2. Review of Literature

2.1. Taxonomy of *Bipolaris oryzae*

The genus ‘*Helminthosporium*’ formerly comprised a large, heterogenous group of fungi with dark phragmosporous conidia. Segregation of the genera *Bipolaris, Curvularia, Drechslera* and *Exserohilum* from the genus ‘*Helminthosporium*’ has not been accepted universally (Alcorn, 1988). Taxonomic classification of the genus ‘*Helminthosporium*’ is based on the teleomorph, morphological observations of conidia, mode of conidiophores and conidia formation (Sivanesan, 1987). These graminicolous species, originally described as ‘*Helminthosporium*’ were subsequently transferred to the genera *Cochliobolus* (anamorph *Bipolaris*), *Pseudocochliobolus* (anamorph *Curvularia*) (Tsuda et al., 1977), *Pyrenophora* (anamorph *Drechslera*) (Sivanesan, 1987).

The shape and septation of the ascospores produced during the sexual stage of the fungi has been used to distinguish among these genera. The ascospores of *Cochliobolus* are long, slender, coiled and transversely septate in contrast to the ascospore of *Setosphaeria*, that are shorter, fewer (usually 3) with transverse septation. Ascospores of *Pyrenophora* are characterized by both longitudinal and transverse septa, whereas those of *Pseudocochliobolus* are morphologically less defined in which the ascospores are loosely coiled (Berbee et al., 1999).

Conidial morphology forms the principal criteria for the separation of species within the genera *Bipolaris* and *Curvularia* (Alcorn, 1983; Sivanesan, 1987). *Bipolaris* and *Curvularia* are morphologically very similar and cannot be easily distinguished by taxonomic criteria (Sivanesan, 1987). However, these two genera have a few morphological differences such as septal structure; conidia of *Curvularia* species are euseptate (Ellis, 1971) and those of *Bipolaris* are disto-septate. Tsuda and Ueyema (1987) observed that variations do occur in the conidial morphology due to changes in the environmental conditions and thus conidial morphology is no longer a strong criteria to be considered for the taxonomic classification of fungal species.

*Helminthosporium* species with cylindrical conidia exhibiting amphigenous conidial germination were defined as *Drechslera* (Ito, 1930). Genus *Drechslera* was
redefined as *Bipolaris* based on the fusoid spores with bipolar germination with slightly protruding hilum (Shoemaker, 1959). Species germinating in bipolar fashion with protruding hilum at the base of the conidium were included in genus ‘*Exserohilum*’ (Leonard and Suggs, 1974). The taxonomy of *B. oryzae* is confusing as frequent nomenclatural changes and refinements have occurred based on conidial characters. The name *Helminthosporium oryzae* was changed to *Bipolaris oryzae* because of the bipolar conidial germination (Shoemaker, 1959). The fungus was described as *Drechslera oryzae* based on the amphigenous conidial germination (Subaramanian and Jain, 1966).

**Identification and detection of *Bipolaris oryzae***

The ‘brown spot’ agent in rice (*Oryza sativa* L.) is *Bipolaris oryzae* (Breda de Haan) Shoemaker, 1959 [syns. *Drechslera oryzae* (Breda de Haan) Subramanian and Jain, 1966 and *Helminthosporium oryzae* (Breda de Haan). The teleomorph of this species is *Cochliobolus miyabeanus* (Ito and Kuribayshi, 1942). Presently, the only reliable method to identify *B. oryzae* is to observe them on incubated seeds subjected to standard blotter test (ISTA, 2005). The habit characters of the fungi on the seed surface are observed using a stereo-binocular microscope and are confirmed based on the conidia characters under the compound microscope.

**Symptom based identification of *B. oryzae***

Brown spot disease caused by *B. oryzae* and blast disease caused by *Pyricularia oryzae* (teleomorph *Magnaporthe oryzae*) are the two major diseases of rice reported in all rice growing regions of the world. Conventional method of identification of brown spot of rice involves analysis of typical lesion on leaves, on nodes, on different parts of the panicle and on the rice grains. Disease symptoms produced on rice by these two fungi are often non-discriminating and frequently mistaken by farmers. Thus, field diagnosis of *B. oryzae* is difficult.

**Culture based identification of *B. oryzae***

Fungal plant pathogens are commonly identified by culture based morphological approaches. Morphological characters are unstable and can vary with culture conditions and overlap of phenotypes. Conventional methods of identification
of brown spot of rice involve analysis of typical symptoms on infected leaves and seeds. In rice, *B. oryzae* can be recovered from both disease and asymptomatic or apparently healthy seeds. Screening of seed samples through standard blotter test and pathogenicity tests have been used for routine detection of *B. oryzae* in rice seeds. Morphology based identification is difficult to interpret, slow, labor-intensive and require skilled personnel. Accurate diagnosis is not easy because rice seeds are prone to infection by a large number of other fungal species, thus complicating pathogen identification. Some isolates of *B. oryzae* sporulate sparsely or produce only mycelia (mycelia type) under laboratory conditions. Thus, identification based on phenotypic characters is impossible when this occurs and has led to false diagnosis. Identification based on genotypic characters would be more advantageous and potentially more accurate, reproducible, simple and rapid. Therefore, it is desirable to develop a reliable, rapid and sensitive diagnostic method for detecting seed borne infection of *B. oryzae* in rice.

Conventional identification of *B. oryzae* based on the above two criteria are not always reliable as they are often time consuming, laborious, require extensive taxonomical knowledge and delay timely disease management decisions (Leivens and Thomma, 2005). There is currently no reliable method available for detecting *B. oryzae* in rice. For this reason, the availability of fast, sensitive and accurate methods for detection and identification of *B. oryzae* is increasingly necessary to improve disease control decision making. These limitations have led to the development of molecular approaches with improved accuracy and reliability.

In the last two decades, molecular approaches have revolutionized the detection, identification and quantification of plant pathogens. The polymerase chain reaction (PCR) assay is one approach that allows detection of extremely small quantities of specific DNA in complex environments. The utility of PCR as a specific and sensitive assay for identification of plant pathogens is well documented (Henson and French, 1993). The PCR is highly sensitive and reproducible for amplification of diagnostic molecular markers and could easily be used for identification and detection if species-specific primers are available.

Development of specific PCR primers will aid to confirm the identification and measure *B. oryzae* levels on seed stocks, especially in seed health evaluations. It
can also be applied to test seed consignment at quarantine stations, plant breeders and therefore to make decisions regarding disease prevention or control strategies such as avoiding planting susceptible cultivars in fields with a high risk of disease caused by seed borne \textit{B. oryzae}.

2.2 Polymerase Chain Reaction (PCR) in Agriculture and Plant Pathology

PCR is the most important and sensitive technique for the detection of a wide range of plant pathogens (White \textit{et al.}, 1990; Zhang \textit{et al.}, 2005a, b). PCR allows the use of specific oligonucleotides to provide millions of copies of the target gene (Mullis and Faloona, 1987). PCR overcomes the limitations of conventional method of identification by being much faster, more specific, more sensitive and more accurate.

PCR is commonly used in the field of Agriculture and Plant pathology due to the following reasons:

- Easy to perform and requires no taxonomical expertise
- Allows detection and identification on non-culturable microorganisms
- Method is sensitive and can detect minute quantity of pathogen DNA (Lee and Taylor, 1990)
- Early detection of disease, before symptom is visible
- Specificity of the reaction can distinguish closely related organisms at different taxonomic levels
- Accurate detection of pathogen using target specific primers aids in understanding the biology of the plant pathogen, pathogen population structure and dynamics, host-pathogen interactions, gene flow in pathogen population and inoculum movement (McCartney \textit{et al.}, 2003)

**Gene targets in PCR diagnostics and phylogenetics of fungal plant pathogens**

Generally, conserved genes with enough sequence variation are selected for designing PCR assays and performing phylogenetic analysis. The most common DNA region targeted for these purposes is ribosomal DNA (rDNA). These gene sequences are arranged as tandemly repeated clusters in the genome. They contain the
transcribed segments namely 18S, 5.8S and 28S rRNA’s, which are separated by two internal transcribed spacer segments namely Internal Transcribed Spacer 1 and Internal Transcribed Spacer 2 (ITS1 and ITS2) (Bruns et al., 1991). The rRNA genes (18S, 5.8S and 28S) contain highly conserved sequences adequate for genus- and species-consensus primer designing. The ITS regions (ITS1 and ITS2) flanked on either side of the smaller 5.8S gene, forms highly variable region in the fungal genome (White et al., 1990). The low rate of intra-specific polymorphism and high inter-specific variability in the rDNA transcription unit allows characterization of the rDNA of each species and makes this DNA useful for inter-specific comparison (Martin and Rygiewicz, 2005).

Traditionally, molecular identification of plant pathogenic fungi is accomplished by PCR amplification of ITS regions, followed by restriction analysis (Duran et al., 2009) or by using amplified DNA as probe (Ward and Gray, 1992) or by direct sequencing and BLAST searching against GenBank or other databases (White et al., 1990). ITS region is relatively short (500-800 bp) and can be easily amplified by PCR using universal single primer pairs that are complementary to conserved regions within the rDNA subunit genes (White et al., 1990). Following sequence analysis it is possible to design taxon-specific PCR primers by targeting DNA sequences that are unique to the organism(s) in question.

During the last 15 years, nuclear rDNA, particularly the ITS region have been targeted and are widely used as a genetic marker in primer designing and for exploring functional diversity (Bruns et al., 1991) in a number of fungal species (Cullen et al., 2002; Nilsson et al., 2005; Abd-Elsalam et al., 2006). Each coding region of rDNA repeat shows distinct evolution rates and is in routine use to study the taxonomical relationship between fungal species and also between different genera (Manamgoda et al., 2011; Manamgoda et al., 2012).

Oligonucleotide specific primers/probes targeting the ITS region have been demonstrated to selectively detect several agriculturally important fungal genera like Verticillium (Nazar et al., 1991), Phytophthora (Lee et al., 1993), Fusarium (O’Donnell, 1992; Schilling et al., 1996; Edel et al., 2000; Dass et al., 2010), Alternaria (Iacomi-Vasilescu et al., 2002; Konstantinova et al., 2002; Pavon et al., 2012), Botrytis (Chilvers et al., 2007), Colletotrichum (Tapai-Tussell et al., 2008).
Sequences of the rDNA genes, in particular the spacer regions ITS1 and ITS2 surrounding the 5.8s gene, have been used to develop species-specific primers for the detection of phytopathogenic fungi namely *Rhynchosporium secalis* (Lee et al., 2001), *Colletotrichum coccodes* (Cullen et al., 2002), *Fusarium oxysporum* f. sp. *vasinfectum* (Moricca et al., 1998; Abd-Elsalam et al., 2006), *Alternaria brassicae* (Guillemette et al., 2004), *Macrophomina phaseolina* (Babu et al., 2007), *Colletotrichum capsici* (Torres-Calzada et al., 2011).

Ribosomal DNA-ITS region is also used in the characterization of several fungal isolates. Photita *et al.* (2005) characterized *Colletotrichum* species using primer amplifying the rDNA-ITS region. Almeida *et al.* (2008) used ITS region to characterize powdery mildew strains isolated from soybean, bean, sunflower and weeds. Similarly, based on the ITS sequences, McKay *et al.* (2009) characterized *Colletotrichum acutatum*, the causal agent of Anthracnose of Almond.

**Design of species-specific primers**

PCR methods are based on the use of specific oligonucleotides/primers that specifically hybridize with the DNA target and that are required to initiate the synthesis of the new DNA chain. The first step for designing primers consists of the alignment of the sequences of interest by the BLAST program (Altschul *et al*., 1997) using sequences of many fungal genes available at the National Center for Biotechnology Information (NCBI).

Consensus sequences are used to design primers for detection of members of same genus or species. Cullen *et al.* (2002) designed genus-specific primers to detect *Colletotrichum* species based on the conserved sequences of ITS1 and ITS2 regions. Species-specific primers are designed by amplification of rDNA sequences of fungal isolates using universal ITS1 and ITS4 primers (White *et al*., 1990), followed by sequencing and alignment of such sequences with previous EMBL and GenBank DNA database sequences (Moricca *et al*., 1998; Cullen *et al*., 2002; Udayashankar *et al*., 2012).

Sequences are aligned and analyzed for divergences using the software Clustal v package (Higgins *et al*., 1992), BLAST program (Altschul *et al*., 1997) or BioEdit program (Altschul *et al*., 1990). Based on the uniqueness of the target sequence,
several primers can be designed manually or using primer 3. v.0.4.0. version software (Rozen and Skaletsky, 2000). Before synthesis, specificity of the designed primer(s) is confirmed using BLAST with DNA database sequences of other plant pathogenic fungi and bacteria.

**Primer specificity and sensitivity**

Specificity of the designed primers is confirmed by heterogeneity in the sequence alignment. Forward primer designed at the ITS1 region revealed a mismatch of 3 nucleotides between *Fusarium oxysporum* f. sp. *vasinfectum* and *F. sambucinum* (Moricca et al., 1998).

Specific primers (ITS-Fu-f and ITS-Fu-r) designed for the detection of *Fusarium* genus were tested for non-specific amplification with other fungi associated with cotton such as *Rhizoctonia solani* and *Macrophomina phaseolina*. PCR showed no cross-reaction with DNA from other fungal isolates, indicating the specificity of the primers to detect only *Fusarium* species (Abd-Elsalam, 2003).

**rDNA-ITS in fungal phylogeny**

Analysis of rDNA sequences has become a common tool in modern systematic and has been used to establish molecular phylogenetic relationships within many groups of fungi (White et al., 1990). Analysis of the ITS1, ITS2 and 5.8S rDNA sequence has been used to describe family-genus relationship with the *Pleosporaceae*, and has revealed a close phylogenetic relationship between *Helminthosporium*, *Bipolaris* and *Curvularia* (teleomorph *Cochliobolus*), *Drechslera* (teleomorph *Pyrenophora*) and *Exserohilum* (teleomorph *Setosphaeria*) (Berbee et al., 1999; Olivier et al., 2000; Zhang and Berbee, 2001). Analysis of ITS1, ITS2 and 5.8S rDNA, clustered 49 fungal isolates into 3 clades in the phylogeny tree, namely, *Drechslera* (*Pyrenophora*), *Exserohilum* (*Setosphaeria*), *Curvularia/Bipolaris* (*Cochliobolus*). The *Curvularia/Bipolaris* (*Cochliobolus*) clade is further divided into two subclades, viz., *Cochliobolus* Group 1 subclade with only *Bipolaris* isolates and *Cochliobolus* Group 2 subclade with intermingled isolates of *Bipolaris* and *Curvularia* (Paz et al., 2006).
Bipolaris oryzae isolates from different rice field of Philippines exhibited different patterns of conidial and clustered with B. oryzae (GenBank X78122). The clustering of these isolates in Bipolaris (Cochliobolus) Group 1 subclade indicates all are B. oryzae, irrespective of whether the culture originated from a condition with a bipolar, intercalary or monopolar germination type. New ITS sequences (not previously analyzed) in the dendrogram revealed unexpected relationship of fungal isolates with their respective teleomorphs. Drechslera brizae is a synonym of B. oryzae with Cochliobolus as its teleomorph [Index Fungorum (http://www.indexfungorum.org)]. However, Paz et al. (2006) suggest D. brizae as the correct name, due to its clustering with Pyrenophora. Similarly, Curvularia heteropogonicola is a synonym of Exserohilum heteropogonicola with Setosphaeria as its teleomorph [Index Fungorum (http://www.indexfungorum.org)]. Clustering with Cochliobolus indicates Curvularia heteropogonicola as the correct name. Bipolaris tetramera (not previously analyzed) is described as Cochliobolus spicifera in Index Fungorum. It is consistent with the clustering of B. tetramera with B. spicifera, which has also Cochliobolus spicifera as its teleomorph (Paz et al., 2006).

Other genes in fungal detection and phylogeny

Currently, housekeeping genes with higher variability are being extensively used as targets for diagnostics and phylogenetic analysis of fungi. They include nuclear genes such as β-tubulin (McKay et al., 1999), translation elongation factor 1α (TEF1α) (Kristensen et al., 2005), mitochondrial smaller subunit (mtSSU) (Pryor and Gilbertson, 2000; Stewart et al., 2006), calmodulin (Mule et al., 2004), avirulence genes (Lievens et al., 2009), glyceraldehydes-3-phosphate dehydrogenase (gpd) (Berbee et al., 1999), Bnr1 gene (Shimizu et al., 1998), cytb (Chung et al., 2008), polyketide synthase (PKS) (Baird et al., 2008), xylanase (Emami and Hack, 2002), toxin genes such as FUM1 (Bezuidenhout et al., 2006) and AM-toxin gene (Johnson et al., 2000).

Kristensen et al. (2005) and Stewart et al. (2006) performed sequence of the TEF1alpha and mtSSU genes to study the diversity of Fusarium species allowing differentiation of virulent and avirulent species. Zhang and Steffenson (2001) characterized Cochliobolus sativus based on mating type (MAT) genes. MAT genes provide significantly better resolution among Colletotrichum species complex (Du et
al., 2005) and Gibberella fujikuroi species complex (Steenkamp et al., 2000). Molecular phylogeny analysis of Brn1 reductase melanin biosynthesis gene sequences revealed the separation of Bipolaris into two major clades with respect to Pseudocochliobolous and Cochliobolus (Shimizu et al., 1998).

To enhance the specificity of diagnostic assay, a combination of multiple diagnostic regions is recommended. Many authors have followed this multi-locus diagnostic strategy, eg. ITS and gpd genes provide sufficient phylogenetic information for the separation of Cochliobolus species into two groups viz., Cochliobolus and Pseudodo Cochliobolus (Berbee et al., 1999). The multi-locus phylogenetic tree generated based on combined genes of ITS, gpd, LSU, EF1alpha sequence data resolved two well-supported group (Bipolaris and Curvularia) in Cochliobolus complex. The study also revealed that Bipolaris and Curvularia cannot be combined into a single monophyletic genus (Berbee et al., 1999; Goh 1998; Shimizu et al., 1998).

**Conventional and Real-time PCR assays**

Seed-borne pathogens present a serious threat to seedling establishment. Close association with seeds facilitates the long-term survival, introduction into new areas and widespread dissemination of pathogens. Under greenhouse conditions, the risks of significant economic losses due to diseases are great because factors including high temperatures and overhead irrigation promote explosive plant disease development. Under these conditions, the most effective disease management strategy is exclusion which is accomplished by using healthy seed lots before planting.

Testing seeds for plant pathogens is a difficult task, because infested seeds may be asymptomatic making visual detection impossible. Apart from this, the inoculum level on seeds may be low or unevenly distributed within a lot. Assays such as visual examination, selective media, seedling grow-out tests and serological techniques are used for detection of different seedborne pathogens.

An ideal seed health testing method should be sensitive, specific, rapid, robust, inexpensive and simple to implement and interpret. One such assay is Polymerase Chain Reaction (PCR). Because of its great potential, over the past 20 years many
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PCR based assays have been reported for seedborne fungal pathogens (Moricca et al., 1998; Zhang et al., 1999; Udayashankar et al., 2012).

**Specificity and Sensitivity of PCR assays**

The designed primer sets to target pathogen are tested for amplification of the predicted size fragments and for its specificity at different annealing temperatures (Lee et al., 2001; Abd-Elsalam et al., 2006). To reveal the specificity of the designed primers, they are used to amplify DNA from isolates of the target fungal species from a range of geographical origins and from different host cultivars. DNA from isolates of contaminants isolated from host seeds, taxonomically related species and microbes from the same ecological niche are used to validate primer specificity (Johanson et al., 1998; Lee et al., 2001; Abd-Elsalam et al., 2006; Udayashankar et al., 2012).

Based on their performances or efficiency, the primers are further tested using real-time PCR. The *Fusarium oxysporum* f. sp. *vasinfectum* specific primers, passed the specificity test in both conventional and real time PCR using SYBR Green dye, thus eliminating further specificity test using *TaqMan* Probes (Abd-Elsalam et al., 2006). In contrast, the conventional PCR primers developed for specific detection of *Rhizoctonia solani* (Johanson et al., 1998) were not suitable for use in real-time PCR, because of too long amplicon size (550 to 1200 bp). This led to the design of real-time PCR primers for specific detection of *R. solani* in rice tissues (Sayler and Yang, 2007), that had an amplicon size of 137 to 140 bp compared to the previous primers developed by Johanson et al. (1998).

The real-time PCR primers detected sheath blight pathogen-*Rhizoctonia solani* in infected, symptomatic and asymptomatic rice tissues. These primers did not amplify DNA from sheath rot pathogen – *Rhizoctonia oryzae* or the sheath spot pathogen – *Rhizoctonia oryzae-sativa*, providing evidence for their specificity (Sayler and Yang, 2007). The early, rapid and reliable detection and quantification of *Rhizoctonia solani* in infested rice tissues by real-time PCR may provide breeders and other researchers with an unbiased method for monitoring fungal growth in rice plants. It will also improve the timing and implementation of control measures for the disease, especially fungicide application (Sayler and Yang, 2007). Similarly, real time PCR using SYBR Green dye enabled the quantification of fungal genomic DNA from
known amounts of propagules of *Fusarium solani* f. sp. *phaseoli* in both sterile and non-sterile substrates (Filion *et al*., 2003).

Despite the potential improvements over conventional assays, PCR-based seed assays have not been widely adopted by commercial and government testing agencies in the US. This is due to the presence of PCR inhibitors (*eg*., tannins, phenolic compounds) in the seed extracts. However, these compounds can be eliminated by the use of chloroform and phenols during the seed extraction procedure. Use of such chemicals is laborious and harmful, leading to reduced sensitivity and efficiency of the assay. This led to the development of an advanced rapid-cycler real-time PCR that is currently considered the gold standard method for detection of plant pathogens.

Real-time PCR eliminates the risk of cross-contamination and the need for post-PCR steps such as gel electrophoresis. The technique allows the monitoring of the reaction during the amplification process by the use of fluorescent signal that increases proportionally to the number of amplicons generated and to the number of targets present in the sample. The method, with its improved sensitivity and specificity allows the accurate quantification of the target pathogen, by interpolating the quantity measured to a standard curve with known amounts of target copies. This quantification characteristic is very useful in phytopathology in order to correlate the amount of fungus in a biological sample with the disease state, or to monitor the progress of the disease in an infected plant (Garrido *et al*., 2009). To date, real-time PCR detection of seed borne pathogens in seeds has been reported for several fungal pathogens (Table 2.1).

The detection limit of the designed primers varies with the kind of PCR employed. *Alternaria helianthi* specific primers designed by Udayashankar *et al*. (2012) detected 10 pg of genomic DNA through conventional PCR assay. *Rhynchosporium secalis* specific primers detected 1 % scald infection in barley seeds (Lee *et al*., 2001). PCR product of the expected size was amplified from as little as 100 fg of purified *R. solani* genomic DNA. The sensitivity of real-time PCR allows the identification of 200 fg of genomic DNA. Real-time PCR assay is more sensitive than conventional PCR assay. Schilling (1996) has previously reported that 50 pg of *Fusarium culmorum* and 5 pg of *Fusarium graminearum* DNA were sufficient to yield PCR products with species-specific primers. For DNA extracted from *Fusarium*
oxysporum f. sp. vasinfectum mycelium, 100 ng to 50 ng was found to be suitable (Moricca et al., 1998). Colletotrichum coccodes DNA was detected in apparently healthy potato tubers, and the lowest concentration of target DNA was detected in peel extract from symptomless tubers at concentrations of 3.26 and 6.20 pg (Cullen et al., 2002).

Detection sensitivity is also calculated based on the infection in seed batches. The seed powder obtained from artificially infested seed batches are used to test the detection limit of the primers (Guillemette et al., 2004; Udayashankar et al., 2012). Alternaria helianthi is detected in artificially infested seed powder, mixed by A. helianthi infected seed powder with known amount of uninfected seed powder. Alternaria helianthi is consistently detected from 100 to 2 % infection. Variable results were obtained in detection of A. helianthi with 1 % seed powder samples (Udayashankar et al., 2012).

Konstantinova et al. (2002) reported sensitive detection of 1 carrot seed infected with Alternaria alternata and A. radicina in 100 carrot seeds through PCR analysis. Pryor and Gilbertson (2001) obtained positive results for detection of A. radicina for 0.1 % of infestation with carrot seeds. The TaqMan-based real-time assay was able to quantify Fusarium culmorum in root tissue from both growth-chamber and field studies down to 61 pg (Strausbaugh et al., 2005).
### Table 2.1. List of seed borne pathogens detected by real-time PCR

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium avenaceum</em></td>
<td>Cereals</td>
<td>Kulik et al., 2011</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>Cereals</td>
<td>Kulik et al., 2011</td>
</tr>
<tr>
<td><em>F. tricinctum</em></td>
<td>Cereals</td>
<td>Kulik et al., 2011</td>
</tr>
<tr>
<td><em>Pyrenophora graminea</em></td>
<td>Barley seed</td>
<td>Taylor et al., 2001</td>
</tr>
<tr>
<td><em>Macrophomina phaseolina</em></td>
<td>Chickpea, Soybean, Pigeon pea</td>
<td>Babu et al., 2011</td>
</tr>
<tr>
<td><em>F. solani f.sp. glycines</em></td>
<td>Soybean roots</td>
<td>Gao et al., 2004</td>
</tr>
<tr>
<td><em>F. culmorum</em></td>
<td>Wheat stem</td>
<td>Hogg et al., 2007</td>
</tr>
<tr>
<td><em>F. pseudograminearum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tilletia indica</em></td>
<td>Wheat</td>
<td>Frederick and Synder, 2001</td>
</tr>
<tr>
<td><em>Tilletia walker</em></td>
<td>Ryegrass</td>
<td></td>
</tr>
<tr>
<td><em>Tilletia caries</em></td>
<td>Wheat seed</td>
<td>McNeil et al., 2004</td>
</tr>
<tr>
<td><em>Botrytis species</em></td>
<td>Onion seed</td>
<td>Chilvers et al., 2007</td>
</tr>
<tr>
<td><em>Phialophora gregata</em></td>
<td>Soybean</td>
<td>Malvick and Impullitti, 2007</td>
</tr>
<tr>
<td><em>Colletotrichum coccodes</em></td>
<td>Potato</td>
<td>Cullen et al., 2002</td>
</tr>
<tr>
<td><em>F. graminearum</em></td>
<td>Wheat</td>
<td>Reischer et al., 2004</td>
</tr>
<tr>
<td><em>Alternaria brassicae</em></td>
<td>Cruciferous seeds</td>
<td>Guillemette et al, 2004</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em></td>
<td>Rice</td>
<td>Sayler and Yang, 2007</td>
</tr>
</tbody>
</table>
2.3. Genetic diversity of fungal populations

Characterization of pathogenic variation in a pathogen population is time consuming and does not provide information on the genetic relatedness of the evaluated strains (Baysal et al., 2010). Molecular methods involving use of PCR have been proposed to resolve genetic variation in various organisms (Arabi and Jawhar, 2007). Commonly used genome characterization techniques include RAPD (Random Amplified Polymorphic DNA) (Williams et al., 1990), AFLP (Amplified Fragment Length Polymorphism) (Vos et al., 1995), RFLP (Restriction Fragment Length Polymorphism) and ISSR (Inter-Simple Sequence Repeats) (Reddy et al., 2002). One of the recent and the most direct approach is SNP (Single Nucleotide Polymorphism).

RAPD (Random Amplified Polymorphic DNA)

RAPD analyses rely on PCR amplification of the pathogen genome with short arbitrary sequences (usually decamers) that are used as primers. These primers find distinct complementary sequences in the genome producing specific binding patterns. The resulting PCR fragments are then separated by electrophoresis to obtain fingerprints that may distinguish fungal species or strains (Williams et al., 1990). Some of the specific DNA fragments detected in a profile may be cut out of the gel and sequenced to obtain a SCAR (Sequence-Characterized Amplified Region), into which specific primes can be designed for a more precise PCR detection. SCAR primers have been used for instance to specifically diagnose R. solani (Nicholson and Parry, 1996), Microdochium nivale (Parry and Nicholson, 1996), Fusarium subglutinans (Zaccaro et al., 2007); to identify different lineages of Magnaportha grisea (Srinivasachary et al., 2002), to study the phylogenetic lineage of Alternaria species complex (Andrew et al., 2009) and to analyze the virulence complexity of M. grisea (Singh and Kumar, 2010).

RAPD results are also useful for the analysis of genetic diversity among fungal populations. Fingerprints are scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrices. Data are analyzed to obtain statistic co-efficient among the isolates that are then clustered to generate dendrograms. RAPDs have been used to analyze the genetic diversity among different species and races of Fusarium species (Arici and Koc, 2010) and genetic
diversity among races of *Setosphaeria turcica*. RAPD have been used to study the heterogeneity, intra- and inter-specific variability among populations from different as well as from the same geographic origin, namely *Magnaporthe grisea* (Chadha and Gopalakrishna, 2005), *Exserohilum turcicum* (Abadi *et al*., 1996), *Bipolaris victoriae* (Motlagh and Kaviani, 2008), *Bipolaris oryzae* (Kumar *et al*., 2010; Motlagh and Anvari, 2010), *Colletotrichum lindemuthianum* (Barcelos *et al*., 2011). Isolates of *B. sorokiniana* were analyzed by RAPD to determine the amount of intra-specific genetic variability and to study the host-pathogen interactions (Oliveira *et al*., 2002; Muller *et al*., 2005). Irham and Ahmad (2004) employed RAPD to correlate morphological, aggressiveness and genetic variation among isolates of *B. sorokiniana*. Isolates of *Fusarium oxysporum* f. sp. *vasinfectum* were differentiated into three main groups based on their virulence and geographic origin.

The RAPD technique is rapid, inexpensive and does not require any prior knowledge of the DNA sequence of the target organism. Results obtained from RAPD profiles are easy to interpret because they are based on amplification or non-amplification of specific DNA sequences. In addition, RAPD analyses can be carried out on large numbers of isolates without the need for abundant quantities of high-quality DNA (Nayaka *et al*., 2011). Disadvantages of this include poor reproducibility between laboratories, and the inability to differentiate non-homologous co-migrating bands. In addition, RAPDs are dominant markers, so, they cannot measure the genetic diversity affected by the number of alleles at a locus, nor differentiate homozygotes and heterozygotes individuals. RAPD was deployed to identify strains (Jawhar *et al*., 2000), to characterize races and to investigate genetic changes in phytopathogenic fungi (Malvick and Grau, 2001).

**Restriction Fragment Length Polymorphism (RFLP)**

RFLP involves restriction enzyme digestion of the pathogen DNA, followed by separation of the fragments by electrophoresis in agarose gels to detect differences in the size of DNA fragments. Polymorphisms in the restriction enzyme cleavage sites are used to distinguish fungal species. PCR-RFLP combines the amplification of a target region with the further digestion of the PCR products obtained. RFLPs have been used as a tool for detection, identification and phylogenetic studies of different fungal species (Babu *et al*., 2007; Nascimento and Sand, 2008). PCR-RFLP analysis
of the ITS region demonstrates the presence of different Anastomosis Group (AG) within isolates of *Rhizoctonia solani* and reveals heterogeneity among *Pyrenophora graminea* isolates (Arabi and Jawahar, 2007). It also allowed the differentiation of pathogenic and non-pathogenic isolates of *Fusarium oxysporum* (Apple and Gordon, 1996). RFLP has been used to study genetic variation among *Fusarium solani* isolates (Gupta *et al*., 2010), *Pyrenophora teres* (Wu *et al*., 2003), *Pyrenophora graminea* (Aminnejad *et al*., 2009), *Cochliobolus heterostrophus* (Gafur *et al*., 2003) isolates from diverse geographic regions. This technique has also been applied to differentiate fungal isolates according to their host plant (Weikert-Oliviera *et al*., 2002).

**Amplified Fragment Length Polymorphism (AFLP)**

AFLP analysis consists the use of restriction enzymes to digest total genomic DNA followed by ligation of restriction half-site specific adaptors to all restriction fragments. Then, a selective amplification of these restriction fragments is performed with PCR primers that have in their 3’ end the corresponding adaptor sequence and selective bases.

AFLP has been used efficiently to study genetic variability of many pathogenic fungi. AFLPs have been used to distinguish species and phylogenetic groups in a number of *Fusarium* species (Zeller *et al*., 2003; Belabid *et al*., 2004) including *Fusarium oxysporum* (Baayen *et al*., 2000a; Belabid *et al*., 2004).

Zhong and Steffenson (2001) employed AFLP to study the virulence and molecular variability of *Cochliobolus sativus*. Comparison of AFLP banding patterns of the four *Cochliobolus* species showed greater differences between species than among isolates within a species. The AFLP banding patterns of the *C. carbonum* and *C. victoriae* were more similar than for any other pair of species, suggesting that these two species are closely related and may be derived from the same progenitor species. Unique AFLP profiles between isolates of *Fusarium oxysporum* and *F. commune* revealed these isolates to be genetically different (Stewart *et al*., 2006).

AFLP produces substantially more robust polymorphic amplification products per experiment than other marker system (Jones *et al*., 1997). The disadvantages of AFLPs are that they require high molecular weight DNA, more technical expertise
than RAPDs, and that AFLP analyses suffer the same analytical limitations of RAPDs (Idrees and Irsahd, 2014).

**Inter-simple sequence repeats (ISSR) and Simple Sequence Repeats (SSR)**

ISSR is a molecular marker that amplifies inter-microsatellite sequences at multiple loci throughout the genome. ISSR primers are based on di-, tri-, tetra-, or penta-nucleotide repeats with 5’ or 3’ anchored base(s). ISSR techniques are almost similar to the RAPD techniques. The ISSR primer sequences are designed from microsatellite regions and the annealing temperatures are more than those used for RAPD markers. ISSR markers are easily generated and are used to study fungal populations (Abd-Elsalam et al., 2011a). ISSR is a variant of the SSR approach that amplifies the DNA sequence between two SSR loci (Zietkiewicz et al., 1994). ISSR technique has been employed for the characterization of genetic variations of *Colletotrichum capsici* (Ratanacherdchai et al., 2010), *Pyrenophora teres* (Statkeviciute et al., 2010).

Microsatellites (SSR) have been used in several epidemiological studies (Raboin et al., 2007) and to establish genetic relationship among phytopathogenic fungi (Mahfooz et al., 2012). SSR primers have been used as diagnostic tool for the detection of *Macrophomina phaseolina* in cotton and also in genetic analysis of *M. phaseolina* isolates (Arias et al., 2011).

ISSR is cheaper than AFLP, and both do not require prior-knowledge of DNA sequence. ISSR is more reproducible and provides higher polymorphism than RFLP (Williams et al., 1990; Winter and Kahl, 1995). In contrast to RAPD and ISSR, SSR is more sophisticated, requires knowledge of DNA sequence and based on construction of enriched genomic libraries (Tautz, 1989).

**DNA sequencing**

Sequencing of DNA is one of the direct approaches for the detection, identification and phylogenetic analysis of many fungal pathogens (Henson and French, 1993; Oliver, 1993; Taylor et al., 2000). It involves amplification of a target gene with universal primers, followed by sequencing and comparison with the available databases such as EMBL and GenBank, using computer programs such as
BLAST and FASTA. These programs identify the target fungi based on the closest match to the previous deposits. Programs available can determine the likely phylogenetic relationships between the unknown fungus and other organism and therefore help confirm its taxonomic status.

Recent advances in DNA sequence technologies and analytical methods have revolutionized fungal systematic. Nuclear rDNA, gpd, LSU, Brn1 gene sequence data have been used to establish anamorph-teleomorph connections (Berbee et al., 1999; Goh, 1998), infer phylogenetic relationships of form-genera of anamorph-rich clades, and anamorphic generic-complexes (Zhang and Berbee, 2001) and in species delineation of monophyletic groups of anamorphic taxa (Manamgoda et al., 2011; Manamgoda et al., 2012).

2.4. Plant Pathogen Interaction

Plant-pathogen interactions can be classified as non-host interactions, compatible, and incompatible interactions. In a non-host interaction, a plant species is resistant to all strains of a given pathogen, therefore, the putative pathogenic microbes are unable to reproduce and colonize it (Heath, 2000; Mysore and Ryu, 2004). Incompatible interactions between a resistant host and an avirulent pathogen result in a hypersensitive response (HR, Hammond-Kosack and Jones, 1996). The HR is characterized by localized cell death at the site of infection, thus confining the spread of the infected microbes (Van Loon, 1997; Hammond-Kosack and Jones, 1996; Fritig et al., 1998). Compatible interactions occur when a virulent pathogen infects a susceptible or moderately resistant host, resulting in severe damage or death due to colonization of the plants (Agrios, 2005). Following pathogen infection, the initial plant response is a change in plasma membrane permeability, influx of calcium and protons, and efflux of chloride (McDowell and Dangl, 2000). Subsequently, ion fluxes in plant-pathogen interactions lead to production of reactive oxygen species (ROS) in the apoplast. Superoxide anion, hydrogen peroxide and hydroxyl free radical are designated ROS due to their highly oxidizing potentials. High production of ROS is harmful to plants, however, regulated production of ROS in biotic and abiotic stresses can trigger plant defense responses. In a plant-pathogen interaction, the production of ROS is catalyzed by a plasma membrane-located NADPH oxidase and/or apoplastic-localized peroxidases (Somssich and Hahlbrock, 1998). The initial
transient production of ROS, an oxidative burst, can trigger a complex but highly organized signaling pathway for defense responses, including hypersensitive response induction and defense gene expression (Piffanelli et al., 1999; Lamb and Dixon, 1997; Alvarez et al., 1998). The burst of ROS, especially hydrogen peroxide, at the plant cell surface induces the cross-linking of the structural proteins in the cell wall, which fortifies the cell surface against pathogen infiltration (Scheel, 1998). The oxidative burst also triggers plant defense responses by inducing signal molecules, including salicylic acid (SA), jasmonate (JA), or ethylene (ET), which may then move into the nucleus (Van Wees et al., 2000; Devadas et al., 2002).

**Hormone Defence Signalling in plants**

SA plays a central role in plant defense against pathogens (Kunkel and Brooks, 2002). The accumulation of SA is observed in the immediate vicinity of incompatible interactions and exogenous application of SA induces higher levels of resistance to a broad range of pathogens. SA is derived from the phenylpropanoid pathway, and can lead to disease resistance by encoding pathogenesis-related (PR) proteins. JA is produced from linolenic acid, and is released from membrane unsaturated fatty acids by the action of membrane-located phospholipases; the phospholipases are induced following pathogen attack. Linolenic acid is catalyzed to JA and related molecules via a series of enzymatic steps. Ethylene (ET), the only gaseous plant hormone, is produced at high levels during compatible and incompatible interactions. ET preferentially induces basic pathogenesis-related proteins. These signal molecules affect each other’s signalling potential positively or negatively, therefore, the cross talk among signaling molecules allows plants to effectively regulate defence responses. The delivered signals control up-/or down regulated transcription factors, resulting in reprogramming of cellular metabolic pathway for pathogen defense.

**Jasmonic acid**

Jasmonic acid is a growing class of lipid-derived signalling molecule that acts as a potent regulator of genes involved in cell growth, biotic and abiotic stress responses (Kazan and Manners, 2008). The importance of JA in defense against pathogenic microorganisms has only been envisaged in the last few decades. JA plays
a central role in the complex signalling network leading to disease resistance. The involvement of JAs in defense responses against pathogens was evidenced by the fact that JAs often accumulate in response to pathogen attack, the altered susceptibility/resistance of mutant plants affected in JA biosynthesis or signalling, and the effects of exogenous application of JA on plant resistance (Pozo et al., 2005). It is evident that JA plays a major role in basal and induced resistance against necrotrophic pathogens. However, JA also influences resistance to hemibiotrophs and certain biotrophs, which are generally thought to be resisted exclusively through SA-dependent defenses. Thus, the concept that JA-dependent defense responses are predominantly effective against pathogens with a necrotrophic lifestyle, whereas SA-dependent defense responses are mostly effective against pathogens with a (hemi)biotrophic lifestyle, is not universal and should be used with caution.

Over the years, increasing evidence has pointed to the involvement of JA in plant-pathogen interactions (Cohen et al., 1993; Pozo et al., 2005; Kazan and Manners, 2008). Despite the fact that the effects of JA on plants have been well characterized, the precise mechanism by which JA acts are not fully understood (Xu et al., 2002). Genetic analysis of plant mutants and transgenics has revealed both positive and negative regulatory mechanism of JA in plant disease resistance. In Arabidopsis, the JA-deficient COII mutants showed increased susceptibility to Alternaria brassicicola, Botrytis cinerea and Plectosphaerella cucumerina compared to wild-type plants (Thomma et al., 1998).

In addition, the exogenous application of MeJA has been shown to enhance resistance against several necrotrophic fungal species. For instance, wheat (Triticum aestivum) pre-treated with MeJA showed delayed symptom development against Fusarium pseudograminearum (Desmond et al., 2005; El-Wakeil et al., 2010) and enhanced resistance to infection by Stagnospora nodorum (Jayaraj et al., 2004). Systemic protection against virulent barley powdery mildew strain, Blumeria graminis f.sp. hordei was induced by exogenous application of MeJA (Walters et al., 2002). JA application has also been shown to enhance maize resistance to the necrotrophic pathogen Rhizopus microspores and Colletotrichum graminicola (Schmelz et al., 2011).
The antagonism between JA and the well-characterized salicylic acid (SA) or ethylene (ET) or abscisic acid (ABA) defense pathways, so far, has been proposed to be associated with JA-regulated resistance. Activation of SA-mediated responses most often results in the inhibition of JA/ET signalling and vice versa (Gupta et al., 2000; Kunkel and Brooks, 2002). Induction of the SA pathway by Pseudomonas syringae suppressed JA signalling and rendered infected leaves more susceptible to the necrotrophic fungus Alternaria brassicicola (Spoel et al., 2007). The induction of both SA and JA-mediated signalling has been shown to synergistically enhance resistance to powdery mildew fungal biotroph, Golovinomyces cichoracearum, as well as bacterial and insect pathogens (Ellis et al., 2002). Deficiency in the JA-signaling pathway leads to an increase in susceptibility to G. cichoracearum (Fabro et al., 2008).

Mutations linked to JA signalling/synthesis can lead to an increase in susceptibility to several necrotrophic fungi. For example, the tomato mutants def1, jai1 and pht1 are more susceptible to Verticillium dahlia, B. cinerea and F. oxysporum respectively (Thaler et al., 2004; AbuQamar et al., 2008; Kidd et al., 2009). In contrast, recent studies have shown some JA mutants with increased resistance to several necrotrophic fungi. For instance, the tomato mutant def1 shows increased resistance to Alternaria alternata (Egusa et al., 2009). Additionally, mutations in opr3, coi1 and jar1 have been shown to exhibit hyper-resistant response when infected with F. graminearum (Makandar et al., 2010). Similarly, mutations in JIN1/MYC2 are known to exhibit resistance to both B. cinerea and P. cucumerina (Kachroo and Kachroo, 2009). Mitogen activated protein kinase 4 (MPK4) induces JA/ET signalling responses and represses SA-mediated defence (Petersen et al., 2000; Brodersen et al., 2006; Kazan and Manners, 2008; Gao et al., 2008; Wang et al., 2009). mpk4 mutants show an enhanced resistance to hemibiotrophic bacterial pathogen, Pseudomonas syringae pv. tomato DC3000 (Qui et al., 2008; Cui et al., 2010). All the above data highlight the complexities of JA-mediated signalling and defense responses. Recent experimental evidence suggests that JA-mediated signalling plays a role in defense against both biotrophic and necrotrophic fungi (Antico et al., 2012).

Salicylic acid
In rice plants very high basal levels of SA is present (Silverman et al., 1995), however, SA does not appear to act as an effective signal molecule to activate many defense genes and induce disease resistance. This high level of endogenous SA may not act as secondary signal molecule for induced resistance in rice, but may serve as a preformed chemical barrier with direct and/or indirect antioxidant responses to minimize oxidative damage caused by various biotic and abiotic factors (Yang et al., 2004). Depletion of endogenous SA in tobacco and Arabidopsis typically abolishes or greatly reduce pathogen-induced expression of defense genes, leading to reduced disease resistance. In contrast, SA deficiency in rice does not measurably affect the induction of PR genes and other defense genes (Lee et al., 2001), suggesting that induction of many defense genes is independent of SA in rice.

Based on findings by Daw et al. (2008), depletion of SA in rice plants might attenuate their ability to detoxify reaction oxygen intermediates, and SA plays a role in modulating the expression of enzymes resistant to oxidative stresses. In addition the finding revealed that the protection effects of SA treatment could last for at least 3 days against Magnaporthe oryzae. Exogenous application of SA could induce a range of defense responses including the oxidative enzymes that are associated with tolerance of oxidative stress and modification of cell wall, phytoalexin and antifungal conjugates. The results were consistent with the notion that SA is a key signal in plant defense signaling pathway (Zhang and Klessig, 2001; Shah, 2003) and added new insight into the molecular bases of SA-mediated defense mechanism in rice plants.

When exogenously applied to rice plants, SA is a poor activator of PR gene expression and induced resistance. In contrast, Arabidopsis and Tobacco show boosted endogenous SA signaling production and induces SAR responses against pathogens including expression of PR genes that are implicated in disease resistance (Delaney et al., 1994; Summermatter et al., 1995; Metraux et al., 1992). A possible role for SA in signaling disease resistance was first suggested by White and coworkers, who demonstrated that injecting leaves of resistant tobacco with SA or aspirin stimulated PR protein accumulation and enhanced resistance to tobacco mosaic virus (TMV) infection, manifested by a 90% reduction in lesion number (Klessig and Malamy, 1994; Malamy and Klessig, 1992; Raskin, 1992). SA
pretreatment was subsequently found to induce PR gene expression and/or resistance to viral, bacterial and fungal pathogens in plant species.

Highly elevated SA levels found in certain plants and genetic mutants correlated with their enhanced resistance to pathogen infection. The strongest evidence supporting SA’s role as a critical defense signal has come from analyses of plants in which endogenous SA levels were altered. The first of these studies utilized transgenic tobacco or Arabidopsis expressing the bacterial nahG gene, encoding the SA-metabolising enzyme salicylate hydroxylase. Following pathogen infection, these plants were unable to accumulate high SA levels, and they failed to develop SAR or express PR genes in the systemic leaves; instead, they displayed heightened susceptibility to both virulent and avirulent pathogens (Vernooji et al., 1994). Both disease resistance and PR expression were restored in these plants by treatment with the SA syntetic analog, 2,6-dichloro-isonicotinic acid (INA) (Vernooji et al., 1995). Subsequent studies revealed that plants defective for SA biosynthesis displayed similar phenotype. Tobacco or Arabidopsis with suppressed PAL expression or mutations in SID2/EDS16 (encodes ICS1) or SID1/EDS5 (encodes a member of the MATE transporter family required for SA accumulation) displayed enhanced pathogen susceptibility and/or failed to develop SAR or systemically express PR genes (Nawrath et al., 2002). Like nahG plants, resistance and PR expression were restored by treatment with SA or INA. Overexpression of enzymes involved in SA metabolism, including SA glucosyltransferase 1 (AtSGT1) or SA methyltransferase (OsBSMT1), in transgenic Arabidopsis also led to reduced endogenous SA levels, reduced PR expression and enhanced susceptibility to pathogens (Song et al., 2008). By contrast, over-expression of bacterial SA biosynthetic genes in transgenic tobacco conferred highly elevated SA levels, constitutive PR expression, and enhanced resistance (Verberne et al., 2000).

Although SA’s role as a defense signal is well established in dicots, its role in monocots is less well understood. SA or its synthetic analogs INA or BTH induce PR expression and/or resistance in maize (Morris et al., 1998), rice (Hwang et al., 2008), barley (Kogel et al., 1994) and wheat (Gorlach et al., 1996).

SA levels similarly failed to increase in rice inoculated with P. syringae or the fungal pathogens Magnaporthe grisea (causal agent of rice blast) or Rhizoctonia
solani (Silverman et al., 1995), and pathogen-induced PR expression was unaffected in SA-deficient, nahG-expressing transgenic rice (Yang et al., 2004). However, because rice has constitutively high levels of SA, signaling might occur via altered sensitivity of downstream components to SA, rather than via actual changes in SA levels (Chen et al., 1997). Supporting a role for SA as a defense signal in rice are the combined findings that (a) nahG-expressing rice exhibits increased susceptibility to M. grisea (Yang et al., 2004), (b) over-expression of NON-EXPRESSOR OF PATHOGENESIS-RELATEDGENES1 (NPR1, a critical SA signal transducer in Arabidopsis and tobacco) or its rice homolog enhances resistance to the bacterial blight-causing Xanthomonas oryzae pv. oryzae (Yuan et al., 2007), (c) rice/Arabidopsis NPR1 interacts with rice TGA transcription factors, one of which binds a cis-element required for SA responsiveness in the rice RCH10 promoter (Chern et al., 2001), (d) SA or BTH treatment induces expression of several rice WRKY genes (members of the Arabidopsis WRKY transcription factor family participate in defense responses (Liu et al., 2007) and (e) expression of WRKY45 is required for BTH-induced resistance to blast disease (Shimono et al., 2007). Taken together, these results suggest that SA is a signal for defense responses in at least some monocots and that many of the components involved in this pathway(s) are analogous to those utilized in dicots.

**Pseudomonas as biotic elicitors**

In recent years, there are interests in biocontrol and biofertilizers. Among the various biocontrol agents, fluorescent Pseudomonads are known to survive both in rhizosphere (Parke, 1991) and phyllosphere (Wilson et al., 1992). Fluorescent Pseudomonads are known to induce disease resistance against foliar disease (Riu et al., 1995). Pseudomonas fluorescens is used as a biocontrol agent to manage disease caused by Rhizoctonia solani under field conditions (Bautista et al., 2007). In recent years, the heterotrophic rhizobacteria Pseudomonas fluorsesces has been successfully used as seed inoculants for biological control of several plant pathogens. The ability of these bacteria to reduce disease and promote plant growth is believed to be due in part to antibiosis in the rhizosphere and subsequent displacement of root-colonizing microflora (Kloepper and Schroth, 1981). In recent years, fluorescent Pseudomonas have drawn attention worldwide because of production of secondary metabolites such
as siderophores, antibiotics, volatile HCN compounds, enzymes and phytohormones which suppress the growth of phytopathogenic fungi (Gupta et al., 2001). *Pseudomonas fluorescens* (AUPF25) produce protease, IAA and siderophore showed inhibition of mycelial growth of *Pyricularia oryzae* a causal organism of blast disease of rice.

*Pseudomonas fluorescens* as an effective biocontrol agent that reduced several plant diseases (Manikandan et al., 2010). The mechanism of pathogen suppression varies from different host pathogen interaction. DAPG (2-4-diacetylphloroglucinol) an antibiotic produced from *Pseudomonas fluorescence* suppress the growth of *Xanthomonas oryzae* pv. *oryzae*, a causal agent of bacterial blight of rice both under greenhouse and field conditions. Similarly, antibiotic Phenazine-1-Carboxylic acid (PCA) suppress the leaf and neck blast of rice. Rice diseases caused by *Sarcothalamium oryzae* and *Rhizoctonia solani* are highly sensitive to *P. fluorescens* due to production of antibiotics.

Siderophore mediated suppression has been implicated in the biocontrol of wilt diseases caused by *Fusarium oxysporum*. Siderophores are iron binding extracellular compound with low molecular weight and high affinity for ferric iron that are secreted by microorganisms to take up iron from the environment. Fluorescent *pseudomonads* are characterized by the production of yellow-green pigments termed pyoveridnes which fluoresce under UV light and function as siderophores. The role of siderophores produced by fluorescent *Pseudomonas* in plant growth promotion was first reported by Kloepper and Schroth (1981) and was later reported to be implicated in the suppression of plant pathogens. Siderophores produced by *Pseudomonas* showed good antifungal activity against plant deleterious fungi, viz. *Aspergillus niger*, *A. flavus*, *A. oryzae*, *F. oxysporum*, *Sclerotium rolfsii*, *Alternaria* and *Colletotrichum capsici*. Recently, the pseudobactin siderophore of *P. fluorescens* WCS374r was found to be an important determinant of ISR against blast disease of rice.

Studies on induction of antioxidant enzymes, POX, PPO and SOD, revealed that POX activities significantly were induced in tomato plants treated only with bacteria and bacterized plants challenged with pathogen. Highest POX activity was observed in bacterized plants challenged with fungi, whereas there were not any
significant differences between pathogen inoculated and control plants. Research has indicated that the activities of antioxidant enzymes, as an important part of defense mechanisms, were induced by applied *Pseudomonas fluorescens* CHA0 as a bioagent (Girlanda *et al*., 2001). The enhanced activities of defense enzymes may contribute to bioprotection of plants against pathogen. Study on the activity of PAL, the key enzyme of phenylpropanoid metabolism, illustrated that there were not any significant differences of PAL activity between treatments. It is probable that changes in PAL occurred earlier and declined to the control level at the time of analysis. It is also possible that induction of PAL is a long term response and may be found later. It is now clear, that any stress condition or significant change in environment is associated with up- or down-regulation of hundreds of genes (Cheeseman, 2007). Induction of defense proteins positively correlates with defense against pathogen invasion in different plants (Ramamoorthy *et al*., 2002).

### 2.5. Management of rice diseases

There are very few reports on the biocontrol againsts against brown spot disease. Fungi from *Trichoderma* genus are among the biological control agents of *Bipolaris oryzae* also bacteria belonging to *Pseudomonads* and *Bacillus* genus have also been used (Singh *et al*., 2005). Seed treatments with *Trichoderma viride* or *T. harzianum* have reduced disease by 70 % (Biswas *et al*., 2010). Over 70% disease reduction has been achieved too from the use of selected *Pseudomonas* spp. isolates (Joshi *et al*., 2007; Ludwig *et al*., 2009).

### Systemic Acquired Resistance (SAR)

SAR, the most well characterized systemic defense response, confers resistance to a broad spectrum of pathogens and can be induced by treatment of SA or its analogs, such as benzothiadiazole (BTH) (Friedrich *et al*., 1996; Gorlach *et al*., 1996) and methyl-2,6-dichloroisonicotinic acid (INA) (Delaney, 1997). The state of pathogen resistance that is created by SAR can be activated by weakly virulent infections, incompatible plant pathogen interactions and/or exogenous treatment with synthetic compounds isolated from natural sources. The resistance conferred by SAR activation is effective against a broad range of pathogens. Application of one of these SAR-inducing chemicals to a susceptible host prior to pathogen infection leads to
very rapid induction of HR and PR gene expression upon pathogen infection, resulting in no or reduced disease (Lawton et al., 1996).

Although SAR has been shown to be activated through incompatible plant-pathogen interactions, the presence of specific pathogen derived factors is sufficient to elicit SAR. PAMPs are pathogen-derived molecules conserved across a large class of pathogens. Recognition of PAMPs by plants is a key part of non-host pathogen resistance. A number of PAMPs are known activators of SAR. This defensive response is termed PAMP/pattern-triggered immunity (PTI) (Jones and Dangl, 2006). Another broad form of pathogen-induced immunity distinct from PTI, called effector-triggered immunity (ETI), depends on a class of pathogen-associated factors termed effectors, and is associated with the activation of SAR (Katagiri et al., 2002). ETI occurs when a specific host-based resistance protein recognizes specific pathogen-associated effectors, either directly or indirectly.

**Gene expression changes associated with SAR**

As SAR produces significant changes to the susceptibility of a host plant toward a wide spectrum of pathogens, there should be gene expression changes that underlie these systemic changes in susceptibility. Pioneering work reported that SAR activation is correlated with the production of proteins throughout the systemic tissues of the SAR-expressing plant.

A more diverse set of proteins have been found to be produced upon pathogen infection. These proteins have been named pathogenesis-related (PR) proteins (Bol et al., 1990). Though the classification of PR-proteins was originally applied to proteins were only induced during pathogen infection, the term has since been applied to describe proteins that are normally present in healthy tissues but show an increase in expression during pathogen infection. Activation of SAR responses with various SAR-activators such as pathogens, exogenous application of phytohormones (eg. SA) or synthetic compounds (eg. BTH) increases expression of PR protein coding genes.
Table 2.2. List of SAR mediated resistance during host-pathogen interactions

<table>
<thead>
<tr>
<th>Host</th>
<th>SAR type</th>
<th>SAR source</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabaccum</td>
<td>Incompatible pathogen</td>
<td>Ralstonia solanacearum</td>
<td>Increase in resistance against TMV, bacterial pathogen <em>P. syringae</em> pv. <em>tabaci</em> and powdery mildew fungal pathogen <em>Oidium</em> sp.</td>
<td>Nakashita et al., 2002</td>
</tr>
<tr>
<td><em>N. tabaccum</em></td>
<td></td>
<td><em>Ralstonia solanacearum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Solanum lycopersicum</em></td>
<td></td>
<td><em>Phytophthora cryptogea</em></td>
<td></td>
<td>Attitalla et al., 2001</td>
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<tr>
<td><em>Oryza sativa</em></td>
<td></td>
<td><em>Fusarium oxysporum</em> f.sp. <em>lycopersici</em></td>
<td></td>
<td>Smitha and Metraux, 1991</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td></td>
<td><em>P. syringae</em> pv. <em>Syringae</em></td>
<td>SAR against <em>Pyricularia oryzae</em></td>
<td>Morris et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Puccinia sorghi</em> and <em>Bipolaris maydis</em></td>
<td>Increased expression of SAR-related genes</td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>Synthetic compounds</td>
<td><em>Erypsiphe graminis</em> f.sp. <em>tritici</em></td>
<td>Increased resistance to pathogens <em>Erysiphe graminis</em> f.sp. <em>tritici</em></td>
<td>Gorlach et al., 1996</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>Saccharin</td>
<td><em>Microdochium nivele</em></td>
<td>SAR related defense response</td>
<td>Hofgaard et al., 2005</td>
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<td></td>
<td></td>
<td><em>Septoria nodorum</em></td>
<td></td>
<td>Jayaraj et al., 2004</td>
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<tr>
<td></td>
<td></td>
<td><em>Functional analogs of SA</em></td>
<td>Defense against pathogens <em>Rhynchosporium secalis</em>, <em>Blumeria graminis</em> f.sp. <em>hordei</em>, <em>Ramuleria collocygni</em></td>
<td>Walters et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>INa and BTH</em></td>
<td>Signalling activation of SAR</td>
<td>Gorlach et al., 1996</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>PAMPs</td>
<td><em>Bacterial flagellin</em> and lipopolysaccharides</td>
<td>Increased resistance to <em>P. syringae</em> pv. <em>tomato</em></td>
<td>Mishina and Zeier, 2007a</td>
</tr>
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<td></td>
<td></td>
<td><em>Blumeria graminis</em> f.sp. <em>hordei</em></td>
<td>Increased resistance to <em>Peronospora parasitica</em></td>
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<td><em>Hordeum vulgare</em></td>
<td></td>
<td><em>Fungal cell-wall derived Chitosan</em></td>
<td>Induction of SAR related defense gene expression</td>
<td>Faoro et al., 2008</td>
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Induced Systemic Resistance (ISR)

A type of induced resistance distinct from SAR is activated by the colonization of plant roots by certain species of non-pathogenic plant-growth-promoting rhizobacteria, such as *Pseudomonas fluorescens* (Van Peer *et al*., 1991). The ability to activate ISR appears to be dependent on the strain of PGPR and the species of host plant. The exact mechanism for such specificity between PGPR and host plant is not entirely clear, it has been suggested that bacterial markers such as outer membrane lipopolysaccharides and iron-siderophores may be involved in the plant-microbe recognition process that activates ISR (Van Loon *et al*., 1998).

Although ISR and SAR have a similar end result of increased resistance, the signalling pathway associated with ISR appears to be distinct from SAR. ISR signaling is dependent on JA but, activation of ISR is not correlated with increased levels of JA either locally/systemically (Pieterse *et al*., 2000). This has led to the suggestion that ISR activation is associated with an increase in sensitivity towards JA, rather than an increase in the levels of this signalling molecule.

Exogenous application of JA to ISR-activated plants results in significant enhancement of certain JA-responsive gene expression after challenge inoculation by pathogen (Van Wees *et al*., 1999).

2.6. Host Gene expression upon pathogen infection

Mitogen-activated protein kinase (MAPK) cascades

It is well known that MAPKs respond to diverse external stimuli and are involved in stress signaling of many plants including rice (Zhang and Klessig 2001; Nakagami *et al*., 2005). There are a number of studies that demonstrate the activation of rice MAPKs to various extracellular stimuli, including biotic and abiotic stresses (Agrawal *et al*., 2003b; Reyna and Yang 2006).

Mitogen-activated protein kinase (MAPK) cascades play important roles in transmission of extracellular signals to downstream components through protein phosphorylation. A MAPK cascade minimally consists of three kinases: a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Increasing evidence has
shown that MAPKs play an important role in plant signal transduction related to biotic and abiotic stresses. Activation of MAPKs has been observed in plants exposed to pathogens (Suzuki and Shinshi, 1995; Adam et al., 1997; Ligterink et al., 1997; Zhang and Klessig, 1997, 1998b; He et al., 1999). Plant MAPKs also can be activated by fungal elicitors (Suzuki and Shinshi, 1995), salicylic acid (Zhang and Klessing, 1997) and jasmonic acid (Seo et al., 1999). MAPKs expressed in response to fungal infection and host cell death as well as defense signaling molecules such as JA, SA, ABA and ET has been studied during rice-M. grisea interaction (Reyna and Yang, 2006).

**OsMPK5**

*OsMPK5* falls under the group A with TEY motif (Reyna and Yang, 2006). It is the most extensively studied among all of the rice MPKs. It is also identified by different names such as *OsMAPK2, OsMSRMK2, OsMAP1* and *OsBIMK1*. Agrawal et al. (2002) observed an induction of *OsMPK5 (OsMSRMK2)* in detached rice leaf segments by wounding and various signaling molecules (e.g. JA, SA, ABA, ET and H$_2$O$_2$) as well as salt, drought, ozone, UV-C irradiation, heavy metals and at high temperatures.

*OsMPK5 (OsBIMK1)* was also demonstrated to be activated by wounding, pathogen infection and SAR inducers such as BTH, INA and PBZ, indicating the potential role of *OsMPK5* in the activation of defense responses (Song and Goodman, 2002). It was shown that expression of *OsMPK5 (OsBIMK1)* was further induced during the first 36 h after inoculation with *M. grisea* in BTH-treated rice seedlings and in incompatible interactions, but not in compatible interaction between rice and *M. grisea*, thus reasonably suggesting that *OsMPK5 (OsBIMK1)* may mediate the blast disease resistance signaling pathway.

In a more comprehensive study, Xiong and Yang (2003) reported that *OsMPK5* activity was induced by fungal infections. Transgenic studies (Reyna and Yang, 2006; Xiong and Yang, 2003) suggests that *OsMPK5* is a positive regulator of abiotic stress tolerance, but a negative regulator of the PR-gene expression and broad spectrum disease resistance.

**OsMPK7**
OsMPK7 belongs to group D with TDY motif. It is found to be associated with biotic and abiotic stress responses. The expression of OsMPK7 is inducible by JA and M. grisea during early stage of fungal infection (Reyna and Yang, 2006). Transgenic studies suggest that kinase is involved in JA signaling and the rice defense responses (Reyna, 2004).

OsMPK12

OsMPK12 belongs to group E with TDY motif. It is the largest rice MAPK group. He et al. (1999) first isolated OsMPK12 (OsBWMK1) and showed its induction in rice leaves by M. grisea infection 4 hpi and at 30 min after mechanical wounding. Reyna and Yang (2006) also reported the induction of OsMPK12 by M. grisea and defense signal molecules including JA, ABA, SA and ET. In addition, Agrawal et al. (2003c) demonstrated a significant induction of OsMPK12 with 15-30 min of wounding or other treatments such as JA, SA and fungal elicitor chitosan.

Peroxidase

Various abiotic and biotic inducers of resistance stimulated activity of POX and PAL in treated plants (Ruiz et al., 1999). Peroxidase (POX) catalyses the oxido-reduction of various substrates using hydrogen peroxide. Many reports have suggested that POXs play role in resistance to pathogens such as lignification and suberization, cross-linking of cell wall proteins, xylem wall thickening, generation of reactive oxygen species, hydrogen peroxide scavenging, phytoalexin synthesis, antifungal activity of POX itself and auxin metabolism. Peroxidases are ubiquitous plant enzymes that are encoded by a large number of related genes within a plant genome (Passardi et al., 2004) and have been implicated in a myraid of plant developmental process and responses to biotic and abiotic stress (Almagro et al., 2009; Cosio and Dunand, 2009). An essential function for peroxidases is to protect the cellular membranes against oxidative damage. Peroxidases are players in both reactive oxygen species (ROS) removal and ROS generation. Peroxidases have been documented as ROS scavengers in plants stressed by pathogens.

Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL) is the first enzyme of phenylpropanoid pathway that catalyzes the conversion of L-phenylalanine to trans-cinnamic acid. It is
the key enzyme in the synthesis of several defense-related secondary compounds like phenols and lignins (Hemm et al., 2004). The presence of phenolic compounds in plants and their synthesis in response to infection is associated with disease resistance.

Following pathogen recognition and signal transduction, defense responses are activated that protect plants from infection. These responses include cell wall reinforcement, accumulation of antimicrobial secondary metabolites such as phytoalexins, and expression of PR proteins. PR proteins are classified into 17 groups (PR1-PR17) based on their amino acid sequence, serological relationship, and enzymatic activities. In rice, only a few groups of PR genes, including PR1, PR8, and PR10, have been reported to be induced following bacterial or fungal infections (Wu et al., 2011). Several studies suggest that some PR genes are regulated pathogen species-dependently while some are not.