Review of Literature:

Rice (*Oryza sativa L*.) is one of the major nutritional source and a staple food for more than half of the population of the world. Rice cultivation dates back to 3000 B.C. in South Asia. Rice is produced at a worldwide level of approximately half a billion tonns annually. Half of the world's human population derives their major calorie intake from rice consumption (Goff *et al*. 1999). African rice (*Oryza glaberrima*) and Asian rice (*Oryza sativa*), two species of rice were domesticated and the different histories led to the different ecological niches for the two variety of rice.

With less land available to expand rice growing areas and with competing demands for urbanisation and industrialisation on existing rice lands, increase in productivity will have to be from the existing land. Globally, 20 - 35% rice yield is lost due to insects and pests and 10 - 15% due to diseases caused by different rice pathogens, which becomes a major constraint to higher rice yield (Ou *et al*. 1985). There are over 80 reported rice diseases and some of them have been shown to be major limitation to rice yield in specific environments of different rice ecosystem (Mew *et al*. 1991). Among all the pathogens, fungi account for nearly 30 diseases of rice.

Rice blast is one of the major diseases affecting rice. It causes heavy crop losses up to 30% of the harvested rice. The scale of the problem is well illustrated by a disease outbreak in Bhutan in 1995 (Talbot 2003). Rice blast is caused by *Magnaporthe oryzae* (Herbert Barr) (Formerly known as *Pyricularia grisea*, *Pyricularia oryzae*). It is perhaps the most widely distributed plant disease as it occurs in 85 countries worldwide (Ou *et al*. 1985), causing up to 70-80% of crop

loss during epidemic season (Ou *et al.* 1985). Losses due to this fungal disease accounted for up to $157 million between the year 1975 and 1990 (Zeigler *et al*. 1994). During 2003, in India, loss of more than 266,000 tons of rice was recorded which was calculated to about 0.8% of the total rice yield in India. Approximately 865,000 hectares of rice fields affected by rice blast each year in Japan. More than 50% of rice yield is lost each year in Philippines due to rice blast (IRRI 2003). *M. oryzae* infects more than 50 types of grasses, including economically important crops like barley, wheat, and millet, but individual field isolates are limited to infecting one or few host species (Rossman *et al*. 1990).

M. oryzae is highly adaptable to environmental conditions and can be found in irrigated lowland, rain-fed upland, or deepwater rice fields (Rao *et al*. 1994). In spite of a great deal of research on the pathogen and the disease, blast still remains a serious constraint in rice production in all irrigated and upland environments. Blast was first reported in Asia more than three centuries ago (IRRI 2002). Control strategies like the use of resistant cultivars and application of fungicides have not allowed complete eradication of the disease. The fungus has been able to develop resistance to both chemical treatments and genetically resistant rice cultivars developed by plant breeders. As such, a detailed understanding of the infection mechanism may help in the development of new strategies to control this disease.

Rice blast disease:

Rice blast is a leaf spot disease which is spread by splash dispersal. Heavy dew conditions contribute to infection and all the aerial parts of the plant can be affected; leaf surfaces become

speckled with oval lesions, plant are liable to lodging if stems are infected and if the panicle is infected then a severe yield loss results (Ou 1985).

Symptoms:

1. Initial symptoms are white to grey-green lesions or spots with dark borders produced on all parts of shoot.
2. Older lesions are elliptical or spindle shaped and whitish to grey with necrotic borders
3. Lesions wide in the centre and pointed towards either end
4. Lesions enlarges and coalesce to kill the entire leaves
5. Larger lesions (2 cm long) at reproductive stage on younger plants (less than 1 cm long)
6. Symptoms are observed on leaf collar, culm, culm nodes, and the panicle neck node.
7. Internodal infection of the culm occurs in a banded pattern with a 3 cm blackened necrotic culm and 3cm healthy tissue in succession.
8. Few or no seeds, or white heads, when neck is infected or rotten.

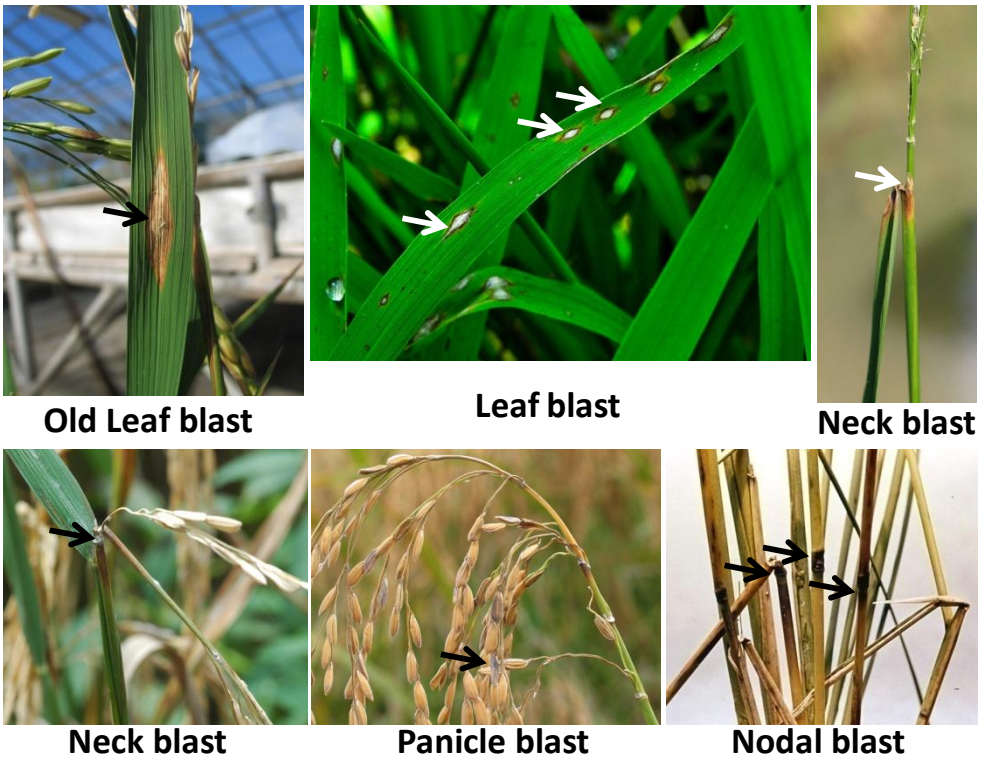
The fungus attacks all aerial parts of the plant as well as the root system, at all stages of growth. Depending on the stage the disease is named as:

Leaf blast: After an incubation period purple spots can be observed after fungal attack and changes into a spindle shape which has a grey centre with a purple-to-brown border, and then surrounded by a yellow zone as time passes. Brown spots are shown by only on the older leaves

or leaves of resistant cultivars (Fig. 1). In young or susceptible leaves, lesions coalesce and cause withering of the leaves, especially at the seedling and tillering stages.

Neck and panicle blast: On nodes blackening extends both ways up to 1-2 cm. Infection of the neck node produces triangular purplish lesions, followed by lesion elongation to both sides of the neck node (Fig. 1). Invasion of young neck node results in whitening of panicles and called as 'white head' that is sometimes misinterpreted as insect damage. Further infection causes incomplete grain filling, and poor grain quality. Infection may spread to the panicle branches and glumes (Fig. 1). Panicle blast is most dangerous as it causes total loss of grain. Panicle hangs down and can even break off.

Node infections: *M. oryzae* attacks stem nodes and results in to a brown or black node which ultimately leads to the death of the upper part of stem (Fig. 1).



(http://www.knowledgebank.irri.org)

Figure 1: Various blast lesions caused by *Magnaporthe oryzae* in the field

Rice blast fungus:

The rice blast fungus can be found referenced in the literature under several names. *Pyricularia oryzae* was used to refer to the asexual stages of rice blast fungus as it was found in the field. The rice pathogen was morphologically indistinguishable from pathogens of other hosts, and the entire group was defined under the name *Pyricularia* (or *Piricularia) grisea* (Rossman 1990). The sexual stage was named *Magnaporthe grisea* until it was shown by phylogenetic analysis and inter-strain fertility tests that *Magnaporthe* isolates should be separated into species that infect *Digitaria spp.* (crab grass) (*M. grisea*), whereas *M. oryzae* collectively refers to the other characterised isolates, including the rice pathogen (Daniel 2007).

Classification of *Magnaporthe oryzae*:

Domain	:	Eukaryota
Kingdom	:	Fungi
Phylum	:	Ascomycota
Subphylum	:	Pezizomycotina
Class	:	Sordariomycetes
Order	:	Sordariomycete sincertae sedis
Family	:	Magnaporthaceae
Genus	:	*Magnaporthe*
Species	:	*oryzae*

Genome of *Magnaporthe oryzae:*

M. oryzae whole genome sequence is available and is the first fungal plant pathogen whose genome has been fully sequenced. With the availability of rice genome sequences published earlier in 2005, the new information about *M. oryzae* will help to develop better and cheaper methods of protecting the rice than currently available fungicides. The *M. oryzae* draft sequence includes more than 90 percent of the fungal genome representing 7-fold coverage with long continuous stretches of overlapping DNA.

Genome features: General genome feature	**Value**
Size (bp)	39,878,070
Chromosomes	7
(G+C) %	51.6
Protein coding genes	12,841
Percent coding	40.5
Average size (bp)	1,683
Average intergenic distance (bp)	1,503
Predicted protein sequences	
Conserved hypothetical protein	8,868 (79%)
Predicted proteins	2,233 (20%)

Infection cycle of *M. oryzae*:

To efficiently control the fungal infection, the path of infection and its molecular basis must be discovered and studied. The infection cycle of the fungus has been well understood though the complete molecular basis of pathogenesis still remains to be discovered.

Dispersal of Spores: The infection is spread by means of wind, water, or infected seeds. Even after dissemination from primary host factors such as rainfall, wind, and competition from other pathogens can result in displacement from the infection court (Mercure 1995). *M. oryzae* is able to infect and colonise the root also (Daniel 2007). Infested seed could initially lead to disease through root colonisation with subsequent lesion formation and aerial dispersal of conidia. Each lesion from a susceptible host can give rise to more than 20000 conidia over several days, serving as a source for secondary dispersal (Daniel 2007). The release of spores is an active process. The small stalk cell formed at the base of conidia builds turgor pressure until it ruptures, thus releasing spores.

Spore attachment mechanisms: In *M. oryzae* moist air or dew is required for hydration and extrusion of spore tip mucilage, which serves to attach the conidium by its apex (Fig. 2) to the hydrophobic plant surface (Sara 2001). Adhesion in *M. oryzae* is a passive process involving the release of a preformed adhesive, α-linked-mannosyl and glucosyl residues in addition to protein and lipid components. The adhesives are immediately released upon hydration and allow attachment to leaf surface without any expenditure of energy.

Formation of germ tube: After an hour of landing on leaf surface conidia gives rise to a narrow germ tube. The germ tube can emerge from the apical cell or from sub-apical cell of the conidia

but only one germ tube develops in to a mature appressoria. Hydration stimulates the process of germination. Hydrophobin rodlet Mpg1 is expressed during germination (Sara 2001). Hydrophobins self assemble to form monolayers of rodlet proteins, with one side being hydrophobic and the opposite side being hydrophilic. On an extremely hydrophobic surface, such as a rice leaf, the Mpg1 hydrophobin may contribute to strong attachment to the leaf surface by providing an adaptor surface between the hydrophilic surface of the germ tube and the hydrophobic rice leaf. Adhesion of germ tubes to the substrate involves synthesis of an adhesive extracellular matrix produced during germ tube growth (Daniel 2007).

Appressorium formation: Within four hour of landing to the leaf surface, spore start germinating and swell at its apex. The tip of germ tube flattens against the rice leaf surface. The germ tube apex develops into a swollen melanised dome-shaped cell, called the appressorium (Wang *et al*. 2005). The mitosis is always observed in the germ tube after spore germination and before appressoria formation, it is believed that morphogenesis of appressoria is tightly coupled with cell cycle.

Induction by a set of cues like surface hardness, hydrophobicity, cuticular wax and absence of exogenous nutrients gives rise to the development of an appressorium (Talbot 2003, Gilbert *et al*. 2006, Wang *et al*. 2005, Ebbole 2007). Soon after the fungal spore germinate and attaches the leaf surface the cAMP pathway is triggered which signals the germ tube to develop into appressorium. MAP kinase (mitogen- activated protein kinase) cascade is also required for the development and maturation of appressoria (Wang 2005). Cell cycle and various signalling pathways govern the progression of appressorium development in *M. oryzae* during leaf

infection. Mitosis is a prerequisite for appressorium development in *M. oryzae* (Veneault-Fourrey *et al*. 2006) and three cell cycle check point govern the appressorium-mediated plant infection (Saunders *et al*. 2010). Morphogenesis of appressoria in *M. oryzae* is tightly regulated by cell cycle, cAMP and protein kinaseA (Choi and Dean 1997, Xu and Hamer 1996, Adachi and Hamer 1998, Thines *et al*. 2000). Involvement of a G-protein–coupled receptor, *Pth11*, and cognate G-α and G-βγ-subunit proteins has been reported in appressorial development (Wilson and Talbot 2009). MAP-kinase pathway proteins, Mst11, Mst7 and Pmk1 proteins are necessary for the appressorium formation and subsequently invasive growth of appressoria (Park *et al*. 2006, Zhao *et al*. 2005, 2007a, 2007b).

After maturation, appressoria separated from germ tube and conidia by a thick layer of melanin which is formed in the cell wall of appressoria. Melanin is necessary to maintain turgor pressure by providing an effective barrier to solute movement. Water can diffuse across the melanin layer, but the melanin serves as a semi permeable barrier that prevents ions and other small molecules from moving into or out of the cell.

Appressoria is generally formed at the leaf surface in presence of water droplet and due to rapid influx of water in the cell a high turgor pressure is generated in the appressoria. Water is the pre-requisite for the appressoria germination and it is also required for the turgor generation to breach the plant cell wall. The movement of water in the appressoria is against the solute gradient. The accumulation of compatible solute (e.g. glycerol) in the appressoria is the reason for the high concentration gradient. Appressorial turgor generation is accompanied by *CPKA* (**c**atalytic subunit of **p**rotein **k**inase A) and *PMK1* (mitogen activated protein kinase 1) dependent

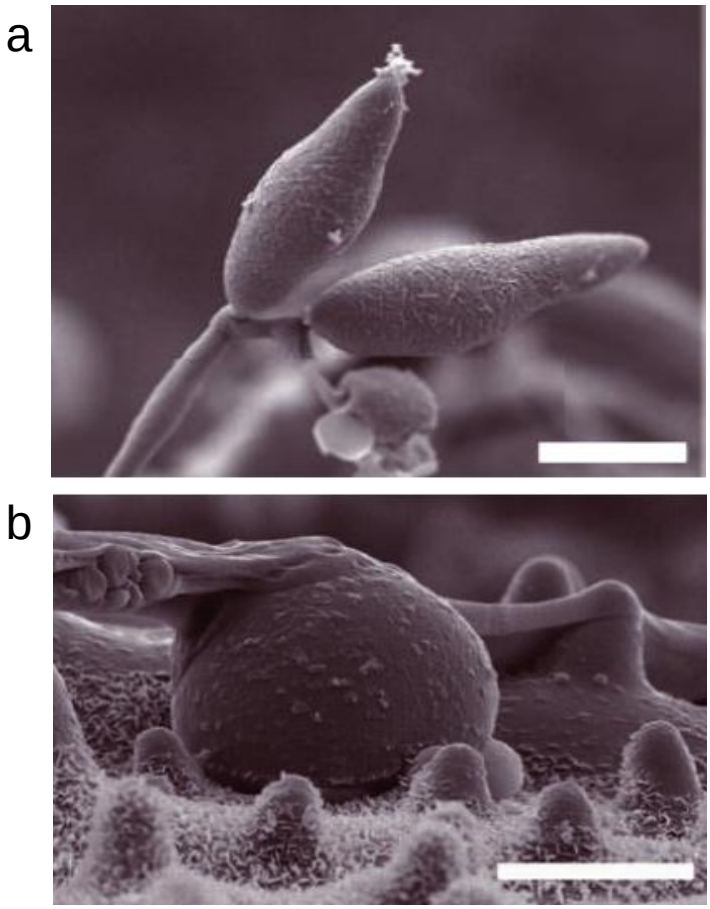
mobilisation of carbohydrate and lipid reserves, which accumulate in developing appressoria before being degraded rapidly in mature cells prior to plant infection (Thines *et al*. 2000, Weber *et al*. 2001). Carbohydrates and lipids stored in the conidium are mobilised to the appressorium. Subsequent breakdown of the lipid and carbohydrate stores results in a large increase in solute concentration inside the appressorium. Biochemical analysis of *M. oryzae* appressoria demonstrated that accumulation of glycerol in the cell is notably high up to 3M in concentration. Since the glycerol cannot escape the melanin layer, tremendous turgor pressure is generated when water is available. This turgor pressure has been measured and is generally accepted to be approximately 80 atmospheres of pressure (Daniel 2007).

To coordinate the process with metabolism, trehalose disaccharide is accumulated in appressoria. Trehalose may play a regulatory role in metabolism that interferes with proper carbon metabolism such as gluconeogenesis that may be important for accumulation of glycerol in the appressorium. Lipid metabolism also plays a role in providing a source for glycerol synthesis. At the early stage of germ tube emergence and appressoria development deposition of lipid bodies takes place at the tip of incipient appressoria. Subsequently, during appressoria maturation all the lipid bodies internalised in to the vacuoles resembling a process known as autophagy. Triacylglycerol lipase activity is induced by cAMP mediated signalling pathway during maturation. The activity leads to fatty acid breakdown that yield acetyl-CoA that could generate glycerol from the glyoxylate cycle and gluconeogenesis. Glycogen breakdown also contributes to the synthesis of glycerol. Glycogen is present in conidia and is rapidly broken down during germination (Daniel 2007).

Formation of Penetration peg: The plant infection through appressorium proceeds via generation of narrow penetration peg from a pore at the base of appressorium (Fig. 3). The pore is without a wall and the fungal plasmalemma attached to the plant surface. Prior to penetration peg formation, a double layered appressorium pore cover forms and the peg than emerges into the substratum bound by a single cell wall layer. The peg contains numerous microtubules and microfilaments, and cytoskeletol rearrangement is a key step in peg formation. Cuticle penetration results from a sustained application of physical force. The *punchless* (*PLS1*) gene encodes a tetraspanin-like protein that is required for penetration peg development. Tetraspanins are membrane proteins and associates with other membrane protein such as integrins. This provide mechanical force at the appressorium pore and orchestrates network of actin protein at the site of peg emergence. It also plays a role in integrin mediated attachment and acts as a signal transduction molecule (Talbot 2003). A MAPK is required for the regulation of penetration peg formation called as *MPS1* (Fig. 4). The *MPS*1, also regulates cell wall biosynthesis at the emergence of the penetration peg and its downstream effectors include a large number of morphogenic proteins required to synthesise a functional penetration peg.

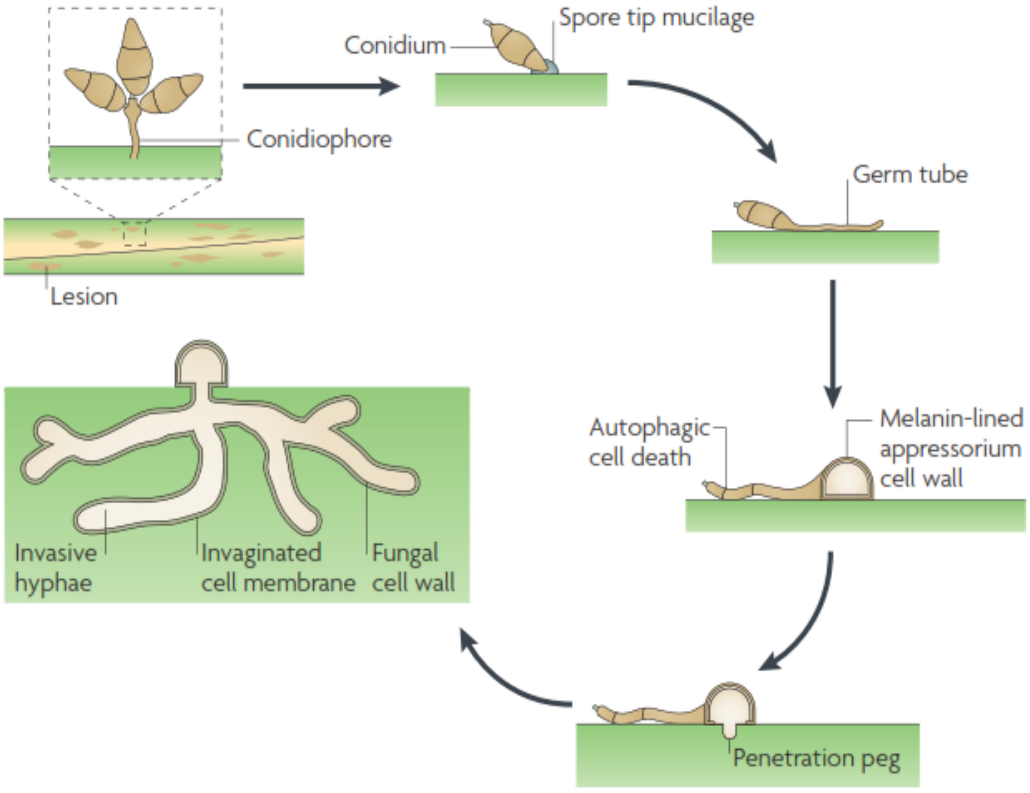
Invading the Host: The penetrating hyphae differentiate into a series of bulbous branched infectious hyphae soon after plant infection. After filling the initial epidermal cells, longer, cylindrical hyphae ramify out into adjacent cells and the leaf tissue is severely affected. An ATP-driven efflux pump (*ABC*1) gene is involved in the transport of various substrates and critical in pathogenesis. It is induced by a number of metabolic poisons and antimicrobial agents, including

a rice phytoalexin, thus providing tolerance to plant defence compounds produced against pathogenic fungi. (Talbot 2003).



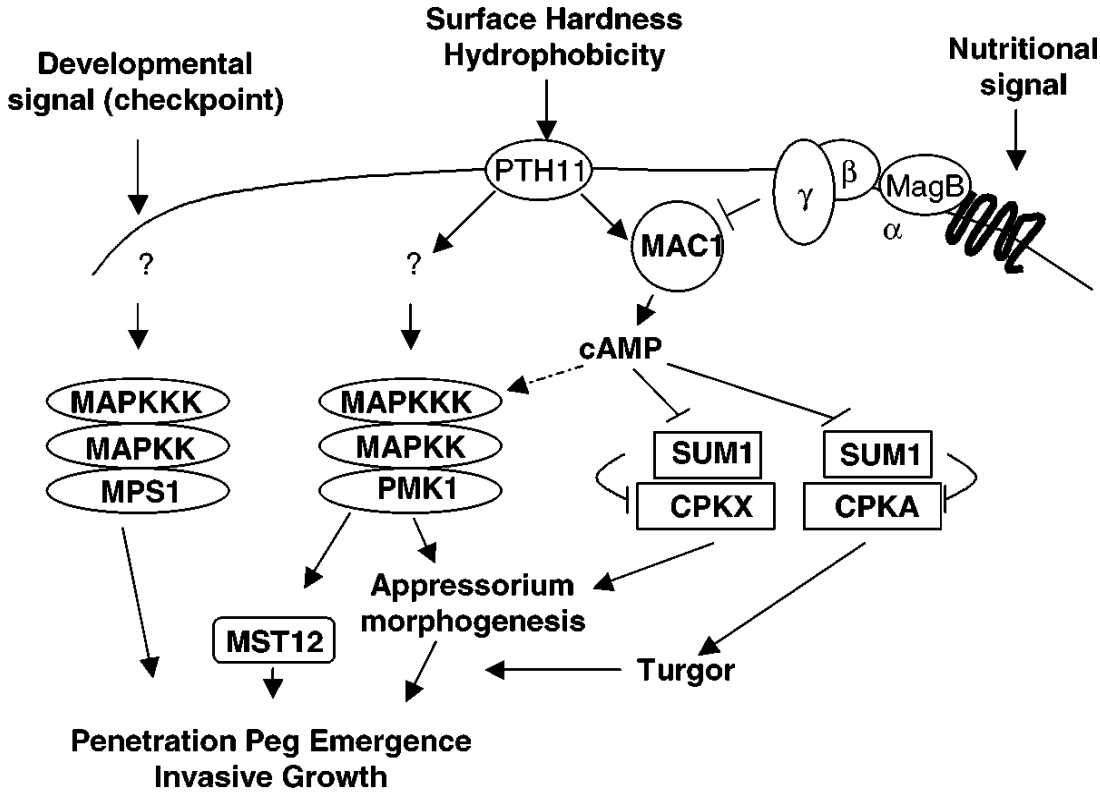
(Wilson and Talbot, 2009)

Figure 2: (a) Scanning electron micrograph of conidia of *M. oryzae* and **(b)** a dome-shaped appressorium on the rice leaf surface



(Wilson and Talbot, 2009)

Figure 3: Infection cycle of *M. oryzae*



Talbot et al. 2003

Figure 4: Model for signal transduction pathways that operate to regulate appressorium morphogenesis and penetration peg formation

***Magnaporthe oryzae* as a model organism:**

M. oryzae is an excellent model organism to study fungal pathogenicity and host-pathogen interaction with the availability of its whole genome sequence. The majority of fungal pathogens belong to its taxonomic class and exist as related asexual forms. *M. oryzae* is also related to the non-pathogenic fungus *Neurospora crassa*, a leading model organism for studying eukaryotic genetics and biology. Unlike mildews and rust of rice, *M. oryzae* can be cultured on defined and minimal media. This facilitates the biochemical and molecular analysis of the fungus manifold. *M. oryzae* has been utilised as a model fungal pathogen for understanding the molecular basis of plant-fungus interactions due to its genetic and molecular tractability (Talbot *et al*. 1995). It is a filamentous ascomycete fungus that is heterothallic. The presence of two mating alleles MAT-1 and MAT-2 in *M. oryzae* provide the opportunity to study the diploid stage of its life cycle. When fertile isolates of opposite mating types are paired together on an appropriate growth medium (oatmeal agar) and at low temperature (20°C), within 21 days they will form sexual fruiting bodies called as 'perithecia'. Perithecia are flask-shaped sexual fruiting bodies and carry asci-bags filled with ascospores (Talbot *et al*. 2003). The important feature which makes *M. oryzae* as model organisms are:

1. *M. oryzae* is not an obligate parasite and can be grown in a variety of culture media. Due to the availability of fertile strains, studies on sexual cycle under laboratory conditions have become possible.
2. Various genomic and cDNA libraries are available and genetic maps with the availability of genome sequence of the fungus have been developed.

3. Transformation protocols have been developed and various auxotrophic and drug resistance markers are available.
4. The formation of infection related structures (appressoria, germ tubes and infection hyphae) can be followed *in vitro* using artificial surfaces and plant surfaces.
5. Infection can be carried out in a variety of hosts.
6. Availability of complete genome sequence (Dean *et al*, 2005).

To study genes involved in the infection cycle of this fungus might prove valuable in using them as potential drug targets to control the disease. But to use such targets for antifungal drugs it must be assured that they are absent in plants and animals so as to avoid harmful side effects. Such targets are identified by sequence alignments, using tools of bioinformatics.

Molecular Approaches to study gene function

Functional genomics

Functional genomics can be referred as the characterisation of a gene functions by developing a genome wide experiment and application of entire nucleotide sequence information of the organism provided by structural genomics. This is the combination of large scale experimental methodologies and computational analysis of the results (Hieter and Boguski 1997). Compared to classical investigation of a single gene and its involvement in the development of the organism, modern functional genomics approaches examine a large number of gene at a time and its expression as a function of development. With the availability of whole-genome sequences of

M. oryzae, there is an increasing need for large-scale analysis to determine the function of thousands of genes.

With the progress in structural genomics, *in silico* approach to identify a large number of genes is the major undertaking in fungal genomics. **Reverse genetics** approaches, which have been widely employed to study the infection strategies of necrotrophic and hemibiotrophic fungal pathogens at the molecular level. A significant contribution have been made by reverse genetic approach to unravel the molecular mechanisms essential for fungal development (sporulation, conidia germination, infection structure formation or appressorium morphogenesis and penetration), fungal nutrition (nutrients uptake e.g., phosphorus and iron from the host environment) and the plant-pathogen interactions (compatible or incompatible interactions) between fungi and their host plants. Approaches like expressed sequence tags (EST) sequencing (Kamakura *et al*. 1999; Kim *et al*. 2001; Rauyaree *et al*. 2001), serial analysis of gene expression (SAGE) (Irie *et al*. 2003), microarray (Takano *et al*. 2003) and protein analysis (Kim *et al*. 2004) have helped in analysing the genes responsible for pathogenicity at large scale.

Reverse Genetics Approaches

1. **Targeted gene disruption/ Knock-out**

   Targeted gene disruption is a powerful approach for dissecting gene function in phytopathogenic fungi. This approach allows the study of phenotypes of the various mutants where the genomic locus has been changed either by gene insertion or by gene replacement with a transforming DNA fragment (Timberlake and Marshall 1989, Wendland *et al*. 2003).

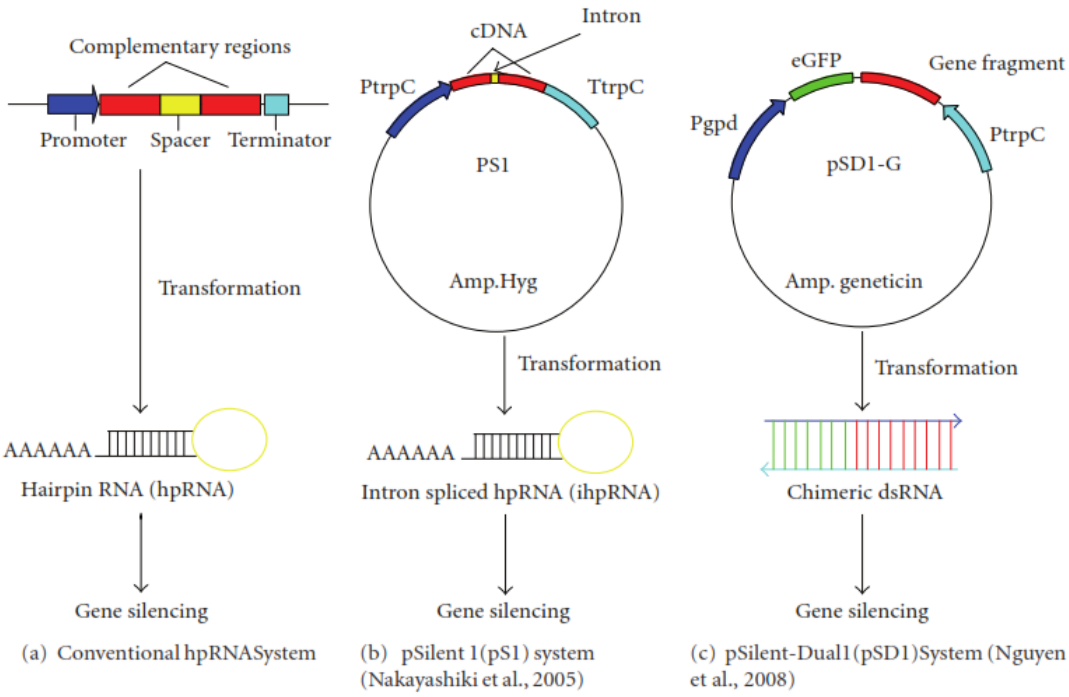
These reverse genetic approaches have become more straightforward since increasing numbers of genomic information have become available to a large number of phytopathogenic species. The targeted gene disruption or gene replacement enables inactivation of the gene by homologous DNA recombination. The homologous recombination results in a reciprocal exchange of DNA sequences found in between the two chromosomes carrying the same genetic loci. The homologous recombination between the target gene and mutated plasmid DNA allele results in to a targeted knock-out of the gene. The knock-out approach was first established for the *Saccharomyces cerevisiae* to study gene function (Scherer and Davis 1997). Now it is the best choice of techniques to study several phytopathogenic fungi including *M. oryzae*.

A major benefit of gene knock-out is its preference to target a specific genetic region. However, due to the time required for the construction of replacement vectors and the poor efficiency of homologous recombination due to nonhomologous (ectopic) integration, the significance of this approach is restricted. The majority of fungi consist of multinuclear hyphae, and some of them have heterokaryon. Heterokaryon is the condition where cytoplasm contains two or more genetically different nuclei. These characteristics of fungi make gene targeting difficult and inefficient.

2. RNA interference (Knock down)

Post-transcriptional gene silencing in plants by RNA interference and RNAi in animals have been used for decade for reverse genetics (Waterhouse *et al*. 1998). The double-stranded RNA (dsRNA) is responsible for the degradation of a specific homologous mRNA, thereby

diminishing the gene expression. The RNAi was first discovered in *Caenorhabditis elegans* as a response to double stranded RNA, and further resulted in sequence-specific gene silencing (Fire *et al*. 1998). The generation of dsRNA is a prerequisite of this technique. The dsRNA molecules act as a template for Dicer ribonuclease III enzyme which produces small interfering RNAs (siRNAs). Further these siRNAs are incorporated into a silencing complex known as RNA-inducing silencing complex (RISC). There they serve as sequence-specific guide RNA which target specific corresponding mRNA molecules for destruction (Hammond *et al*. 2001, Hannon *et al*. 2002). In the last decade, RNA-mediated post-transcriptional gene silencing methods have been identified in a small number of economically important phytopathogens like *M. oryzae* (Kadotani *et al*. 2003, Nakayashiki *et al*. 2008) and *P. infestans* (West *et al*. 1999, Latijnhouwers *et al*. 2004, Whisson *et al*. 2005). As a matter of fact the antisense RNA, dsRNA or sense transgene are more commonly used in the functional biology study. The silencing using dsRNA and sense transgene in plant is called as co-suppression where as in fungi it is known as quelling. RNAi has became an important tool in functional biology and has been extensively used for the elucidation of the function of many unknown genes and also for the identification of genes essential for pathogenic development (DeBacker *et al*. 2002). The application of RNAi in phytopathogenic fungi is limited to a few species. There are three RNAi strategies that have been used in functional analysis of phytopathogenic fungi to the date. The conventional hairpin RNAi, introns-spliced hairpin RNAi and the chimeric double stranded RNA mediated silencing (Fig. 5).



Bhadauria et al. 2009

Figure 5: Plasmid based RNAi strategies applied in *M. oryzae*

3. Insertional mutagenesis

Insertional mutagenesis is a potential tool to investigate molecular mechanism in phytopathogens. The main advantage of this technique is that it does not require prior information about genome sequence of the organism. Insertional mutagenesis therefore can be applied to several phytopathogenic fungi, whose genomes have not been sequenced yet. Classical genetic analysis approaches, chemical mutagens or ultraviolet light have provided a huge amount of information on pathogenic development. A large number of mutagens generate base pair deletions or substitutions. Genetic analysis of mutant strains is time consuming because of laborious isolation of the mutated gene from genomic DNA library of wild type strain by complementation assays. The use of a selectable marker in the transforming plasmid DNA is necessary to isolate the gene of interest and to establish link between the insertion and the observed phenotype. It also helps in recovering the mutated allele for cloning and subsequent sequence analysis (Brown and Holden 1998). Thus, the use of insertional mutagenesis and chromosomal integration with the plasmid DNA has been increase. The plasmid DNA based chromosomal integration performed by two most accepted techniques known as *Agrobacterium tumefaciens*-mediated transformation (ATMT) and restriction enzyme mediated integration (REMI).

Agrobacterium tumefaciens-mediated transformation is a well established molecular techniques during the recent years. Transformation in filamentous fungi results in a heterologous integration in to the genome because the transforming plasmid DNA does not show any homology with the fungal genome. This transforming DNA can be used as an

insertional mutagen to disrupt genes (Mullins *et al*. 2001, Michielse *et al*. 2005, 2008). The exploitation of ATMT has been continues for transforming a large number of phytopathogenic species, such as *Botrytis cinerea* (Rolland *et al*. 2003), *Colletotrichum gloeosporiodes* (deGroot *et al*. 1998), *F. oxysporum* (Khang *et al*. 2005), *M. oryzae* (Khang *et al*. 2005, Rho *et al*. 2001, Jeon *et al*. 2007), *Mycosphaerella graminicola* (Zwiers and Waard. 2001), *Venturia inaequalis* (Fitzgerald *et al*. 2003), *Pythium ultimum*, and *Phytophthora* spp. (*P. infest ans* and *P. palmivora*) (Vijn and Govers 2003). Recently, ATMT mutant library of the *M. oryzae* KJ201 have been generated to identify pathogenicity genes in *M. oryzae* (Jeon *et al*. 2007).

The REMI technique was developed for *S. cerevisiae* (Schiestl and Petes 1991). REMI has also been used in pathogenic fungi where a linearised vector DNA mixed with fungal protoplast and is integrated into fungal genome in the presence of a unique restriction enzyme (RE). The restriction enzyme generates double stranded breaks in the genome after in the nucleus. The ends of these breakages recombine with the linearised plasmid, integrating the vector into the genome at the recognition site of the RE (Riggle and Kumamoto 1998). Although REMI is an efficient tool for tagging and cloning various pathogenicity genes from pathogens, a significant number (20 to 100%) of mutants appears to be untagged. In spite of this, REMI has been used to mutate several phytopathogenic species, including *M. oryzae* (Shi *et al*. 1995, Sweigard *et al*. 1998, Mitchell and Dean 1995), *Cochliobolus heterostrophus* (Yang *et al*. 1996), *U. maydis* (Bolker *et al*. 1995),

Colletotrichum spp. (*C. lindemuthianum* and *C. graminicola*) (Redman and Rodriguez 1994) and *Pyrenophora teres* (Maier and Schafer 1999).

4. TILLING

Targeting Induced Local Lesions In Genomes (TILLING) was introduced (McCallum *et al*. 2000) as a new reverse genetic strategy for plants that combines the efficiency of ethyl methanesulfonate (EMS) induced mutagenesis with denaturing high-performance liquid chromatography (DHPLC) which utilise heteroduplex analysis to detect base pair changes like G/C to A/T transition. The specific mutants of *Phytophthora* spp have been generated using TILLING by constructing a library of 2400 mutants of *P. sojae* by ethylnitrosourea (ENU). These mutants have been screened for induced point mutations in the genes which encode a necrosis-inducing protein (*PsojNIP*) and a *Phytophthora*-specific phospholipase D (*PsPXTMPLD*) protein (Lamour *et al*. 2006). Chemical mutagenesis like EMS treatment causes a high mutation frequency without any noticeable preferences for specific genomic regions. Using this method a large number of alleles can be generated, facilitating the recovery of null phenotypes (Meinke *et al*. 1995). Although the development of novel mutagenesis techniques may ultimately make TILLING outdated, at present, but for medium to high-throughput reverse genetics analysis TILLING remains the technique of choice (Gilchrist and Haughn 2005).