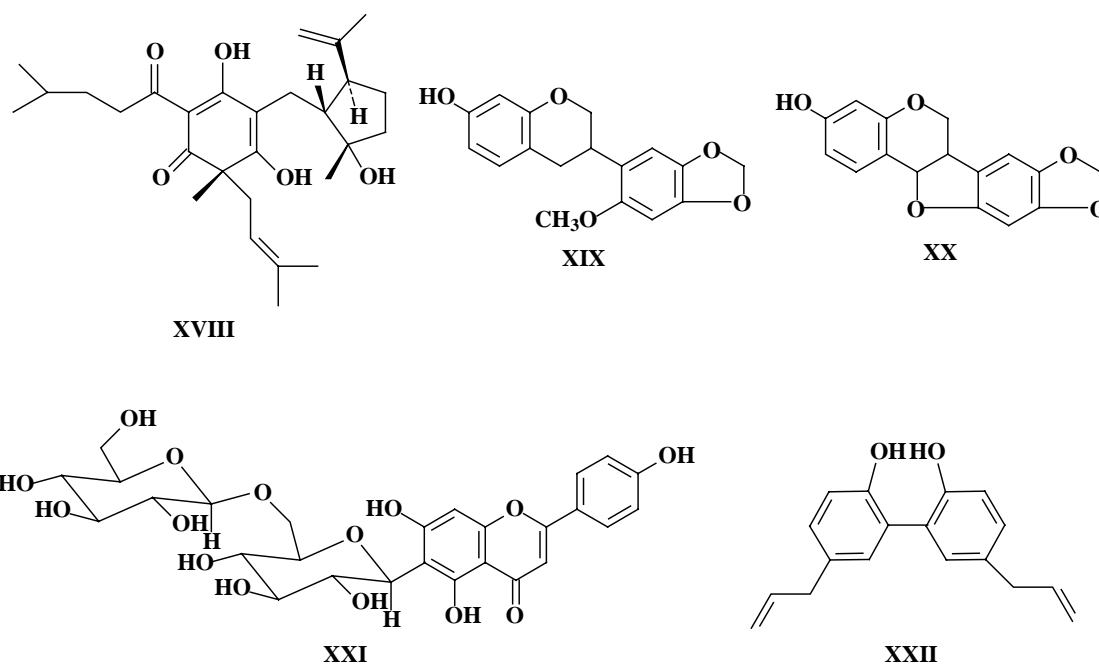
A well-known example of simple phenolics being feeding barriers to insect herbivores is represented by salicylates in *Salix* leaves and their role in the feeding and growth of the polyphagous larvae of *Operophtera brumata*. It has been observed that the levels of salicylates correlated negatively with growth: larvae exposed to leaves rich in these compounds grew slowly and consumed less material, and this suggests that salicylates could be considered as antifeedants for *O. brumata* [248]. Some authors [249] observed that the flower of *Hypericum calycinum*, which appears uniformly yellow to humans, bears an UV pattern, presumably visible to insects. Two categories of phenolic pigments, flavonoids (quercetin-3-*O*- β -D-glucuronide and the dimeric flavonoid, I3-II8-biapigenin) and dearomatized isoprenylated phloroglucinols (hypercalin A (XVIII), hypercalin B, hypercalin C, chinesin I, and chinesin II), are responsible for the UV demarcations of this flower. The finding that isoprenylated phloroglucinols are present in the anthers and in the ovarian wall of the flower raised the possibility that these compounds also served a second function. It made sense to presume that both pollen and developing seeds might be in need of protection, and the finding that hypercalin A was deterrent and toxic to *Utetheisa ornatix* larvae suggested that the second function was defense.

Chlorogenic acid, a phenylpropanoid derivative, showed antifeeding properties in the same system, even if, because of its widespread occurrence in the plant kingdom, this compound is less likely to be a useful defensive agent in that many insects may become adapted to it and hence would be expected to tolerate its dietary presence. The effects of pure chlorogenic acid on insect feeding behaviour were tested using four common leaf beetle species, which are in the field mainly found on willows with low-chlorogenic acid leaves. One species, *Lochmaea capreae* L., was invariably deterred by pure chlorogenic acid applied in naturally occurring concentrations on the willow leaves. Accordingly, in 2-choice laboratory feeding trials *L. capreae* was found to prefer low-chlorogenic acid leaves of four willow species over high-chlorogenic acid leaves of *Salix pentandra* L. and *S. myrsinifolia* Salisb. When presented on the leaves of *S. phylicifolia* L, pure chlorogenic acid inhibited also the feeding by *Phratora polaris* Sp.-Schn. Instead, chlorogenic acid had no significant effect on *Ph. polaris* when it was presented on the leaves of another willow *S. cinerea* L. Thus, the response of *Ph. polaris* to chlorogenic acid seems to depend on the plant species. To two other leaf beetle species, *Galerucella lineola* F. and *Plagioderma versicolora* Laich., chlorogenic acid is an ineffective deterrent even at unnaturally high concentrations. Thus, among

four studied leaf beetle species, only *L. capreae* seems to be clearly affected by this phenolic. Therefore, the overall importance of chlorogenic acid as a defence against willow-feeding leaf beetles appears to be very limited [250].

Most plants contain an array of flavonoids, whose fingerprints often differ among families, genera and species [25]. The fact that phytophagous insects usually differentiate among families suggests that flavonoids play a role in host selection. Plant flavonoids affect the behaviour, development and growth of a number of insects [248, 251, 252]. Some flavonoids are feeding stimulants for the boll weevil, *Anthonomus grandis*, [253], or oviposition stimulants of a Citrus-feeding swallowtail butterfly, *Papilio xuthus* L., [254] or, finally, antibiotic substances efficient against phytophagous insects [244, 246, 255-261]. In comparison to many other secondary metabolites, flavonoids are apparently not very toxic to and have a low physiological activity in most insects. Nevertheless, many flavonoids can act as feeding deterrents to phytophagous insects at relatively low concentrations. So, the concentrations of flavonoids in plants are normally far higher than those needed to have a deterrent effect on aphid feeding. However, aphids tend to feed from tissues such as the phloem, which are generally low in flavonoids, and thus they will normally only encounter high levels while probing the plant tissues for phloem sap, and not while feeding [262].

Four isoflavonoids (judaicin (XIX), judaicin-7-*O*-glucoside, 2-methoxy-judaicin, and maackiain (XX)) isolated from wild relatives of chickpea, *Cicer arietinum*, were shown to deter larval feeding by *Helicoverpa armigera* at 100 ppm. All four isoflavonoids showed a dose-dependent decrease in activity, with judaicin and maackiain retained their antifeedant activity at 50 ppm and



10 ppm, respectively. The isoflavonoids were tested in combinations and with chlorogenic acid: the combinations containing judaicin and maackiain were most active, and chlorogenic acid enhanced the antifeedant activity of all four isoflavonoids. *H. armigera* was the only one of four noctuids to be deterred by all four isoflavonoids. *Spodoptera littoralis* was deterred by judaicin alone and *S. frugiperda* by maackiain alone. *Heliothis virescens* and *S. exigua* were not deterred from feeding by any of the isoflavonoids. When incorporated into a diet, isoflavonoids decreased the weight gain of early stadia larvae of *H. armigera* more than they did later stadia, and maackiain and judaicin were most potent. These data suggest that these isoflavonoids, especially maackiain and judaicin, could play a role in decreasing the susceptibility of *Cicer* to attack by *H. armigera* [252]. The host plants of the native American butterfly, *Pieris napi oleracea*, include most wild mustards. However, garlic mustard, *Alliaria petiolata*, a highly invasive weed that was introduced from Europe, appears to be protected from this insect. Although adults will oviposit on the plant, most larvae of *P. n. oleracea* do not survive on garlic mustard. By using feeding bioassays with different larval stages of the insect to monitor the isolation and identification of two bioactive constituents that could explain the natural resistance of this plant, Renwick *et al.* [263] found that a cyanopropenyl glycoside, alliarinoside, strongly inhibits feeding by first instars through an apparent post-ingestive feedback mechanism, while a flavone glycoside, isovitexin-6''-D- β -glucopyranoside (XXI), acts as a direct feeding deterrent that is perceived by taste receptors on the mouthparts of late instars. Interestingly, the first instars are insensitive to isovitexin-6''-D- β -glucopyranoside, and the late instars are little affected by alliarinoside.

Most of the researches concerning insect antifeedants have concentrated on their effects upon agricultural pests, in order to determine how flavonoids can confer resistance to crops against insect attack. In this connection, flavonoid HPLC analyses of cultivated and wild species of genus *Vigna*, an important food legume in many countries in sub-Saharan Africa and Latin America, showed that cultivated lines of cowpea (*Vigna unguiculata* L. Walp.) are very similar from a qualitative point of view, always showing three flavonoid aglycones: quercetin, kaempferol and isorhamnetin. In addition, a positive relationship between resistance/susceptibility characteristics against aphids (*Aphis craccivora* Koch) and flavonoid glycoside content of cowpea lines was found. The resistant lines showed a flavonoid content higher than susceptible ones. *In vitro* bioassays proved that, amongst endogenous flavonoids, quercetin and isorhamnetin possess a good inhibitory aphid reproduction rate. On the contrary, flavonoid HPLC analyses of wild *Vigna* species supported evidence for the existence of different flavonoid chemotypes in some species of section *Vigna*. There are kaempferol chemotypes, kaempferol being the main aglycone detected, quercetin chemotypes, containing only quercetin

glycosides, and two isorhamnetin chemotypes. From an ecological point of view the most interesting chemotypes are some accessions, belonging to the same species, which permits to study, *ceteris paribus*, the role of endogenous flavonoids in plant resistance to aphids. Amongst *V. marina* accessions, two chemotypes were found: *V. marina* var. *oblonga* TVnu 1174 (isorhamnetin chemotype) contained two isorhamnetin glycosides and traces of two kaempferol glycosides, while *V. marina* var. *marina* TVnu 717 (kaempferol chemotype) contained only kaempferol glycosides. *V. luteola* accessions also showed two different chemotypes: the accession TVnu 475 contained only quercetin glycosides, while the other two accessions TVnu 172 and TVnu 905, kaempferol chemotypes, contained robinin (kaempferol-3-robinoside-7-rhamnoside). When the resistance characteristics to aphids in different chemotypes of the same species were tested, it became evident that quercetin or isorhamnetin chemotypes showed a higher level of resistance compared to the kaempferol chemotypes of the same species, thus demonstrating a direct involvement of quercetin or isorhamnetin in the resistance mechanism [264].

Here it must be stressed that the salivary secretions of phytophagous insects contain various enzymes, playing a fundamental role in food digestion of sucking-piercing insects. Among these enzymes, polyphenol oxidases (PPO) [E.C. 1.10.3.1.] and peroxidases (POD) [E.C. 1.11.1.7], oxidoreductases metabolizing plant phenolics, are also present. Both enzymes have been identified in the salivary secretions of several aphid species and various functions have been proposed for these enzymes. At first, it was suggested that they are involved in the chemical stabilisation of the stylet sheath. Secondly, they were thought to enable phytophagous insects to overcome plant defences by neutralising phenolics and their derivatives. In fact, it has been observed that when the stylets of grain aphids penetrate plant tissues, the ruptured cells may become partially filled with aphid saliva. Thus, the salivary sheath material may absorb plant metabolites from plant tissues. As a result, the grain aphid PPO secreted in its saliva can react with the phenolics accumulated around the aphid stylet sheath, thus oxidizing and polymerizing them. Natural occurring phenolic toxins are thus converted into less toxic substances [265].

It is also true that the effectiveness of phenolics as a resistance factors to insect feeding is enhanced by oxidation to polymers, which reduce digestibility, palatability and nutritional value. Thus high levels of PPO, the major phenolic oxidising enzyme of plants, can be correlated with plant resistance mechanisms against insects. Polyphenol oxidases, presumed antiherbivore enzymes, are antinutritive enzymes that decrease the nutritive value of wounded plant tissues by cross-linking proteins or catalyzing the oxidation of phenolic toxic metabolites to reactive and polymerizing quinones. The inducible expression of PPO was examined in hybrid poplar: following mechanical wounding, simulating insect damage, PPO activity increased in

wounded and unwounded leaves on wounded plants beginning at 24h and 48h, respectively. Feeding by forest tent caterpillar also induced PPO expression. This wound- and herbivore-induced expression of PPO in hybrid poplar supports the defensive role of this enzyme against insect pests. The results obtained showed that: (i) PPO induction is systemic, i.e. induced PPO functions in reducing additional herbivore damage rather than in wound repair; (ii) PPO induction is mediated by increased mRNA accumulation, which is typical of a variety of induced defence proteins [266, 267].

Flavonoids are often phagostimulatory for polyphagous insects, at least at low concentrations, and it has been suggested that for some species this may be related to the attraction for novelty with the potential benefits of exploiting new foods. For example, the flavonoid glycoside, rutin (quercetin-3-*O*- β -rutinoside), is a phagostimulant for *Schistocerca americana* even at 10% of the dry weight of a glass-fiber disc, and 2% dry weight added to 10% dry weight of sucrose enhances phagostimulation. At least at low concentrations, rutin is also a phagostimulant for two other polyphagous species, *Schistocerca albolineata* (Thomas) and *Melanoplus differentialis* (Thomas). The insects appear able to stabilize their intake and, at high concentrations, rutin ceases to be a phagostimulant after two days. Much of the rutin is hydrolyzed in the gut and excreted, but some is absorbed and metabolized to β -3-*O*-glucoside. This is sequestered in the cuticle, where it imparts a yellowish tinge to the exocuticle and exuviae [233, 251]. In addition, some flavonoids are oviposition stimulants of a *Citrus*-feeding swallowtail butterfly, *Papilio xuthus* L. [254]. Narirutin (naringenin-7-*O*- β -rutinoside), hesperidin (hesperetin-7-*O*- β -rutinoside) and rutin isolated from the leaves of *Citrus unshiu* were found to play a role in the ovipositional behaviour of *P. xuthus*. Each of these compounds is inert on its own and, even when the glycosides are mixed together in similar concentrations to those occurring in *Citrus* leaves, they only evoke a weak response from *P. xuthus*. When the glycosides are mixed with two other water-soluble (organic bases) constituents from *C. unshiu* leaves, the ovipositional behaviour of *P. xuthus* is comparable to that induced by intact leaves, thus demonstrating a synergistic action.

The herbaceous perennial plant, *Phryma leptostachya* L., widely distributed in the Himalayas, temperate Asia, and northern East America has been traditionally used as natural insecticide in East Asia. From the root extracts of this plant, various lignans of the 3,7-dioxabicyclo-[3.3.0]octanes (furofuran) type, showing insecticidal activity against larvae of some species of lepidopterous insects, have been isolated. Leptostachyol acetate, a lignan isolated from the roots of *P. leptostachya* var. *asiatica* was lethal to third instar larvae of *Culex pipiens pallens*, *Aedes aegypti*, and *Ocheratatos togoi*, three mosquito species, at 10 ppm [268]. Another toxic lignan that has been implicated as defensive agent against herbivores is magnolol (XXII) isolated

from leaves of *Magnolia virginiana*, which resulted to be toxic to larvae of the moth *Callosomia promethean* [234].

In addition, tannins are generally considered to be deleterious to phytophagous insects. Tannins may affect the growth of insects in three main ways: they have an astringent taste, which affects palatability, and decreases feed consumption, they form complexes of reduced digestibility with proteins and they act as enzyme inactivators. Tannins are present at high levels in most plant seeds and grains, where they negatively impact the use of seeds and grain in insect feed [269]. In cowpea, condensed tannins (proanthocyanidins) contribute to resistance to infestation by insects such as the cowpea weevil *Callosobruchus maculatus* (F.) [270]. An increased level of proanthocyanidins during seed and grain development and maturation has been studied in a number of different plant species. A significant increase in the degree of polymerization of proanthocyanidins has been observed during maturation of sorghum grains [271]. In *Phaseolus vulgaris* a gradual increase in condensed tannins and α -amylase inhibitory activity, both considered defensive tools against phytophagous insects, was observed from anthesis to seed maturity [272]. In this connection, two accessions of stored cowpea seeds, both classified as susceptible accessions, showing a different degree of bruchid damage in storage, were analysed for their condensed tannins and α -amylase inhibitors contents as defensive compounds against cowpea weevil: IT 84E-1-108 exhibited an elevated degree of infestation (about 30%), while Vita 7 did not show damages caused by cowpea weevil larvae. Surprisingly, no α -amylase inhibitory activity was found in cotyledons of Vita 7 seeds, while IT 84E-1-108 exhibited a moderate level of inhibitory activity. On the contrary, the seed coat tannin content was found thirteen times higher in not damaged Vita 7 seeds than in IT 84E-1-108 infested seeds. These last results support the hypothesis that, if bruchids infest cowpea when the grain is stored after harvest, seed coat tannins are effectively involved in the biochemical defence mechanisms, which can deter, poison or starve bruchid larvae that feed on cowpea seeds [270].

Even if tannins are generally considered to be deleterious to herbivores, more recent work suggests that insects can overcome this problem by modification of gut structure or the midgut environment. Some polyphagous grasshoppers generally have the ability to tolerate the ingestion of gallotannins. Thus, they are able to tolerate the reactive oxygen species of the tannin generated by polyphenol oxidation. Alternatively, they rely on rapid and extensive hydrolysis of the tannin to avoid any damaging effects [273]. Some grasshoppers with different feeding habits have thick peritrophic envelopes that adsorb tannins and do not permit the passage of tannic acid so that it does not reach the tissues. In addition, the midgut environment of *Schistocerca gregaria* contains surfactants that greatly reduce the formation of tannin-protein complexes except at very high tannin concentrations [233].

3. Final remarks

Plant phenolics are secondary metabolites involved in the defence mechanisms of plants against fungal pathogens and insect herbivores. Plants respond to diverse environmental enemies with a bewildering array of responses, which use constitutive and induced phenolic substances affecting the susceptibility/resistance characteristics of the attacked plant. The production of the resistance traits is likely to be costly if fitness-limiting resources are invested and allocated to regrowth (tolerance) or to the production of compounds that directly affect the attacking enemies. Plants currently bred for increased levels of phenolics might well influence different aspects of plant/pathogen and/or plant/insect interactions. Studies on these interactions are needed in order to have a better understanding of how increasing the expression of an endogenous phenolic compound or a class/subclass of phenolics in plants could influence plant resistance characteristics against pathogens and phytophagous insects.

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