Epidemiology, phytopathological and molecular differentiation and leaf infection processes of diverse strains of *Magnaporthe* spp. on wheat and rice

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Abbreviations

µl	microlitre
µM	micromolar
bp	base pairs
AP	alkaline phosphatase
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
℃	degree celsius
cm	centimeter
CTAB	hexadecyltrimethylammoniumbromid
DNA	deoxyribonucleic acid
dNTP	deoxnucleotide-5`-triptophate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
et al.	*et alii* (lat.)
g	gram
h	hour
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
min	minute
ng	nanogram
PCR	polymerase chain reaction
pg	picogram
RNAse	ribonuclease
rpm	revolutions per minute
s	second

spp.	species pluralis
TBE	tris-Borate-EDTA buffer
TE	tris-EDTA buffer
Tris	tris hydroxymethyl aminomethane
U	unit
UV	ultraviolet light
V	volt
v/v	volume by volume
%	percent

1 Introduction

1.1 Wheat and wheat blast

Wheat (*Triticum aestivum*) is a cereal grain, originally from the Levant region of the Near East but now cultivated worldwide. Wheat is grown on more than 701.5 million hectares, thus being larger than any other crop (The Statistics Portal, 2014). In 2013, world production of wheat was 713 million tons, making it the third most-produced cereal (FAOSTAT, 2013). Wheat is the primary staple food in North Africa and the Middle East, and is growing in popularity in Asia. The four largest producers of wheat in 2013 were China (121.7 million tons), India (93.5 million tons), USA (58 million tons) and Russia (52 million tons), (FAOSTAT, 2013). In terms of total production used for food, it is currently second to rice as the main human food crop and ahead of maize, which is also extensively used in animal feed.

Wheat blast ("brusone") caused by *Magnaporthe grisea* (Hebert) Barr (anamorph: *Pyricularia grisea* (Cooke) Sacc.) is a relatively new disease on wheat (*Triticum aestivum* L.). After initial reports of its occurrence in 1985 in Northern Paraná State in Brazil, the disease has been found in other wheat-growing areas of Paraná and has caused severe yield losses (Igarashi *et al.*, 1986). Subsequently, it has quickly spread out to a number of major wheat producing regions of Brazil including Sao Paulo; Mato Grosso do Sul, Goias and the Central Cerrados of Brazil (Piccinini & Fernandez, 1989; Goulart *et al*., 1990; Prabhu *et al*., 1992; Dos Anjos *et al*., 1996). Within a few years, the disease has spread to several neighboring countries where it has caused serious damage (40-100%) in wheat fields of Paraguay in 1989. In the lowlands of Bolivia, it was responsible for a loss of 90,000 hectares of wheat between 1997 and 2000. In 2007, the disease was seen in summer-sown experimental wheat trials in Chaco, Argentina, and although researchers in Uruguay have not observed the disease in wheat, they have found the fungus on barley. In 2009, an outbreak cut Brazilian wheat production by up to 30%. (Igarashi, 1990; Prabhu *et al.*1992; Goulart & Paiva, 2000; Shoharak, 2003; Viedma, 2005; Alberione *et al.*, 2008). In some regions of South America, *Magnaporthe grisea* already leads to an annual yield reduction of 10-100% of wheat, especially when there are favorable climatic conditions to coincide with critical growth stages for infection (Talbot, 2003). Recently, UK soil scientist Lloyd Murdock found wheat blast on a single wheat head in 2011 at a UK Research and Education center research plot in Princeton, which is the first known occurrence of wheat blast outside of South America (Pratt, 2012).

Duveiller *et al.* (2010) addressed that there are some wheat-producing regions in the world where wheat blast has not been reported so far, but which have climatic conditions very similar to the regions in South America where wheat blast attack is quite frequent. There is an opinion that wheat blast could not become a serious problem in European countries, as the normal conditions of humidity and temperature in these countries are below the optimal levels for the development of the disease. However, with the effects of global warming, changes in rainfall may create environmental conditions favorable to wheat blast conducive to the spread of wheat blast as in other parts of the world such as South Asia or Africa. The potential spread of the wheat blast pathogen to those locations should be considered. The features of wheat

blast, a broad host range and sexual reproduction, would accelerate the speed of its propagation, and make it a potential threat to wheat production in the whole world.

The typical symptom of wheat blast is the head infection, which is common and destructive in the field. It consists of blighting of immature spikelets. Infection of the rachis blocks the translocation of photosynthates to the part of the spike above the point of necrosis, resulting in partial or total sterility of the spike. The kernels of the affected spikelets are often shrivelled (Prabhu *et al.*, 1992). The affected spikelets above the infection point exhibit a bleached straw color (Fig.1 c), which can be easily distinguished from normal green color of the healthy spikelets (Urashima & Kato, 1994). On the leaves, the symptoms of wheat blast are elliptical to elongate lesions with light to dark green centers and yellow borders (Fig.1 d). However, the occurrence of disease symptoms on the leaves or stems before the heading stage is rare. Most of blast disease is transmitted by seeds. The seed-transmitted rate depends on the time of infection in relation to heading. Early infection leads to low seed-transmission rates. Transmission is greater when seed infection occurs later in the grain-filling period (Cunfer *et al.*, 1993).

Magnaporthe grisea is able to attack many graminaceous plants, including economically important crops like barley (*Hordeum vulgare* L., Brett & Linda, 2002; Zellerhoff *et al.*, 2006), finger millet (*Eleusine coracana* L., Singh & Kumar, 2010), oat (*Avena sativa*, Oh *et al.*, 2002) and is reported to be very common in perennial ryegrass in the United States (Carver *et al.*, 1972). The fungus subsequently develops different pathotypes according to the original host plant from which they were isolated. Therefore, each pathotype has a restricted host range while commonly they are virulent to their original host (Tosa *et al.*, 2004). Viji *et al.* (2001) reported that isolates from wheat showed notable similarity to the perennial ryegrass (*Lolium perenne* L.) isolates based on *Pot2* fingerprints and one wheat isolate was highly virulent on perennial ryegrass. The wheat isolates produced sporadic lesions on Italian ryegrass and perennial ryegrass and the isolates derived from perennial ryegrass showed the same ITS2 sequence as wheat isolates and millet isolates (Tosa *et al.*, 2004)

1.2 Rice and rice blast

As a cereal grain, rice (*Oryza sativa* L.) is the most widely consumed staple food for a large part of the world population, especially in Asia. Rice represents the third most produced agricultural commodity, after sugarcane and maize. The world dedicated 106.1 million hectares for rice cultivation (The Statistics Portal, 2014) and the total production has risen steadily from 212 million tons in 1961 to about 745.2 million tons in 2013 (FAOSTAT, 2013). It is estimated that its production will increase by 40% until 2030 (Ribot, 2008). Developing countries account for 95% of the total rice production, with China and India alone being responsible for nearly half of the world output. Since a large portion of maize crops is grown for purposes other than human consumption, rice is the most important grain with regard to human nutrition and caloric intake, providing more than one fifth of the calories consumed worldwide by humans (Smith, 1998).

The ascomycete fungus *Magnaporthe oryzae* B. Couch (anamorph: *Pyricularia oryzae* Cavara) is a pathogen on many graminaceous plants. It is one of the most serious and widespread

diseases of rice, due to its widespread occurrence and destructiveness under conducive conditions. The domestication of rice started from its wild relative *Oryza rufipogon* about 7,000 years ago in the middle Yangtze valley in China (Crawfor & Shen, 1988; Higman & Lu, 1998), and spread to the hills of the Indian Himalayas around 3,000 BP. The first report of blast disease, which was designated as rice fever disease, was in China in 1637, followed by a record as `Imochi-byo` by Tsuchiya from Japan in 1704. In Italy, a disease called `brusone` been considered as blast was reported by Astolfi in 1828 and by Brugnateli in 1838. In 1960, Metcalf in the USA named it as `blast`. In 1968, rice blast (*Magnaporthe oryzae*) has been recorded in more than 80 countries worldwide by the Commonwealth Mycological Institute.

This disease destroys rice crops to a great extent, particularly in temperate, flooded and tropical upland ecosystems (Ou, 1985). From the year of 1900 to 1960, publications document the outbreak of rice blast besides in Asia also in Australia, the Middle East (Iraq), Madagascar, Southern Europe (Portugal and Spain), as well as in Africa (Morocco, Uganda, Senegal and South Africa) and in the Americas (Costa Rica, Argentina and Brazil) (Parthasarathy & Ou, 1965). Surveys confirmed that blast remains among the most serious constraints to yield in South Asia (Widawsky & O`Toole, 1990; Geddes & Iles, 1991). In Japan, blast causes annual yield losses corresponding to 275,000 tons of rice (25% of production) and requires the extensive use of fungicides (1.8 million euros in 2000, Ribot, 2008). In Brazil, the records reveal about 60% damage in upland rice crops (Prabhu *et al.*, 2003). In recent years, rice blast epidemics have occurred in China (5.7 million hectares of rice were destroyed from 2001 to 2005), Korea, Japan, Vietnam and the United States (Richard & Nicholas, 2009). The region of Indian Himalayas forms a heterogeneous mountainous area where rice production takes place by different cultivation systems, including intercropping with millets. Compared to other regions (Americas and Europe), the Indian Himalayas show the highest diversity in population structure of *Magnaporthe oryzae* host-specific on rice (Kumar *et al.*, 1999).

The rice blast fungus attacks rice plants at all stages of development and may infect all parts of the rice plant: leaf, collar, node, neck, stems, peduncles, panicles, seeds, and even roots.

The most common and diagnostic symptoms of rice blast occurs on the leaves. The symptoms on leaves may vary according to the environmental conditions, the age of the plant, and the levels of resistance of the host cultivars. On susceptible cultivars, initial symptoms may appear as white to gray-green lesions or spots with dark green borders on all parts of the shoot, while older lesions on the leaves are elliptical or spindle-shaped and whitish to gray centers with red to brownish or necrotic border (Fig.1 e). Some resemble diamond shape, wide in the center and pointed toward either end. Lesions can enlarge and coalesce, growing together, to kill the entire leaves. On resistant cultivars, lesions often remain small in size (1-2 mm) and brown to dark brown in color. Rice can have blast in all growth stages. However, leaf blast incidence tends to lessen as plants mature and develop adult plant resistance to the disease.

Symptoms of infection of the collars consist of a general area of necrosis at the union of the two tissues. Collar infections can kill the entire leaf and may extend a few millimeters into and around the sheath. The fungus may produce spores on these lesions (APS, 2012). Node and neck blast commonly occur together and have similar symptoms. Node infection occurs in a banded pattern. Lesions on the node are blackish to grayish brown. Infected nodes can

cause the culm or the part of the plant that holds the panicle to break. Lesions on the neck are grayish brown and can cause girdling, making the neck and the panicle fall over. If infection of the neck occurs before milky stage no grain is formed, whereas infection at later stages, leads to poor quality grains. After the flowering stage, the fungus can also infect the panicles as the seeds form. Lesions can be found on the panicle branches, spikes, and spikelets. The lesions are often gray brown discolorations of the branches of the panicle, and over time the branches may break at the lesion (IRRI, 2014). The fungus has often been isolated from the pedicels of the seeds. Seeds are not produced when pedicels become infected, a condition called blanking. Symptoms of rice blast on leaves themselves consist of brown spots, blotches, and occasionally the diamond-shaped lesions are often seen. The process and the time during which infection of seeds by spores of the pathogen occurs has not been fully described but recent information shows that the fungus can infect seeds by infecting the florets as they mature into seeds, and it is believed that this is the main way seed infection develops (APS, 2012).

Due to the distribution of rice around the world, the pathogen was adapted to other hosts. It has been found that *M. oryzae* can infect and survive in/on more than fifty species of grasses, many of which are of agricultural importance (Ou, 1985), such as barley (Zellerhoff *et al.*, 2006), Italian ryegrass and corn (Prabhu *et al.*, 1992) and millet. But no infection to perennial ryegrass, results for copy numbers, fingerprints of *Pot2* and *MGR586* and ITS region sequencing indicate that rice isolates are genetically distinct from the isolates derived from perennial ryegrass (*Lolium perenne*) and none of the isolates from rice caused the disease on perennial ryegrass, and *vice versa* (Viji *et al.*, 2001). And Barbara V. (2013) demonstrated that rice isolates are genetically distinct from wheat isolates and unlikely to cross over to infect wheat in the field.

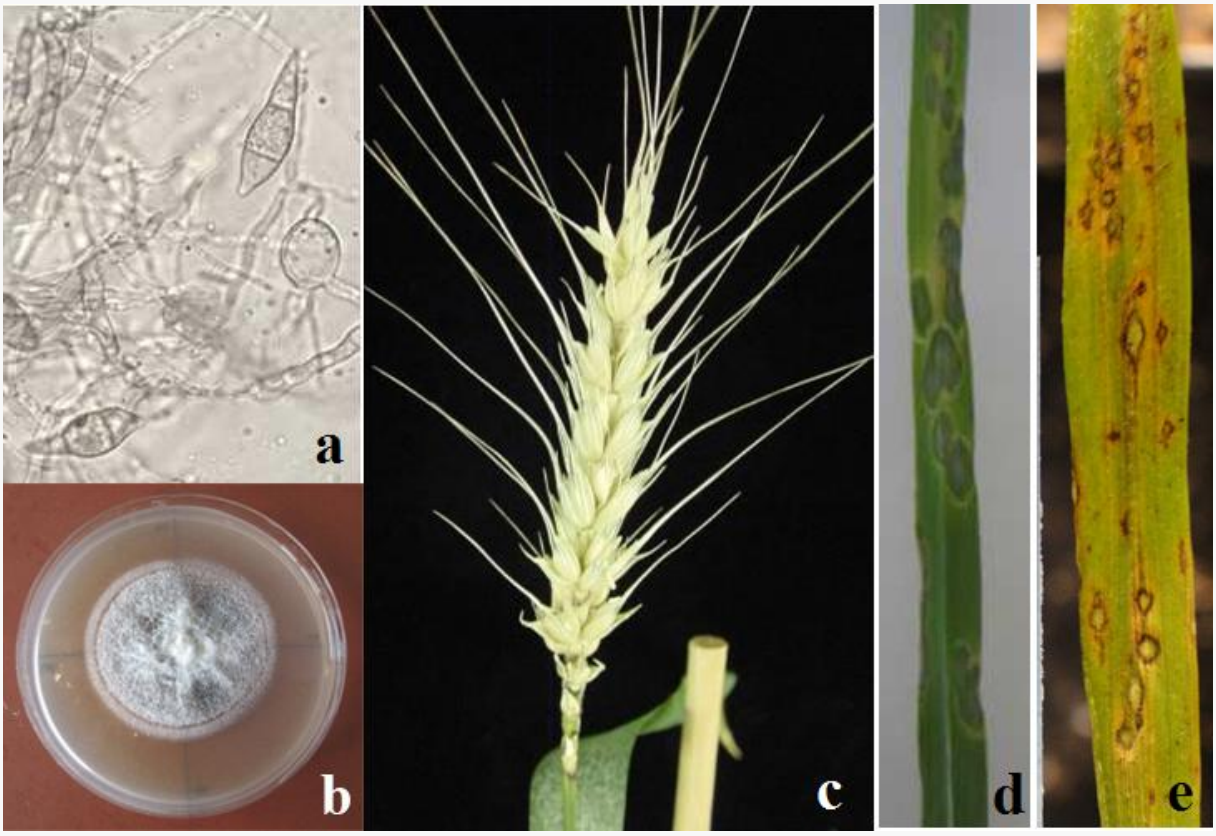

Figure 1 Conidia and colony of *Magnaporthe* spp. and infected plant tissues. (a) Conidia and mycelia of *Magnaporthe* spp.; (b) Colony on an agar plate with fleecy grayish appearance; (c) Bleached wheat ears with straw color, the kernels are often shrivelled; (d) Elliptical lesions with yellow margin on the leaf of wheat seedlings; (e) Elliptical or spindle-shaped and whitish to gray centers with red to brownish or necrotic border on the leaf of rice seedlings.

1.3 Taxonomy and relationship of *Magnaporthe grisea* and *Magnaporthe oryzae*

It is now widely accepted, that the taxon *Magnaporthe oryzae* refers to the isolates of the pathogen from cultivated cereals including rice, while *Magnaporthe grisea* represents isolates from the members of the genus *Digitaria* (Chuma *et al.*, 2009). Based on morphological observations, Rossmann *et al.* (1990) demonstrated that *M. grisea* and *M.oryzae* are morphologically not distinguishable and that their isolates are interfertile. *Pyricularia grisea* was suggested as the correct name for the pathogen. However, Kato *et al.* (2000) confirmed that isolates from crabgrass (*Digitaria sanguinalis*) are genetically distinct from those of rice, finger millet (*Eleusine coracana*) and other crop species. In the description of the anamorph, two form-species names have been applied in *Magnaporthe*. *Pyricularia oryzae* Cavara was described from rice and *P. grisea* (Cooke) Saccardo was described from grasses and cereals. *Pyricularia oryzae* was distinguished from *P. grisea* based on its sparse, usually nonseptate hyphae and larger, biseptate conidia (Brett & Linda, 2002). The use of the names *P. grisea* and *P. oryzae* has generally reflected the host from which the fungus was isolated rather than any morphological differences, with the name *P. oryzae* applied to isolates from rice and *P. grisea* to isolates from cereals and other grasses (Sprague, 1950).

Although the two blast pathogens from wheat and rice have very similar characteristics, the knowledge about wheat blast compared to rice blast is still very limited, especially its genetic and epidemiological aspects. The relationship between the blast of wheat and rice has always generated questions about the origin of the first, considering wheat blast as a disease of relatively recent occurrence. Initially, rice blast was considered as the source of wheat blast. However, genetic studies have found significant differences among isolates from the two plant species and confirmed that the wheat pathogen *Magnaporthe grisea* found in Brazil has not originated from rice (Urashima *et al.*, 2005; Valent & Chumley, 1994) and differs to a large extent from the rice pathogen, *Magnaporthe oryzae* (Couch & Kohn, 2002). Using microsatellite markers, Ceresini *et al.* (2011), compared sympatric populations of *Magaporthe* spp. adapted to either wheat and rice in Brazil and revealed that there was very low historical migration between the two different populations. Reports from fingerprints with repetitive DNA elements also showed the same results (George *et al.*, 1998; Urashima, 1999). Dobinson & Hamer (1993) addressed repetitive DNA called MGR586 and MGR583 common in the genome of the rice pathotype, where they are found in a more frequent number than in isolates of wheat and other grasses. It is concluded that the wheat pathotype is not a descendent of the rice pathotype. Urashima (1993) tested the host range, mating type and fertility of wheat isolates in Brazil and found that the wheat isolates are distinguishable from the rice isolate or other host-specific pathotypes but similar to the *Eleusine* isolate. Urashima *et al.* (2004a) has confirmed that the fungus *Magnaporthe grisea*, adapted to wheat, probably originated from a strain on *Digitaria insularis.*

1.4 Epidemiology and life cycle of *Magnaporthe* spp.

1.4.1 Epidemiology

1.4.1.1 Wheat blast

Limited data are available on the environmental determinants for optimal wheat blast (*Magnaporthe grisea*) infection. Based on previous observations, it is known that the intensity of damage caused by wheat blast has been directly proportional to the level of rainfall during the heading stage of wheat. Brazil has suffered one of the most serious epidemics of wheat blast in 2009 when there was a period of high precipitation during the heading stage (Maciel, 2011). Cardoso *et al.* (2008) developed a warning system model to predict the intensity of wheat blast based on temperature and the duration of wheat spike wetness. The investigation was carried out at a temperature range of 10-35˚C and duration of spike wetness of 0-40 hours. It is concluded that wheat blast intensity could exceed 85% at 25˚C and 40 h wettness period. Alves and Fernandes (2006) revealed that production of conidia by *Magnaporthe grisea* was favored by high relative humidity (≥ 90%) and temperatures around 28˚C.

1.4.1.2 Rice blast

The vastness of the outbreaks of the blast disease differs according to environmental conditions. Long periods of leaf wetness, high humidity, moderate temperatures between 17-23˚C during the night, low solar radiation as well as overcast skies, high moisture and little or no wind at night are some of the environmental factors favoring blast disease (Bonman, 1992; Maciel, 2011). Severe epidemics of blast in rice cultivation are generally associated with wet

weather. Frequent and long periods of rain are more prone to cause infection than short periods of heavy rain. All the vital processes of the disease cycle require free water and night time dew provides it. The longer the dew remains on the leaves, the more lesions are produced. In the tropics, the temperature is always optimal at night, and the disease is practically determined by the presence and duration of dew (Ou, 1980). There are five broad categories of rice production environments: irrigated, rain-fed lowland, upland, deep-water and tidal wetland (Khush, 1984). Upland environment is the most affected by the disease with a large production of dew on the leaf surface. Bonman (1992) and Ou (1980) demonstrated that blast severity depends largely on the cropping system adopted in rice fields. In the tropics, lowland rice fields flooded with water have shorter dew periods than upland fields. This is because the sun heats the water in the field to 42˚C. During the night, the warm water releases the absorbed heat slowly, delaying dew formation; the closer to the water surface, the shorter the dew period. Besides, protein and sugar contents are also higher in upland than in lowland rice.

Important processes in the disease cycle include spore release, spore germination, infection and spore production. Spores are produced and released under high relative humidity (RH) conditions, with no spore production below 89% RH. The temperature for spore germination, lesion formation and sporulation is 23 to 30˚C, and the minimum of leaf wetness is 4 h. In water, conidial germination may occur within 3 h. Based on previous studies, the optimal conditions for blast conidial germination were 92 to 96% relative humidity, temperatures of 25 to 28˚C and leaf wetness of 7 to 14 h. Under optimal conditions, conidiophores and the first conidia were produced 4 to 6 h after dew formation and the conidia were released shortly thereafter (Greer & Webster, 2001).

1.4.2 Life cycle

The plant pathogenic fungus *Magnaporthe* is a genus of the ascomycetes, the class of Sordariomycetes and the family of Magnaporthaceae. There are five species in the widespread genus of *Magnaporthe* (Kirk, 2008), where *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* (Cooke) Sacc.) and *Magnaporthe oryzae* B. Couch (anamorph *Pyricularia oryzae* Cavara) are the two species studied in the present work.

1.4.2.1 Life cycle of wheat blast

The life cycle of blast is well studied on rice isolates (Valent and Khang, 2010; Wang and Valent, 2009; Wilson and Talbot, 2009), but it appears wheat isolates also shared the same disease cycle (Tufan *et al.*, 2009). Disease cycle begins when a blast spore infects and produces a lesion on wheat plant and ends when the fungus sporulates and disperses many new airborne spores to nearby healthy plant tissues or other plants.

Magnaporthe spp. are extremely effective plant pathogens as they can reproduce both sexually and asexually to produce specialized infectious structures. The life cycly of *Magnaporthe* spp. undergo sexual and asexual reproduction is shown in Fig. 2 (Dean *et al.*, 2005).

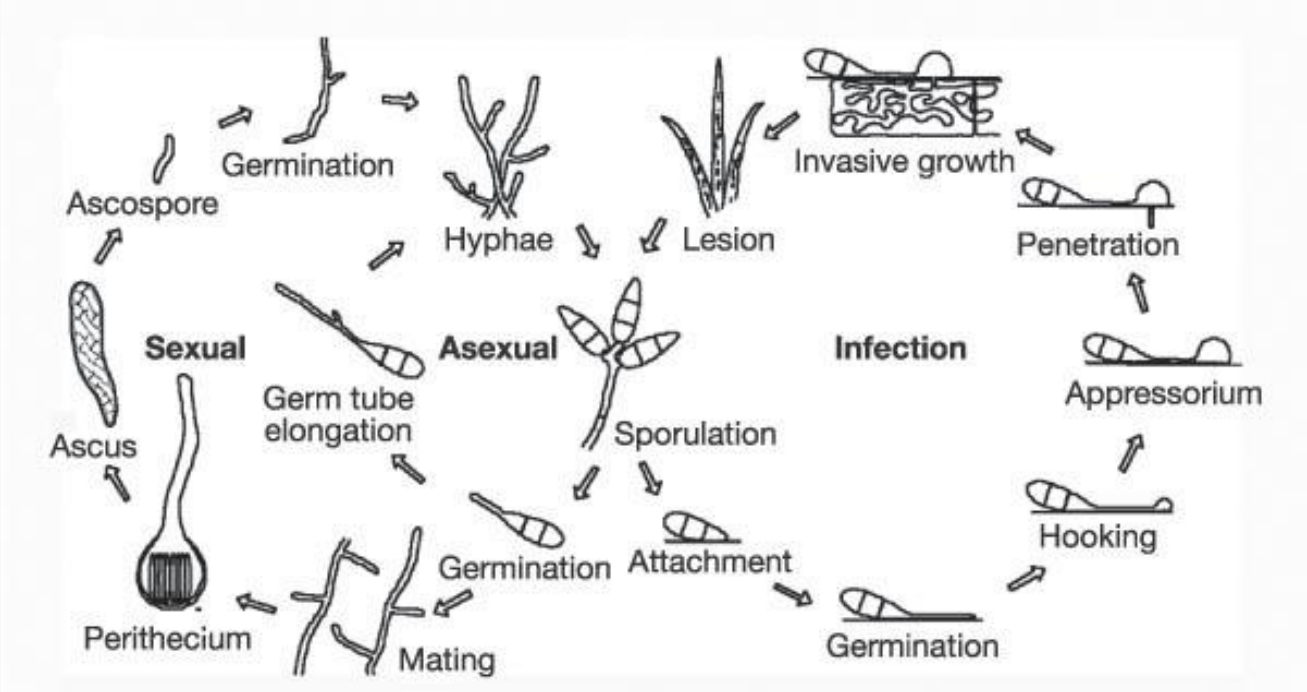

Figure 2 Life cycle of *Magnaporthe* spp. including sexual and asexual reproduction and infection cycle (Dean *et al.*, 2005). Infection cycle consists of germination of asexual spores (conidia), formation of appressorium and penetration, host colonization with hyphae and sporulation of new conidia. In sexual reproduction, ascospores (asci) develop in perithecia after fusion of two opposite mating types. Once released, ascopores can develop appressoria and infect host cells.

Wheat blast isolates show high levels of sexual fertility, functioning as hermaphrodites and crossing to produce abundant viable ascospores (Urashima *et al.*, 1993). Sexual reproduction of the ascomycete fungi requires two different compatible mating types, at least one of which is female-fertile. The sexual or teleomorph stage of the blast pathogen can be produced *in vitro* undergo sexual crosses if isolates of opposite mating types are paired, but this occurs rarely in the field on any host (Yaegashi & Udagawa, 1978). This level of fertility in the laboratory raises the possibility that the wheat blast may undergo sexual recombination in the field, which is in contrast to the infertility of most rice isolates (Bruno & Urashima, 2001). Fully fertile isolates are self-sterile hermaphrodites (functioning both as females and males), with compatibility for mating governed by alternate alleles of the mating type locus *MAT*1. Two additional spore forms, ascospores and microconidia, are produced by sexually fertile isolates (Barbara V., 2013). Ascospores belong to the diagnostic pyriform condia and are produced in unordered asci within perithecia with long necks in the characters of hyaline and fusiform (spindle-shaped with tapering ends) with three septae and a single mitotically-derived nucleus. Perithecia produce asci within 2 to 3 weeks when strains of opposite mating type are incubated on oatmeal agar in the light at 20℃. Mature asci are extruded through the perithecial necks and ascospores are released into a viscous liquid (Yaegashi & Udagawa, 1978). So water is required for release of conidia from conidiophores to reinitiate the disease cycle. Ascospores produce appressoria for plant penetration. The melanin layer in the appressorium is essential to build-up the very high pressure required to puncture the outer plant surface and gain access to host tissue. Pressure build-up requires immersion of the appressorium

in water, explaining in part the requirement for extended periods of rain or dew for this disease (Barbara V., 2013). Sexually fertile isolates also produce small, crescent-shaped microconidia, 6 micrometers in length and 0.7 micrometers in width, which are hypothesized to function as spermatia. Microconidia are produced from phialides, and conditions under which they germinate are not known (Chuma *et al.*, 2009).

All nuclei in a conidium are derived through mitosis from a single nucleus, and consequently, a fungal strain can be purified by isolation of a single spore. In greenhouse, the fungus could sporulates from dead or dying coleoptiles of plants grown from infected seeds, while in the field, infested seeds left on the soil surface can readily produce spores for more than several weeks after planting.

1.4.2.2 Life cycle of rice blast

The overwintering sources of spores that comprise the primary inoculum consist of host seed, crop residue and secondary hosts (Greer & Webster, 2001). A single cycle can be completed in about a week under favorable conditions. In addition, a single lesion can generate hundreds to thousands of spores in one night with the ability to continue to produce the spores for over twenty days. Under favorable moisture and temperature conditions, the fungus can go through many disease cycles and produce a tremendous load of spores by the end of the season. This high inoculum level can be devastating to susceptible crops. The number of cycles and the number of spores that are produced on each individual lesion can be influenced by many factors, including the temperature, rainfall, the depth of the water in the paddy, the amount of nitrogen used to fertilize the rice, and the level of genetic resistance in the cultivar that is infected (TeBeest *et al*., 2007).

Magnaporthe oryzae, an Ascomycete causing blast disease on rice, reproduces mostly asexually in nature. Sexual reproduction is possible *in vitro* and requires two strains of opposite mating types. The asexual reproduction is initiated by the attachment of asexual spores (conidia, Fig.1 a) disseminated by wind or splashed raindrops on infected plant organs. Spore release is regulated separately from production of conidia on conidiophores. The release of conidia is triggered by a one- to two- hour exposure to darkness and decreases with continued darkness (Daniel J.E., 2007). Ingold C.T. (1964) suggested that the small stalk cell formed at the base of the conidium builds turgor pressure until it ruptures, thereby launching the conidium, but this has not been confirmed by photos.

A single polarized germ tube emerges from the spore, normally from its tapering end, and grows across the leaf surface before differentiating into the dome-shaped appressorium which is apparent on the epidermis (Veneault-Fourrey *et al.*, 2006). On the leaf surface, the fungus may also respond to cutin monomers, ascis-9, 10-epoxy-18-hydroxyoctadecanoic acid or lipid monomers, which are effective inducers of appressorium development (Talbot, 2003). Formation of appressoria requires a hard, hydrophobic surface and can be induced in the laboratory using hydrophobic plastic surfaces, such as polytetrafluoroethylene (Richard & Nicholas, 2009). Hours after development, the swollen appressorium is melanized, caused by an impermeable layer in between the cell wall containing melanin which is derived from a polyketide precursor. This characteristic enables the glycerol filled appressorium to develop a turgor pressure up to 8.0 MPa. The highly concentrated glycerol is responsible for the

formation of a penetration peg, which ruptures through the leaf cuticle by mechanical force, allowing invasion of the underlying leaf tissue (de Jong *et al.*, 1997; Talbot, 2003). Germination and penetration may be accomplished in 7-8 hours.

After penetration, the fungus forms specialized biotrophic invasive hyphae that successively colonize living rice cells without visible symptoms for the first 4 days (Valent and Khang, 2010). Subsequent invasion of hyphae results in a biotrophic and a necrotrophic phase, indicating *Magnaporthe* to be a hemibiotrophic pathogen. During the biotrophic phase at the early stages of infection, primary hyphae differentiate to bulbous intracellular invasive hyphae that fill up the plant cell lumen and directly contact the membrane of the infected cell. Colonization of host tissues by the fungus occurs through the perforation of cell walls from adjacent cells without causing cell death, likely using plasmodesmata as penetration points, or through hyphal growth in the apoplast. After a few days of biotrophic growth within plant tissues, the fungus switches to the necrotrophic phase, during which it colonizes the plants by degrading the cell wall at a later period of invasion (Heath *et al*., 1990; Ribot, 2008). Nevertheless, depending on the *Magnaporthe* strain, the severity of disease induced in the invaded host plants may differ.

The colonization spreads with fungal hyphae rapidly growing and ramifying within and between cells in the plant tissue, resulting in visible lesions that are symptomatic of blast disease after 5 to 7 days. Aerial conidiophores produce a sympodial arrangement of conidia from disease lesions under conditions of high humidity, allowing the disease to spread to adjacent plants and initiate a new cycle (Ou, 1985; Talbot, 2003).

The peak of spore release occurs at midnight, allowing the spores to disseminate while humidity is high and dew is forming. Rain splash and strong wind also lead to dispersal at other times during the day. Conidia are coated with a rodlet layer of hydrophobin making them hydrophobic such that spores are not easily wetted and thus do not readily fall to the ground with the water droplets. Rather, they are launched into the air by the force of rain dropping on the leaf surface (Ebbole, 2007).

Under favorable conditions, the fungus sporulates in the center of the lesions on infected plant tissues. The spores, called conidia, are produced abundantly on the tips of denticulate conidiophores that extend beyond lesion surfaces. A conidiophore may bear twenty or more conidia, and a typical leaf lesion produces 4,000-6,000 conidia every night (Ou, 1980). Conidia are produced after several hours of high humidity and are easily released or liberated near mid-day, especially under windy conditions. Most conidia travel only one or two meters from their source before landing on the plant (TeBeest *et al*., 2007).

The teardrop-shaped and three-celled conidium sticks to the plant organ of its host plant by means of special adhesive released from an apical compartment in the tip of each spore during hydration. This adhesive provides the spore with a way to anchor itself tightly to the hydrophobic rice surface and allows germination to start (Hamer *et al.*, 1988).

1.5 **Management of blast on wheat and rice**

1.5.1 Management of wheat blast

The control of wheat blast requires the integration of a series of measures. In general, these tools mentioned in integrated management of wheat crop disease are similar to these recommended below to control rice blast, such as early sowing, avoidance of irrigation in the early morning and evening, deep plowing and eliminating alternative hosts like grass weeds (Pannwitt, 2012). The following management measures are described in detail for the wheat-*Magnaporthe grisea* pathosystem.

The first is the use of healthy seeds. Reis *et al.* (1995) have verified that the pathogen from wheat has high efficiency in transmission by wheat seeds, besides the ability to remain viable on seeds up to 22 months. Hence, the use of healthy seeds or seeds treated with fungicides could also be seen as an important measure to restrict the initial establishment of wheat blast in the field.

The second point is that the wide host range on cereal crops of wheat blast greatly limits the implementation of a crop rotation strategy to control the disease.

As most of the wheat cultivars demonstrate high susceptibility to blast, the use of fungicides to protect the spikes becomes one of the main alternatives for wheat growers, especially in regions with historical occurrence of the disease. However, there are only few active ingredients registered for chemical control of blast on wheat spikes. In Brazil, wheat producers use fungicides to protect their crop during the heading stage, with an effect of maximum 50% protection (Igarashi, 1990). Etienne Duveiller, wheat pathologist and associate director of CIMMYT`s global wheat program, also mentioned that there are places where farmers are using four fungicide applications with no results, which suggests that current chemicals are not effective against the fungus, or are not properly applied (Corporate Communications, 2010).

The most desired and cost effective tool for controlling wheat blast is resistant cultivars. Preliminary results from CIMMYT international nurseries (2004) in Brazil suggest that several genotypes may have some level of blast tolerance. Yet, since the ascomycete fungus has a high genetic variability, major resistance genes are broken easily. Cultivars such as BR18, IPR 85 and CD113 moderate levels of resistance, while Milan, the cultivar from the CIMMYT line, reveals a high level of resistance (Kohli *et al.*, 2010). It is more difficult to screen or breed resistant cultivars for wheat than rice. Urashima and Kato (1994) have analyzed the response of forty-three wheat cultivars from Brazil, Japan, United States, and Bulgaria, added to seven species of *Triticum* and eighteen lines of *Aegilops* under greenhouse conditions. All cultivars showed susceptible reactions except two lines from *Aegilops* that presented some promising results. This similar varietal resistance has also been reported in 2004 by Urashima *et al.*, when twenty wheat (*Triticum aestivum*) cultivars were inoculated with seventy-two monoconidial isolates of *Magnaporthe grisea* obtained from the States of Mato Grosso do Sul and Paraná None of the wheat cultivars were resistant to all isolates of *M. grisea*, and the cultivar BR18 was suggested to have a broad resistance in relation to the rest of the tested cultivars to the isolates of *M. grisea* in the States of Mato Grosso do Sul and

Paraná BR18 seems to carry a combination of several major genes for resistance reflected in the best RSR. However, the resistance of this cultivar in our experiment showed differential resistance to seventy *Magnaporthe* isolates. Approximately 35% of wheat isolates showed a strong pathogenicity to BR 18, and more than 80% of the leaf area had symptoms, but 45% of isolates induced less than 50% of lesions on the leaves. The mutation and parasexuality (Ou, 1980; Zeigler *et al.*, 1997; Zeigler, 1998) would explain why some cultivars resistant in the previous studies, BH1146, BR11, CNT8 (Igarashi *et al*, 1986; Barros *et al.*, 1989; Goulart & Paiva, 1992; Goulart & Paiva, 1993) were not resistant in the present work. It is important to continue looking for new sources of resistance to blast and study the factors associated with this trait such as checking for new avirulence genes from the pathogen and selecting the resistance genes which are most effective. Maciel (2011) mentioned that there is an initiative to control wheat blast by transformation of wheat plants with major resistance genes for rice blast, which are associated with complete resistance, as well as gene pyramiding.

1.5.2 Management of rice blast

In order to most effectively control infection by rice blast, an integrated management should be implemented to avoid overuse of a single control method and fight against genetic resistance. The main cultural practices used to control rice blast in upland environments are the following: good soil preparation with deep plowing, uniformity in planting, seeding at the appropriate time which means sowing seeds early after the onset of the rainy season, management of the amount of water supplied to the crops limits spore mobility thus dampening the opportunity for infection, practice of recommended plant-spacing and timely weed management, restricted use of nitrogen topdressing and application of balanced doses of other fertilizers (IRRI, 2014). More details are described below.

Elimination of crop residues could reduce the occurrence of overwintering and discourage inoculation in subsequent seasons. If possible, diseased straw and stubble should be destroyed by burning. This is an important control measure, but will not provide complete control by itself. Burning diseased crop residues will reduce overwintering inoculum in a given field and region, but will not protect the field from other inoculum sources.

Silicon fertilizers (e.g., calcium silicate) can be applied to soils that are silicon deficient to reduce blast. However, because of its high cost, silicon should be applied efficiently. Cheap sources of silicon, such as straw of rice genotypes with high silicon content, can be an alternative. Care should be taken to ensure that the straw is free from blast as the fungus can survive on rice straw and the use of infected straw as a silicon source can spread the disease further.

Crop rotation is one simple and effective technique that is highly recommended simply because it provides a mechanism that separates viable spores in crop residues from the newly emerging seedlings.

Use of high quality healthy seeds is important. Infected seeds left on the soil surface provide inoculum from which epidemics develop and transmission of infected seeds by intercontinental trade or distribution of rice varieties from the breeder to the farmer could be the reason for the introduction of rice blast to different continents.

Applications for blast control techniques in irrigated environments are very similar to those of the upland. The main differences are related to the proper management of irrigation itself. Continuous flooding is recommended to limit blast development. Field drainage, especially for extended periods, should be avoided. Since it allows the formation of nitrate and may cause drought stress. Since shallow water favors the disease more than deep water, moderate water (4-5 inches) and deep water (6-8 inches) are suggested for early and late season, respectively (Scardaci, 1997).

Pathogenicity is often used for screening and breeding of resistant cultivars. DNA fingerprint groups specific to a particular geographical region were also obtained by Sharma *et al.* (2002) in the pathotype analyses of *Magnaporthe grisea* populations from the north-western Himalayan region of India. A pathogen population consisting of 119 isolates from the north-western Himalayan region has been classified into fifty-two pathotypes on the basis of disease reaction in the international differential rice lines. The lowest frequency of virulence was recorded on the rice line Tadukan (Pi−ta and/or Pi−ta^2) and BL-1(Pi−b and Pi−sh), while it was highest on Caloro (Pi−k^s) followed by NP125 (Pi−? and K-60 (Pi−k^p). Rice line Tetep (Pi−k^h+?) was highly effective in the north-western Himalayan region since none of the isolate could infect this line. Nevertheless, virulence rarely occurred on Fukunishiki (Pi−z^s). These blast resistant rice lines can be used in resistance breeding for the effective management of rice blast in this region of India.

However, in spite of the development of resistant cultivars being a cheap alternative for the farmer with low environmental impact, management of rice blast in existing resistant cultivars does not have a successful long-term strategy. Evolution of the pathogen results in the emergence of new virulent isolates, which make those resistant cultivars carrying resistance genes without specificity against the new types susceptible to blast. Resistant rice cultivars lose their effectiveness after 2 to 3 years of widespread use in commercial production (Ou, 1980). This is a common event in rice cultivars and depends largely on how much the newly released resistant cultivars are adopted by farmers and come to occupy major proportions of areas of a given region, state or country. Zhu *et al.* (2000) suggested to plant a mixture of resistant and susceptible varieties of rice in the field by the use of crop heterogeneity. This practice leads to greater yield than fields planted in monoculture in Yunnan Province, China. This kind of rice cultivation seems to be very efficient in avoiding breaking resistances of rice cultivars by rice blast.

The use of chemical fungicides to control the disease has long been viewed as a last resort for rice blast. There are two basic techniques that can be used to manage diseases with the chemical fungicide strategy. The first one is seed treatment to prevent infection of seedlings after germination. The second is using fungicides to prevent infection in leaves and panicles during the growing season by making one or two applications of fungicides to the foliage to protect the panicles when they are emerging from the boot. This technique attempts to reduce the incidence of rice blast on the panicle necks and panicles. Systemic fungicides like triazoles and strobilurins can be used judiciously to control blast. The melanin layer in the appressorium is essential to build-up the very high pressure required to puncture the outer plant surface. This accounts for the special class of fungicides, the melanin biosynthesis inhibitors (tricyclazole, pyroquilon, phthalide, and carpropamid), which are specific for controlling rice blast

disease (Barbara V., 2013). In Brazil, the most-used fungicide for spraying the aerial parts of the plant is tricyclazole, which has performed reasonably well in controlling rice blast, but has no effect on other rice diseases (Maciel, 2011).

1.6 Objectives of the studies

The overall aim of this thesis was to study the optimum conditions for the development of wheat blast under controlled conditions and distinguish *Magnaporthe* spp. from wheat and rice. The specific objectives are the following:

- Identification of the optimal conditions for wheat blast infection. The culture conditions of *Magnaporthe* spp. *in vitro* were firstly tested and then the optimal conditions for the development of *Magnaporthe grisea* on wheat ears were found out in the climate chamber.

- Blast isolates from wheat and rice are considered as different taxonomic groups, therefore pathogenicity distinction was performed with isolates from both hosts. The disease phenotype of *Magnaporthe* spp. on wheat and rice leaves was recorded in the climate chamber.

- Development of molecular markers to distinguish different groups among *Magnaporthe* spp. The genotypic differentiation was analyzed with AFLP and MLST. Establishment of a precise disease assessment (quantitative real-time PCR) for specific detection of *Magnaporthe* spp. biomass on inoculated leaf samples.

- Observation of leaf infection processes by fluorescence microscopy and confocal laser scanning microscopy (CLSM).

- Study of the impact of phytotoxin of *Magnaporthe* spp. on wheat and rice leaves.

2 Materials and methods

2.1 Chemicals

Agar	AppliChem, Darmstadt, Germany
Agarose	AppliChem, Darmstadt, Germany
Ammonium nitrate (NH_4NO_3)	SIGMA-Aldrich, Saint Louis, USA
Ammonium tartrate ($(NH_4)_2C_4H_4O_6$)	SIGMA-Aldrich, Saint Louis, USA
ATP (100mM)	Thermo Scientific, Schwerte, Germany
Calcium chloride ($CaCl_2.2H_2O$)	SIGMA-Aldrich, Saint Louis, USA
Calcium carbonate ($CaCO_3$)	Merk, Darmstadt, Germany
Chloroform	Applichem, Darmstadt, Germany
DNA Loading Dye (6 ×)	Fermentas, St. Leon-Rot, Germany
dNTPs (10mM)	Fermentas, St. Leon-Rot, Germany
Dream *Taq*-buffer (10x)	Fermentas, St. Leon-Rot, Germany
EDTA	AppliChem, Darmstadt, Germany
Ethanol	AppliChem, Darmstadt, Germany
Ethidium bromide	AppliChem, Darmstadt, Germany
FastAP (1U/µl)	Thermo Scientific, Schwerte, Germany
FastDigest BanmHI (10x)	Thermo Scientific, Schwerte, Germany
FastDigest PstI (10x)	Thermo Scientific, Schwerte, Germany
FastDigest Smal (10x)	Thermo Scientific, Schwerte, Germany
FastDigest SmaI Buffer (10x)	Thermo Scientific, Schwerte, Germany
Glycerol	Roth, Karlsruhe, Germany
GeneRuler™ 100 bp Plus DNA Ladder	Fermentas, St. Leon-Rot, Germany
Isoamyl	Merk, Darmstadt, Germany
Lambda DNA Standard (300ng/µl)	Fermentas, St. Leon-Rot, Germany
Magnesium chloride ($MgCl_2$)	Fermentas, St. Leon-Rot, Germany

β-Mercaptoethanol	AppliChem, Darmstadt, Germany
PCR-buffer (10x)	Bioline, Luckenwalde, Germany
PEG 4000 (50%)	Thermo Scientific, Schwerte, Germany
Pfu Buffer (10x) with $MgSO_4$ (25mM)	Thermo Scientific, Schwerte, Germany
Pfu DNA Polymerase (2.5U/µl)	Thermo Scientific, Schwerte, Germany
Phenol	AppliChem, Darmstadt, Germany
Potassium phosphate monobasic (KH_2PO_4)	Merk, Darmstadt, Germany
Proteinase K	Analytik Jena, Jena, Germany
Restriction enzyme *EcoR*I (10U)	Thermo Scientific, Schwerte, Germany
Restriction enzyme *Mse*I (*Tru*I, 10U)	Thermo Scientific, Schwerte, Germany
RNAse A (10mg/ml)	Applichem, Darmstadt, Germany
Sodium chloride (NaCl)	Merk, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	AppliChem, Darmstadt, Germany
Streptomycin sulphate	Duchefa Biocheme, Harrlern, Netherlands
Sucrose	AppliChem, Darmstadt, Germany
SureClean™ Plus	Bioline, Luckenwalde, Germany
SYBR Green I (1:1000)	Invitrogen, Karlsruhe, Haarlem, Germany
T4 DNA ligase (5 U/µl)	Thermo Scientific, Schwerte, Germany
T4 DNA ligase buffer (10x) with ATP (0.5mM)	Thermo Scientific, Schwerte, Germany
T4 Polynucleotide Kinase (10U/µl)	Thermo Scientific, Schwerte, Germany
Tango buffer (10x)	Thermo Scientific, Schwerte, Germany
Taq DNA Polymerase (BioTAQ™) (5U/µl)	Thermo Scientific, Schwerte, Germany
TBE buffer	AppliChem, Darmstadt, Germany
TE buffer	AppliChem, Darmstadt, Germany
Tris (pH 8)	Merk, Darmstadt, Germany
Tween 20	NeoLab Migge, Heidelberg, Germany
Vegetable juice	Granini, Nieder-Olm, Germany

Yeast extract	Roth, Karlsruhe, Germany

2.2 Media and buffers

Media and buffers were autoclaved at 121 ℃, 103.4 kPa pressures for 20 min.

V8 agar medium

Vegetable juice	100ml
$CaCO_3$	2g
Agar	15g
Distilled water	900ml
Streptomycin sulfate (after autoclaving, Duchefa Biochemi)	200mg

Complete medium agar (CM)

Yeast extract	3g
Casamino acid	3g
Sucrose	5g
Agar	15g
Distilled water	1L
Streptomycin sulfate (after autoclaving)	200mg

OA medium

Oat flour	20g
Sucrose	2g
Agar	15g
Distilled water	1L
Streptomycin sulfate (after autoclaving)	200mg

PDA

PDA extract (Potato Glucose Agar)	39g
Distilled water	1L

Liquid Fries Complete medium

Sucrose	30 g
Ammonium tartrate (NH^{-4} Tartrate)	5 g
Ammonium nitrate (NH_4NO_3)	1 g
Potassium phosphate KH_2PO_4	1 g
Magnesium sulfate ($MgSO_4.7H_2O$)	0.5 g
Sodium chloride (NaCl)	0.1 g
Calcium chloride ($CaCl_2.2H_2O$)	0.13 g
Yeast extract	1 g
$FeSO_4.7H_2O$	0.02g
Distilled water	1 L
Streptomycin sulfate (after autoclaving)	200mg

SOC medium

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
KCl (250mmol/L)	10 ml
Distilled water	1 L
Glucose (1M) (after autoclaving)	20 ml
$MgCl_2.6H_2O$ (2mol/L) (before using)	5 ml

$MacConkey_{AP}$ medium

MacConkey	54 g
Distilled water	1 L
Ampicillin (100mg/ml) (after autoclaving)	1 ml

LB_{AP} medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Distilled water	1 L
Ampicillin (100mg/ml) (after autoclaving)	1 ml

$Terrific\ broth_{AP}$ medium

Tryptone	12 g
Yeast extract	24 g
Glycerol	4 ml
Distilled water	1 L
KH_2PO_4(0.17M)+K_2HPO_4(0.72M) (after autoclaving)	100 ml
Ampicillin (100mg/ml) (after autoclaving)	1 ml

CTAB buffer

CTAB (1%)	10g
NaCl (0.7M)	40g
Tris/HCl (50mM, pH 8.0)	50ml
EDTA (10mM)	20ml
PVP (1%)	10g
LiCl (100mM)	25ml
Distilled water	Up to 1L

1 ×TE buffer

Tris (10mM)	0.5ml
EDTA (1mM)	0.1ml
Distilled water	Up to 50ml

PBS (Phosphate Buffered Saline) buffer (pH 7.4)

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Tween 20	0.5ml
Distilled water	Up to 1L

2.3 Isolation and *in vitro* cultivation of *Magnaporthe grisea*

2.3.1 Isolation of *Magnaporthe grisea* from infected wheat tissues

Isolation from kernels

Infected wheat kernels were surface-disinfested in 3% NaOCl for 1 min, washed twice in sterilized distilled water and incubated in moist chambers on filter paper at 25 ℃ under constant illumination. The disinfested kernels were cut in two halves and aseptically incubated on Complete Medium Agar (CM) at 25 ℃ in a 12-h light/dark cycle for 4 d. Single colonies were transferred on V8 agar plates supplemented with 200 mg/L streptomycin sulfate. As a result, a total of thirty-six isolates from different locations were obtained and used in the experiment (Table 1). Isolates were microscopically checked for their purity.

Isolation from leaves

The surface of infected wheat leaves was washed with sterilized distilled water and incubated on two layers of moistened filter paper in Petri dishes at 25 ℃ for 2 d. Fungal material was scraped from the surface of symptoms with a sterile needle, transferred on CM agar and incubated at 25 ℃ for 2 d. This isolation procedure was repeated three times.

Storage

For long term storage of samples, pure cultures of relevant fungal isolates were grown on V8 agar plates covered with small sterilized filter paper discs (9 mm diameter). After 10 days of incubation at 25 ℃ and 12 h light/dark, the filter papers colonized by mycelium from the respective isolates were desiccated and stored at -20 ℃.

2.3.2 *In vitro* cultivation

2.3.2.1 Medium selection

One piece of filter paper from each of the wheat isolates MG 1.2 and MG 5 were cultured on CM at 25 ℃ and 80% relative humidity for 7 d. One 5mm diameter agar block with fungal mycelium punched out from the margin of a colony was transferred to the center of CM, OA, PDA and V8 plates and incubated in the same conditions as before. Colony morphology, colony diameter and sporulation were recorded at 7 dpi. There were three replications prepared for each medium.

2.3.2.2 Cultural characteristics on V8 agar medium

Mycelium agar discs were punched out with a sterile cork borer (5 mm diameter) from the surface of 7-day old colonies on CM agar and transferred onto the center of V8 agar plates upside down with a sterile needle. Each plate had one mycelium disc and three replications were prepared for each of the seventy isolates from different hosts (Table 5). The plates were incubated in a climate chamber at 25 ℃ and 80% humidity in a 12-h light/dark cycle for 7 d. The cultures from these plates were used to determine the characteristics of colonies. Colony morphology were determined by visual observation while the colony diameter was measured daily using a Vernier calliper by taking an average of four radial measurements. After mycelium exposure under UV light for 2 d and washing with 100 µl of distilled water in each plate, one drop of each conidial suspension was evaluated for sporulation under a binocular microscope.

2.3.2.3 Mycelium dry weight in Liquid Fries Complete medium

All seventy isolates (shown in Table 1), collected from wheat and rice, were grown in flasks (500 ml) containing 200 ml of sterilized Liquid Fries Complete medium. Flasks were inoculated with one agar block which was punched out from the margin of a 7-day old colony on V8 agar. The cultures were placed on a rotary shaker at 100 rpm and incubated in the dark at 25 ℃ for 10 d. Mycelia were harvested by filtration through two layers of filter paper in a porcelain filter funnel, dried in a vacuum freeze dryer for 48h and stored at -20 ℃ until further use. The dry weight of each mycelium was registered.

2.3.2.4 Mycelial growth after cultivation at low temperature

Five isolates of *Magnaporthe grisea* from different wheat fields (MG 27 and MG 51 from Brazil, MG 1.2 and MG 5 from Bolivia; 1836-3/0-12 from Japan) were selected, due to their strong growth ability based on the experiment of cultural characteristics. Those isolates were transferred on V8 agar at 25 ℃ for 3d. In present, *Magnaporthe* strains are mainly conserved as the form of fungus-colonized filter papers under -20 ℃, which is very infective but a little bit complicated. In order to figure out the possibility of long term cryopreservation of *Magnaporthe* strains directly on the medium with high infectivity, the low terperature treatment was carried out. Three Petri dishes of each isolate were kept at 4 ℃ or -20 ℃, respectively, for 2 d, and another three dishes of each isolate incubated at 25 ℃ served as control. After 2 days of cold treatment, all dishes were placed in the same climate chamber at 25 ℃ for another 3 d to test mycelia activity. The colony diameters of all treatments were recorded daily by Vernier calliper and sporulation was checked in the end.

2.4 Plant material, fungal strains and inoculation methods

2.4.1 Plant material and cultivation

2.4.1.1 Wheat plants

The wheat cultivar BR 18, a widely grown moderately resistant variety, was used in this study. Plants were grown from seeds in 9 × 9 cm plastic pots filled with a sieved mixture of sand, peat and compost (1:1:2). Two kernels were sown in one pot and after 2 weeks, each healthy

seedling was transplanted into one pot filled with the same mixed soil. All plants were grown in the greenhouse at 16:8 h light/dark cycle at 23 ± 2 ℃ and 60% relative humidity. Plants were well watered and no fertilizer was applied.

2.4.1.2 Rice plants

The rice cultivar CO 39 (susceptible indica variety) is strongly susceptible to *Magnaporthe* spp. under field conditions. Rice seeds were soaked in water, pre-germinated in a 9cm Petri dish on two layers of filter paper which had been moistened with sterile distilled water and incubated in the dark at 32 ℃ for 7 d in an incubator. After 7 d, the pre-germinated seeds were transferred into 9 × 9 cm plastic pots containing a mixture of sandy loam soil, sand and organic soil in a ratio of 1:1:1 and kept at the same conditions in the greenhouse as mentioned above. Each pot was planted with three seedlings. Nitrogen fertilization with 10 g/L of YaraVita™ and TENSO™Iron (EC Fertiliser, CA Vlaardingen, The Netherlands) was applied 5 weeks after sowing.

2.4.2 *Magnaporthe* spp. isolates

Seventy *Magnaporthe* spp. isolates (Table 1) from different geographical and host origins were used in the experiment. Thirty-seven isolates from wheat were isolated in our laboratory; thirty isolates from rice came from thirteen fields in three West-African countries and were provided by Geoffrey Onaga (Ph.D. student, Division of Plant Pathology and Crop Protection, Georg-August-University Göttingen, Germany). One wheat isolate, one finger millet isolate and two perennial ryegrass isolates were kindly provided by Prof. Dr. Yokio Tosa (Dept of Agrobioscience Graduate School of Agricultural Science, Kobe University, Japan).

Table 1 List of seventy *Magnaporthe* spp. isolates used in this study according to host plant and geographic origin

Isolate name	Host plant	Geographic origin
T-4 / Br48**	*Triticum aestivum* (wheat)	Brazil
MG 1.2*	*Triticum aestivum* (wheat)	Bolivia
MG 5* / **	*Triticum aestivum* (wheat)	Bolivia
MG 8	*Triticum aestivum* (wheat)	Bolivia
MG 11	*Triticum aestivum* (wheat)	Bolivia
MG 14	*Triticum aestivum* (wheat)	Bolivia
MG 27	*Triticum aestivum* (wheat)	Brazil
MG 44*	*Triticum aestivum* (wheat)	Brazil
MG 51	*Triticum aestivum* (wheat)	Brazil
MG 52	*Triticum aestivum* (wheat)	Brazil
MG 5.1	*Triticum aestivum* (wheat)	Bolivia
MG 5.2	*Triticum aestivum* (wheat)	Bolivia
MG 8.1	*Triticum aestivum* (wheat)	Bolivia
MG 8.2	*Triticum aestivum* (wheat)	Bolivia
MG 20.3	*Triticum aestivum* (wheat)	Brazil
MG 29	*Triticum aestivum* (wheat)	Brazil
MG 9* / **	*Triticum aestivum* (wheat)	Bolivia
MG 12	*Triticum aestivum* (wheat)	Bolivia
MG 16*	*Triticum aestivum* (wheat)	Bolivia

Isolate name	Host plant	Geographic origin
MG 38	*Triticum aestivum* (wheat)	Brazil
MG 50	*Triticum aestivum* (wheat)	Brazil
MG 52	*Triticum aestivum* (wheat)	Brazil
MG 53	*Triticum aestivum* (wheat)	Brazil
MG 54	*Triticum aestivum* (wheat)	Brazil
Ca 89*	*Oryza sativa* (rice)	Philippines
43	*Oryza sativa* (rice)	Philippines
JMB8401	*Oryza sativa* (rice)	Philippines
AGT211*	*Oryza sativa* (rice)	Philippines
M36-1-3-10-1**	*Oryza sativa* (rice)	Philippines
C9228-37	*Oryza sativa* (rice)	Philippines
CBN9214-1*	*Oryza sativa* (rice)	Philippines
B90103(BN111)	*Oryza sativa* (rice)	Philippines
B90099	*Oryza sativa* (rice)	Philippines
M39-1-2-21-2	*Oryza sativa* (rice)	Philippines
V850256	*Oryza sativa* (rice)	Philippines
V86010**	*Oryza sativa* (rice)	Philippines
Br116.5 / T-7**	*Triticum aestivum* (wheat)	Brazil
Ken 15-15-1* / **	*Eleusine coracana* (finger millet)	Japan
TP / L-2* / **	*Lolium perenne* (perennial ryegrass)	Japan
FI5 / L-5*	*Lolium perenne* (perennial ryegrass)	Japan
1836-3 / 0-12* / **	*Triticum aestivum* (wheat)	Japan
MG 19	*Triticum aestivum* (wheat)	Brazil
MG 21	*Triticum aestivum* (wheat)	Brazil
MG 25	*Triticum aestivum* (wheat)	Brazil
MG 28*	*Triticum aestivum* (wheat)	Brazil
MG 30	*Triticum aestivum* (wheat)	Brazil
MG 31*	*Triticum aestivum* (wheat)	Brazil
MG 32	*Triticum aestivum* (wheat)	Brazil
MG 33	*Triticum aestivum* (wheat)	Brazil
MG 39*	*Triticum aestivum* (wheat)	Brazil
MG 48	*Triticum aestivum* (wheat)	Brazil
482RWA09	*Oryza sativa* (rice)	Rwanda
503UGA09	*Oryza sativa* (rice)	Uganda
520UGA09	*Oryza sativa* (rice)	Uganda
523UGA09	*Oryza sativa* (rice)	Uganda
524UGA09	*Oryza sativa* (rice)	Uganda
492RWA11* / **	*Oryza sativa* (rice)	Rwanda
500RWA11	*Oryza sativa* (rice)	Rwanda
528UGA11*	*Oryza sativa* (rice)	Uganda
531UGA11	*Oryza sativa* (rice)	Uganda
552UGA11	*Oryza sativa* (rice)	Uganda
556UGA11	*Oryza sativa* (rice)	Uganda
559UGA11*	*Oryza sativa* (rice)	Uganda
561UGA11	*Oryza sativa* (rice)	Uganda
MG 10	*Triticum aestivum* (wheat)	Brazil
563UGA11	*Oryza sativa* (rice)	Uganda
564UGA11* / **	*Oryza sativa* (rice)	Uganda

Isolate name	Host plant	Geographic origin
RWA 11.2*	*Oryza sativa* (rice)	Rwanda
507RWA11	*Oryza sativa* (rice)	Rwanda
511RWA11	*Oryza sativa* (rice)	Rwanda

* Isolates used on MLST test

** Isolates used to test the specificity and sensitivity of primers by PCR

2.4.3 Inoculum preparation and inoculation of plant ears and leaves

2.4.3.1 Inoculum preparation

Filter papers colonized by *Magnaporthe* spp. were cultured in Petri dishes containing 20 ml of V8 agar under sterile conditions. The cultures were placed in a growth chamber with a 16 h/8 h (light/dark) cycle and a constant temperature of 25 ℃ for 7 d, followed by exposure under NUV light for 2 d to promote conidiation. Conidia were harvested by rinsing the surface of cultures with 100 µl sterile distilled water and scraping it using a glass slide. The conidial suspension was filtered through two layers of gauze to remove mycelia and agar. The number of conidia in the suspension was determined using a haemocytometer and a spore concentration of 1.0×10^5 conidia/ml was used for inoculation. Before inoculation, 0.01% of the nonionic surfactant Tween 20 was added to the conidial suspension.

2.4.3.2 Inoculation

Wheat ear inoculation

Ear inoculations were performed by spraying 20 ml of the conidial suspension on wheat ears (flowering stage BBCH 61-65, Table A1) using a glass sprayer connected to an air compressor until drop-down. The inoculated ears were enclosed with plastic bags to maintain 100% relative humidity and outside of the plastic bag were covered with kraft paper bags to keep it in darkness. As Barksdale & Asai (1961) illustrated, conidiation requires a period of darkness and conidia are released in a diurnal pattern. The covered ears were incubated at 25 ℃ for 24 h. After incubation, wheat plants with inoculated ears were kept in controlled climatic chambers for 12 h light/dark at 25 ℃ and 80% relative humidity. The ear inoculation was repeated twice and five ears were used for each isolate.

Plant seedling inoculation

Seedling inoculations were performed on the whole plant of wheat seedlings (3-leaf stage) and rice seedlings (4-leaf stage) by spraying with conidial suspensions. To promote spore germination, all inoculated seedlings were covered by plastic bags and kept in a dark moist chamber at 25 ℃ and 100% relative humidity for 24 h equipped with a humidifier, and then moved to the micro-climate room with 12 h/12 h light/dark at 25 ℃ and 90% relative humidity. Three seedlings were inoculated with each isolate, and the experiment was repeated once. Seedlings sprayed with sterile distilled water served as control.

2.5 Microclimatic requirements of wheat blast infection on wheat ears

Since wheat blast is seed-borne and able to survive on alternate hosts, with uncertainties related to climate and absence of satisfactory genetic resistance, more information is needed to forecast epidemics and protect other regions and cropping systems from infection. The wheat pathogen is considered to be favored by warm and humid conditions, but the detailed epidemiological studies should be done to determine potential risk areas for this new pathogen. Changes in temperature and relative humidity can affect pathogen infection on host plant, so for the purpose of epidemiological study, the optimal conditions for infestation of *Magnaporthe grisea* by spray inoculation was conducted on wheat ears.

2.5.1 Variation of microclimatic conditions

The isolates of *Magnaporthe grisea* MG 1.2 and MG 5 were isolated from infected wheat kernels, originating from St. Cruz, Bolivia, at the Division of Plant Pathology and Crop Protection, Göttingen, 2008. Conidial suspensions from these two isolates with high sporulation (Table 5) were mixed before inoculation and the density in the mixture was adjusted to 1.0×10^5 conidia/ml. To test environmental factors, the mixed conidial suspensions were sprayed on wheat ears (BBCH 61-65) and incubated as described above. The covering kraft paper bags were firstly removed after 24h, followed by plastic bag covers which were removed depending on the treatment (covered for 24 h, 48 h, 72 h and 96 h). For the temperature treatment, 5 different incubation temperatures were designed: 20 ℃, 23 ℃, 26 ℃, 29 ℃ and 32 ℃ during the day, separately and 18 ℃ in the night. Plants were kept in 5 different climate chambers for the different temperature treatments; humidity treatment, which ears were sealed in different length of time, had five replicates and totally twenty plants in each climate chamber.

2.5.2 Disease symptom assessment for infected wheat ears

At 14 days post-inoculation (dpi), the blast severity level was determined according to a scoring system from 0-5 (Division of Plant Pathology and Crop Protection, Göttingen, 2008). 0 = absence of symptoms; 1 = less than 5% of ear surface bleached; 2 = 5% - 35% ear surface bleached; 3 = 35% - 65% ear surface bleached; 4 = 65% - 80% ear surface bleached; 5 = more than 80% ear surface bleached (Fig. 3).

0 **1** **2** **3** **4** **5**

Figure 3 Disease scores of *Magnaporthe grisea* on wheat ears (pictures from Department of Crop Sciences, Georg-August-University of Göttingen)

2.6 Pathogenicity differentiation of *Magnaporthe* spp. on seedling leaves

2.6.1 Inoculation and incubation conditions

Inoculation with seventy *Magnaporthe* spp. isolates was individually performed using the 3-leaf stage of wheat seedlings (3 weeks old) and the 4-leaf stage of rice seedlings (6 weeks old). After harvesting conidia from fungal cultures on V8 agar using sterile distilled water, the concentration was adjusted to 1×10^5 conidia/ml. Three seedlings were sprayed with 20 ml of conidial suspension using a sprayer and covered by plastic bags to keep 100% relative humidity. The inoculated seedlings were kept in a climate chamber at 25 ℃ in the dark for 24 h. Subsequently the plastic bags were removed and the plants were transferred to a growth chamber at 25 ℃,16 h/8 h light/dark and 80% relative humidity. This experiment was performed two times.

2.6.2 Evaluation of disease incidence and severity on infected leaves

Lesions were registered from four leaves per isolate. Disease incidence (percent leaves showing lesions) was recorded. Additionally, a disease index for blast pathogen was used to estimate the pathogenicity of isolates. Lesion types reflecting disease severity were assessed 6 days after inoculation according to the rating index described by Murakami *et al.* (2000). The disease severity was categorized by 6 progressive grades (Table 2).

Table 2 Rating scale for the percentage of symptom development on wheat and rice leaves infected by *Magnaporthe* spp.

Disease index	Percentage of symptom development
1	no visible symptoms
2	expansion of lesions less than 5%
3	expansion of lesions 6% ~ 20%
4	expansion of lesions 21% ~ 50%
5	expansion of lesions 51% ~ 80%
6	expansion of lesions more than 80% of the whole leaf area

2.7 Genotypic differentiation with AFLP and MLST

2.7.1 Phylogenetic studies of *Magnaporthe* spp. with Amplified Fragment Length Polymorphisms (AFLP)

Amplified fragment length polymorphism (AFLP) technology has the capability to detect polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. Therefore, to estimate the association with geographical origin, host species and genetic diversity, AFLP is used in the study for identification of genetic relationships among the seventy *Magnaporthe* spp. isolates (Table 1).

2.7.1.1 DNA extraction from mycelium

DNA extraction

Total genomic DNA extraction was performed according to the CTAB method used in the laboratory. Freeze-dried mycelium was ground to a fine powder with the help of liquid nitrogen using a pre-cooled ceramic mortar and pestle. Approximately 30 mg of powder was suspended and lysed with 500 µl CTAB buffer in a 2 ml Eppendorf tube. The samples were vortexed until the viscosity of suspension was significantly reduced and the formation of froth indicated the detachment of DNA from polysaccharides. 1 µl β-mercaopoethanol and 1 µl proteinase K were added, the sample was briefly vortexed incubated for 10 min at 65 ℃ by occasional gentle mixing. Fifty µl of 20% sodium dodecyl sulphate (SDS) was added by gently inverting the tube and the sample was incubated at 37 ℃ for 1 h. To facilitate the precipitation of most polysaccharides, proteins and cell debris, 75 µl of 5 M NaCl solution and 65 µl of CTAB/ NaCl (10% CTAB in 0.7m ol/L NaCl) solution were added and incubated at 65 ℃ for 15 min after components have been mixed by inverting the tube several times. An approximately equal volume of phenol/chloroform/isoamyl (25/24/1) was added and mixed thoroughly by shaking vigorously in a shaker for 5 min. The suspension was centrifuged at 10,000 rpm for 15 min at 4 ℃ after incubation, and the aqueous supernatant was immediately transferred to a fresh 1.5 ml fresh tube. The DNA in the aqueous supernatant was precipitated by adding an equal volume of ice cold isopropanol. The tube was placed in a

-20 ℃ freezer for at least 1 h before centrifugation at 10,000 rpm for 15 min. The precipitated DNA pellets were washed and centrifuged at 15,000 rpm for 5 min twice with 500 µl 70% ice cold ethanol and the remaining ethanol was carefully removed with the help of a pipet. The DNA was dried in a SpeedVac at 30 ℃ for 2 min. The dried DNA was dissolved in 50 µl TE buffer with 3 µl RNAse A (10mg/ml) to digest RNA, and further incubated at 37°C for 30 min. All DNA samples were stored at -20 ℃.

Quantification of extracted DNA

DNA was quantified by running on 1% agarose gel. The agarose gel was prepared as follows: Agarose was dissolved in 0.5 × TBE buffer (boric acid 55.03 g/L (0.89 M); EDTA-$Na_2.2H_2O$ 7.44 g/L (0.02 M); Tris 107.81 g/L (0.89 M) in a microwave oven with gentle shaking. Then 0.0001% v/v ethidium bromide (EB) was added to the solution after the melted agarose had cooled to 60 ℃. Liquid agarose was poured on a gel support with a comb and remained for 30min before putting it into a 0.5 × TBE buffer tank. Two µl of DNA aliquot mixed with 1 µl of 6 × DNA loading dye was loaded into 1% agarose gel and run at 45 V for 60 min, along with Lambda DNA as standards with different amounts ranging from 50, 100, 150, 200 and 300 ng. Gel documentation and analysis were performed with the Multi-Analyst software (Version 1.1, Bio-Rad Laboratories, Hercules, USA).

2.7.1.2 AFLP analysis

Sample preparation and amplification for AFLP were performed according to Laurentin H.E (2006) with minor modifications. Electrophoresis of PCR products and data analysis was according to Tredway *et al.* (2004), Weiberg A. (2009) and Splivallo *et al.* (2012).

Restriction digestion of genomic DNA

Genomic DNA (250 ng) was digested with two restriction enzymes *EcoR*I and *Mse*I in a total volume of 20 µl reaction solution by adding the first enzyme 1 unit of *EcoR1I* (10 U) with 4 µl of 10 × Tango buffer and completing the volume with sterile distilled water. Reaction solutions were spun briefly and incubated at 37 ℃ for 6 h. Then 0.5 units of *MseI1*(10 U) was added, reaction solutions were spun again and further incubated at 65 ℃ for 3 h followed by heat deactivation of enzymes at 65 ℃ for 10 min. The double-digested DNA was separated by 0.8% agarose gel in TAE buffer at 3 V/cm distances of electrodes for 3 h.

Ligation of adapters

Adapter-ligation-solution was prepared for all DNA samples as follows: 5µl of forward and reversed *EcoR*I adapter and 50 µl of both strand *Mse*I adapters (Table 3) were mixed with 100 µl T4 ligase buffer (10 ×) in a total volume of 1000 µl premixed solution by completing with sterile distilled water. 10 µl of adapter-ligation-solution was ligated with an equal volume of each double-digested sample by the help of 1unit T4 DNA ligase (5 U/µl). Samples were incubated at room temperature overnight and then diluted 10-fold with sterile distilled water.

Pre-amplification reactions of ligated DNA

Pre-amplification reactions were conducted in a TPersonal thermocycler (Biometra, Göttingen, Germany) with primers complimentary to the adapter sequences with one selective nucleotide, *EcoR*I-A and *Mse*I-C (Table 3). Diluted ligation reaction (1 µl) was added to 2.5 µl of 10 × PCR buffer, 3 mM of $MgCl_2$, 1 unit of *Taq* DNA polymerase, 10 pmol of *EcoR*I-A primer, 10 pmol of *Mse*I-C primer, 0.2 mM of each dNTPs and water to a final volume of 25 µl. Thermal cycling conditions included an initial denaturation step at 94 ℃ for 30 s followed by 25 cycles at 94 ℃ for 30 s, 56 ℃ for 1 min, and 72 ℃ for 1 min, and a final extension step at 72 ℃ for 1 min. The pre-amplification products were diluted 10-fold for use in selective amplification reactions.

Selective amplification of pre-amplified DNA

Selective amplification reactions were performed with *EcoR*I and *Mse*I primers that included three selective nucleotides (provided by Prof. Petr Karlovsky, Division of Plant Pathology and Crop Protection, Georg-August-University Göttingen, Germany). A single *Mse*I primer (*Mse*I-CAC) was used in combination with three *EcoR*I primers (*EcoR*I-AGA, *EcoR*I-ACC and *EcoR*I-ACA) to yield three primer pair combinations. Each *EcoR*I primer included a 5′fluorescent label (Dy680-labelled *EcoR*I-AGA, Dy750-labelled *EcoR*I-ACC and Dy635-labelled *EcoR*I-ACA) for detection of PCR products by automated sequencing equipment. Twenty µl of selective amplification contained 1 µl of diluted pre-amplification product, 5 pmol of *EcoR*I primer, 5 pmol of MseI primer, 0.3 units of Taq DNA polymerase, 1 × PCR buffer, 3 mM $MgCl_2$, and 0.2 mM each dNTPs. Selective amplifications were programmed for an initial cycle at 94 ℃ for 30 s, 65 ℃ for 30 s, and 72 ℃ for 1 min, followed by a touchdown phase where the annealing temperature was lowered 0.7 ℃ each cycle for 11 cycles, and finally 30 cycles at 94 ℃ for 30 s, 56 ℃ for 1min, and 72 ℃ for 1 min. Pre-amplification and selective amplification was performed twice for each isolate from the same ligated DNA solution.

Table 3 Sequences of adapters and primers used in the AFLP analysis

Adapter	Sequence
Forward EcoRI adapter	5’- CTCGTAGACTGCGTACC - 3’
Reversed EcoRI adapter	3’- CATCTGACGCATGGTTAA-5’
Forward MseI adapter	5’- GACGATGAGTCCTGAG - 3’
Reversed MseI adapter	3’- CTGCTACTCAGGACTCAT-5’
Primer	**Sequence**
EcoRI-A	GACTGCGTACCAATTC-A
MseI-C	GATGAGTCCTGAGTAA-C
EcoRI-AGA	GACTGCGTACCAATTC-AGA
EcoRI-ACC	GACTGCGTACCAATTC-ACC
EcoRI-ACA	GACTGCGTACCAATTC-ACA
MseI-CAC	GATGAGTCCTGAGTAA-CAC

Electrophoresis of PCR products

For each *Magnaporthe* spp. isolate, 2.5 µl of selective amplification product from each of the three primer combinations was mixed with 30 µl of separation loading buffer (98% formamide, 10 mM EDTA and 0.025% bromophenolblue) and 1 µl of IRD800 labelled internal size standard 600 (Beckman Coulter Ltd). The mixture was overlaid by approximately 10 µl of mineral oil and separated by denatured linear polyacrylamide capillary electrophoresis CEQ™ 8000 (Beckman Coulter GmbH, Krefeld, Germany) under the following conditions: running temperature at 50 ℃, denaturation step at 95 ℃ for 2 min. Sample injection was at 2.0 kV for 30 s and separation was at 4.8 kV for 1 h.

Analysis of AFLP data

The raw data were imported and analyzed with the CEQ 8000 software (Beckman Coulter, Fullerton, CA, USA). Fragment recognition was performed with the following parameters: a maximum bin-width of three nucleotides, a slope threshold of 50%, and a relative peak height threshold of 10%. The confidence level was set at 95%. The applied model for calibration was the quartic curve model, which is recommended by Beckman when the Standard 600 is used. During gel evaluation, a binary matrix was constructed where each band was treated as a single character, and the band which presence in an individual was designated as 1 and the band which absence in another individual was designated by 0. The binary data were exported into a spreadsheet. All numerical taxonomic analyses were conducted using NTSYSpc 2.21 software (Exeter Software, New York, USA). Similarity matrices from binary data were derived with 'Qualitative Data' Program in Dis/similarity and genetic similarity were estimated using a Dice similarity coefficient. Clustering analysis was done with 'SAHN' program clustering by the unweighted pair group method with arithmetic averages (UPGMA). Based on genetic similarities, the dendrogram was constructed by the 'Tree plot' program.

The robustness of the dendrogram was assessed by bootstrap analysis with the Winboot software and 1,000 repeated samplings with replacement. The bootstrap values, reflecting the frequency with which each group is formed in repeated cycles of dendrogram construction, were used as a measure of the relative stability of the clusters of strains. And the cophenetic correlation for a cluster tree is defined as the linear correlation coefficient for the cophenetic distances obtained from the tree and the original distances (or dissimilarities) used to construct the tree. Thus, it is a measure of how faithfully the tree represents the dissimilarities among observations.

2.7.2 Multilocus gene genealogy analysis with Multilocus Sequence Typing (MLST)

MLST was used to investigate the phylogenetic relationships among *Magnaporthe* isolates from different hosts by analyzing DNA sequences of three housekeeping genes (actin, ß-tubulin and calmodulin). Based on the geographical origin and cultural characteristics (Table 5), 20 *Magnaporthe* spp. isolates were selected for this experiment (Table 1, isolates marked with an asterisk).

2.7.2.1 Gradient PCR reaction

Gradient PCR

Fragments of the actin, ß-tubulin, and calmodulin genes were amplified using the primer pairs ACT-512F (5'-ATGTGCAAGGCCGGTTTCGC-3') and ACT-783R (5'-TACGAGTCCTTCTGGCCCAT-3'), CAL-228F (5'-GAGTTCAAGGAGGCCTTCTCCC-3') and CAL-737R (5'-CATCTTTCTGGCCATCATGG-3') (Carbone & Kohn, 1999), and Bt1a (5'-TTCCCCCGTCTCCACTTCTTCATG-3') and Bt1b (5'-GACGAGATCGTTCATGTTGAACTC-3') (Glass & Donaldson, 1995), synthesized by Invitrogen™ (Life Technologies GmbH, Darmstadt, Germany). PCR amplification reactions were performed in a total volume of 30 µl containing 1 µM of each primer (10 µM), 1 × PCR buffer with $MgSO_4$ (2 mM), 0.2 mM of each dNTP, and 1.25 units of *Pfu* DNA Polymerase and 50 ng of template DNA. The gradient PCR reactions were performed on TProfessional basic Gradient Thermocycler (Biometra, Germany), using thermal cycling conditions, which consisted of an initialization step at 95 ℃ for 5 min, 35 cycles of denaturation at 95 ℃ for 1 min, annealing at 50-70 ℃ for 1 min, elongation at 72 ℃ for 1 min, a final extension at 72 ℃ for 10 min and hold at 10 ℃.

Agarose gel electrophoresis

After amplification, 5 µl of PCR products under different annealing temperatures for three genes were electrophoresed in three 1.5% agarose gels prepared in 0.5 × TBE buffer, respectively. 6 × DNA loading dye was mixed in a 5/1 (v/v) ratio to the amplified products. Then, 0.0001% v/v ethidium bromide (EB) was used in the gel to stain DNA bands. Electrophoresis was performed at 45 V for 2 h in 0.5 × TBE buffer. 300 ng of 100 bp plus DNA ladder was also loaded in the same gel to estimate the molecular weight of the amplified products and PCR products were visualized under UV light.

2.7.2.2 PCR amplification and purification for the actin gene

PCR amplification

PCR amplification for the actin gene was performed under the same condition as gradient PCR with an annealing temperature at 57 ℃. The amplified PCR products of all twenty isolates were separated by 1% agarose gel to determine the target fragment 334 bp of the actin gene.

Purification

To increase the sensitivity of sequencing, PCR products from the actin gene had to be purified from undesired proteins and unknown inhibitors. Therefore a volume of 40 µl SureClean Plus™ was added into each PCR product to precipitate DNA and spun briefly following the manufacturer's instructions. The mixture was incubated at room temperature for 10 min followed by centrifugation at 14,000 rpm for 10 min. The supernatant was decanted carefully and the pellet was rinsed twice with 70% ethanol. The remaining ethanol was removed and the pellet was air-dried under the laminar flow clean bench for 30 min. Finally the pellet was re-suspended in 50 µl sterile distilled water. 2 µl of purified DNA was quantified by

electrophoresis through a 1.5% agarose gel. To determine the quantity, a dilution series of Lamda DNA was run on the same gel with DNA samples. The identification of band for the actin gene and the amount of DNA was carried out by densitometry using the Multi-Analyst program™. The forward and reverse strands of the PCR products were sequenced, using the same primers as in the amplification reactions (ACT-512F and ACT-783R) on ABI Prism 3730XL DNA Sequencer (Macrogen Europe, Amsterdam Zuid-oost, The Netherlands).

2.7.2.3 PCR amplification and blunt ended cloning for the ß-tubulin and calmodulin genes

PCR amplifications

PCR amplifications were performed as described above with the annealing temperature at 52 ℃ for ß-tubulin and 61 ℃ for calmodulin. The PCR products of both genes were run in an 1% agarose gel. The fragments for the ß-tubulin gene (550 bp) and the calmodulin gene (516 bp) were observed in all twenty isolates.

Blunt ended cloning

Since the first sequencing results of PCR products of both genes were unclear, blunt ended cloning for the ß-tubulin gene and the calmodulin gene were performed to improve the quality of fragments and reduce errors caused by the amplification. To combine the vector and PCR products, the PCR products were phosphorylated in a total volume of 50 µl reaction, consisting of 25 µl PCR products, 0.5 × *Pfu* Buffer with $MgSO_4$, 4 units T4 Polynucleotide Kinase and 0.05 mM ATP, and incubated at 37 ℃ for 30 min and inactivated at 75 ℃ for 10 min, while the vector pBluescript SK^{-minus} was dephosphorylated and digested in the 50 µl reaction solution, consist of 5 µg pBluescript SK^{-minus}, 1 × FastDigest SmaI buffer, 2.5 FDU FastDigest Smal and 5 U FastAP (Thermosensitive Alkaline Phosphatase), and then incubated and inactivated under the same conditions as the PCR products. The vector pBluescript SK^{-minus} and PCR products were purified by PCl (phenol: chloroform: isoamyl = 25:24:1) and Cl (chloroform: isoamyl = 24:1) solutions. Equal volumes of PCl solution were mixed with vector and PCR products individually for 5 min; the upper phase was transferred carefully into a new Eppendorf tube by pipet after centrifuged at 15,000 rpm for 10 min. The purification was repeated with the Cl solution. Vector and PCR products were cleaned up and separated by running in a 1% agarose gel without EB in TAE buffer for 16 h. The agar slices containing the unstained fragments of vector and PCR products were cut out from agarose gel on a UV-transilluminator and each was transferred into a separate 2 ml Eppendorf tube. DNA of vector and PCR products were extracted from agar slices by using the glass milk method. Agar slices were melted in tubes with three volumes of DNA-binding buffer at 50 ℃ for 15 min and 6 µl of thoroughly re-suspended glass milk was added to permanently mix the components at room temperature for 10min. Glass milk sediments were re-suspended in 500 µl DNA-binding buffer after centrifugation at 13,000 rpm for 30s and the supernatant was discarded. Sedimentation of glass particles was performed by re-suspending twice in 25 volumes of washing buffer and centrifugation at 13,000 rpm for 30 s. The DNA pellets of vector and PCR products were individually re-suspended in 20 µl sterile distilled water for 5 min at 50 ℃ and controlled in 1% agarose gel for 3 h with 100 bp plus DNA ladder. The ligation of vector and PCR products (molar ratio, vector: PCR product = 1: 3) was performed

on ice in a final volume of 20 µl reaction with 20 ng pBluescript SK^{-minus}, 1 × T4 DNA ligase buffer (included FC 0.5 mM ATP), 1 unit T4 DNA ligase, 5% PEG 4000 and PCR products (11 ng for ß-tubulin and 10 ng for calmodulin). The reaction solution was incubated at 4 ℃ for 2 d. Five µl of each ligation reaction was used for the transformation in competent cells of *E.coli* which were just prepared from our group. Those competent cells of *E.coli* were took out from the -70°C refrigerator and thawed on ice. Fifty µl *E.coli* was mixed with each ligation reaction and placed on ice for 30 min. For DNA uptake by heat shock, the mixtures were incubated at 42 ℃ for 60s, and immediately placed on ice for 2 min. Then each mixture was transferred into 400µl preheated SOC medium with 2 µl $MgCl_2$ at 37 ℃ for 1 h. Two hundred µl of each suspension was placed on $MacConkey_{AP}$ plates with the help of a Drigalski spatula, and incubated at 37 ℃ overnight. Eight single white colonies were selected and incubated on LB_{AP} medium at 37 ℃ for 8 h as stock cultures. A bit of cell mass from stock culture was transferred into a glass vial containing 3 ml TB_{AP} medium and incubated at 37 ℃ overnight on a rotary incubator. Plasmid DNA was extracted from bacterial suspensions. Cells were harvested via centrifugation of 2 ml of each bacterial suspension, and re-suspended and incubated in 200 µl ice-cold GLC buffer (50 mM Tris pH 8.0, 50 mM glucose and 10mM Na-EDTA) with lysozyme (2 mg/ml) at room temperature for 20 min. Then 300 µl of LYZ solution was added (1:1 mix of 2% SDS and 0.4 N NaOH) and the sample was incubated at room temperature for 10 min. Two hundred and fifty µl HS buffer was added (147 g potassium acetate and 19.16 ml formic acid were dissolved in 500 ml sterile distilled water) and the sample was kept on ice for 30 min. The supernatants were transferred into new Eppendorf tubes after centrifugation at 14,000 rpm at 4 ℃ for 10 min. Nucleic acids were precipitated in 0.6 volumes of isopropanol at room temperature for 30 min and collected after centrifugation. The pellets were re-suspended in 50 µl TE buffer supplemented with 1 µl of an RNAse A solution (10mg/ml) for at least 30 min, washed twice with 500 µl 70% ethanol and air-dried under the Laminar Flow clean bench for 30 min. For checking the successful uptake of DNA-fragments, 500 ng of each plasmid DNA was double digested in a total volume of 20 µl reaction solution by FastDigest enzyme PstI (5 U) and BanmHI (5 U) at 37°C for 1 h, inactivated at 80 ℃ for 5 min and controlled by running in an 1% agarose gel for 3 h.

Purification

Recombined plasmid DNA was purified using QIAquick spin columns and buffers (QIAprep Miniprep, Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The both strands of PCR products from two genes were sequenced by the primer pairs T7 (AATACGACTCACTATAG) and M13R (GCGGATAACAATTTCACACAGG) provided by Macrogen (Macrogen Europe, Amsterdam Zuid-oost, The Netherlands).

2.7.2.4 DNA sequence alignment and phylogenetic analysis

The sequence data for three genes from 20 *Magnaporthe* isolates were compared with the complete sequence data published in NCBI. Accumulation of neutral mutations is an important measure of evolutionary distance among lineages, these differences are fixed by genetic drift, and ideal sequences should not underly selection. In general, mutations occur at the same rate in both exons and introns, but are removed more effectively from the exons by adverse selection. However, in the absence of the constraints imposed by a coding function,

an intron is able quite freely to accumulate point substitutions and other changes. Hence, the sequences for introns and exons were separated to do the analysis. The intron sequences from three genes were concatenated for each isolate to perform the analysis of multiple alignment, as well as the concatenated exon sequences.

Using Mega 6.0.5 software (molecular evolutionary genetics analysis version 6.0.5; Tamura *et al.*, 2013), multiple sequence alignments were conducted by ClustalW progress and phylogenetic analyses were constructed by Neighbor-joining. The bootstrap analysis (Felsenstein 1985) was performed with 1,000 replicates for the assessment of the robustness of dendrogram topology and cophenetic correlation was used as an estimation of the faithfulness of cluster analysis.

2.7.2.5 Genetic diversity

To study the genetic structure of *Magnaporthe* spp. from different hosts, the 20 isolates were grouped in four sets according to their original host plant. Gene diversity indices such as total diversity (H_T), diversity within group (H_S), absolute differentiation among groups (D_{ST}) and coefficient of relative population differentiation (G_{ST}) (Nei, 1987) were calculated for each band and then averaged for the total set. All Nei's parameters were calculated using Popgene v. 1.32 software, which was used to calculate all genetic parameters. The sequence data were manually converted in a text format for Popgene software.

2.8 Detection and quantification of fungal biomass in infected wheat and rice leaves by qPCR

2.8.1 Sample collection and preparation

Sample collection

Wheat seedlings at the 3-leaf stage and rice seedlings at the 4-leaf stage were individually inoculated with the isolates MG 5 and MG 31 from wheat and the isolate Ca 89 from rice. Those isolates were selected according to the different genetic groups from AFLP results (Fig. 10). Infected leaves which had been collected at different time points (0, 2, 4 and 6 dpi) and the mock-inoculated control were used to investigate fungal biomass accumulation over time in each interaction.

Sample preparation

All samples were frozen immediately in liquid nitrogen after harvest and kept at -80 ℃. Genomic DNA from dried mycelium, inoculated leaves and control leaves were extracted by a modified CTAB method according to 2.7.1.1. The extracted DNA samples were purified with SureClean Plus™ and quantified by electrophoresis.

2.8.2 Evaluation of primer specificity and sensitivity by PCR

Primer

For this protocol to be successful, the PCR primers had to selectively amplify a region of the *Pot2* transposon in blast isolates, without amplifying DNA from the plant itself or other

pathogens. The *Pot2* transposon is believed to be present only in *Magnaporthe* spp. from various plants and with 100 copies in the genome which is advantageous for PCR detection (Kachroo *et al.*, 1994; George *et al.*, 1998; Harmon *et al.*, 2003). Primers for the *Pot2* region were chosen based on the specificity to the genome of *Magnaporthe* spp. The forward primer *pfh*2a (5´-CGTCACACGTTCTTCAACC-3´) and reverse primer *pfh*2b (5´-CGTTTCACGCTTCTCCG-3´), synthesized by Invitrogen™ (Life Technologies GmbH, Darmstadt, Germany), were designed to amplify the 687 bp region of repetitive DNA fragment, the *Pot2* transposon, which is shared by both rice and non-rice pathogens of *Magnaporthe* spp. (Kachroo *et al.*, 1994).

PCR reaction

To determine primer sensitivity and primer ability to amplify *Magnaporthe* spp., the primers *pfh*2a and *pfh*2b were evaluated by applying PCR on eleven *Magnaporthe* spp. (Table 1, isolates marked with two asterisks) which were used in this study from different locations, combining with another primer specificity test performed by Heike (2012) using the same primers tested on 8 different pathogens. A semiquantitative polymerase chain reaction (PCR) was carried out using TProfessional basic Gradient Thermocycler with an initial denaturation step at 95 ℃ for 2 min, followed by 33 cycles of denaturation for 1 min at 95 ℃, annealing for 1min at 57 ℃ and elongation for 1 min at 72 ℃, and a final extension step for 10min at 72 ℃. A reaction mixture of 25 µl consisted of the following components: 2.5 µl PCR-buffer (10 ×), 2 µl dNTPs (10 mM), 2.5 µl of each primer (10 µM), 0.75 µl $MgCl_2$ (25 mM), 0.2 µl BioTAQ™ DNA Polymerase (2 U/µl) and 10 ng template of each isolate. Ten µl of each PCR amplification product were separated on 1.3% of ethidium bromide stained agarose gel at 45 V for 2 h and visualized under UV-light on a gel documentation system (Quantity One, Version 4.5.0 Bio-Rad Laboratories, Hercules, USA).

2.8.3 Establishment of DNA standard curves

DNA standard curve

Quantitative real time polymerase chain reaction (qPCR) was used to quantify the exact amount of pathogen which is responsible for disease symptoms. The PCR program was performed on a CFX384 Touch™ Real-Time PCR Detection System using 384-well Quali-PCR-Plates transparent (Kisker Biotech GmbH & Co. KG, Steinfurt, Germany) and the AB-0558 Adhesive PCR Film (Fisher Scientific, Schwerte, Germany). Amplifications were performed in 10 µl containing 7 µM of each primer and 0.6 mM dNTPs in 1 × PCR buffer with 2 mM $MgCl_2$, 0.2 U DNA polymerase (BioTAQ), 1 × SYBR Green I and 1µl of fungal DNA template. Standard curves were constructed and determined by comparing a standard series of known fungal DNA templates, which consisted of 1 pg, 10 pg, 100 pg, 1 ng and 10 ng diluted in sterile distilled water with three technical replications per standard, to serve as reference to calculate the unknown DNA amounts in the samples. The PCR amplification analysis was performed with the Bio-Rad CFX Manager (Version 2.0, Bio-Rad Laboratories, Hercules, USA).

Comparison of standard curves

To eliminate effects from plant DNA, equal amounts of DNA from mock-inoculated wheat and rice leaves were mixed artificially with fungal material and used as templates in the real-time PCR. The results were compared with standard curves from pure fungal DNA. As a negative control, pre-mixed reaction solution was mixed with sterile distilled water. The PCR reaction was performed in an iCycler system (BioRad, Hercules, CA, USA) according to the following program: an initial denaturation step at 95 ℃ for 15 min, followed by 35 cycles of a denaturation step for 15 s at 95 ℃, annealing for 25 s at 58 ℃ and elongation for 45 min at 72 ℃. Fluorescence was detected after each elongation step and the program was completed with a final elongation step of 10 min at 72 ℃. Melting curve analysis was performed at a denaturation step at 95 ℃ for 10 s, followed by a renaturation step at 55 ℃ step for 10 s and subsequent measurements within a range of 65 ℃ to 95 ℃ (every 10 s in 0.5 ℃ temperature increments), followed by a cooling step at 15 ℃. In the real-time PCR application, the accumulation of the amplicon is monitored in each cycle based on the emission of fluorescence. The average threshold cycle (C_T) values calculated from three replications were fit by linear regression to derive the template DNA concentration.

2.8.4 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR was carried out in a total volume of 10 µl (Table 4) to detect and quantify the biomass of three isolates on wheat and rice leaves at different time points. The primers *pfh*2a and *pfh*2b were used to amplify the DNA samples. Sterile distilled water was used instead of sample DNA as a negative control. A series of known fungal DNA (1 pg, 10 pg, 100 pg, 1 ng and 10 ng) was inserted as standard curve to quantify the amount of sample DNA. Three simultaneous replicates were used for each sample to confirm the reproducibility and reliability of the results. The quantitative real-time PCR was performed in the same conditions as described in 2.8.3. Melting curve analysis was performed at the end of each reaction to monitor primer-dimer formation and the amplification of gene-specific products.

Table 4 Quantitative real-time PCR reaction mixture in a total volume of 10 µl

Reagent	Final concentration	µl per reaction
Distilled water	--	5.46
10 × PCR buffer	1 ×	1
$MgCl_2$ (50mM)	2mM	0.4
dNTPs (10mM)	6mM	0.6
F-Primer (*pfh*2a, 10µM)	7µM	0.7
R-Primer (*pfh*2b, 10µM)	7µM	0.7
BioTAQ™ DNA Polymerase (5 U/µl)	0.2U	0.04
SYBR Green I (1:1000)	1 ×	0.1

DNA template	--	1

Detection and calculation were performed on Bio-Rad CFX Manager (Version 2.0, Bio-Rad Laboratories, Hercules, USA), using a mathematical model to determine the relative quantification of fungal biomass from infected samples compared with the standard curve. The average threshold cycle (C_T) value for each interaction was calculated from duplicate samples for each experiment.

2.9 Cytological investigations of infection processes of heterologous and homologous strains of *Magnaporthe* spp. on wheat and rice leaves

2.9.1 Sample preparation for cytological investigations

The isolates MG 5 and MG 31 from wheat and Ca 89 from rice were also used in cytological investigations. The conidial suspensions (1.0×10^5 conidia/ml) from three isolates were sprayed on the whole wheat seedlings at the 3-leaf stage and rice seedlings at the 4-leaf stage as described in 2.4.3.2. For cytological investigations, the second true leaves of wheat and leaf sheaths of rice were collected after infection.

2.9.2 Cytological studies with confocal laser scanning microscopy (CLSM)

The ability of different strains to infect plants showed differences, which was analysed by CLSM to observe the process of pathogen infection. The inoculated samples were harvested at 12, 24, 48 and 72 hours post inoculation (hpi). Time points were chosen with respect to the biology of infection development (Kankanala *et al.*, 2007; Ribot *et al.*, 2007). The second true leaves from wheat and rice leaf sheaths were cut into 2 cm segments and immersed in the clearing solution (0.15% (w : v) trichloroacetic acid in ethanol/chloroform (4/1, v/v)) at room temperature for 2 d. After washing away the cleaning solution attached to the sample surface by PBS buffer twice, samples were dried on paper towel briefly and immersed in the staining solution (1900 µl distilled water, 40µl propidium iodide (20 µg/ml) and 60 µl WGA Alexa Fluor 488 (30 µg/ml) in each beaker. All beakers from different time points and interactions were wrapped with aluminum foil and placed in a desiccator connected with a vacuum pump. Samples were infiltrated for 20 min under vacuum (800-900 mbar), subsequently washed with PBS buffer and mounted in 50% (v /v) glycerol (Roth, Karlsruhe) on glass slides in the dark for further observation. Attacked cell sites were located beneath appressoria and examined under a confocal laser scanning microscope (TCS SP5, Leica, Wetzlar, Germany). Fluorescence was excited with an argon laser at 488nm and detected at wavelengths 580-630 nm. Images were processed and arranged using CLSM-LAS-AF software (Leica). Five wheat leaves or rice leaf sheaths were used to analyze fungal development at different time points in epidermal cells. There were three repetitive experiments.

Fungal growth stages

Development of fungi in the host plant was grouped into four stages. In the first stage, the spores germinate and form an appressorium; the second stage is the intracellular growth of primary invasive hyphae in the first infected epidermal cell after the infection peg penetrates; in the third stage, primary infection hyphae have extensively invaded the first invaded cell and are trying to reach the adjacent living cells through plasmodesmata; in the fourth stage, the

newly formed secondary infectious hyphae colonize the intracellular space of adjacent cells and other hyphae grow in the intercellular space (Ribot *et al.*, 2008). Differences between isolates and time points were further compared by the least significant difference Tukey HSD test analysis.

2.9.3 Hetero- and homologous interactions studied with autofluorescence microscopy

Observations under an autofluorescence microscope

Samples used for studying hetero- and homologous interactions were harvested at 48 h after inoculation, and evaluated under an auto-fluorescence microscope to investigate plant responses to infection at the cellular level. Infected wheat leaves and rice sheaths were destained in the clearing solution as mentioned above (2.9.2) for 2 d, and immersed in diluted cotton blue solution in darkness overnight. Samples were subsequently placed on glass slides and stored in 50% (v/v) glycerol after washing twice with PBS buffer to remove excess dye. Fungal structures stained with cotton blue were observed under a bright-field microscope. Autofluorescence of epidermal cells was observed under an epi-fluorescence microscope (excitation filter BP 515-560 nm, dichromatic mirror 580 nm, suppression filter LP590 nm; TCS SP, Leica Microsystems, Wetzlar, Germany). A total of 200 infection sites were inspected and scored independently. Five wheat leaves or rice leaf sheaths were analyzed for each interaction. Independent repetitive experiments were carried out three times.

The types of cytological response

Cytological responses visualized by autofluorescence microscopy could be categorized into four types (Fig. 21). Type A showed no reaction on epidermal cells. In this case the fungal appressorium seemed to have no capacity to attempt penetration. Type B referred to papilla formation with yellow fluorescence beneath the appressorium, which stopped the appressorial penetration of cell walls first attacked by the fungus. Type C comprised hypersensitive reaction of the epidermal cell. Such cells were usually accompanied by strong yellow fluorescence of cell walls or cytoplasm. Type D exhibited no resistance reactions to the appressorial penetration. The appressorium successfully invaded the epidermal cell, followed by the development of infection hyphae (Murakami *et al.*, 2000). The incidences of cellular responses between host and nonhost type of interactions were compared by Tukey HSD test.

2.10 Assessments of phytotoxin effects on rice and wheat leaves

Necrotrophic phytopathogenic fungi synthesize a wide range of phytotoxic compounds, some of which induce lesion formation on plant leaves (Park *et al.*, 2009). Therefore, we conducted a preliminary study for the phytotoxin effects on wheat and rice leaves.

2.10.1 Testing of fungal culture filtrate (CF) on leaves

The preparation of fungal culture filtrate

Culture filtrates were prepared following Park *et al.* (2009) with minor modifications. The wheat isolates (MG 5 and MG 31) and rice isolate (Ca 89) were selected for this test as for the inoculation tests. One filter paper carrying each isolate (see above) was transferred to 500ml

flasks containing 250 ml of potato dextrose broth (PDB). Cultures were incubated at room temperature in the dark for 10 d with shaker agitation at 120 rpm. The crude culture filtrates were separated from the mycelia with the help of sterilized three layers of gauze and subsequently filtered through a 0.20 µm Millipore filter (Minisart®, Sartorius AG, Göttingen, Germany) to eliminate the conidia. The final culture filtrates (CF) were diluted with distilled water into two concentrations: 1/2 and 1/4. As a positive control, conidial suspensions were obtained as follows. Mycelium was scraped from V8 agar after growing 7 d and suspended in sterile distilled water, and then the concentration was adjusted to 1.0×10^5 conidia/ml with sterile distilled water. The filtrate of fresh PDB was applied as a negative control. 2% Methyl cellulose (1 g methyl cellulose in 50 ml H_2O) was added to each treatment.

The effects of fungal culture filtrate on detached leaves

Non-inoculated leaves of wheat cultivar BR 18 and rice cultivar CO 39 were used to study the effect of the filtrate. The second complete leaves detached from 3-week old wheat plants and 5-week old rice plants were placed in plastic boxes on two layers of filter paper and inoculated with conidial suspension (1.0×10^5 conidia/ml) by drop inoculation. Five drops of different treatments, including three concentrations (1/4, 1/2 and final CF) of pathogen filtrate from each isolate, pure conidial suspensions and PDB filtrate, were dropped on rice and wheat detached leaves. Some water was left in the plastic boxes to keep the high humidity close to 100% and the lid was covered to prevent evaporation. The leaves were incubated at 25 ℃ and 16h light/8h dark in the climate chamber and observed at 3 dpi. Each treatment was repeated three times, and three detached leaves were used for each treatment.

2.10.2 Assessment of leaf responses to infected leaf extract leachate

Some fungi liberate toxins early during spore germination as an aid for penetration and establishment, while others are produced much later in the infection process and many enhance the senescence of the plant tissue leading to its more rapid death (Isaac, 1998). Therefore, the extraction of toxic compounds from lesions was performed.

Preparations of infected leaf extracts

Leaves of wheat and rice were harvested after 7 days post noculation with three isolates, individually and cut to small pieces. The small fragments from different interactions were immersed in sterile distilled water with stirring from time to time for 1 h and filtered on two layers of gauze to remove those fragments. The lesion filtrates were serially diluted to two concentrations: 1/2 and 1/4. The positive control of pure conidial suspension and negative control with sterile distilled water were prepared as described above.

Effects of infected leaf extracts on detached leaves

The incubation was performed following Talbot *et.al.* (1997) with modifications. The second complete leaves from the same stage as before were used. The bottom of three detached leaves was placed in one 2 ml Eppendorf tube containing either lesion filtrates or conidial suspension or sterile distilled water. The tubes were wrapped with Parafilm (Glasgerätebau Ochs, Bovenden/Lenglern, Germany) to prevent excess evaporation and aerial contamination, and incubated in the same conditions as above.

2.11 Statistical data analysis

Processing of raw data and drawing in charts and tables were done by Microsoft Office Excel 2007. Data were further analyzed using STATISTICA 9.1 (Stat Soft, Inc., Tulsa, Oklahoma, USA). Comparisons on cultural characteristics were performed by analysis of one-way ANOVA and Tukey HSD. Data from experiments on low temperature storage, microclimatic requirements, pathogenicity differentiation and fungal biomass by qPCR were processed by two-way ANOVA with multi-comparison by Tukey HSD test. For all analyses, P-values $\leq$ 0.05 were considered significant. Different letters in the graphs indicate significant differences at a significance level of 5%.

3 Results

3.1 *In vitro* growth of *Magnaporthe* spp.

3.1.1 Isolation of *Magnaporthe grisea* from kernels and leaves

The overwintering sources of spores that comprise the primary inoculum of blast disease consist of seed, crop residue and secondary hosts (Greer & Webster, 2001). Hence blast disease is also seed-transmitted. Kernel samples from infected wheat plants in fields were used for the isolation of *Magnaporthe grisea*. In total, we obtained thirty-six strains from infected kernels, including twenty-four strains from Brazil and twelve strains from Bolivia.

Wheat leaves with lesions collected from the same fields were used for isolation, however, no aerial hyphae emerged on any lesions of the leaves, which were kept on CM agar for 2 days. The isolation from lesions on wheat leaves was unsuccessful and no spores were obtained from lesions. The reason could be that the leaves were too dry for hyphae to survive.

3.1.2 Medium selection

The colonies of wheat isolates MG 1.2 (Fig. 4) and MG 5 (Fig. A1) showed similar features on four different media (V8, CM, OA and PDA), while differences in morphology were observed between each media. Fig. 4 shows the colonies of MG 1.2 at 7 dpi on different media. Abundant vegetative mycelia have been generated on V8 and formed a white-gray colony with a mean diameter of 50.2 ± 0.76 mm. V8 is the best choice for further study, showing the highest sporulation after microscopic examination. White vegetative mycelia were also generated on CM (45.3 ± 0.58 mm) from mean diameter, but with less sporulation than on V8. Pigmented mycelia were produced on OA and PDA with mean diameters of 49.3 ± 0.58 mm and 48.5 ± 0.50 mm respectively, and low amount of spores were observed under microscopy.

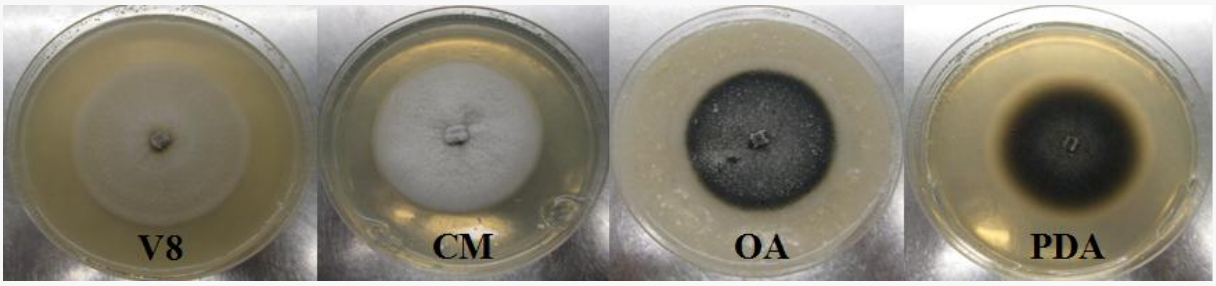

Figure 4 Colonies of wheat isolate MG 1.2 on different media: V8, CM, OA and PDA at 7 dpi.

3.1.3 Cultural characteristics of *Magnaporthe* spp.

Magnaporthe isolates, regardless of host species, exhibited high variability in cultural characteristics on V8 medium (Table 5). Description on colony morphology followed Breakwell *et al.* (2013). The surface of the colonies exhibited a variety of features with flat, convex and raised shapes, and accompanying flocculent, dull and rough textures. The colors of mycelia were observed to vary from white to gray and even black, sometimes taking on a translucent or cloudy state. Most of the colors on the substrate were black or darker yellow (data not show). The growth of aerial mycelium was classified as luxuriant, moderate and scanty with 48.6%, 37.1% and 14.3% of the isolates respectively. This showed that most of the *Magnaporthe* isolates could easily form aerial hyphae on a V8 medium. Sporulation intensity was divided into three levels: "++" ($\geq 1.0 \times 10^6$ conidia/ml), "+" (1.0×10^6 conidia/ml ~ 1.0×10^4 conidia/ml) and "-" ($\leq 1.0 \times 10^4$ conidia/ml). The percentages of sporulation intensity were 58.6% ("++"), 27.1% ("+") and 14.3% ("-"). This suggests that the amount of aerial hyphae formation and sporulation are positively correlated. There were significant differences ($p \leq 0.05$) in radial mycelium growth rate (colony diameter) among the isolates on the V8 medium at 7 dpi. The percentage of isolates exhibiting slow growth ($\leq$ 41.0 mm) was 15.7%, those with moderate growth (41.1 ~ 46.0 mm) made up 37.1% and fast growth ($\geq$ 46.1 mm) comprised the remaining 47.1%. There was no relationship between sporulation and colony growth in Fig. 4. This can be seen with isolates MG 19 and 531RWA11 which both had the same high sporulation ("++") but different colony diameters (53.8 mm and 38.9 mm, respectively).

Mycelial dry weight also showed significant differences ($p \leq 0.05$) after 10 days incubation on a Liquid Fries Complete media (see Method and materials 2.3.2.3). The mycelium growth was grouped into slow growth ($\leq$ 1.20 g), moderate growth (1.21 g ~ 1.60 g) and fast growth ($\geq$ 1.61 g), and the proportion of each group was calculated to be 18.6%, 67.1% and 14.3%, respectively. The majority of isolates presented similar growth rates on Liquid Fries Complete medium.

From the perspective of the host and geographical origin to compare the differences, these isolates have demonstrated some variability in cultural characteristics. For the specific description, the variability of all characteristics has shown within and among wheat isolates from different geographical origins (Brazil, Bolivia and Japan). Rice isolates from three countries (Philippines, Uganda and Rwanda) displayed similarities in colony morphology (raised/convex and flocculent features, white to gray color and moderate to luxuriant aerial mycelium) but differences were observed in sporulation, colony diameter and mycelial dry weight. The isolates of finger millet and perennial ryegrass from Japan had similar aerial mycelium formation, sporulation, colony diameter and mycelial dry weight, but varied in colony features and mycelium color. This suggested that there is no association between colony characteristics and host species or geographical origin.

Table 5 Cultural characteristics of *Magnaporthe* spp. isolated from different hosts and origins

Isolate	Colony morphology*			Sporulation	Diameter at 7 dpi (mm)**	Mycelial dry weight (g)**
	Feature	Color of mycelium	Aerial mycelium			
T-4 / Br48	Flat, dull	gray, translucent	Scanty	-	43.2 f-t	1.52 b-k
MG 1.2	Raised, flocculent	White	Luxuriant	++	51.2 a-d	1.21 j-s
MG 5	Convex, flocculent	White	Luxuriant	++	49.3 a-f	1.30 g-q
MG 8	Flat, dull	gray, translucent	Scanty	-	41.7 j-t	1.31 g-q
MG 11	Raised, flocculent	White	Moderate	++	47.0 a-n	1.50 b-l
MG 14	Raised, dull	White to gray, translucent	Moderate	+	46.7 b-p	1.17 l-s
MG 27	Raised, flocculent	White to gray, translucent	Luxuriant	++	41.0 n-t	1.53 b-j
MG 44	Flat, dull	gray, translucent	Scanty	-	42.5 h-t	1.13 m-s
MG 51	Flat, flocculent	White to gray, translucent	Scanty	+	49.7 a-e	1.00 q-s
MG 52	Raised, flocculent	White to gray, cloudy	Moderate	+	48.5 a-h	1.01 p-s
MG 5.1	Raised, flocculent	White	Luxuriant	++	47.7 a-k	1.29 h-q
MG 5.2	Flat, dull	White to gray, translucent	Scanty	+	45.3 c-q	1.18 l-s
MG 8.1	Flat, dull	White to gray	Scanty	+	47.8 a-j	1.13 m-s
MG 8.2	Flat, dull	White to gray, translucent	Scanty	-	47.2 a-m	0.90 rs
MG 20.3	Flat, dull	White to gray	Scanty	-	46.8 a-o	1.22 j-s
MG 29	Flat, dull	White to gray, translucent	Moderate	+	46.7 b-p	1.31 g-q
MG 9	Raised, flocculent	White	Luxuriant	++	44.0 e-t	1.29 h-q
MG 12	Raised, flocculent	White	Luxuriant	++	46.2 c-p	1.33 f-q
MG 16	Convex, flocculent	White to gray	Luxuriant	++	49.0 a-g	1.34 e-q
MG 38	Raised, flocculent	White	Luxuriant	++	53.3 ab	1.35 d-p
MG 50	Flat, rough	White to gray	Moderate	-	45.8 c-p	0.88 s
MG 52	Flat, rough	White to gray	Scanty	-	47.2 a-n	1.04 o-s
MG 53	Raised, flocculent	White to gray	Luxuriant	++	47.8 a-l	1.26 i-q
MG 54	Raised, flocculent	White	Luxuriant	+	47.8 a-j	1.28 i-q
Ca89	Raised, flocculent	gray	Moderate	++	44.3 e-s	1.67 a-e
43	Raised, flocculent	White to gray	Moderate	+	42.8 g-t	1.58 a-i

Isolate	Colony morphology			Sporulation	Diameter at 7 dpi (mm)	Mycelial dry weight (g)
	Feature	Color of mycelium	Aerial mycelium			
JMB8401	Raised, flocculent	White	Moderate	-	41.5 k-t	1.66 a-f
AGT211	Flat, flocculent	White	Moderate	+	40.7 p-t	1.75 ab
M36-1-3-10-1	Flat, flocculent	White to gray	Moderate	-	35.3 u	1.72 a-c
C9228-37	Raised, flocculent	White to gray	Luxuriant	+	41.5 k-t	1.52 b-k
CBN9214-1	Raised, flocculent	White to gray	Luxuriant	+	42.0 i-t	1.57 b-i
B90103 (BN111)	Raised, flocculent	White to gray	Moderate	++	44.7 d-r	1.62 a-h
B90099	Raised, flocculent	White to gray	Moderate	+	44.3 e-s	1.67 a-e
M39-1-2-21-2	Raised, flocculent	White	Moderate	+	46.3 c-p	1.90 a
V850256	Raised, flocculent	White to gray	Moderate	++	43.7 e-t	1.72 a-c
V86010	Raised, flocculent	White	Moderate	++	42.7 g-t	1.68 a-d
Br116.5/T-7	Flat, dull	White to gray	Scanty	-	39.8 q-u	1.38 d-o
Ken 15-15-1	Flat, rough	White to black	Moderate	++	46.2 c-p	1.50 b-l
TP / L-2	Raised, flocculent	White to black	Luxuriant	++	42.7 g-t	1.48 b-l
FI5 / L-5	Raised, flocculent	White to green	Moderate	++	46.5 b-p	1.39 c-n
1836-3/0-12	Convex, flocculent	White	Luxuriant	++	42.7 g-t	1.08 n-s
MG 19	Raised, flocculent	White and black	Luxuriant	++	53.8 a	1.37 d-o
MG 21	Raised, flocculent	White	Luxuriant	++	48.3 a-h	1.46 b-m
MG 25	Raised, flocculent	White	Luxuriant	++	52.2 a-c	1.36 d-o
MG 28	Raised, dull	White to gray	Moderate	+	49.0 a-g	1.41 c-n
MG 30	Raised, flocculent	White	Luxuriant	++	48.3 a-h	1.46 b-m
MG 31	Convex, flocculent	White	Luxuriant	++	51.0 a-d	1.44 b-m
MG 32	Raised, flocculent	White and black	Luxuriant	++	46.7 b-p	1.29 h-q
MG 33	Raised, flocculent	White and black	Luxuriant	++	43.3 e-t	1.25 i-r
MG 39	Convex, flocculent	White to gray	Luxuriant	+	39.8 q-u	1.17 l-s
MG 48	Convex, flocculent	White	Luxuriant	++	47.3 a-m	1.31 g-q

Isolate	Colony morphology			Sporulation	Diameter at 7 dpi (mm)	Mycelial dry weight (g)
	Feature	Color of mycelium	Aerial mycelium			
482RWA09	Convex, dull	White to gray	Moderate	++	40.7 p-t	1.44 b-m
503UGA09	Convex, flocculent	White	Luxuriant	++	46.7 b-p	1.52 b-k
520UGA09	Convex, flocculent	White	Luxuriant	++	48.0 a-i	1.47 b-m
523UGA09	Convex, flocculent	White	Luxuriant	++	47.2 a-m	1.58 a-i
524UGA09	Convex, flocculent	White	Luxuriant	++	49.3 a-f	1.63 a-g
492RWA11	Raised, flocculent	White	Moderate	++	43.7 e-t	1.19 k-s
500RWA11	Flat, rough	White to gray	Moderate	++	43.3 e-t	1.09 n-s
528UGA11	Raised, flocculent	White and black	Luxuriant	++	39.5 q-u	1.53 b-j
531UGA11	Raised, flocculent	White	Luxuriant	++	38.8 s-u	1.54 b-j
552UGA11	Convex, flocculent	White	Luxuriant	++	40.8 o-t	1.34 e-q
556UGA11	Convex, flocculent	White and black	Luxuriant	++	41.2 m-t	1.48 b-l
559UGA11	Raised, flocculent	White and black	Luxuriant	+	38.5 tu	1.52 b-k
561UGA11	Raised, flocculent	White	Luxuriant	++	41.7 j-t	1.36 d-o
MG 10	Raised, flocculent	White	Luxuriant	++	49.0 a-g	1.23 j-s
563UGA11	Raised, flocculent	White	Moderate	+	39.3 r-u	1.42 b-n
564UGA11	Raised, flocculent	White	Moderate	+	39.3 r-u	1.48 b-l
RWA 11.2	Raised, flocculent	White	Moderate	++	41.5 l-t	1.29 h-q
507RWA11	Flat, flocculent	White to gray	Moderate	++	44.2 e-t	1.28 i-q
511RWA11	Flat, flocculent	White to gray	Moderate	+	41.8 i-t	1.32 g-q

* Description of colony morphology following Breakwell *et al.* (2013)

**The values are the means of three replications, which are followed by different letters indicating significant differences ($p \leq 0.05$) between the isolates. These are calculated by the Tukey HSD (honestly significant difference) test.

3.1.4 Mycelial growth rate after exposure to low temperature

The colonies were exposed to low temperatures (4 ℃ or -20 ℃) for 2 d. The effect of low temperature on colony growth trend of the 5 selected isolates was similar (Fig. 5). However,

there were significant differences ($p \leq 0.05$) on colony diameters among different isolates and treatments. The characteristic differences between the five isolates are described in Table 5. After 8 days of incubation, the colony diameters for all five isolates were smaller than the control after treatment at 4 ℃. Meanwhile, the isolates treated at -20 ℃ were observed the minimum value for its diameter. Sporulation under microscopy showed a large reduction at -20 ℃, and fewer spores were produced at 4 ℃ compared to the control.

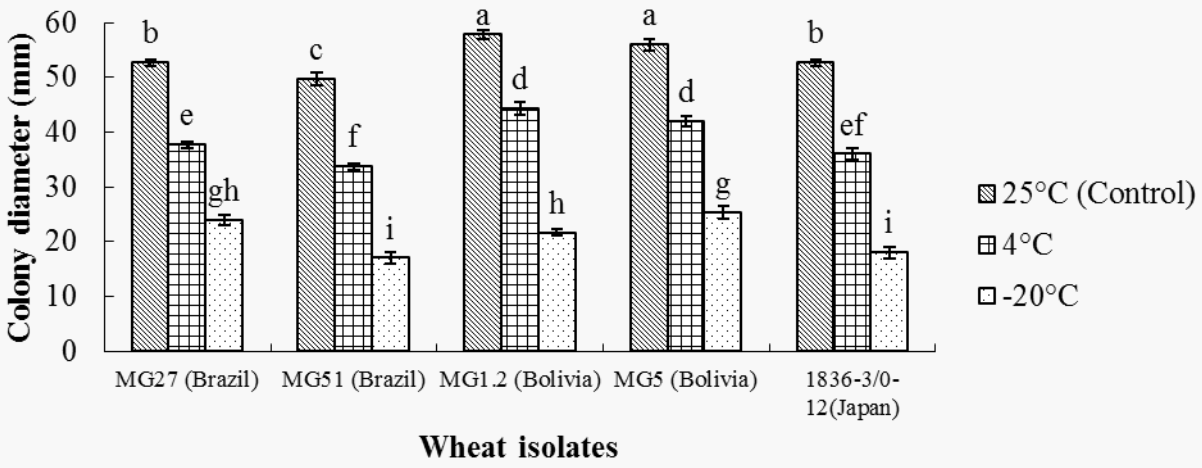

Figure 5 Average growth rates of wheat isolates (*Magnaporthe grisea*) on V8 from different origins at 8 dpi after low temperature storage treatment for two days. Each value is the average of 3 individual plates per isolate.

The growth trend of the five isolates were similar prior to the temperature treatment. For this reason, only the growth curves of MG 5 from 8 d measuring data are shown in Fig. 6. The same growth rate was observed from 1d to 3d at 25 ℃ in all Petri dishes. The cold treatments were performed at 4 d and 5 d and the rate of colony growth was slowed down at 4 ℃ and completely prevented at -20 ℃ because the media were frozen. From 6 d, mycelia slowly began to grow faster after being taken from 4 ℃ to 25 ℃ in the climate chamber; in the meantime, mycelia, which were kept at -20 ℃, started to slowly grow again with the thawing media at 25 ℃.

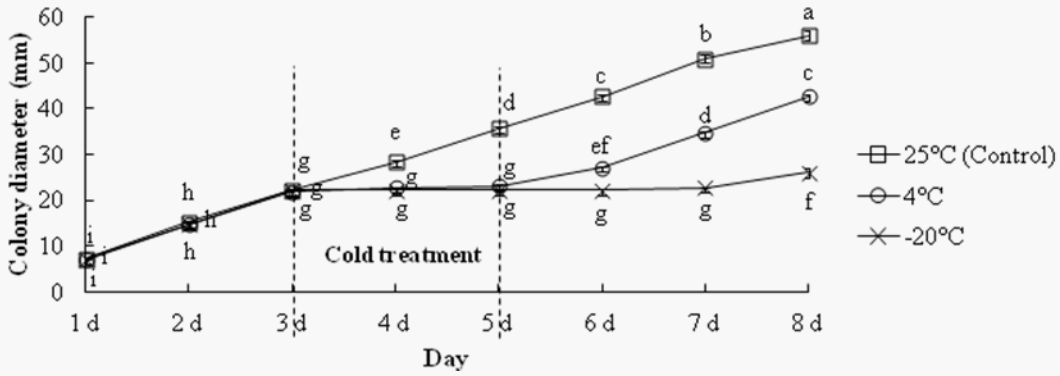

Figure 6 Colony growth of wheat isolate MG 5 measured for eight consecutive days on V8 after the low temperature treatments. Each value is the average of 3 individual plates. The values followed by different letters indicate significant differences at $p \leq 0.05$, calculated by the Tukey HSD test.

3.2 Microclimatic requirements for wheat blast infection on wheat ears

The objective of this experiment was to find out the influence of different environmental factors on the development of *M. grisea* on wheat ears, and determine the optimum conditions, especially under varying temperature and spike-wetness time. Starting from 7dpi, clear symptoms could be identified on infected ears. Initial symptoms for *M. grisea* were observed as the spikelets took on a black color with pale yellow dots. These dots then became larger until most of or even the whole spikelet became bleached.

Symptoms were recorded at 14 dpi for all treatments. The effects of varying temperature and spike-wetness time on the disease severity are shown in Fig. 7. The differences were significant ($p \leq 0.05$) among the different temperatures. Ear bleaching was more severe at temperatures above 26 ℃ compared to lower temperatures. As the temperature rose, disease severity increased up to a value of 5 and more than 80% of wheat ears were bleached. However, when temperatures were higher than 23 ℃, the upward trend of disease severity slowed down, and similar severity was observed at both 29 ℃ and 32 ℃. The results suggest that 26 ℃ is conducive to the development of *M. grisea* on wheat ear.

The main symptom of wheat blast is ear bleaching, as is also the case for other pathogens such as *Fusarium* head blight or unfavorable abiotic environmental conditions such as high temperature. In the control group, wheat ears - which were sprayed with distilled water and incubated at the same conditions - still remained green without any visible symptoms. At maturity, the control plants were still able to produce kernels. This proved that the wheat plants were not affected by other pathogens or high temperature.

For the environmental factor of spike-wetness time, it was noted that as the wetness time increased, disease severity showed a corresponding slow increase. However, there was insignificant ($p \leq 0.05$) difference between different time treatments. Mean disease severity values from four treatments were similar and ranged from three to four. When the wetting time lasted up to 24 h (a period in which most spores would germinate), disease severity had

reached 3.33 and more than half of all wheat ears were bleached. Hence, a spike-wetness time of 24h is sufficient for infection by *M. grisea* in our further experiments.

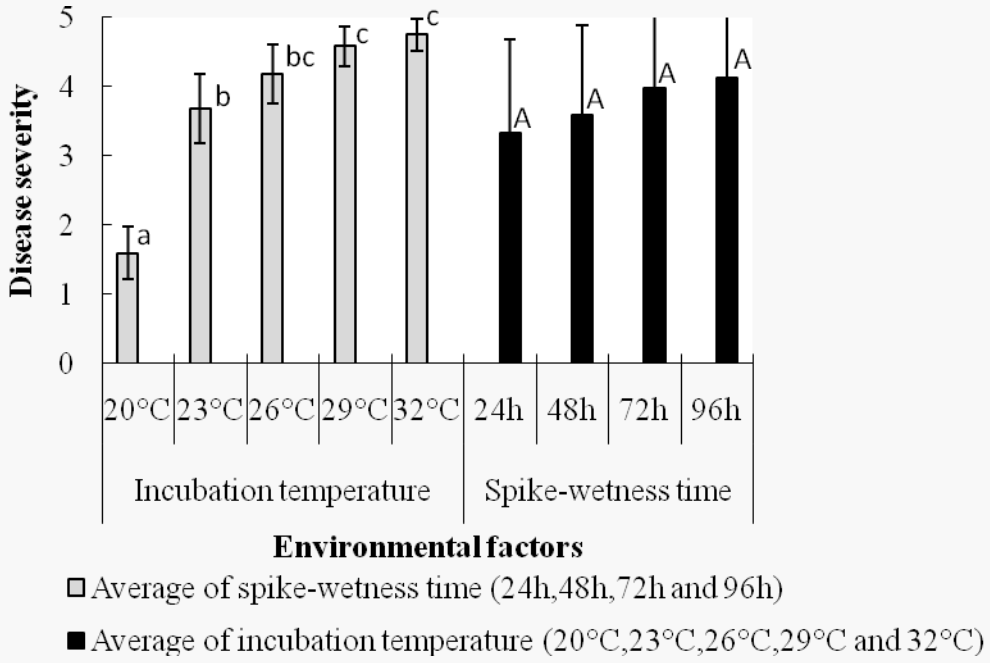

Figure 7 Disease severity of *Magnaporthe grise* (MG 1.2 and MG 5) on wheat ears (BR 18) under various treatments of incubation temperature and spike-wetness time at 14 dpi. Each gray bar represents the mean of four spike-wetness time values and each black bar represents the mean of five temperature values. The values followed by different capital or small letters indicate significant differences at $p \leq 0.05$, calculated by the Tukey HSD test.

The effects of the two environmental factors temperature and spike-wetness time on disease severity at 14 dpi are shown in Table 6. The difference was highly significant (P = 0.000) for each factor separately, but was insignificant (P = 0.833) for the interaction of two factors. In Table 6, there was no significant difference between the four wetness times in the lowest treatment (20 ℃). This proves that lower temperatures are not conducive to the growth of *M. grisea* on wheat ears, regardless of the duration of humidity. Comparing disease severity in the treatments between 20 ℃ and 26 ℃, the value of severity (1.94 ± 0.73) under lower temperature and longer wetness (20 ℃*96h) was much smaller than that (3.61 ± 0.74) under higher temperature and shorter wetness (26 ℃*24h). This shows that as the temperature rises, disease severity increases. Significant differences were observed in the treatments of 23 ℃ and 26 ℃, as disease severity was shown to be higher under a wetting time of 96 h than it was for 24 h. This indicates that a longer duration of humidity would promote infection in warm conditions and suggests that the inoculated plants should be kept under high humidity conditions (>80%) after inoculation. However, differences were shown to be insignificant in the temperature range 26 ℃-32 ℃. The disease severity of 4.11 (± 0.65) under 26 ℃*48h was quite similar to that of 4.50 (± 0.66) under 32 ℃*24h. This seems to indicate that after the temperature reaches the optimum point it no longer influences disease severity. Overall,

results illustrate that temperature is the main factor in influencing disease severity, but it is also very important to maintain the infected plants in high humidity after inoculation. Therefore, optimum conditions for the infection of *M. grisea* were chosen to be 26 ℃ with the plants kept in high humidity conditions for 24h after inoculation.

Table 6 Disease severity of wheat blast as affected by two environmental factors (temperature and spike-wetness time) on wheat ears (BR 18) which inoculated with mixed spore suspension from MG 1.2 and MG 5 at 14 dpi, calculated from the mean of nine leaves (± standard deviation) per group.

Spike-wetness / Time / Temperature	24 h	48 h	72 h	96 h
20 ℃	1.17 (±0.56) g*	1.39 (±1.08) g	1.89 (±0.60) fg	1.94 (±0.73) fg
23 ℃	3.06 (±0.68) e-g	3.56 (±0.92) d-f	3.89 (±0.60) b-e	4.22 (±0.75) a-d
26 ℃	3.61 (±0.74) c-e	4.11 (±0.65) a-e	4.39 (±0.65) a-d	4.61 (±0.42) a-c
29 ℃	4.33 (±0.50) a-d	4.33 (±0.66) a-d	4.78 (±0.26) ab	4.89 (±0.22) a
32 ℃	4.50 (±0.66) a-d	4.61 (±0.70) a-c	4.94 (±0.17) a	4.94 (±0.17) a

*Each value represents the mean of three replications. All values followed by different letters indicate significant differences at $p \leq 0.05$, calculated by the Tukey HSD test.

3.3 Pathogenicity differentiation on seedling leaves

Pathogenicity tests for *Magnaporthe* spp. were performed to confirm cross-infectivity between wheat and rice. Leaves of wheat and rice seedlings were inoculated with seventy *Magnaporthe* isolates from different geographical origins, of which thirty isolates were from rice and forty were from wheat and other grasses. The typical blast symptoms (Fig.1 d) on wheat leaves are elliptical, elongated lesions with a light to dark green center and yellow margin which corresponds to the region colonized by the fungus. The symptoms on rice leaves vary according to the environmental conditions. Expanding elliptical or spindle-shaped lesions and whitish to gray centers with red to brownish or necrotic margins were observed in our controlled conditions (Fig. 1 e).

Disease incidence was recorded at 6 dpi and is presented in Fig. 8. All isolates have produced typical lesions on their host plants, but there were significant differences ($p \leq 0.05$) in the ability of the infection among the seventy isolates. For example, wheat isolate MG 8 caused 100% of disease incidence on wheat, yet another wheat isolate - MG 44 - colonized only 10% of wheat leaves. The same differences also occur on rice isolates: 507RWA11 and CBN9214-

1 showed 62.5% and 12.5% of incidence, respectively, on rice seedling leaves. This indicates that there is a variability of pathogenicity on *Magnaporthe* isolates.

In addition, thirty-six out of forty isolates from wheat and other grasses were able to form small reddish to brown pin-point lesions on rice leaves (disease incidences were 5% - 32.5%), but isolation of mycelium from the lesions failed. Considering the response of wheat leaves to the infection from rice isolates at 6 dpi, only a few rice isolates were able to cause spindle-shaped white to gray lesions, yet no pathogen was isolated from those lesions. Thus, thirty-five isolates from wheat and other grasses and eleven rice isolates were able to cause lesions on both wheat and rice. Meanwhile five other isolates from wheat and other grasses were only pathogenic to wheat and nineteen rice isolates were only pathogenic to rice.

Differences were also observed in the response of wheat and rice leaves to the same isolates. Wheat leaves were highly infected by isolates MG 19 and MG 33, and disease incidence reached 100% so that the entire wheat leaf gradually withered with an inwardly curling edge until it was completely dead. However, incidences were found to be very low for rice leaves - 0% and 10%, respectively. In contrast, isolate 531UGA11 could not infect wheat leaves, but it colonized 55% of rice leaves. However, isolate 492RWA11 caused a similarly high disease incidence on wheat and rice leaves, 42.5% and 45%, respectively, and isolate Br116.5 / T-7 showed similarly low infection in both hosts, 5% on wheat leaves and 7.5% on rice leaves.

There was a significant difference ($p \leq 0.05$) of disease severity in the ability of fungal strains to infect host and non-host plants (Fig. 9 and Table 7). In compatible interactions between pathogen and host plant, the vast majority of wheat isolates caused a disease severity value of at least 4. More specifically, all wheat isolates were able to infect wheat leaves and 87.5% of these could colonize more than half of the leaves at 6 dpi. Indeed, 35% of isolates were able to colonize over 80% of the leaf area. For other pathogen-host interactions, 93.3% of rice isolates successfully reached disease severity values between 4 and 5 on rice leaves, meaning that 50% - 80% of rice leaves were infected by their compatible pathogens. In the incompatible interactions (i.e. post-penetration stopping of fungal invasion) between pathogen and non-host plant, most isolates from wheat caused 20% - 50% symptoms on rice leaves with a severity of between 3 and 4; yet, most rice isolates developed less than 5% of lesions on wheat leaves, with a corresponding disease severity value of between 1 and 2.

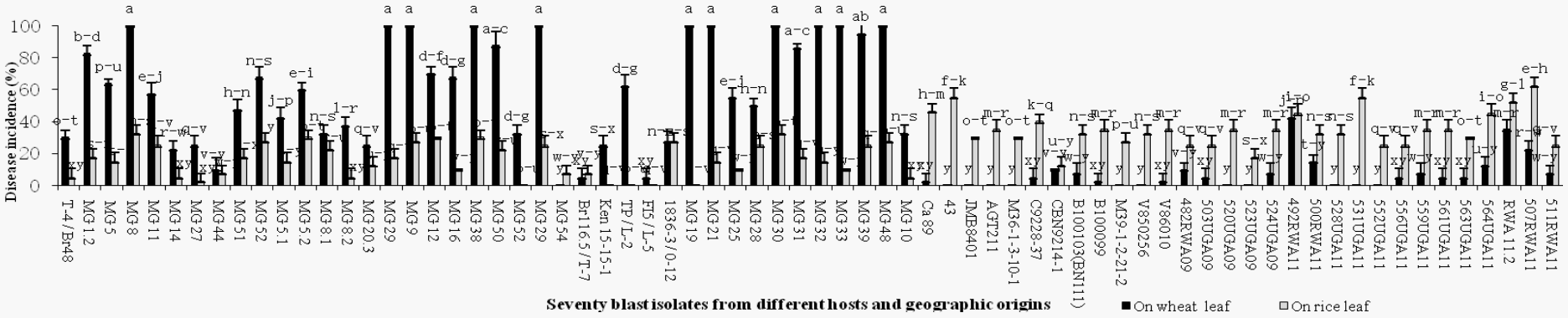

Figure 8 Disease incidence of blast on wheat (BR 18, black bars) and rice (CO 39, gray bars) seedlings inoculated with seventy *Magnaporthe* spp. isolates at 6dpi. Disease incidence was expressed as the percentage of coverage by the lesion of the whole leaf, and the values are the means of three replications. The values followed by different letters indicate significant differences at $p \leq 0.05$, calculated by the Tukey HSD test.

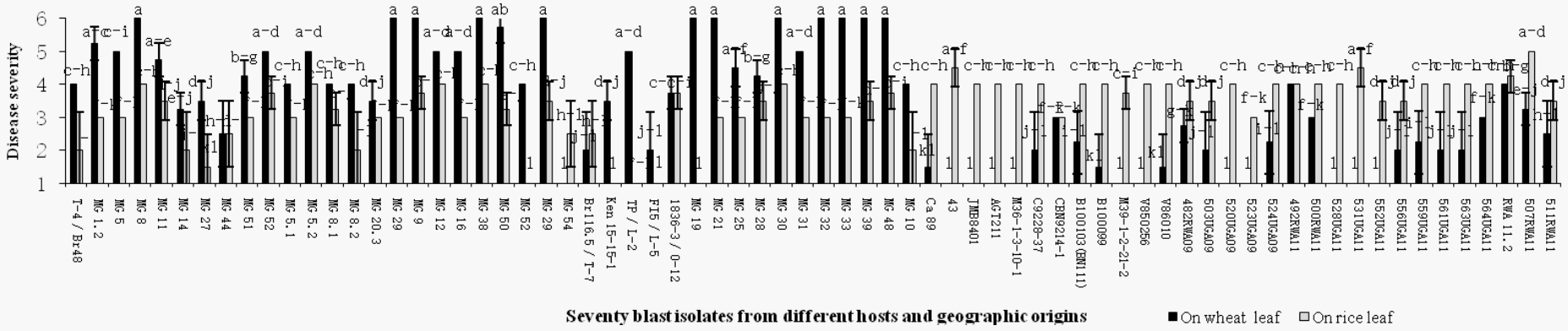

Figure 9 Disease severity of blast on wheat (BR 18, black bars) and rice (CO 39, gray bars) seedlings inoculated with seventy *Magnaporthe* spp. isolates at 6 dpi. Disease severity was assessed according to Murakami *et al.* (2000) with 6 levels: 1 = no lesion, 2 = lesion ≤ 5%, 3 = lesion 6% ~ 20%, 4 = lesion 21% ~ 50%, 5 = lesion 51% ~ 80%, 6 = lesion ≥ 81%. The values followed by different letters indicate significant differences at $p \leq 0.05$, calculated by the Tukey HSD test.

Table 7 Comparison of disease severity on wheat and rice seedlings by seventy *Magnaporthe* spp. isolates at 6 dpi.

Group of isolates	Disease index*	Number and percentage of isolates pathogenic on	
		Wheat (%)	Rice (%)
Magnaporthe grisea (40 isolates collected from wheat, finger millet and perennial ryegrass)	1	1 (2.5%)	5 (12.5%)
	2	2 (5.0%)	5 (12.5%)
	3	2 (5.0%)	17 (42.5%)
	4	13 (32.5%)	13 (32.5%)
	5	8 (20%)	0 (0%)
	6	14 (35%)	0 (0%)
Magnaporthe oryzae (30 isolates collected from rice)	1	11 (36.7%)	0 (0%)
	2	11 (36.7%)	0 (0%)
	3	6 (20.0%)	2 (6.7%)
	4	2 (6.7%)	25 (83.3%)
	5	0 (0%)	3 (10.0%)
	6	0 (0%)	0 (0%)

* Disease index was according to Murakami *et al.* (2000) with 6 progressive grades: 1 = no lesion, 2 = lesion ≤ 5%, 3 = lesion 6% ~ 20%, 4 = lesion 21% ~ 50%, 5 = lesion 51% ~ 80%, 6 = lesion ≥ 81%

3.4 Phylogenetic relationships among *Magnaporthe* spp. isolates from different hosts and geographical origins addressed by AFLP

DNA fragments from seventy *Magnaporthe* spp. isolates were specifically amplified using three AFLP primer combinations. A band was recorded only when it was present in two or more isolates to avoid manual errors. A total of 175 AFLP bands were selected for the calculation of genetic distance and the phylogenetic analysis. Bands were considered polymorphic if the frequency of one of its states (present or absent) was no less than one. Using this definition, approximately 85% of bands were found to be polymorphic.

Fig. 10 displays a phylogenetic tree (UPGMA) obtained by using similarity coefficients. The cophenetic correlation coefficient (0.95) indicated little distortion between the original similarity values from the similarity matrix and the values used to construct the dendrogram. Two distinct groups were identified in Fig. 10 at a similarity value of 0.57 by bootstrapping (100%). Within group I, two subgroups (A and B) were observed, which were joined by a node at the 74% similarity level.

The phylogenetic tree (Fig. 10) revealed that all seventy isolates were clustered and each group was associated with its host plant. Populations from wheat dominated (92.5%) group I, however the group also contained one isolate from finger millet and two isolates from perennial ryegrass; group II was composed of all thirty rice isolates. Isolates derived from finger millet and perennial ryegrass appeared to be more closely related to wheat isolates and shared 87.5% of similarities with wheat isolate MG38, MG 50 and 1836-3/0-12.

In group I, subgroup A was composed of the isolates from Brazil (50%) and Bolivia (50%). Whereas, subgroup B contained 73%, 23% and 3% of wheat isolates collected from Brazil, Bolivia and Japan, respectively. In group II, 12 rice isolates from the Philippines were separated into three subgroups (58%, 9% and 33%, respectively) with 0.79 and 0.83 of similarities to each other. The isolates derived from Uganda were divided into several small subgroups with an average similarity coefficient of around 0.83, while 6 isolates collected from Rwanda shared a similarity range between 0.79 and 0.85.

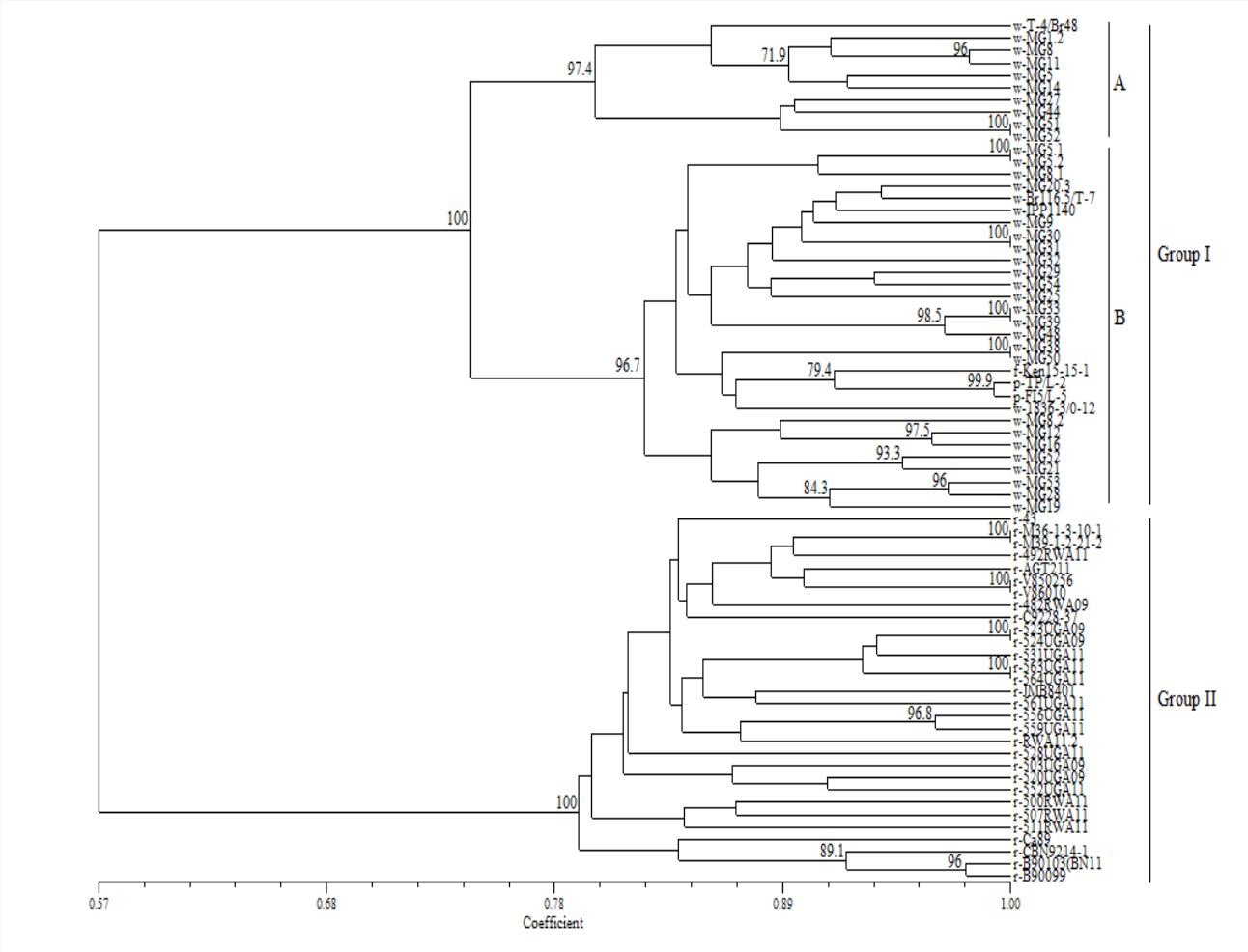

Figure 10 Phylogenetic tree (unweighted pair group method with arithmetic mean, UPGMA) produced from amplified fragment length polymorphism (AFLP) fingerprint patterns observed in seventy *Magnaporthe* spp. isolates. Coefficient scale indicates the horizontal distance corresponding to genetic similarity as measured by the Dice similarity coefficient. Bootstrap values greater than 70 are indicated adjacent to the nodes and are based on 1,000 replications.

3.5 Multilocus gene genealogy analyzed by MLST

3.5.1 Sequence preparation from three genes for the phylogenetic analysis

The sequence information for fragments of three housekeeping genes - actin, ß-tubulin and calmodulin from *Magnaporthe* spp. - were generated and compared with complete sequence data published in NCBI. Introns and exons of these three genes were identified and labeled (Fig. 11). Sequence lengths for the final analysis were 294 bp (gene actin), 684 bp (gene ß-tubulin) and 597 bp (gene calmodulin). The mutation frequency for introns and exons is greatly variable, hence they were separately analyzed. In addition, introns for actin and exons for ß-tubulin in each isolate are too short for analysis, therefore the introns from three genes were concatenated for each isolate, while the same method was used for the exons. The total sequence length for concatenated introns and concatenated exons was 906 bp and 669 bp, respectively, for each isolate. The concatenated data set, comprising twenty *Magnaporthe* spp, was then analyzed by MLST.

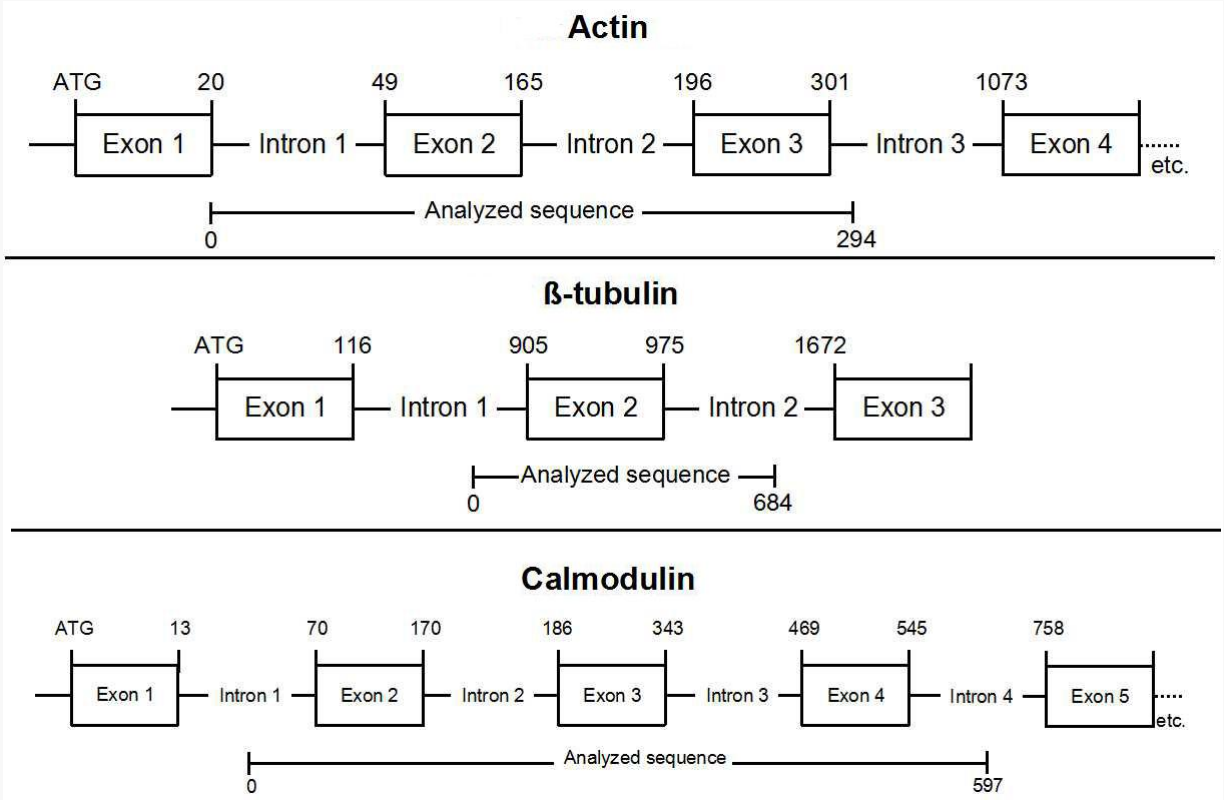

Figure 11 Diagram of three genes (actin, ß-tubulin and calmodulin) with labeled intron and exon positions. The length of analyzed sequences for actin, ß-tubulin and calmodulin were 294 bp, 684 bp and 597 bp, respectively.

3.5.2 Dendrograms composed by concatenated intron sequences

To determine genetic differences in the population of twenty *Magnaporthe* isolates derived from wheat and rice, phylogenetic analysis of three housekeeping genes was performed.

Multiple sequence alignment (Fig. A3 and A4) by ClustalW and dendrograms for concatenated intron sequences (Fig. 12) by neighbor-joining were performed by Mega 6.0.5. As shown in Fig. 12, twenty *Maganaporthe* isolates were divided into four groups according to their concatenated intron sequences with genetic distances. Group Ai was composed of the isolates from wheat (43%), rice (43%) and perennial ryegrass (14%). The three rice isolates were close to each other with a bootstrap value of 99%. Meanwhile, the three wheat isolates were grouped together with 92% bootstrapping and were associated with one isolate from perennial ryegrass. In group Bi, three wheat isolates which clustered together and were close to another perennial ryegrass isolate with 74% bootstrap support. Group Ci only contained two rice isolates with 99% bootstrapping. Moreover, group Di was distinct from the other three groups and had 100% bootstrap support. However, it has a similar clustering structure to group Ai, and the isolates included in group Di were derived from rice, wheat and finger millet (43%, 43% and 14%, respectively.) These seven isolates were also separated into two subgroups according to the different hosts, and the isolate (Ken15-15-1) from finger millet was associated with three wheat isolates. Each group contained isolates from different hosts and geographical origins.

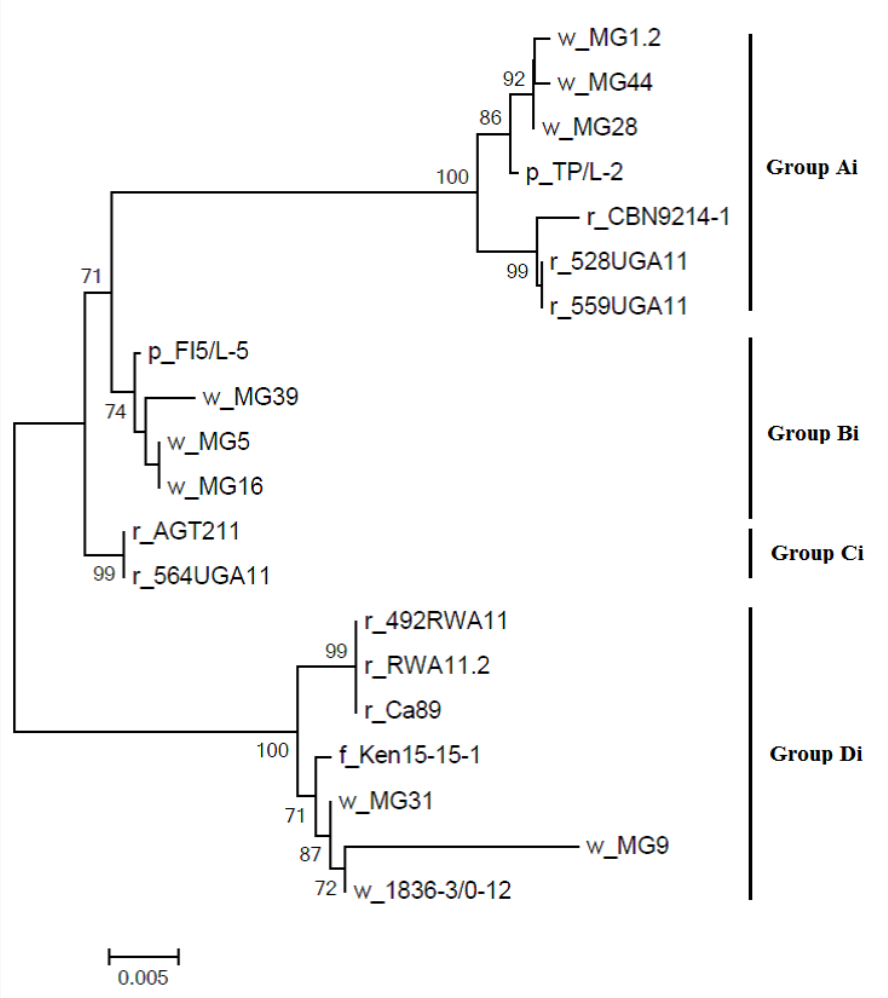

Figure 12 Dendrogram (Neighbor-joining) produced from concatenated introns of three housekeeping genes (actin, ß-tubulin and calmodulin) for twenty *Magnaporthe* spp. isolates derived from different hosts. Scale bar (0.005) indicates the genetic horizontal distance corresponding to the distance matrix by Neighbor joining. Bootstrap values greater than 70 are indicated adjacent to the nodes and are based on 1,000 replications.

3.5.3 Dendrograms composed by concatenated exon sequences

The sequences from exons are highly conserved, therefore very low diversity was detected from their sequence data (Fig. 13). Similar results were also observed in clusters of introns. Two distinct groups were formed. Group Ae contained isolates from different hosts, with 46% of isolates from wheat, 39% from rice and 15% from perennial ryegrass; meanwhile, group Be contained three wheat isolates and three rice isolates, as well as one isolate from finger millet. The sequences of all *Magnaporthe* isolates within each group showed a high degree of consistency.

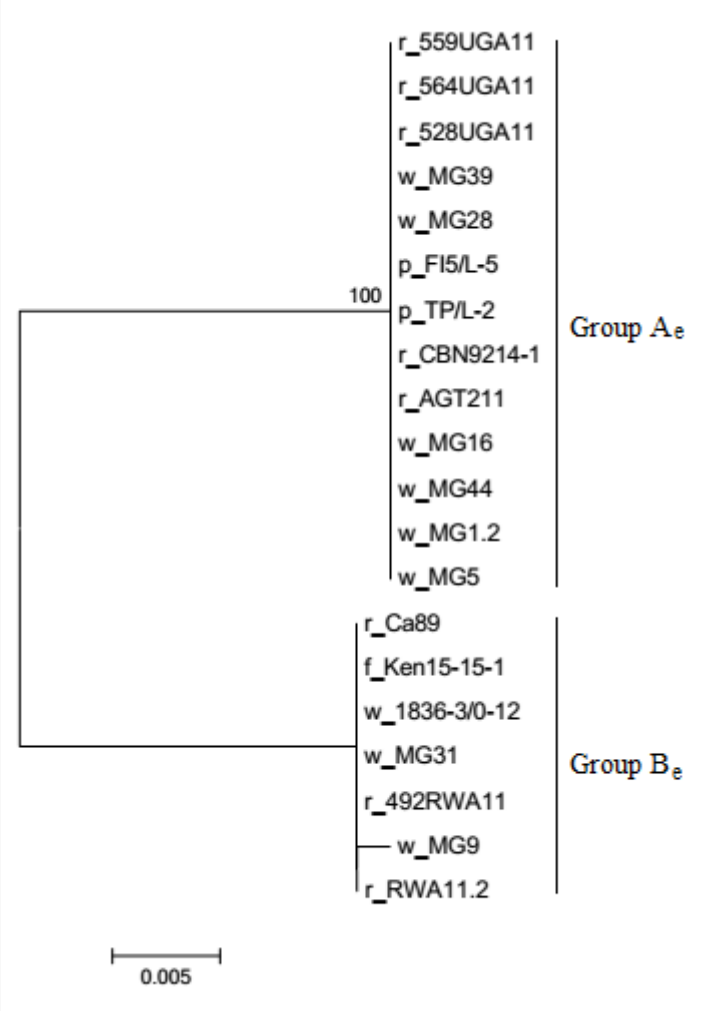

Figure 13 Dendrogram (Neighbor-joining) produced from concatenated exons of three genes (actin, ß-tubulin and calmodulin) for twenty *Magnaporthe* spp. isolates derived from different hosts. The scale bar (0.005) indicates the genetic horizontal distance corresponding to the distance matrix by Neighbor joining. Bootstrap values greater than 70 are indicated adjacent to the nodes and are based on 1,000 replications.

3.5.4 Genetic diversity

The parameters for the Nei equation were estimated by Popgene software among four population groups (wheat, rice, finger millet and perennial ryegrass) related to genetic diversity. Polymorphic loci and genetic diversity from intron data is shown in Table 8, while exon data is shown in Table 9.

Table 8 estimated Nei's parameters according to the concatenated intron data, showing that 41% of relative variation (G_{ST}) in allele frequencies corresponds to differences among groups with 0.0179 (SD $\pm$0.0048) of total diversity (H_T). The percentages of polymorphic loci for the four groups ranged from 0% - 7.84%. Average diversities within the groups (H_S) were, respectively, 0.03 $\pm$ 0.1072 for wheat, 0 for finger millet, 0.0143 $\pm$ 0.0835 for perennial ryegrass and 0.0273 $\pm$0.1091 for rice. The diversity values both inside and outside the groups demonstrated that genetic drift does not frequently occur in these fungi within host-species groups, but differences between groups are still very evident. In addition, the number of

isolates from finger millet and perennial ryegrass was very low, only 1 and 2, respectively. Therefore, low diversity coefficients (H_S) for these two groups do not represent the actual diversity of their population, and only denote a control parameter in this test.

Table 8 Polymorphic loci and genetic diversity of twenty *Magnaporthe* isolates from four populations, according to their intron data.

Population	Polymorphic loci	%	H_S
Wheat	71	7.84	0.03 ± 0.1072
Finger millet	0	0	0
Perennial ryegrass	26	2.87	0.0143 ± 0.0835
Rice	54	5.96	0.0273 ± 0.1091

Average H_S = 0.0179 ± 0.0048; H_T = 0.0303 ± 0.0122; G_{ST} = 0.4096

Very low diversities within the group were estimated from concatenated exon data (Table 9). The relative differentiation among all four groups (G_{ST}) was 53% and the total diversity (H_T) was 0.0072 ± 0.0016. The diversity within groups (H_S) was shown to be 0% in the finger millet and perennial ryegrass groups, 1.42% in the wheat group and 1.47% within the rice group. The H_S values in the finger millet and perennial ryegrass groups were zero, therefore the diversity index among the four groups increased. The diversity values derived from concatenated exon data suggests that in the exon sequences, the frequency of mutation is very low and proves that the exon is much more conservative. It should be concerned that there were only one isolate from finger millet and two from perennia ryegrass, results of the analysis would be more accurate when there are more isolates from different hosts.

Table 9 Polymorphic loci and genetic diversity of twenty *Magnaporthe* isolates from four populations, according to their exon data.

Population	Polymorphic loci	%	H_S
Wheat	22	3.29	0.0142 ± 0.0779
Finger millet	0	0	0
Perennia ryegrass	0	0	0
Rice	21	3.14	0.0147 ± 0.0818

Average H_S = 0.0072 ± 0.0016; H_T = 0.0154 ± 0.0073; G_{ST} = 0.5311

3.6 Fungal growth in plant tissue measured with qPCR

3.6.1 Primer sensitivity

The accuracy of PCR-based assays can be significantly affected by the specificity and sensitivity of DNA primers. Before the amplification of the sample DNA, the size of amplified fragments of *Pot2* transposon using primers *pfh*2a and *pfh*2b was checked on an agarose gel to determine the fragments' abilities to amplify a specific DNA fragment from *Magnaporthe* spp.. This was achieved using the molecular tool PCR by Mullis & Faloona (1987), which amplifies a specific DNA fragment *in vitro* and is therefore a common technique for detecting and identifying pathogens with high sensitivity. Eleven *Magnaporthe* isolates, of which four were from wheat (MG 5, MG 19, MG 44and Br116.5 / T-7), four from rice (Ca 89, V86010, 528UGA11 and 507RWA11), one from finger millet (Ken 15-15-1) and two isolates from perennial ryegrass (TP / L-2 and FI5 / L-5), were tested for primer sensitivity and the presence of *Pot2*. Gel electrophoresis in Fig. 14 demonstrated the sensitivity of primer pair *pfh*2a and *pfh*2b. They successfully produced the diagnostic amplicon of the *Pot2* transposon in all eleven *Magnaporthe* isolates which were collected from different hosts and geographical origins, and the amplified DNA fragments were the expected size, 687bp of the chromosome of *Pot2*.

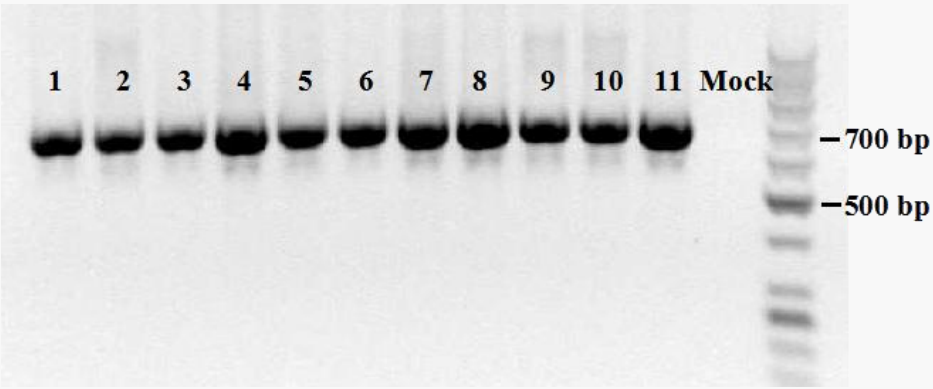

Figure 14 Detection of primer sensitivity for *pfh*2a and *pfh*2b by PCR on eleven *Magnaporthe* isolates. Lanes 1 to 4 were wheat isolates, lanes 5 to 8 were rice isolates, lane 9 was the finger millet isolate, lanes 10 and 11 were perennial ryegrass isolates and lane 12 was the water control. The DNA fragments are 687 bp.

Pannwitt (2012) also detected the specificity of primers *pfh*2a and *pfh*2b on eight different pathogens, *Fusarium oxysporum* 242/1, *Alternaria solani* 158, *Magnaporthe grisea*, *Phytophthora infestans*, *Fusarium oxysporum* f.sp. *lycopersici*, *Hansfordia pulvinata*, *Rhizoctonia solani* and *Colletotrichum gloeosporioides*. The result was that the single band of 687 bp was amplified only from the *Magnaporthe grisea* isolate with the absence of this product in any other fungus tested.

Therefore, the primer pairs of *pfh*2a and *pfh*2b were used in the further detection of quantitative real-time PCR because of their specificity and sensitivity to *Magnaporthe* spp..

3.6.2 DNA standard curve

The isolates selected were MG 5 and MG 31 from wheat and Ca 89 from rice. The standard curve formed by the amplification products of these three isolates is shown in Fig. 15. A linear relationship was established when the logarithm values of DNA concentrations (Log Starting Quantity) were plotted against Ct (cycle threshold) values. Fig. 15 clearly indicates the differences between the three isolates in Ct value under the same concentration. The correlation coefficient of this standard curve was 0.673. Interestingly, the Ct values from the two wheat isolates were very close to each other, and are both separated from the Ct value of the rice isolate. The initial concentration of each isolate was adjusted to the same concentration (10ng/µl) before the amplification reaction, so it is reasonable to exclude the possibility that there were any differences in the sample concentration. Further study into this separation is possible, to discover whether this is due to the copy number or amplification ability between wheat and rice isolates. Consequently, three standard curves were created using each isolate and calculating the biomass separately.

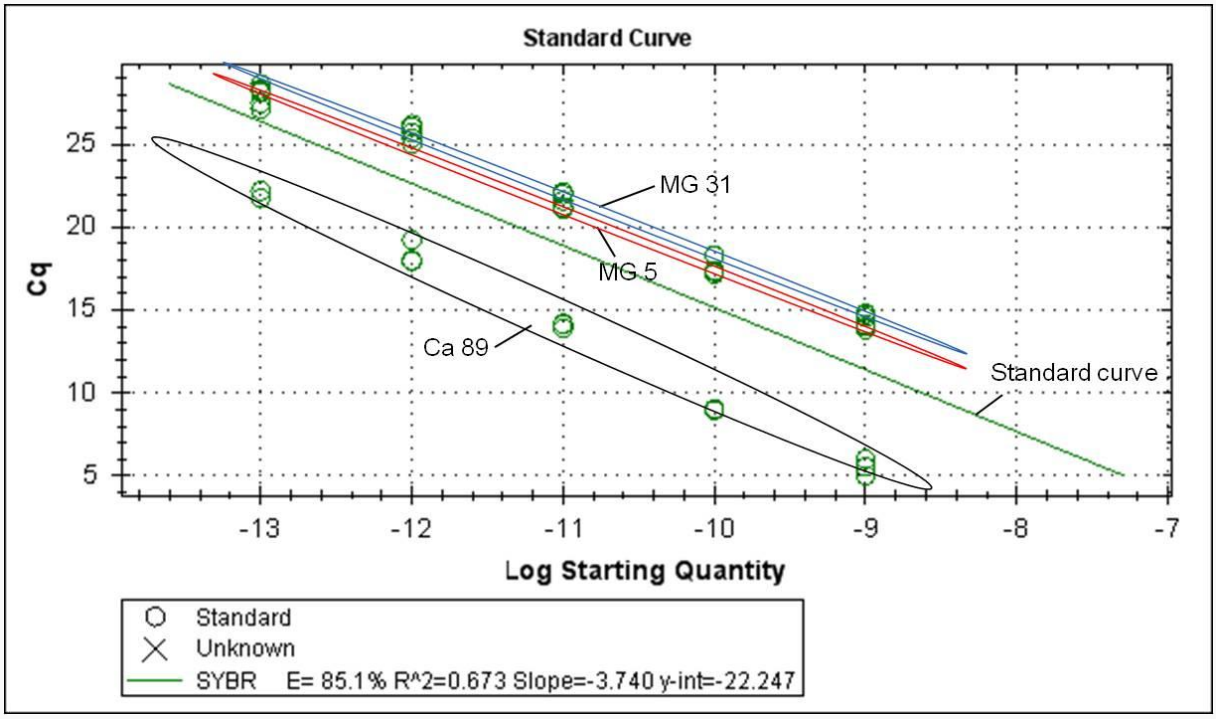

Figure 15 Standard curve formed by the amplification products of three *Magnaporthe* isolates (MG 5, MG 31 and Ca 89). The amplified products of five concentrations from wheat isolates (MG 5 framed in red oval and MG 31 framed in blue oval) were above the standard curve, meanwhile the amplified products of five concentrations from rice isolate (Ca 89 framed in black oval) were under the standard curve.

In order to exclude the impact of plant DNA on PCR detection, the fungal DNA was mixed with both plant DNAs (wheat and rice), separately. The comparison of amplifications between pure fungal sample and mixed samples is shown in Fig. 16. Each 10-fold difference in initial DNA amounts is represented by approximately 3.5 cycle differences in the Ct value. A standard curve was obtained with a good correlation coefficient (R^2 = 0.997) and 95.8% efficiency (an average from three tests of different DNA sample preparations), indicating that the individual data points were highly correlated with the calculated linear regression. There were no differences presented in the Ct values between the pure fungal sample and the mixed sample. Therefore, the presence of plant DNA did not interfere with the final detection of qPCR when the infected leaf samples were prepared using the same method with fungal DNA extraction.

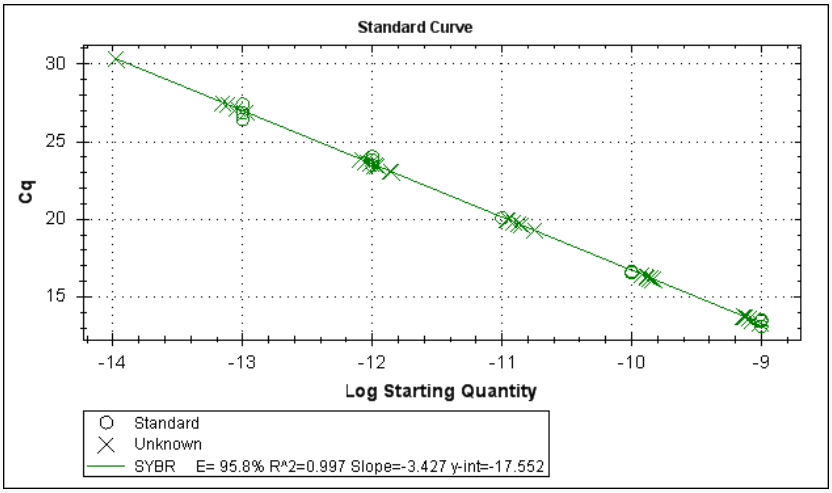

Figure 16 Standard curve formed by pure fungal DNA, and the comparison with mixed DNA samples (fungi DNA mixed with plant DNA). “o” represents the Ct values from pure fungi DNA, and “×” represents the Ct values from mixed DNA samples.

3.6.3 Quantitative real-time PCR detection

In order to quantify the degree of colonization of infected leaves and to observe the course of infection of *Magnaporthe* spp. in host and non-host plants, we employed quantitative real-time polymerase chain reaction (PCR) using total DNA extracted from infected leaves at 2 dpi, 4 dpi and 6 dpi. Ct values were calculated and the biomasses of fungal DNA for three isolates were estimated using their own standard curves.

Fungal biomass in infected plant leaves at 6 dpi was calculated using DNA standard curves and is shown in Fig. 17. In accordance with the disease severity observed in infected leaves, the highest amount of fungal DNA (approximately 199 pg of fungal DNA per 1 mg of plant sample) was found in the interaction between wheat isolate MG31 and its host plant. Slightly less in biomass (174 pg/mg) was present in wheat leaves infected by another wheat isolate MG 5, congruent with the reduced disease symptoms on wheat leaves. The lowest amount of fungal DNA (133 pg/mg) was detected in the interaction between rice isolate Ca 89 and its host plant.

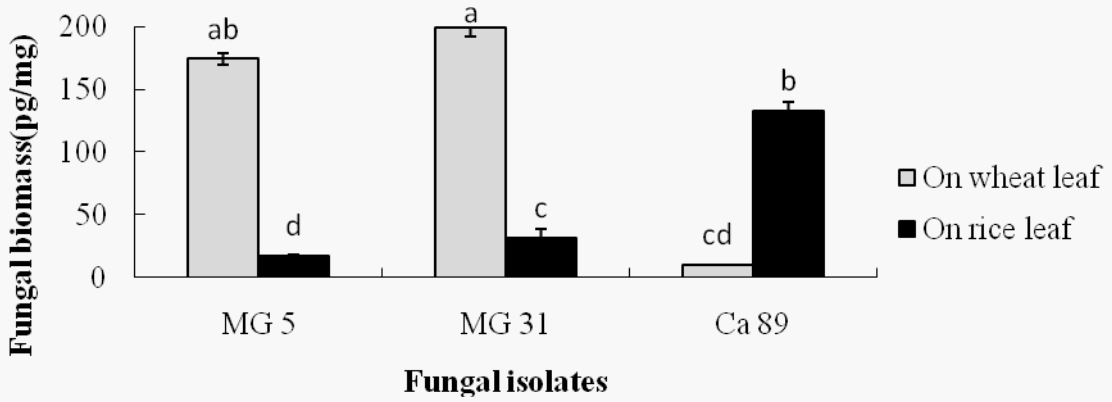

Figure 17 Quantification of fungal biomass detected by quantitative real-time PCR with the primer pair *pfh*2a and *pfh*2b on wheat and rice leaves which were inoculated with three *Magnaporthe* isolates at 6 dpi. Each value represents the average and standard deviation of three DNA samples and is followed by different letters indicating significant differences at $p \leq 0.05$, calculate by the Tukey HSD test.

Fig. 18 illustrates the fungal infection trend of *Magnaporthe* spp. in the host and non-host plants at 0, 2, 4 and 6 dpi. Fungal DNA was detected at 0 and 2 dpi with similar amounts for all interactions, confirming that a considerable amount of inoculum had remained on the leaf surfaces while preparing samples. Depending on the different interactions, fungal biomass increased with varying degrees over time. In the infection of wheat isolates with their host plant, a rapid development occurred between 2 and 4 dpi and was followed by a reduction in the growth. However, the rice isolate Ca 89 began to rapidly grow on the rice leaves after 4 days of inoculation. On the other hand, the infections were restricted to incompatible interactions and the biomass of them was much lower than in the case of infection of the pathogen with their host plants. This is due to the inhibition of non-host resistance to the development of pathogens.

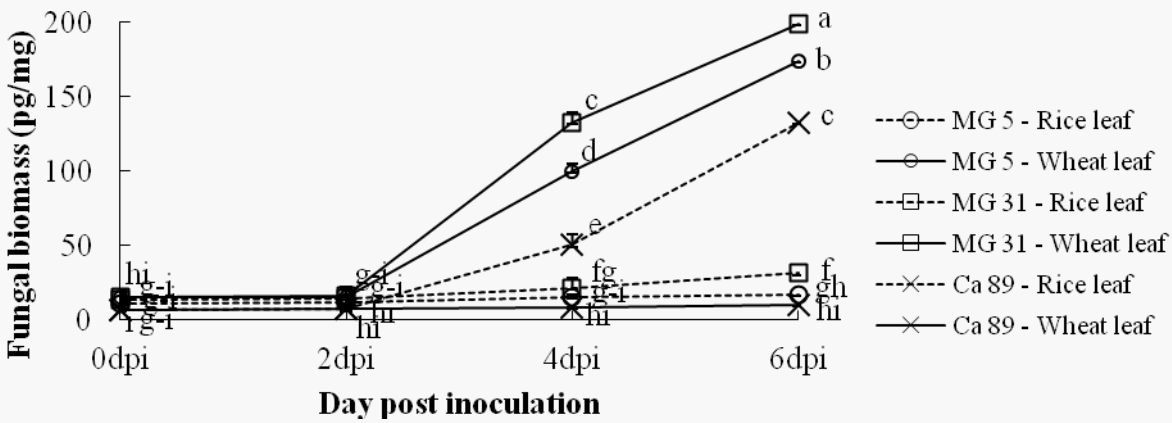

Figure 18 Growth of *Magnaporthe* spp. in wheat and rice leaves measured by fungal DNA biomass using quantitative real-time PCR. Each data point represents the average and standard deviation of three DNA samples at a specific time (0, 2, 4, or 6 dpi).

3.7 Pathogen development on leaves of wheat and rice studied with CLSM

To determine the development of *Magnaporthe* spp. (MG5, MG 31 and Ca 89) on host and non-host plants, the infection process was microscopically evaluated on wheat (BR 18) and rice (CO 39) by confocal laser scanning microscopy (CLSM). The frequency of each cellular interaction phenotype was scored after inoculation at different time points (12 , 24, 48 and 72 hpi). These time points were selected for examination based on the infection of *Magnaporthe* on rice and were predicted to cover pre-invasion and post-invasion stages of pathogen development (Kankanala *et al.*, 2007). The growth of hyphae was classified into four stages: stage one is the appressorium formation; stage two is the initial growth of the primary hyphae into the cells; stage three is the extensive invasion of the initially invaded cells; and stage four is the colonization of hyphae in the adjacent cells and intercellular spaces. All the samples from wheat leaves and rice leaf sheaths were double stained by WGA-AlexaFluor488 conjugate and propidium iodide (PI). Stained images are shown in Fig. 19 (A) and Fig. 19 (B). Fungal structures (conidia, germination tubes and appressorium) were stained by WGA-AlexaFluor488 (yellow-green), and the infection hyphae in the cells were highlighted by PI (red). However, plant structures were also stained with this nonspecific stain (Ramonell *et al.*, 2005).

All three *Magnaporthe* isolates produced infection on wheat leaves (BR 18) (Fig. 20) and rice leaf sheaths (Fig. 21). Almost all spores germinated and formed an appressorium (stage one) at 12 hpi on the surface of wheat leaves and rice leaf sheaths. Even the non-adapted isolates for each plant were able to produce appressoria and attempted to infect the non-host plant as effectively as the adapted isolate.

Significant differences ($p \leq 0.05$) on wheat leaves (Fig. 20) were found at 24 hpi when hyphae from both wheat isolates grew in the initially invaded cells. At 24 hpi, 33% of MG 5 and 26% of MG 31 reached stage two, and 18% of MG 5 and 30% of MG 31 were growing in the cells (stage three). Furthermore, at 24 hpi the transition from penetration to invasion occurred more

rapidly with isolate MG 31, but for the non-adapted isolate Ca 89, there was a lack of appressorium penetration for the rice isolate and only 9% of hyphae grew into the cells. Similar differences have also been demonstrated on rice leaf sheaths (Fig. 21). Insignificant differences ($p \leq 0.05$) were visible at stage two with 21% from MG5, 28% from MG 31 and 30% from Ca 89, but the differences were significant at stage three at 24 hpi: 19% of the rice isolate (Ca 89) grew in the rice leaf sheaths, and only 0% of MG 5 and 3% of MG 31 were found.

Hyphae were well developed after 48 hours of inoculation and all the adapted isolates had reached the fourth stage indicating that hyphae were colonizing the adjacent cells and intercellular spaces. Wheat isolates MG 5 and MG 31 showed a similar growth trend from stage one to stage three at 48 hpi, but a slightly higher percentage of isolate MG 31 (9%) at stage four was observed on wheat leaves (Fig. 20). At this time point, the majority of appressoria from rice isolate Ca 89 were arrested in their development at the first stage, and only 19% of them penetrated into the cells of wheat leaves. Turning our attention to the response of the rice leaf sheaths at 48 hpi (Fig. 21), most of the hyphae from isolate Ca 89 were growing in the epidermal cells, and 7% of them had even colonized the adjacent cells or occupied the intercellular spaces. However, for the non-adapted wheat isolates, only 5% and 13% of MG 5 and MG 31, respectively, could reach the third stage.

At the last observation time point of 72 hpi, the number of infection sites defined by appressorium formation decreased over time in all interactions, with only 29% (MG 5), 22% (MG 31) and 21% (Ca 89) of infection sites remaining on their host plants from stage one. For interactions on the wheat leaves (Fig. 20), significant differences ($p \leq 0.05$) were recorded at all four stages between wheat isolate and rice isolate. Most notably, at the fourth stage 42% and 50% of multicellular infections were observed from isolates MG 5 and MG 31, respectively, in contrast to 0% of hyphae detected from rice isolate Ca 89: This is clearly indicative of a successful infection. The infection from Ca 89 were seen to form hyphae (22%) within epidermal cells (stage two) at 72 hpi, but it seemed to rarely develop hyphae (5%) in the cells (stage three). However, rice isolate Ca 89 successfully infected rice with 32% of hyphae detected at the fourth stage (Fig. 21). In the meantime, 60% (MG 5) and 49% (MG 31) of the other two isolates still remained in the first stage, but 4% of hyphae from wheat isolate MG 31 were observed in development on adjacent cells. The arrest of appressoria was occasionally accompanied with the formation of papilla, which could happen on both compatible and incompatible interactions. More precise observations on these structures were made by using autofluorescence microscopy.

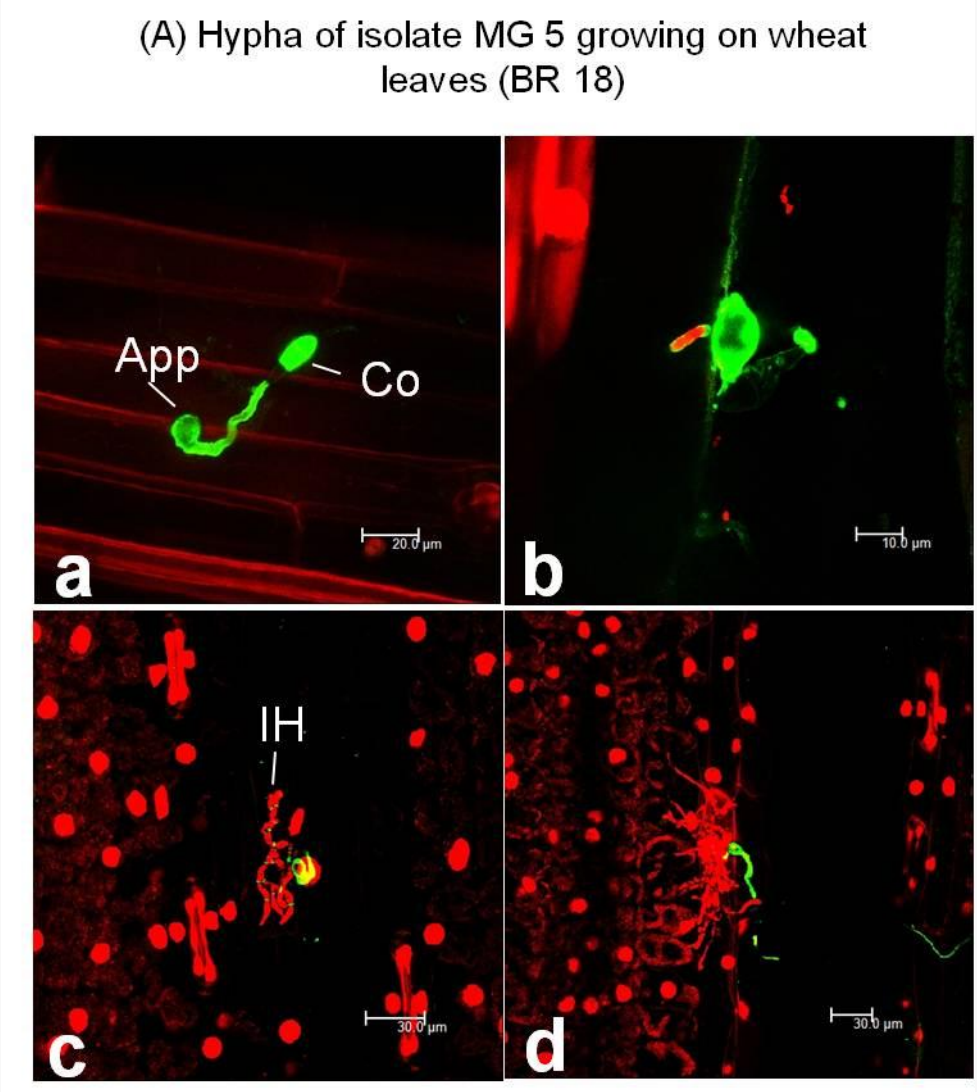

Figure 19 (A) Detection of hyphal growth of isolate MG 5 at different time points (12, 24, 48 and 72 hpi) on wheat leaves (BR 18) by confocal laser scanning microscopy (CLSM). Stage one (a) was detected at 12hpi when appressoria formed; stage two (b) was at 24hpi when hyphae started to grow within the cells; stage three (c) was at 48hpi when hyphae extensively invaded the initial cell; and stage four (d) was at 72 hpi when hyphae colonized the adjacent cells and intercellular spaces. All the samples were stained with WGA-AlexaFluor488 and PI for 48 h. Conidia, germination tubes and appressoria were stained with WGA-AlexaFluor488 (yellow-green), and infection hyphae in the cells and plant structures were stained with PI (red). App: appressorium; Co: conidia; IH: infection hyphae.

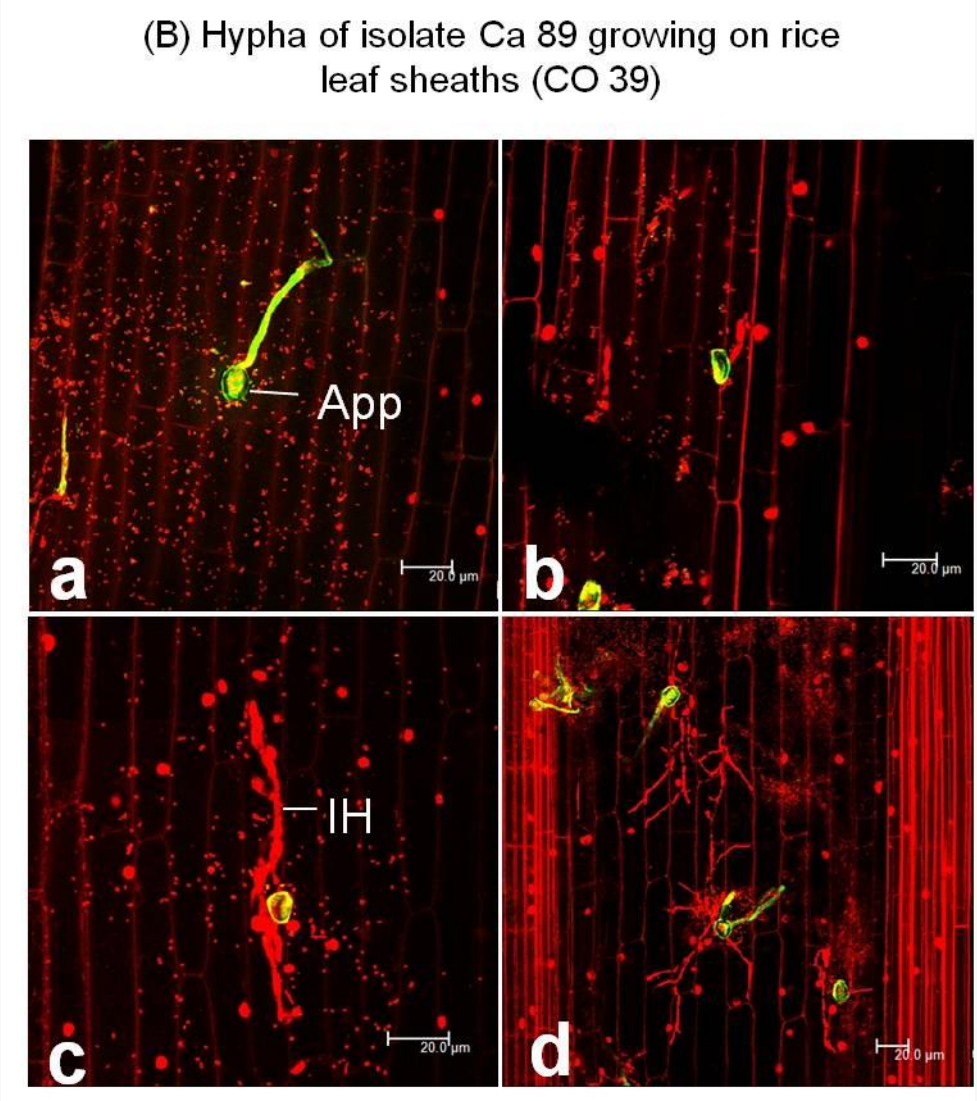

Figure 19 (B) Detection of hyphal growth of isolate Ca 89 at different time points (12, 24, 48 and 72 hpi) on rice leaf sheaths (CO 39) by confocal laser scanning microscopy (CLSM). Stage one (a) was detected at 12hpi when appressoria formed; stage two (b) was at 24hpi when hyphae started to grow within the cells; stage three (c) was at 48hpi when hyphae extensively invaded the initial cell; and stage four (d) was at 72 hpi when hyphae colonized the adjacent cells and intercellular spaces. All the samples were stained with WGA-AlexaFluor488 and PI for 48 h. Conidia, germination tubes and appressoria were stained with WGA-AlexaFluor488 (yellow-green), and infection hyphae in the cells and plant structures were stained with PI (red). App: appressorium; IH: infection hyphae.

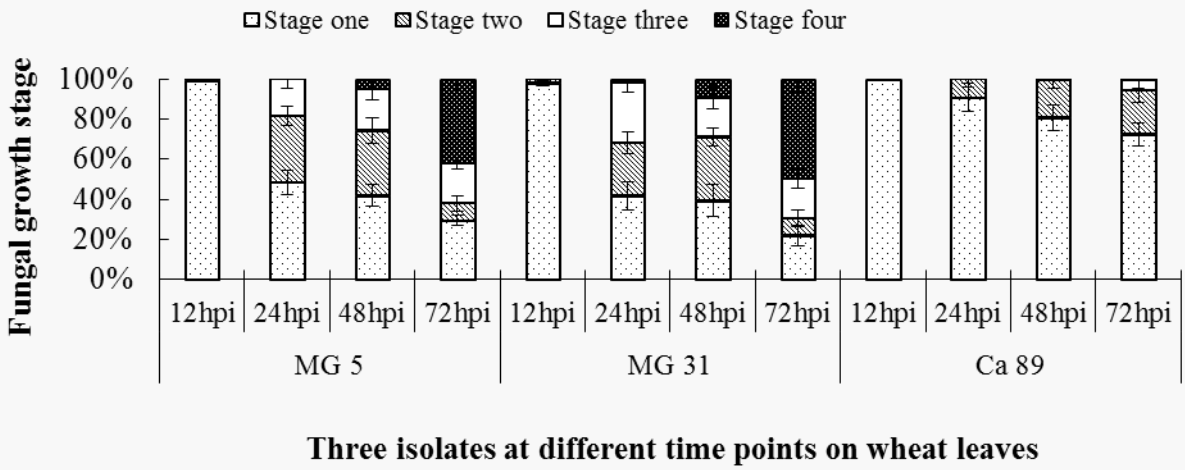

Figure 20 Distribution of wheat / *Magnaporthe* interaction stages over time. Wheat (BR 18) leaves were inoculated with two adapted wheat isolates MG5 and MG 31, as well as a non-adapted rice isolate Ca 89, and monitored at 12, 24, 48 and 72 hpi. Bars represent percentage averages, with standard deviations from five wheat leaves in three independent experiments. Stage one is the formation of appressoria; stage two describes when the primary hyphae start to grow within the cells; stage three represents the extensive invasion of hyphae into the initially invaded cells; and stage four characterizes the colonization of hyphae in the adjacent cells and intercellular spaces.

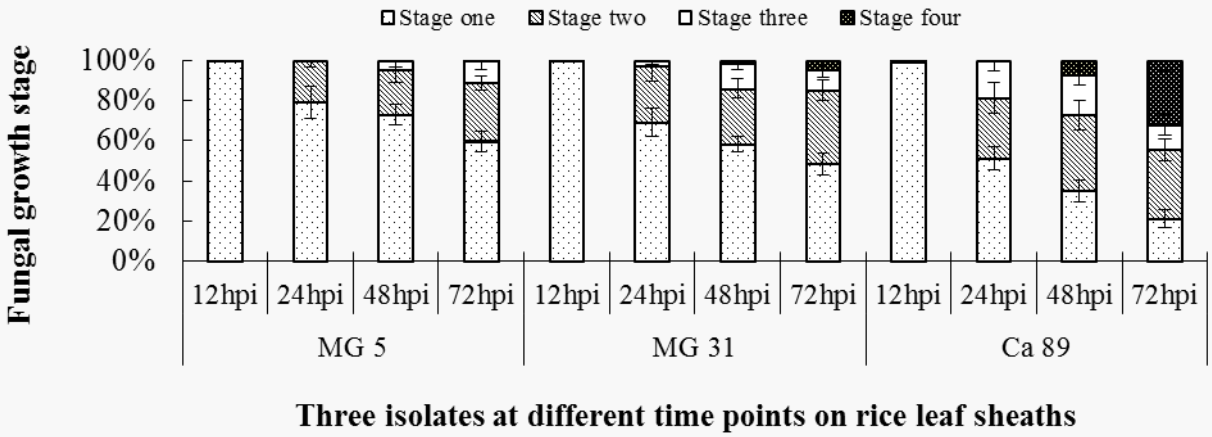

Figure 21 Distribution of rice / *Magnaporthe* interaction stages over time. Rice (CO 39) leaf sheaths were inoculated with two non-adapted wheat isolates MG5 and MG 31, as well as an adapted rice isolate Ca 89, and were evaluated at 12, 24, 48 and 72 hpi. Bars represent percentage averages, with standard deviations from five wheat leaves in three independent experiments. Stage one is the formation of appressoria; stage two describes when the primary hyphae start to grow within the cells; stage three represents the extensive invasion of hyphae into the initially invaded cells; and stage four characterizes the colonization of hyphae in the adjacent cells and intercellular spaces.

3.8 Cytological responses on leaves recorded by autofluorescence

The response of wheat and rice leaves to infection from adapted and non-adapted *Magnaporthe* isolates was investigated using microscopy at the cellular level. The same isolates (MG5, MG 31 and Ca 89) used before were inoculated on wheat (BR 18) and rice (CO 39) seedling leaves. Microscope observations were performed under bright-field and epi-fluorescence microscopy. Four different cellular interaction stages are shown in Fig. 22 (A) and Fig. 22 (B). Picture A from Fig. 22 (A) and E from Fig. 22 (B) refer to type A (no reaction on epidermal cells), B and F show type B (papilla formation with fluorescence). C and G are comprised of hypersensitive reactions of epidermal cells (the whole cell autofluorescence was taken as a reliable marker for cell death (Koga, 1994)) while D and H describe the successful infection in epidermal cells. The frequency of each cellular interaction was scored after inoculation of three isolates on wheat and rice at 48 hpi (Fig. 23).

Microscopic investigations under bright field revealed that spore germination and appressorium formation of these three isolates can be performed normally on wheat and rice tissues. Many appressoria was just halted in this stage and there was no reaction detected on epidermal cells (type A), 17.7% of appressorium from rice isolate Ca 89 were found on wheat leaves, and similarly 15.8% from wheat isolate MG 5 and 13.8% from wheat isolate MG 31 were detected in this stage (Fig. 23).

One of the earliest cellular responses toward pathogen penetration is the plant immune system. The plant would form papillae by thickening the cell wall to prevent the invasion of pathogens, classified as type B. Significant differences ($p \leq 0.05$) were apparent between compatible and incompatible interactions for papilla formation. On wheat leaves, approximately 69.3% of epidermal cell responded with a papilla to penetration with the rice isolate Ca 89, while only 22.5% and 21.2% responded to wheat isolates MG 5 and MG 31, respectively. This shows that wheat plant (BR 18) could effectively prevent the invasion by the rice isolate at the beginning. In regards to the response of rice leaves, the penetration of wheat isolates MG 5 and MG 31 were prevented by 55.7% and 39.7%, respectively. However, only 21.5% of the adapted isolate Ca 89 were stopped by papilla, showing the significant differences for the adapted and non-adapted isolates. However, successful penetration through ineffective papillae allowed the establishment of infection hyphae in epidermal cells and resulted in strong autofluorescence in the cell walls in both host and non-host combinations.

In type C, isolates triggered hypersensitive reactions (HR) of epidermal cells: the whole cell died with accumulated fluorescent materials on the cell walls (Koga *et al.*, 1988). This autofluorescence was observed in 12% of the penetration sites on wheat leaves infected by Ca 89, and is insignificantly different with 20.3% and 17.3% from MG5 and MG 31, respectively. Insignificant differences were also demonstrated on rice leaves with their responses to MG5, MG 31 and Ca 89 (21.2%, 27% and 17.3%).

When the plant failed to prevent the development of the pathogen, hyphae grew into the cells (type D). In the compatible interactions, 41.3%, 47.7% and 43% of isolates MG 5, MG31 and Ca 89 successfully formed and developed hyphae in the cells of their host plants. Only 1% of rice isolate Ca 89 and 1.5% of wheat isolate MG 5 reached type D on their non-host plants.

Interestingly, wheat isolate MG 31 showed high frequency (13.2%) in type D, which indicates that MG 31 is capable of infecting non-host rice plants. Lesions had also been observed on rice leaves after 7 days of inoculation.

In all host-type interactions between pathogens and their host plants, a relatively low frequency of papilla formation and cell autofluorescence occurred together with a high frequency of hyphae development in the cell. There were no significant differences between the three isolates and their hosts. However, for the non-host interactions, the opposite occurred, with a high frequency of papilla formation and very low frequency of hyphae growth. However, there was a significant difference between the invasion of wheat isolate MG 31 and the other two isolates. Overall, autofluorescence microscopy revealed that there were significant differences in host and non-host interactions. Both plants (wheat and rice) demonstrated active defense responses to the invasion of *Magnaporthe* spp.. The wheat isolate MG 31 is more aggressive when infecting rice, as non-host plant.

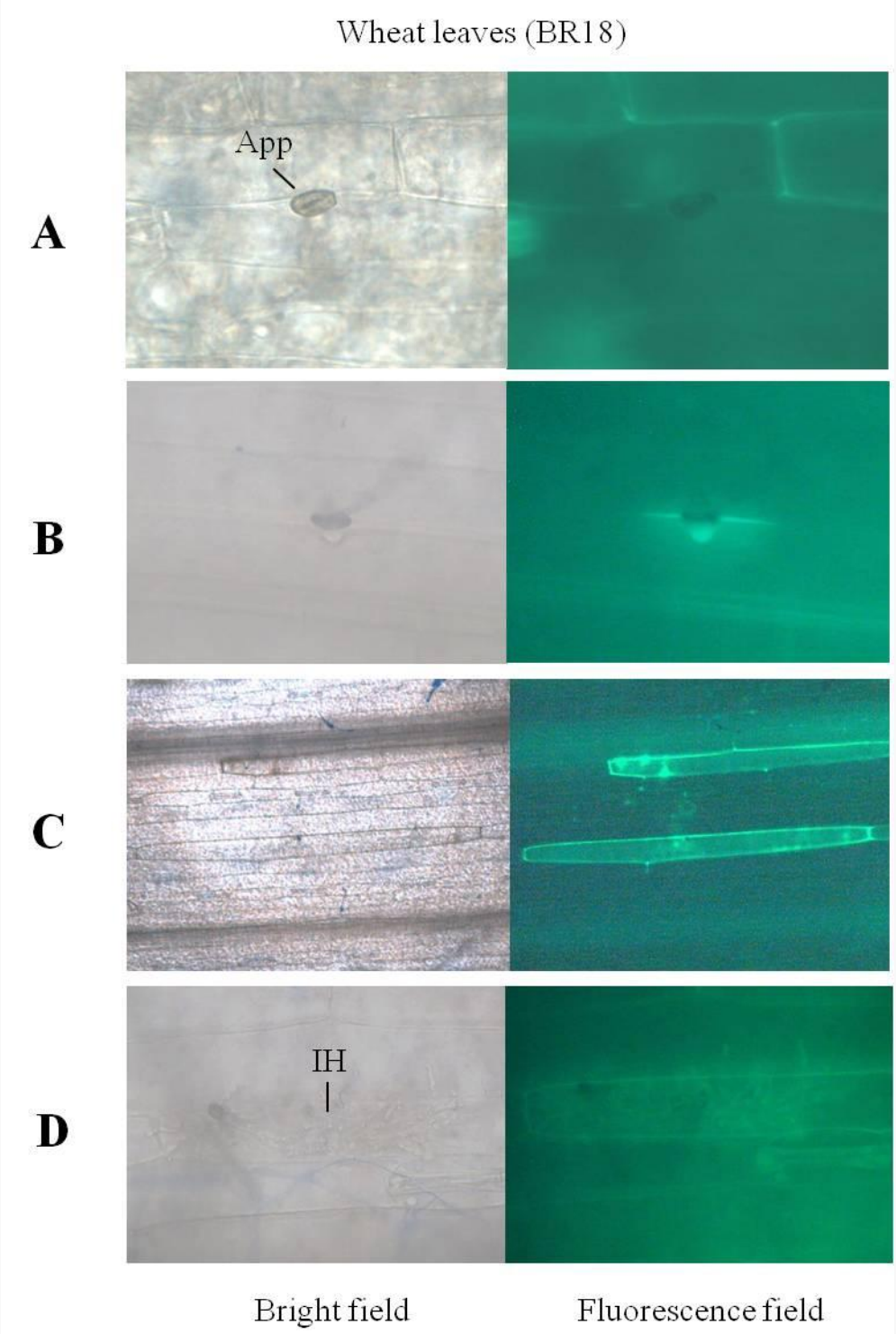

Figure 22 (A) Cellular interaction of wheat leaves inoculated with wheat isolates (MG 5 and MG 31) and the rice isolate (Ca 89) of *Magnaporthe* spp. at 48hpi. The pictures on the left side were detected under bright field, and the pictures on the right side were detected under fluorescence light. Four different types of cellular responses were classified on wheat and rice. Type A (picture A) represents appressorium formation with no plant response, type B (picture B) represents papilla formation with autofluorescence, type C (picture C) represents hypersensitive responses associated with whole cell autofluorescence and type D (picture D) represents successful penetration in epidermal cells. App: appressorium; IH: infection hyphae.

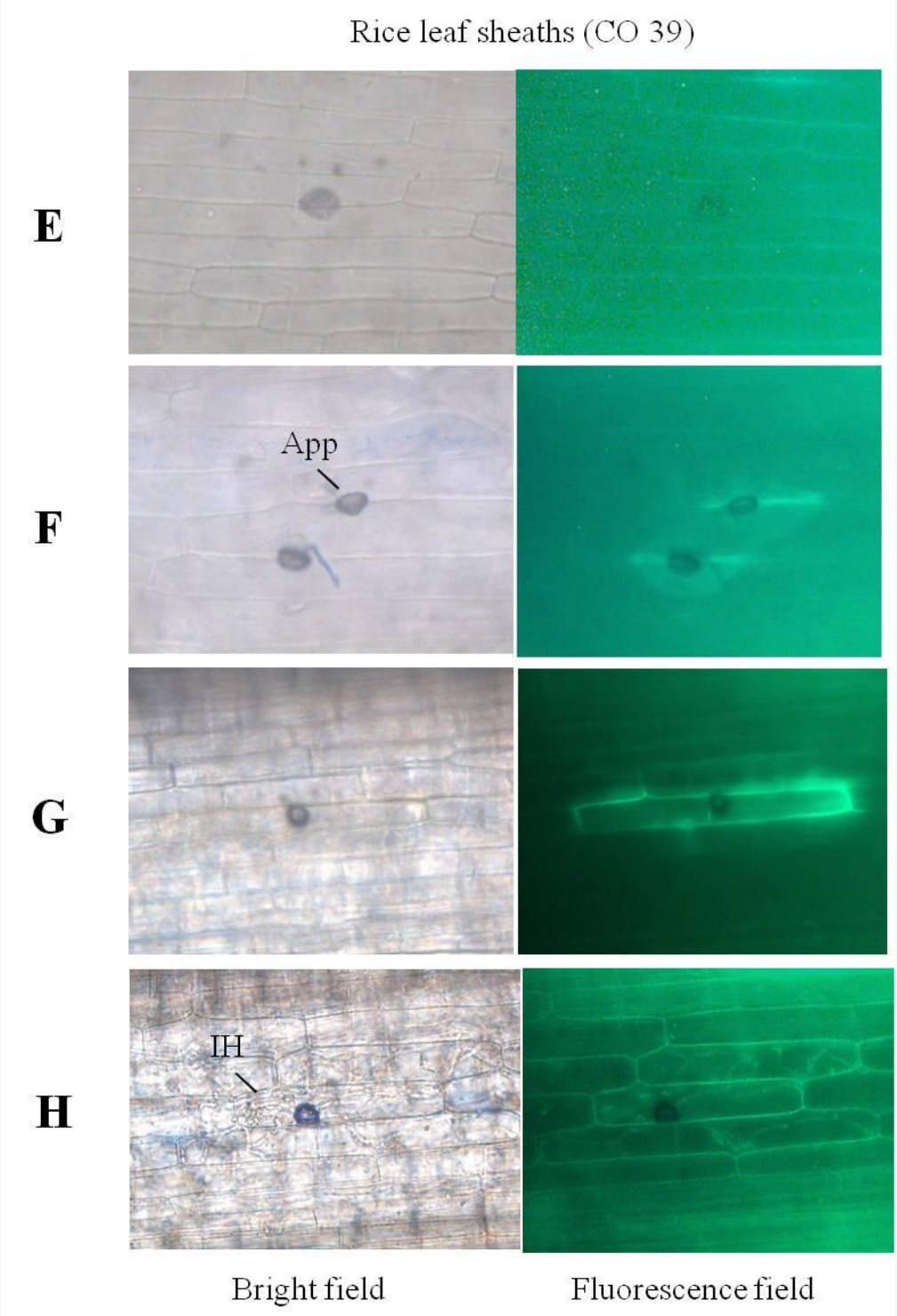

Figure 22 (B) Cellular interaction of rice leaf sheaths inoculated with wheat isolates (MG 5 and MG 31) and the rice isolate (Ca 89) of *Magnaporthe* spp. at 48hpi. The pictures on the left side were detected under bright field, and the pictures on the right side were detected under fluorescence light. Four different types of cellular responses were classified on wheat and rice. Type A (picture E) represents appressorium formation with no plant response, type B (picture) represents papilla formation with autofluorescence, type C (picture G) represents hypersensitive responses associated with whole cell autofluorescence and type D (picture H) represents successful penetration in epidermal cells. App: appressorium; IH: infection hyphae.

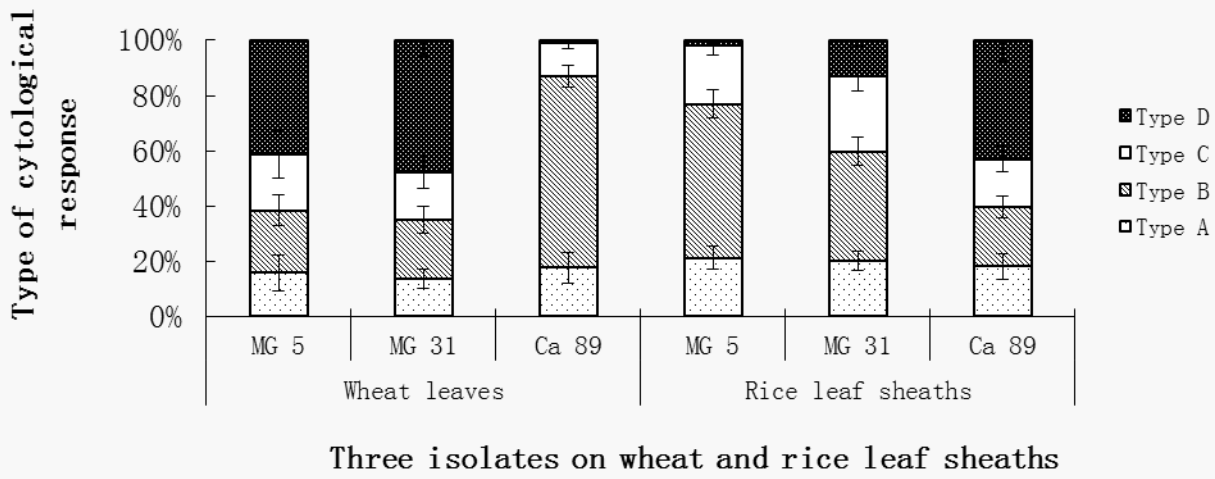

Figure 23 Autofluorescence microscopic analyses of wheat and rice seedlings interacting with isolates MG 5 and MG 31 from wheat and Ca 89 from rice at 48 hpi. Columns represent the mean values of percentages and standard deviations are calculated from five wheat leaves or rice leaf sheaths with approx. 200 interaction sites inspected per sample. Type A represents no reaction on epidermal cells, type B represents papilla formation with fluorescence, type C represents hypersensitive reactions with whole cell autofluorescence and type D represents successful infection in epidermal cells.

3.9 Effects of phytotoxins

3.9.1 The effects of fungal culture filtrate on detached leaves

Conidial suspensions of *Magnaporthe grisea* prepared from wheat isolates MG 5 and MG 31 induced lesions on wheat leaves (Fig. 24 a-II and b-II) after 3 days of inoculation. On leaves which were inoculated with MG 31, serious infection was clearly visible – far more than those inoculated with MG 5. However, neither the filtrate of fresh potato dextrose broth (PDB; Fig. 24 a-I and b-I) nor culture filtrates from *Magnaporthe grisea* with a series of three concentrations (Fig. 24 a-III, IV, V and b-III, IV, V) produced any visible symptoms. Furthermore, effects from the rice isolate (Ca 89) failed to cause any lesions in all treatments (Fig. 24 c).

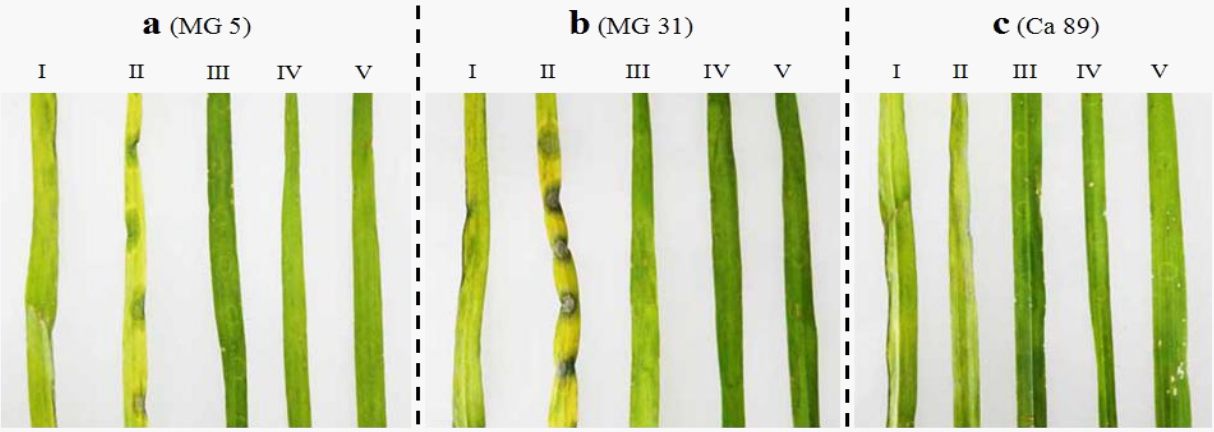

Figure 24 Effects of fungal culture filtrates on wheat leaves. Detached wheat seedling leaves were drop-inoculated with fresh PDB as control (I), conidial suspension (II), culture filtrate (III), 1/2 of culture filtrate (IV) and 1/4 of culture filtrate (V). The conidial suspensions were prepared from wheat isolates MG 5 (a) and MG 31 (b) and rice isolate Ca 89 (c).

In Fig. 25 c-II, typical necrotic lesions were evident on rice leaves which were inoculated with conidial suspensions of *Magnaporthe oryzae* (rice isolate Ca 89) at 3 dpi. However, no visible symptoms were noticeable in the treatments with either the filtrate of PDB (Fig. 25 c-I) nor three concentrations of culture filtrates (Fig. 25 c-III, IV, V). HR lesions were observed in the interaction between rice leaf and wheat isolate MG 31 (Fig. 25 b-II), but no symptoms were found on other treatments of MG 31 (Fig. 25 b-I, III, IV, V). The wheat isolate MG 5 did not induce any lesions with conidial suspension (Fig. 25 a-II) or culture filtrates (Fig. 25 a-III, IV, V) as well as the PDB control (Fig. 25 a-I).

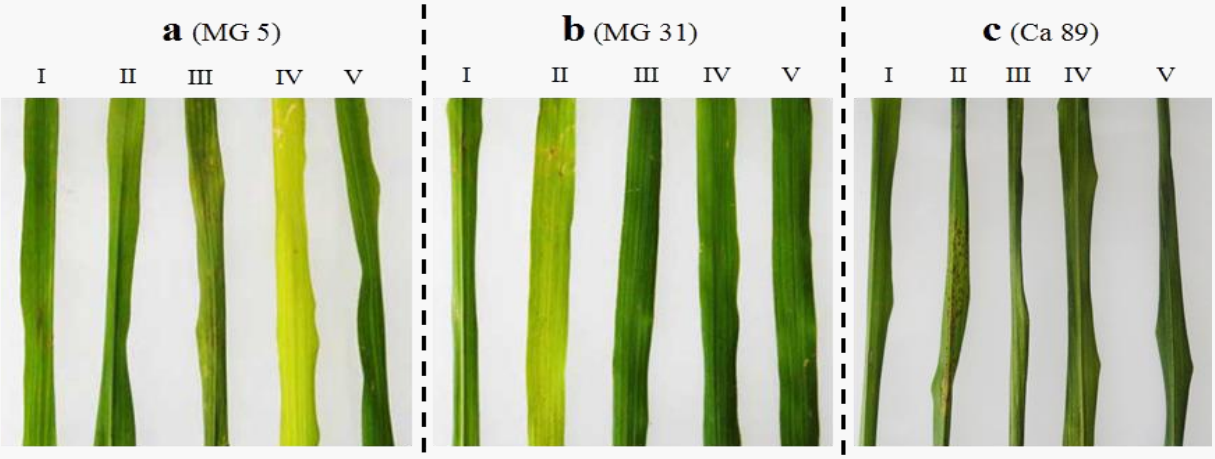

Figure 25 Effects of fungal culture filtrates on rice leaves. Detached rice seedling leaves were drop-inoculated with fresh PDB as control (I), conidial suspension (II), culture filtrate (III), 1/2 of culture filtrate (IV) and 1/4 of culture filtrate (V). The conidial suspensions were prepared from wheat isolates MG 5 (a) and MG 31 (b) and rice isolate Ca 89 (c). Symptoms of necrotic lesions appeared on rice leaves inoculated with conidial suspension from the rice isolate.

3.9.2 The effects of extracts from infected leaves on detached leaves

On wheat leaves, the effects of infected leaf extract leachate showed similar results as those of fungal culture filtrate. Wheat leaves did show any response to infected leaf extract leachate, but a less severe incidence of the disease was observed in the inoculation of spore suspensions. Specifically, the symptoms were only seen on the bottom of detached leaves, where they had been immersed in the conidial suspensions obtained from wheat isolates MG 5 (Fig. 26 a-II) and MG 31 (Fig. 26 b-II) in 3 dpi. The treatments of both isolates from three concentrations of culture filtrate (Fig. 26 a-III, IV, V and b-III, IV, V) did not cause phytotoxicity on leaves compared with the PDB control (Fig. 26 a-I and b-I). The non-host isolate Ca 89 also failed to induce lesions on wheat leaves (Fig. 26 c).

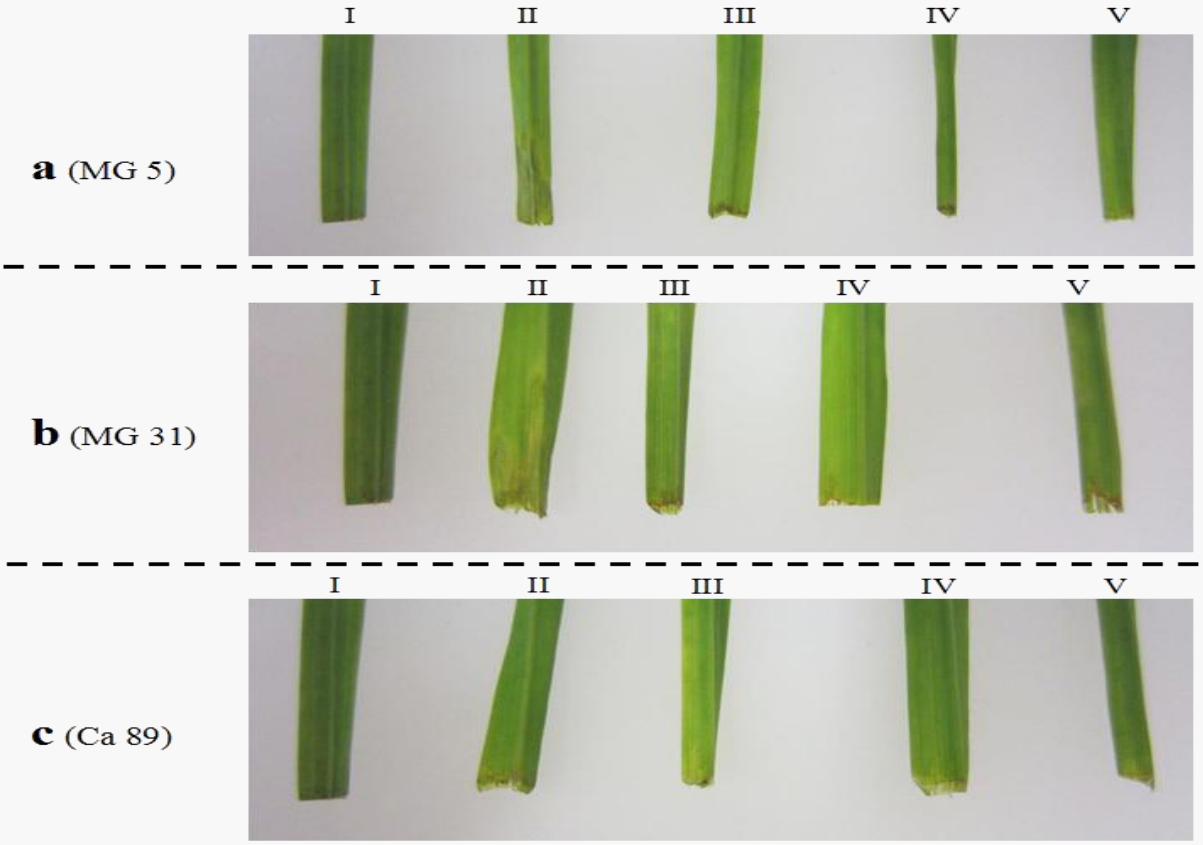

Figure 26 Effects of infected leaf extracts on wheat leaves. Detached wheat seedling leaves were wound-inoculated with fresh PDB as control (I), conidial suspension (II), leaf extract leachate (III), 1/2 of leaf extract leachate (IV) and 1/4 of leaf extract leachate (V). The conidial suspensions were prepared from wheat isolates MG 5 (a) and MG 31 (b) and rice isolate Ca 89 (c).

On rice leaves, no symptoms were observed in all treatments from the isolates of *Magnaporthe grisea* MG 5 (Fig. 27 a) and MG 31 (Fig. 27 b) after 3 days of inoculations. The senescence symptoms of the detached leaves, as descriped in Talbot *et al.* (1997), appeared as the leaf rolling, with pronounced desiccation and yellowing. On the bottom of the rice leaves,

there were only two or three necrotic lesions shown (Fig. 27 c-II), far less than after drop-inoculation.

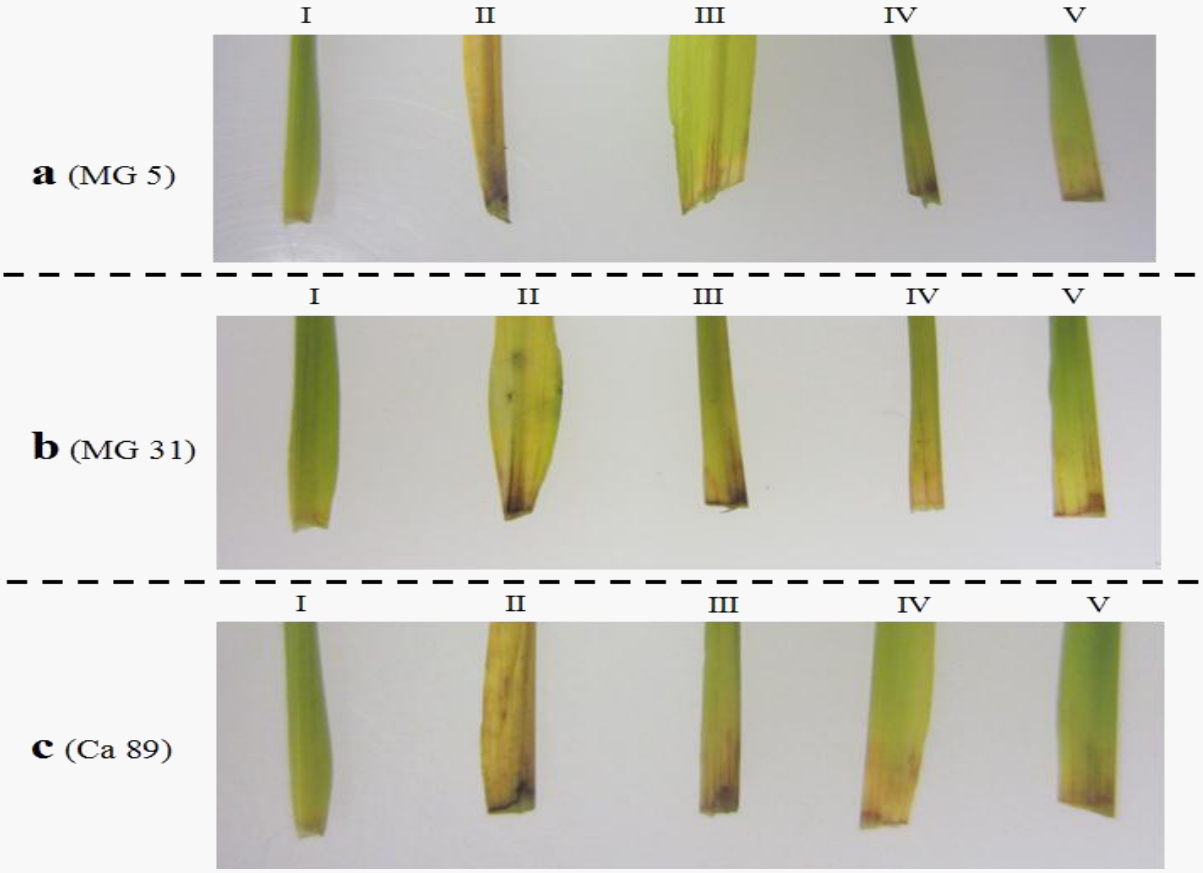

Figure 27 Effects of infected leaf extract leachates on rice leaves. Detached rice seedling leaves were wound-inoculated with fresh PDB as control (I), conidial suspension (II), leaf extract leachate (III), 1/2 of leaf extract leachate (IV) and 1/4 of leaf extract leachate (V). The conidial suspensions were prepared from wheat isolates MG 5 (a) and MG 31 (b) and rice isolate Ca 89 (c).

Through the above observation, it can be seen that regardless of the method which was used to extract phytotoxic substances or indeed the inoculation method, similar results were evident. Results showed that on both wheat and rice seedling leaves, lesions were only induced by the treatment with conidial suspension from compatible isolates and not by fungal culture filtrate, extracts from inoculated leaves or PDB filtrate (control).

4 Discussion

4.1 Growth conditions required by *Magnaporthe* spp. *in vitro* and *in vivo*

The inoculum concentration influences the disease severity, so it is important to obtain a sufficient amount of spores for artificial inoculations. Different fungal species and isolates from the same species are able to show significant differences in their sporulation ability. Factors such as the medium, temperature and environment for incubation would also affect spore production. Partridge-Metz & Chandra (2011) verified that rapid vegetative mycelial growth of *Magnaporthe oryzae* occurred using V8, PDA and OMA, while the culture media producing the highest amount of pigmentation were found to be V8, OMA and STAA. The highest yield of conidia was obtained using St. Augustinegrass agar (STAA). Vanaraj *et al.*, (2013) suggested that potato dextrose and malt extract agar were also suitable for culturing different isolates of *Pyricularia oryzae*. The blast disease is more frequent in wheat producing regions above the 24 °S parallel. Kalbia and José (2006) have studied the influence of temperature and relative air humidity on the sporulation of *Magnaporthe grisea*. The highest rates of infection and production of conidia from *Magnaporthe* spp. occur around 28 °C with high relative humidity (≥90%). They also revealed that the effect of temperature and humidity was significant ($P<0.001$), but not the interaction. This is consistent with our results from epidemiological studies. The influence of temperature on the colony growth of *Magnaporthe* isolate on Petri dishes has been detected in our laboratory (Fig. A5). The maximum diameter of colony growth was measured between 24 °C and 28 °C. Latterell & Rossi (1986) mentioned that occasionally isolates from *Pyricularia oryzae* - representing broadly pathogenic races, which is valuable in cultivar screening - sporulated poorly; e.g. isolate 825 (IB-1) from Costa Rica. They tested three methods to enhance sporulation and proved that several isolates yielded improved sporulation capacities following UV irradiation of 24 h germinating spores in quartz tubes and no changes in pathogenic specialization occurred. Furthermore, several cultural changes were observed in X-radiation treatments. Eventually, in accordance with previous research and our results from medium screening, the colonies of all seventy *Magnaporthe* isolates were incubated on V8 agar medium at 25 °C for 7 d, followed by exposure under UV light for 2 d.

Studies on low temperature effect on blast disease were only reported for plant resistance response (Koga *et al.*, 2004) or *Magnaporthe* cold acclimation (Li *et al.*, 2014). However, the influence of low temperature on *Magnaporthe* to grow and sporulation has not been studied *in*

vitro. Significant differences ($p \leq 0.05$) on colony diameters among five isolates (MG 27, MG 51, MG1.2, MG5 and 1836-3/0-12) and three treatments (25 ℃, 4 ℃ and -20 ℃) were shown in Fig. 5. The growth rate of the colonies was slowed down at 4 ℃ and halted at -20 ℃; microscopy showed fewer spores at 4 ℃ and a large reduction at -20 ℃. This suggests that the colony of wheat isolates could be stored at 4 ℃ for short term storage, but that there is an associated effect on sporulation.

The climate has a strong influence on the appearance of blast epidemics (Suzuki, 1975). Asai *et al.* (1966) demonstrated that the weather is an important factor in the variability of disease development. When there are no fluctuations in relative humidity and temperature, there appears to be no change in the number of diseases. The infection condition of blast disease on rice has been studied by many researchers. However, the environmental conditions needed for wheat plants have not yet been clearly defined. It is well-known that temperature and spike wetness are two of the most important environmental factors affecting the interaction between plant and pathogen. The pathogens which can infect crops are considered to be favored by warm and humid conditions (rain in the flowering stage). Kranz & Hau (1980) have pointed out that optimum condition studies under controlled conditions (climate chamber) provide a solid base for understanding the effects of environmental factors on the development of plant epidemics. Rotem (1988) also confirmed that the data obtained under controlled conditions would provide an indication of what may happen in the field. However, they cannot simulate with accuracy the development of the disease in nature, because a model is just a simplified representation of reality in a climate chamber and is a modified representation of the atmospheric conditions occurring during cultivation in the field. The objective of our experiment on microclimatic requirements was to find out the effect of different environmental factors on the development of *M. grisea* on wheat ears, and determine the optimum temperature and spike-wetness. Ear bleaching was more severe at temperatures above 26 ℃ compared to lower temperatures (Fig. 7). At 14 hpi, spike wetness significantly contributed to disease severity (Table 6). However, there was no significant effect of spike wetness on disease severity for the wetness durations ≥24 hpi. These results indicate that temperatures above 26 ℃ increase disease severity, whereas ear bleaching is less affected by wetness duration after 24hpi. Moreover, leaf wetness had no significant effect on disease severity at 20 ℃, even at 14 hpi.

Temperature generally accelerates biological processes, and both plants and their pathogens require a minimum temperature for growth and development. Infections do not normally start

during the cold seasons, and the development of ongoing infections will be halted during such periods. However, when other environmental conditions become favorable, pathogens are far more aggressive and will easily infect susceptible plants with increasing temperatures (Reis & Bresolin, 2004). The effect of temperature on the development of disease depends on the type of the pathogen-host association. Normally, a disease develops more rapidly at the optimum temperature for the pathogen which is generally above or below the optimum temperature for the host because the time required in completing the life-cycle of the pathogen is much shorter. However, when temperatures are below or above the optimum temperature for the pathogen or are close to the optimum for the host, the development of the disease is slower (Reis & Bresolin, 2004). Indeed, there is a maximum temperature for plant growth. Cardoso *et al.* (2008) verified that under controlled conditions, blast detection on wheat cultivar BR 23 was unable to be processed at 35 ℃, and all spikes (even in the control group) turned white, preventing evaluation. Perhaps the clearest possible reason points to the conditions of the experiment: the temperature (35 ℃) was simply too high for the wheat crop. However the growth of wheat at high temperatures showed different responses in our experiment. Wheat (BR 18) growth was not affected by temperatures between 20 ℃ - 32 ℃, and ears in controls remained green during the whole experiment. This might be due to the different requirements of the cultivars in the growth environment. Wheat blast is more frequent in wheat producing regions above the 24 °S parallel and prefers warmer conditions. In lower temperature regions, we can effectively reduce the rate of wheat blast outbreak (Kalbia and José, 2006). The blast severity was highly influenced by temperature. When the temperature rose, the disease severity increased relatively. When the temperature rises from 20 ℃ to 23 ℃, a significant difference in severity was observed (1.60 at 20 ℃ compared to 3.68 at 23 ℃).

The wetness time refers to the time that a plant organ is exposed to liquid water, and the occurrence of liquid water on the surface of a plant constitutes an important factor in disease development (Sutton *et al.*, 1984). The humid environment could lead to spore germination of the pathogen, and this response is automatic and irreversible. Infection would then be successful if wetting occurs for a sufficient time and at a suitable temperature to allow colonization of the host to be initiated. This interaction has been called the ‘critical period’ (CP) by Zadoks & Schein (1979). Therefore, when the infection occurs during a critical period, the surface of a spike may get dry but the life cycle of the pathogen will continue, which results from the establishment of parasitism. As described by Rossi *et al.* (2001), frequent rainfall, high humidity, and heavy dew which coincides with a crop sensitive period favors infection. This “critical period” is particularly evident in the field environment: the

rainfall will directly affect the duration liquid water spends on the plant surface. In controlled conditions, no symptoms of *M. grisea* on wheat ears were observed when wetting time was less than 10 hours (Cardoso *et al.*, 2008). According to the results at different wetness times in our experiment, when high humidity is maintained over 24 hours for newly inoculated wheat ears, there were insignificant differences between disease severities shown as the extension of wetness time. This result suggested that humidity would no longer be the main factor affecting disease development when the initial high humidity was longer than 24 hours.

Disease forecasting systems which use climatic models are based on the temporal and spatial presence of three decisive disease factors: a host, a plant pathogen and favorable atmospheric conditions which affect the infectious process (Sutton, 1988). The data from the interactions between temperature and spike wetness time could be used to create a basic climatic model for forecasting systems. This interaction has been used in many early warning systems (Stutton, 1988; Cardoso *et al*., 2008). According to the climatic model created by Cardoso *et al*. (2008), the variations as a function of temperature of blast intensity were explained by a generalized beta model; the variations as a function of the duration of spike wetness were analyzed by the Gompertz model. Disease intensity was modeled as a function of both temperature and the duration of spike wetness, and the resulting equation provided a precise description for the response of *P. grisea* to temperature and the duration of spike wetness. Tables were constructed using the models so the intensity of wheat blast (*P. grisea*) can be predicted based on temperature and the duration of wheat spike wetness obtained in the field. However, the effect of interactions between temperature and spike-wetness time on infection under controlled conditions can cause distortions when applied to the field. Possible reasons for these distortions could be temperature fluctuations, interruptions in the duration of wetness time, different inoculum concentrations, variation in spore germination, host factors (different growth stages or organs) and presence of nutrients or pesticides on the phylloplane and antagonistic biological activity of phylloplane microorganisms (Sutton, 1988). Hence, the absolute infection frequency is difficult to predict under field conditions.

One solution which was suggested to avoid these problems was to divide data on the response of infection into daily probability of infection (DPI) values. Krause and Massie (1975) and Madden *et al.* (1978) developed a disease forecasting system using a climatic model to construct the table of critical periods containing four arbitrary selected categories of infection efficiency and denominated daily severity values (DSV). A computer program has been developed to automatically combine the mean of temperature values and wetness-period

values collected in the field from meteorological stations, and to calculate both daily probability of infection (DPI) values and the sum of the DPI (DDPI) values over a specified period. The DPI tables are different for each plant pathogen and host combination because there are different critical periods for each pathosystem (Madden *et al.,* 1978). The outcome of our experiment would generate basic information to develop an early-warning system for wheat blast and strategies of blast disease management under practical field conditions. Cardoso *et al*. (2008) provided some data for the validation of the wheat blast warning system. In field conditions, the lesions on leaves, auricles and ligules can provide sufficient spores to infect the panicle, which is the main organ, resulting in the loss of wheat production (Thomas, 1930; Ono & Suzuki, 1960; Hori, 1963). In addition, several climatic models for other diseases have been published and they also used the critical period concept (Zadoks & Schein, 1962) to predict infection, including apple scab (Mills & La Plante, 1951), potato blight (Wallin, 1962), Alternaria infection of tomato (Madden *et al.*, 1978), tomato septoriosis (Scaloppi, 1999), soybean rust (Reis *et al*., 2004) and wheat scab (Reis & Blum, 2004).

Sutton (1988) addressed the fact that fungal spores do not germinate synchronously for the response to temperature and moisture, so environmental factors such as temperature and the duration of the wetting period would affect the ability of individual spores to successfully complete infection along with the final intensity of fungal diseases. Castejón-Muñoz (2008) investigated the effects of temperature and relative humidity on the airborne concentration of *Pyricularia oryzae* spores and the development of rice blast in the field. They reported that a relative humidity of 95% and an average temperature of 26-27 ℃ were optimum for infection and substantially favored spore release, suggesting that the peak spore concentration in August could be used to forecast panicle blast. The incubation period for pathogens varies depending on the temperature, relative humidity and humectation period (Maheswari Amma & Raj, 1973). Castejón-Muñoz (2008) added that the crop growth stage and the airborne spore concentration will affect the development of the disease. Kato *et al.* (1970) noted that a rapid increase in the number of spores was recorded during the heading stage. The disease intensity may be more sensitive to the fluctuation of temperature in field conditions. A rise in temperature of 1 ℃ in early August (midtillering stage) may have caused an increase in mean leaf blast intensity in terms of the AUDPC (Castejón-Muñoz, 2008).

4.2 Differences in infectivity of *Magnaporthe* spp.

Plants and pathogens that are in constant competition during their co-evolution have undergone changes in relative resistance/susceptibility and virulence/avirulence, respectively

(Zellerhoff *et al.*, 2008). *Magnaporthe* spp. exhibited a broad range of virulence on wheat and rice supported by previous studies (Choi *et al.*, 2013). Controversial results are also revealed in scientific literature in the case of the infection ability of blast pathogens and cross-infection between wheat and rice.

It is believed that wheat isolates are capable of infecting rice but the reverse scenario has not been confirmed (Mehta, 2014). Igarashi *et al.* (1986) described positive pathogenicity of the wheat isolates towards rice. Furthermore, Odile *et al.* (2008) pointed out that the wheat isolate BR 32 could induce a hypersensitive response (necrotic flecks) with varying degrees of pathogen growth inside the invaded cell according to the rice genotype. However, other researchers hold a contrary opinion. Urashima (1993) verified that the eleven wheat isolates did not infect rice cultivars of the Japanese differential or Brazilian lowland and upland. Prabhu *et al.*(1992) reported that all isolates from rice were pathogenic to wheat cultivars and barley, but none of the ten wheat and seven grass isolates infected any of the thirty rice cultivars. Interestingly, some researchers claim that there is no cross-infection between wheat and rice. Tosa *et al.* (2004) discovered that two wheat isolates (Br 48 and Br 3) were avirulent on ten rice cultivars and the rice isolates (Ken 53-33 and 1836-3) also failed to infect wheat cultivars. Pratt (2012) found the blast isolates which infect rice do not infect wheat and vice-versa in Kentucky, thus rice producers who grow wheat in extreme southwest Kentucky and surrounding states are not at increased risk from the wheat blast fungus. Ou (1985) proposed that these contradictory results might come from different genetic backgrounds of the isolates and hosts or environmental conditions in the tests.

To elucidate the diversity of *Magnaporthe* spp. in our experiment, seventy isolates were obtained from different hosts and locations to provide more representative data of the actual diversity in South America. The hypersensitive response (HR) is a mechanism used by plants to prevent the spread of infection by pathogens. The HR is characterized by the rapid death of cells in the local region surrounding an infection and it serves to restrict the growth and spread of pathogens to other parts of the plant. Dead cells resulting from HR would form small spots on the leaf surfaces, but these spots would not expand because of the successful prevention from further infection. On the other hand, the successful infection should be able to spread from an initially infected cell to neighboring cells which causes the expansion of lesions. Normally the aerial conidiophores would start to produce conidia on the lesions under conditions of high humidity after 7 days of inoculation. The results from our pathogenicity test showed that 87.5% of wheat isolates successfully caused lesions on rice leaves with

different severity, some of these lesions were slightly expanded over time, but no spores were obtained from the lesion surface. The reason may be that conidiophores have not been formed at 6 dpi under our controlled conditions, and some wheat isolates induced HR on rice. Indeed, 63.3% of rice isolates also formed spots on wheat leaves with slight severity, but these lesions did not expand. We were unable to isolate any pathogens from these lesions when incubated in humid Petri dishes. These observations suggest that, in the present experiment, some strongly aggressive isolates from wheat had the ability to infect the susceptible rice cultivar CO 39 and some isolates resulted in HR on rice. The existence of different populations of *M. grisea* causing wheat blast in Brazil was first suggested by Urashima *et al.* (1993), one composed of isolates employed by Igarashi *et al.* (1986) which infected rice, and another one used by other scientists which were non pathogenic to rice (Prabhu *et al.* 1992; Urashima *et al.* 1993; Urashima *et al.* 2004b).

Most rice isolates were only able to trigger HR on wheat leaves to prevent the further spread of infection. Kato *et al.* (2000) have also tested pathogenicity on eighty-five *Pyricularia* isolates, which were collected from twenty-nine host species of Gramineae, Bambusideae and Zingiberaceae plants in Brazil, Uganda, Ivory Coast, India, Nepal, China, Indonesia and Japan. Based on the pathogenicity to eight differential gramineous plants, seven pathotypes were classified from these isolates: finger millet type, foxtail millet type, common millet type, rice type, crabgrass type, Italian ryegrass/ weeping lovegrass type, and non-cereal/grass type. In another study, seventy-two monoconidial isolates of *Magnaporthe grisea* obtained from the States of Mato Grosso do Sul and Paraná were inoculated on seedlings of twenty wheat (*Triticum aestivum*) cultivars in greenhouse conditions by Alfredo *et al.* (2004). In total, they identified fifty-four distinct virulence patterns on twenty test cultivars among the isolates collected from these two States which are the main wheat producing areas. Approximately 22.2% of these isolates (sixteen isolates) showed a similar virulence pattern. The virulence diversity of *M. grisea* has been attributed to mutation and parasexuality in rice (Ou, 1980; Zeigler *et al.*, 1997; Zeigler, 1998), as well as the contribution of sexual recombination (Kumar *et al.*, 1999; Mekwtanakarn *et al*, 2000). Moreover, Urashima *et al.* (1993) have characterized *M. grisea* from wheat as highly sexually fertile and hermaphroditic, sexually compatible with isolates from other hosts and both *MAT1-1* and *MAT1-2* mating types exist in a single field (Bruno & Urashima, 2001).

However, some wheat isolates of *M. grisea* collected from different locations presented similar pathogenicity in our experiment. Wheat isolates MG 8 from Bolivia and MG 29 from

Brazil presented the same high pathogenic levels on their host plant (100% of disease incidence). Rice isolates C9228-37 from Philippines, 492RWA11 from Rwanda and 564UGA11 from Uganda showed similar disease incidence, 40%, 45% and 45%, respectively. One of the possibilities for the presence of common pathogenicity in different geographical origins may be the movement of seeds which are infected with a specific isolate of *M. grisea* from one region to another. The outbreak of blast disease caused by seed contamination and transmission in new wheat-growing areas has been reported (Goulart & Paiva 1991; Urashima *et al.*, 1999).

In addition, pathogenicity on finger millet and perennial ryegrass has also been studied. Phylogenetic analysis showed that among twenty isolates from sixteen host species including perennial ryegrass, only the isolates from wheat (*Triticum aestivum*) and triticale (× *Triticosecale*), showed notable similarities with the perennial ryegrass isolates based on their *Pot2* fingerprints. The copy number and fingerprints of *Pot2* and repetitive DNA (MGR586) in isolates of *P. grisea* from perennial ryegrass are genetically distinct to the isolates derived from rice (*Oryza sativa*) in the United States (Farman, 2002). The perennial ryegrass isolates also had the same sequence in the internal transcribed spacer (ITS) region of the genes encoding ribosomal RNA as that of the wheat and triticale isolates, and exhibited rice isolate sequence polymorphisms (Harmon, 2003). Tosa *et al.* (2004) also studied the population of perennial ryegrass isolates on the ITS region. All of the perennial ryegrass isolates they collected were interfertile with Triticum isolates and clustered into the same group (CC group) on a dendrogram constructed from rDNA-internal transcribed spacer two sequences. DNA fingerprinting with *MGR586, MGR583,* and *Pot2* showed that perennial ryegrass isolates are divided into two distinct populations, TALF isolates and WK isolates. The TALF isolates were virulent only on *Lolium* species, whereas the WK isolates were less specific and were able to infect wheat and rice. In pathogenicity assays, Viji *et al.* (2001) reported that all the isolates of *P. grisea* from Legacy II perennial ryegrass caused characteristic blast symptoms on Marilee soft white winter wheat, Bennett hard red winter wheat, Era soft white spring wheat, and Presto triticale. An isolate from wheat and one from triticale (from Brazil) were also highly virulent on perennial ryegrass. None of the isolates from perennial ryegrass caused the disease on Lagrue rice, and vice versa. Kato *et al.* (2002) reported that the isolates from cereal crops were generally capable of mating with finger millet strains and constituted a closed mating compatibility group. The transmission of *M. grisea* isolates occurs in natural agroecosystems between finger millet and *Eleusine africana*, goosegrass or *Bambusa arundinacea*, and between rice and tall fescue, Italian ryegrass, sweet vernalgrass, reed

canarygrass or *Oryza longistaminata* with a combined analysis of the pathogenicity and genetic similarity. In our experiment, only two perennial ryegrass isolates from Japan were included, and they showed specific virulence on wheat, but not rice. However, due to the small number of isolates collected from perennial ryegrass, these characteristics of virulence and avirulence are not representative for all perennial ryegrass isolates.

Due to the lack of resistant wheat cultivars against *M. grisea*, farmers have been forced to rely on other control methods. In recent years, wheat blast has not caused yield loss in large areas as has happened in the past, but large yield losses have been observed in small areas in different parts of Brazil (Mehta *et al.,* 1992). One reason for the reduction in yield loss is delaying planting in epidemic areas until 10th of April. This strategy allows the heading stage to occur when the environment gets drier and is not favorable for the outbreak of blast disease. The other reason is the withdrawal of highly susceptible cultivars like Anahuac, Candeias and Ocepar7 (Urashima *et al.,* 2004). Fungicide spray is another widely used method in the field to control blast disease. Urashima and Kato (1994) reported that fungicides, probenazole and tricyclazol (spray) provided good protection at the vegetative stage but not at the head level. New products like blacin, code numbers SSF-126 ((E)-2-methoxyimino-N-methyl-2-(-2-phenoxylphenyl) acetamide), SSF-129 and 28S-28 72 seem to be promising with good protection of the head.

It has been noticed that there is varying severity resulting from different inoculation methods with the same amount of inoculum. Berruyer *et al.* (2006) used four inoculation methods (spray, mist, dip, and sheath) to evaluate the growth of blast pathogen on rice and demonstrated that the type of infection method has an effect of several orders of magnitude on the quantity of fungus growing in the plant at the earliest time points using the same inoculum. Spray inoculation is used routinely for studying the rice–*M. oryzae* interaction (Ou, 1985), which produces both susceptible and resistant interactions that closely resemble the situation in the field. However, Berruyer *et al.* (2006) verified that fungus could not be detected in spray-inoculated rice leaf tissues until 96 hpi, even using the repetitive *Pot2* sequence to maximize the potential for detection of fungus genes, which limits the possibilities for studying early infection stages. The results were consistent with our experiment; the detection by qPCR were performed at 0, 2, 4 and 6 dpi for the interaction of *Magnaporthe s*pp. and host plants. No increase in fungal biomass untill 2 dpi was detected, but differences between host- and nonhost- interactions were demonstrated at 4 dpi. As for the other two inoculation methods described by Berruyer *et al.* (2006), mist inoculation gave both larger infection ratios

and denser infection points resulting in rapidly coalescing symptoms. It caused the highest fungus/plant ratio on the rice leaf sheath assay during early stage. The reason for mist inoculation resulting in more infection sites is probably due to the higher density of smaller drops of inoculum deposited on the plant surface. Interestingly, for dip inoculation the inoculated detached leaves showed more natural infection than the inoculated detached sheaths.

The concentration of conidial suspension can affect the infection results. Berruyer *et al.* (2006) verified that normal fungus behavior was seen in the resistant interaction when using drop inoculation with conidial concentrations of 10^2-10^4 conidia/ml, but resistance was overcome and sporulation occurred when using an inoculum concentration of 10^5 conidia/ml. The ability to overcome the resistance seemed to be directly proportional to the inoculum concentration. To reduce errors arising from the concentration of inoculum, 1.0×10^5 conidia/ml was used in all of our experiments, as well as most inoculation experiments with *Magnaporthe* spp. (Tosa *et al.*, 2003; Berruyer *et al.*, 2005).

4.3 Molecular genetic differentiation of *Magnaporthe* spp.

DNA fingerprinting has been used to identify genotypes that are associated with various hosts in the genus *Magnaporthe*, such as *Magnaporthe grisea* repeat (MGR 586), grasshopper retroelement, and *Magnaporthe gypsy* element (Hamer *et al.*, 1989; Dobinson *et al.*, 1993; Farman *et al.*, 1996) and to characterize genetic variability within *Magnaporthe* spp. (Levy *et al.*, 1993; Chen *et al.*, 1995; Zeigler *et al.*, 1995). Two molecular typing methods in our studies were used to estimate phylogenetic relationships within seventy *Magnaporthe* strains. from wheat, rice, finger millet and perennial ryegrass. All isolates were genotyped by amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST). AFLP has been successfully used to analyze the population structure and genetic diversity in plant pathogenic fungi (Vos *et al.*, 1995; Brown, 1996; Reineke *et al.*, 1999; Tredway *et al.*, 2004). As compared with classical DNA-fingerprinting techniques, the AFLP method does not require prior knowledge of DNA sequences to detect variation over the entire genome and has proved to be robust and reliable because it uses stringent reaction conditions (Vos *et al.*, 1995; Savelkoul *et al.*, 1999). Recently, the concordance of multilocus DNA sequence typing (MLST) data has become popular among filamentous fungi (Taylor *et al.*, 2000; Taylor & Fisher, 2003). In *Magnaporthe* spp., Couch & Kohn (2002) performed phylogenetic analyses using actin, ß-tubulin and calmodulin gene sequences, and identified isolates from crabgrass as a distinct phylogenetic group from the other isolates from rice and other grasses.

Comparing the two molecular tools used in our phylogenetic analysis, AFLP is cheaper, faster, and easier to perform than MLST; but interlaboratory comparison by AFLP analysis will be difficult because complex banding patterns are PCR based and therefore prone to variation. MLST is more expensive, but it does result in solid DNA sequence data that are not subject to experimental variation.

Both molecular typing methods resulted in similar genetic clustering indicating that most of the isolates were associated with their host species. Schouls *et al.* (2002) supported that the clustering analysis yielded a similar grouping of isolates by either AFLP or MLST, and haplotypes of most isolates were associated with the host origins. The results indicated that these methods, both of which are based on characterization of multiple loci in the genome, are equally suited in disclosing genetic relationships between *Campylobacter jejuni* isolates (Schouls *et al.*, 2002). In our experiment of MLST, although isolates from wheat and rice were separated into different groups, they were still clustered together in each group. The association of each group with different hosts indicates ecological specialization. A multilocus gene genealogy analysis was reported by Brett & Linda (2002) in which forty-nine *Magnaporthe* isolates from five hosts were clustered and each group contained the isolates from different hosts. *Digitaria* species are ubiquitous weeds and often occur adjacent to rice cultivation, yet host association is maintained. They explained that this host association may result from ecological factors such as the life history or microhabitat occupied by the host. Alternatively, host association may be maintained by the inability to overcome resistance genes present in other grass hosts.

There are also many other molecular techniques that have been used to study the genetic relationship between blast isolates. Using random amplified polymorphic DNA (RAPD) analysis, Sharma *et al.* (2002) collected 250 isolates of *Magnaporthe grisea* from the north-western Himalayan region and these isolates were separated into twenty-five DNA fingerprint groups or lineages. Thirteen groups were exclusive to isolates obtained from Himachal Pradesh (HP), four from Uttaranchal and one from Jammu and Kashmir (J & K). The seven remaining groups were composed of isolates from different locations. Although RAPD analysis revealed high genetic variability among *M. grisea* populations from HP and J & K, genetic variation was low in the isolates collected from Uttaranchal. Bernardo *et al.* (1993) also used the RAPD method to fingerprint 120 Philippine isolates of the blast pathogen. The primer J-06 and MGR586 defined groups corresponding to each other, but this correspondence appeared to be population specific, and problems inherent with the RAPD

technique limited the reproducibility of the fingerprints in different laboratories. The internal transcribed spacer (ITS) regions of thirteen representative isolates were also amplified by PCR to construct a dendrogram for *Pyricularia* isolates. Isolates from agronomically important crops (rice, foxtail millet, common millet, finger millet, wheat and oats) and their relatives were classified into a single cluster, isolates from weeds (crabgrass and buffelgrass) were located outside the crop isolate cluster, and Japanese bamboo or bamboo grass isolates were farthest from the crop isolate cluster (Kusaba *et al.*, 1999). Kato *et al.*(2000) collected eighty-five *Pyricularia* isolates from 29 host species of Gramineae, Bambusideae and Zingiberaceae plants in Brazil, Uganda, Ivory Coast, India, Nepal, China, Indonesia and Japan. Genetic variation among these isolates was assessed by RFLP analysis with two restriction enzymes and nine single-copy DNA probes isolated from a finger millet strain. A UPGMA dendrogram based on RFLP fingerprinting with MGR 586 has revealed that the eighty-five isolates could be classified into seven major groups. Isolates from cereal crops (finger millet, foxtail millet, common millet, wheat and rice) and a grass, *Brachiaria plantaginea*, were clustered into a single group and further divided into six subgroups corresponding to the pathotypes. These results suggested that the isolates from cereal crops form a single group with a common ancestor although they are pathogenic to taxonomically diverse plants. However, the high investment in time and resources for RFLP analysis has seriously limited the application of DNA fingerprinting for large-scale studies of pathogen ecology and evolution. Adreit *et al.* (2007) developed 18 microsatellite markers for rice blast (*Magnaporthe grisea*) population studies. These 18 markers were used in multiplex PCR to characterize six populations from different geographical origins and a large range of polymorphism was found. The average number of alleles per locus across populations ranged from 1.2 to 7 and the total number of alleles detected from 2 to 19. DNA samples from *Magnaporthe grisea* isolates were fingerprinted by using repetitive element-based PCR (rep-PCR) with two outwardly directed primer sequences from *Pot2* by George *et al.* (1998). Because *Pot2* occurs at approximately 100 copies per haploid genome and is dispersed among the chromosomes, a number of intervening sequences were expected to be within amplifiable distance, generating sufficient polymorphism to detect genetic diversity. Cluster analysis from *Pot2* rep-PCR fingerprints distinguished rice- and non-rice infecting isolates; isolates from rice formed a large and diverse cluster, while isolates from other hosts were much more distantly related.

Ramanatha (2002) mentioned that distribution of genetic diversity in plant species depends on its evolution and breeding system, ecological and geographical factors and often on human

activities. Meanwhile, there is a traditional assumption that genotype selection from different geographical origins will maximize the diversity available to a breeding project, but it does not apply in all cases. In the present studies, geographical factors have not played an important role in the evolution of the pathogen *Magnaporthe*, since there is no association between genetic diversity and geographical origin. Isolates selected from the same location were separated into different groups which have been shown in our phylogenetic tree of AFLP. Wheat isolates MG 31 and MG 44 were both collected from Brazil and belong to different subgroups in group I of AFLP with a similarity of 74%, as well as those separated in MLST. The subgroup B in AFLP was composed of isolates from different locations: Brazil, Bolivia and Japan. Similarly, three wheat isolates - MG 9 (Bolivia), MG 31 (Brazil) and 1836-3/0-12 (Japan) - showed insignificant differences in genetic diversity after MLST. In AFLP, host species were associated with groups: all wheat isolates were included into group I and all rice isolates were classified into group II. This indicated that there is an obvious relationship among the hosts, but not between geographical origins. Many researchers have also studied pathogen population structure using AFLP. Tredway *et al.* (2004) used AFLP to determine the genetic structure of *Magnaporthe grisea* populations associated with tall fescue and St. Augustinegrass in Georgia; the results showed that five lineages were isolated from tall fescue, St. Augustinegrass comprised 99.8% of the population in lineage C and populations from crabgrass were dominated (98%) by lineage K. Hence, the conclusion was made that host species was the primary factor determining population structure according to analysis of molecular variance, and host cultivar and geographical region had no significant effect on diversity. In another study of a sesame germplasm collection, it was reported that there was no association between geographical region and AFLP patterns, suggesting that there was considerable gene flow among diversity centers (Laurentin & Karlovsky, 2006). The dendrogram derived from intron and exon sequences by another molecular tool (MLST) revealed that the isolates collected from wheat and rice are distributed throughout clusters in Neighbor-joining analysis. The genetic diversity assessed by Nei`s parameters supported the fact that the percentage of polymorphic loci in intron sequences was higher than that in exon sequences, suggesting that there is a higher mutation rate occurring in introns. Although isolates from wheat and rice were divided into three groups, respectively, the isolates from the same hosts were clustered together with 74%-99% bootstrap supporting. Those isolates from the same host in each group were derived from different locations. On the other hand, the diversity within groups, which differs depending on the geographical origin, was lower than that among four groups classified by their host plants, especially the G_{ST} values on intron

(0.41) and exon (0.53). Overall, the cluster and genetic diversity produced by MLST suggested that in three home-keeping genes, most of the variation is explained by genetic diversity between host species rather than geographical origin, which supported the results from AFLP. Geotge *et al.* (1998) reported that cluster analysis from *Pot2* rep-PCR fingerprints distinguished rice- and non-rice infecting isolates; isolates from rice formed a large and diverse cluster, while isolates from other hosts were much more distantly related. Among the non-rice infecting isolates, there was divergence in some isolates from the same host. Phylogenetic analyses of multilocus sequences and DNA fingerprinting tested by Choi *et al.* (2013) also demonstrated that the haplotypes of most *Magnaporthe grisea* isolates were associated with their hosts. Notably, Caicedo *et al.* (2000) had mentioned that heterogeneity values or gene diversity indices might overestimate the number of real loci, whereas the number of alleles per locus is underestimated. Thus, heterogeneity measures are only relative values and cannot be compared with values obtained from other molecular markers.

There is another interesting comparison between molecular clusters and pathogenicity. Urashima *et al.* (1993) suggested that there were two distinct populations of wheat blast in Brazil, one composed of isolates which infected rice and another one was non pathogenic to rice. Although the isolates derived from wheat in the present experiment were also divided into two subgroups by AFLP (Fig. 10), there was no correlation between subgroups and pathogenicity. Highly pathogenic strains of *Magnaporthe* spp. (Fig. 8) were widely distributed within groups / subgroups. Wheat isolates MG5 and MG 31, which were mainly tested in other experiments, were clustered into different groups or subgroups in MLST and AFLP. Similarly, differences in their ability of infection were also shown on pathogenicity and cytological reactions. However, those wheat isolates also exhibited high pathogenicity to rice, but did not cluster into the same group with MG 31. For example, 82.5% of the highly pathogenic wheat isolate MG 1.2 was grouped with MG 5 (27.5%) in AFLP and showed similarity with MG 5 in MLST, in contrast to the similarly highly pathogenic isolate MG 31 (86.3%). From another point of view, isolates which are in the same group (group B) as MG 31 in AFLP demonstrated significant differences in pathogenicity. For example, wheat isolates Br116.5/T-7, MG 28 and MG 48 exhibited their pathogenicity with 5%, 50% and 100%, respectively, to their host plants. The same situation also occurs in rice isolates: those isolates with similarly high pathogenicity showed low similarity level in AFLP. Rice isolates 531UGA11 and 507RWA11, which showed 55% and 62.5% of pathogenicity to their host, had a similarity of 79.5% in group II. This suggested that the differences among *Magnaporthe* isolates shown on molecular level are not correlated to their pathogenicity ability.

Isolates from finger millet and perennial ryegrass were clustered together with wheat isolates in both AFLP and MLST, which suggested that the phylogenetic relationships with those three host species are close, and the pathogen *Magnaporthe* could be shifted among them. It also suggests that in wheat field, the probability of infection by *Magnaporthe* will increase with the presence of these two plant species. In 1971, a severe epidemic occurred on annual ryegrass (*Lolium multiflorum* Lam.) pastures in Mississippi and Louisiana (Bain *et al.*, 1972; Carver *et al.*, 1972). In 1991, *M. grisea* was first reported as a pathogen of perennial ryegrass (*L. perenne* L.), when a localized epidemic occurred on golf course fairways in Pennsylvania (Landschoot & Hoyland, 1992). Since then, gray leaf spots on perennial ryegrass have been observed throughout the United States wherever this species is cultivated (Dernoeden, 1996; Schumann & Jackson, 1999; Uddin *et al.*, 1999; Vincelli, 1999). Tredway *et al.* (2004) supported the idea that isolates from perennial ryegrass were closely related to the isolates from wheat. Rice isolates differ from wheat isolates in containing a distinct set of transposable elements, but wheat and ryegrass isolates share the same set of transposable elements according to rDNA sequences and transposon-detected restriction fragment length polymorphisms (RFLPs) (Viji *et al*., 2001; Farman, 2002). The susceptibility of wheat to the *Eleusine* isolate was reported previously in Japan (Kato, 1978). In this study all wheat isolates caused serious damage in seven lines of finger millet from different countries and one line of *E. africana* from Uganda. The pathogenic pattern of the wheat isolates from various gramineous plants showed a similarity to the pattern of the isolates from *E. africana* (Kato & Yamaguchi, 1980). The mating behavior of wheat blast isolates collected from Brazil has been determined with fertile tester lines from finger millet by Urashima *et al.* (1993). Fourteen wheat isolates out of sixteen were classified as *MAT1*-1. Fertility was determined by crossing the wheat isolates with isolates of other gramineous plants. Mature ascospores can be produced when wheat isolates are crossed with isolates from *Eleusine coracana*. The compatibility with mating type and fertility indicated the similarity between wheat and finger millet isolates.

4.4 Quantitative real-time PCR detection

Commonly, the evaluation for fungal pathogenicity, host resistance or strain aggressiveness is based on a disease rating or measurement of lesion number and size or sporulation intensity (Yeh & Bonman, 1986). However, these methods only provide a visual estimation rather than an accurate measurement of fungal growth in plants. Various methods for detection and quantification of fungal pathogens in plant or soil have been developed. Fungal biomass has

been quantified by measuring levels of fungal constituents such as ergosterol or chitin (Pacovsky & Bethlenfalvay, 1982; Gretenkort & Ingram, 1993), or by immunological methods (Harrison *et al.,* 1990; Newton & Reglinski, 1993; Dewey *et al.,* 1997; Karpovich-Tate *et al.,* 1998), and also by the GUS (a bacterial ß -glucuronidase gene) - transformed strain (Couteaudier *et al.,* 1993; Liljeroth *et al.,* 1993; Thrane *et al.,* 1995; Bao *et al.,* 2000). However, these methods are more or less defective. In recent years, quantitative real-time PCR (qPCR) has been developed as a molecular tool to detect and identify fungal pathogens (Horevaj *et al.,* 2011; Farman *et al.,* 1996; George *et al.,* 1998). This technique contains thermal cycling with fluorescence detection for PCR amplification and records the specific cycle number. The term of cycle threshold (Ct value) has been regarded to be the most reliable parameter to detect and quantify target DNAs with qPCR (Heid *et al.,* 1996). On the other hand, specific primers can be designed to distinguish between closely related fungal species and offers higher sensitivity and specificity than many other methods (Henson & French, 1993). qPCR has had numerous applications in viral, bacterial, and fungal plant pathogen research thanks to its characteristics of fast, specific, reproducible and sensitive results (Schaad *et al.,* 1999; Böhm *et al.,* 1999; Frederick *et al.,* 2000; Bate *et al.,* 2001; Harmon *et al.,* 2003; Horevaj *et al.,* 2011). qPCR is primarily used to quantify the pathogen in planta, generally for a diagnostic purpose, as it accurately measures the relative growth and absolute biomass of pathogens (Schaad *et al.,* 2003; Berruyer *et al.,* 2005). It was also applied to detect genetically modified organism contamination in food and gene expression analysis (Gachon *et al.,* 2004). Mason *et al.* (2002) has quantified insert number in transgenic plants by qPCR. Berruyer *et al.* (2005) remarked that such a biological quantification method does not work for fungi that grow as cohesive mycelial hyphae, because it is hard to separate them into single, viable cells from plant tissue.

The interactions between pathogens and their non-host plants showed significant differences with the compatible interactions (Fig. 17). In incompatible interactions, the qPCR reaction still detected small amounts of fungal DNA from infected plant leaves. This may be due to spores which are left on the leaf surfaces after inoculation, or the mycelium which have grown into the plant cells, considering that the HR lesions were found on leaves. The fact that the biomass of isolate MG 31 on rice leaves was still higher than others may indicate that there was a development of wheat isolate MG 31 on the rice leaves. This result suggests that there are differences among the various isolates of *Magnaporthe* spp. with both the infection capacity and the speed of development.

The amount of fungal growth in plants may not always be proportional to the development of disease symptoms elicited by different blast isolates (Qi & Yang, 2002). Therefore, both quantification of the fungal growth and phenotypic assessment of disease symptoms are needed to more precisely evaluate virulence or aggressiveness of different blast isolates or mutants. The results of three isolates MG 5, MG 31 and Ca 89 from qPCR detection in our experiment are related to cytology observations and pathogenicity tests. Insignificant differences of fungal biomass from MG 5, MG 31 and Ca 89 on their host plants were detected at 2 dpi in qPCR, and 26%, 29% and 27% of hyphae from those three isolates started to grow in the initially invaded cells on the host plants at the same time point for cytology observations. This indicated that the early growth of primary hyphae from different isolates is similar. At 6 dpi, the fungal biomass of isolates MG 5, MG 31 and Ca 89 was 174.26, 199.36 and 132.79 pg/mg, respectively, from qPCR detection. 63.8%, 86.3% and 46.3% of disease incidence from pathogenicity tests were correspondingly recorded for those isolates. The corresponding differences reflected in the different host species illustrates that detection results of fungal biomass from qPCR in the early stage of infection could indirectly reflect the disease severity of *Magnaporthe* infection in the late stage. Therefore, this qPCR detection method can be used as an effective and efficient tool for reliable assessment of fungal pathogenicity and host resistance, and for monitoring disease progression.

In this research, primers used in qPCR amplification were the regions of the *Pot2* transposon (EMBL Accession No. Z33638). *Pot2* is a putative transposable element from *Magnaporthe grisea*. The element is 1857 bp in size, has 43 bp perfect terminal inverted repeats (TIRs) and 16 bp direct repeats within the TIRs. A large open reading frame, potentially coding for a transposase-like protein, was identified by Kachroo *et al.* (1994). This putative protein coding region shares general structural features with a group of transposons, including the transposable element *Fot1* from *Fusarium oxysporum* (Migheli *et al.,* 1999), *Tc1* from *Caenorhabditis elegans* (Harris *et al.*, 1988), *Tcb1* from *Caenorhabditis briggsae* (Brownlie *et al.*, 2005), *Uhu* from *Drosophila heteroneura* (Brezinsky *et al.,* 1992) and *Mariner* from *Drosophila* (Lidholm *et al.,* 1993). The fact that *Pot2* is present in both rice- and nonrice-infecting isolates of *M. grisea* at equal copy numbers broadens its utility relative to other host-specific repetitive elements (George *et al.,* 1998). *Pot2* has been reported in many studies and it is believed to be only present in the isolates of *Magnaporthe* spp. from various hosts (Farman *et al.,* 1996; George *et al.,* 1998; Harmon *et al.,* 2003). It represents one of the major repetitve DNAs shared by both rice and non-rice pathogens of *Magnaporthe*. Kachroo *et al.* (1994) demonstrated that the *Pot2* transposon has been found in all of 50 perennial ryegrass

isolates of *M. oryzae* and is more specific than other potentially useful sequences. *Pot2* is believed to have originated in an ancestor of these fungi, before the evolution of host specificity, and was estimated to be present in 100 copies in the *M. oryzae* genome (Kachroo *et al.,* 1994). Harmon *et al.* (2003) reported that the limit of detection of purified DNA was found to be between 5 and 50 pg. The ability to detect a relatively small amount of DNA enhances the efficiency of the procedure and increases the likelihood of successful detection. Interestingly, pathogen detection for naturally infected turfgrass samples from golf course fairways was implemented. There was no pathogen to be detected from symptomatic perennial ryegrass leaf blades that were dried and stored at 23℃ for an extended period of more than 3 years. This suggests that symptomatic leaf blades should be processed fresh without drying or storing the plant material for long periods of time (Harmon *et al.,* 2003).

4.5 Cytological investigations of *Magnaporthe* infections on host and nonhost plants

Cytological studies on the process of blast infection or the responses from plants have been mainly focused on rice plants (Arase *et al*., 1983; Koga & Kobayashi, 1982; Tomita & Yamanaka, 1983; Peng & Shishiyama, 1988 and 1989; Koga, 1994). The responses of wheat leaves to the blast fungus were examined in this investigation, and the similarities or differences between wheat and rice plants which respond to *Magnaporthe* infection were discovered. At the beginning stages (germination), all the conidia showed similar ability to germinate and form appressoria on host or nonhost plants, wheat or rice. The differences between host and nonhost interactions were observed at 24 hpi: more appressoria successfully penetrated the cell wall and attempted to penetrate into the cell on host interactions which occurred similarly on both wheat and rice plants, but most of the appressoria were arrested by papilla formation at this time. The differences between host and nonhost interactions increased over time. Some hyphae infested the entire initial invaded cells at 48 hpi on host interactions, while only a few hyphae broke through the control from the papilla and penetrated into the cells. The different responses to *Magnaporthe* infection between wheat and rice started to appear at this time. For the host interaction, the hyphae grew slightly faster in wheat leaves at 48 hpi. The percentage of successful infection in epidermal cells of host plants for wheat isolate MG 31 and rice isolate Ca 89 were 47.7% and 43%, respectively. Meanwhile, the difference in nonhost interactions was more obvious. The percentages for successful infections in nonhost plants for MG 31 and Ca 89 were 13.2% and 1%, respectively. At 72 hpi, clearer differences occurred in both host and nonhost interactions between wheat and rice. In comparison to the percentage of successful infections in epidermal

cells, approximately 62%, 70% and 44% of wheat isolates MG 5 and MG 31 and rice isolate Ca 89 were recorded for their host interactions. Similarly, 11%, 14% and 5% of isolates MG5, MG 31 and Ca 89 were found in their nonhost plants. These results suggest that the largest differences happened with host and nonhost interactions or host-specific pathotypes, followed by the resistant response of plant species. Interestingly, the responses of wheat and rice tissues to the infection of *Magnaporthe* at the microscopic level were correlated with those at the macroscopic level in out experiment. More infection hyphae of the three isolates were observed under microscope at 72 hpi in the host interactions than were observed in nonhost interactions. A higher incidence and severity were correspondingly discovered in the pathogenicity test for host interactions at 6 dpi. For the response of different plant species, about 70% and 44% of infection in the host plant was noticed for wheat isolate MG 31 and rice isolate Ca 89 in cytology observations. Correspondingly, 86.3% and 46.3% of disease incidence in pathogenicity was recorded for MG 31 and Ca 89, respectively. Comparing these results reveal that there is a positive correlation between cytology investigation and pathogenicity.

Blast disease, caused by the fungal pathogen *Magnaporthe*, is one of the most destructive diseases occurring in crop cultivation. Breeding for resistance against blast has not achieved a lasting success because resistance of newly developed cultivars was quickly overcome by a shift in the pathogen population to new virulent races (Hamer *et al.,* 1993). Plants in natural and agricultural ecosystems are exposed to bacteria, fungi, viruses and insects which have the potential to cause diseases. However, a given plant species is not a general host for all plant pathogenic organisms. The phenomenon that all cultivars of a plant are resistant to infection by all genotypes within a pathogen species is referred to as nonhost resistance (Heath, 1991; Nürnberger & Lipka, 2005). Nonhost disease resistance is one of the common forms for defense response. Because of its durability, nonhost resistance is regarded as a robust protection against the majority of potentially pathogenic microorganisms. In both monocot and dicot species, it was proposed that nonhost resistance should be classified in two types. The first is constitutive or passive and applies when the pathogen lacks the necessary pathogenicity factors, without producing any visible symptoms. The second type of nonhost resistance has been described as inaccessibility, for example, when a preformed antimicrobial substance cannot be overcome by the pathogen or when inducible, active defenses keep the pathogen in check. It is always associated with brown spots at the leaf surface because of a rapid and localized hypersensitive response (HR) with cell death (Holub and Cooper 2004; Mysore & Ryu, 2004). Thus, nonhost resistance could be viewed not only as a host property

but also as an inadequacy in the pathogen in terms of limited host specificity (Heath 2001). There is much research about nonhost disease resistance (Koga & Kobayashi, 1982; Arase *et al.*, 1983; Koga, 1994). A nonhost resistance in barley was reported by Zellerhoff *et al.*(2006), the nonhost type of resistance for blast disease was detected in barley against isolates derived from genera *Pennisetum* (fontaingrass) or *Digitaria* (crabgrass). Rice-*Magnaporthe* interactions have been studied extensively and have become a reference system to studying plant-pathogen interactions at the molecular level (Caracuel-Rios & Talbot, 2007; Ebbole, 2007; Xu *et al.*, 2007). The nonhost resistance observed in our cytological investigations could correspond to the two types of resistance described above. Approximately 87% of appressoria from rice isolate Ca 89 failed to penetrate the wheat cell walls at 48 hpi and no visible symptoms were observed on wheat leaves after 6 dpi. This corresponds to the first nonhost resistance type that the pathogen lacks the pathogenicity factors. Conversely, HR reactions with autofluorescence which refer to the second type of nonhost resistance were recorded on rice epidermal cells, and were triggered by 21.2% and 27% of primary infection hyphae from wheat isolates (MG 5 and MG31, respectively) with the presence of small necrotic spots on rice at 6 dpi. Heath *et al.* (1992) proposed a reason that the failed penetration of a non-adapted *M. oryzae* isolate on goosegrass was associated with the deposition of a silica-based granular deposition beneath the appressoria. There is only limited research available on the comparison of nonhost resistance on rice and wheat challenged with *Magnaporthe* spp. isolated from different species. The characterization of this nonhost pathosystem and comparison between compatible or incompatible interactions of rice and wheat with isolates derived from wheat and rice was conducted in our experiment at the cellular level. Host interactions on wheat and rice were successfully achieved and typical symptoms appeared on the leaves of the host plant. However, the responses of nonhost interactions between rice (CO 39) - wheat isolate MG 31 and wheat (BR 18) - rice isolate Ca 89 were presented in two types as described previously. Jackson and Taylor (1996) revealed that during the course of infection, these pathogens engage in many sophisticated but poorly understood activities that redirect nutrient flow in plant tissues and alter the growth and morphology of the plant. Changes in the morphology of the pathogen are also evident during pathogenesis, and these developmental modifications offer great future potential for molecular genetic analyses, biochemical studies, and cell biological investigations of infection.

Defense of plants against infection is based on diverse strategies, thus plants have evolved mechanisms to perceive pathogen attacks using both preformed defenses (e.g. antimicrobial compounds) and inducible defense responses (Odile *et al.*, 2008). Gene-for-gene resistance is

considered to be the best characterized form of resistance. The defense responses mediated by gene-for-gene interactions include rapid localized cell death, known as the hypersensitive response (HR; Glazebrook, 2001; Rate & Greenberg, 2001), the production of phytoalexins (Modolo *et al.*, 2002) and other antimicrobial secondary metabolites, and the expression of pathogenesis-related (PR) proteins (Narasimhan *et al.*, 2001). The first *M. grisea* gene was detected expressed during the infection and identified using Northern hybridizations, was an ubiquitin-encoding gene (*UEP3*) (McCafferty & Talbot, 1998), and gene *MPG1* encoding a class I hydrophobin, which is one of the most expressed *M. grisea* genes (Talbot *et al.*, 1993), it is strongly expressed during the colonization of the plant by the fungus. Since then, many approaches such as expressed sequence tag (EST) or SAGE analysis have been implemented and discovered many novel *M. grisea* genes expressed during a susceptible infection from infected rice leaves (Kim *et al.*, 2001; Rauyaree *et al*., 2001; Matsumura *et al.*, 2003). Most of these genes encode proteins involved in protein synthesis, primary metabolism or cytoskeleton proteins, suggesting that the fungus is actively growing in the infected tissues. Ribot *et al.* (2007) have developed a genome-wide transcriptomic study of a susceptible infection using Agilent microarrays representing the 13,666 genes of the *M. grisea* genome. RNA from infected and noninfected rice leaves were hybridized on Agilent chips, and statistical analysis showed that 1851 fungal genes are significantly expressed in infected leaves in comparison to non-infected leaves. Among these 1851 genes, 34% have unknown functions, and 50% are involved in DNA, RNA, amino acid and protein synthesis, and energy production. These genes reflect the fact that the fungus is actively growing in infected tissues, requiring a significant biosynthesis of novel DNA, proteins and primary metabolites. Kiyosawa (1976) identified 13 rice genes conditioning the resistance to *Magnaporthe oryzae*. Urashima *et al.* (2004) demonstrated that the isolates of *M. grisea* with the ability to infect all 20 wheat cultivars could overcome a great number of resistant genes, because each of the test cultivars possessed different resistance genes against the wheat blast fungus. The results from investigations by Urashima & Kato (1994) and from the rice blast study by Correa-Victoria & Zeigler, 1993 revealed that the *Magnaporthe* isolates which were able to overcome many resistant genes, are present over an extensive geographical area, retained over a large period of time, and in other pathossystems. It is important to identify resistant genes with the help of markers. Once a gene is tagged with a molecular marker, it can be transferred selectively into different genetic backgrounds by marker assisted selection. Ten random amplified polymorphic DNAs (RAPD) and two sequence characterized amplified region (SCAR) primers were used to identify blast resistant genes by Kumar *et al.* (2010). Markers OPA-05,

OPF-06, OPF-09, OPF-17, OPG-17, OPG-18, OPG-19, OPH-18, OPK-12, P-265-550 and P-286-350 found linked to blast resistance in most of the resistant genotypes could be considered as potential molecular markers in the selection of blast resistant genotypes. Amplification with RAPD and SCAR primers revealed a nonallelic relationship among resistant genotypes and thus, there is a good possibility of obtaining enhanced resistance through gene pyramiding.

Some studies suggested that the plant species specificity of *Magnaporthe* is governed by only a few genes (one or two) and these genes differ according to plant species or the type of cultivar attacked. This variation could result from the variation of resistance genes carried by the plants and cultivars. Avirulence genes from pathogens can be recognized by specific genotypes (cultivars) of the host species that contain corresponding resistance genes. Ebbole (2007) had mentioned that the genome sequence of *M. oryzae* 70-15 contains three members of the *pwl* family. The *pwl* genes do not encode avirulence factors toward any known rice cultivar, although functional copies of *pwl* are recognized by weeping lovegrass (*Eragrostis curvula*). Thus, the *pwl* genes appear to act as avirulence factors at the host species level, rather than at the cultivar level. Tosa *et al.* (2006) concluded that five loci (*Pwt1, Pwt2, Pwt3, Pwt4* and *Pwt5*) were involved in the specific pathogenicity of *Setaria, Triticum, Avena* and *Oryza* isolates on wheat. The *Oryza* and *Setaria* isolates were shown to share two avirulence genes, *PWT1* and *PWT2*, while the oat isolate carries other avirulence genes. The wheat strain carries none of the five avirulence genes. Conversely, Murakami *et al.* (2000) have shown that specific pathogenicity of *Setaria* and *Triticum* isolates on wheat was controlled by two genes (*PWT1* and *PWT2*) located at different loci and no PCR products were amplified from those isolates with primers designed based on conserved DNA sequences of *PWL* genes. It is suggested that these genes had weak or no homology with the *PWL* genes family isolated from *Eleusine* and *Oryza* isolates that prevent the blast fungus from infecting weeping lovegrass (Kang *et al.*, 1995; Sweigard *et al.*, 1995). The primary role of *Pwt1* assumed by Murakami *et al.* (2000) was that *Pwt1* is the determination of pathogenicity and that the lesion color is merely a superficial result of this determination. At a lower temperature the hypersensitive reaction controlled by the *Pwt1* locus can stop the fungal penetration efficiently and produce brown pinpoint or small lesions without the help by *Pwt2s*. At a higher temperature the hypersensitive reaction mediated by the *Pwt1* locus cannot stop it efficiently under the absence of *Pwt2s*, so it is induced continuously and extensively, resulting in the browning and shriveling of the whole leaf. Host species specificity of *Magnaporthe*

toward foxtail millet was also studied (Murakami *et al.,* 2003) and revealed the involvement of two genes only (*PFM1* and *PFM2*).

Inducible defenses can be activated upon recognition of elicitors, which derived from subpopulations of a pathogen species and evoked defense reactions in a cultivar-specific way (Odile *et al.,* 2008). Several PR proteins such as chitinases and ß-1, 3-glucanases have well-recognized antifungal activity on account of their hydrolytic action. For example, chitin is a major component of fungal cell walls and causes rice blast disease from *Magnapothe oryzae*. Plants produce chitinases upon pathogen attack and chito-oligomers (Oligosaccharide elicitor) induce defense responses in plants. The chitinases could release fragments of the cell wall of the fungal pathogen as a result of hydrolysis of cell wall components. These fragments may act as elicitors of active plant defense reactions. Chitin and its fragments, *N*-acetylchitooligosaccharides, are representative microbe-associated molecular patterns of fungi that trigger various defense responses in plants at subnanomolar levels (Shibuya & Minami 2001). They can degrade the pathogen cell wall or disrupt its deposition and thus block the infection process. Thus, pathogenic fungi are very likely to have mechanisms to avoid generating the chitin elicitor during the infection process. Mochizuki *et al.*(2011) have reported that invasive hyphae in the first-invaded rice cell can be stained with fluorescently labeled wheat germ agglutinin (WGA), a probe to detect chitin. However, in the second-invaded cell, hyphae showed strong fluorescence, as cell-wall chitin is hydrolyzed by the plant chitinase. Recent studies have revealed that cell wall chitin is physically masked by other cell wall components. Fujikawa *et al.* (2009) showed that α-1,3-glucan masks both chitin and ß-1,3-glucan in the cell walls of *M. oryzae* at 24 hpi. They also demonstrated that the presence of α-1,3-glucan results in increased tolerance to chitinase digestion. Thus, masking chitin by α-1,3-glucan is a major strategy of *M. oryzae* to evade attack by chitinase. The PR-1 proteins are often used as markers of the enhanced defensive state induced by the pathogen, but their biological activity has not yet been clearly elucidated. Peroxidases could act in cell wall reinforcement by catalysing lignification, enhancing resistance against multiple pathogens. PBZ1 is induced by probenazole (3-allyloxy-1, 2- benzisothiazole-1, 1-dioxide), an effective inducer of plant defenses against rice blast disease. This has an important role in disease resistance in rice (Komatsu *et al.,* 2004). Plants are able to recognize microbial invaders through specific surface determinants, collectively called pathogen-associated molecular patterns (PAMPs) and to react through defense signaling cascades (Jones & Dangl, 2006). Defense-gene analysis performed by (Odile *et al.,* 2008) suggested that PR-type and similar proteins are part of an immune surveillance mechanism that protects

the plant primarily against invasion by microorganisms that are generally perceived as nonhost. They speculated that the strategies deployed by a nonhost fungus to attempt penetration of the epidermal cells are less efficient than those of a virulent host pathogen and thus are rapidly counteracted by the defense of the attacked plant. The encoded proteins could then hydrolyse the fungal cell wall before rupture of the leaf cuticle by the turgor pressure developed in the appressorium, which occurs at 24–30 h (Kankanala *et al.*, 2007).

For the microscopic observation, a double staining method with WGA-AlexaFluor488 and PI was used on wheat and rice samples under CLSM. As previously described, the fungal structures and plant tissues can be stained into different colors with WGA-AlexaFluor488 and PI which is more distinguishable for observation. There are many other staining methods for fungal structures that were performed in the interaction of *Magnaporthe* isolates with plants. Tufan *et al.* (2009) stained fungal structures with Uvitex-2B, and plant autofluorescing cellular structures were differentiated using a Uvitex-2B specific filter and an autofluorescence-specific filter, respectively (fungal structures showed green and autofluorescence showed red). The staining method with lactophenol cotton blue solution was also tested (Fig. A2) in autofluorescence microscopy. The staining results revealed that only conidia, germination tubes and appressoria were able to be stained blue and not hyphae in the epidermal cells which is not helpful for the observation of fungal growth within cells. Normally, the cotton blue staining is just used for the observation on conidia germination and appressorium formation (Gupta & Chattoo, 2007; Ghatak *et al.*, 2013). Zellerhoff *et al.* (2008) used trypan blue to stain harvested barley leaves and the accumulation of autofluorescent material was observed using epifluorescence microscopy, but both fungal structures and plant tissues were stained blue which is not quite distinguishable. Ramonell *et al.* (2005) pointed out that trypan blue is good to stain the fungal hyphae and conidiophores on the leaf surface, and has the same staining results as cotton blue.

Some researchers also performed studies investigating penetration into the mesophyll by *Magnaporthe* spp.. Hyphal growth was almost invariably coupled with autofluorescence and cytoplasmic granulation of the first invaded epidermal cell. This cytoplasmic granulation in the first invaded epidermal cell has been associated with race-specific resistance to adapted *Magnaporthe* isolates in rice (Koga & Kobayashi, 1982; Tomita & Yamanaka, 1983). Nonadapted isolates are rarely observed escaping from the first invaded cell, whereas hyphae from the adapted isolates would move on to infect multiple cells, resulting in collapse and autofluorescence of the adjacent mesophyll cells. Jarosch *et al.* (2003) confirmed that attacked

mesophyll cells collapsed and underwent cell death that was thought to allow development of the facultative biotroph fungus *M. grisea*. Autofluorescence was observed in both compatible and incompatible interactions in our experiment. On the one hand, HR of epidermal cells can accumulate fluorescence materials in both interactions, but on the other hand, the collapsed mesophyll cells could also display autofluorescence in compatible interactions. Heath *et al.* (1990b) supported that, in highly compatible interactions of weeping lovegrass (*Eragrostis*) or rice with *M. oryzae*, fungal progression into the mesophyll is accompanied by cell collapse and autofluorescence. In contrast, autofluorescent mesophyll cells of the incompatible interaction reported kept their regular shape and were not invaded by the pathogen. Quantitative real-time PCR analysis further confirmed that, in the nonhost interaction, fungal colonization was halted at very low levels of fungal biomass (Fig. 17), indicating the effectiveness of cellular defense reactions.

There is another view about secondary infectious hyphae. While primary infection hyphae extensively invade the first infected epidermal cell, they seem to search the plant cell wall for plasmodesmata and breach it to reach the neighboring cells, forming secondary infectious hyphae. Interestingly, Ribot *et al.* (2008) mentioned that this process takes place independently of the amount of primary infection hyphae present in the first invaded cell and is rather time dependent. In addition, there are several advantages on microscope observation to use rice leaf sheaths. The rice leaf sheaths can be trimmed easily to exclude lower plant cell layers not yet invaded by the fungus. Meanwhile, the infected sheath tissue is optically clear, allowing detailed visualization of the fungus–plant interactions before attempting further studies on the sample. Nevertheless, the intact leaf sheath assay diverged slightly from nature in the later stages of infection, especially at higher inoculum concentrations. Koga *et al.* (2004b) made morphological comparisons of fungal growth between excised and intact leaf sheaths for the selection of samples. For technical reasons, most of these cytological studies were performed on rice leaf sheaths or onion/barley epidermal layers. As these tissues are very susceptible to blast, some of these observations may reflect excessive susceptibility and lead to somewhat artificial effects as the plant defense response is highly reduced. Berruyer *et al.* (2006) also reported similar observations that the difference between excised and intact leaf sheath was seen 48 h after inoculation, suggesting that only the early infection process mimics what occurs in the field.

4.6 Role of fungal phytotoxins

Plant pathogenic fungi produce a range of toxic compounds (phytotoxins), usually secondary metabolites, that affect the physiological function of plants and often induce lesion formation in plant leaves (Stone *et al.*, 2000). Phytotoxins often cause wilting, chlorosis and necrosis, but the importance and actual role in disease establishment is variable and intensly disputed for some diseases (Isaac, 1998). There are also other factors such as hormones, enzymes, genetic determinants and compounds liberated by the disruption of plant tissues. These toxins appear to target critical biochemical pathways, and their action can have pleiotropic effects on plant metabolism (Jackson & Taylor, 1996). Phytotoxins are low molecular weight secondary metabolites which are capable of disturbing the vital activity of plant cells or causing their death at concentrations below 10mM. Toxins are formed by diverse organisms (bacteria, plants and even phytophagous insects). Nevertheless, fungi are well known as phytotoxin producers, particularly those which are phytopathogenic. They are a very diverse group of molecules, including polypeptides, glycoproteins, phenolics, terpenoids, sterols and quinones (Isaac, 1998). Certain lipids regulate a wide range of important cellular processes in plants, including the regulation of ROS production (Sang *et al.*, 2001) and the activation of defense gene expression (Farmer & Ryan, 1992). Nishimura and Scheffer (1965) first reported that a host-specific toxin, HV-toxin, was released from germinating spores of *Helminthosporium victoriae*. Xiao *et al.* (1991) later emphasized the importance of 'toxin release' during spore germination for infection. Fujita *et al.* (1994) confirmed that susceptibility-inducing activity incubated in spore germination fluids of *P. oryzae* increased concomitantly with the incubation time. Results indicated that the toxins were released as soon as spores germinated. In addition, Isaac (1998) mentioned that some fungi would liberate toxins in the early stage when they just encounter the host plants as an aid to penetration and establishment, while others are produced much later in the infection process. This may enhance the senescence of the plant, leading to its more rapid demise. Phytotoxin formation is sensitive to a number of diverse factors, e.g. the composition of the medium, its acidity and the duration and conditions of culturing. Most of them are not identified in advance as being able to affect the process. Microorganism strains are genetically unstable and their storage or reinoculation may adversely affect the ability to produce toxins (Berestetskiy, 2008).

The effect of phytotoxins on plants is characterized by the appearance of specific symptoms; wilting and general growth suppression, as well as chloroses, necroses and spotting of aerial portions are the most common (Berestetskiy, 2008). Arase *et al.* (1990a) reported that the

spore germination fluids of *Pyricularia oryzae* induced infection by non-pathogenic *Altemaria altemata* (Fr.) Keissler of the leaves of rice cv. Sekiguchi-asahi which is a mutant of cv. Asahi, and caused a characteristic necrosis on the leaves of cv. Sekiguchi-asahi. This necrosis seems to be the same as that caused by inoculation with the fungus (Arase *et al.* 1990b). Heath (1981) suggested that specific compatibility between *P. oryzae* and host species seems to be determined through two steps. The first is to establish compatibility at the species level (basic compatibility) and the second at race-cultivar level. Susceptibility or resistance of rice plants to *P. oryzae* is determined by the race-cultivar interaction. Fujita *et al.* (1994) confirmed that the toxins produced by *Pyricularia oryzae* during spore germination induced susceptibility to infection by non-pathogenic *Altemaria alternata* of rice leaves. The induced susceptibility was independent of the compatibility between the races of blast fungi used for obtaining the toxins and the rice cultivars used for bioassay. Nishiuchi *et al.* (2006) have reported that *Fusarium* phytotoxins have elicitor-like activity in Arabidopsis, causing the induction of defensive genes, the accumulation of SA and ROS, and lesion formation. However, the production of phytotoxins does not appear to be the sole factor determining virulence in plants, and other virulence factors are probably involved in successful colonization. Park *et al.* (2009) mentioned that the severity of symptoms caused by culture filtrate treatment did not always correlate with the degree of disease severity in certain ecotypes of Arabidopsis and suggested that several mechanisms operate during pathogenesis in Arabidopsis.

Magnaporthe spp. displays a considerable capacity for the production of phytotoxins. Susceptibility-inducing activity of the toxin(s) was recognized in all isolates tested, regardless of compatibility between fungal races and rice cultivars. The toxin(s) also induced susceptibility in other host plants of *P. oryzae* such as barley, Italian ryegrass, perennial ryegrass and wheat plants, but not in non-host species. Thus, the specificity of susceptibility-inducing activity of this toxin(s) corresponded with the host range of *P. oryzae*. The results indicate that the toxin(s) were host-selective disease determinants to establish a basic compatibility (Fujita *et al.*, 1994).

The phytotoxins produced by *Magnaporthe* spp. are a series of salicylaldehyde-type phytotoxins including pyriculol, pyricuol and pyriculariol, and some of these have known structures. So far, however, only very limited information is available concerning the role of specific compounds in disease (Arase *et al.*, 1998; Ebbole, 2007). One of the best characterized metabolites is tenuazonic acid, a photosystem II inhibitor. Strains producing low

levels of tenuazonic acid have smaller lesions than those with high levels of the phytotoxin (Lebrum, 1990). Arase *et al.* (1998) demonstrated that the toxin of the rice blast fungus inhibited root growth in rice seedlings cultivar-nonspecifically and induced necrosis formation on the leaves of rice cv. Sekiguchi-asahi. The induction of susceptibility by the toxin was also observed on other host plants, but not on nonhost plants. It was therefore suggested that the toxin is not playing an important role in specificity at the race-cultivar level, but it is host-selective and determines the host specificity at plant species level.

Park *et al.* (2009) demonstrated that in response to treatment with a crude culture filtrate of *M. oryzae*, lesion formation and induction of PR gene expression were observed in Arabidopsis but not in rice, suggesting that *M. oryzae* employs virulence factors (i.e. phytotoxins) unique to infecting Arabidopsis. *Fusarium* phytotoxins have elicitor-like activity in Arabidopsis and cause the induction of defensive genes. However, the *M. oryzae* mutants lack the involvement in appressorium formation. Park *et al.* (2009) reported that a culture filtrate (CF) of *M. oryzae* KJ201 caused lesion formation in Arabidopsis leaves within 48 h of treatment and the severity of the lesions was correlated with the filtrate concentration. In contrast, the CF failed to produce any visible symptoms on the leaves of rice cultivar Nakdongbyeo at 1 dpi; and faint HR-like lesions were visible around the inoculation sites at 3 dpi. The application of CF induced the expression of three PR genes in Arabidopsis within 48 h of treatment even without visible symptoms, but failed to do so in rice at the same concentration. Our results showed that the culture filtrates from *Magnaporthe* spp. did not cause any lesions by phytotoxins on wheat and rice leaves at 3 dpi. A possible reason for this could be that the concentration of phytotoxin production from *Magnaporthe* spp. is too low to affect the host plants. Furthermore, the factors mentioned above and the extraction methods may influence the toxin formation, resulting in a low production. Conversely, it may be possible that the filtrates produced by those three isolates used in this study are not effective on those two plants.

In our preliminary study, the phytotoxins from germinating conidia of three *Magnaporthe* isolates in the liquid media PDB and from infected plant tissues were simply extracted by double filtration using three layers of gauze and a 0.20 μm Millipore filter to remove conidia and hyphae, and diluted with distilled water. However, a lot of research on the selection for host resistance to pathogen toxins was carried out using purified toxin or culture filtrate for the high content of the target compounds (Daub, 1986; Zhane *et al.*, 2011). The following are the most commonly used extraction method for fungal culture filtrate (Fujita *et al.*, 1994; Park

et al., 2009). The spore germination fluids from liquid medium cultures were filtered through gauze or filter paper to remove mycelia and subsequently through a 0.20-0.22 μm Millipore filter to eliminate the conidia, or the toxins were extracted with ethyl acetate (EtOAc). After the removal of liquid medium or EtOAc by evaporation, the culture filtrate was dissolved in acetone. The solution was then evaporated under reduced pressure to remove acetone and concentrated to 1/50 or 1/100 of the original spore suspension volumes. Then a series of different concentrated toxin solutions are ready to be used for phytotoxin analysis. However, Isaac (1998) reminded us that toxins produced in culture may not necessarily be formed in a host plant. It is important to detect the naturally infected plant tissues to establish a role for toxins in the development of a plant's disease. However, this may be difficult since active concentrations are often very low. In our preliminary test, we attempted to extract phytotoxins from lesion tissues by artificial inoculation.

Further purification of phytotoxic compounds has been conducted to determine the chemical structure and biological activity of the phytotoxic metabolites produced by *Magnaporthe* spp.. The method for purification of phytotoxic compounds was based on a large-scale culture of isolates on plant grains prepared in flasks. Park *et al.* (2009) has extracted three phytotoxic compounds using this method and identified compound 1 was the fatty acid (9,12-octadecadienoic acid). They were unable to identify the other two compounds due to insufficient quantities for subsequent chemical analyses. Deighton *et al.* (1999) indicated that necrotrophic pathogens use oxidative bursts to invade and destroy plant tissues. The 9, 12-octadecadienoic acid may be a precursor of biologically active oxylipins in plants with JA as a terminal signal (Blechert *et al.*, 1995). The production of excess 9, 12-octadecadienoic acid by *M. oryzae* may disrupt cellular homeostasis and lead to cell death. Recovery of disease resistant plants by *in vitro* selection at the cellular level using partially purified toxin or culture filtrate produced by a plant pathogen, has been reported (Daub M., 1986; Arcieni et al., 1987; Frame et al., 1991). The effect of phytotoxins derived from *Magnaporthe* spp. on wheat and rice leaves in our experiment is just a preliminary test. Further purification of culture filtrate has to be performed in order to identify toxic metabolites of *Magnaporthe* spp. involved in pathogenicity. The potential use of culture filtrate of *Magnaporthe* spp. should also be investigated to determine structural and biochemical barriers involved in defense mechanisms and its usefulness for resistance induction in cells and tissue cultures.

With the exception of the observation of lesions, the effect of culture filtrate on membrane permeability is another way to study the role of the phytotoxins. Iacobellis & Bottalico (1981)

suggested that the absence of non-specific substances in the filtrate have been related to changes in membrane permeability in infected plants. Changes in cell permeability of plant leaves were determined with a conductivity method which measures ion leakage and has been regarded as one of the earliest host responses to a variety of plant pathogens (Wheeler, 1976; Misaghi, 1982). A significantly higher electrolyte leakage was detected on potato leaves treated with filtrate of *Fusarium eumartii* compared to Richard's filtrate used as control, and both conidia and filtrate of *F. eumartii* increased ion leakage in potato leaves while no significant changes appeared in tomato leaves (Botta *et al.,* 1994). Wheeler & Black (1963) also reported that only the host-selective *Helminthosporium victoriae* toxin causes permeability changes in oat tissues identical to those caused by the pathogen in experimentally infected plants.

However, none of several evaluating points proposed to determine whether a toxin has a role in plant disease has any importance by itself. Yet in combination, confidence may increase (Yoder, 1980). Isaac (1998) reported that *Fusicoccum amygdali* is the causal agent of wilt diseases of almond and peach. This disease is caused by complex interactions between cell wall degrading enzymes, plant growth regulators and the toxin Fusicoccin produced by *Fusicoccum amygdali*. It is more likely that this toxin is a secondary determinant of the disease. Botta *et al.* (1994) suggested that the use of toxic metabolites instead of the pathogen to evaluate plant responses might be valuable, mainly when the disease expression is highly influenced by the environment or isolation of the pathogen is difficult.

The most common approach to develop systems for selecting for disease resistance in culture has been to use of pathogen toxins as the selecting agent. Many investigators have succeeded in selection or breeding some cultivars with resistance to a specific pathogen toxin. The first resistance gene against toxins from plant was cloned and named *Hm1*, which confers resistance to HC- maize. *Hm1* was cloned by transposon tagging, and homologs are present in severa1 HC-toxin-insensitive grasses. An enzyme from maize, called HC-toxin reductase, which deactivates HC-toxin by pyridine nucleotide-dependent reduction of an essential carbonyl group, is detectable only in extracts of maize that are resistant to *C. carbonum* race 1 (genotype *Hm/Hm* or *Hm/hm*) (Meeley *et al.,* 1992). In most cases, the toxin resistance expressed in regenerated plants is correlated with significant increase in the levels of disease resistance in the plants. Moreover, these changes were genetic and able to transmit the resistance to the progeny of the selected plants. In addition, Daub (1986) found that host

response to the toxin was not correlated to pathogenicity. Therefore, there are still many problems in the study of phytotoxins that need to be solved.

Summary

Wheat blast (causal agent *Magnaporthe grisea*) is a novel and serious disease of wheat, causing high yield losses which has become a critical problem for wheat production in South America. This study aimed to improve the knowledge of characterization, epidemiology and pathogenicity of *Magnaporthe* spp., and to distinguish *Magnaporthe grisea* from wheat with another blast pathogen from rice (*Oryza sativa* L.), *Magnaporthe oryzae* in terms of phylogenetic relationships and interaction with its hosts and non-hosts. The results from this research would provide useful information for the development of strategies for the future control of wheat blast under practical field conditions.

Initially, cultural characteristics of seventy *Magnaporthe* isolates were characterized *in vitro*. Diversity of colony morphology of these isolates was shown on V8 medium and significant differences in colony growth diameter and mycelial dry weight were demonstrated. These differences were significant among isolates from the same host, but insignificant between different hosts.

Effects of temperature (20, 23, 26, 29 and 32 ℃) and spike-wetness periods (24h, 48h, 72h and 96h) on ear infection at flowering stage of wheat (BR 18) were studied. The results showed that higher temperatures (>26 ℃) are conducive for the growth of *M. grisea*. Differences in spike-wetness periods were insignificant, but disease severity increased in warm conditions with extended wetness periods. Therefore, the optimum inoculation conditions for further studies was 26 ℃ and 24h wetness duration after inoculation.

Pathogenicity tests were carried out in the greenhouse. Seventy *Magnaporthe* isolates collected from different host plants and geographical origins were inoculated on wheat and rice seedlings under controlled conditions to evaluate their infection capacity on wheat and rice leaves. All isolates produced typical lesions on their host plants, but there were significant differences in disease incidence and severity between the different host-isolate combinations. Thirty-six isolates from wheat and other grasses and eleven rice isolates were able to cause lesions with varying severity on both wheat and rice while the rest of the isolates were pathogenic only on their host plants. In the compatible interactions, 55% of the wheat isolates and 93.3% of the rice isolates successfully induced disease (severity between 4 to 5), which means the pathogen has developed on between 50% and 80% of the host leaves, and 32.5% of wheat isolates infected over 80% of wheat leaves. For the incompatible interactions, very low levels of disease severity were found on both host plants.

Molecular research tools have also been developed and applied. Amplified fragment length polymorphism (AFLP) was used to detect genetic diversity within seventy isolates of *Magnaporthe* spp. from different hosts and geographical origins using three AFLP primer combinations. The isolates clustered into two groups corresponding to their original host. *Magnaporthe grisea* isolates from wheat were grouped together with 74% similarity, and resulted in the formation of two subgroups. Isolates from finger millet and perennial ryegrass were closely related to wheat isolates at a similarity level of 87.5%. Rice isolates (*Magnaporthe oryzae*) also clustered together with 79% similarity. However, isolates which originated from the same host but were collected from different countries were distributed in

different subgroups or clades, instead of clustering together. The assessment of phylogenetic relationships using AFLP revealed that the variation assessed by genetic diversity studies within *Magnaporthe* spp. is mainly dependent on the host species, rather than the geographical origin.

Furthermore, another molecular genotyping method, multilocus sequence typing (MLST), was established. A phylogenetic analysis among twenty *Magnaporthe* isolates from different hosts was performed by inferring dendrograms for the concatenated sequences of three home-keeping genes, actin, ß-tubulin and calmodulin. Due to the differences in mutation frequency, the concatenated sequences were divided into two parts, concatenated introns and concatenated exons, and their diversity was calculated separately. Dendrograms constructed using introns revealed three groups which contained the isolates from different hosts. Similar clusters were also found in the dendrogram derived from exons. The genetic diversity assessed by Nei`s parameters revealed that the percentage of polymorphic loci in the intron sequences was higher than that in the exon sequences. The diversity within the group, which differs depending on the geographical origin, was lower than that among four groups classified by their host plants. The clusters and genetic diversity produced by MLST suggested that in three house-keeping genes, most of the variation is explained by genetic diversity between host species rather than geographical origins, which is in accordance with the results of AFLP.

In addition, a DNA based quantitative real-time polymerase chain reaction (PCR) was evaluated to quantify fungal biomass of *Magnaporthe* spp. in wheat and rice leaves. Samples from inoculated wheat and rice leaves were collected at different time points (0, 2, 4 and 6 dpi) and their *Pot2* transposon was amplified with primers *pfh*2a and *pfh*2b. At the same time point, the amount of DNA biomass from the wheat isolate MG 31 infection on wheat leaves was higher than the other two isolates with their host plants. At 6 dpi, the biomass was 199 pg/mg for MG 31, 174 pg/mg for MG 5 and 133 pg/mg for Ca 89. For the non-host reaction, a very small increase of biomass was detected at 6 dpi in rice leaves infected by MG 31, but not in the two other incompatible interactions.

Cytological investigations were performed with three *Magnaporthe* isolates selected from wheat (MG 5 and MG 31) and rice (Ca 89) for evaluating their capacity to infect the leaves of host and non-host plants. Microscopic observations were carried out by confocal laser scanning microscopy (CLSM) at different time points (12, 24, 48 and 72 hpi) and the growth of hyphae was classified into four stages. Spore germination and appressorium formation (stage one) were observed at 12 hpi in all interactions. At 24 hpi, only adapted isolates grown on their host were found and the percentages grown were 18%, 30% and 19% for isolates MG 5, MG 31 and Ca 89, respectively. At 48 hours post inoculation, some of the hyphae from adapted isolates (26% from MG 5, 29% from MG 31 and 27% from Ca 89) extensively grew in the primary invaded cells, and attempted to enter the adjacent cells. Multicellular infection (stage four) from isolate MG 5 (42%), MG 31(50%) and Ca 89 (32%) were observed at 72 hpi. For the invasion of adapted isolates, MG 31 appeared to grow more rapidly in its host plant than the other two isolates. The nonhost resistance was restricted in the interactions for rice isolate Ca 89 on wheat and for wheat isolate MG 5 on rice, but another wheat isolate MG 31 showed a certain degree of invasion in rice.

In a parallel study, cellular responses of wheat (BR 18) and rice (CO 39) to both adapted and non-adapted isolates of the blast fungus *Magnaporthe* were investigated by autofluorescence microscopy at 48hpi and grouped into four response types. The observations revealed that most of the compatible interactions between adapted isolates and their host plants reached type D (41.3% for MG 5, 47.7% for MG 31 and 43% for Ca 89) at 48 hpi, when hyphae successfully occupied the whole invaded cell and even grew into the adjacent cells. Inoculation with non-adapted isolates resulted in a lack of effective penetration, and resistance was detected in wheat leaves against rice isolate Ca 89 (81.3%) and in rice leaves against the wheat isolate MG 5 (76.9%). Partial resistance occurred as a response in rice to isolate MG 31 derived from wheat where only 13.2% of hyphae from MG 31 were able to invade rice epidermal cells. The initial cellular defense towards the non-adapted isolates was associated with the formation of papillae (type B) and hypersensitivity responses (type C) which occurred in the initially invaded cells by showing strong autofluorescence and preventing further invasion from hyphae.

Finally, a preliminary test on phytotoxins has been implemented. Detached seedling leaves of wheat and rice were treated with culture filtrate, infected leaf extract leachate and conidial suspension of *Magnaporthe* spp. as well as the filtrate of fresh potato dextrose broth (PDB) as a control. Symptoms were only evident on leaves treated with conidial suspension from compatible isolates. This result suggests that phytotoxins do not play a role in infection with three isolates (MG 5, MG 31 and Ca 89), neither on the seedling leaves of wheat nor on rice.

Overall, the differences within *Magnaporthe grisea* isolates derived from wheat were evidenced in terms of phylogenetic characterization and pathogenicity. Genetic and cytological studies demonstrated differences between the two blast pathogens, *Magnaporthe grisea* (wheat) and *Magnaporthe oryzae* (rice). The results support the assumption that *Magnaporthe grisea* isolates (wheat) are distinct from those of rice, but some aggressive isolates may exhibit pathogenicity to rice. In order to further investigate the differences and relationships among *Magnaporthe* spp., more isolates should be collected from other hosts and geographical origins. In addition, research should also be expanded to practical field conditions and associated with the study of wheat blast management.

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Appendix

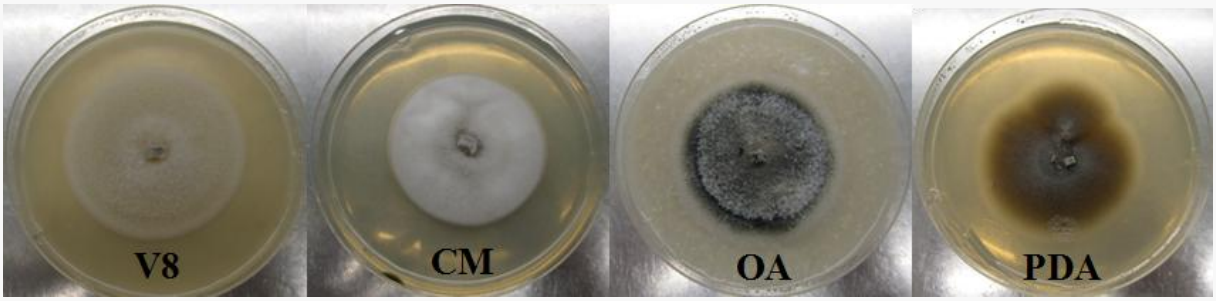

Figure A1 The colony of wheat isolate MG 5 on the media of V8, CM, OA and PDA at 7 dpi.

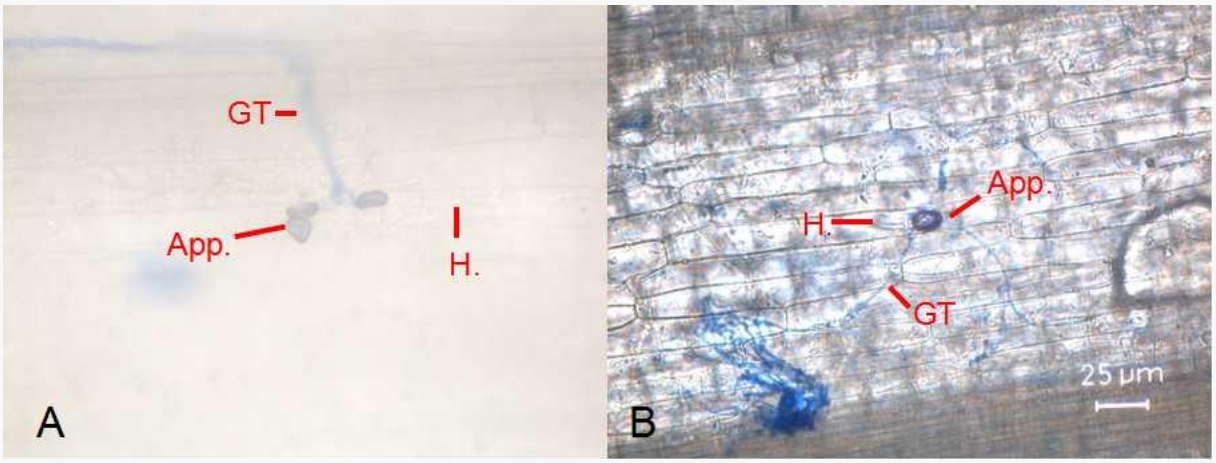

Figure A2 Cotton blue staining on wheat leaves (A) and rice leaf sheaths (B) infected by adapted *Magnaporthe* isolates. Only conidia, germination tubes and appressorium were stained into blue, but not hyphae in the epidermal cells. App., appressoria; H., hypha; GT, germination tube.

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w_MG1.2        C G G T G A C G A T G C T C C C C G C G C C G T C T T C C C G T C C A T T G T C G G T C G C C C T C G T C A C C A T G G T
w_MG5          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . .
w_MG9          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG16         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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r_564UGA11      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG1.2         A G T G G C C C T C A C C G C G G T G G C G G C C G C T C T A G A C T A G T G G A T C C C C C T T C C C C C G T C T C C A
w_MG5           . T . A A . . . . . . . . T G . . . A C . . . . . C . . . . . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
w_MG44          . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9           . T . A A . . . . . . . . T G . . . A C . . . . . C . . . . . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
w_MG16          . T . A A . . . . . . . . T G . . . A C . . . . . C . . . . . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
r_Ca89          . T . A A A G . . . . . . T G . . . A C . . . . . C . . . G . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
r_AGT211        . T . A A A G . . . . . . T G . . . A C . . . . . C . . . G . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
r_CBN9214-1     . T . . . A G . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1    . T . A A . . . . . . . . T G . . . A C . . . . . C . . . G . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
p_TP/L-2        . T . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5       . T . A A . . . . . . . . T G . . . A C . . . . . C . . . G . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
w_1836-3/0-12   . T . A A . . . . . . . . T G . . . A C . . . . . C . . . . . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
w_MG28          . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31          . T . A A . . . . . . . . T G . . . A C . . . . . C . . . . . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
w_MG39          . T . A A . . . . . . . . T G . . . A C . . . . . C . . . . . . G . . . T C . A . . T . . . . . . . . . . . . . . . . . .
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r_492RWA11    . T . A A A G . . . . . T G . . . A C . . . . . C . . . G . . G . . . T C . A . . T . . . . . .
r_528UGA11    . T . . . A G . . . . . G . . . . . .
r_559UGA11    . T . . . A G . . . . . G . . . . . .
r_564UGA11    . T . A A A G . . . . . T G . . . A C . . . . . C . . . G . . G . . . T C . A . . T . . . . . .
r_RWA11.2     . T . A A A G . . . . . T G . . . A C . . . . . C . . . G . . G . . . T C . A . . T . . . . . .

w_MG1.2       T C G C T C C T T T G A C C A G C C G T G G T G C C C A C T C T T T C C G C G C T G T C A C C G T T C C C G A G T T G A C
w_MG5         . . . . . .
w_MG44        . . . . . .
w_MG9         . . . . . G . . . . . .
w_MG16        . . . . . .
r_Ca89        . . . . . .
r_AGT211      . . . . . .
r_CBN9214-1   . . . . . .
f_Ken15-15-1  . . . . . .
p_TP/L-2      . . . . . .
p_FI5/L-5     . . . . . .
w_1836-3/0-12 . . . . . .
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w_MG28        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


w_MG1.2       C C A A G A A C A T G A T G G C T G C T T C T G A C T T C A G G A A T G G T C G T T A C C T G A C C T G C T C T G C C A T
w_MG5         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . A . . . . . . . . . . . . . . .
w_MG16        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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p_TP/L-2	. . . .
p_FI5/L-5	. . . .
w_1836-3/0-12	. . . .
w_MG28	. . . .
w_MG31	. . . .
w_MG39	. . . .
r_492RWA11	. . . .
r_528UGA11	. . . .
r_559UGA11	. . . .
r_564UGA11	. . . .
r_RWA11.2	. . . .

w_MG1.2	C C A T G A A G G A G G T C G A G G A C C A G A T G C G C A A C G T C C A G A A C A A G A A C T C G T C G T A C T T C G T
w_MG5	. . . .
w_MG44	. . . .
w_MG9	. . . . T . . . .
w_MG16	. . . .
r_Ca89	. . . .

```
r_AGT211      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG1.2       A C A T C C A G A C C G C T C T C T G C T C T A T C C C G C C C C G A G G C C T C A A G A T G T C G T C G A C T T T C A T
w_MG5         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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w_MG9	C		A
w_MG16			
r_Ca89		C	
r_AGT211		C	
r_CBN9214-1		C	
f_Ken15-15-1			
p_TP/L-2			
p_FI5/L-5			
w_1836-3/0-12			
w_MG28			
w_MG31			
w_MG39			
r_492RWA11		C	
r_528UGA11		C	
r_559UGA11		C	
r_564UGA11		C	
r_RWA11.2		C	

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w_MG1.2        T C C A G G A G C T G T T C A A G C G T G T C G G T G A G C A G T T C A C T G C C A T G T T C A G G C G C A A G G C T T T
w_MG5          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9          . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . T . . . . . . . .
w_MG16         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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r_564UGA11      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG1.2         G T G A G G G T A T G G A C G A G A T G G A G T T C A C T G A G G C C G A G T T C A A C A T G A A C G A T C T C G G G A A
w_MG5           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
w_MG44          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . G .
w_MG16          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
r_Ca89          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
r_AGT211        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
r_CBN9214-1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
p_TP/L-2        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
w_1836-3/0-12   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
w_MG28          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
w_MG39          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
```

```
r_492RWA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
r_528UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .
r_RWA11.2      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .

w_MG1.2        G G C C G G T A C C C A G G T G A G G G T T A A T T C G C T C C A G T C A A G G A G G C C T T C T C C T T G T T T G A C A
w_MG5          . . . . . C C . . . . . . . . . . . . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9          . . . . . C C . . . . . . . . . . . C C . C . . . . . C . C . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . .
w_MG16         . . . . . C C . . . . . . . . . . . . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         . . . . . C C . . . G C . . . . G A . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211       . . . . . C C . . . G C . . . . G A . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . G C . . . . G A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . . . . . C C . . . G C . . . . . . . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . C C . . . G . . . . . . . . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . . . . . C C . . . G . . . . . . . . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . .
```

```
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . . . . . C C . . . G . . . . . . . . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39         . . . . . C C . . . G . . . . . . . . C . C . . . . . . . C . . . . . . . . T . . . . . . . . . . . G G . . . . . . . . .
r_492RWA11     . . . . . C C . . . G C . . . . G A . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11     . . . . . . . . . . G . . . . . G A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . G . . . . . G A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11     . . . . . C C . . . G C . . . . G A . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2      . . . . . C C . . . G C . . . . G A . C . C . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG1.2        A T C A C C A C C A A G G A G C T C G G C A C T G T C A T G C G C T C C C T C G G C C A G A A C C C T T C C G A G T C G G
w_MG5          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG16         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```

```
p_TP/L-2        . . . . . .
p_FI5/L-5       . . . . . .
w_1836-3/0-12   . . . . . .
w_MG28          . . . . . .
w_MG31          . . . . . .
w_MG39          . . . . . .
r_492RWA11      . . . . . .
r_528UGA11      . . . . . .
r_559UGA11      . . . . . .
r_564UGA11      . . . . . .
r_RWA11.2       . . . . . .

w_MG1.2         A A C G A G G T C G A T G C T G A C A A C A A C G G C A C C A T C G A C T T T C C C G A G T T C C T C A C C A T G A T G G
w_MG5           . . . . . .
w_MG44          . . . . . .
w_MG9           . . . . . .
w_MG16          . . . . . .
r_Ca89          . . . . . .
```

```
r_AGT211       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


w_MG1.2        T C C A C T A G T T C T A G A G C G G C C G C C A C C G C G G T G G A G C T C C A A T T C G C C
w_MG5          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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```
w_MG9          . T . . T C . A G C T . . T C . A T A . . . T . . . . T . . A G . . G . G G . . C G G . A C . .
w_MG16         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         . T . . T C . A G C T . . T C . A T A . . . T . . . . T . . A G . . G . G G . . C G G . A C . .
r_AGT211       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . T . . T C . A G C T . . T C . A T A . . . T . . . . T . . A G . . G . G G . . C G G . A C . .
p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . T . . T C . A G C T . . T C . A T A . . . T . . . . T . . A G . . G . G G . . C G G . A C . .
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . T . . T C . A G C T . . T C . A T A . . . T . . . . T . . A G . . G . G G . . C G G . A C . .
w_MG39         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11     . T . . T C . A G C T . . T C . A T A . . . T . . . . T . . A G . . G . G G . . C G G . A C . .
r_528UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2      . T . . T C . A G C T . . T C . A T A . . . T . . . . T . . A G . . G . G G . . C G G . A C . .
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Figure A3 Multiple sequence alignments of concatenated intron sequences from twenty *Magnaporthe* isolates conducted by ClustalW using Mega 6.0.5

```
w_MG1.2        GT AA GT GCCCCGCAATTTCAATATGACAACCGTCAGCTCCCGGTTTGTCGACAGATGTGCT
w_MG5          ..........................................................................
w_MG44         ..........................................................................
w_MG9          ..........................................................................
w_MG16         ..........................................................................
r_Ca89         ..........................................................................
r_AGT211       ..........................................................................
r_CBN9214-1    ..........................................................................
f_Ken15-15-1   ..........................................................................
p_TP/L-2       ..........................................................................
p_FI5/L-5      ..........................................................................
w_1836-3/0-12  ..........................................................................
w_MG28         ..........................................................................
w_MG31         ..........................................................................
w_MG39         ..........................................................................
r_492RWA11     ..........................................................................
r_528UGA11     ..........................................................................
r_559UGA11     ..........................................................................
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r_564UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG1.2        C T T G G T C A G A A T G A G A G A C T G A T A A T T T T G C G A T T A G G T T A G T A C T C C A T T T C C C A G G G C C
w_MG5          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG16         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```

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r_492RWA11   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

r_528UGA11   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

r_559UGA11   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

r_564UGA11   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

r_RWA11.2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


w_MG1.2      C C A A  GG C C C T A G C C C C T G G A T G A T G A T A T C C A T CGC G T CGG C G A G A T C T G A C A G T T T G C T A

w_MG5        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG44       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG9        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG16       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

r_Ca89       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

r_AGT211     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

r_CBN9214-1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

f_Ken15-15-1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

p_TP/L-2     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

p_FI5/L-5    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_1836-3/0-12. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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w_MG28       .............................................................
w_MG31       .............................................................
w_MG39       .............................................................
r_492RWA11   .............................................................
r_528UGA11   .............................................................
r_559UGA11   .............................................................
r_564UGA11   .............................................................
r_RWA11.2    .............................................................

w_MG1.2      GGACCCTCATGGTTCGAGGATTTGAACGTCAACTGACAATAATCACCATAATAGAGCGCAA
w_MG5        .............................................................
w_MG44       .............................................................
w_MG9        .....................................................AG...A.T
w_MG16       .............................................................
r_Ca89       ......................................................G...A.T
r_AGT211     .............................................................
r_CBN9214-1  .............................................................
f_Ken15-15-1 ......................................................G...A.T
```

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p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . A . T
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . A . T
w_MG39         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . A . T
r_528UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . A . T


w_MG1.2        A C C G GC C C C T C G A G G T C T A T C G A A T T C C G T A A G T T T C C T A G C C T T G T T T A T G T G A C T G T C G
w_MG5          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9          G G . . . . G . . . T . . A A . . . G T . G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG16         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         G G . . . . G . . . T . . A A . . . G T . G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```

```
r_AGT211        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1    G G . . . . . G . . . T . . A A . . . G T . G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12   G G . . . . . G . . . T . . A A . . . G T . G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31          G G . . . . . G . . . T . . A A . . . G T . G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11      G G . . . . . G . . . T . . A A . . . G T . G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2       G G . . . . . G . . . T . . A A . . . G T . G . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


w_MG1.2         C C A T G C T T T C T A C C A G G T T A C T G A T A G A G G A C C T T G A T A T C A A C A G G T T A G T C T A C A C C C C
w_MG5           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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w_MG9 . . . . . .

w_MG16 . . . . . .

r_Ca89 . . . . . .

r_AGT211 . . . . . .

r_CBN9214-1 . . . . . .

f_Ken15-15-1 . . . . . .

p_TP/L-2 . . . . . .

p_FI5/L-5 . . . . . .

w_1836-3/0-12 . . . . . .

w_MG28 . . . . . .

w_MG31 . . . . . .

w_MG39 . . . . . .

r_492RWA11 . . . . . .

r_528UGA11 . . . . . .

r_559UGA11 . . . . . .

r_564UGA11 . . . . . .

r_RWA11.2 . . . . . .

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w_MG1.2        C A C A  C C G A T T G C G C A T T C T C A G C G A C G T C C T C A C C G C G A G A A C T A G A C G T T T G C G A T C G C T
w_MG5          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG16         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_492RWA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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r_564UGA11      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


w_MG1.2         C C C G C C T C A C G G A A T G C A G A A A C T A A A A C A A T A C C C T T A T C G A T C A G G T A G G C A A A C A G C C
w_MG5           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG16          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG28          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG31          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG39          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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r_492RWA11       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_528UGA11       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_559UGA11       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_564UGA11       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_RWA11.2        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

w_MG1.2          G A T T A A G C C T T G A G C T G G T G G G G C T T T G C T G A C A G C T C C C A A C A G
w_MG5            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG44           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG9            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_MG16           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_Ca89           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_AGT211         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
r_CBN9214-1      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
f_Ken15-15-1     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_TP/L-2         . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
p_FI5/L-5        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
w_1836-3/0-12    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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w_MG28      ..............................................
w_MG31      ..............................................
w_MG39      ..............................................
r_492RWA11  ..............................................
r_528UGA11  ..............................................
r_559UGA11  ..............................................
r_564UGA11  ..............................................
r_RWA11.2   ..............................................
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Figure A4 Multiple sequence alignments of concatenated exon sequences from twenty *Magnaporthe* isolates conducted by ClustalW using Mega 6.0.5

Figure A5 The influence of temperature on the colony growth of *Magnaporthe* isolate in Petri dishes

Table A1 The phenological growth stages and BBCH (**B**ASF, **B**ayer, **C**iba-Geigy and **H**oechst) -identification keys of cereals (including wheat) (Lancashire *et al.*, 1991). The wheat seedlings on BBCH 12-13 were used in leaf inoculation, and wheat ears on BBCH 61-65 were used in ear inoculation.

Growth stage	Code	Description
0: Germination	00	Dry seed (caryopsis)
	01	Beginning of seed imbibitions
	03	Seed imbibition complete
	05	Radicle emerged from caryopsis
	06	Radicle elongated, root hairs and/or side roots visible
	07	Coleoptile emerged from caryopsis
	09	Emergence: coleoptile penetrates soil surface (cracking stage)
1: Leaf development[1], [2]	10	First leaf through coleoptiles
	11	First leaf unfolded
	12	2 leaves unfolded
	13	3 leaves unfolded
	1 .	Stages continuous till . . .
	19	9 or more leaves unfolded
2: Tillering[3]	20	No tillers
	21	Beginning of tillering: first tiller detectable
	22	2 tillers detectable
	23	3 tillers detectable
	2 .	Stages continuous till . . .
	29	End of tillering. Maximum no. of tillers detectable
3: Stem elongation	30	Beginning of stem elongation: pseudostem and tillers erect, first internode begins to elongate, top of inflorescence at least 1 cm above tillering node
	31	First node at least 1 cm above tillering node
	32	Node 2 at least 2 cm above node 1
	33	Node 3 at least 2 cm above node 2
	3 .	Stages continuous till . . .

	37	Flag leaf just visible, still rolled
	39	Flag leaf stage: flag leaf fully unrolled, ligule just visible
4: Booting	41	Early boot stage: flag leaf sheath extending
	43	Mid boot stage: flag leaf sheath just visibly swollen
	45	Late boot stage: flag leaf sheath swollen
	47	Flag leaf sheath opening
	49	First awns visible (in awned forms only)
5: Inflorescence emergence, heading	51	Beginning of heading: tip of inflorescence emerged from sheath, first spikelet just visible
	52	20% of inflorescence emerged
	53	30% of inflorescence emerged
	54	40% of inflorescence emerged
	55	Middle of heading: half of inflorescence emerged
	56	60% of inflorescence emerged
	57	70% of inflorescence emerged
	58	80% of inflorescence emerged
	59	End of heading: inflorescence fully emerged
6: Flowering, anthesis	61	Beginning of flowering: first anthers visible
	65	Full flowering: 50% of anthers mature
	69	End of flowering: all spikelets have completed flowering but some dehydrated anthers may remain
7: Development of fruit	71	Watery ripe: first grains have reached half their final size
	73	Early milk
	75	Medium milk: grain content milky, grains reached final size, still green
	77	Late milk
8: Ripening	83	Early dough
	85	Soft dough: grain content soft but dry. Fingernail impression not held
	87	Hard dough: grain content solid. Fingernail impression held
	89	Fully ripe: grain hard, difficult to divide with thumbnail
9: Senescence	92	Over-ripe: grain very hard, cannot be dented by thumbnail
	93	Grains loosening in day-time
	97	Plant dead and collapsing
	99	Harvested product

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Declaration

I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen, 10.12.2014

Tingting Wei