Chapter 3

Results:

Isolation and characterization of Indian field strains of the blast fungus

3.1 Collection and isolation of *Magnaporthe* field strains from various host plants

3.1.1 Collection of blast infected tissues from various geographic locations across India

Magnaporthe oryzae is known to cause blast disease in over 50 grass species and it could infect all aerial parts of the crop plants. We collected the blast disease infected host tissues from various hosts like rice, millets and grasses and from different plant parts such as leaf, neck and panicle/finger in case of rice/millet respectively **(Fig. 3.1)**. Blast disease symptoms are often visible on the onset of monsoon completion i.e. late September-October months in India, considering the high humidity as a major factor for disease occurrence and further development. Thus, we collected most of the samples during the aforementioned time period. Sampling sites included blast hotspots such as Sikkim and the Dangs region of Gujarat, where blast occurs frequently on different hosts **(Table. 3.1)**. In total, we were able to collect the blast infected tissues from 15 different geographical locations across India **(Table 3.1, Fig. 3.2)**.

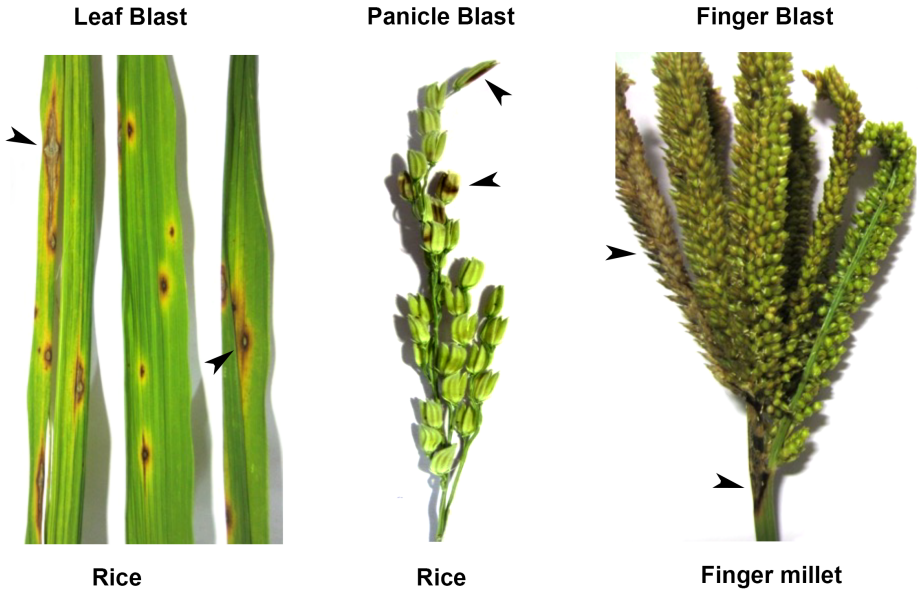


Figure 3.1: Representative images of blast disease infected host tissues. The arrowheads display the infection lesions on different parts – leaf, neck and panicle/finger - of the plants.

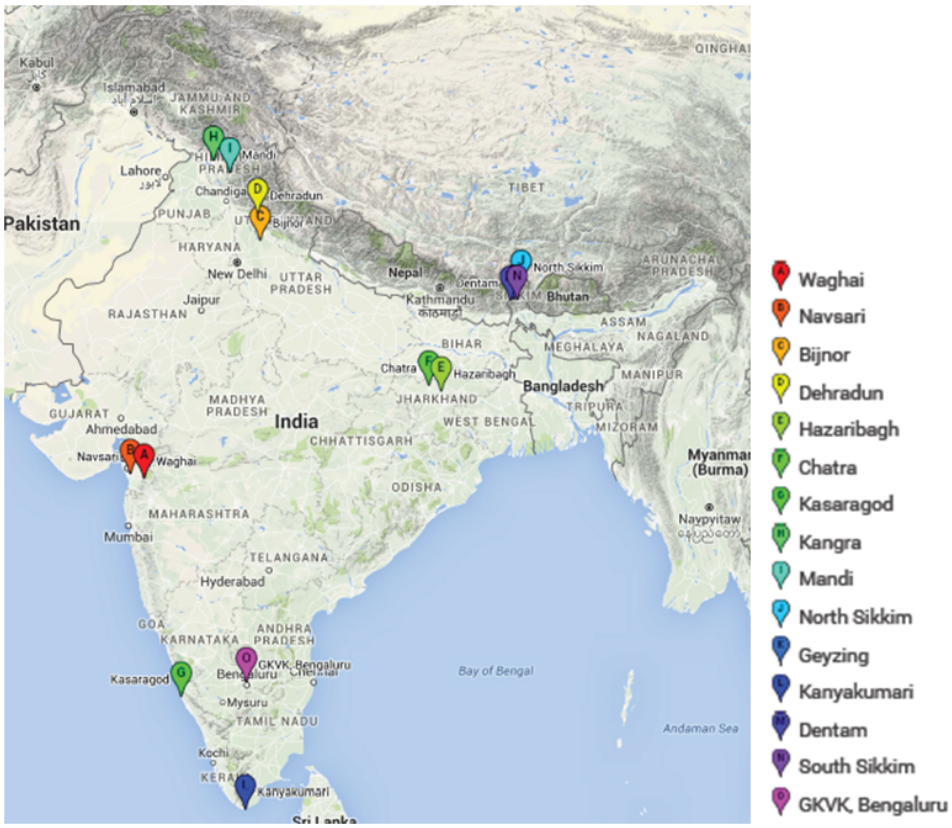


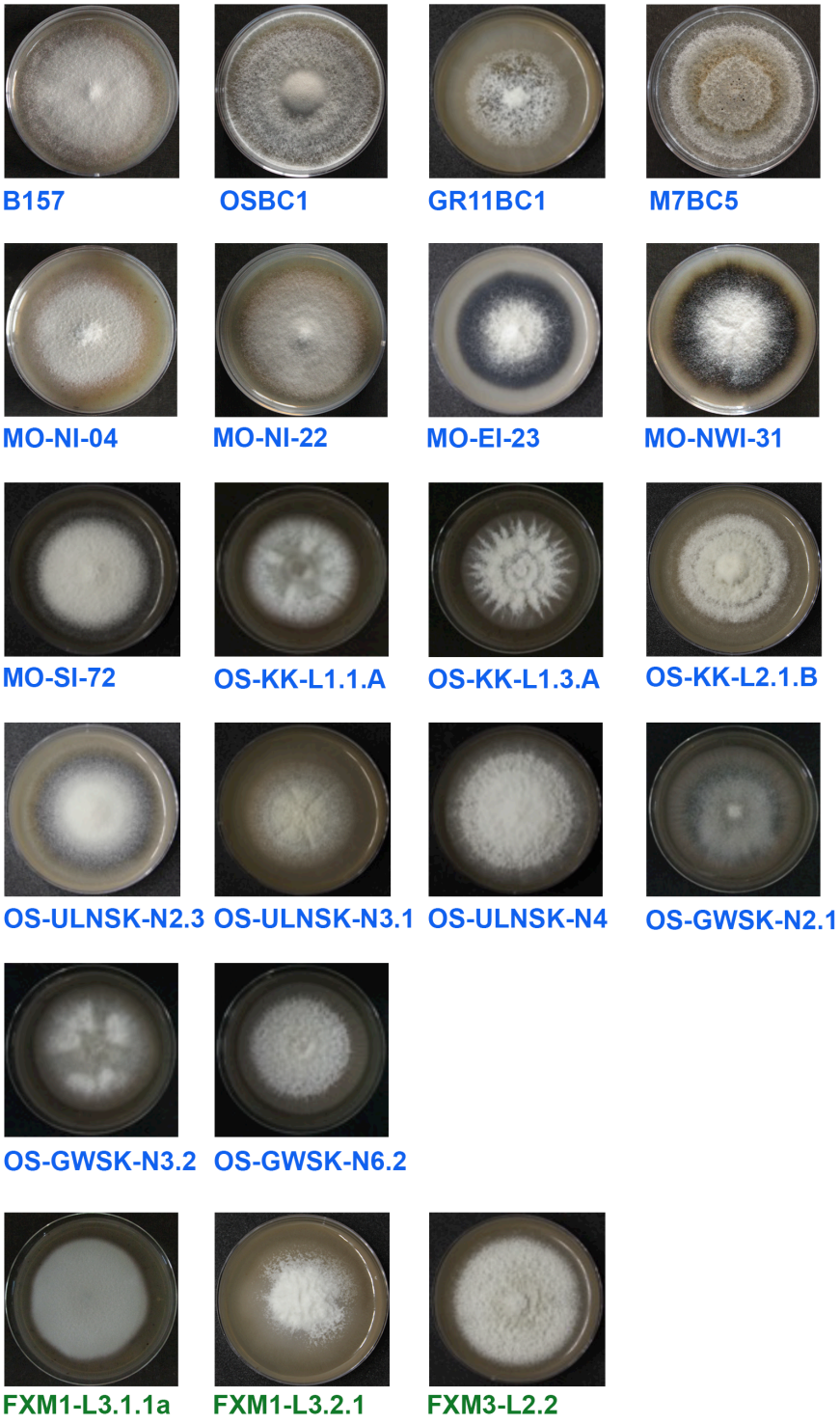
Figure 3.2: Sampling sites of blast disease in the current study. The sampling sites are depicted as locations pins on 15 different geographic locations across India.

3.1.2 Mono-conidial isolation of *M. oryzae* field strains from infected plant materials

Pure fungal cultures are fundamental to the identification of taxa as well as phenotypic characterization such as culture morphology, mating type, degree of virulence and host-specificity. Thus, all the collected samples were subjected to mono-conidial isolation technique to obtain the pure fungal isolates. **Table 3.1** summarizes the total number of field strains isolated from infected plant tissues. Colonies obtained as a result of mono-conidial isolation were different in terms of mycelial morphology, melanization content and aerial mycelial growth **(Fig. 3.4)**. Further, some colonies obtained from the same lesion often showed distinct morphologies. Isolates in panel L5 **(Fig. 3.3)** show differences in colony morphology and growth as well. Some other isolates such as GPU67 panel also showed

Table 3.1: List of *Magnaporthe* isolates collected from different geographic locations

Hosts	Geographic locations	Strains	Infection type	No. of Isolates
Rice	Bijnore, UP	Mo-ni-004	Leaf blast	1
	Dehradun, UT	Mo-ni-022	Leaf blast	1
	Hazaribag, JH	Mo-ei-23	Leaf blast	1
	Chatra, JH	Mo-ei-48	Leaf blast	1
	Kasaragod, KL	Mo-si-72	Leaf blast	1
	Kasaragod, KL	Mo-si-74	Leaf blast	1
	Kangra, HP	Mo-nwi-31	Leaf blast	1
	Mandi, HP	Mo-nwi-209	Leaf blast	1
	Dangs, GJ	OSBC1	Leaf blast	1
	Dangs, GJ	GRIIBC1	Leaf blast	1
	Dangs, GJ	M7BC5	Leaf blast	1
	Navsari, GJ	NvsR 6029-2	Leaf blast	1
	Upper Linghten, SK	OS-ULNSK-N	Neck blast	5
	Gyalsing, West SK	OS-GWSK-N	Neck blast	6
	Kanyakumari, TN	OS-KK-L	Leaf blast	10
Finger Millet	Dangs, GJ	GNBC8	Leaf blast	1
	Dangs, GJ	FMGPU 45-1	Leaf blast	1
	Dangs, GJ	Millet BC1	Leaf blast	1
	Dangs, GJ	Millet BC2	Leaf blast	1
	Waghai, Dangs, GJ	WWN25	Leaf blast	13
	Waghai, Dangs, GJ	MR6	Leaf blast	14
	Waghai, Dangs, GJ	L5	Leaf blast	4
	Waghai, Dangs, GJ	PR202	Leaf blast	11
	Waghai, Dangs, GJ	GPU48	Leaf blast	5
	Waghai, Dangs, GJ	GPU67	Leaf blast	8
	Dentam, SK	EC-DWSK-F	Finger blast	7
	South Sikkim, SK	EC-SSK-N	Neck blast	8
	Bangalore, KA	EC-GKVK-L	Leaf blast	3
	Bangalore, KA	EC-GKVK-F	Finger blast	9
	Bangalore, KA	EC-GPU66-GKVK-N	Neck blast	3
Wild Ragi	Bangalore, KA	WR-GKVK-L	Leaf blast	2
Foxtail Millet	Bangalore, KA	FXM1-GKVK-L	Leaf blast	3
	Bangalore, KA	FXM3-GKVK-L	Leaf blast	4
Grass	Bangalore, KA	G-GKVK-L	Leaf blast	2
Total				**133**


B157
OSBC1
GR11BC1
M7BC5
MO-NI-04
MO-NI-22
MO-EI-23
MO-NWI-31
MO-SI-72
OS-KK-L1.1.A
OS-KK-L1.3.A
OS-KK-L2.1.B
OS-ULNSK-N2.3
OS-ULNSK-N3.1
OS-ULNSK-N4
OS-GWSK-N2.1
OS-GWSK-N3.2
OS-GWSK-N6.2
FXM1-L3.1.1a
FXM1-L3.2.1
FXM3-L2.2

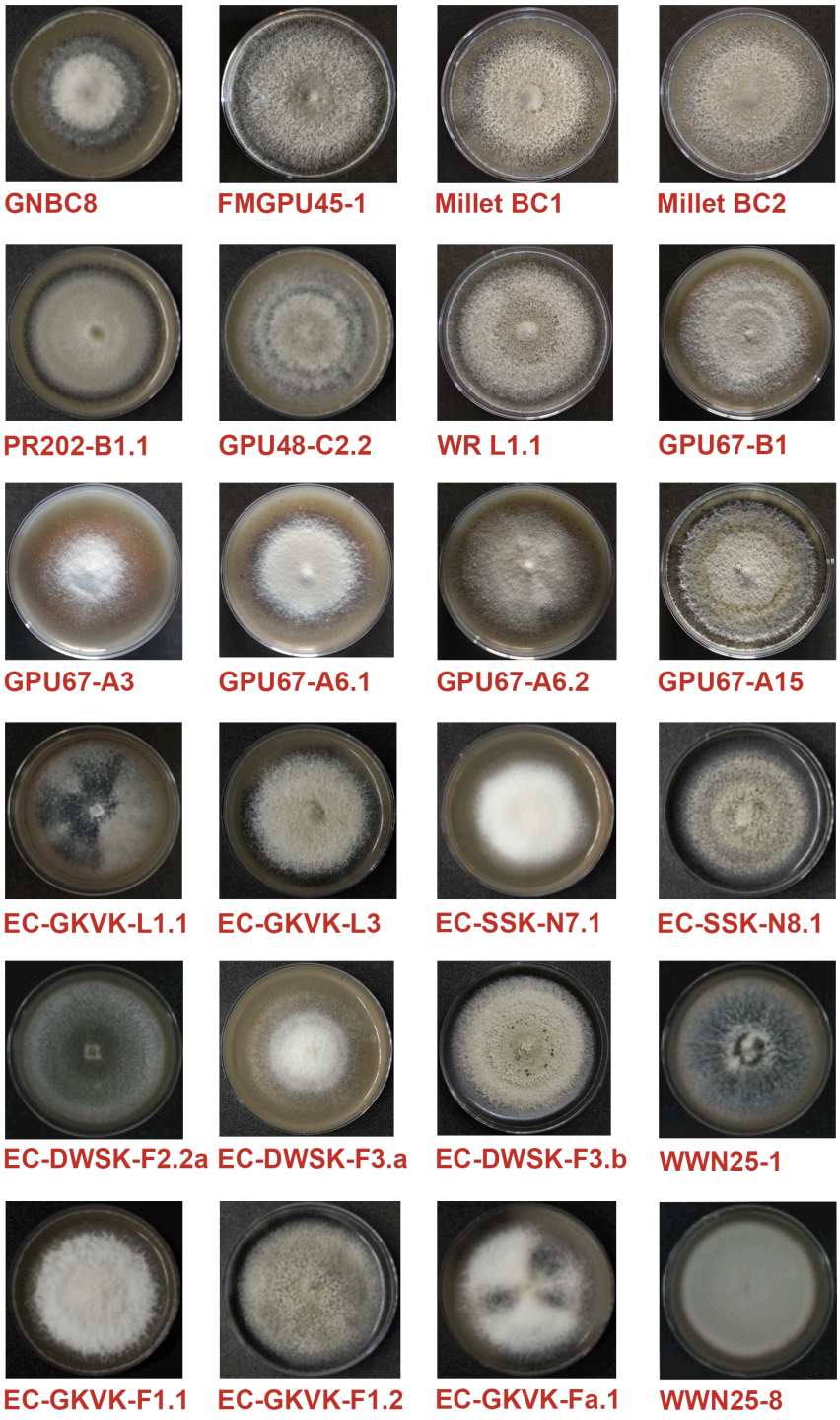


Figure 3.3: Colony morphology of different strains of *M. oryzae*. The labels of each strain are color-coded according to their host of origin (rice-blue, finger millet – red and foxtail millet – green).

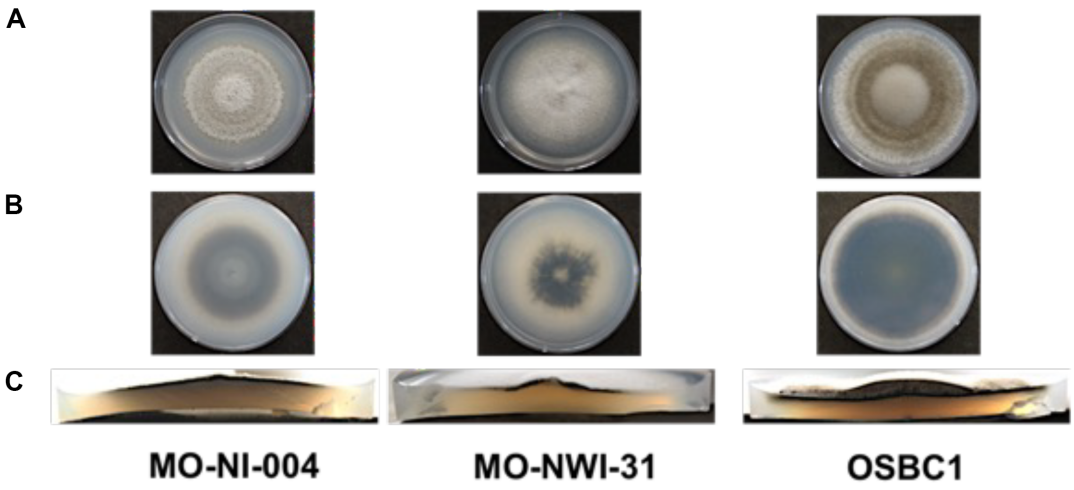


Figure 3.4: Colony characteristics of representative strains of *M. oryzae*. Variations in (A) colony morphology, (B) melanization content and (C) aerial mycelial growth.

Differences in terms of mycelial colour and sectoring formation. Colonies obtained from the same lesion often showed distinct morphologies even upon repeated subculturing. It is worth studying how such morphological plasticity exist in the clonal population being isolated from the same lesion.

3.1.3 Establishment of *Magnaporthe* strains repository

Long-term stocks were prepared from a total of 133 *M. oryzae* strains collected in the current study. It is important that the cultures are sporulating in order to maintain the virulence of these strains for long-term. The cultures were grown on oat meal agar plates containing filter paper discs for 8-10 days in continuous light conditions to allow sporulation. Upon completion of the incubation, filter papers covered with fungal biomass were appropriately desiccated and placed in vacuumized poly bags and stored at -80 °C.

3.2 Diversity assessment using molecular markers and phenotypic characterization

3.2.1 Molecular characterization of field strains of *M. oryzae*

We believe that study of fungal diversity from various cultivation zones is an important step towards understanding the disease at the field level. Detailed information about the genetic diversity of *Magnaporthe* strains prevalent in different cultivation zones would provide a better understanding of the variation in pathogenicity factors, avirulence genes, mating type and repetitive sequences, and consequently can provide insights into the origin, evolution and spread of strains across hosts. Further, isolates obtained provide a resource for further research on the virulence factors underlying the mechanism behind adaptation to hosts, facilitating the selection of resistant crop varieties. Molecular analysis may also throw up diagnostic molecular markers associated with virulent strains or host specificity.

3.2.1.1 DNA fingerprinting using repetitive elements (Pot2 & Mg-SINE)

One of the objectives of this study was to use Pot2 and Mg-SINE Rep-PCR fingerprinting to determine the genetic structure of populations of *M. oryzae*. Pot2 Rep-PCR fingerprinting has been found to be reliable, reproducible, and highly discriminatory for assessing diversity in large collections of *M. oryzae* strains (George et al., 1998). Pot2 and Mg-SINE both are known to be present in ~100 copies per haploid genome in rice and non-rice isolates (Kachroo et al., 1994, 1995). Presence of both, Pot2 and Mg-SINE in a population, is strong indication of common ancestry of isolates before the divergence of rice and non-rice pathogens.

Further, we investigated the genetic relatedness of the *M. oryzae* field strains based on the fingerprinting pattern based on Pot2 and Mg-SINE Rep-PCR **(Fig. 3.5 and Fig. 3.6)**. We found that the strains isolated from the same cultivars of the host plant showed the exact same pattern of transposon distribution. In contrast, the pattern of fingerprinting differed among the strains belonging to different host plants, even if the host plants were grown besides each other at the same geographic location. While the geographic locations could be one of the factors shaping genetic architecture of the field isolates of *M. oryzae*, our observation suggests that it was likely influenced more significantly by the host plants/cultivars they adapted to.

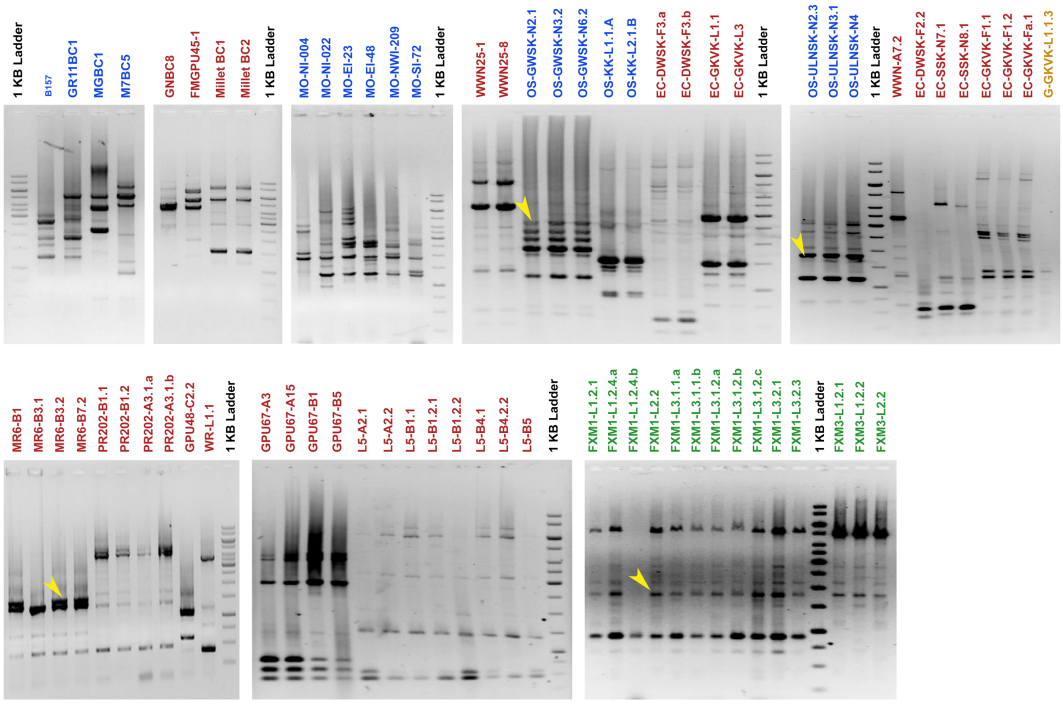


Figure 3.5: Molecular fingerprinting based on Pot2 Rep-PCR. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Yellow arrowheads display the representative amplified genomic region between any two Pot2 elements in a given strain.

3.2.1.2 Presence/Absence polymorphisms in Avirulence genes

A systematic analysis of the distribution of AVR genes across diverse isolates of *M. oryzae* is required to understand how the repertoire of effectors encoded by individual strains might be related to their ability to infect certain specific host plants. Avirulence genes in *Magnaporthe* are recognized specifically by the corresponding resistance (R) genes in its host species. So far, 85 blast *R* genes have been identified from rice but only nine AVR genes have been identified from the fungal pathogen. We studied the presence/absence variations in AVR genes (AVR-Pita, ACE1, AVR1-CO39, AVR-Pik and AVR-Pizt) and host specificity factor (PWL2), were evaluated using gene-specific PCR amplification in field strains of *M. oryzae* isolated from rice, finger millet, foxtail millet and grass host plants.

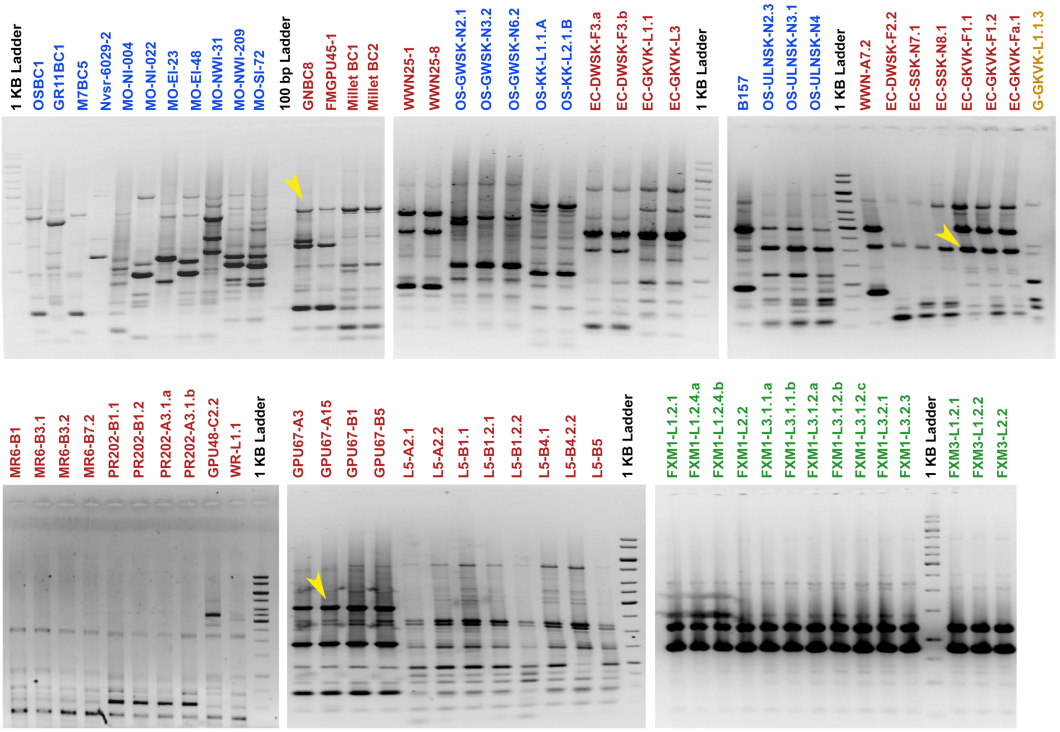


Figure 3.6: Molecular finger printing based on Mg-SINE Rep-PCR. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Yellow arrowheads display the representative amplified genomic region between any two Mg-SINE elements in a given strain.

The AVR-Pita gene, encodes a putative zinc metallopeptidase and located in the telomeric region. AVR-Pita suppresses pathogen-associated molecular pattern (PAMP)-triggered immune (PTI) responses and a key regulator of mitochondrial reactive oxygen species in rice (Han et al., 2021). AVR-Pita has been reported to be found in various *M. oryzae* isolates from wheat, millets and other related species in addition to rice. This gene is highly diverse and confers variability in the virulence via rapid changes such as deletions, transposon insertions, point mutations. We were able to identify its presence in a few of the rice infecting strains (MO-NI-004, MO-NI-022, MO-EI-23, MO-EI-48) and all the strains from foxtail millet **(Fig. 3.7)**.

Magnaporthe isolates carrying the Avirulence Conferring Enzyme1 (ACE1) gene is specifically recognized by the rice cultivars carrying R gene Pi33. *ACE1* encodes a putative

hybrid polyketide synthase and non-ribosomal peptide synthetase (PKS-NRPS) **(Fig. 3.8)**. The resulting secondary metabolite product is expressed at onset of appressorium penetration during host invasion (Böhnert et al., 2004; Collemare, Pianfetti, et al., 2008; Fudal et al., 2007). We used ß-ketoacyl CoA synthase (KS) and acyl transferase (AT) domains to identify the presence of gene by PCR. Presence of ACE1 gene in all the isolates (except MO-NWI-209, MO-SI-72 and MO-SI-74) suggests its essential role in the pathogenesis **(Fig. 3.9)**.

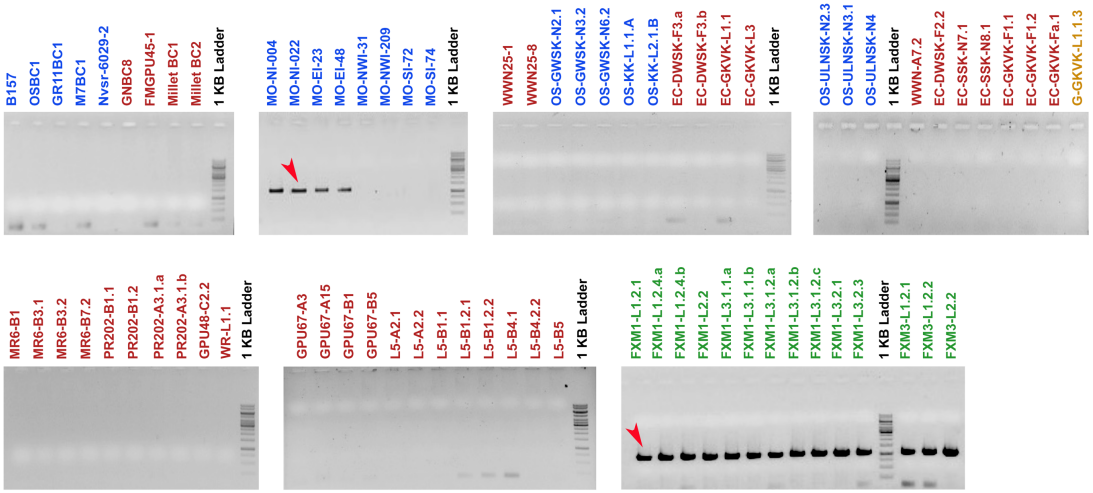


Figure 3.7: PCR amplification of AVR-Pita gene from various rice and non-rice *M. oryzae* isolates. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red arrowheads display the representative amplified product of gene-specific PCR, depicting the presence of AVR-Pita gene in a certain strain.

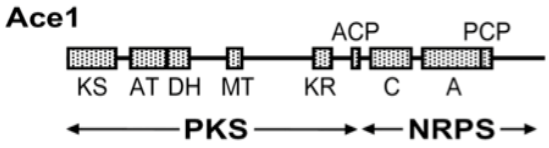


Figure 3.8: Organization of enzymatic domains in the hybrid PKS-NRPS product of ACE1 gene. The enzymatic domains are depicted as KS – ß-ketoacyl CoA synthase, AT – acyl transferase, DH – dehydratase, MT – methyl transferase, KR – ß-keto reductase, ACP – acyl carrier protein, C – condensation, A – adenylation and PCP – peptidyl carrier protein.

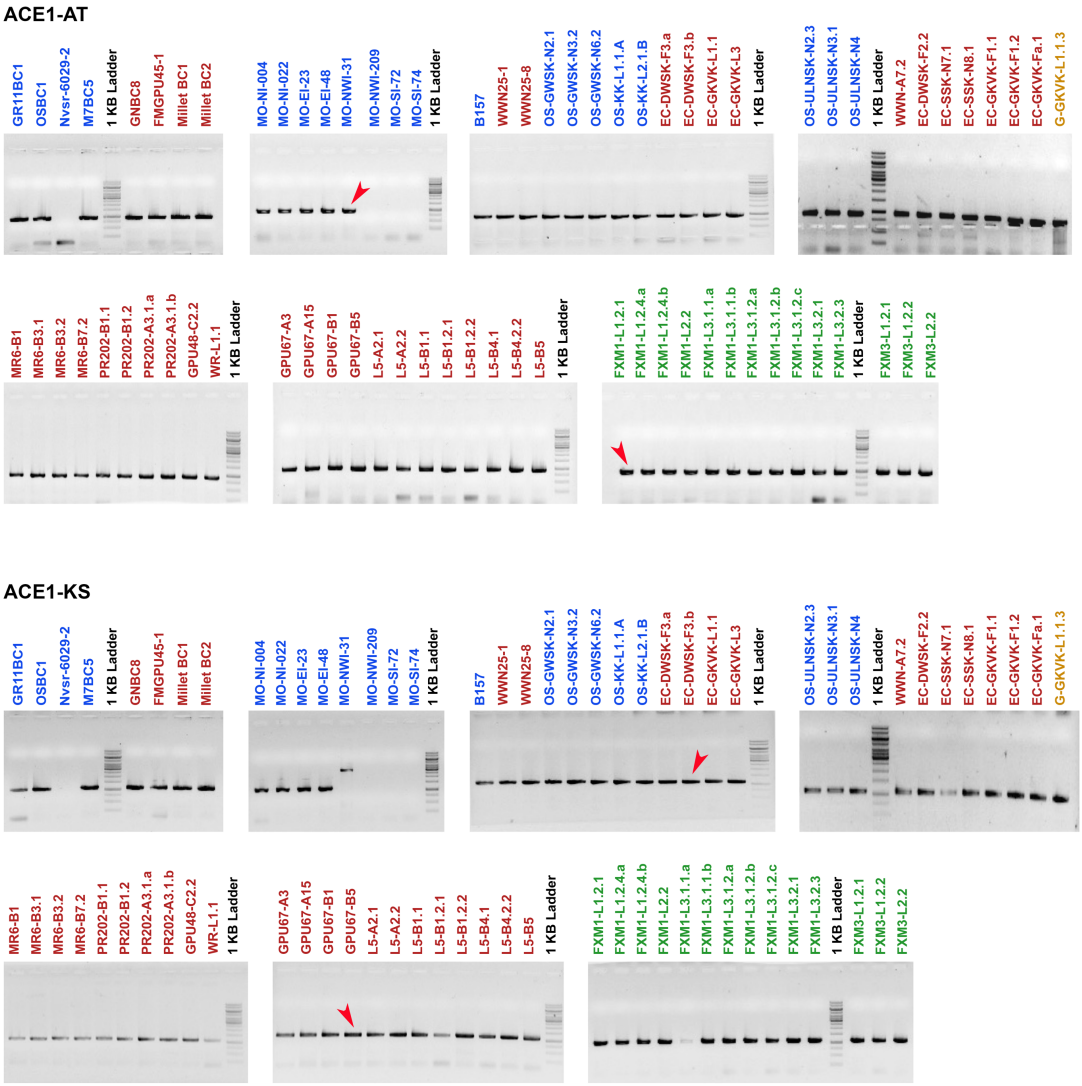


Figure 3.9: PCR amplification of ACE1 gene from various rice and non-rice *M. oryzae* isolates. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red arrowheads display the representative amplified product of gene-specific PCR, depicting the presence of ACE1 gene in a certain strain. AT - acyl transferase and KS – keto synthase.

AVR1-CO39 came from a *M. oryzae* isolate infecting weeping love grass and the corresponding resistance gene in rice is Pi-CO39. The gene-specific amplified product was found absent in most of the rice isolates, except a few - OSBC1, M7BC5 and OS-KK isolates **(Fig. 3.10)**. This observation is concordant with the earlier reports of AVR1-CO39 being

absent in the rice-infecting *M. oryzae* isolates collected from Brazil, China, Japan, India, Indonesia, Mali and Philippines (Couch, 2005)(Farman et al., 2002).

The AVR-Pik gene was identified from field isolate Ina168 after genome sequencing along with two others and its corresponding *R* gene in rice is Pik gene (Yoshida et al., 2009). AVR-Pik gene was not present in most of the millet isolates but found to be present in most of the rice isolates **(Fig. 3.11)**.

This avirulence gene can induce immune response if recognized by resistant gene Piz-t. AVR-Pizt manipulates the host immune responses by targeting ßZIP transcription factor in host cells to promote tissue necrosis and modulating potassium channel activity (Shi et al., 2018; R. Wang et al., 2016). The presence of AVR-Pizt in most of the isolates was in check with earlier reports, considering its essential role in host invasion/colonization. However, no amplification product was observed in three rice isolates MO-NWI-31, MO-NWI-209, MO-SI-74 and foxtail millets FXM3 isolates **(Fig. 3.12)**.

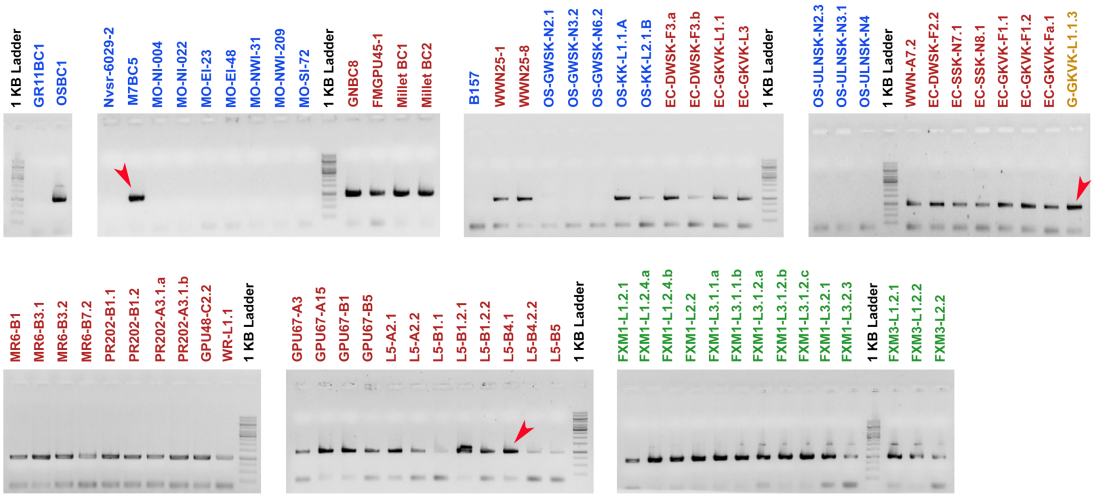


Figure 3.10: PCR amplification of AVR1-CO39 gene in different isolates. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red arrowheads display the representative amplified product of gene-specific PCR, depicting the presence of AVR1-CO39 gene in a certain strain.

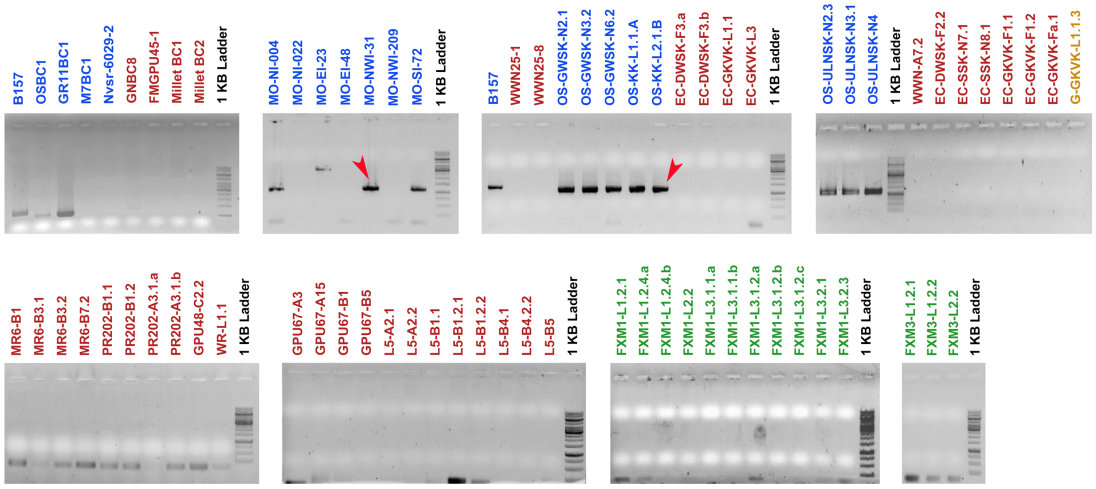


Figure 3.11: PCR amplification of AVR-Pik gene in various isolates. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red arrowheads display the representative amplified product of gene-specific PCR, depicting the presence of AVR-Pik gene in a certain strain.

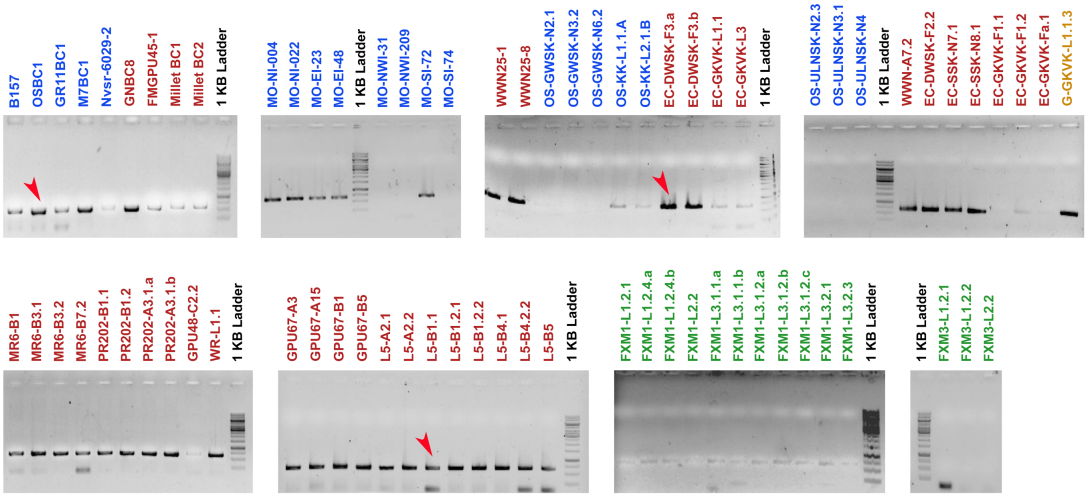


Figure 3.12: PCR amplification of AVR-Pizt gene in different *M. oryzae* strains. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red arrowheads display the representative amplified product of gene-specific PCR, depicting the presence of AVR-Pizt gene in a certain strain.

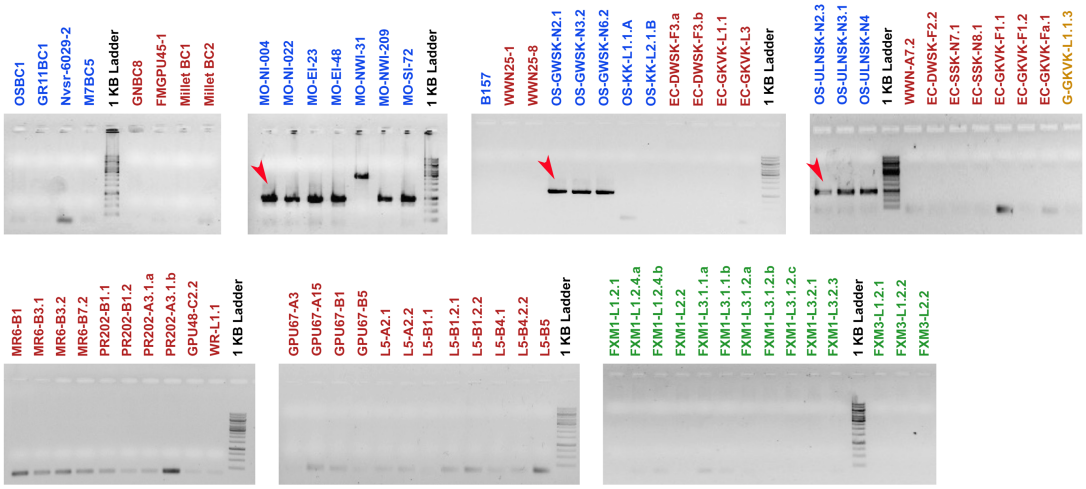


Figure 3.13: PCR amplification of PWL2 gene in different strains. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red arrowheads display the representative amplified product of gene-specific PCR, depicting the presence of PWL2 gene in a certain strain.

The PWL gene family has four members – PWL1, PWL2, PWL3 and PWL4. These genes are species-specific and confer avirulence towards *Eragrostis curvula* (Kang Seogchan et al., 1995). The PWL2 gene belongs to rice isolates, and it prevents pathogen to infect second host weeping lovegrass. PWL2 is biotrophic interfacial complex (BIC)-associated secreted effector known to be translocated the primary infected cell into neighbouring uninfected cells by invasive hyphae (Khang et al., 2010). Although we were able to identify presence of the PWL2 gene only in a few rice isolates **(Fig. 3.13)**, as this gene is particularly belonging to pathotypes infecting rice.

3.2.1.3 Identification of Mating-types

M. oryzae is a heterothallic fungus, with two distinct mating types (MAT1-1 and MAT1-2). Both MAT 1-1 and MAT 1-2 are idiomorphs of a single mating type locus MAT. Sexual reproduction is possible only between the two opposite mating types. Sexual reproduction can enhance and influence the genotypic variability of *M. oryzae* populations, where the recombinant progeny may have new capabilities to infect different host cultivars (Adarisini

et al., 1999). Assessment of mating type alleles has been used as a marker to measure population diversity in this pathogen.

We found both the mating types in the isolates from all the three hosts. Rice isolates were distributed in 40% MAT 1-1 and 60% MAT 1-2 mating types, whereas 53% and 47% finger millet isolates were of MAT 1-1 and MAT 1-2 types respectively **(Fig. 3.14, Table 3.2)**.

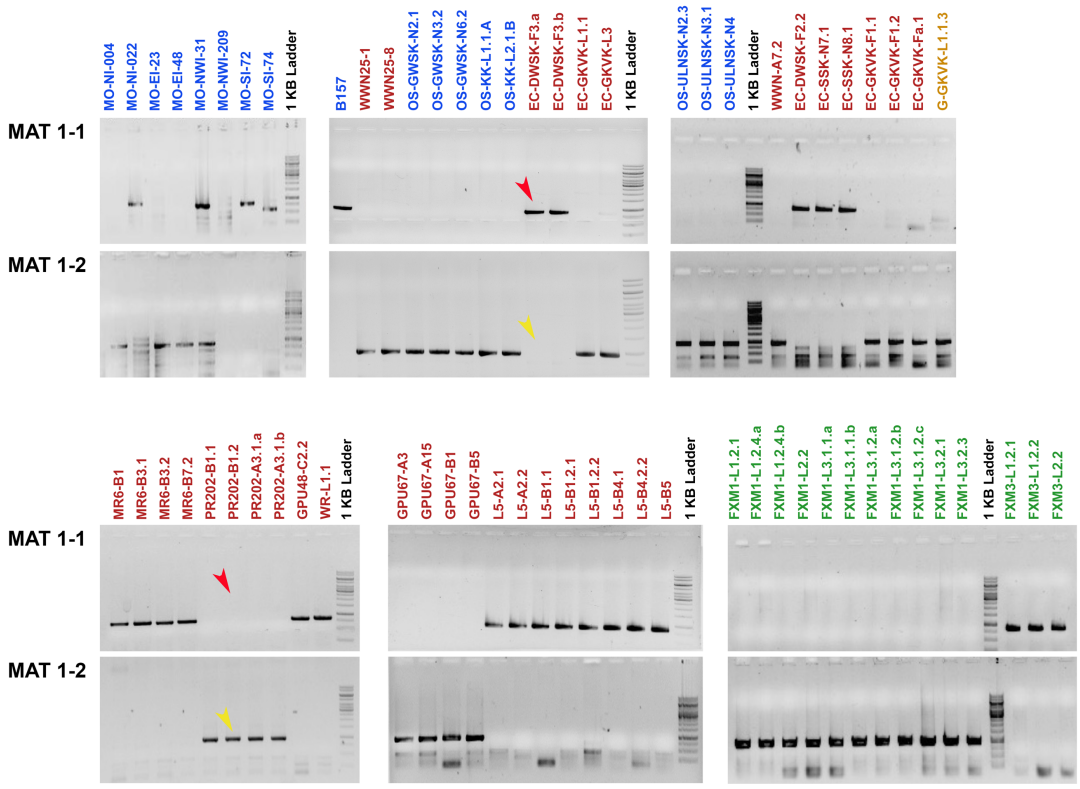


Figure 3.14: PCR amplification confirming the mating types in various isolates from rice and non-rice hosts. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red and yellow arrowheads display the representative amplified product of Mating type PCR, depicting either the presence/absence of MAT 1-1 and MAT 1-2 genes in a certain strain, respectively.

Table 3.2: Scoring displaying presence/absence variations in AVR genes and mating type in *M. oryzae* isolates

Host	Strains	AVR-Pita	AVR-Pik	AVR-Pizt	ACE1	PWL2	AVR-CO39	Mating Type
Rice	B157	-	+	+	+	-	-	MAT 1-1
	OSBC1	-	+	+	+	-	+	MAT 1-2
	GR11BC1	-	+	+	+	-	-	MAT 1-1
	M7BC5	-	+	+	+	-	+	MAT 1-1
	NVSR 6029-2	-	-	+	-	-	-	ND
	MO-NI-004	+	+	+	+	+	-	MAT 1-2
	MO-NI-022	+	-	+	+	+	-	MAT 1-1
	MO-EI-23	+	+	+	+	+	-	MAT 1-2
	MO-EI-48	+	-	+	+	+	-	MAT 1-2
	MO-NWI-31	-	+	-	+	+	-	MAT 1-1
	MO-NWI-209	-	-	-	-	+	-	MAT 1-2
	MO-SI-72	-	+	+	-	+	-	MAT 1-2
	MO-SI-74	-	+	+	-	+	-	MAT 1-1
	OS-GWSK-N2.1	-	+	-	+	+	-	MAT 1-2
	OS-GWSK-N3.2	-	+	-	+	+	-	MAT 1-2
	OS-GWSK-N6.2	-	+	-	+	+	-	MAT 1-2
	OS2-KK-L1.1A	-	+	+	+	-	+	MAT 1-2
	OS2-KK-L2.1B	-	+	+	+	-	+	MAT 1-2
	OS-ULNSK-N3.1	-	+	-	+	+	-	MAT 1-2
	OS-ULNSK-N2.3	-	+	-	+	+	-	MAT 1-2
	OS-ULNSK-N4	-	+	-	+	+	-	MAT 1-2
Finger millet	GNBC8	-	+	+	+	-	+	MAT 1-2
	FMGPU 45-1	-	-	+	+	-	+	MAT 1-2
	MILLET BC1	-	-	+	+	-	+	MAT 1-1
	MILLET BC2	-	-	+	+	-	+	MAT 1-1
	MR6 B1	-	-	+	+	-	+	MAT 1-1
	MR6 B3.1	-	-	+	+	-	+	MAT 1-1
	MR6 B3.2	-	-	+	+	-	+	MAT 1-1
	MR6 B7.2	-	-	+	+	-	+	MAT 1-1
	PR202 B1.1	-	-	+	+	-	+	MAT 1-2
	PR202 B1.2	-	-	+	+	-	+	MAT 1-2
	PR202 A3.1.A	-	-	+	+	-	+	MAT 1-2
	PR202 A3.1.B	-	-	+	+	-	+	MAT 1-2
	GPU48 C2.2	-	-	+	+	-	+	MAT 1-1
	GPU67 A3	-	-	+	+	-	+	MAT 1-2
	GPU67 A15	-	-	+	+	-	+	MAT 1-2

	GPU67 B1	-	-	+	+	-	+	MAT 1-2
	GPU67 B5	-	-	+	+	-	+	MAT 1-2
	L5 A2.1	-	-	+	+	-	+	MAT 1-1
	L5 A2.2	-	-	+	+	-	+	MAT 1-1
	L5 B1.1	-	-	+	+	-	+	MAT 1-1
	L5 B1.2.1	-	-	+	+	-	+	MAT 1-1
	L5 B1.2.2	-	-	+	+	-	+	MAT 1-1
	L5 B4.1	-	-	+	+	-	+	MAT 1-1
	L5 B4.2.2	-	-	+	+	-	+	MAT 1-1
	L5 B5	-	-	+	+	-	+	MAT 1-1
	WWN25-1	-	-	+	+	-	+	MAT 1-2
	WWN25-A7.2	-	-	+	+	-	+	MAT 1-2
	WWN25-8	-	-	+	+	-	+	MAT 1-2
	EC-DWSK-F3.A	-	-	+	+	-	+	MAT 1-1
	EC-DWSK-F3.B	-	-	+	+	-	+	MAT 1-1
	EC-DWSK-F2.2	-	-	+	+	-	+	MAT 1-1
	EC-GKVK-L1.1	-	-	+	+	-	+	MAT 1-2
	EC-GKVK-L3	-	-	+	+	-	+	MAT 1-2
	EC-GKVK-FA.1	-	-	-	+	-	+	MAT 1-2
	EC-GKVK-F1.1	-	-	-	+	-	+	MAT 1-2
	EC-GKVK-F1.2	-	-	+	+	-	+	MAT 1-2
	EC-SSK-N7.1	-	-	+	+	-	+	MAT 1-1
	EC-SSK-N8.1	-	-	+	+	-	+	MAT 1-1
Wild ragi	WR L1.1	-	-	+	+	-	+	MAT 1-1
Foxtail millet	FXM1 L1.2.1	+	-	+	+	-	+	MAT 1-2
	FXM1 L1.2.4.A	+	-	+	+	-	+	MAT 1-2
	FXM1 L 1.2.4.B	+	-	+	+	-	+	MAT 1-2
	FXM1 L 2.2	+	-	+	+	-	+	MAT 1-2
	FXM1 L3.1.1.A	+	-	+	+	-	+	MAT 1-2
	FXM1 L3.1.1.B	+	-	+	+	-	+	MAT 1-2
	FXM1 L3.1.2.A	+	-	+	+	-	+	MAT 1-2
	FXM1 L3.1.2.B	+	-	+	+	-	+	MAT 1-2
	FXM1 L3.1.2.C	+	-	+	+	-	+	MAT 1-2
	FXM1 L3.2.1	+	-	+	+	-	+	MAT 1-2
	FXM1 L3.2.3	+	-	+	+	-	+	MAT 1-2
	FXM3 L1.2.1	+	-	-	+	-	+	MAT 1-1
	FXM3 L1.2.2	+	-	-	+	-	+	MAT 1-1
	FXM3 L2.2	+	-	-	+	-	+	MAT 1-1
Grass	G-GKVK-L1.1.3	-	-	+	+	-	+	MAT 1-2

3.2.1.4 Dendrogram based on molecular markers

Based on the molecular characterization (presence/absence of *AVR* genes and mating types) of a total 75 different isolates, a dendrogram was generated using distance-based method – UPGMA (Unweighted pair group method with arithmetic mean). We observed distinct clades of isolates based on their host of origin. Interestingly, there was a high degree of variability seen particularly in rice isolates associated with different geographic locations (**Fig. 3.15**). However, due to lack of enough information, it is difficult to assess whether this variability was due to different rice varieties cultivated or geographic locations or both. While isolates belonging to different host plants fell under different clades, one clade specifically stood out with both rice and finger millet isolates (OSBC1, GNBC8 and OS-KK) clustered together **(Fig. 3.15)**. Overall, these observations suggest that the host selection pressure is likely the major determinant of the genetic diversity in the *M. oryzae* strains. A total of 15 isolates belonging to three different hosts were selected for whole genome sequencing, based on the grouping in the dendrogram **(marked with stars, Fig. 3.15)**. The nomenclature of the selected isolates was changed for the convenience in the further study.

3.2.2 Phenotypic characterization of field strains

3.2.2.1 Pathogenicity tests with different host plants

Representative isolates from each group were picked and subjected to pathogenicity tests by checking their cross-infectivity on different host plants. We used two methods – host invasion on leaf sheaths and whole plant infection assays.

For leaf sheaths invasion assay, spore suspension of ~10^4 conidia/ml was inoculated in hollow cylindrical leaf sheaths. The invasive hyphae of the pathogen in the epidermal layer of plant host cell were visualized under microscope at ~36 hours post inoculation (hpi), as an indication of pathogen's ability to invade the host plants. Not all the isolates were able to invade in all four hosts – rice, finger millet, wheat and barley **(Fig. 3.16)**. All the strains under study were able to invade their host of origin, additionally they could infect certain other hosts as well. Rice isolate B157 were able to invade all four hosts, although a little less invasion was observed in case of finger millet host. Similarly, rice isolate GR11BC1 was not able to infect finger millet host but could invade the other three hosts. Finger-millet

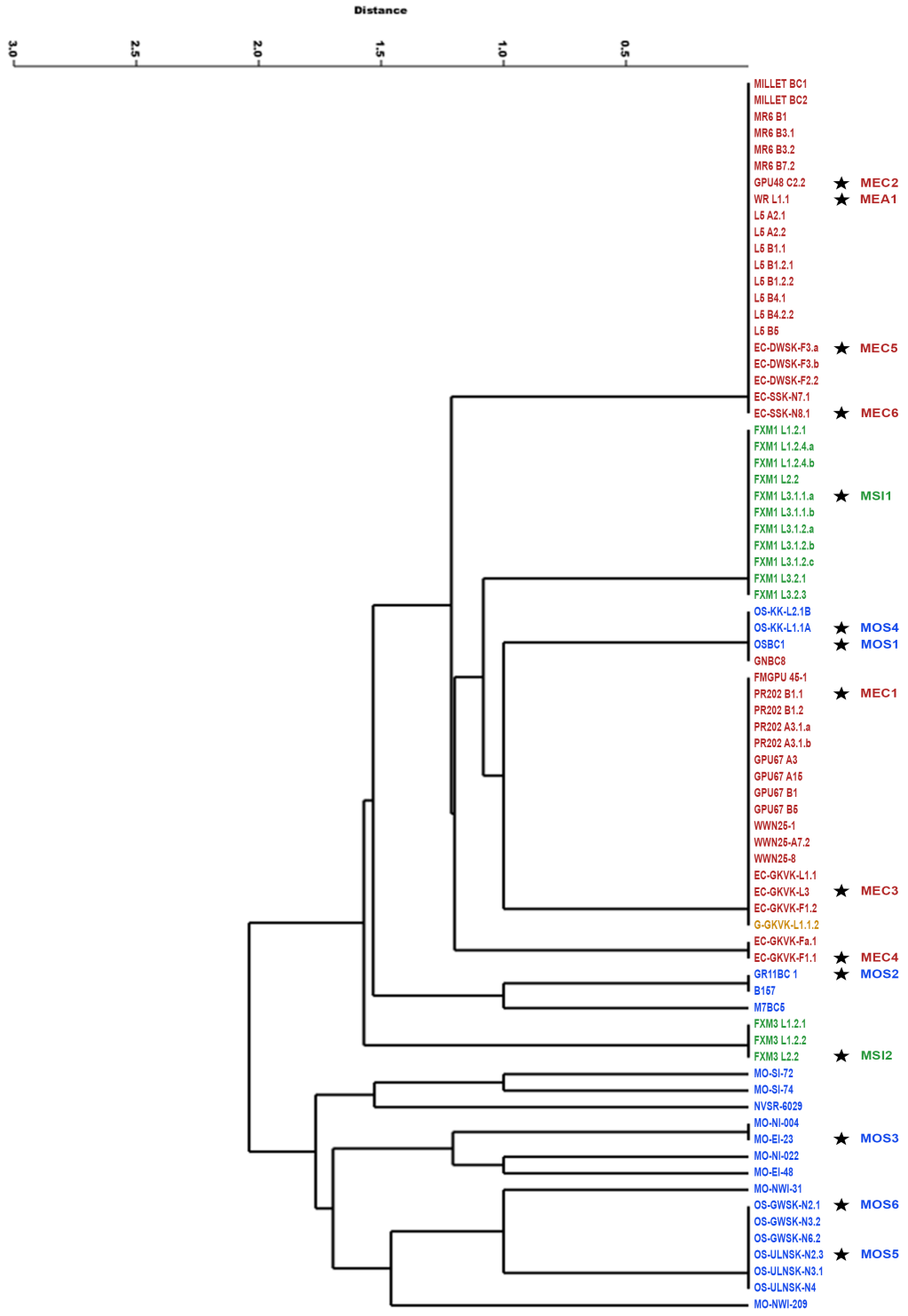


Figure 3.15: Dendrogram generated by taking into account presence/absence of AVR genes and mating types. The labels on each lane are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). The stars represent the strains selected for whole genome sequencing.

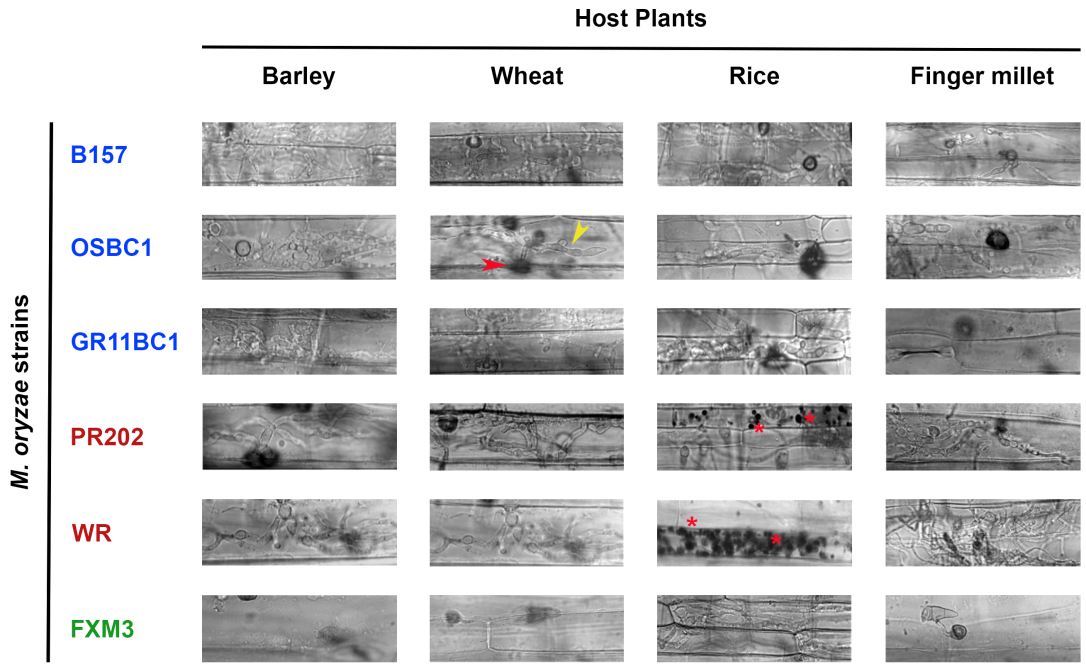
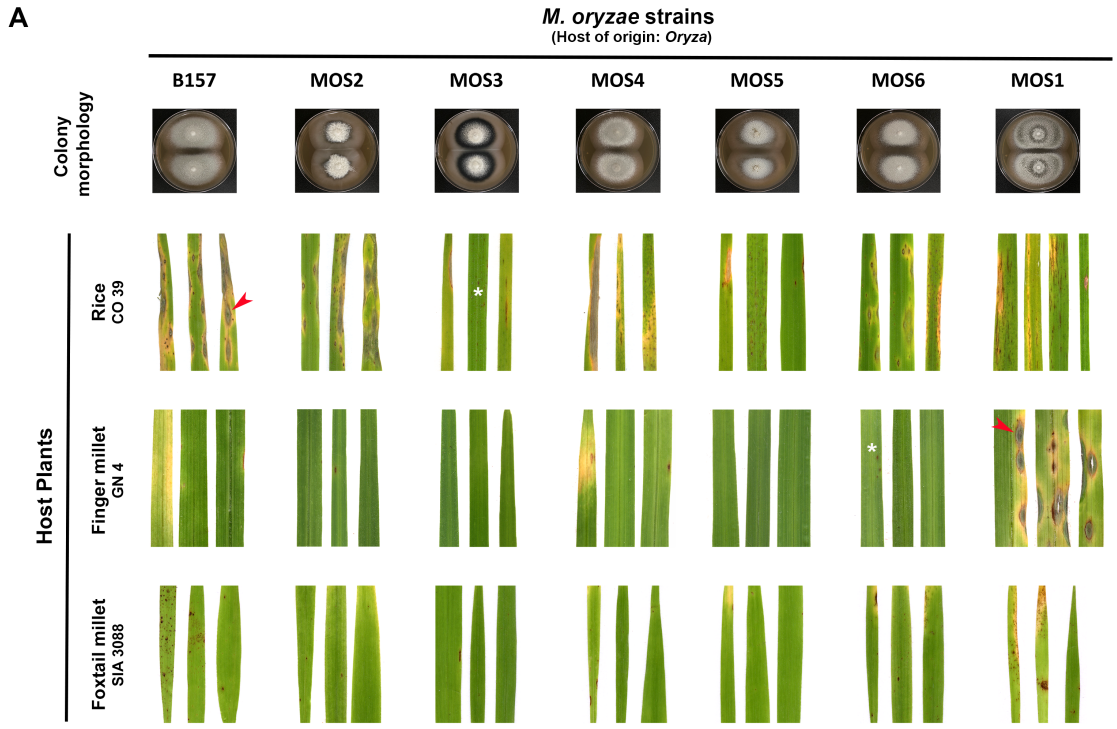
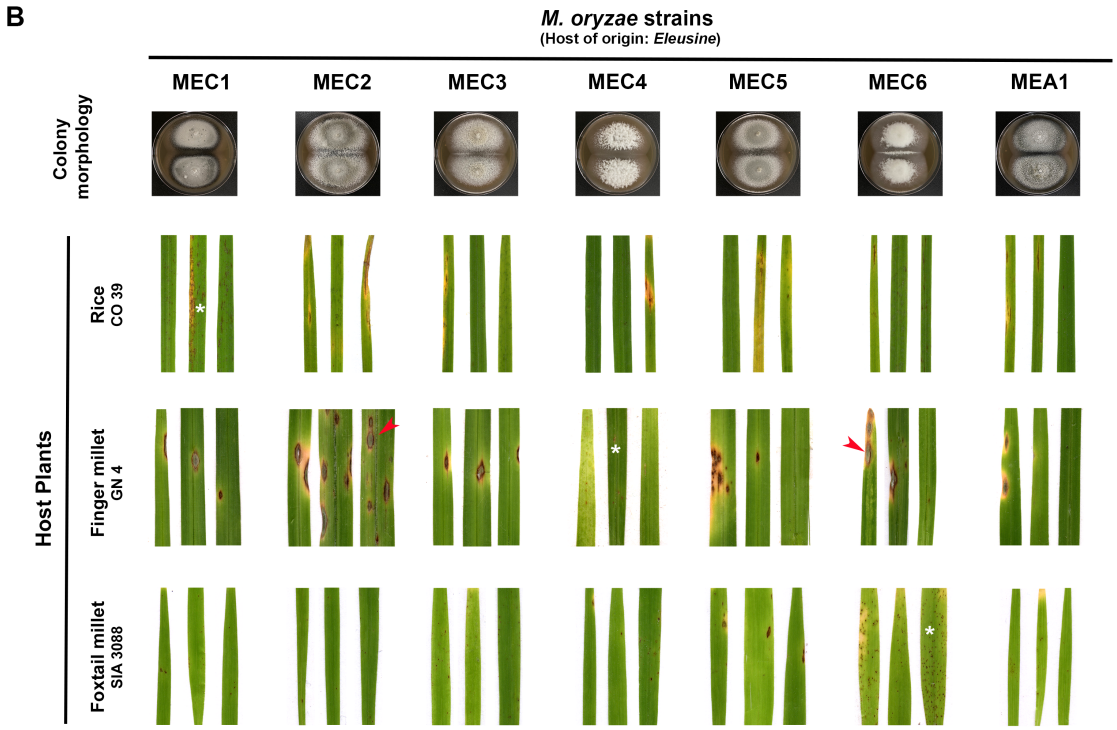


Figure 3.16: Host invasion of representative field strains of *M. oryzae* on leaf sheaths of four different host plants. The microscopic observations for the host invasion were made at ~36 hpi. The strain labels are color-coded according to their host of origin (rice-blue, finger millet – red, foxtail millet – green and grass - yellow). Red and Yellow arrowheads depicts the Appressoria and Invasive hyphae respectively. Red * represents the inclusions localized around invasive hyphae as a result of host innate immune response.

isolates PR202 B1.1 and WR L1.1 were able to invade all four hosts, although their invasion in rice host induced innate immune response (inclusions). Foxtail-millet isolate FXM3 L2.2 was not able to show any cross-infectivity on any of the four hosts, however innate immune response could be observed when isolate invaded the rice host **(Fig. 3.16)**.

Further, the leaf sheaths are detached from the living plant, so it might affect the physiology of plant part used for the experiment. Thus, in order to maintain the physiology of plants as close to nature as possible, we performed infection assays to check the pathogenesis of 16 Indian isolates of *M. oryzae* on three hosts – rice, finger millet and foxtail millet. We found that all isolates but one induced clear blast lesions on their host-of-origin, while they triggered no or few symptoms on non-hosts **(Fig. 3.17)**. In several cases, small dark necrotic spots were observed, indicative of a hypersensitive response caused by recognition of the


A
M. oryzae strains
(Host of origin: Oryza)
B157
MOS2
MOS3
MOS4
MOS5
MOS6
MOS1
Colony morphology
Host Plants
Rice CO 39
Finger millet GN 4
Foxtail millet SIA 3088


B
M. oryzae strains
(Host of origin: Eleusine)
MEC1
MEC2
MEC3
MEC4
MEC5
MEC6
MEA1
Colony morphology
Host Plants
Rice CO 39
Finger millet GN 4
Foxtail millet SIA 3088

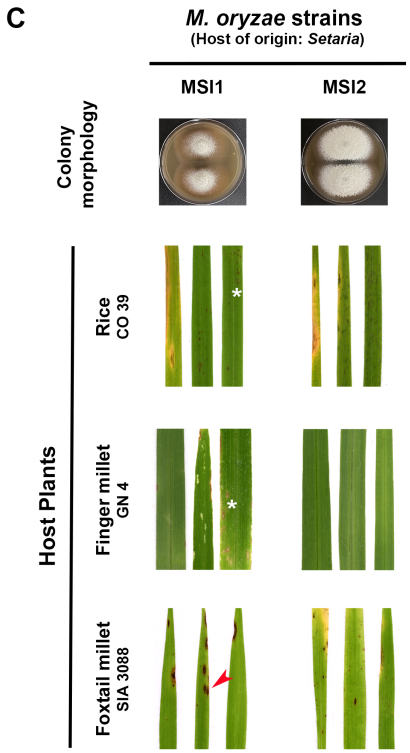


Figure 3.17: Pathogenicity tests of field strains of *M. oryzae*. The strains were isolated from different hosts and geographic locations in India. Whole-plant infection assays depicting differential virulence of *M. oryzae* isolates from rice (A), finger millet (B) and foxtail millet (C) on different indicated host plants. Assay results were photographed on 5 to 7 days post inoculation. Differential vegetative colony characteristics of these strains are shown in top panels. Red arrowheads represent the typical susceptible lesions and white * depicts the moderately-resistant lesions of the blast disease.

strain **(Fig. 3.17)**. Intriguingly, MOS1 and MOS4, two strains isolated from infected rice leaf tissue, were found to induce differential virulence pattern. In case of MOS1, inoculated finger millet and rice plants developed highly-susceptible and moderately-resistant lesions, respectively, similar to other isolates from finger millet **(Fig. 3.17A and B)**. The MOS4 strain also isolated from rice plant grown in field showed similar virulence pattern as MOS3 and MOS5, with clear interaction with rice. However, only few lesions were observed, and small necrotic spots could indicate recognition by the rice cultivar CO39. These results suggest that either these field strains have other primary hosts, or they are the examples of host range-expansion.

3.2.2.2 Mating assays

To check the mating ability for sexual reproduction, we investigated fertility status of a few *M. oryzae* strains belonging to rice and finger millet host plants. We used TH12 (MAT1-1) and GUY11 (MAT1-2) as reference strains and GR11BC1 (rice isolate); FMGPU45-1, MR6 and PR202 (finger millet isolates) as test strains. *M. oryzae* is a heterothallic fungus and sexual mating is only possible between the strains belonging to opposite mating types.

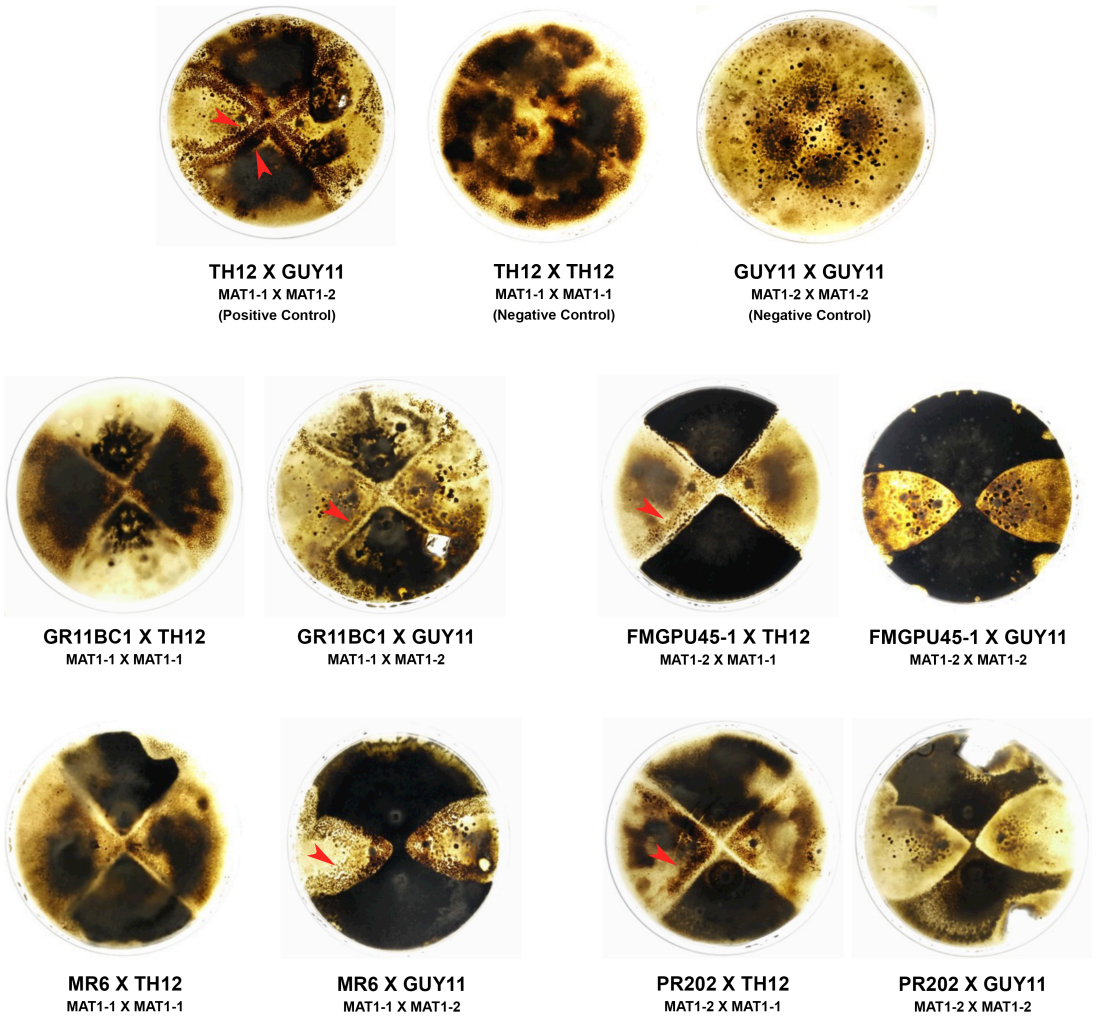


Figure 3.18: Mating assay displaying sexual crosses of representative field strains. The sexual crosses of test strains were carried out with the female fertile strains TH12 (MAT1-1) and GUY11 (MAT1-2). The perithecia (depicted with red arrowheads) were formed as a result of successful sexual reproduction.

Positive control showed the two lines of perithecia in sexual cross between TH12 (MAT1-1) and GUY11 (MAT1-2), while crosses among themselves did not produce any perithecia (negative control, **Fig. 3.18**). Two lines of perithecia indicate that both reference strains (TH12 and GUY11) in the sexual cross are male and female fertile (Saleh, Xu, et al., 2012). Each test strains when crossed with the corresponding opposite mating type, produced a single line of perithecia **(Fig. 3.18, Fig. 3.19)**. Single perithecia line produced in the test strains, is suggestive of the male fertility, while they are not female-fertile strains. We were able to check the sexual ability for only four field strains and all of them were found to be male-fertile and female-sterile.

Further we also made microscopic observation and checked the presence of asci in perithecia produced. We stained the released asci with calcofluor white (CFW) dye, which is useful for staining fungal cell wall. We were able to observe the ascus comprising of eight ascospores **(Fig. 3.19)**, which aligns with the fact that *M. oryzae* is ascomycetous fungus.

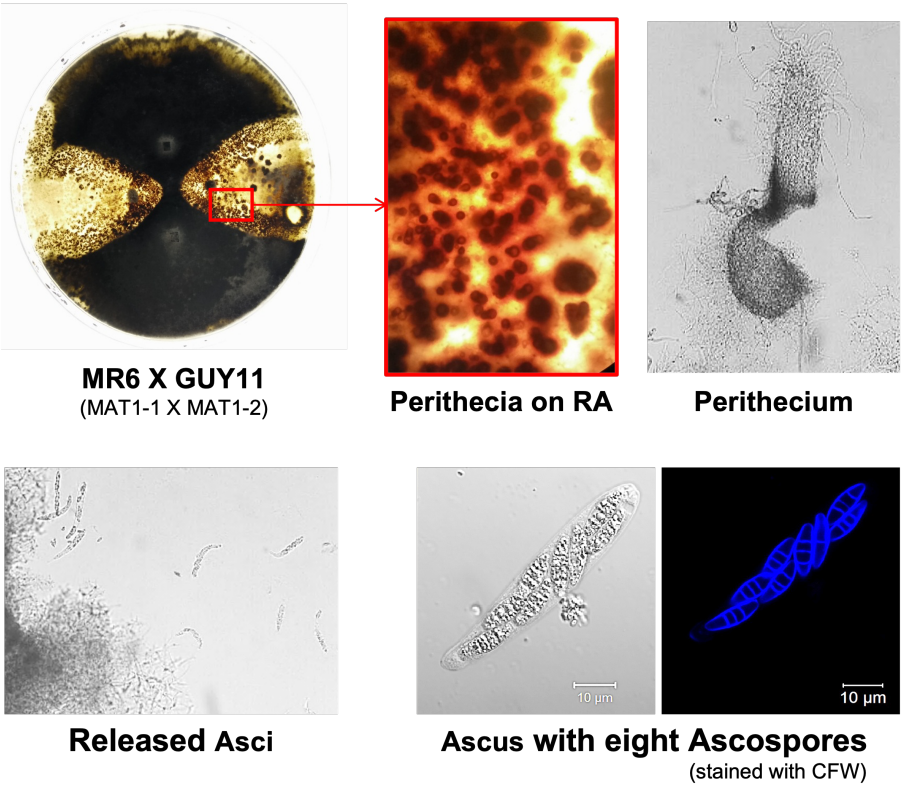


Figure 3.19: Perithecia development as a result of successful mating between opposite mating type strains. The perithecia were dissected and observed under the microscope. The ascus with the eight ascospores were observed with CFW staining.

3.3 Selection of representative strains for further comparative genomics analyses

We isolated a total of 133 field strains of *M. oryzae* from blast infected host plant tissues collected from fifteen different cropping zones across India. The sampling host plant species included rice, finger millet, foxtail millet and grass. The total of 75 field strains were characterized based on molecular markers encompassing transposons, AVR genes and mating type, which displayed significant genetic variability in natural populations of *M. oryzae*. Field strains were also subjected to phenotypic assessment to check their ability to infect other plants than the host-of-origin and sexual reproduction. Based on all these factors **(Fig. 3.20)**, we selected a total of 15 field strains for whole genome sequencing using next generation sequencing (NGS) technology.

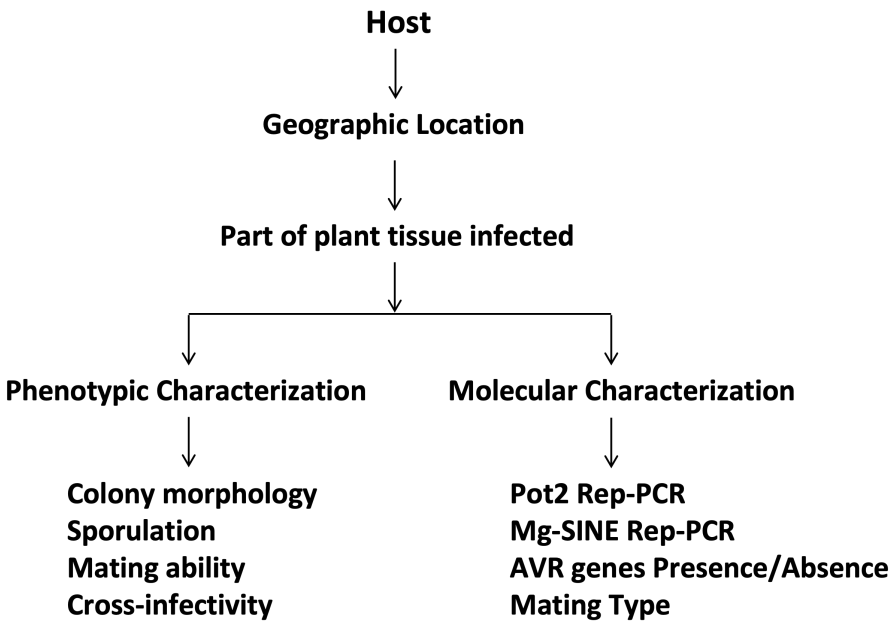


Figure 3.20: Key factors considered for classification of *M. oryzae* field strains and subsequent selection for comparative genomics analyses.