Chapter 1:

Review of Literature

Chapter 1: Review of Literature

1.1 Plant-pathogen interactions

Microbial plant pathogens including bacteria, oomycetes, fungi, nematodes and viruses cause disease to a variety of economically important plants and thereby posing a major threat to global food security. Pathogenic bacteria and viruses enter the plant via stomatal openings or water pores (hydathodes) or wounds and thereby, multiply in the plant apoplast (intercellular gaps). Aphids and nematodes feed by introducing a stylet into a plant cell. Fungi and oomycetes, can infiltrate the plasma membrane of the host cell through invading feeding structures called haustoria (Jones and Dangl, 2006). To infect plants, fungal pathogens must detect the presence of a compatible host by activating intracellular signalling pathways, which leads to the development of infection structures required to gain access into host tissue (Hamel et al., 2012). Pathogens derive nutrients from their host and depending on their lifestyle, they have been categorized into three broad classes: Biotrophic, Necrotrophic and Hemibiotrophic. Biotrophs survive within the living host tissue by suppressing host immunity and thereby derive nutrients from the living host cells. On the other hand, Necrotrophs grow within the host tissue by producing toxins and enzymes that eventually kill the cells and thereby acquire nutrients from dead and dying host tissues. Whereas, Hemibiotrophs initially grows within a host in a biotrophic manner and derive nutrients, which later turns into necrotrophic lifestyle resulting in death of the plant cells (Fernandez and Orth, 2018).

1.1.1 Plant defense mechanisms – physical and chemical

Plants deploy a variety of physical and chemical defense responses to protect against the invading microbial pathogens.

Physical defense –

Majority of the microbial pathogens infect the aerial parts of the plant such as leaf and stem. Plants possess a number of structural components that acts as a physical barrier against invading pathogenic microbes. Moreover, some of these physical structures could also sense the pathogen-attack and relay downstream signals to activate the plant immune system. **Cuticle** is an outermost covering of plants, present on leaves, non-woody stem, flowers and fruits. It provides protection against various abiotic stresses, mechanical injuries and pathogen/pest infection. Cuticle is mainly composed to cutin matrix (an insoluble esterified-polymer) and embedded wax (soluble lipids; Ziv et al., 2018). **Plant cell wall** is a primary barrier and acts as a first line of defense against invasion and expansion of bacterial and fungal pathogens. It is mainly composed of structural polysaccharides such as cellulose, hemicellulose and pectin, which provides strength and rigidity to the cell (Freeman et al., 2008). Moreover, plant cell wall spanning cuticle and epidermal cell membrane form a continuum responsive to various pathogen infections (Ziv et al., 2018). **Callose**, a polysaccharide complex, is another structural defense employed by plant cells at the site of infection/mechanical injury. Accumulation of callose, known as papillae, is a part of plant basal defense reaction that hinders cellular perforation at the infection site. Callose is synthesized and deposited between cell wall and cell membrane in response to microbial invasion (Jacobs et al., 2003). **Guard cells** are found within the stomatal pores, which facilitate entry of carbon dioxide into the leaf for photosynthesis by limiting water-loss from

plant. Guard cells function in defense system by closing the stomatal openings and thereby restricting pathogen entry into the host plant (Lee et al., 1999).

Chemical defense –

Plant pathogen interaction is a two-way process wherein plant recognizes the pathogen-attack to activate the defense response whereas pathogen targets specific plant proteins to evade the host immune response. Generally, these interactions are highly specific and therefore the pathogens can infect specific host plants. However, host expansion due to host switching events has also been observed in emerging fungal diseases. Plant innate immunity involves two strategies (Fig. 1.1A): **1)** recognition of microbial elicitors such as bacterial flagellin and fungal chitin called **pathogen-associated molecular patterns (PAMPs)** by plant's pattern recognition receptors (PRRs), present on the cell surface induce **PAMP-triggered immunity (PTI)**. PTI involves responses such as burst of reactive oxygen species (ROS), Ca^{2+} influx, and a three-tiered mitogen-activated protein kinase (MAPK) activation, expression of defense related genes, and callose deposition (Cheng et al., 2019; Zipfel and Oldroyd, 2017; Boutrot and Zipfel, 2017). Plant MAPKs have been linked in immune signalling that enhances defence responses during plant–microbe interactions, whereas fungal MAPKs regulate mechanical and enzymatic penetration of host tissues (Hamel et al., 2012). In contrast to PAMPs, pathogen infection often results into generation of DAMPs (Damage-Associated Molecular Patterns) in the host. These DAMPs, are the plant's self-molecule(s) produced as a result of cell/tissue-damage, act as "danger" signals, triggering immune responses. Various biomolecules including proteins, carbohydrates, lipids, and nucleotides are known to act as the plant DAMPs (Bacete et al., 2018; Boutrot and Zipfel, 2017). **2)** Pathogen secretes **virulence factors (effectors)** secreted to evade and suppress PTI. However, these effectors often get recognized via plant's polymorphic intracellular

NB-LRR (Nucleotide Binding Leucine Rich Repeat) proteins, thereby inducing **effector-triggered immunity (ETI**; Dangl et al., 2013**)**. NB-LRR activation results in deployment of differential plant response by regulating balance of NADPH-oxidase dependent ROS and salicylic acid-jasmonic acid/ethylene signaling (Torres et al., 2005; Glazebrook, 2005). ETI is generally stronger, faster and often gives rise to hypersensitive response (HR), a localized cell death, that prevents systemic spread of disease (Fig. 1.1B; Jones and Dangl, 2006). Triggering of HR makes plant tissues highly resistant to a broad range of pathogens for an extended time-period in a phenomenon called **System Acquired Resistance (SAR)** (Durrant and Dong, 2004). Moreover, ETI is induced against well adapted pathogens as opposed to PTI which is relatively active against non-adapted pathogens in a phenomenon called non-host resistance (Ham et al., 2007).

Plants also synthesize and secrete variety of **secondary metabolites** involving terpenoids, phenolics and alkaloids that prevent pathogen invasion (Agarwal et al., 1999 Book chapter). Additionally, plants can respond against viruses via an **RNA silencing** mediated genetic defense mechanism, resulting into degradation of foreign (viral) DNA or RNA (Freeman and Beattie, 2008). Plant **defensins**, small cysteine-rich proteins, are found to exhibit anti-microbial activity by inhibiting key enzymes in invading bacteria and fungi. Moreover, plant produce hydrolytic enzymes such as **chitinases** and **glucanases** to degrade cell wall of invading pathogenic fungi (Broekaert et al., 1995; 1997).

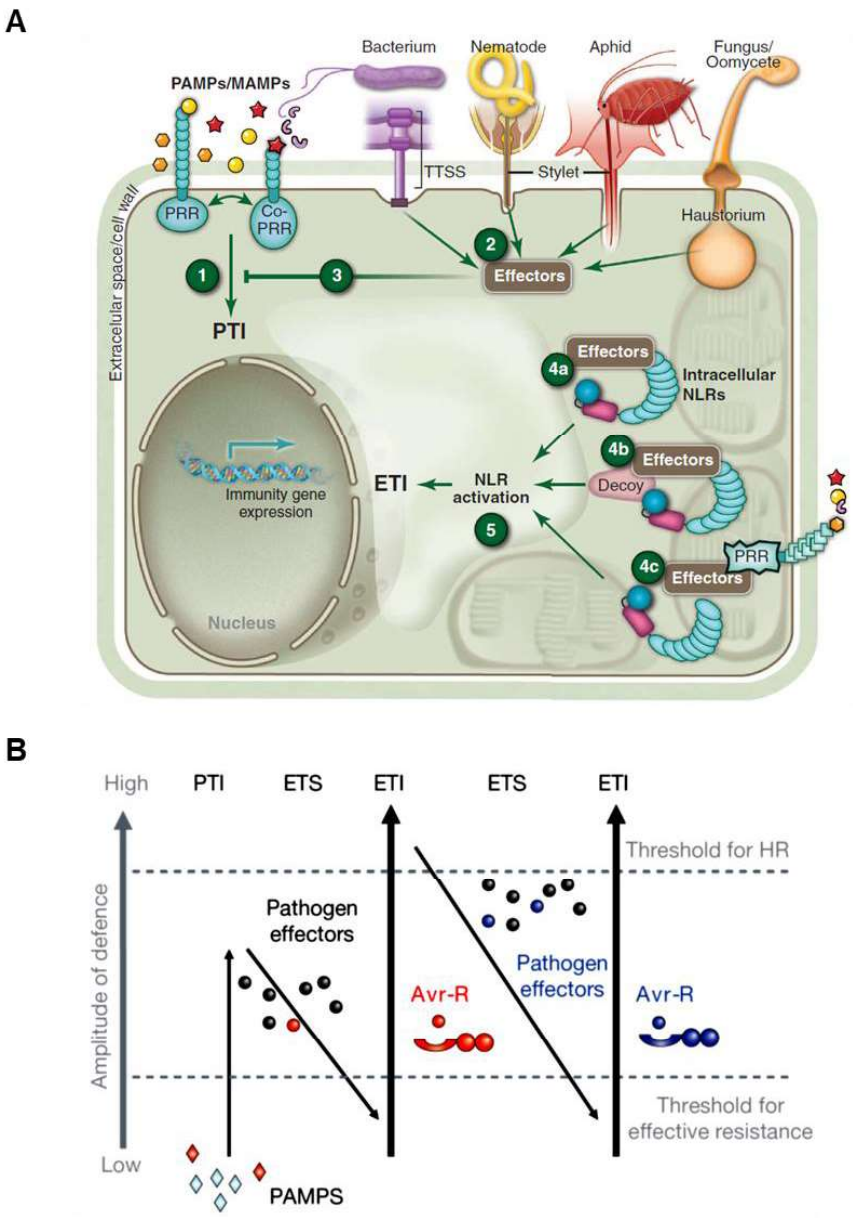

Figure 1.1. Schematic showing strategies and extent of the plant immune system. (A) Pathogens express PAMPs/MAMPs (pathogen/microbial associated molecular patterns) recognized by plant PRR (pattern-recognition receptor) inducing PTI (PAMP-triggered immunity; step 1). Pathogens secrete virulence effectors to block PTI (step 2 and 3). Intracellular plant's NLR (nucleotide-binding leucine-rich repeat) receptors can sense effectors either by direct receptor-ligand interaction (step 4a) or via a structural mimic of effector target, a decoy receptor-effector interaction (step 4b) or by sensing altered host effector target such as cytoplasmic domain of PRR (step 4c), each resulting in NLR-dependent ETI (step 5; adapted from Dangl et al., 2013). **(B)** Model showing quantitative output of plant immune response, in a phase-wise manner: phase 1, plants detect PAMPs (red diamonds) to trigger PTI; phase 2, pathogen-effectors interfere PTI resulting in effector-triggered susceptibility (ETS); phase 3, one effector, acting as avirulence factor (Avr; denoted in red) gets recognized by plant NLR receptor (R protein), activating ETI, an

amplified version of PTI; and phase 4, pathogen isolates that have lost the Avr (red) effector infects and suppresses ETI, while new plant NLR alleles get selected resulting in ETI again (Adapted from Jones and Dangl, 2006).

1.1.1.1 Plant cell wall – the primary physical barrier

The plant cell wall is mainly composed of three polysaccharides: cellulose, hemicellulose (consists of xylan and xylan derivatives) and pectin. A mesh-like interconnected network of cellulose microfibrils and hemicellulose is embedded within the pectin matrix (Cosgrove, 2005). Additionally, plant cell wall also often contains lignin (phenolic polymer) and glycoproteins (Zhang et al., 2021), however, considering the relevance to the present study, here only the structural polysaccharides and their arrangement in plant cell wall has been reviewed in detail.

Cellulose, hemicellulose and pectin are interconnected to each other via an ester bond formed between various small phenolic compounds such as ferulic acid, sinapic acid, p-coumaric acid, caffeic acid, etc. These ester-bonds between phenolic compounds and polysaccharide imparts elasticity and plasticity to the plant cell wall. The mesh-like network is required for providing strength and maintaining integrity of the plant cell wall.

Cellulose is the most abundant polysaccharide, comprising of repeating units of unbranched (1,4)-linked β-D-glucosyl residues. Cellulose microfibrils are formed by joining of discrete cellulose chains via inter- and intrachain hydrogen bonds (Zhang et al., 2015). These crystalline microfibrils provides mechanical strength to the cell wall and are also highly resistant to enzymatic hydrolysis (Fig. 1.2A; Cosgrove 2005).

Hemicellulose binds to cellulose and has a backbone made up of (1,4)-β-D-glycans, similar to cellulose. However, it contains branches that prohibit them from forming microfibrils on

their own. Two of the most common hemicelluloses are xyloglucan and arabinoxylan. The backbone of xyloglucan is similar to that of cellulose, but three out of four glucose residues are attached with xylose branches. Galactose and fucose residues can be serially added to the xylose. The backbone of arabinoxylan is a (1,4)-linked β-D-xylan attached with arabinose branches. Mannans, like xyloglucan and arabinoxylan, are also found in primary cell walls and are thought to have similar functions. In arabinoxylans, which are particularly prevalent in cereal grasses, other residues such as glucuronic acid and ferulic acid esters (FA) are also attached (Fig. 1.2A, Cosgrove 2005).

Pectins comprises of complex and diverse polysaccharides that possess α-(1,4)-linked galacturonic acids backbone and mainly have three types of domains: unbranched homogalacturonan, rhamnose-alternated rhamnogalacturonan I, and complex branched rhamnogalacturonan II (Zhang et al., 2021). Homogalacturonan is made up of a linear chain of galacturonic acid residues, whereas xylogalacturonan has xylose branches added to it. Carboxyl groups of homogalacturonan and xylogalacturonan are frequently methyl-esterified, which blocks the acidic group and inhibits their capacity to form gels. Rhamnogalacturan I is made up of alternating galacturonic acid and rhamnose residues, with side branches containing additional pectin domains. Rhamnogalacturonan II is a complex pectin domain with 11 sugar residues that forms dimers by borate esters. The acidic pectins are also connected to the neutral arabinans and arabinogalactans, and it has been claimed that they increase wall flexibility (Fig. 1.2A, Cosgrove 2005).

Cellulose microfibrils in the plant cell wall may be interlinked directly or indirectly with hemicellulose and pectin in five different ways (Fig. 1.2B), a) Hemicellulose such as xyloglucan may be connecting the cellulose microfibrils, b) Portion of the xyloglucan might become entrapped during cellulose microfibril formation and remaining free end could get

connected to the other matrix polysaccharides, c) Coating of cellulose microfibrils with xyloglucans, which might also get adhered to other matrix polysaccharides, d) Xyloglucan also possibly attaches to pectin polysaccharides forming a complex that in turn anchors cellulose microfibrils and e) Crosslinking of arabinoxylans (hemicellulose) to the cellulose microfibrils and also to pectins by ferulic acid esters. Moreover, formation of large pectin networks includes rhamnogalactouronan I, which serves as the backbone and other pectin domains attached as branches. Here, rhamnoglactouronan II are crosslinked via boron dister linkages, while homogalactouronans are crosslinked by calcium via ionic interactions (Vincken et al. 2003). Many plant cell-types also contains (but not always) a secondary wall, laid down inside the primary wall, marked by a halt of the cell expansion (Cosgrove, 2005). Altogether, these different interactions and crosslinking between cellulose microfibrils, hemicellulose and pectins forms a compact plant cell wall that acts a major structural barrier for invading plant pathogens especially fungi.

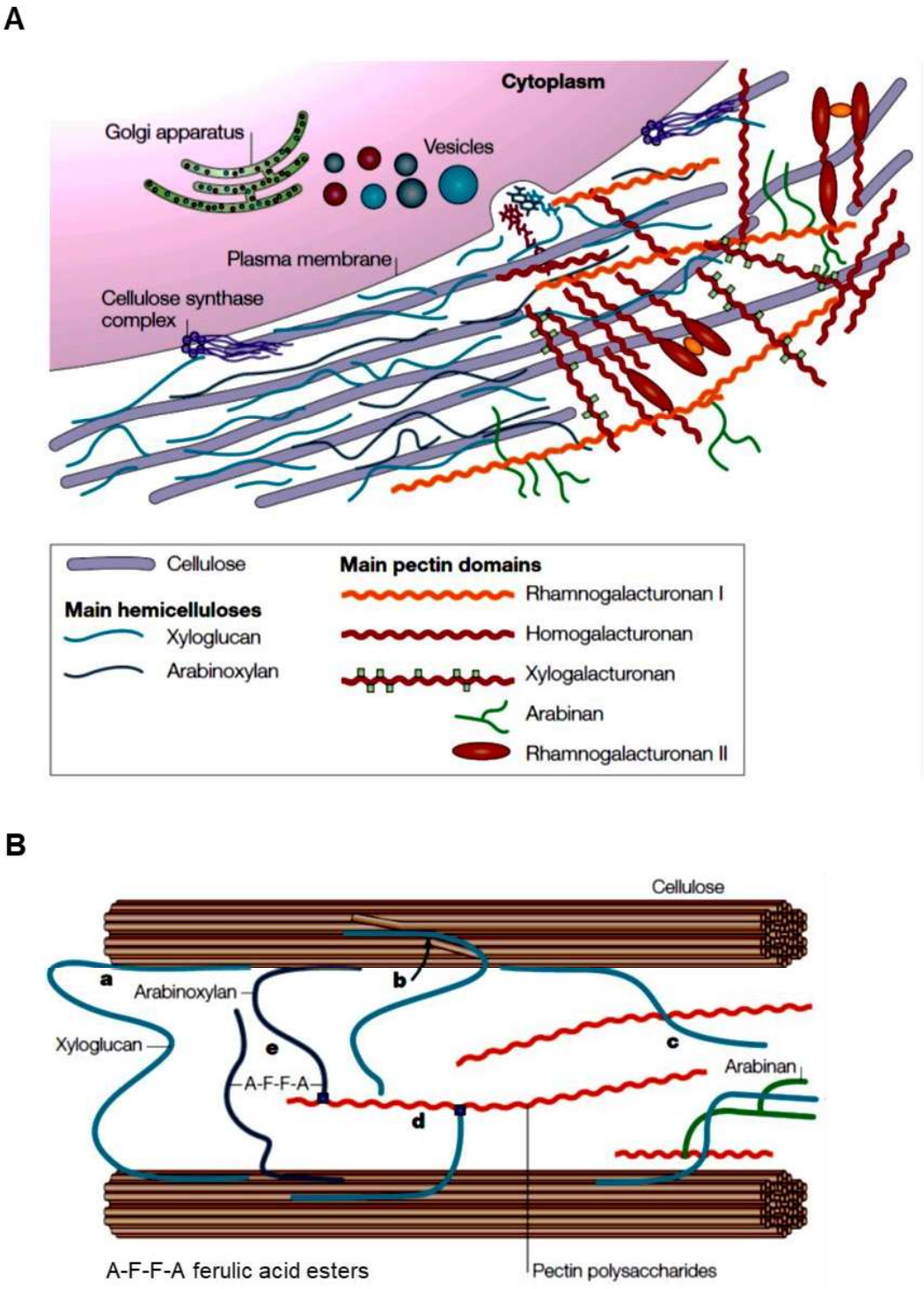

Figure 1.2. Schematic representation showing **(A)** structure of growing primary plant cell wall and **(B)** cross-linking of hemicellulose/pectin with cellulose microfibrils forming a polysaccharide network of the plant cell wall (adapted from Cosgrove, 2005).

1.2 Plant cell wall degrading enzymes (CWDEs)

Phytopathogens secrete CWDEs upon contact with the plant cell walls and studies have shown that during plant-pathogen interaction, genes encoding CWDEs are upregulated. Identification of transcriptional activators that control the expression of CWDE-encoding genes would be a good way to evaluate the importance of these in plant disease (Martinez et al., 2013; Mathioni et al., 2011). In phytopathogenic fungi, studies on global regulatory circuits, such as carbon catabolite repression (CCR), which ensures utilization of easily metabolizable carbohydrates (e.g., glucose) first and also pH dependent regulation of gene expression, has demonstrated the importance of CWDE-expression in fungal pathogenesis. For instance, Snf1 (sucrose non-fermenting protein) and XlnR (xylanolytic regulator) dependent activation of CWDE-expression have been studied with respect to fungal invasion and pathogenesis (Brunner et al., 2007; Seiboth et al., 2012; Tonukari et al., 2000). However, only a few CWDEs have been shown to be necessary for virulence by CWDEs. There is no single mechanism that can explain the behaviour of all plant pathogenic fungus, yet even within the same genus, there are substantial variances. Therefore, understanding the genetic basis of plant-fungal pathogen interaction is critical for designing successful disease mitigation methods.

1.2.1 CWDEs produced by fungi

CWDEs act on various complex polymers of the plant cell wall such as xylan, pectin and cellulose and degrades them into their monomeric constituents that can be readily absorbed and utilized by the fungus. Plant CWDEs are produced by different fungi ranging from saprotrophic fungi such as *Aspergillus spp.*, *Neurospora spp.*, etc. to hemibiotrophic fungi such as *Fusarium graminearum*, *Magnaporthe oryzae*, *Cochliobolus sativus*, etc. and

necrotrophic fungi such as *Fusarium oxysporum*, *Magnaporthe poae*, *Gaeumannomyces graminis*, etc. These CWDEs produced by fungi are mainly involved in cellulose degradation including exocellulase (cellobiohydrolases), endocellulase (endo-β-1,4-glucanases) and β-glycosidases, hemicellulose degradation including xylanases, xyloglucanases, α-/β-galactosidases and accessory enzymes such as feruloyl esterases, acetyl esterases, glucuronyl esterases, etc. and pectin degradation including polygalacturonases and pectin/pectate lyases (Kubicek et al., 2014; Kubicek, 2013).

Cellulase: Hydrolysis of cellulose occurs due to synergistic or overlapping actions of three major cellulases such as 1) endo-β-1,4-glucanase (EC 3.2.1.4) acting on both soluble and crystalline cellulose chain, 2) exoglucanase such as cellobiohydrolases (EC 3.2.1.91) that acts on ends of the cellulose chain resulting in release of cellobiose and sometimes glucose, and 3) β-glucosidase (EC 3.2.1.21), acts on cellobiose to release D-glucose. Fungi contain varying degree of different types of cellulases that act synergistically (Moreira et al., 2011). Fungal cellulases usually belong to different Glycoside hydrolase (GH) family such as GH 1, 3, 5, 6, 7 and 45. (Kubicek et al., 2014).

Xylanase: For hydrolysis of the backbone chain of xyloglucan, fungi utilise endo-β-(1→4)-glucanases and 1,4-β-xylosidase. The glycosidic linkages in the xylan backbone are cleaved by endo-1,4-β-xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8). The majority of fungal xylanases belong to GH10 and GH11 family, wherein GH10 xylanases are abundant in plant-pathogenic and saprobic fungi (Kubicek et al., 2014).

Pectinase: Pectin being primarily composed of sugar acid; the hydrolysis of glycosidic bond is carried out by polygalacturonases (EC 3.2.1.15 and 3.2.1.67), while pectin lyases (EC 4.2.2.10) and pectate lyases (EC 4.2.2.2) cleaves the pectin polymer via a nonhydrolytic β-elimination reaction (Kubicek et al., 2014). Plant pathogens produce pectinases such as

endo- and exo-pectate lyase, endo- and exo-polygalacturonase and pectin methylesterase. Mutations in genes encoding pectinases lead to a significant effect on fungal pathogenesis (Walton, 1994). Both, endo- and exo-acting pectinases belong to the GH28 family of polygalacturonidases (Kubicek et al., 2014).

Accessory CWDEs act on the side chains of the polysaccharide backbone, thereby enabling access to the aforementioned polysaccharide degrading enzymes. As described in detail by Kubicek et al., 2013, a wide-range of hemicellulose-degrading accessory enzymes have been reported especially in fungi, such as endoarabinases (acts on arabinan side chains in pectin), α-L-arabinofuranosidases (cleaves α-1,2- or α-1,3-L-arabinofuranose side chain in xylan), endo- β-1,4- and β-1,6-galactanases (breakdown of pectin's side chains), exo-β-1,3-galactanases (hydrolyse β-1,3-galactan main chains), α-xylosidases (hydrolyse backbone of oligoxyloglucans), α-fucosidases (cleave fucose branches to xyloglucans), α-glucuronidases and glucuronan lyases (cleaves glucuronic acid branches of xylan) and α-L-rhamnosidases (hydrolyse rhamnosides attached to pectin). Other accessory enzymes; **Feruloyl esterases** are able to hydrolyze the ester linkages of ferulic- and diferulic-acid, crosslinking the plant cell wall polysaccharides. **Acetylxylan esterases** de-esterify plant xylans and are mainly found in anaerobic fungi. **Pectin esterases** involved in removal acetyl and methyl residues from the pectins, comprise of three classes: pectin methyl- and acetyl-esterases and rhamnogalactouronan esterases. **Glucuronyl esterases** hydrolyse ester connections between glucuronoxylan residues and lignin aromatic alcohols. A simplified phylogenetic relationship between different accessory CWDEs is shown in Fig. 1.3 (Kubicek, 2013).

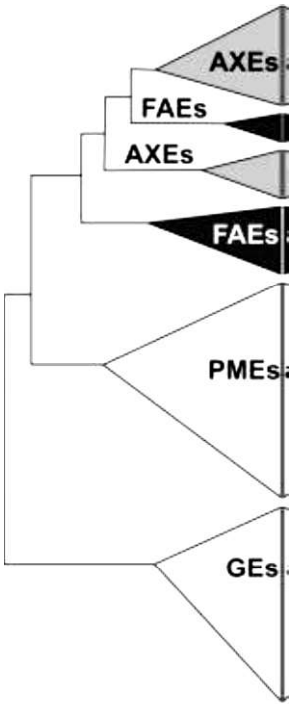

Figure 1.3. Phylogenetic relationship between different accessory plant CWDEs; acetylxylan esterase (AXE), ferulic acid esterase (FAE), pectin methylesterase (PME) and glucuronoyl esterase (GE; adapted from Kubicek, 2013).

A large number of CWDEs has resulted from the arms race between plants and phytopathogenic fungi. The majority of fungus can only infect dicots or monocots, which have differing cell wall compositions. Biotrophic fungi contain the fewest CAZymes and no GH6 cellobiohydrolases in their genomes. *Cladosporium fulvum*, causal agent of tomato leaf mold, has significantly more CAZymes in the β-glucosidase family GH3, GH31, and GH43 in its genome. This shows that various subsets of ancestral paralogs may have been lost in the evolution of some fungal taxa (Zhao et al., 2013; Kubicek et al., 2014). When compared to all other sequenced fungal genomes, *Macrophomina phaseolina*, one of the most destructive necrotrophic fungal infections, had the highest number of carbohydrate esterases (Islam et al., 2012; Kubicek et al., 2014). Due to redundant nature and presence of a large number of fungal CWDEs, raises a question whether or not they constitute virulence factors. However, for instance, plants produce polygalacturonases-inhibiting proteins (PGIPs) found in the plant cell wall, highlights the role of polygalacturonases in fungal

attack (Lorenzo et al., 2001). Fungal CWDEs belonging to GH10 and GH11 families have been shown to act as virulence determinants (Vu et al., 2012).

1.3 Feruloyl esterases (Fae)

Feruloyl Esterases (Fae; EC 3.1.1.73), a subclass of carboxylic acid esterases (EC 3.1.1.1), are a group of enzymes involved in the degradation of xylan and pectin, from which they release ferulic acid and other aromatic acids. The enzymatic reaction catalysed by Fae is as follows –

Feruloyl-polysaccharide + H_2O → Ferulic acid + polysaccharide

Molecular analysis of Fae from *Aspergillus spp.* has revealed that many of these enzymes is modular, comprising a catalytic domain and a non-catalytic cellulose-binding domain (Hermoso et al., 2004).

1.3.1 Role of Fae in plant cell wall degradation

Fae hydrolyses the ester bonds (formed between ferulic acid/diferulate and polysaccharide) and thus dissociating the complex into ferulic acid and the constituent polysaccharide (Fig. 1.4). Breakage of the ester bonds leads to weakening of the plant cell-wall, that acts as a barrier protecting from invading microbial pathogens. Majority of Fae acts synergistically with other CWDEs such as xylanases, cellulases and pectinases (Faulds and Williamson, 1995; Kroon et al., 1996).

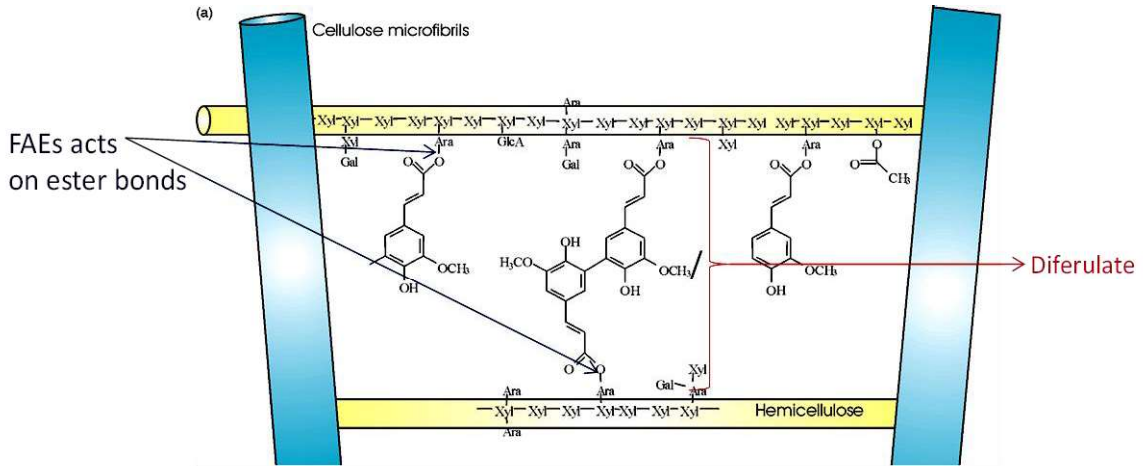

Figure 1.4. Schematic representation of plant cell wall containing feruloylated heteroxylans crosslinking hemicellulose chains by ester-linked monomeric ferulic acid and 5, 5'-diferulic acid, forming network with cellulose microfibrils. Blue-arrows indicate the ester-bonds between ferulic acid and polysaccharide, that gets hydrolysed by feruloyl esterases (adapted from Hermoso et al., 2004).

The catalytic triad (S133-H247-D194) is present in Fae, and the active-site consisting of a lid and a loop, provides plasticity to the substrate-binding site. The lid, which is mostly made up of polar residues and a unique N-glycosylation site, stabilises the enzyme in an open conformation and gives it the typical esterase property. Fae are structurally related to lipases, however, a minor amino acid and conformational modifications have provided this protein with new enzymatic capabilities, indicating a functional convergence of Fae family during evolution (Hermoso et al., 2004).

Crepin et al. (2003) showed that Fae enzyme activities were regulated by type of the primary substrate which determines the extent of substrate breakdown. Ester linkage between polysaccharide and phenolic acids are found to be mimicked by synthetic substrates. The four conventional synthetic substrates used for functional characterization of feruloyl esterases are methyl p-coumaric acid (MpCA), methyl caffeic acid (MCA), methyl ferulic acid (MFA) and methyl sinapic acid (MSA; Puchart et al., 2007). Accordingly, Fae were

classified, type A to D, on the basis of the primary amino acid sequence and substrate specificity exhibited against four model substrates (Crepin et al., 2003). Hernandez et al. (2010) performed a phylogenetic analysis of Fae sequences and found five major clades (I–V): clade I contains the three characterized Fae from *A. niger*, *A. awamori*, and *A. tubingensis*, belonging to Fae-type A. Clade II contains sequences from both from Sordariomycetes and Eurotiomycete, but the clade does not reflect any phylogenetic structure. Clades III and IV are phylogenetically poorly resolved and contain genes that were classified as type B and type C. Clade IV includes mainly Fae type B and type C belonging to *Fusarium*, *Phaeosphaeria*, *Pyrenophora,* and *Magnaporthe* species. However, clade V is basal to I–IV and contains sequences from different taxonomic classes.

1.3.2 Industrial applications of fungal Fae

Filamentous fungi, are a well-known source of the enzymes such as cellulases and hemicellulases. Fae constitutes an interesting group of enzymes with potentially broad range of applications in the food, biofuel, pharmaceutical, and paper-pulp industries. Fae cleaves ester bonds between hydroxycinnamic acids esterified to arabinoxylans (AXs) and certain pectins present in plant cell walls. Presently, industrial enzymes are used for a wide variety of applications, with an estimated market of more than US $ 8.2 billion. In biomass degradation, Fae is an integral part of an enzyme system that acts collectively and synergistically with a variety of other cellulolytic and xylanolytic enzymes to increase the total yield of sugars. One of the implications of this is their role in improving the quality of animal feedstocks. These enzymes can be utilized in the enhancement of biomass degradation (Dilokpimol et al., 2016).

Fae from *Aspergillus spp.* have been used for biotechnology applications and in breakdown of plant polysaccharides of plant biomass. Fae type A and B are used to make phenolic components from agro-industrial waste (Benoit et al., 2006). Fae has a wide range of applications in the pharmaceutical and food industries due to its ability to hydrolyse ester-linkage between lignin and phenolic acids (Koseki et al., 2009; Hegde and Muralikrishna, 2009).

Ferulic acid (FA), product of Fae activity, has been used in a variety of industrial processes. FA also has a potential application in cosmetics due to its photoprotective quality and ability to inhibit melanin biosynthesis. Moreover, FA has an ability to block the cytotoxic enzymes such as caspases, cyclooxygenase-2, and nitric oxide synthase, leading to its potential use for development of drugs against diseases such as diabetes, cancer and neurodegeneration. FA can also stop bone deterioration in osteoporosis patients. Because of its ability to improve genetic change of phenolic constituents and digestibility of monocot cellular walls via vacuolar selection *A. niger* Fae has been employed in agriculture to release FA (Pinto, 2015).

1.4 Rice-blast pathosystem

Rice is a staple food crop for more than half of the world's population (Sasaki & Burr, 2000). However, due to a variety of microbial diseases, considerable rice-yield losses have been occurring. Rice blast disease caused by *Magnaporthe oryzae* is one of the most serious challenges to rice production (Zhang et al., 2016). Owing to the pathosystem's scientific and economic importance, extensive research has been conducted to understand the cellular and molecular basis of rice blast disease and resistance (Ebbole, 2007, Liu et al., 2014, Wilson & Talbot, 2009).

Rice blast disease has expanded throughout Sub-Saharan Africa, where demand for rice has risen rapidly in recent years (Kihoro et al., 2013). Due to a lack of resistance and adequate fungicides, a separate lineage of *M. oryzae* that infects wheat was discovered in Brazil in 1985, and has been one of the most serious threats to wheat production (Igarashi et al., 1986). Wheat blast disease has also spread to Bangladesh in Asia, posing a severe danger to the continent's wheat production (Islam et al., 2016, Malaker et al., 2016).

Magnaporthe oryzae (also known as *Pyricularia oryzae*) is a hemi-biotrophic filamentous ascomycetes fungus that causes blast disease in rice and other economically important cereals like wheat, barley and millet (Talbot, 2003; Wilson & Talbot, 2009; Valent and Khang, 2010; Zhang et al., 2016). *M. oryzae* was identified as a new species, distinct from the morphologically similar crabgrass-infecting *Magnaporthe grisea* (Couch & Kohn, 2002). It severely damages the rice yield worldwide. So, it is a matter of great concern to study and control this disease.

1.4.1 A model system to study host-microbe interactions

Rice blast pathosystem has emerged as a model system to study host-pathogen interactions (Ebbole, 2007; Patkar et al., 2015) due to various salient features like –

1. Availability of genome sequences of both host (rice) and the pathogen (*M. oryzae*; Dean et al., 2005).
2. Well-established methods to study infection related development under *in vitro* conditions.
3. Well-developed transformation and genetic manipulation methods.
4. Availability of selectable markers.
5. Availability of genomic and cDNA libraries.
6. Advancement due to CRISPR/Cas9 technology for *M. oryzae*, allows better gene targeting efficiency (Arazoe et al., 2015).
7. Understanding host-pathogen interactions using live-cell imaging by fluorescently labelled fungal and plant proteins (Giraldo & Valent, 2013).

As a result, the *M. oryzae*-rice interaction is a good genetic system for understanding more about the molecular basis of fungal cell development and pathogenicity (Wilson & Talbot, 2009). Importantly, *M. oryzae*'s infection process shares some similarities with those of other major plant infections, such as appressorium development and tissue invasion (Perez-Nadales et al., 2014), which could help the researchers in identifying common disease determinants that might be targeted for disease control.

1.4.2 *Magnaporthe oryzae* infection cycle and host invasion strategies

M. oryzae is a haploid organism that reproduces predominantly by asexual reproduction. Infection cycle of *M. oryzae* (Fig. 1.5; Skamnioti and Gurr, 2009) starts upon landing of a three-celled asexual spore, called conidium, via wind and/or water splashes, on the plant leaf surface. Conidium adheres to the host-surface via mucilage secreted from the spore tip. The tapering end of the conidium begin to germinate under high humidity conditions. Two hydrophobin proteins, Mpg1 and Mph1, are involved in host-surface sensing, condium adherence and also cutinase action required to overcome a tough leaf cuticle barrier (Pham et al., 2016, Talbot et al., 1993, Giraldo et al., 2013). Further, elongation and swelling of the germ tube occurs along the substratum, forming a hook at its tip. Hooking is a surface recognition characteristic that occurs prior to a morphogenetic change, wherein germ tube's growth shifts from polarised to isotropic, resulting in the formation of a dome-shaped infection structure called appressorium (Mitchell & Dean, 1995). However, specific pre-existing host cues such as hard hydrophobic surface, high humidity and nutrient-limiting conditions are required for appressorium formation.

Appressorium accumulates a high concentrations of compatible solutes like glycerol (upto 3 M) which generates enormous turgor pressure within the cell. Melanin present in the cell-wall of appressorium provides rigidity and strength to withstand high pressure. It has been shown that mutants defective in melanin production are able to form appressorium but are less virulent or non-pathogenic. During appressorium maturation, the conidium undergoes a programmed cell-death by autophagy, transferring its content including high concentrations of lipids and other compatible solutes and one of the terminal nuclei into the appressorium. The turgor pressure (up to 8 MPa) within the melanized appressorium will soon be converted to a mechanical force pushing a fine penetration peg through the rice

surface (Howard et al., 1991; Howard and Valent, 1996; de Jong et al., 1997). The penetration peg, again changing to a polarised growth, develops into a tubular primary hypha, which further give rise to bulbous invasive hyphae.

For a prolonged period, invasive hyphae grow inside living plant cells in a biotrophic manner, without causing any apparent damage to host tissues (Kankanala et al., 2007). Inside the host plant, fungus comes into contact with a well-developed, multi-layered plant immune system. To support its growth, the fungus uses a variety of tactics to escape and suppress host immunity (Mosquera et al., 2009). The fungal hyphae spreads from one plant cell to another probably via plasmodesmata and colonizes the host tissue by around 72 hours after infection. Then after, disease lesions appear on the leaf surface when the fungus undergoes a necrotic development, killing the host cells. *M. oryzae* is categorized as a hemibiotrophic pathogen, which means it grows parasitically in living host tissue for a period of time before killing it (Kankanala et al., 2007). This gives rise to a typical spindle-shaped lesions with grey centre and brown margin on the plant leaf surface. A single leaf lesion carrying multiple conidiophores can discharge up to 20,000 conidia (asexual spores) per night for up to 20 days. Epidemic disease is caused by the vast numbers of conidia emanating from disease lesions on rice; the disease is polycyclic, with a spore-to-spore cycling time of 7 days. Wind and dewdrop splash distribute the asexual spores produced by the lesions quickly to nearby plants (Shi et al., 1998).

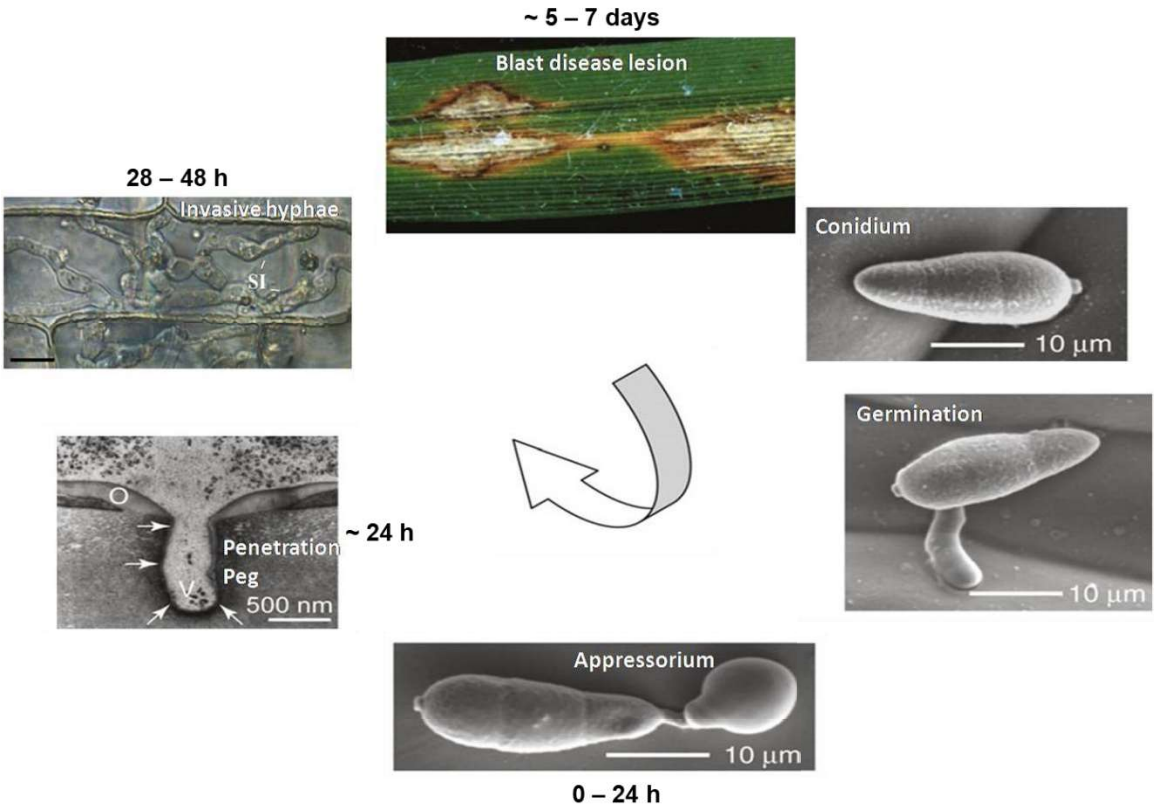

Figure 1.5. Infection (asexual) cycle of *Magnaporthe oryzae*, showing different pathogenic stages of the fungal development (in a clockwise direction shown by an arrow) starting with three-celled asexual spore, condium landing on the host-surface, finally leading to development of typical disease lesions. Disease cycle gets completed in 5-7 days of infection (modified from Skamnioti and Gurr, 2009).

In addition to the mechanical pressure-mediated fungal penetration, various CWDEs produced by *Magnaporthe* are also shown to play an important role in breaching and overcoming host physical barriers such as leaf cuticle and cell wall. CWDEs such as endo-xylanases and cellulases are required for pathogenicity in *M. oryzae* (Nguyen et al., 2011; Vu et al., 2012). Moreover, a cutinase (Cut2) mediates formation of penetration peg and is required for full virulence in *M. oryzae* (Skamnioti and Gurr, 2007). Moreover, during fungal attack, plant generate ROS (reactive oxygen species) near the infection sites, while for successful infection, fungus must overcome this plant basal defenses. In *M. oryzae SSD1*

gene, a regulator of cell wall biogenesis, is found to circumvent the induction of plant basal defense responses and is therefore required for successful infection (Tanaka et al., 2007). Importantly, *M. oryzae* also generate ROS production via NADPH oxidases (Nox1 and Nox2), which are required for appressorium mediated penetration and successful infection (Egan et al., 2007). Furthermore, *M. oryzae* metallothionein-like protein Mmt1 acts as a strong antioxidant and likely plays a role in oxidative cross-linking of the fungal cell wall during infection. Loss of function of Mmt1 in *M. oryzae* leads to impaired penetration and makes the fungus non-pathogenic (Galhano and Talbot, 2011). Another fungal cell wall integrity maintaining protein, catalase B in *M. oryzae* is also required during plant invasion. Pathogenicity of *catB*Δ is severely reduced upon exposure to ROS (Skamnioti et al., 2007). Importantly, nitronate monooxygenase (Nmo2) in *M. oryzae* enable host-colonization by protecting the fungus from nitrooxidative stress during nitrate metabolism and also prevents accumulation of host-ROS (by an unknown mechanism) and thereby maintains redox homeostasis (Marroquin-Guzman et al., 2017).

During biotrophic phase of rice-*Magnaporthe* interaction, for successful host colonization, fungus evades and suppress the plant immunity. Post-penetration, fungus forms invasive hyphae leading to disease progression in susceptible (compatible) rice cultivars, as oppose to resistant (incompatible) cultivars, wherein plant's resistance (R) proteins recognize fungal avirulence (AVR) effectors and induces hypersensitive response and thereby blocks the disease development (Mosquera et al., 2009). Upon entry into the host, fungal invasive hyphae get surrounded by a layer of plant-derived membrane called the extra invasive hyphal membrane (EIHM). Interestingly, a plasma membrane-bound rice immune receptor OsCERK1 involved in chitin-triggered plant immunity, seems to be excluded from EIHM (Kouzai et al., 2014). *M. oryzae* secretes effector proteins to suppress plant innate immunity.

Cytoplasmic effectors (e.g., Pwl2, Bas1) in *M. oryzae* are accumulated and translocated into the plant cells via a plant membrane-rich biotrophic interfacial complex (BIC; Valent and Khang, 2010), while apoplastic effectors (e.g., Slp1, Bas4) are secreted into EIHM matrix via conventional ER-golgi pathway (Fig. 1.6; Giraldo et al., 2013; Wilson, 2021). *M. oryzae* effector Avr-Pii restricts ROS production in rice by directly inhibiting NADP-malic enzyme activity (Singh et al., 2016). Slp1 (LysM protein) is a well-known apoplastic effector in *M. oryzae* which sequesters free chitin oligosaccharides and accumulates it in the apoplastic region between fungal cell wall and rice plasma membrane. Slp1 thus, prevent free chitin to bind CEBiP, a chitin sensor in rice cells and thereby, suppress chitin-triggered plant immune response (Mentlak et al., 2012). AvrPiz-t, a BIC-dependent cytoplasmic effector, suppresses host ROS and expression of defense related genes, by directly interacting with rice ubiquitin ligase E3, APIP6 (Park et al., 2012).

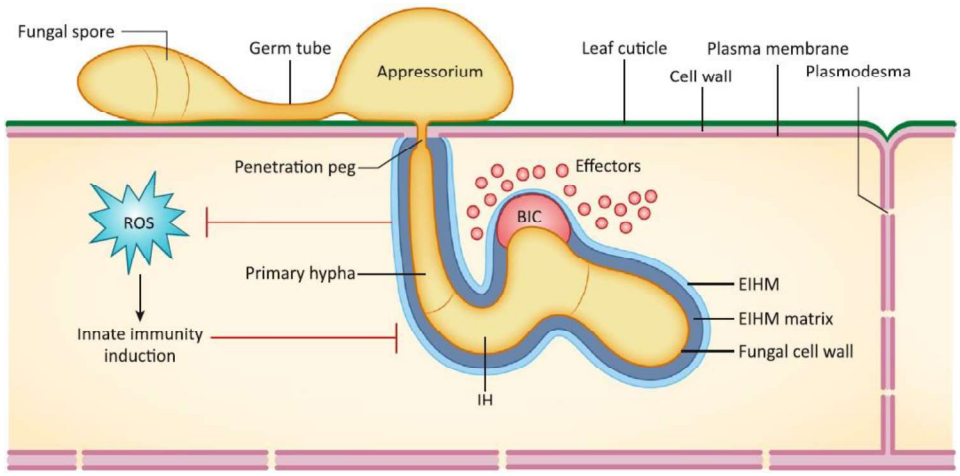

Figure 1.6. Schematic representation of biotrophic host invasion by *M. oryzae*. T-bars indicate inhibition of host ROS response or fungal biotrophic growth; IH- invasive hyphae, BIC- biotrophic interfacial complex, EIHM- extra-invasive hyphal membrane (adapted from Wilson, 2021).

Interestingly, in addition to secreted protein effectors, *M. oryzae* also produces many plant hormones or hormone-mimicking compounds to evade host immune responses. Fungal pathogens target plant hormones such as salicylic acid, jasmonic acid or ethylene, that are involved in host defense response or regulates growth hormones such as indole 3-acetic acid (IAA, auxin), abscisic acid, cytokinin or gibberellin (Patkar and Naqvi, 2017). For instance, *M. oryzae* cytokinin-deficient mutants (*CKS1*), are unable to suppress expression of defense genes and ROS burst in rice. Moreover, fungal cytokinin is also found to induce relocation of host nutrients (such as sugars and amino acids) at the infection site (Chanclud et al., 2016). Furthermore, fungal IAA positively regulates rice IAA, which in turn induce expression of expansins in rice, protein that is associated with plant cell wall loosening and thereby making plant more susceptible to fungal-infection (Tanaka et al., 2011). *M. oryzae* also produces abscisic acid that plays an important role in host invasion and pathogenicity, likely by suppressing plant immunity (Spence et al., 2015).

Intriguingly, as shown by Patkar et al., 2015, a phytohormone analog secreted by *M. oryzae* plays a crucial role in suppressing rice immunity. Fungal antibiotic biosynthesis monooxygenase (Abm) secreted by *M. oryzae*, evade host defense and enables host colonization. Jasmonic acid (JA) signalling is involved in plant growth and immunity. However, *M. oryzae* Abm converts endogenous as well as plant jasmonic acid (JA) to 12-hydroxyjasmonic acid (12OH-JA), a phytohormone-mimic, which prevents accumulation of methyl-JA in plants and thereby perturbing JA-mediated plant defense during fungal invasion. Interestingly, fungus secretes endogenous 12OH-JA before the host penetration, acting as a chemical-effector enabling successful host invasion, while Abm, serves as a peptide effector, secreted during post-penetration stage, facilitating subsequent host colonization (Fig. 1.7; Patkar et al., 2015; Patkar and Naqvi, 2017).

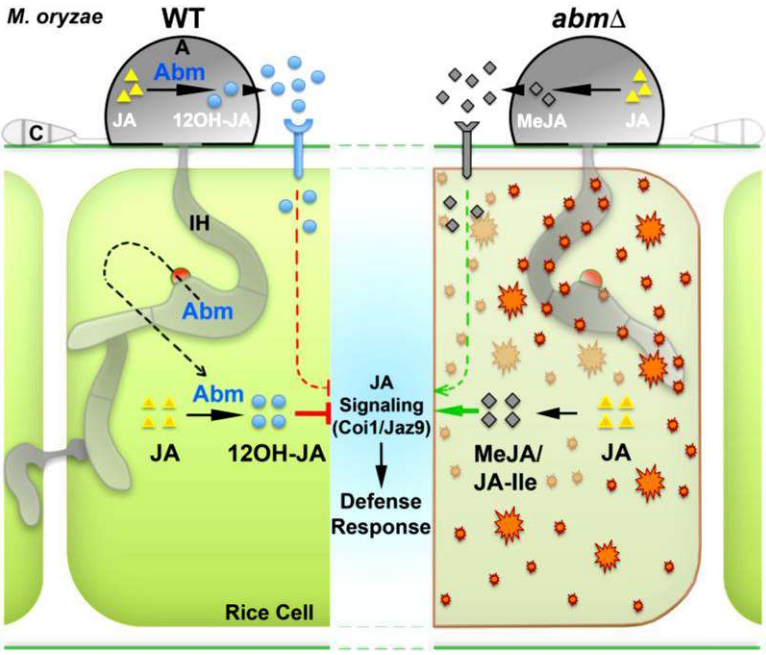

Figure 1.7. Schematic representation of *M. oryzae* Abm-mediated disabling of JA-defense signalling in rice. Brownish orange inclusions depict the sites of methyl JA-induced innate immunity that blocks the *abm*Δ strain of *M. oryzae* in the first invaded rice cell. A, appressorium; C, conidium; IH, invasive hypha; JA-Ile, isoleucine conjugate of JA; Jaz9, jasmonate-ZIM domain repressor protein 9; MeJA, methyl JA; WT, wild-type *M. oryzae*. Hypothetical receptors or transporters for the fungal JA derivatives have been depicted on the host cell surface (adapted from Patkar and Naqvi, 2017).

During fungal infection, especially tissue colonization via plant plasmodesmata, plant defense response closes intercellular plasmodesmata channel by callose deposition (Faulkner et al., 2013; Cheval and Faulkner, 2018). In *Magnaporthe*-rice interaction, mitogen-activated protein kinase (MAPK) signalling plays a key role in appressorium morphogenesis and regulate mechanical and enzymatic penetration of host cells, whereas plant MAPK signalling is responsible for immune response (Hamel et al., 2012). A key MAPK in *M. oryzae*, Pmk1 is found to play an important role in suppression of host ROS and callose deposition. Importantly, Pmk1 MAPK regulates invasive hyphal morphogenesis and promote plasmodesmal cell-to-cell spread, independent of host defense suppression.

Additionally, Pmk1 facilitates fungal adaptation to nutrient availability during biotrophic growth by regulating expression of sugar transporters and carbohydrate degrading enzymes (Sakulkoo et al., 2018).

Overall, this chapter summarizes various studies highlighting different mechanisms underlying the plant defense responses and pathogen's ability to counteract such defenses by employing variety of effector molecules and hydrolytic enzymes, shaping an arms race between the host and fungal pathogen.