

# Induced plant secondary metabolites for phytopathogenic fungi control: a review

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## Abstract

Pathogenic fungi constitute one of the main infectious agents in plants, causing alterations during developmental stages including post-harvest. Phytopathogenic fungi are controlled by synthetic fungicides; however, the use of these is progressively restricted due to both, the harmful effects of pesticides on the environment and human health and the appearance of highly resistant fungal strains. Therefore, there is a great demand for novel natural fungicides. Higher plants are rich source of bioactive secondary metabolites of wide variety such as tannins, terpenoids, saponins, alkaloids, flavonoids, and other compounds, reported to have *in vitro* antifungal properties. Thus, secondary metabolites with antifungal activity represent an alternative for achieving a sustainable control of phytopathogenic fungi and to reduce the heavy reliance of synthetic pesticides used to control them. Plant antifungal metabolites may be preformed inhibitors that are present constitutively in healthy plants (phytoanticipins), or they may be synthesized *de novo* in response to pathogen attack or another stress conditions (phytoalexins). These molecules may be used directly or considered as a precursor for developing better fungicidal molecules. This review presents a selection of antifungal agents induced in plants during fungal attack that can be potentially used for phytopathogenic fungi control in crops.

**Keywords:** phytopathogenic fungi, antifungal activity, secondary metabolites, phytoalexins.

## 1. Introduction

Phytopathogenic fungi are one of the major biotic stresses that contribute substantially to the overall loss in yield among crop plants. In fruit and vegetables, there is a wide variety of fungal genera that cause quality problems related to aspect, nutritional value, organoleptic characteristics, and limited shelf life (Agrios, 2004). In fact, according to current estimates, almost 10 to 20% of staple foods and cash crops are destroyed by phytopathogenic fungi (Hewitt, 2000). The most of the control strategies for this fungus have been based on the use of synthetic fungicides. Nevertheless, this method has become less effective and more restricted due to the appearance of highly resistant isolates (Yourman and Jeffers, 1999) and to the negative environmental effects that derive from the indiscriminate use of these products (Paulitz and Bélanger, 2001). This, in addition to the increasing demand by environmentally safe agrochemicals generated by alternative agriculture systems that promote the sustainable and healthy production of foods, has stimulated the development of research oriented to provide alternative control strategies, being the use of plants as a source of secondary metabolites with antifungal properties, one of them (Fiori *et al.*, 2000). One of the most important plant defences against fungal pathogens is the production of secondary metabolites (Singh *et al.*, 2003). Plant secondary metabolites possess a wide range of biological activities leading to diverse types of interactions with other organisms (Rizvi and Rizvi, 1992; Einhellig, 1995). In this context, plant natural products offer a pool of structurally diverse antifungal agents, and may provide an alternative to synthetic fungicides to control phytopathogenic fungi (Duke 1990, Singh *et al.*, 2003). Such compounds are active against a limited number of specific target species, are biodegradable to non-toxic products, and are potentially suitable for their use as agrochemicals

in integrated pest management programs (Kim *et al.*, 2004). This review highlights the chemical diversity of induced natural plant secondary metabolites that can be used as a sustainable tool on plant fungal disease management.

## 2. Plant secondary metabolites with antifungal activity

Plants are a rich source of thousands of secondary metabolites (SM). These consist of low-molecular weight compounds that are regarded as not essential for sustaining life, but as crucial for the survival of the producing organism (Hadacek, 2002). These compounds are frequently accumulated by plants in smaller quantities than are primary metabolites (Croteau *et al.*, 2000, Dewick 2002). To the date, the number of described structures exceeds 100,000. However, the real number is certainly much more higher due to only less than 20% of all plants have been studied (Wink, 2010). Plants secondary metabolites are synthesized in specific pathway and sites of production can vary between kinds of compounds as well as between plant species. Moreover, some molecules can be synthesized by all plant tissues, whereas others are produced in a specific tissue or even cell-specific fashion (Yazdani *et al.*, 2011). The site of synthesis for SM is not certainly the site of accumulation. This, hydrophilic compounds are mainly stored in the vacuole while the lipophilic SM are commonly sequestered in resin ducts, laticifers, oil cells, trichomes, or in the cuticle (Engelmeier and Hadacek, 2006).

Secondary metabolites in plants can be divided into three main groups according to their biosynthetic origin: terpenoids, nitrogen-containing compounds (alkaloids, glucosinolates and cyanohydrins) and phenylpropanoids also known as phenolic compounds

(Croteau *et al.*, 2000). The most important building blocks employed in the biosynthesis of SM are derived from: acetyl coenzyme A, shikimic acid, mevalonic acid, and 1-deoxyxylulose-5-phosphate and, these are utilized respectively in the acetate, shikimate, mevalonate and deoxyxylulose phosphate pathways (Croteau *et al.*, 2000; Dewick 2002) (Figure 1).

Plant SM can play important roles in plant defense and chemical signalling (Inderjit and Weston 2003).

Behavioural messages are delivered by a wide array of chemical compounds. In some cases, they may facilitate communication between the members of a single species (e.g., pheromones) or between members of different species (e.g., allelochemicals) (Nordlund, 1981; Singh *et al.*, 2003). These interactions include largely a negative effect on germination, growth, development, distribution and behaviour of other organisms (Rizvi and Rizvi 1992, Einhellig 1995).

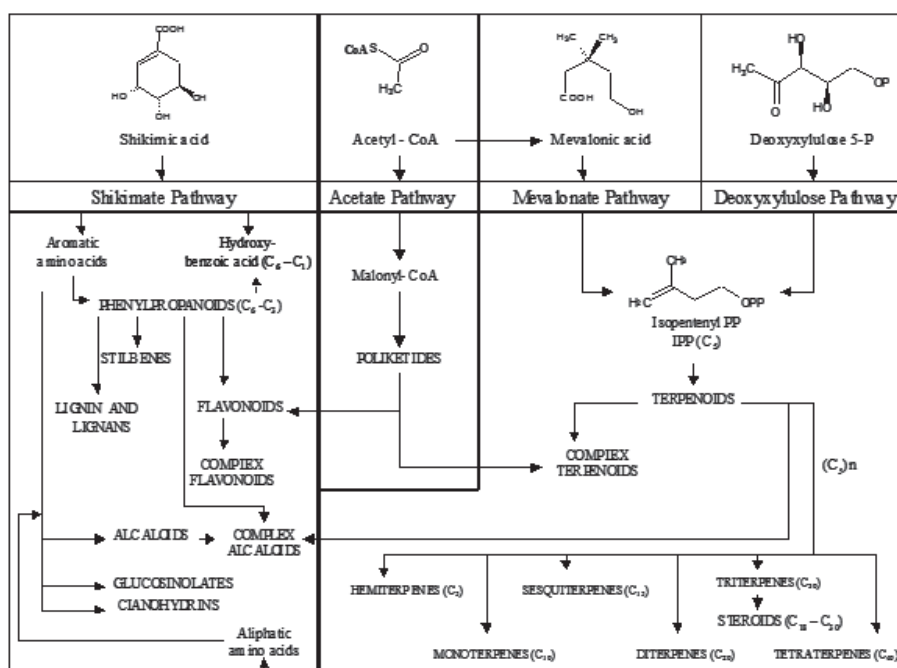


Figure 1. Biosynthetic pathways of plant secondary metabolites.

Secondary metabolites with antifungal activity derived from plants may be preformed inhibitors that are present constitutively in healthy plants (phytoanticipins), or they may be synthesized *de novo* in response to pathogen attack or another stress conditions (phytoalexins) (Morrissey and Osbourn, 1999; Os-

bourn, 1996, 1999; Dixon, 2001). These definitions are based on the dynamic of the synthesis of the antifungal molecule, not on its chemical structure, which can be unclear sometimes due to the same compound, can act as phytoalexins in one plant and as phytoanticipin in another. In addition, the same molecule can be

a phytoalexin or a phytoanticipin in different organs of the same plant (Grayer and Kokubun, 2001).

### 3. Induced local defence by means of phytoalexins

The synthesis of phytoalexins requires transcriptional and/or translational activity in the plant once the pathogen has been detected. Furthermore, the induced response mechanism includes the trafficking and secretion of these compounds to the infection site (Bednarek and Osbourn, 2009). González-Lamothe *et al.* (2009) indicated that this definition avoids the appointment of a role in plant disease resistance for these molecules, due to, although a function in plant defence is assumed, this role cannot always be easily verified.

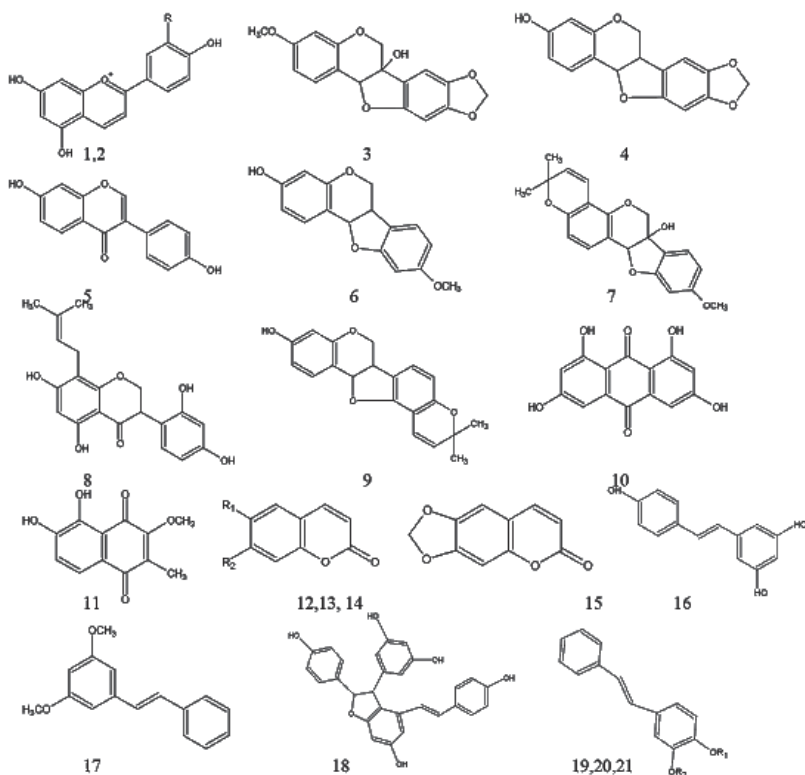
Phytoalexins are a group of structurally diverse molecules that are generally lipophilic and nonspecific in their antifungal activity (Smith, 1996; Morrissey and Osbourn, 1999; González-Lamothe *et al.*, 2009). As mentioned above, the term “phytoalexins” is usually restricted to compounds that required the *novo* expression of genes involved in the metabolic pathway. This is an economical way to counteract fungal pathogens, because phytoalexin synthesis occurs only during the early period of infection, and usually localized in a certain area (Morrissey and Osbourn, 1999). Then, unchallenged plants use the energy consumed in phytoalexins synthesis, for the normal metabolic processes of the plant (Grayer and Kokubun, 2001; Commun *et al.*, 2003). Although both, disease-resistant and –disease-susceptible plants may respond to pathogen attack by producing phytoalexins, these compounds generally accumulate more rapidly at high levels in resistant plants (Morrissey and Osbourn, 1999). It is important to mention that the accumulation of phytoalexins in plants can be induced by all,

microorganisms, insects and abiotic stresses such as freezing, salts, heavy metals or ultraviolet radiation (Pellicice *et al.*, 2000). Most phytoalexins are less fungitoxic than synthetic fungicides, but they can accumulate in large quantities within plant tissues, exceeding the concentrations needed to inhibit fungal growth (Jeandet *et al.*, 2002).

#### 3.1 Phenolic phytoalexins

This group includes metabolites derived from the condensation of acetate units, those produced by modifications of aromatic acids, phenolic compounds such as phenolic acids, coumarins, stilbenes, flavonoids, isoflavonoids, quinones and xanthenes (Grayer and Harborne, 1994; Morrissey and Osbourn, 1999; González-Lamothe *et al.*, 2009). Some examples of phenolic phytoalexins are shown in Figure 2.

Cereal plants are reported to accumulate various flavonoids upon infection by a range of pathogens. In response to fungal infection by both pathogenic and nonpathogenic fungi, sorghum plants (*Sorghum bicolor*) produces a complex mixture of flavonoids (Nicholson *et al.*, 1987; Snyder and Nicholson, 1990). In this specie, the accumulation of 3-deoxyanthocyanidins has been shown to be toxic to the pathogen *Colletotrichum graminicola* (Nicholson *et al.*, 1987; Snyder and Nicholson, 1990). The major components of this mixture are a group of structurally related compounds, the 3-deoxyanthocyanidins apigeninidin (**1**), luteolinidin 5- methyl ether, apigeninidin 7-methylether and the caffeic acid ester of arabinosyl 5-O apigeninidin (Warton and Nicholson, 2000). In sugarcane, luteolinidin (**2**) and one of its glycosides are detected, but the activity of these compounds against the sugarcane red rot disease, *Colletotrichum falcatum*, is minimal (Brinkr and Seigler, 1991).



**Figure 2.** Representative phenolic phytoalexins : Apigeninidin (1 R=H), Luteolinidin, (2 R=OH) Pisatin, (3), Maackiain (4), Daidzein (5), Medicarpin (6), Glyceollin (7), Keivitone, (8), Phaseollin, (9), Emodin-a (10), Malvone A (11), Umbelliferone (12 R<sup>1</sup>=H, R<sup>2</sup>=OH), Scopoletin, (13 R<sup>1</sup>=MeO, R<sup>2</sup>=OH), Scoparone (14 R<sup>1</sup>=MeO, R<sup>2</sup>=MeO), Ayapin (15), Resveratrol (16), Pterostilbene (17), ε-Viniferin (18), Pinosylvins, (19 R<sup>1</sup>=R<sup>2</sup>=H), Pinosylvin monomethyl ether (20 R<sup>1</sup>=H, R<sup>2</sup>=Me), Pinosylvin dimethyl ether (21 R<sup>1</sup>=R<sup>2</sup>= Me).

The isoflavonoid phytoalexins have been characterized especially from bean (*Phaseolus vulgaris*), soybean (*Glicine max*), pea (*Pisum sativum*), and alfalfa (*Medicago sativa*) (Dewick, 2002). Pea plants produce pisatin (3), while chickpea plants produce maackiain (4) (Morrisey and Osbourn, 1999). The isoflavone daidzein (5) is the precursor of the major phytoalexins including medicarpin (6) and glyceollins (7), which are produced in alfalfa and soybean, respectively (Graham, 1995). The isoflavone genistein with antifungal activity (Graham and Graham, 2000),

is the precursor to the phytoalexin keivitone (8) produced by bean, and it is involved in the pathway leading to the glyceollin response of soybean cells (Shanker *et al.*, 2003). Another interesting example is founded in Colombian bean cultivars. In resistant plants to *Colletotrichum lindemuthianum* fungus, the causal agent of anthracnose disease, phaseollin (9) production is higher than in susceptible plants (Durango, 2002).

Emodin (10), an anthraquinone, isolated from *Rhammus triquetra* bark was highly effective against

spore germination of 17 tested fungi species, including seven species of *Alternaria* and three species of *Fusarium*, and was also highly inhibitory against a pathogenic basidiomycete fungus *Fomes annosus* (Izhaki 2002). Malvone A (**11**) (2-methyl-3-methoxy-5,6-dihydroxy-1,4-naphthoquinone) has been identified as a phytoalexin induced in *Malva sylvestris* L. by the plant pathogen *V. dahliae*, showing antifungal activity against this pathogen (Veshkurova *et al.*, 2006).

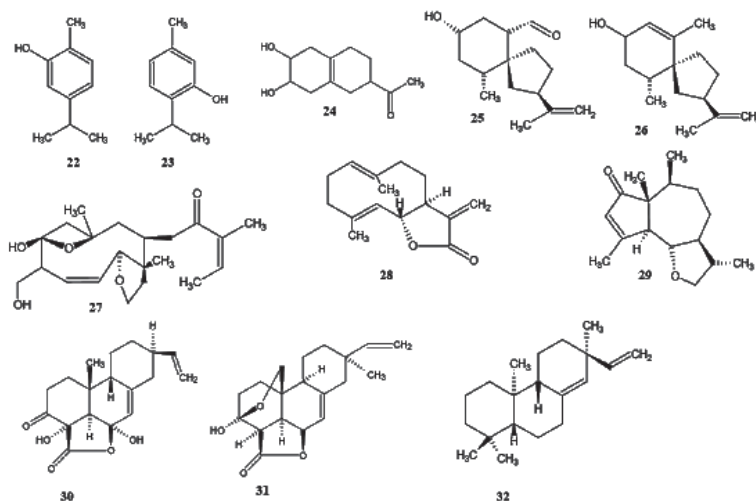
In response to infection for some fungi such as *Ceratostyxis fimbriata*, coumarins are produced at an early state in sweet potato tissue. The main constituents induced were identified as umbelliferone (**12**) and scopoletin (**13**), and the minor ones esculetin and the two  $\beta$ -D-glucosides such as scopolin and skimminn (Uritani and Hoshiya, 1953; Minamikawa *et al.*, 1962; Tanaka *et al.*, 1983; Minamikawa *et al.*, 1964). In addition, several authors have been reported that scoparone (6,7-dimethoxycoumarin) (**14**) is the main phytoalexin involved in induced defense mechanism of citrus against pathogens such as *Phytophthora parasitica* (Afek, *et al.*, 1986), *Guignardia citricarpa* (De Lange *et al.*, 1976), *Penicillium digitatum* (Kim *et al.*, 1992) and *Diaporthe citri* (Arimoto *et al.*, 1986). Detached leaves, leaf disks, stems and hypocotyls of sunflower (*Helianthus annuus*) plants accumulate scopoletin and ayapin (**15**), in response to both pathogenic (*Alternaria helianthi*) and nonpathogenic (*Helminthosporium carbonum*) fungi (Tal and Robeson, 1986).

Leaves and fruits of grapevines synthesize antifungal metabolites in response to fungal infection and abiotic treatment (reviewed in Morrissey and Osbourn, 1999; Jeandet *et al.*, 2002). After fungal attack of grapevine and berries leaves produce resveratrol (*trans*-3,5,4'-trihydroxystilbene, **16**) (Langcake and Pryce, 1976) and related compounds which have antifungal activity toward a number of fungal pathogens, including *Rhizopus stolonifer*, *Plasmopara viticola*, and *Botrytis cinerea* (Jeandet *et al.*, 2002). The ability of *B. cinerea* to infect grapevine has been as-

sociated with its capacity to degrade stilbene phytoalexins. Laccase-mediated oxidation of resveratrol by a number of enzymes has been described (Adrian *et al.*, 1998). Resveratrol have been also recognized as phytoalexin produced by *Arachis* and *Trifolium* (Leguminosae) and, *Broussonetia* (Moraceae), *Festuca*, *Saccharum* (Gramineae) and *Veratrum* (Liliaceae) (reviewed in Grayer and Harborne, 1994). Other related compounds that have been found in grapevine as a result of infection or stress treatment are, pterostilbenes (3,5-dimethoxy-4'-hydroxy stilbene) (**17**) and viniferins, in which resveratrol units are coupled together to produce compounds containing aryl dihydrobenzofuran units (Jeandet *et al.*, 2002; Commun *et al.*, 2003; Bala *et al.*, 2000). Pterostilbene, is 5-fold more active than resveratrol. The differences in the antifungal activities of two related stilbene compounds indicate that the *in vivo* methylation of hydroxyphenyl groups can potentially lead to an increased biocidal activity in phenolics (Pezet and Pont, 1990).  $\epsilon$ -viniferin (**18**) has an antifungal activity upon germination of *B. cinerea* conidia very similar to pterostilbene, which is the most toxic stilbene (Dercks and Creasy, 1989). On the other hand, the stilbenes pinosylvins (**19**), pinosylvin methyl ether (**20**) and pinosylvin dimethyl ether (**21**), extracted from white spruce (*Picea glauca*), jack pine (*Pinus banksiana*), and red pine (*Pinus resinosa*) pine cones, inhibit growth of white-tot fungi (*Trametes versicolor* and *Phanerochaete chrysosporium*) and brown-rot fungi (*Neolentinus lepideus*, *Gloeophyllum trabeum* and *Postia placenta*) (Lange *et al.*, 1994).

### 3.2 Terpenoids act as phytoalexins

Terpenoids (mono, di, tri and sesquiterpenes) are frequently highly hydrophobic substances, stored in resin ducts, oil cells or glandular trichomes (Wink, 2010). The chemical structures of the main terpenoids with antifungal activity described in this review are shown in Figure 3.



**Figure 3.** Representative terpenoids phytoalexins: Carvacrol (**22**), Thymol (**23**), Rishitin, (**24**) Lubimin (**25**), Solavetivone (**26**), Niveusin-B (**27**), Costunolide (**28**), Cichoralexin (**29**), Lactucin (**30**) Momilactone A (**31**), Momilactone B, (**32**).

It has been described that the monoterpenes carvacrol (**22**) and thymol (**23**) are the main antifungal compounds found in *Satureja thymbra* and *Thymbra spicata*, respectively (Müller-Riebau *et al.*, 1995). These SM showed effective antifungal activity when compared with some commercial fungicides. In a concentration of 100 mg ml<sup>-1</sup> there was a complete mycelium growth inhibition of *Rhizoctonia solani*, *Fusarium moniliforme*, *Sclerotinium sclerotiorum* and *Phytophthora capsici* (Müller-Riebau *et al.*, 1995). The strong antifungal activity of carvacrol and thymol was also confirmed on the ubiquitous phytopathogenic fungi *B. cinerea* (Bouchra *et al.*, 2003; Tsao and Zhou, 2000; Camele *et al.*, 2012). Also, the monoterpene  $\gamma$ -terpinene, assayed *in vitro* in gaseous state, showed a high inhibitory activity against *B. cinerea* (Espinosa-Garcia and Langenheim, 1991). Moreover, the monoterpene citral has been reported as a potent antimicrobial compound against *Penicillium italicum* (Saddiq and Khayyat, 2010), *B. cinerea* (Tsao

and Zhou, 2000) and *Colletotrichum acutatum* (Alzate *et al.*, 2009).

The sesquiterpenes rishitin (**24**), lubimin (**25**) and, solavetivone (**26**) are the major phytoalexins that accumulate in potato tubers in response to fungal infection (Kúc 1995). The sesquiterpene lactones, *niveusin B* (**27**), *ethoxyniveusin B* and, leptocarpin described from the different species of *Helianthus*, are among the most active fungicidal compounds (Spring *et al.*, 1982). In addition, costunolide (**28**) and cichoralexin (**29**) have been also recognized as phytoalexins in lettuce (Takasugi *et al.*, 1985) and chicory (Grayer and Harborne, 1994), respectively.

Examples of diterpenoids acting as phytoalexins are compounds isolated from rice (*Oryza sativa*). These phytoalexins exhibit antifungal activity against *M. grisea* a filamentous ascomycete fungus that is the causal agent of rice blast disease. *M. grisea* also has been observed to infect wheat, barley, and millet crops, produ-



cing a similar blast disease and loss of grain production (Talbot, 2003). The first identified rice phytoalexins were the momilactones A and B (30, 31). These compounds were originally isolated and identified as plant growth inhibitors from rice seed (Kabuto *et al.*, 1973). Later it was demonstrated that at least momilactone B acts as an allelochemical, inhibiting seed germination of other plant species (Kato-Noguchi *et al.*, 2002). Specifically, the momilactones exhibit antifungal activity against *M. grisea* and only appear in rice leaves after infection (Kodama *et al.*, 1988). Another group of diterpenoid phytoalexins are oryzalexins (32), also isolated from rice (Kato *et al.* 1993, Tamogami *et al.*, 1993) and, mansonones A-F synthesised in sapwood of *Ulmus americana* (Dumas *et al.*, 1983).

### 3.3 Nitrogen- and sulphur-containing compounds phytoalexins

The most important nitrogen-containing secondary products are the alkaloids, founded in 20% of higher plants (Dewick, 2002). Some chemical structures of nitrogen- and sulphur-containing plant metabolites act as phytoalexins are shown in Figure 4. Surprisingly, very few alkaloids have been identified as phytoalexins, one exception is sanguinarine (33), which is obtained by elicitation of *Papaver bracteatum* cell suspension cultures. Their synthesis is stimulated by both fungal elicitation and hormonal deprivation (Cline and Coscia, 1988).

The *Brassica rapa* and *B. napus* produce a group of sulfur-containing phytoalexins following infection with pathogens (Morrissey and Osbourn, 1999). Brassinin (34) is of great interest in the interaction of crucifers with their fungal pathogens due to both its biological activity and as intermediary in the biosynthetic pathway of other relevant phytoalexins such as cyclobrassinin (35), brassilexin (36) and brassicanal A (Pedras and Jha, 2006). Another exam-

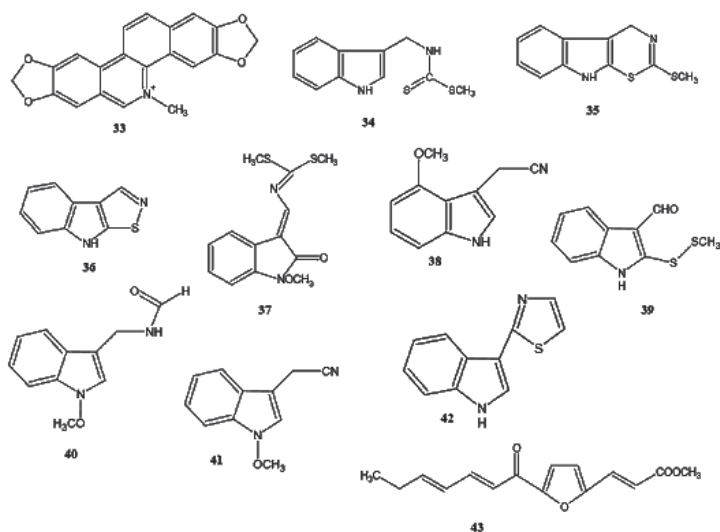
ple of crucifer phytoalexins are wasalexins A and B (37), isolated from *Wasabia japonica*, produced in response to the blackleg fungus *Leptosphaeria maculans* (Desm.), the causative agent of one of the most damaging diseases of oilseed crop, canola (*B. napus* and *B. rapa*) (Pedras and Sorensen, 1998; Pedras *et al.*, 1999). In the last years four new related chemical structures have been reported: arvelexin (38) isolated from *Thlaspi arvense* (stinkweed) (Pedras *et al.*, 2003), and isalexin, brassicanate A, and rutalexin isolated from *B. napus*, ssp. *rapifera* (rutabaga) (Pedras *et al.*, 2004). These indole-based phytoalexins appear to have significant antifungal activity and are formed in sufficient quantity to act as a barrier to infection (Monde *et al.*, 1990 or 91). Cauliflower (*B. oleracea* var. *botrytis*) produce the phytoalexins caulilexins A (39), B (40), and C (41). These compounds are active against the economically important pathogenic fungi *Leptosphaeria maculans*, *R. solani* and *S. sclerotiorum* (Pedras *et al.*, 2006). Other known phytoalexins in this specie are isalexin (Pedras *et al.*, 2004), S-(–)-spirobrassinin (Takasugi *et al.*, 1987; Kutschy *et al.*, 2001), 1-methoxybrassinin (Takasugi *et al.*, 1988) and brassicanal C (Monde *et al.*, 1991).

Although only a few wild crucifers have been examined, it appears that their phytoalexins profiles are different from those of cultivated species. With the exception of *T. arvense* (Pedras *et al.*, 2003) and *Erucastrum gallicum* (Pedras and Ahiahonu, 2004), wild crucifer species appear to produce only type-camalexin metabolites (Pedras *et al.*, 2000). Indeed, camalexin (42) is recognized as the best studied phytoalexin. Camalexin refers to the molecule 3-thiazol-2'-yl-indole which was isolated from the leaves of the crucifer *Camelina sativa* infected with *Alternaria brassicae* (Glawischmig, 2007). In addition, high concentrations of camalexin have been observed at the infection site of *Alternaria alternata* (Schuhegger *et al.*, 2007) and in the proximity to the lesions induced



by *Botrytis* species (Kliebenstein *et al.*, 2005). Similarly, the infection of *Arabidopsis thaliana* leaves with both biotrophic and necrotrophic plant pathogens induce camalexin formation (Thomma *et al.*, 1999, Roetschi *et al.*, 2001). The camalexin biosynthesis is not restricted to leaves, but also to roots infected with the oomycete *Phytophthora sylvaticum* (Bednarek *et al.*,

2005). In addition, the resistance of the wild crucifers *Capasella sativa* and *C. bursa-pastoris* to different Brassica pathogens including *Alternaria brassicae* has been associated with the production of camalexin as the major antifungal compound, as well as smaller quantities of a related compound, 6-methoxycamalexin (Jejelowo *et al.*, 1991; Jimenez *et al.*, 1997;).



**Figure 4.** Representative nitrogen- and sulfur containing compounds phytoalexins: Sanguinarine (33), Brassinin (34), Ciclobrassinin (35), Brassilexin (36), Wasalexin A (37), Arvelexin (38), Caulexin A (39), Caulexin B (40), Caulexin C (41), Camalexins (42), Wyerone acid (43).

### 3.4 Poliketides as phytoalexins

Phytoalexins also may be derived from poliketides (metabolites built primarily from combinations of acetate units). *Vicia faba* tissues produce, as a post-infection defense response against fungal pathogens, low-molecular-weight secondary metabolites, such as wyerone acid and wyerone furanoacetylenic phytoalexin. Wyerone acid (43) accumulates in *B. cinerea* lesions, whereas in *Botrytis fabae* lesions the phytoalexin starts to accumulate but later tends to decrease.

The greater ability of *B. fabae* to colonize broad bean tissues seems to be related to its capacity to detoxify broad bean phytoalexins and to reduce their toxic effects (Buzi *et al.*, 2003).

## 4. Mode of action of some plant antifungal compounds

Plant secondary metabolites can affect the phytopathogenic fungi via interference with molecular targets in their organs, tissues and cells. The major

targets include: biomembranes, proteins and nucleic acids (Engelmeier and Hadacek, 2006). More specifically, it has been described that some natural plant products can cause inhibition of cutinases and laccases production by phytopathogenic fungi (Goetz *et al.*, 1999, Bostock *et al.*, 1999). The resistance of young berries plants results from several conjugated processes being the inhibition of the stilbene oxidase activity of *B. cinerea* laccases one of them (Jeandet *et al.*, 2002). Phenolic compound isolated from grape berries, such as catechin, epicatechin-3-*O*-gallate, *trans*-caftaric, *trans*- and *cis*-cautaric and *trans*-coumaric acids, taxifoline-3-*O*-rhamnoside and quercetin-3-*O*-glucuronide were identified as potent *B. cinerea* stilbene oxidase inhibitors (Goetz *et al.*, 1999). Chlorogenic and caffeic acids are the major phenolics in the epidermis and subtending cell layers of peach fruits (*Prunus persica*). Their concentrations are especially high in peach genotypes with a high level of resistance to the brown rot fungus, *Monilia fructicola* (Bostock *et al.*, 1999).

The production of *p*-nitrophenyl butyrate esterases and laccase by *B. cinerea* is stimulated by the diterpene 3 $\beta$ -hydroxy-kaurenoic. This compound induced efflux of phosphorous from *B. cinerea* mycelium, suggesting that their effect is mainly by affecting membrane permeability (Cotoras *et al.*, 2004).

The tetracyclic triterpenoids, cucurbitacins I and D are inhibitors of the induction of extracellular laccase formation of *B. cinerea* (Bar-Nun and Mayer, 1989). Extracts of *Ecballium elaterium* (a ready source of cucurbitacins) or cucurbitacin I applied to cucumber fruits or plants or to cabbage leaves, prior to inoculation with *B. cinerea*, prevented infection of the tissue, the infecting fungus being restricted to the site of infection. The protective effect is due to the ability of cucurbitacin I to inhibit induction of laccase formation by *B. cinerea* (Bar-Nun and Mayer, 1990). This effect is because that

cucurbitacin specifically reduced the amount of mRNA coding for laccase (Gonen *et al.*, 1996).

Luo *et al.* (2002) proposed a possible action mechanism for the monoterpene citral against *Aspergillus flavus*, indicating that after it penetrates cell wall, irreversibly damages plasma membrane and DNA with consequent spore loss germination. Moreover, it has been shown that citral is able of forming charge transfer complexes with tryptophan, a good electron donor (Kurita *et al.*, 1979). Apparently, the antifungal actions of the aldehydes, including citral, are due to their capacities to form charge transfer complexes with electron donors as well as to their reactivity with SH groups (Kurita *et al.*, 1979).

The major mechanism of antifungal activity of saponins (triterpenoids, steroids or steroidal alkaloids glycosylated; Papalopodou *et al.*, 1999), which despite acts mainly as phytoanticipins also can be induced (Zhao *et al.*, 2010), is apparently due to their ability to complex with sterols in fungal membranes and to cause loss of membrane integrity (Keukens *et al.*, 1995). Electron microscopic analysis and electrical conductivity measurements suggest the formation of transmembrane pores (Seeman *et al.*, 1973; Armah *et al.*, 1999), although steroidal glycoalkaloids have been proposed to interfere with membrane integrity by extracting sterols from membranes (Keukens *et al.*, 1992). Aggregation of the saponin-sterol complexes in the membrane may be mediated by interactions between the sugar residues of the saponin molecules (Keukens *et al.*, 1995; Armah *et al.*, 1999). The sugar chain attached to C-3 is usually critical for both the membrane-permeabilizing and antifungal properties of saponins, and removal of these sugar residues often results in loss of biological activity (Keukens *et al.*, 1995; Armah *et al.*, 1999). The saponin tomatine has been thought to form a complex with membrane sterols with free 3 $\beta$ -hydroxyl groups of fungi, and the

complexes result in pore formation and loss of membrane integrity (Ito *et al.*, 2005).

Stilbenes produce cell abnormalities in *B. cinerea* conidia, including the formation of curved germ tubes, cessation of growth of some germ tubes with protoplasmic retraction in the dead hyphal tip cell, cytoplasmic granulation of conidia, disruption of plasma membrane, or regrowth of a secondary or tertiary germ tube from the surviving conidium (Adrian *et al.*, 1997). Pezet and Pont (1990) reported that pterostilbene added to dormant conidia of *B. cinerea* induces strong modifications of the endocellular membrane system; it causes the rapid destruction of endoplasmic reticulum, and of nuclear and mitochondrial membranes, all these phenomena synchronously appearing with a complete cessation of respiration (i.e., 5 to 10 min after pterostilbene addition). Within 30 min, the cytoplasm is coagulated into numerous vacuoles and mitochondria are clear with a complete disorganization of the cristae. Destruction of the conidium ends with the disruption of the plasma membrane (Pezet and Pont, 1990).

Zoospores of some fungi such as, *Phytophthora infestans*, *P. porri*, and *P. cactorum*, can develop cytoplasmic granulations, plasma membrane disruptions, and the leakage of cellular contents in presence of four terpenoid-type phytoalexins (rishitin, phytuberin,  $\beta$ -rotunol, and solavetivone) (Harris & Denis 1976, 1977). These kind of effects have also been observed in fungal cells treated with isoflavonoid phytoalexins, such as phaseolin and keivitone (VanEtten and Bateman, 1971).

It has been suggested that hydroxystilbenes (especially those presenting methoxy-groups or electron-attracting substituents such as chlorine at the 3-, 3,4-, or 3,5-positions of the stilbene ring) play an important role in the formation of charge transfer complexes, favoring contact and affinity with (membrane) proteins and acting as uncoupling agents of electron transport

and photophosphorilation (Jeandet *et al.*, 2002). It has recently been reported that hydroxystilbenes, such as resveratrol and piceatannol, are capable of inhibiting some fungal ATPases and inducing the dissociation of chaperones and co-chaperones, two proteins frequently associated with the cytoskeleton (Kindl, 2000). Some phytoalexins (e.g., glyceollin from *G. max* soybean) are specific inhibitors of complex of the mitochondrial electron-transport chain (Lamber *et al.*, 2000).

## **5. Biotechnological approaches to obtain plant antifungals**

Some of the above described phytoalexins provide a potentially interesting weapon to be used in agriculture at commercial scale. In fact, the final goal of the researches studying the significance of phytoalexins in plant defence response is oriented to develop biotechnological applications in crop protection. Nevertheless, the use of phytoalexins in phytoprotection however entails some disadvantages that have to be overcome (González-Lamothe *et al.*, 2009). Production of natural products by plants is not always satisfactory. Some plants are difficult to cultivate, or may grow very slowly (*Taxus* sp). For these reasons, different biotechnological tools can be used to produce plant secondary metabolites of commercial interest (Verpoorte *et al.*, 2002).

An approach would be the production of plants that express a higher quantity of phytoalexins induced by both, spraying with phytoalexin elicitors or by genetic transformation (González-Lamothe *et al.*, 2009). One problem of the first approach is that plants continuously elicited to produce phytoalexins results in plants with reduced plant yield (Grayer and Kokubun, 2001). Thus, an adequate regulation of the production of phytoalexins could help resolving the problem. In the same context, transfer of genes involved in the synthesis of phytoalexins in order to obtain more resistant

cultivars has been verified in tobacco plants in which genetic transformation with the enzyme stilbene synthase, involved in the resveratrol synthesis, provided plant resistance to *B. cinerea* (Hain *et al.*, 1993).

Plant cell culture on a large scale has been shown to be feasible for industrial production. However, only a few processes have been developed. Secondary metabolites occur in many plant species, but only in low concentrations. The biotechnology focuses on alternative production systems for these natural compounds, because the plant *in vitro* cultivation has several advantages over collecting plants from fields (Calva-Calva *et al.*, 2000, Vanisree *et al.*, 2004). Growing plant cells permit a stricter control of the quality of the products as well as their regular production without dependence on the variations of natural production resulting from climate and socio-political changes in their countries of origin. Problems connected with gathering, storing (in special conditions), processing and disposal of huge amounts of biomass, connected with extraction of active substances from *in vivo* plants are also solved (Alfermann and Petersen, 1995; Calva-Calva *et al.*, 2000; Vanisree *et al.*, 2004). Suspension cultures are of special interest due to their high growth rate and short cycle of reproduction. Another advantage is the fact that undifferentiated plant cells, maintained in a liquid medium possess a high metabolic activity due to which considerably high yields of secondary products can be achieved in short terms (from one to three weeks of cultivation) (Verporte *et al.*, 2002).

Metabolic engineering is also an important tool to improve the plant cell factory for the production of secondary metabolites. It is interesting to note that the enzyme for stilbene production, (stilbene synthase), has been successfully transferred from plant producing stilbene phytoalexins to one that does not and that the resulting transformed plant makes resveratrol in addition to its normal phytoalexins. Transforming

tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum* Mill.) and alfalfa (*M. sativa*) plants with a stilbene synthase (from *Vitis vinifera* L.) enabled the transformants to synthesize the grapevine phytoalexin resveratrol. The transformants showed an increase in the resistance to *B. cinerea* (Hain *et al.*, 1993), to *Phytophthora infestans* (Thomzik *et al.*, 1997), and to *Phoma medicaginis* (Hipskind and Paiva, 2000). In addition, two resveratrol synthase genes *vst1* and *vst2* from grapevine (*V. vinifera*) and the pinosylvin synthase gene *pss* from pine (*Pinus sylvestris* L.) have been stably transferred into bread wheat. Their expression in transgenic plants caused accumulation of novel stilbenes and improved disease resistance against two wheat-specific pathogens, *P. recondita* f. sp. tritici and *S. nodorum* Berk (Serazetdinova *et al.*, 2005).

## 6. Conclusions and future directions

Although relatively few of the several tens of thousands of known, secondary metabolites have been analyzed biochemically and ecologically, we can nevertheless generalize that many of them are compounds that serve as defense compounds against pathogens.

The vast repository of fungicidal secondary compounds from higher plants could serve as biopesticides or as templates for the synthesis of new pesticides based on their chemistry. On the other hand, crop cultivars with higher antifungal compounds content can be screened and improved through various molecular genetic approaches. The work in this direction has already begun. In this context, crop cultivars with enhanced antifungal activity can be obtained by the selection among the existing cultivars, through traditional breeding methods or by genetic manipulation. A better knowledge of the biochemical pathways (enzymes and genes) involved in production of putative secondary plant products, the methods of storage and transport to the soil, the molecular target

sites of antifungal compounds from plants, their detoxification, and the potential in vivo interactions of these compounds, will provide the physiological basis for improved understanding of the role of these compounds in ecosystems, both agricultural and natural. To use genomics to identify genes responsible for the biosynthesis of plant natural products, it is necessary to interfere with their production.

Key areas of antifungal plant metabolites research are: selectively to enhance defensive traits of crop cultivars in breeding programs; to transfer genes into commercial cultivars through modern biotechnology; to enhance their pest-killing capability; and to identify and characterize those substances involved in strong antifungal activity and to use them either directly as natural fungicides, or as models for developing new and environmentally friendly agrochemicals. With the rapid development of analytical techniques and biotechnology, research on these areas will be enhanced. Natural plant products will become an important component in the development of future integrated fungal plant diseases management strategies.

Finally, in order to demonstrate the importance of plant natural products in defence the following criteria must be used: a) The restriction of the pathogen must be associated with secondary metabolites accumulation and/or production. b) Secondary metabolites must accumulate to antifungal levels at the infection site in resistant plants that could result the cessation of the pathogen growth. c) There must be strong evidence that the secondary metabolites have vital importance in resistance and absence of these compounds would result enhanced susceptibility.

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