International Journal of Botany

ISSN: 1811-9700

science alert

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OPEN ACCESS International Journal of Botany

ISSN 1811-9700
DOI: 10.3923/ijb.2017.82.102

Review Article
Host Defense Mechanisms During Fungal Pathogenesis and how these are Overcome in Susceptible Plants: A Review

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Abstract

The interaction between plants and fungal pathogens comprises a range of mechanisms that determine the outcome of the interaction, i.e., compatible leading to susceptibility or incompatible leading to resistance. Several host defense mechanisms act both in susceptible and resistant plants. Yet, in the case of a compatible interaction involving a susceptible host and a virulent pathogen, the latter is able to win the battle and cause disease. By documenting several interactions between plant and fungal pathogens, this review describes some of the mechanisms which plants use for defense such as the reinforcement of the cell wall and the accumulation of pathogenesis-related (PR) proteins and of secondary metabolites and ways by which pathogens overcome plant defense.

Key words: Phenolics, lignin, suberin, secondary metabolites, cell wall, PR proteins, phytoalexins, susceptibility, plant defense mechanisms, fungal pathogens

Citation: Mirella Aoun, 2017. Host defense mechanisms during fungal pathogenesis and how these are overcome in susceptible plants. Int. J. Bot., 13: 82-102.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Molecular communication between plant and pathogens starts almost immediately after the pathogen makes contact with the plant surface. In this battle, susceptibility or resistance is determined by the winner. If the pathogen is able to overcome the toxic environment in the plant tissue, the disease develops and if the plant is able to ward off the pathogen's toxic weapons, disease resistance develops. Plant cells recognize the presence of spores of fungal pathogens on the surface and initiate defense-associated responses even within 30 min of contact with pathogen spores. Plant cells then launch diverse defense mechanisms. Here it is described three important aspects of host defense mechanisms during fungal pathogenesis: (1) Reinforcement of the cell wall, the first barrier against fungal pathogens, (2) The induction and accumulation of new readily detectable proteins, called pathogenesis-related (PR) proteins and (3) The accumulation of antifungal secondary metabolites. In this review, focus was placed on the way these defenses are counteracted in the compatible interaction leading to susceptibility.

CELL WALL REINFORCEMENT DURING FUNGAL PATHOGENESIS

Reinforcement of plant cell wall by phenolics: Plant cell walls respond to invasion by fungal pathogens by accumulating phenolics (Fig. 1) and phenolic polymers such as lignin[1,2]. Ferulic acid, p-coumaric acid and sinapic acid are the predominant cell wall-bound phenolics, whereas, lignins are the wall-bound polymerized phenolics[3]. Bound forms of ferulic acid can be dimerized by peroxidases to form cross-links between arabinoxylan chains that strengthen the cell wall[4]. The pathway of synthesis of major cell wall-bound phenolics is presented in Fig. 2.

Accumulation of wall-bound phenolics in response to fungal invasion: In histology, the appearance of yellow autofluorescence (at 365 nm) and of autofluorescence under blue light excitation in diseased plant tissues is considered to be a result of the presence of phenolic compounds that accumulate in the tissues as the host attempts to limit the development of the pathogen[7,8]. Tomato cell cultures inoculated with *Verticillium albo-atrum* accumulated up to five fold more of wall-bound phenolics than were found in uninoculated control cultures[9]. The analysis of this cell wall-bound material revealed that two populations of phenolic

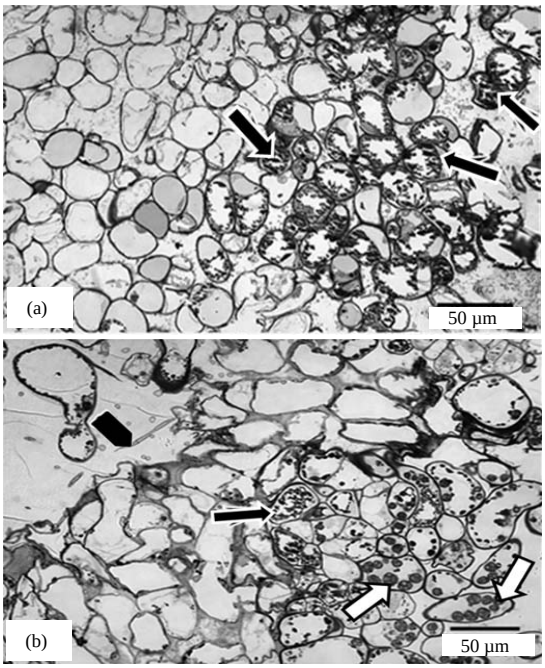

Fig. 1(a-b): (a) Phenol accumulation and (b) Starch deposits, in *U. americana* callus cells after inoculation with *O. novo-ulmi*

Friable callus samples at 48 and 72 h post-inoculation (hpi), respectively. Phenols (black arrows) appear dark-stained with toluidine blue. Starch grains (B, white arrows) are obvious in host cells located just beneath the front line of dead cells in direct contact with the fungus (arrow head)at the surface (S) of the callus. Source: Aoun *et al.*[5]

material existed. The first comprised esterified compounds and the second comprised nonbase-labile polymeric material. Several studies[5,10,11] have shown that the increase by hosts of the levels of phenylalanine ammonia lyase (PAL), the first enzyme in the phenylpropanoid pathway, was a direct response to attempted penetration by the fungus. PAL expression was relatively stable in water-treated *Ulmus americana* callus samples and it did not exceed 1.5 times the level observed in healthy callus sample, whereas, it reached 7.2 [144 h post inoculation (hpi)] times in callus samples inoculated with the fungal pathogen *Ophiostoma novo-ulmi* (Fig. 3). Heavy accumulation of tannin oligomers and monomers (e.g., catechins) were also observed in these inoculated callus tissues[5].

Shiraishi *et al.*[10] observed increases in PAL activity at two different times in barley cultivars inoculated with *Blumeria graminis* regardless of the resistance or susceptibility of the barley cultivar to the fungus. The first increase began at 3 hpi and was followed by a second increase between 12 and

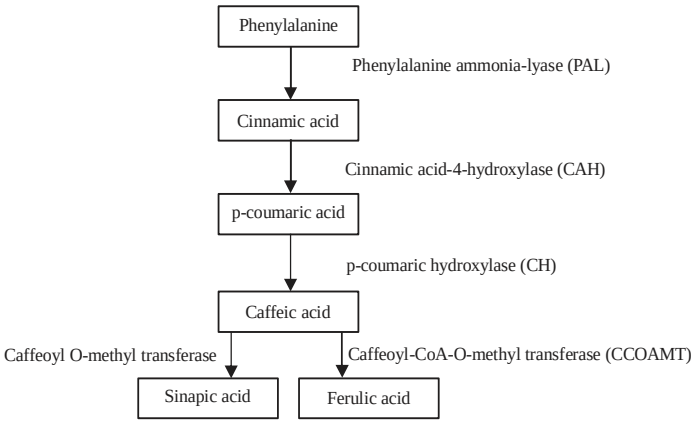

Fig. 2: Biosynthesis of wall-bound phenolics. Adapted from Vidhyasekaran[6]

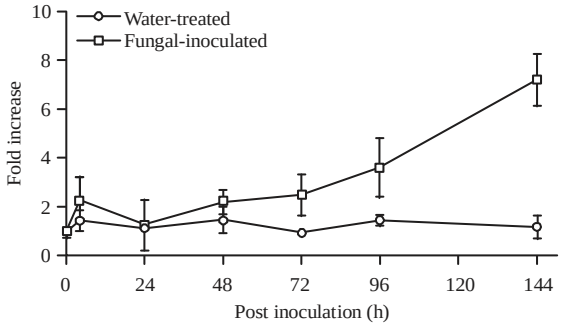

Fig. 3: Fold difference in PAL gene expression in water-treated and fungal-inoculated *Ulmus americana* callus cultures. X-axis: Time in hour, Y-axis: Fold increase. Adapted from Aoun *et al*.[5]

15 hpi. The conidium produced a primary germ tube that attempted penetration beginning about 2 hpi and an appressorium that attempted to penetrate beginning from 9-10 hpi. Clark *et al*.[11] showed that an initial accumulation of PAL transcripts occurred in barley cultivars inoculated with *B. graminis* between 4 and 6 hpi. This accumulation declined to near constitutive levels by 8-10 hpi. A second peak was observed from 10-12 hpi and then declined until 15-18 hpi. The first increase occurred in response to contact with the fungal germ tube, whereas the second increase was because of appressorial contacts[11].

Besides PAL, other enzymes implicated in synthesis of wall-bound phenolics, such as cinnamyl alcohol dehydrogenase (CAD) and Caffeoyl-COA-O methyltransferase (CCoAOMT), showed transient increase in transcript activity after treatment with fungal elicitors or upon fungal infection[12-14].

Potato tuber treated with a fungal elicitor from the incompatible pathogen *Trichothecium roseum* could systemically induce, total phenolic content, flavonoid content and defense enzymes, including three keys of phenylpropanoid pathway (PAL, 4CL and C4H). The fungal elicitor also enhanced the up-regulation of the transcription and expression of PAL, C4H, 4CL, GLU and CHT genes[15].

How does the pathogen overcome the cell wall-bound phenolics to cause disease?: Suppression of accumulation of phenolics in the host cell wall and delay of synthesis of wall bound phenolics are two ways that help the pathogen overcome and cause disease.

When comparing resistant and susceptible hosts, higher accumulation of phenolics were found in resistant hosts suggesting that successful pathogens may be able to suppress accumulation of phenolics in plant cell walls of susceptible hosts. Constitutively higher levels of phenolic compounds were measured in the resistant *M. truncatula* accession during the interaction of *Medicago truncatula* with the fungal necrotrophic pathogen *Phoma medicaginis* in leaf tissue of susceptible and resistant accessions[16]. Higher levels of cell wall-bound phenolics were found in the resistant cultivar of pineapple (*Ananas comosus* var. comosus) inoculated with conidia suspension of *Fusarium subglutinans* f. sp. *anana*. p-coumaric and ferulic acids were shown to be the major phenolics bound to the cell walls of inoculated mature leaves of pineapple and were found in higher amounts in the resistant cultivar compared to the susceptible one[1].

More than that, suppression of PAL has been shown to induce susceptibility in resistant varieties. Inhibition of PAL by α-Aminooxy-β-phenylpropionic acid (AOPP) suppressed the accumulation of phenolic compounds in epidermal cell walls in barley and wheat and made the resistant hosts susceptible to the pathogens *Blumeria graminis f. sp. hordei* and *B. graminis f.* sp. *tritici*, respectively[17].

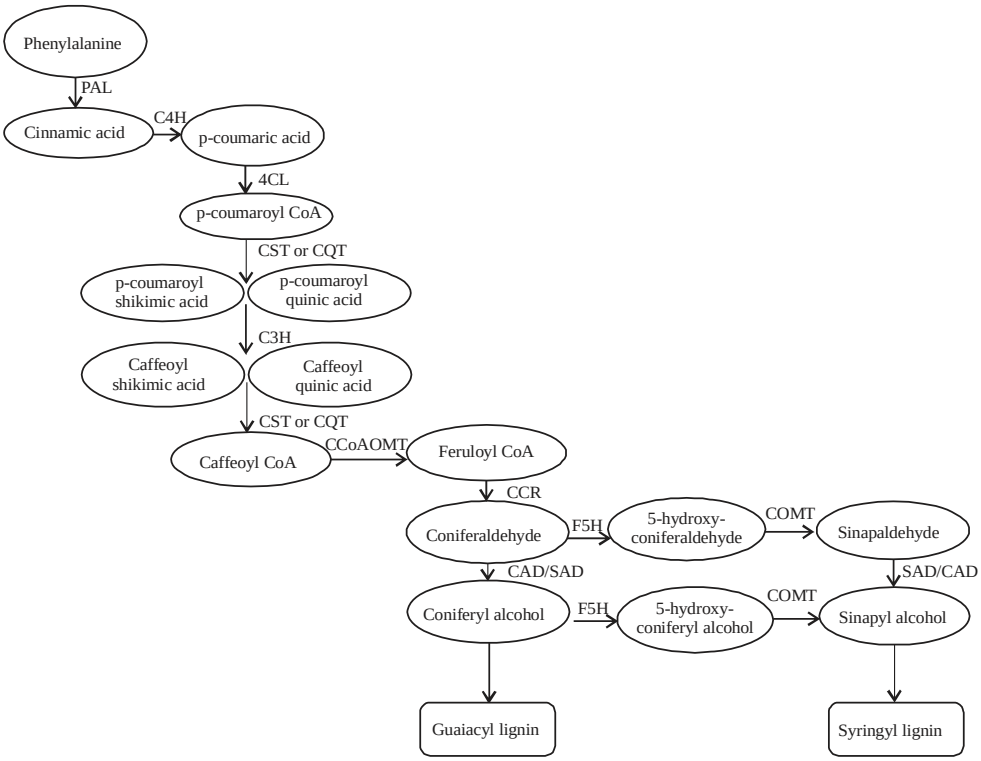

Fig. 4: Key steps in lignin biosynthesis. 4CL: 4-(hydroxy)cinnamoyl CoA ligase, C3H: p-coumarate 3-hydroxylase, C4H: Cinnamate 4-hydroxylase, CAD: Cinnamyl alcohol dehydrogenase, CCoAOMT: Caffeoyl CoA O-methyltransferase, CCR: Cinnamoyl CoA reductase, COMT: Caffeic acid/5-hydroxyferulic acid O-methyltransferase, CQT: Hydroxycinnamoyl, CoA: Quinate hydroxycinnamoyltransferase, CST: Hydroxycinnamoyl, CoA: Shikimate hydroxycinnamoyltransferase, F5H: Ferulate 5-hydroxylase, PAL: Phenylalanine ammonia-lyase, SAD: Sinapyl alcohol dehydrogenase. Adapted from Humphreys and Chapple[19]

Synthesis of cell wall-bound phenolics may be delayed at the fungal penetration site in compatible interactions. Yellow autofluorescence (indicating synthesis of phenolics) after excitation at 365 nm was emitted, by inoculation with *Plasmopara viticola* in the resistant *Vitis rotundifolia* as early as two days after inoculation. In contrast, a few stomatal cells with yellow autofluorescence were detected only 8 days after inoculation in lesions of the susceptible *V. vinifera*[18].

Reinforcement of plant cell wall by lignin: The pathway of lignin synthesis is not yet completely understood and the lignin roadmap has been re-written frequently[19]. Monolignols are the precursor of lignin biosynthesis. Coumaryl alcohol, coniferyl alcohol and sinapyl alcohol are the most important monolignols. The important enzymes involved in the biosynthesis of lignin are: PAL, CCoAOMT, CAD, 4-coumarate: CoA ligase (4CL), coumarate alcohol dehydrogenase, coniferyl-CoA reductase, coniferyl alcohol dehydrogenase, sinapoyl-CoA reductase, sinapoyl alcohol dehydrogenase and peroxidases (Fig. 4).

Lignification in compatible and incompatible interactions: Lignification is a common response to plant infection[20]. Phenolic polymers which accumulate in response to infection have been identified to be lignin and suber in-like polymers[21]. Lignification is common in healthy plants also. However, the increased lignification observed in resistant varieties appears to involve a new type of lignin. A number of histochemical tests, such as phloroglucinol-HCL, toluidine blue O, chlorine-sulfite and the modified chlorine-sulfite are being employed to detect the presence of lignin. When these tests were used to detect the deposition of lignin in infected leaf tissues, additional lignin was observed[22,23]. The additional lignin observed in walls of wheat leaves because of the incompatible interaction with the leaf rust fungus, *Puccinia recondita* f. sp. *tritici*, was considered different from that in uninfected leaves because of its green rather than blue-green response to toluidine blue. The failure to react to phloroglucinol may indicate an absence of cinnamaldehyde groups. The additional lignin formed in the incompatible interaction may not be rich with syringic groups

because of its failure to stain with chlorine-sulfite. This additional lignin was not detected in the susceptible interaction[22,23].

Lignification is often suppressed in several compatible interactions while in incompatible interactions lignification is often predominant. It has been observed that the lignin content in the cell walls of susceptible wheat cultivar tissues infected by *Fusarium culmorum* increased slightly, whereas, lignin accumulated intensely in the host cell walls from infected wheat spikes of resistant cultivars[24].

Resistance of broccoli to *Verticillium dahliae* infection was associated with increase in phenolic and lignin contents after inoculation of broccoli (resistant) and cauliflower (susceptible) by a green fluorescent protein-expressing isolate of *V. dahliae*[25]. Reduced lignin content of rice cells by a peroxidase (POX) inhibitor was associated with increased disease index in rice genotypes inoculated with field isolates of *Alternaria alternata*[26].

How does the pathogen suppress lignification in host cell wall?: Oligogalacturonides with more than eight galacturonosyl residues are endogenous elicitors of lignification in both susceptible and resistant plants[27]. The elicitor active pectic fragments are produced because of partial digestion of the host polygalacturonic acid by fungal enzymes[28]. In the compatible interaction, more rapid degradation of pectic substances by higher concentration of the pectolytic enzymes produced by the pathogen results in complete degradation of the endogenous elicitors and accumulation of pectic polymer of less than eight galacturonosyl residues which do not have elicitor activities and hence no lignification occurs[28,29]. Fungal cell walls also contain molecules that elicit lignification. These molecules are released by host enzymes (such as chitinase) and activation of the host enzymes is under the control of fungal cell wall components[30]. Chitinase activity was induced in cultures of carrot cells incubated with fungal walls of *Chaetomium globosum* and the soluble fragments liberated from the fungal walls stimulated the biosynthesis of phenolic acids which are precursors of the lignin synthesized in cells[30]. Thus, fungal cell wall extracts can induce lignification. However, the release of elicitor containing fungal cell wall components would have been suppressed in compatible interactions[31].

Using metabolite finger printing, Parker *et al*.[32] show that *Magnaporthe grisea*, the causal agent of rice blast disease, dynamically reprograms host metabolism during plant colonization. Identical patterns of metabolic change occurred during *M. grisea* infections in barley, rice and Brachypodium distachyon. Early diversion of the shikimate pathway to produce quinate was observed as well as accumulation of non-polymerized lignin precursors. These data are consistent with modulation of defensive phenylpropanoid metabolism by *M. grisea* and the inability of susceptible hosts to mount a hypersensitive reaction or produce lignified papillae to restrict pathogen invasion[32].

Reinforcement of plant cell walls by suberin:

Suberization of plant cell wall in response to fungal invasion: Suberin, a complex biopolyester organized in a characteristic lamellar structure, comprises a phenolic (aromatic) domain attached to the cell wall and an aliphatic (lipid, hydrophobic) domain attached to the phenolic domain[33]. Historically, the phenolic domain has been likened to lignin and the aliphatic domain was represented as a random network of polyesterified modified fatty acids and alcohols. Recently, however, a new model for suberin has emerged in which a hydroxycinnamic acid-monolignol polyphenolic domain embedded in the primary cell wall was covalently linked to a glycerol based polyaliphatic domain located between the primary cell wall and the plasma membrane[34,35]. The production of suberin coatings was dependent on PAL activity[36]. PAL induces the synthesis of many phenolic acids, which are required for synthesis of suberin[37].Cross-linking of such phenolics forms a polymeric matrix which is made hydrophobic by attachment of aliphatic polyester domains and by deposition of highly non-polar waxes into the layer. The formation of this layer is called suberization[38].The formation of the aromatic matrix is the first step in suberization. The polymerization of the aromatic components of suberin involves an isoperoxidase in a manner similar to that involved in lignin biosynthesis[39].

Suberization is responsible for the reinforcement of cell walls limiting ingress of pathogens in the host and was observed in both susceptible and resistant interactions[40,41]. Suberin is highly resistant to enzymatic degradation by pathogens and hence it is considered as an effective barrier to penetration by many fungal pathogens[42,43].

Suberin was also observed in plant callus cultures in response to fungal inoculation. Aoun *et al*.[5] inoculated friable and hard susceptible *Ulmus americana* callus cultures with the highly aggressive pathogen *Ophiostoma novo-ulmi*. Inoculated callus tissues were compared with water-treated callus tissues. Histological observation showed, for the first time accumulation of suberin with its typical lamellar structure in transmission electron microscopy in inoculated calli (Fig. 5). Expression of the PAL gene monitored by real-time quantitative polymerase chain reaction was correlated with the accumulation of suberin, phenols and lignin in infected callus cultures[5].

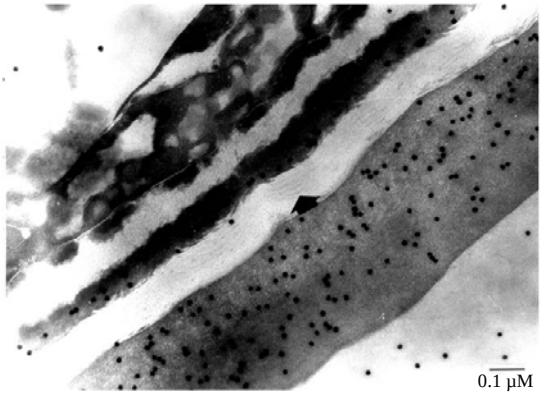

Fig. 5: Typical lamellar structure of suberin (arrow) in infected plant tissues. Suberized cell wall layer (arrow) shown next to primary cell wall intensely labeled with the exoglucanase-gold complex in *Ulmus americana* hard callus cells at 48 h after inoculation with fungal pathogen *Ophiostoma novo-ulmi*. Adapted from Aoun *et al*.[5]

How does the pathogen overcome suberization of host cell walls?: During pathogenesis, suberization appears to be delayed in compatible interactions; this delay would help the pathogen penetrate host tissues. In tomato, one of the earliest defense responses against *Verticillium albo-atrum* was the coating of xylem vessels and pit membranes with suberin[36]. In resistant tomato varieties suberization was found to be very rapid, beginning at 8-10 hpi. In sharp contrast, almost no suberization was visible in susceptible varieties at that same time. After 24 hpi, suberization was visible in susceptible plants but was much less pronounced as compared to resistant plants[37].

The delay in suberin accumulation in compatible interactions appears to be a result of suppression of suberin-synthesizing enzymes by the pathogen. Accumulation of PAL mRNA, the first enzyme involved in suberin synthesis, increased in resistant tomato plants infected with *Verticillium albo-atrum* to about 30% above the constitutive normal level. In contrast, the level did not increase in susceptible plants but proceeded to drop until it was only 30% of the constitutive level after 15 hpi. These results suggest that fungal components may suppress PAL mRNA levels in susceptible plants[37,44].

In the Dutch elm disease pathosystem the DED pathogen induced a large increase in PAL enzyme activity in DED-resistant *U. pumila* suspension cultures[45]. The increase observed in the resistant *U. pumila* reached its maximum at 24 hpi, which is earlier than the first significant increase detected at 72 hpi in susceptible *U. Americana* callus culture[5].

Some fungal pathogens have been reported to penetrate suberized cell walls[46,41]. This can be a result of the action of degrading enzymes. Esterases able to degrade the aliphatic and aromatic domains of suberin have been isolated in several fungal species[47-49]. Suberinase activity was also observed in some cases as shown by Garcia-Lepe *et al*.[50].

The ability of plants to accumulate suberin also appears to determine susceptibility or resistance[51]. Total resistance to fungal infection was attained after completion of deposition of the suberin aliphatic domain within the first layer of suberized cells[33].

INDUCTION OF PATHOGENESIS-RELATED PROTEINS

Variety of pathogenesis-related (PR) proteins: PR proteins may be defined as proteins encoded by the host plants and induced specifically in response to pathogen-attack. These were readily detected in infected but not in uninfected tissues[52]. Proteins that were constitutively expressed were considered as PR proteins when the expression was induced in specific organs of a plant or in specific varieties during infection[52]. A variety of PR proteins were present in infected plant tissues. For example, more than 30 PR proteins have been identified in Norway spruce (*Picea abies*)[53]. The PR proteins have been classified into 17 families based on structure and sequence similarity, rather than on biological activities[54-55]. Table 1 lists recognized PR-protein families and the functions.

Besides these 17 families, some unclassified proteins have also been described. Grenier and Asselin[81] have identified chitosanases as pathogenesis related proteins. Chitosanases act on chitosan[82] but have no activity on chitin. These were distinguished from chitinases that act on chitin without activity on chitosan.

Induction of PR proteins during fungal pathogenesis: When the fungal pathogen invades host tissues, several PR proteins accumulate both locally and systemically[53,83,84]. Accumulation of PR proteins in response to pathogenesis has been detected in compatible as well as in incompatible interactions[85-88]. Genes encoding PR proteins belong to multigene families[89].These have been identified in different plants but were almost silent in healthy plants[89]. Transcriptomic analysis and expression profile of PR genes (Fig. 6) in different pathosystems revealed that these genes were upregulated during fungal infection[14,90,91].

Different signal transduction pathways may exist in triggering induction of PR proteins in plants. The PR protein

Table 1: Pathogenesis-related protein families and associated functions

PR-family	Functions or properties	References
PR-1	Plant cell wall thickening, some inhibitory function on growth of the pathogen	Santen *et al.*[56] and Teixeira *et al.*[57]
PR-2	Show β-1,3-glucanase activity	Menard *et al.*[58] and Balasubramanian *et al.*[59]
PR-3	Chitinases (classes I, II, IV, V, VI and VII) and β-1,4-glucosaminidases	Van Loon *et al.*[60] and Neuhaus[61]
PR-4	• Hevein and wound induced (WIN) proteins • Acidic chitinases	Van Loon[52], Pierpoint[62] and Bertini *et al.*[63]
PR-5	Thaumatin-like (TL) proteins	Koiwa *et al.*[64] and Rout *et al.*[65]
PR-6	Serine proteinases inhibitors, cysteine proteinases inhibitors, aspartic proteinases inhibitors and metalloproteinases inhibitors	Koiwa *et al.*[66] and Sels *et al.*[67]
PR-7	Show endoproteinase activity	Vera and Conejero[68]
PR-8	Class III chitinases	Van Loon *et al.*[60]
PR-9	Peroxidases induced during pathogenesis	Lagrimini *et al.*[69] and Vale *et al.*[70]
PR-10	Intracellular acidic proteins, possess ribonuclease activity	Warner *et al.*[71], Zhou *et al.*[72] and Xu *et al.*[73]
PR-11	Endochitinases	Melchers *et al.*[74] and Younas *et al.*[75]
PR-12	Defensins induced during pathogenesis	Penninckx *et al.*[76] and Sels *et al.*[67]
PR-13	Thionins	Sels *et al.*[67]
PR-14	Lipid transfer proteins (LTPs)	Sels *et al.*[67]
PR-15	Germin-like oxalate oxidase	Zhang *et al.*[77] and Schweizer *et al.*[78]
PR-16	Germin-like proteins without oxalate oxidase activity	Wei *et al.*[79]
PR-17	Peptidases with similarity to the active site and peptide-binding groove of the exopeptidase aminopeptidase N from eukaryotes	Christensen *et al.*[80]

genes PR-1, PR-2 and PR-5 were induced by salicylic acid, whereas the PR-3, PR-4 and PR-12 genes were induced by jasmonic acid in *Arabidopsis thaliana*[92]. Ethylene but not salicylic acid, induces class I chitinase in tobacco[76]. It has been shown that in some cases there may be a synergistic effect of different signals in triggering PR synthesis[73]. But, there may also be antagonistic effects between different signals in inducing PR proteins. Jasmonic acid induced PmPR-10 protein accumulation in Western white pine (*Pinus monticola*), whereas its induction was suppressed by salicylic acid and abscisic acid[93]. In Eucalyptus, the transcript levels of EgrPR2 were decreased in response to high concentrations of methyl jasmonate whereas the expression of EgrPR3 and EgrLOX declined as the concentrations of salicylic acid treatment increased[94].

Role of PR proteins in inhibiting fungal disease development: The role of PR proteins in inhibiting fungal disease development has been demonstrated both *in vitro* and *in vivo*. PR proteins accumulate in both compatible and incompatible interactions[95]. In many instances, these accumulate more in incompatible interactions[96]. However, there were also reports indicating that PR proteins accumulate more in compatible interactions. In fact, some proteins were exclusively induced during disease development and such proteins were not induced in incompatible interactions[97].

Inhibition of fungal growth by PR proteins: Fungal growth is particularly important for the spread of the pathogen in host tissues. Wide-genomic studies of phytopathogenic fungi were also conducted on traits that contribute to parasitic fitness of the fungus such as sexual and asexual propagation and dimorphism switch between spore (yeast-like) and hyphal growth[98-100]. Purified chitinases were shown to be effective inhibitors of spore germination and hyphal growth. Swelling of the hyphal tips and hyphal distortion was also observed[101]. Disruption of chitin macromolecules in the fungal cell wall preceded cell wall breakdown and protoplasm alteration[101]. PR-4 class I protein from tobacco exhibits antifungal activity toward *Trichoderma viride* and *Fusarium solani* by causing lysis of the germ tubes and growth inhibition[102]. The PR-5 group of proteins contains many antifungal proteins[103]. A thaumatin-like protein of *Ocimum basilicum* inhibited mycelial growth of *Scleretonia sclerotiorum* and *Botrytis cinerea* and its ectopic expression in Arabidopsis led to enhanced tolerance against these phytopathogenic fungi[104].

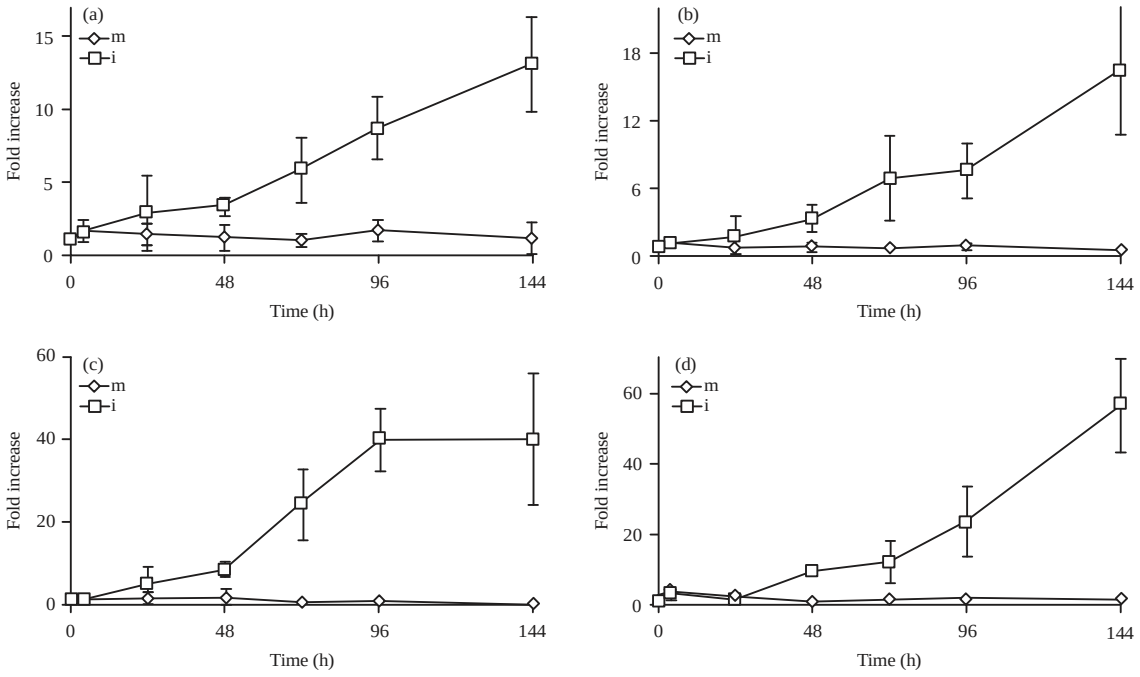

Fig. 6(a-d): Expression profile of PR genes induced during fungal pathogenesis, (a) Class IV chitinase precursor, (b) Pathogenesis-related protein 1a, (c) Pseudo-hevein and (d) Proteinase inhibitor I13

Source: Aoun *et al.*[14]

Transcript levels of *Ulmus americana* uni sequences that were upregulated in *Ophiostoma novo-ulmi* infected American elm callus tissue. Callus tissues were either mock-inoculated (m) with water or inoculated with *O. novo-ulmi* budding cells. The monitoring of gene expression profiles was done by quantitative reverse-transcriptase polymerase chain reaction at 4, 24, 48, 72, 96 and 144 h post-inoculation (hpi)

Defensins (PR-12 proteins) isolated from white spruce (*Picea glauca*) were found to cause extensive growth inhibition of *Cylindrocladium floridanum*, *Fusarium oxysporum* and *Neonectria galligena* at 2.5 μM[105]. Thionins (PR-13 proteins) have been shown to be toxic to fungal pathogens; these penetrate fungal cell membranes and inhibit DNA, RNA and protein synthesis[106]. The PR-14 proteins (LTPs) have also been shown to be fungitoxic. These may insert into the fungal cell membrane and the central hydrophobic cavity may form a pore, allowing efflux of intracellular ions, thus leading to fungal cell death[107]. Some of the PR proteins act synergistically with other PR proteins in inhibiting the growth of fungi. Different chitinases show more antifungal activity when combined with other proteins such as β-1,3-glucanase and PR-4 protein[102,108,109].

Inhibitory action of some PR proteins against fungal pathogens has been demonstrated in the infected tissue itself. PR-1 proteins were found to be associated with host cell wall outgrowths and papillae in infected tobacco. These proteins increase mechanical strength of these defense-related structures and inhibit the development of the fungus[110]. Transgenic plants over expressing PR proteins showed enhanced resistance to fungal pathogenesis and reduction in fungal growth with growth anomalies in hyphae[111-113].

Indirect action of PR proteins in the defense response: In some cases, PR proteins act indirectly in the defense response and not directly on the pathogen. The role of PR proteins involves the release of elicitor molecules in planta and the reinforcement of cell wall structure. Several chitinases release specific oligosaccharides from the plant cell walls, which act as signal molecules in triggering host defense mechanisms[52]. The PR-9 (peroxidases) proteins were involved in biosynthesis of lignin and suberin[34] which act as a cell wall barrier against fungal pathogens. Petioles of carrot plants over expressing a rice cationic peroxidase had higher levels of constitutive lignin accumulation compared to control plants and symptoms reduced by up to 90% when infected with *Botrytis cinerea*[113]. The PR-15 and PR-16 proteins have been suggested to release H_2O_2 necessary for cross-linking of cell wall components during formation of papillae[79].

PR proteins involved in triggering disease resistance: The role of PR proteins in disease resistance has been

demonstrated through inducing mutations resulting in the over expression of PR proteins that conferred resistance to fungal disease[114] and by developing transgenic plants in many pathosystems involving fungus infection[115-116]. Transgenic hybrid poplar leaves over expressing a wheat PR-15 gene showed increased resistance against *Septoria musiva*[117]. Constitutive expression of PpPR-10 in *Physcomitrella patens* moss tissues increased resistance against the oomycete pythium irregulare. The PpPR-10 over expressing moss plants developed less symptoms and decreased mycelium growth than wild type plants[118].

The role of PR proteins in disease resistance has also been demonstrated using chemical or biological elicitors. Thiamine treatment induced three rice PR genes, PR-1, PR-9 (Pox 22.3, a gene encoding peroxidases) and PR-10 (PB 21). Induction of these PR genes resulted in disease resistance against *Magnaporthe oryzae*[119].

However, not all PR proteins are involved in disease resistance since there were reports that transgenic plants over expressing some PR proteins do not show resistance to the pathogen[120,121]. There was no guarantee that a protein that was effective in one host against one pathogen would be effective in a different host against a different pathogen[120].

HOW DO PATHOGENS OVERCOME PR PROTEINS OF THE HOST?

Slower accumulation of PR proteins enable pathogens to escape their antifungal action: The virulent pathogen delays accumulation of PR proteins in the host. Histological observations using antiserum and gold antibodies against the tomato PR-1 (PRP14) protein allowed for detection of PRP14 in the roots of a resistant and a susceptible variety at 48 hpi and 72 hpi, respectively[85]. At 72 hpi, the pathogen had already colonized the root tissues in the susceptible variety[85]. Aoun *et al*.[14] used a large-scale cDNA sequencing approach to study the compatible interaction between *Ophiostoma novo-ulmi* and *Ulmus americana* hard calli. Results from transcript profiling confirmed that several genes encoding pathogenesis-related (PR) proteins and enzymes belonging to the phenylpropanoid pathway were up-regulated. None of the genes studied, however, showed an increase in transcription prior to 48 h after inoculation, which prompted the authors to suggest that the susceptibility of *U. americana* to DED might result partly from a delay in its response to infection[122].

Kumar *et al*.[123] studied the dynamics in root metabolism during compatible and incompatible interactions between chickpea and *Fusarium oxysporum* f. sp. *ciceri*, using quantitative label-free proteomics and NMR-based metabolomics. They showed that higher accumulation of PR proteins occurred in resistant plant[123].

The major causes for slower accumulation of PR proteins in the susceptible hosts may be because of the delayed release of elicitors from the cell wall of fungal pathogens into host tissues[124,125]. Other mechanisms involved include the absence or reduced action of some elicitors to induce accumulation of PR proteins in susceptible varieties. This was thought to be a result of the absence or reduced presence of receptor molecules for binding the available elicitor molecules in those susceptible varieties[126].

Pathogens shed away from the cell wall of the substrate for enzymatic PR proteins and avoid the lytic enzyme action: Chitin is an important structural component in the cell walls of plant pathogenic fungi. It is the substrate for PR proteins with chitinase enzymatic activities which cause lysis of hyphal tips[101]. By excluding chitin from its wall, the fungus may not only resist lysis by host chitinases but also avoid triggering other host defense mechanisms as chitin also acts as an elicitor of plant defense reaction[127]. Several observations have shown the absence of chitin in specialized infection fungal structures[128-129]. To evade recognition by host chitin receptors, several phytopathogenic ascomycetes secrete effector proteins which either compete with the host receptors for binding chitin fragments or reduce the accessibility of cell wall chitin to attack by plant chitinase enzymes which release chitin fragments[130-131] showed that recombinant ChELP1 and ChELP2 from the hemibiotrophic anthracnose fungus, colletotrichum higginsianum bind chitin and chitin oligomers *in vitro* with high affinity and specificity and that both proteins suppress the chitin-triggered activation of two immune-related plant mitogen-activated protein kinases in the host Arabidopsis. Using RNAi-mediated gene silencing, they found that ChELP1 and ChELP2 are essential for fungal virulence and appressorium-mediated penetration of both Arabidopsis epidermal cells and cellophane membranes *in vitro*.

Pathogens produce enzymes that aid in the protection from the fungitoxic action of PR proteins: Chitosan is present along with chitin in the cell wall of fungal pathogens. It arises mainly by deacetylation of nascent chitin which was formed by chitin synthase before the polymer chain aggregates to form fibrils[132]. The chitin deacetylation activity in the infected plant was correlated with hyphal growth in cucumber plants inoculated with *Colletotrichum lindemuthianum*[133]. When deacetylation occurs rapidly, the polymer chains in the form

of partially N-acetylated chitosan polymer would become less accessible to chitinase and remain bound to the hyphae sustaining the rigidity of the fungal wall[133]. N-acetylation of chitin might therefore be a way by which the fungal wall could be partially protected against the fungitoxic action of plant disease.

Pathogens produce enzymes to inhibit the activity of some PR proteins: It has been suggested that virulent pathogens may inhibit plant apoplastic proteases and cause disease in susceptible plants[134-136]. The PR-7 proteins show serine protease activity that confers disease resistance[136]. It has been shown that a protease inhibitor EPI10 secreted by the oomycete *Phytophthora infestans* completely inhibited the protease activity of P69b, a PR-7 protein of *Nicotiana benthamiana*[136].

Less elicitor released from the pathogen's cell wall to activate synthesis of PR proteins: The ability of the virulent pathogen to invade and infect the host may reside in its cell wall structure being less accessible to chitinase, avoiding the mechanism through which elicitor is released. Thus less elicitor is released in the case of a virulent pathogen. When a large amount of extracellular matrix (ECM) was released from the conidia of the non-pathogens *Blumeria graminis* f. sp. *tritici* and *Erysiphe pisi*, these induced more resistance in barley against the pathogen *B. graminis* f. sp. *hordei* than when a small amount of ECM was released from the conidia in barley leaves[137].

PR proteins degraded quickly in the susceptible host tissues: Acidification of the apoplast appears to be important in degradation of PR proteins. Fungal infection can lead to acidification of the apoplast and activation of host aspartyl proteinase enzyme activity that degrades PR proteins[138]. Tomato PR proteins were degraded by an aspartyl proteinase that was constitutively present in healthy and infected leaves at similar levels. However, an acidic pH, attained upon fungal inoculation, was required for its activity[138]. Tobacco leaves were also found to contain an extracellular aspartyl proteinase that endoproteotically cleaves tobacco PR-1a, Pr-1b and PR-1c at an acidic pH[139].

Marcato *et al*.[140] characterized the ability of four necrotrophic plant pathogens Botrytis cinerea, Sclerotinia sclerotiorum, Sclerotinia minor and Sclerotium rolfsii to degrade or sequester two widespread plant PR proteins: a type IV chitinase and a thaumatin-like protein (TLP). They showed that TLP and chitinase are absorbed by fungal mycelia and that fungal proteases are able to decrease chitinase.

Site of accumulation of some PR proteins may determine susceptibility or resistance: Intracellular (i.e., vacuolar) PR proteins generally show antifungal activity. Basic chitinases and glucanases that are found intra cellularly show antifungal activity, whereas those occurring extra cellularly (acidic forms) do not have appreciable antifungal activity[141]. For fungal pathogens that grow exclusively in the intercellular space without penetrating plant cells, chitinase or β-1,3-glucanase may not interact with fungal hyphae[142]. Woloshuk *et al*.[143] used transgenic tobacco plants to demonstrate that if the vacuolar basic proteins were targeted into the apoplast, these might induce resistance.

Adaptation of pathogens to PR proteins: It could be possible that the expression of chitinases does not lead to resistance against fungal pathogens because the fungus has adapted to the defense mechanisms of its host. Basic chitinases and β-1,3-glucanases from tomato were overcome by the pathogen *Cladosporium fulvum*, which was insensitive to these PR proteins[144]. It has been shown that constitutive over expression of a basic vacuolar chitinase gene in tobacco did not lead to increase resistance of transgenic plants against *Cercospora nicotianae*. However, chitinases from unrelated species in transgenic plants could not be overcome by the invading fungus[145]. Enhanced resistance in transgenic tea (*Camellia sinensis* L. O. Kuntze) to blister blight disease caused by the fungus *Exobasidium vexans* was achieved by over expression of class I chitinase gene from potato (*Solanum tuberosum*)[146].

Some PR proteins may not be involved in disease resistance: Some PR proteins may not have any inhibitory action against fungal proteins. For instance, class III chitinases (PR-8 proteins) seem to lack antifungal activity[147]. Also, there were many reports indicating that PR proteins were only stress-induced because of infection and that these may not be involved in host defense mechanisms. In tomato, expression levels of genes encoding PR proteins were correlated with the severity of gray mold disease (*Botrytis cinerea*)[148]. Thus, PR proteins in this case acted as truly pathogenesis-related and not as defense-related proteins[148]. A transgenic wheat line co-expressing a chitinase and a β-1,3-glucanase gene combination and another wheat line expressing a *PR-5* gene were developed. Though these lines showed enhanced resistance in the greenhouse in response to a single application of inoculum, none of these showed resistance under field conditions which provided a continuous inoculum of *Fusarium graminearum*[109].

ACCUMULATION OF SECONDARY METABOLITES

Types of secondary metabolites: Plants produce several secondary metabolites that are distinct from the components of intermediary (primary) metabolism, in that these are generally non-essential for the basic metabolic process of the plant[149]. There are two types of antifungal secondary metabolites: phytoalexins (inducible secondary metabolites) and phytoanticipins (constitutive secondary metabolites)[150]. In general, phytoalexins are defined as the compounds that are synthesized de novo in response to infection, accumulating to antimicrobial concentrations in the area of infection[151]. Whereas phytoanticipins are defined as the compounds that are preformed infectional inhibitors[150]. Phytoalexins and phytoanticipins may belong to the same chemical classes[149] and both accumulate because of infection almost in a similar way[152]. Both of these have been detected in compatible and incompatible interactions[149,153].

Phytoalexins

Chemical structure classes of phytoalexins and site of synthesis: More than 300 phytoalexins have been identified and characterized[152]. Phytoalexins constitute chemically heterogeneous groups of substances[154]. Several phytoalexins belong to the phenylpropanoid structural class such as isoflavonoids. Another major group of phytoalexins belongs to the terpenoid class such as sesquiterpenoids. Some of the phytoalexins are alkaloids, whereas others are nitrogen and sulfur-containing compounds. Some phytoalexins belong to fatty acid derivative compounds[154].

Phytoalexins may be released toward the infection sites by living cells of the host undergoing attack by the pathogen[155]. Subcellular vesicle-like inclusions appear in the host cell and these inclusions were directed to the fungal penetration sites[156]. Nuclear migration, cytoplasmic streaming and intracellular pH provide an environment for inclusion trafficking and release of the phytoalexins to the fungal penetration sites[156]. The phytoalexins synthesized in healthy living cells may be secreted from the cells to accumulate in the adjoining necrotic tissue[157].

Phytoalexins are fungitoxic: Phytoalexins are recognized only based on the antimicrobial activity[150]. Most of these have been reported to be highly fungitoxic. These were found to be inhibitory to fungal spore germination and hyphal growth[158]. Exogenous application of atractylenolide-II, a phytoalexin extracted from *Atractylenolides macrocephala* with the concentration of 200 g mL^{-1} showed a significant inhibitory effect on mycelial growth of *Sclerotium rolfsii* by achieving a 77.23% antifungal activity rate and a minimal inhibitory effect at a concentration of 12.5 g mL^{-1}. The absence of atractylenolide-II in the rhizome of *A. macrocephala* plants made the plant susceptible to *S. rolfsii*[72].

In addition to a general inhibition of fungal growth, phytoalexines such as mansonones have been reported to exhibit several effects on fungal physiology and ultra structure, i.e., ion leakage, respiration rate reduction, cell wall disruption, aggregation of ribosomes and the accumulation of electron-dense material in the mitochondria[159,160]. Phytoalexins may also suppress toxin production by the pathogen[161].

Genes involved in phytoalexins biosynthesis were found to be upregulated during infection of host tissues by fungal pathogens in many pathosystems[14,162,163].

HOW DO PATHOGENS OVERCOME ANTIFUNGAL PHYTOALEXINS?

Pathogens detoxify phytoalexins or suppress their accumulation in compatible interactions: Potential pathogens have been reported to detoxify phytoalexins of the host[164]. These produce specialized enzymes to degrade phytoalexins, such as pisatin demethylase encoded by cytochrome P450 in the pea pathogen *Nectria haematococca*[165] and the hydroxystilbene-degrading enzyme of *Botrytis cinerea*[166]. These enzymes appear to be pathogenicity factors and were shown to be essential for fungal pathogenesis[167,168].

Spores from a virulent race of *Magnaporthe grisea* were able to suppress accumulation of phytoalexins in rice leaves, whereas its elicitors induced phytoalexins faster suggesting that a suppressor or a suppressing system may exist in living cells[169]. Yoshioka *et al.*[170] found that the effect of a suppressor isolated from *Mycosphaerella pinoides* on host defense reactions seemed to result from its inhibition of the ATPase in the host plasma membrane. Some strains of *Peyronellaea curtisii* suppressed phytoalexin production in Hippeastrum scales and were resistant to various concentrations of phytoalexin added to the culture medium[171].

Phytoalexins accumulate less and their induction may be delayed in susceptible hosts: The level of accumulation of Phytoalexins may be less in susceptible hosts. In broad bean (*Vicia faba*) leaves infected with *Botrytis fabae*, rapid accumulation of the phytoalexin wyerone acid was observed in resistant cultivars that reached a level greater than twofold of that in the susceptible cultivars[172]. In elm, inoculation of *U. pumila* with aggressive species *O. novo-ulmi*

led to the accumulation of 90 μg g^{-1} of mansonones E and F. In contrast accumulation reached only 28 μg g^{-1} in inoculated susceptible *U. americana*[173,174].

A delay in the induction of phytoalexins was common in various compatible interactions and the delay helped the pathogen escape from the toxic environment created by the accumulation of phytoalexins at the infection site[175]. In susceptible cultivars of sorghum seedlings, notable amounts of phytoalexins accumulated only 72 hpi but the primary hyphae of the *Colletotrichum sublineolum* pathogen had emerged from infection vesicles by 48 hpi. In contrast, phytoalexins accumulated in considerable amount at about 36 hpi in the resistant cultivar[175].

Proteomics and metabolomics analysis along with expression profiling of candidate genes during compatible and incompatible interactions between chickpea and *Fusarium oxysporum* f. sp. *ciceri* revealed significant increase of phytoalexins (up to 5-fold) in root metabolism of the resistant plants[123].

Some phytoalexins may not have any roles in disease resistance of plants and the highly toxic phytoalexins may not accumulate in susceptible hosts: In some host-pathogen interactions, phytoalexins may not have any role in disease resistance. Accumulation of phytoalexins may be only a metabolic process activated by stress. In pea, the pathogen *Fusarium solani* f. sp. *pisi* induced more pisatin than the non-pathogen *F. solani* f. sp. *phaseoli*[176]. In both sorghum mesocotyls and leaves, the deoxyanthocyanidin phytoalexin accumulates rapidly following attempted fungal infection by pathogens as well as non-pathogens[177].

In vitro bioassays showed that luteolinidin and 5-Methoxyluteolinidin phytoalexins that accumulate in resistant sorghum cultivars exhibit higher toxicity than other phytoalexin components that accumulate in both resistant and susceptible sorghum cultivars and do not have roles in disease resistance[175].

Phytoanticipins

Chemical structural classes of phytoanticipins: Phytoanticipins are low-molecular weight, antifungal compounds that are present in plants before challenge by fungal pathogens or are produced after infection solely from preexisting constituents[150]. Numerous antifungal phytoanticipins have been detected in plants. These belong to several chemical classes including: phenolics, flavonoids, terpenoids and steroids[154].

Phenolics as phytoanticipins and the toxicity: Several phenolics and phenylpropanoids that may act as phytoanticipins have been detected in plants. Phenolics such as isoflavones and isoflavans were highly toxic to fungal pathogens[178]. However, both the toxicity of different phenolics and the sensitivity of pathogens to the phenolics vary[179]. The toxicity of phenolics has been demonstrated by artificially increasing the synthesis of phenolics in some plants. The flavonoid epicatechin plays an important role as phytoanticipin in avocado fruits[180]. Inoculation of freshly harvested avocado fruits with a mutant strain of *Colletotrichum magna* inhibited subsequent decay development by the pathogen *C. gloeosporioides*. The mutant strains induced higher levels of the phenolic epicatechin[181]. Over expression of Polyphenol Oxidase Gene in Strawberry Fruit delayed the Fungus Infection Process of gray mold[182].

HOW DO PATHOGENS OVERCOME THE ANTI-FUNGAL PHENOLICS?

Pathogens degrade phenolics to nontoxic products: Nicholson *et al.*[183] showed that the proline-rich proteins found in the mucilage of spores of some foliar fungal pathogens may protect conidia from toxic phenols that accumulate in the water which is necessary for conidium dispersal and secondary spread of the fungus. The enzymes found in the mucilage (i.e., β-glucosidase and non-specific esterase) may cleave the phenolic esters and glycosides, freeing the aglycones and making these more available for binding to the extracellular proline-rich proteins of the mucilage[184]. Guetsky *et al.*[180] showed that *Colletotrichum gloeosporioides* produced a laccase that may be a pathogenicity factor. This laccase degrades epicatechin in culture and infected avocado fruit tissues. Isolates of the fungus with reduced laccase activity and no capability to metabolize epicatechin showed reduced pathogenicity on ripening fruits[180].

Pathogens suppress production of phenolics in plants: The pathogen may suppress phenolics by: (1) Preventing their accumulation, (2) Suppressing enzymes involved in biosynthesis and (3) Using a suppressor molecule.

A correlation between accumulation of phenolic compounds and resistance in alfalfa stems inoculated with *Colletotrichum trifolii* was reported by Baker *et al.*[185]. In resistant plants, phenolics accumulated to higher levels, whereas in susceptible plants, phenolic synthesis did not seem to be highly induced[185]. Thus, reduced induction of phenolic synthesis may occur in compatible interactions.

Cahill and McComb[179] showed that suppression of PAL using aminooxyacetic acid, a PAL inhibitor, led to a reduction in the synthesis of phenolics which rendered the resistant *Eucalyptus calophylla* susceptible to the oomycete *Phytophthora cinnamomi*. In the susceptible *E. marginata,* the activity of PAL increased only slightly in the first 24 hpi and declined by up to 77% of control level at 96 hpi[179]. Thus, the pathogen may suppress phenol biosynthetic enzymes in compatible interactions.

Vidyasekaran *et al*.[186] showed that a toxin produced by *Helminthosporium oryzae* was able to suppress the phenolic content and PAL activity in rice leaves resulting in severe incidence of the disease. Some pathogens, such as *Alternaria alternata,* possess a tentoxin and were able to suppress oxidation of phenolics by inhibiting PPO with the tentoxin[187].

Twenty one fungal tomato pathogens examined by Sandrok and Van Etten[188], degraded α-tomatine, a tomato phytoanticipin while saprophytes and non-pathogens of tomato tested were sensitive to it. There was a strong correlation between tolerance to α-tomatine, the ability to degrade this compound and pathogenicity on tomato[188].

A protopanaxadiol-type (PPD) ginsenoside from Chinese ginseng inhibited growth of five ginseng nonpathogens tested, while it promoted growth of the ginseng pathogen cylindrocarpon destructans. The ginseng root pathogenic fungus was shown to enzymatically degrade PPD-type ginsenosides by extracellular glycosidase activity and to encounter their toxicity by converting PPD-type ginsenosides into growth or host recognition factors[189]. Thus, in compatible interactions, the pathogen may suppress phenolics metabolism by a suppressor molecule or a toxin.

Bouarab *et al*.[190] presented evidence for a two-component process in which a fungal pathogen subverts the preformed antimicrobial compounds of its host and uses them to interfere with induced defense responses. Antimicrobial saponins are first hydrolyzed by a fungal saponin-detoxifying enzyme. The degradation product of this hydrolysis then suppresses induced defense responses by interfering with fundamental signal transduction processes leading to disease resistance[190].

CONCLUSION

Susceptible plants do not lack defense mechanisms to defend against pathogen attack. Rather, it seems that an anomaly has occurred in the interaction with the microbe. This anomaly is reflected at the molecular level, by a delay, a lack of coordination or an insufficiency in responses, that render the plant susceptible. Thus, gaining a better understanding of the biochemical processes leading to susceptibility would be important and could help develop better control strategies for a given plant disease.

SIGNIFICANCE STATEMENTS

This review shows that susceptible plants do not lack defense mechanisms to defend against pathogen attacks. It gives insights on how virulent fungi are able to counteract plant defense mechanisms to cause diseases and why plant defense products are ineffective in providing the plant with resistance against pathogenic attacks. The review will help the researcher to gain a better understanding of the biochemical processes leading to susceptibility and could help develop better control strategies for a given plant disease.

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