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Pythium spp. affecting aerobic rice cultivation in the Philippines: characterization, virulence strategies and plant defense

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LIST OF ABBREVIATIONS

ABA	abscisic acid
AIC	Akaike's Information Criterion
AFLP	Amplified Fragment Length Polymorphism
AOC	allene oxide cyclase
AOS	allene oxide synthase
AP2/ERF	APETALA2/ETHYLENE RESPONSE FACTOR
ARFs	auxin response factors
AUX(s)	auxin(s)
AWD	Alternate Wetting and Drying
At	Arabidopsis thaliana
BAK1	BRI1-ASSOCIATED KINASE1
BES1	BRI1-EMS-SUPPRESSOR1
BIN2	GSK3-like KINASE BRASSINOSTEROID-INSENSITIVE2
BKI1	BRI1 KINASE INHIBITOR1
BL	24-epibrassinolide
BLE2	BRASSINOLIDE ENHANCED2
BR6ox	BRASSINOSTEROID-6-OXIDASE
BR(s)	brassinosteroid(s)
BRI1	BRASSINOSTEROID-INSENSITIVE1
BRRE	BR-response element
BRZ	brassinazole
BSKs	brassinosteroid signaling kinases
BSU1	BRI1 SUPPRESSOR PROTEIN1
BTH	benzothiadiazole
BZR1	BRASSINAZOLE-RESISTANT1
BZR2	BRASSINAZOLE-RESISTANT2
CAU	Chinese Agricultural University
cDNA	complement DNA (deoxyribonucleic acid)
CDP	*ent*-copalyl diphosphate
CK(s)	cytokinin(s)
CM	chorismate mutase
CN	campestanol
COI1	CORONATINE INSENSITIVE1

CPD	CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM
CPDKs	calcium-dependent protein kinases
CPS	*ent*-copalyl diphosphate synthase
CS	castasterone
CT	cathasterone
Ct	cycle threshold
cv.	cultivar
CWDEs	cell wall-degrading enzymes
D1-3	domains in the LSU
D11	DWARF 11
D2	DWARF2
DAB	3,3'-diaminobenzidine
DAMPs	damage-associated molecular patterns
DMSO	dimethyl sulfoxide
dpi	days post inoculation
DSI	disease severity index
3DT	3-dehydroteasterone
DWF4	DWARF4
EDTA	ethylenediaminetetraacetic acid
EL1	EARLY FLOWERING1
ERF1	ETHYLENE RESPONSE FACTOR1
ESC	Ecological Species Concept
ET	ethylene
ETI	effector-triggered immunity
EtOH	ethanol
ETS	effector-triggered susceptibility
ETYA	5,8,11,14-eicosatetraynoic acid
FAOSTAT	Statistical devision of the Food and Agriculture Organization of the United Nations
flg22	flagellin
FLS2	flg22 RECEPTOR
Fox	*Fusarium oxysporum*
FW	fresh weight
GA(s)	gibberellin(s)
GA3	gibberellin A3
GA13ox	GA 13-HYDROXYLASE
GA20ox	GA 20-OXIDASE

GA2ox	GA 2-OXIDASE
GA3ox	GA 3β-HYDROXYLASE
GB5	Gamborg B5
GGDP	geranylgeranyl diphosphate
GH3	GRETCHEN HAGEN3
GID1	GIBBERELLIN INSENSITIVE DWARF1
GSK3	GLYCOGEN SYNTHASE KINASE-3
H_2O_2	hydrogen peroxide
13-HPOT	(13S)-hydroperoxyoctadecatrienoic acid
hpi	hours post inoculation
IAA	indole-3-acetic acid
IAAld	indole-3-acetaldehyde
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
IAOx	indole-3-acetaldoxime
ICS/SID2	ISOCHORISMATE SYNTHASE
ICS1	ISOCHORISMATE SYNTHASE1
IPA	indole-3-pyruvic acid
IRRI	International Rice Research Institute
ITS	Internal Transcribed Spacer region of the rDNA
JA	jasmonic acid
JA-Ile	JA-isoleucine
JAR1	JA conjugate synthase
JAs	jasmonates
JAZ	JASMONATE ZIM-domain
JMT	JA carboxyl methyltransferase
KAO	ent-kaurenoic acid oxidase
KO	ent-kaurene oxidase
KS	ent-kaurene synthase
LOXs	lipoxygenases
LRR-RK	leucine rich repeat-receptor kinase
LSU	large-subunit of the rDNA
MADS	MADS-box transcription factor
MAPK	mitogen-activated protein kinase
MAMP(s)	microbial-associated molecular pattern(s)
MeJA	methyl jasmonate

MSC	Morphological Species Concept
nahG	salicylate hydroxylase
NaOCl	sodium hypochlorite
NBS-LRR	nucleotide binding site-leucine rich repeat
NCBI	National Center for Biotechnology Information
NERICA	New Rice Varieties for Africa
NHT	N-hydroxytryptamine
NPR1	NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1
OD	optical density
OPC-8	cyclopentanone
OPDA	cis-(+)-12-oxophytodienoic acid
OPR3	OPDA reductase
Os	Oryza sativa
PAL	PHENYLALANINE AMMONIA LYASE
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PIFS	phytochrome interacting factors
PR	PATHOGENESIS-RELATED
PRRs	pattern recognition receptors
PSC	Phylogenetic Species Concept
PTI	PAMP-triggered immunity
qPCR	Quantitative Real-Time PCR
rDNA	ribosomal DNA
RAVL1	RAV-LIKE1 PROTEIN
RGA1	heterotrimeric G protein alfa 1
RKN	root knot nematode
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RRN	root rot nematode
SA	salicylic acid
SAR	systemic acquired resistance
SAUR	small auxin-up RNA
SDS	sodium dodecyl sulfate

SERK	SOMATIC EMBRYOGENESIS RECEPTOR KINASE
SLR1	SLENDER RICE1
SRI	System of Rice Intensification
SSC	Saturated Soil Culture
SLs	strigolactones
TAA	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS
TAM	tryptamine
TE	teasterone
TIR1	TRANSPORT INHIBITOR RESPONSE1
TMV	*Tobacco mosaic virus*
Trp	tryptophan
TSB	Tryptic Soy Broth
TY	typhasterol
UPS	ubiquitin proteasome system
WARDA	West Africa Rice Development Association
WT	wild-type
Xoo	*Xanthomonas oryzae pv. oryzae*
YUC	YUCCA

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Chapter 1
Preface

1.1. Problem statement

Up until now, rice cultivation in flooded 'paddy' fields has been representing the most widely used and highest-yielding cultivation system. With a share of 75% in total rice production, irrigated lowland rice has been feeding billions of people mainly living in Asia (Hibberd et al., 2008; Zhang et al., 2009). Unfortunately, rice paddies consume about 30% of the worldwide and 50% of the Asian fresh water resources (Peng et al., 2006) (Figure 1.1). Fresh water, however, is becoming increasingly scarce due to the global weather changes, rising urban and industrial development, water pollution and the continuously growing human population, which is expected to increase 1.5 billion in Asia over the next 50 years (Hibberd et al., 2008; Yang and Zhang, 2010). Consequently, the sustainability of the paddy field system is severely threatened. By 2025, around 15-20 million hectares of Asia's irrigated rice is estimated to suffer from low water supplies (Tuong and Bouman, 2003), which is already visible around Beijing where paddy fields have been banned since 2001 (Xiaoguang et al., 2005). Since rice yields must be increased by at least 50% over the next 40 years, water scarcity poses a huge problem on the future food security of millions of people in Asia (Hibberd et al., 2008). This urges the need for alternative rice cultivation systems that require less water.

Figure 1.1. The irrigation of rice paddies at the experimental site of the International Rice Research Institute (IRRI, Philippines).

Several strategies have been developed to save water in rice paddies without lowering productivity. Technologies like 'Saturated Soil Culture' (SSC) (Borell et al., 1997), 'Alternate Wetting and Drying' (AWD) (Li, 2001; Tabbal et al., 2002), 'System of Rice Intensification' (SRI) (Stoop et al., 2002) and ground cover systems (Lin et al., 2002), maintain the soil at its saturation point, alternate aerobic with anaerobic conditions, combine wide plant spacing with reduced flooding or reduce evaporation by covering saturated soils. However, water losses remain high in these systems (Peng et al., 2006).

Research activities at the International Rice Research Institute (IRRI) in the Philippines and the Upland Rice Laboratory of the Chinese Agricultural University (CAU) have been focusing lately on the creation of C4-rice (rice with a C4 photosynthesis cycle; Hibberd et al., 2008), beside the on-going breeding for salt-tolerant, submergence-tolerant and aerobic rice varieties. The use of these new rice cultivars is a more promising strategy to save irrigation water, elevate the productivity or expand the available land for rice cultivation. Aerobic rice varieties are high-yielding and drought-tolerant rice cultivars that can be direct seeded in non-puddled and non-flooded fields (Figure 1.2), and that are responsive to nutrients and supplementary irrigation (Atlin et al., 2006; Peng et al., 2006). Most varieties have been developed through crosses of improved lowland and traditional upland cultivars, to combine traits like drought-tolerance and high yield capacity (Prasad, 2011).

Figure 1.2. Aerobic rice field in Paniqui (Tarlac, the Philippines).

The aerobic rice system requires 30-50% less irrigation water than paddy fields, which usually consume 3000 up to 5000 L water/kg rice (Bouman et al., 2002; www.irri.org). Accordingly, it represents a good alternative for irrigated and rainfed areas prone to water-scarcity, lowlands prone to uncontrolled flooding or favorable uplands (Nie et al., 2012). China, Brazil, India, Australia and parts of Southeast Asia and Africa have been intensively investigating the aerobic rice system (Bouman et al., 2006; Humphreys et al., 2005). Yields up to 5-7 t ha^{-1} have been reported from aerobic rice fields in Japan, China and the Philippines (Bouman et al., 2005; Kato et al., 2009; Xiaoguang et al., 2005). This is a lot higher than the productivity of traditional uplands (i.e. 1-2 t ha^{-1}) (Atlin et al., 2006), where rice is grown in unfavorable environments without access to irrigation or external inputs (Bouman et al., 2002). Interestingly, the cultivation of a super-high-yielding lowland-adapted cultivar showed to generate yields of approximately 10 t ha^{-1} in Japanese aerobic soils, suggesting that high yields can be achieved in aerobic rice cultivation without specifically developing varieties that are drought-tolerant (Kato et al., 2009).

Unfortunately, aerobic growth conditions entail soil nutrient deficiencies (Zhang et al., 2009) and are also convenient for numerous pests and diseases (Kreye et al., 2009a), and therefore, continuous monocropping in aerobic rice fields may result in progressive yield decline. Losses from monocultures have been documented in Japan (Nishizawa et al., 1971), Brazil (Pinheiro et al., 2006) and the Philippines (George et al., 2002; Kreye et al., 2009a; Peng et al., 2006), but not in China, where aerobic rice is rotated with winter crops and legumes (Nie et al., 2012). In Brazilian fields, yield losses were attributed to autotoxicity and high yields could only be sustained under crop rotation with soybean (Pinheiro et al., 2006). On the contrary, nematodes have been suggested to underlie the decreasing yields in Japan (Nishizawa et al., 1971). Progressive yield decline in Philippine aerobic rice fields (Figure 1.3) has been associated with low nitrogen or micronutrient availability due to increasing soil pH (Kreye et al., 2009a; Xiang et al., 2009). In these cases, soil fertilization improved yields, but it did not always completely nullify the 2 up to 6 t ha^{-1} yield losses that occurred (Kreye et al., 2009a; Nie et al., 2008; Nie et al., 2009a, 2009b). So, other biotic and/or abiotic factors were assumed to contribute to the observed yield decline. Sampling of Philippine aerobic rice fields revealed the presence of soil-borne pathogens, such as *Pythium*, *Fusarium*, *Rhizoctonia*-like spp. and plant-parasitic nematodes (Kreye et al., 2009b). Root knot nematodes were identified as major yield constraints, but no evidence was found for the involvement of other soil-borne pathogens. More detailed studies on the role of *Pythium* spp. are unfortunately lacking. Nonetheless, *Pythium* spp. have been described to cause poor seedling establishment and stunting in paddy fields (Chun and Schneider, 1998; Cother and Gilbert, 1993; Furuya et al. 2003; Sung et al., 1983) and hence, they might also represent important threats to aerobic rice seedlings.

Figure 1.3. Aerobic rice field at Paniqui (Tarlac, the Philippines) showing progressive yield decline. A, healthy part of the field where rice plants are tall and flowering, **B,** diseased part of the field where rice plants are severely stunted and not flowering.

The International Rice Research Institute (IRRI) in the Philippines collected 115 *Pythium* isolates from diseased aerobic rice fields that were located in different regions on the main island Luzon (Figure 1.5). Preliminary tests elucidated that a lot of the sampled *Pythium* isolates were pathogenic towards rice seedlings, while some appeared to exhibit growth-promoting features. The long-term cooperation between Ghent University and IRRI resulted in a joint project, enabling the detailed investigation of the various biotic and abiotic factors that could underlie the observed yield decline in Philippine aerobic rice fields.

Figure 1.4. The main island Luzon of the Philippine archipelago where aerobic rice fields were sampled.

The present dissertation discusses the results of a PhD project concerning rice-infecting *Pythium* spp. in Philippine aerobic rice fields. This project aimed to answer the following **research questions**: **1)** Which *Pythium* spp. are associated with yield decline in aerobic rice fields? **2)** Which of these species are pathogenic towards aerobic rice seedlings and what are the main disease symptoms? **3)** Do these species show intraspecific variability at the phenotype level and is this reflected in their phylogenetic clustering? **4)** Are they host-specific? **5)** Do they all invade rice tissues and how fast does the infection process proceed? **6)** Which rice root responses accompany *Pythium* invasion? **7)** On which nutrients may *Pythium* spp. grow inside their host? **8)** Which hormonal regulatory network builds up the immune system of rice roots? **9)** How do *Pythium* spp. interfere with root defense to induce susceptibility? Eventually, by disentangling these questions, we also gained insight into the main factors that possibly determine the virulence level of rice-infecting *Pythium* spp..

1.2. Thesis outline

The dissertation starts with an introduction (**Chapter 2**) covering general information on the rice plant and its growth, the genus *Pythium* and its occurrence in rice cultivation, and the role of phytohormones in plant immunity.

Next, four chapters discuss the most important results from our research. In the first phase of this work, we characterized a collection of *Pythium* isolates from diseased aerobic rice fields in the Philippines (**Chapter 3**). *P. arrhenomanes*, *P. graminicola* and *P. inflatum* appeared to be the most frequently isolated species. All isolates were screened for their virulence towards rice seeds and seedlings using *in vitro* and *in vivo* testing systems. In a phylogenetic analysis, we studied the relation of the sampled *Pythium* isolates to several type strains and isolates of other hosts. Accordingly, we identified intraspecific variability at the genotype and phenotype level. Using both techniques, we also examined if rice- and maize-pathogenic *Pythium* spp. were host-specific, thereby evaluating the potential of maize as a crop for rotation strategies.

In **chapter 4** we show the results of an in-depth study that focused on the interaction of rice seedling roots with three isolates of the species *P. arrhenomanes*, *P. graminicola* and *P. inflatum*. Using histopathological and molecular methods we explored the differences among the rice root colonization processes of these oomycetes. Besides, we visualized the induced rice root responses upon *Pythium* infection, involving the production of reactive oxygen species (ROS) and phenolic compounds, and we revealed a regulatory role for jasmonates (JAs). Phenoarrays demonstrated on which specific nutrients *P. arrhenomanes, P. graminicola* and *P. inflatum* could grow. Eventually, we linked rice colonization patterns and nutritional profiles with the virulence level of the three *Pythium* spp. and clarified the reason behind the enhanced fitness of *P. arrhenomanes* in its ecological niche.

In **chapter 5** we investigated the role of brassinosteroids (BRs) in rice defense towards the root-infecting oomycete *P. graminicola*. Brassinosteroids have been widely reported to mediate immune responses towards various biotic and abiotic stresses. Implementing histopathological, molecular, physiological and pathological analyses, we proved that BRs enhance rice susceptibility to *P. graminicola*, at least in part, through their antagonistic crosstalk with resistance-inducing pathways. Furthermore, we obtained compelling evidence that *P. graminicola* strongly manipulates the host BR machinery in rice roots to inflict disease.

In **chapter 6** we assessed if BRs fulfill a similar role in the susceptibility of rice roots to *P. arrhenomanes* and *P. inflatum.* Root applications with hormones and biosynthesis inhibitors, and gene expression analyses, showed that the manipulation of the BR pathway in rice roots probably represents a conserved virulence strategy among rice-infecting *Pythium* species. Using the same techniques, we also expanded our knowledge on the role of auxins (AUXs) and JAs in rice root responses to *P. arrhenomanes, P. graminicola* and *P. inflatum.* AUXs have been frequently implicated as negative regulators of plant immunity to soil-borne pathogens, while JAs are mainly known as positive regulators. Our results illustrated that AUXs might also mediate rice root susceptibility to *Pythium* spp.. On the other hand, JAs did not seem to play key roles in the defense against these oomycetes and the virulence level of the challenging *Pythium* spp. appeared to determine the outcome of an activated JA pathway.

In **chapter 7** we present a literature review on the role of phytohormones in rice root defense. Up until now, most immune-related studies have been focusing on dicots and leaf-pathogen interactions. Therefore, we analyzed how the monocot model crop wards-off root pathogenic microorganisms and illustrated the similarities and/or differences with the dicot model plant Arabidopsis. Similar to the regulation of shoot defense in dicots and monocots, salicylic acid (SA) and jasmonic acid (JA) were identified as the pivotal players in root immunity. In addition, we overviewed the hormonal regulation of root development in both model plants and described how rice pathogens may interfere with these root developmental pathways to suppress SA- and JA-mediated root defense.

In the final chapter (**chapter 8**) we briefly summarize the main findings and propose future research directions.

Chapter 2
General introduction

2.1. Introduction to rice

2.1.1. Rice classification

Rice is classified within the kingdom Plantae, the class Monocotyledons and family Gramineae (or Poaceae). It belongs to the genus *Oryza* of the subtribe Oryzinae within the tribe Oryzeae (Tang et al., 2010). This genus comprises 24 species of which two are cultivated: *Oryza sativa* L., worldwide the dominant species and *Oryza glaberrima*, occurring in Africa (Seck et al., 2012). Due to separate rice domestication events from the ancestral species *O. rufipogon*, *O. sativa* differentiated into two major ecogeographic subspecies: *indica* and *japonica* (Garris et al., 2005). While indica rice is grown in (sub)tropical regions, the 'sticky' japonica rice mainly occurs in temperate up to subtropical regions (Yoshida, 1981; Garris et al., 2005). Furthermore, *O. sativa* comprises the groups *aus* and *aromatic*, of which the drought-tolerant *aus* has a closer evolutionary relationship to *indica* and *aromatic* is more related to *japonica*. Within the *aromatic* rices, *basmati* is extremely popular around the globe (Garris et al., 2005).

Crossings between *O. sativa* L. and *O. glaberrima* resulted in the 1990s in about 3000 new rice lines, designated as New Rice Varieties for Africa (NERICA). These interspecific hybrids were developed by the West Africa Rice Development Association (WARDA), currently known as the Africa Rice Center (AfricaRice, Benin) (www.africarice.org), and combine several unique traits of *O. sativa* and *O. glaberrima*, including high-yield, weed-competiveness, pest resistance and drought-tolerance (Sarla and Swamy, 2005). Their construction led to yield increases of about 50% in Africa, where they have been replacing the traditional rice species *O. glaberrima* (Sarla and Swamy, 2005).

A closely related genus to *Oryza*, *Zizania*, often referred to as wild rice, is also used for human consumption in Asia and North America (Tang et al., 2010). It belongs to the subtribe Zizaniiae of the tribe Oryzeae and contains four species, among which *Z. palustris* is the only species grown for seed production (Tang et al., 2010).

2.1.2. The rice plant and its growth stages

Depending on the cultivated variety and environmental conditions, the growth of a rice plant takes three up to six months from germination to maturity (Figure 2.1; Yoshida, 1981). During the first developmental stage, i.e. the vegetative stage, rice seeds germinate and seedlings emerge above the soil or water surface, which requires temperatures of respectively 27-37°C and 22-31°C. The optimal temperature for the growth of the seedlings lies between 25-30°C. The vegetative stage is also characterized by leaf emergence at regular intervals, active tillering, panicle initiation and gradual increases in plant height (Yoshida, 1981). Tillers are branches that develop from the un-elongated basal node on the main culm or other tillers, and that grow independently from the mother stem (Figure 2.1).

Together with grain filling and the number and size of panicles, the tillering performance of a rice variety determines its grain production and hence, it is an important agronomic trait (Li et al., 2003).

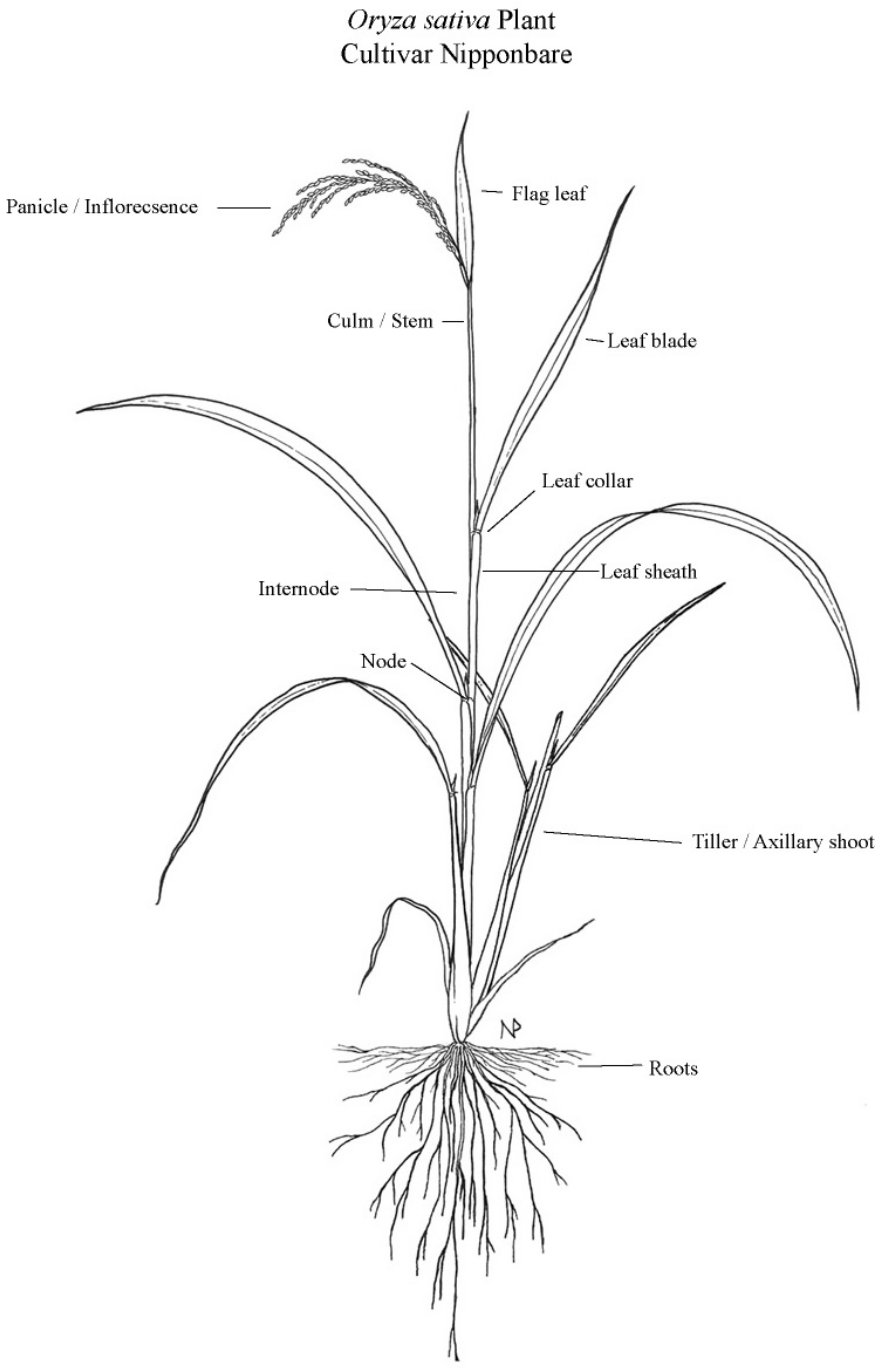

Figure 2.1. Drawing of a mature rice plant (www.gramene.org).

The reproductive stage refers to the period from panicle initiation up to flowering and is characterized by culm elongation, flag leaf emergence, booting (i.e. internode elongation) and heading (i.e. panicle exsertion). In the last stage, the flowering plant starts to ripen, resulting in a mature plant with senescencing leaves and grains that grow and turn more yellow as they mature (Figure 2.2; Yoshida, 1981).

Figure 2.2. Mature rice plants and a detailed picture of their ripe grains.

2.1.3. Rice cultivation systems

Since rice is highly adaptable to its environment, it can be grown in many different countries and varying climates, from high altitudes to below the sea level and from flooded fields to dry land conditions (Seck et al., 2012; Yoshida, 1981). Irrigated rice fields foresee in 75% of the global rice production. In these so-called 'rice paddies' (Figure 2.3), rice seedlings (*O. sativa* ssp. *indica*) are transplanted in bunded plots that are irrigated with 5-10 cm water and fertilized, yielding on average 6-11 t ha^{-1}.

Figure 2.3. Small rice paddies at the IRRI experimental site (Philippines) for genetic resource conservation and research.

Another 20% of the world's rice production comes from rainfed lowlands that are bunded and flooded with rainwater during a part of the cropping season, and have a much lower yield capacity of 1-2.5 t ha^{-1}. Rainfed uplands are not bunded or flooded and therefore, they resemble the fields of other cereals crops. These upland fields yield low amounts of rice (*O. sativa* ssp. *japonica*), and together with deep water or floating rice systems, they have maximal yield capacities of 1-2 t ha^{-1} on average. In West and Central Africa, rice cultivation (*O. glaberrima* and NERICA) in rainfed systems covers 75% of the total rice cultivation area. This contrasts with Asian countries where 56% of rice fields are irrigated (Seck et al., 2012).

2.1.4. The importance of rice

Cereals are the major food source for human consumption. Rice represents the staple food for more than half the world's population (> 3.5 billion), many of whom are living in poverty. Especially in developing countries, rice production is linked with food security and political stability, and the population depends on the crop's production for its livelihood (www.irri.org).

In 2012 the world produced 718.345.380 tons of rice, of which more than 90% was produced and consumed in Asia (FAOSTAT, 2012). The top ten leading countries in rice production include China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil and The United States of America (FAOSTAT, 2012). Asian countries jointly contribute for 50% to the world's rice production, but they still depend on import (14.000.842 tons of rice in 2010) to meet their own demand for rice. The Philippines has worldwide been the largest rice importer, with an import quantity that represented 2.378.045 tons of rice in 2010 (FAOSTAT, 2012). The thriving factors behind this high number are in particular the lack of suitable agricultural land and its fast growing population (www.irri.org). Only 7% of the global rice production is traded internationally, especially coming from Thailand, China, Vietnam, India, USA and Pakistan (www.africarice.org).

Rice has also gained importance throughout Africa (FAO Statistical Yearbook, 2013). Several African countries, including top producers Egypt, Nigeria, Madagascar, Guinea, Mali and Tanzania, jointly contributed to 27.268.806 tons of rice in 2012 (FAOSTAT, 2012). Among these, Madagascar, Eastern Africa and Tanzania belong to the world's leading rice-consuming nations. Despite its increasing rice production, Africa has difficulties to achieve its self-sufficiency in rice production (www.africarice.org) and in 2010 its imported quantity of rice was 9.019.045 ton of which more than 50% destined for the developing countries in West Africa (FAOSTAT, 2012).

Aside from being a primary food source, rice is also used for the production of alcoholic beverages such as beer and sake. Before consumption or further processing, rice seeds are milled. During this process, the hull, germ and bran layers are removed from the seed, which results in a polished white kernel.

The by-products of the milling process can be used to develop new products like cooking oil and fuel, or as ingredients of animal feed (www.irri.org).

In addition to its economic importance, rice turned out to be an ideal model organism for other monocots. It was selected as the first crop for whole genome sequencing because of its modest genome size compared to other cereals (Jung et al., 2008). Rice genomics have been allowing the molecular analysis of rice biological processes, research on rice species domestication and evolution (Jung et al., 2013), and studies on rice tolerance to biotic and abiotic stresses (Cantrell and Reeves, 2002). Besides, they have been enhancing the identification of molecular markers for specific agricultural traits (Swamy and Kumar, 2013). Since grass genomes share a high degree of synteny (Gale and Devos, 1999), rice sequence information is also very important for unraveling the biology of other cereals, analyzing cereal evolution and improving cereal crops (Xu and Zhang, 2004). This, together with its relative ease of genetic transformation, dense genetic maps and large sequence databases (Sasaki and Burr, 2000), make rice highly attractive as a monocot model crop.

2.2. Introduction to *Pythium* species

2.2.1. The oomycete *Pythium* and its life cycle

Pythium spp. are oomycetes, i.e. fungus-like organisms, belonging the family Pythiaceae whithin the order Pythiales of the Kingdom Chromista (Kirk et al., 2008). They are distinguished from true Fungi by several features including the presence of cellulose in their cell walls, the formation of coenocytic hyphae, vegetative diploidy, production of oospores and formation of zoospores with two types of flaggella (van West et al., 2003). Together with *Phytopthora* spp., they represent some of the most destructive plant pathogens and account worldwide for billion dollars of agricultural losses (van West et al., 2003). Plant pathogenic *Pythium* spp. preferentially attack seeds, seedlings and young tissues of mature plants, which may entail rots of fruits, stems or roots, and pre- or post-emergence damping-off (van der Plaats-Niterink, 1981). In addition, *Pythium* infections may also be non-lethal and result in stunted roots and shoots, and less vigorous plants (Martin and Loper, 1999). A wide variety of hosts may be challenged, but sometimes, host ranges of the species are narrow and restricted to one plant species (van der Plaats-Niterink, 1981). Beside plant pathogens, the genus *Pythium* comprises parasites of animals and soil saprophytes (van der Plaats-Niterink, 1981).

The life cycle of plant pathogenic *Pythium* spp. typically consists of an asexual and sexual cycle, which are both stimulated in response to specific environmental conditions (van West et al., 2003) (Figure 2.4). During the asexual cycle, short-lived sporangia are produced that directly germinate by forming mycelia or function as zoosporangia under wet conditions.

The latter generate mobile, biflagellate zoospores that can survive for two up to seven days in the field (Martin and Loper, 1999; van West et al., 2003). Plant seed or root exudates that leak into the spermo- and rhizosphere induce the germination of *Pythium* propagules and trigger chemotaxis of *Pythium* hyphae and zoospores (Martin and Loper, 1999; van West et al., 2003). Attracted zoospores can anchor to an encountered host surface using flagella and adhesives. Upon attachment, they encyst by the production of glycoproteins and other molecules, which finally results in the formation of a germ tube that, like hyphae, penetrates host tissues using appressoria (-like structures) (Martin and Loper, 1999; van West et al., 2003).

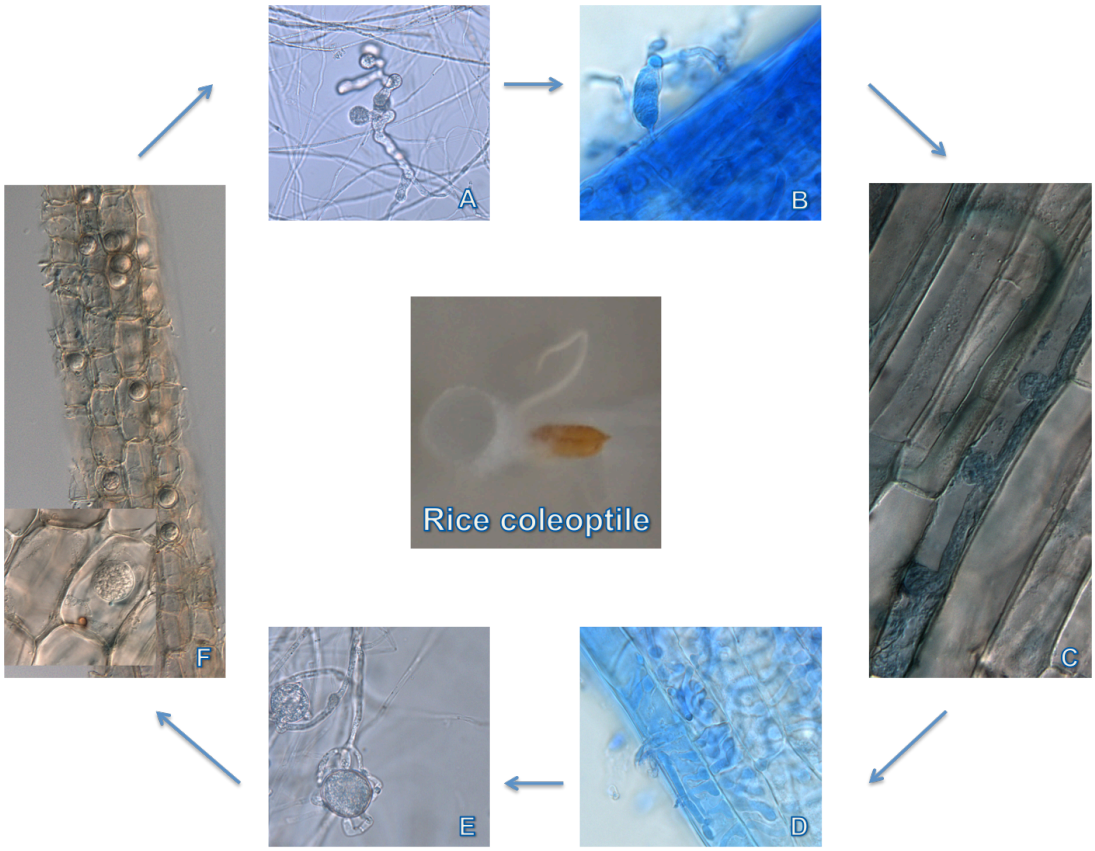

Figure 2.4. The life cycle of *Pythium arrhenomanes* in the rice coleoptile. A, sporangia and hyphae from which the infection may start, **B,** direct penetration of shoot tissue by appressoria (-like) structures, **C and D,** colonization of inner tissues leading to entirely-filled host cells, **E,** interaction between antheridia and an oogonium during oospore formation, **F,** oospores in host cells. A more detailed and general life cycle of plant pathogenic *Pythium* spp. can be found in the review of van West et al. (2003).

Once inside their host, *Pythium* spp. behave as hemi-biotrophic pathogens, keeping host cells alive until nutrients are depleted (Adie et al., 2007), or act as true necrotrophs, killing host cells by the activity of toxins or cell wall-degrading enzymes and feeding on the leftovers (Chérif et al., 1991; Desilets et al., 1994; Mojdehi et al., 1991).

Although rather exceptional, root-infecting *Pythium* spp. may ingress the vascular tissue and block the water transport in their host. Accordingly, they may induce severe wilting symptoms and resemble real vascular pathogens like *Fusarium* and *Verticillium* spp. (Adie et al., 2007; Matta, 1965; Nemec, 1972; Okubara and Paulitz, 2005).

The sexual cycle of *Pythium* spp. is induced when the optimal growth conditions in their ecological niche are disturbed. During this process, female oogonia are fertilized by male atheridia, which results in the formation of typical thick-walled oospores (van west et al., 2003) (Figure 2.4). Most *Pythium* spp. are homothallic, indicating that sexual reproduction mainly occurs within the same individual by selfing (Martin, 2009). This opposes the situation in heterothallic *Pythium* species, where female and male structures are developed from different mating types. In the latter cases, oogonial hyphae of the first mating type produce a hormone that stimulates the formation of antheridia in the other mating type and consequently, both mating types are indispensable for the oosporogenesis (Martin, 2009). The resting spores can show extended dormancy and survival for up to 12 years in the field, during which they resist several unsuitable conditions like extreme temperatures and enzymatic degradation (Martin and Loper, 1999). Driven by light, alternated wetting and drying, specific CO_2-concentrations or plant exudates, oospores may become thin-walled and start to germinate. From this point, the asexual cycle may be recapitulated (Martin and Loper, 1999; van West et al., 2003).

2.2.2. *Pythium* taxonomy and phylogeny

The genus *Pythium* was described in 1858 by Pringsheim, with *Pythium monospermum* as the type species. From then on, the taxonomy of the genus has been revised several times on the basis of morphological characteristics, like the shape and size of sporangia and oogonia, the number of antheridia per oogonium, diclinous versus monoclinous antheridia, or plerotic versus aplerotic oospores (Uzuhashi et al., 2010). Currently, 150 *Pythium* species have been described (Uzuhashi et al., 2010). Because different *Pythium* species often exhibit similar morphological characteristics, molecular identifications and phylogenetic analyses have been executed to validate and/or support their morphological taxonomy (Uzuhashi et al., 2010).

The phylogenetic study by Lévesque and De Cock (2004) divided the genus *Pythium* into 11 clades (A-K) of species (Figure 2.5). To this end, sequence data of the ITS (Internal Transcribed Spacer) region of the rDNA (ribosomal DNA), and the D1 to D3 regions of the large ribosomal subunit (LSU) were combined with the sporangial morphology of *Pythium* spp. A clear evolutionary divergence was detected between clade K, clades A-D and clades E-J. Clade K represents *Pythium* spp. like *P. helicoides* that show similarities with the genus *Phytophthora* and form ovoid sporangia (Figure 2.6, A). The other *Pythium* spp. can be differentiated based on the shape of their sporangia, either being filamentous (clades A-D) (Fig 2.6, B-C) or globose (clades E-J) (Figure 2.6, F-H).

This stressed the importance of the sporangial shape as a taxonomic criterion, while characters like the size of sporangia and oogonia, ornamentation of oogonia, heterothallism, position of antheridia, number of antheridia, oospore wall thickness, appressoria and geographic distribution did not correlate with the *Pythium* phylogeny. Among the species with filamentous sporangia, *P. oligandrum* and *P. acanthicum* (clade D) form sporangia of the contiguous type, i.e. (sub) globose elements that are connected by hyphal segments (Figure 2.6, D-E). This clade was found in between the species with the filamentous and globose sporangia and appears to be an intermediate form.

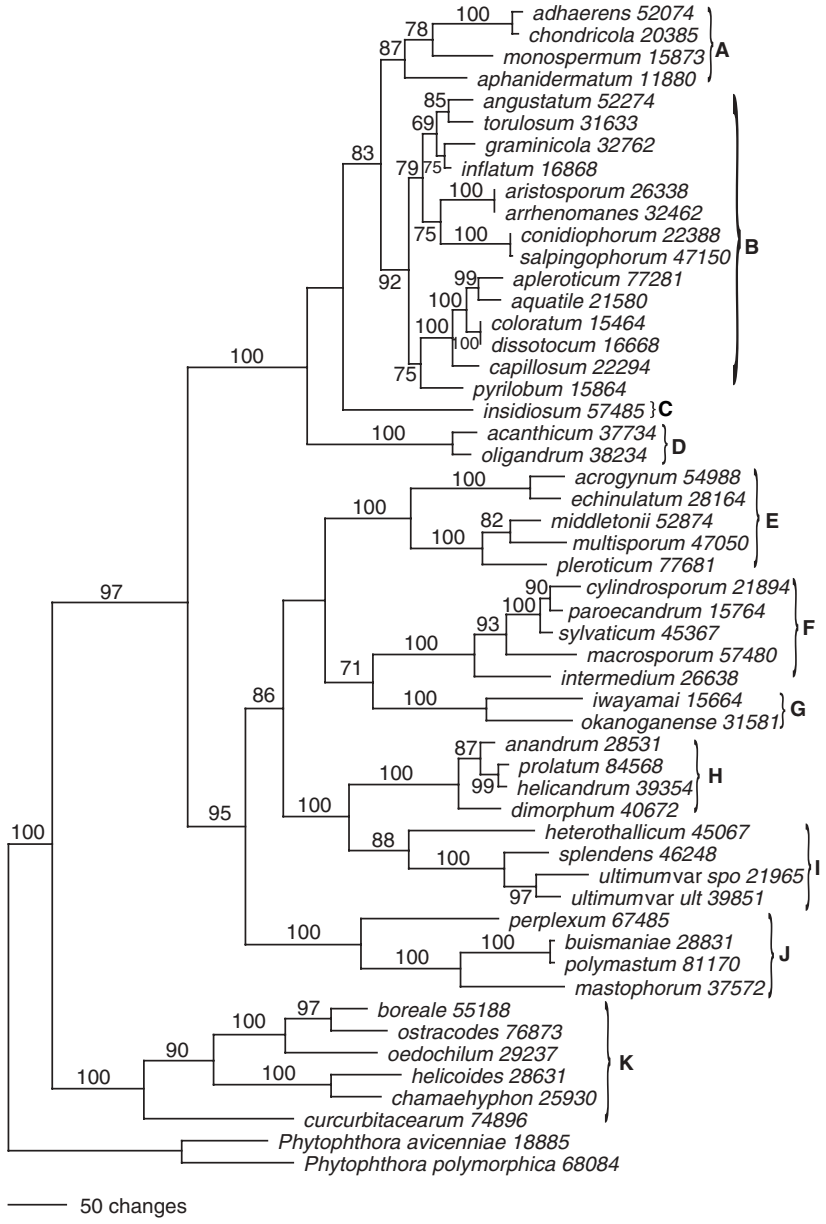

Figure 2.5. *Pythium* phylogeny based on the study by Lévesque and De Cock (2004).

In 2010, Uzuhashi et al. performed a phylogenetic analysis based on the D1/D2 region of the large ribosomal unit (LSU) and the cytochrome oxidase II gene region, and identified five monophyletic clades within the genus *Pythium*, each moderately to well supported by a specific sporangial shape (Figure 2.6). Based on these findings, the authors revised the *Pythium* taxonomy and described four new genera that were separated from the initially described genus including *P. monospermum*: 1) The genus *Ovatisporangium*, formerly known as *Pythium* species of clade K (Lévesque and De Cock, 2004), and characterized by sporangia of various shapes among which the ovoid type is the most common. 2) The genus *Pilasporangium*, based on one species (*P. apinafurcum*) with globose sporangia, strongly branching hyphae and oogonia that often contain two oospores. This species was not included in the study of Lévesque and De Cock (2004). 3) The genus *Globisporangium*, containing the former *Pythium* clades E, F, G, I and J (Lévesque and De Cock, 2004), and typically developing globose sporangia that resemble those of the genus *Pilasporangium*. 4) The genus *Elongisporangium*, originally classified as *Pythium* clade H (Lévesque and De Cock, 2004) and exhibiting large elongated clavate sporangia.

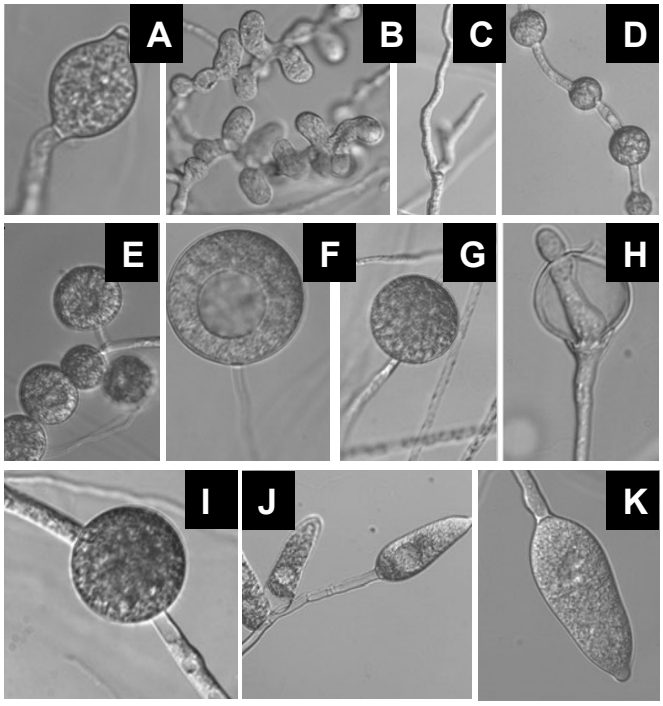

Figure 2.6. Sporangial shapes among *Pythium* species (Uzuhashi et al., 2010). A, Ovoid (genus *Ovatisporangium*), **B**, Filamentous-inflated (genus *Pythium*), **C**, Filamentous non-inflated (genus *Pythium*), **D-E**, Contiguous (genus *Pythium*), **F-G**, Globose (genus *Globisporangium*), **H**, Internally proliferating globose (genus *Globisporangium*), **I**, Intercalary globose (genus *Pilasporangium*), **J**, Elongated clavate (genus *Elongisporangium*), **K**, Terminal elongated clavate (genus *Elongisporangium*).

The present dissertation discusses species from clade 3 (Uzuhashi et al., 2010) or clades A-D (Lévesque and De Cock, 2004), i.e. the genus *Pythium*, which typically form filamentous sporangia with varying morphologies, including non-inflated, lobulate or toruloid inflated, or inflated with catenulate globose elements (Figure 2.6). Within this genus, *P. arrhenomanes*, *P. aristosporum*, *P. graminicola*, *P. inflatum*, *P. periilum*, *P. plurisporium*, *P. vanterpoollii* and *P. volutum* have been mostly isolated from monocots, predominantly grasses. They are characterized by moderately thick hyphae and moderate to high cardinal temperatures, with optimal growth temperatures of 25-30°C and maxima of 30-40°C (Lévesque and De Cock, 2004). Especially *P. aristosporum*, *P. arrhenomanes*, *P. graminicola*, *P. periilum* and *P. volutum* are pathogenic on several gramineous plants, like maize, surgarcane, barley and wheat (Van der Plaats-Niterink, 1981).

2.2.3. *Pythium* and rice

Pythium spp. have also been associated with yield decline in paddy fields, especially during cool and cloudy weather conditions that reduce plant vigor (Chun and Schneider, 1998; Cother and Gilbert, 1993). In most cases, *P. arrhenomanes* was identified as the most dominant and aggressive species. Water-seeded rice fields, i.e. continuous flooded fields in which seeds are directly sown by airplanes, have been suffering worldwide from seedling diseases caused by *Achyla*, *Fusarium* and *Pythium* spp. (Chun and Schneider, 1998). In Australia, *Pythium*-induced seedling death has been accounting for yield losses up to 50% (Cother and Gilbert, 1993). Several *Pythium* species were recovered from these affected fields, but *P. arrhenomanes* and *Pythium* group F were the most frequently isolated. *P. arrhenomanes* showed to be the most virulent species, causing pre- and post-emergence damping-off and severe stunting of rice seedlings, whereas *Pythium* group F appeared not pathogenic (Cother and Gilbert, 1993). Moreover, in approximately 50% of the South-Australian rice fields, *P. arrhenomanes* seemed to be present (Cother and Gilbert, 1992). *P. vanterpoolii* and *P. myriotylum* were also commonly isolated, but *P. vanterpoolii* appeared not pathogenic and the virulence of *P. myriotylum* strongly depended on the environmental conditions (Cother and Gilbert, 1993). In Japanese nursery beds, *P. arrhenomanes* isolates, which have been wrongly identified as *P. graminicola* or *P. aristosporum* by Kobori et al. (2004) and Furaya et al. (2003), also represented the most important constraints on seedling growth. One exceptional case has been described in California, where damping-off of wild rice was attributed to *P. tolurosum* (Marcum and Davis, 2006). However, it has to be noted that wild rice (*Zizania* spp.) is genetically different from the worldwide most cultivated rice species, *O. sativa* L..

Interestingly, the susceptibility of rice seedlings to *Pythium* spp. is plant age-dependent and sharply reduces within eight days after planting, a process that is faster and more abrupt for less aggressive species like *P. myriotylum* and *P. dissotocum* than the highly virulent *P. arrhenomanes* (Chun and Schneider, 1998).

2.3. Plant hormones and host immunity

2.3.1. Principles of the plant immune system

Because of their sessile characteristics, plants continuously encounter a vast array of biotic and abiotic stresses. Water, nutrient, light, temperature and salt stress are among the abiotic factors that can seriously lower plant fitness. Besides, pathogenic microorganisms, nematodes, parasitic plants and herbivores may pose serious constraints to plant growth (Sharma et al., 2013). Each of these attackers tries to establish a parasitic relationship with its host (Pieterse et al., 2009). Fortunately, disease is rather the exception than the rule and most microorganisms are excluded from the host's tissues by effective defense reactions (Kogel et al., 2006). Since each defense response is associated with an ecological fitness cost, plants have evolved a sophisticated and accurately regulated immune system (Pieterse et al., 2009).

The plant immune system is composed of several defense layers, of which the first includes preformed structural and chemical barriers (Bari and Jones, 2009). Pathogens that defeat these pre-invasive defense mechanisms, may be restricted by a second defense line involving the extracellular recognition of pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006), such as hyphal chitin, bacterial flagellin peptides and oomycete elicitins (Hein et al., 2009) (Fig 2.7). In addition, plants may respond to endogenous molecules like cell wall and cuticular fragments that are released by pathogen invasion (damage-associated molecular patterns or DAMPs) (Dodds and Rathjen, 2010). Biochemical dialogues between PAMPs/DAMPs and host-derived pattern recognition receptors (PRRs) activate phytohormone-mediated defense responses, leading to cell wall reinforcement, production of antimicrobial components, reactive oxygen species (ROS) and PATHOGENESIS-RELATED (PR) proteins, and eventually, the establishment of PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Pieterse et al., 2009). Induced mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades, and transcription factors are responsible for the signal transduction during these processes (Dodds and Rathjen, 2010). Unfortunately, pathogenic microorganisms have developed strategies to suppress PTI signaling and cause disease. By delivering effectors (virulence factors) in their host cell, pathogens may evade effective immune responses and gain access to inner tissues and host cell nutrients, a process called effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Bacterial effectors are directly secreted in the host cytoplasm through the type-III secretion system, while fungal and oomycete effectors are secreted by the endomembrane system and delivered into the target cell via unknown mechanisms (Dodds and Rathjen, 2010). On the other hand, plants may specifically recognize these effectors through direct or indirect interaction with nucleotide binding site-leucine rich repeat (NBS-LRR) proteins. This second manner of pathogen perception induces the more specific, qualitatively stronger and faster type of innate immunity, i.e. effector-triggered immunity (ETI), which is also known as 'gene-for-gene' resistance (Dodds and Rathjen, 2010; Jones and Dangl, 2006).

Since it often results in a hypersensitive cell death response (HR), gene-for-gene resistance is ineffective against necrotizing pathogens that kill host cells during colonization (Jones and Dangl, 2006). ETI signaling may be avoided or overcome when natural selection drives the pathogen to change its effector repertoire. But the host may also acquire new NBS-LRR alleles and in its turn, reactivate ETI (Jones and Dangl, 2006). Once ETI or PTI are triggered at the site of infection, a long-lasting and broad-spectrum systemic acquired resistance (SAR) may be induced in distal plant parts (Pieterse et al., 2009).

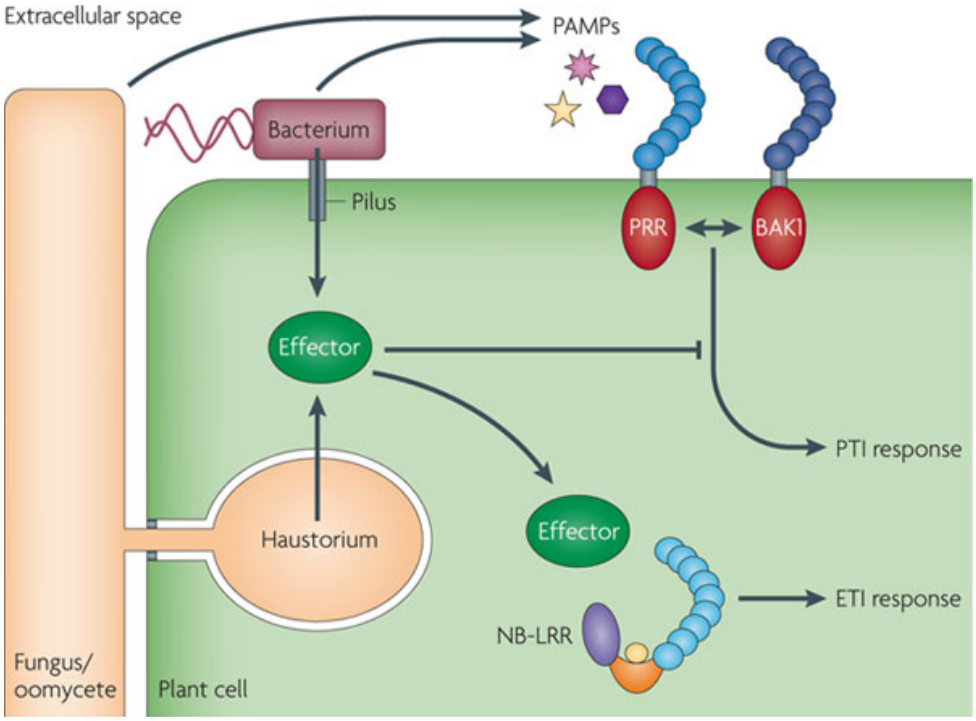

Figure 2.7. PTI and ETI signaling upon pathogen recognition (Dodds and Rathjen, 2010).

2.3.2. Hormone signaling cascades involved in pathogen-induced defense responses

2.3.2.1. The defense model in Arabidopsis

When plants are subjected to abiotic or biotic stress conditions, the concentration or perception of various plant hormones alters and this initiates a whole range of adaptive defense responses (Pieterse et al., 2009). Substantial progress has been made in the dissection of this immune signaling network, especially in light of leaf-pathogen interactions in the dicot model plant *Arabidopsis thaliana*. Salicylic acid (SA) and jasmonic acid (JA) with its bioactive derivatives (jasmonates (JAs)), have been identified as the backbone of the plant immune system.

Other hormones, including ethylene (ET), abscisic acid (ABA), auxins (AUXs), gibberellins (GAs), brassinosteroids (BRs) and cytokinins (CKs), are connected to this backbone and influence disease outcome by modulating its activity (Fig 2.8; Pieterse et al., 2012). Classically, the SA signaling pathway confers resistance to (hemi-)biotrophs, while JA/ET signaling protects plants from necrotizing pathogens (Glazebrook, 2005). SA and JA act mostly mutually antagonistic in plant defense, however, exceptions to this rule have been noted (Glazebrook, 2005; Mengiste et al., 2012; Pieterse et al., 2009, 2012). In addition to JAs, ET, AUXs and ABA are able to attenuate SA-dependent defense responses in Arabidopsis, whereas GAs and CKs stimulate SA signaling and the resistance to biotrophs (Pieterse et al., 2012). This network of cross communicating signaling pathways allows plants to quickly tailor their defense responses to the encountered microorganism in a cost-efficient manner (Pieterse et al., 2009). Besides optimizing immune responses to individual pathogens, the hormonal interplay enables to prioritize one pathway over the other during simultaneous or sequential attacks by several microorganisms (Pieterse et al., 2012).

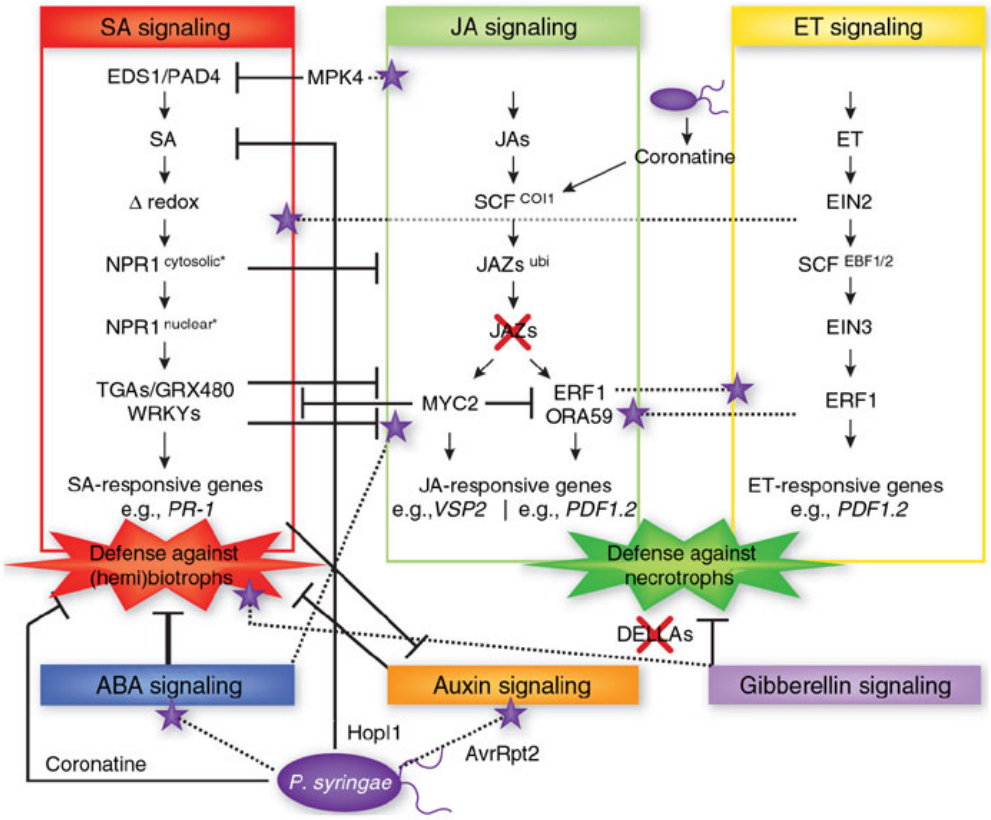

Figure 2.8. Hormonal pathways involved in the dicot immune response (Pieterse et al., 2009). Positive and negative regulatory actions are indicated by arrows and blunt ends, respectively.

2.3.2.2. Rice defense

Here, we will outline the current understanding of pathogen-induced hormone signaling in rice, with a focus on those pathways that are analyzed in chapters 4, 5 and 6. This information is solely based on rice leaf-pathogen interactions, since rice root defense is reviewed in chapter 7. We will include findings from Arabidopsis to provide more details on hormone biosynthesis and signaling components.

The SA pathway

The synthesis of the phenolic compound SA is activated upon PTI and ETI in Arabidopsis, and may proceed via two distinct enzymatic pathways involving the primary metabolite chorismate (Pieterse et al., 2012). In the PHENYLALANINE AMMONIA LYASE (PAL)-pathway, chorismate is first converted to phenylalanine, after which cinnamic acid is formed by the action of PAL and eventually SA is synthesized. The bulk of SA is produced via the ISOCHORISMATE SYNTHASE (ICS)-pathway in the chloroplast (Dempsey et al., 2011), in which chorismate is initially converted to isochorismate by ICS/SID2, which serves as a precursor of SA (Chen et al., 2009). In rice, SA is produced from cinnamic acid via benzoic acid (Silverman et al., 1995), but an isochorismate pathway via ISOCHORISMATE SYNTHASE1 (ICS1) is putatively involved as well (Qiu et al., 2007). Contrary to dicot leaves, rice leaves contain high endogenous levels of free SA that do not significantly alter upon pathogen attack and are suggested to protect rice leaves from oxidative stress (Yang et al., 2004). Nonetheless, rice responds to exogenous SA in an age-dependent manner and especially plant activators like benzothiadiazole (BTH) may induce a broad-spectrum resistance to several rice pathogens irrespective of their lifestyle (De Vleesschauwer et al., 2013).

The signaling downstream of SA in Arabidopsis is controlled by NONEXPRESSOR OF PR GENES1 (NPR1), which fulfills a central role as transcriptional co-activator of *PR* genes (e.g. *PR1*), the latter mostly encoding antimicrobial proteins (Pieterse et al., 2012). NPR1 mainly occurs as oligomers in the cytoplasm of resting plant cells, but when triggered by SA, the redox status of the cell changes and NPR1 monomers are formed. These monomers translocate to the nucleus where they interact with the TGA subclass of the basic leucine zipper family of transcription factors, which bind to the promotors of SA-responsive genes and activate their transcription. Afterwards, phosphorylated NPR1 is removed from the promotor of the target genes to allow their full induction and reinitiate the transcription cycle (Pieterse et al., 2012). Five *NRP1*-like genes have been identified in rice, among which *OsNPR1*, the *AtNPR1* ortholog, activates *PR* gene transcription and confers disease resistance to bacterial blight *(Xanthomonas oryzae* or *Xoo)* and blast (*Magnaporthe oryzae)* (De Vleesschauwer et al., 2013; Yuan et al., 2007). Arabidopsis-related research has also demonstrated that WRKY transcription factors function as transcriptional activators or repressors in SA signaling, with AtWRKY70 operating downstream of NPR1 (Bari and Jones, 2009; Pieterse et al., 2012).

In rice, SA signaling consists of an OsWRKY45- and an OsNPR1-dependent branch, which are both implicated in plant-activator induced resistance (De Vleesschauwer et al., 2013). Recent studies unraveled that OsWRKY45 is continuously degraded by the nuclear ubiquitin proteasome system (UPS) in resting cells, but upon chemical or pathogenic triggers, it starts to accumulate and induces the expression of target genes (De Vleesschauwer et al., 2013).

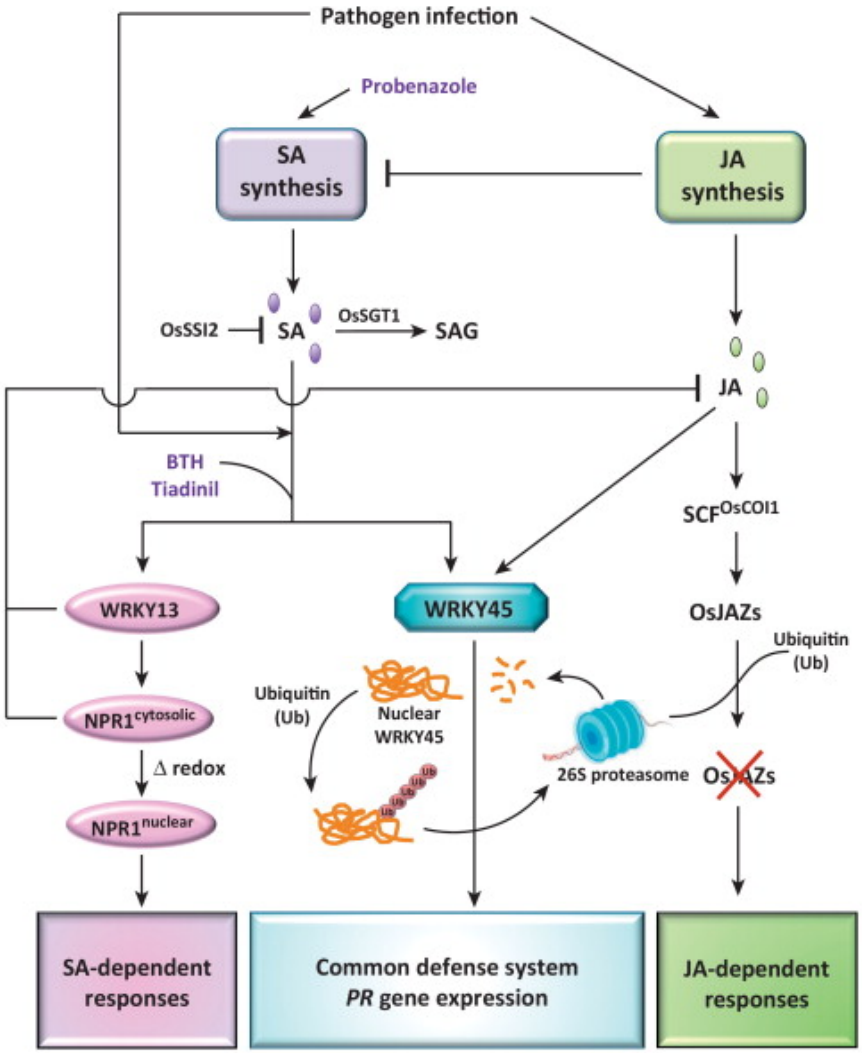

Figure 2.9. The SA signaling and JA signaling pathways in rice, and their role in plant immunity (De Vleesschauwer et al., 2013).

The JA pathway

JAs are lipid-derived hormones that are synthesized via the octadecanoid pathway in different cellular compartments (Turner et al., 2002). In Arabidopsis, the JA biosynthesis pathway starts with the peroxidation of α-linolenic acid in the chloroplasts by lipoxygenases (LOXs). Subsequent steps proceed in the plastids, where allene oxide synthase (AOS) and allene oxide cyclase (AOC) catalyze the conversion of (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT) to cis-(+)-12-oxophytodienoic acid (OPDA).

The latter compound is reduced to cyclopentanone (OPC-8) in the peroxisomes through the action of OPDA reductase (OPR3) and after three cycles of β-oxidation, this finally yields C_{12}-JA (Pieterse et al., 2012). Research with the dicot model plant also revealed that the JA biosynthesis pathway is controlled by a positive feedback loop, with JAs activating JA biosynthetic genes (Turner et al., 2002). The produced JA can be further metabolized to the bioactive methyl jasmonate (MeJA) via JA carboxyl methyltransferase (JMT) or conjugated to amino acids like isoleucine (JA-Ile) via JA conjugate synthase (JAR1) (Pieterse et al., 2012). Comparable molecular components play central roles in the JA biosynthesis pathway of rice (Lyons et al., 2013).

JA-dependent defense responses involve the large-scale reprogramming of several hundreds of genes (Yoshii et al., 2010), including *PR* genes (e.g. *PR10*) (Hashimoto et al., 2004). Research with the dicot model plant showed that these responses are controlled by the JA-Ile receptor, which consists of the F-box protein CORONATINE INSENSITIVE1 (COI1) and JASMONATE ZIM-domain (JAZ) transcriptional repressor proteins in the E3 ubiquitin ligase complex SCF^{COI1}. In presence of a bioactive JA signal, JAZ proteins are degraded, which triggers transcriptional activators like the basic helix-loop-helix leucine zipper protein MYC2 and activates the expression of many JA-responsive genes (Pieterse et al., 2012). JAs also rapidly activate the transcription of JAZ repressors, and this destruction and subsequent resynthesis of JAZs would reset the JA signaling pathway, avoiding a run-away response (Kazan and Manners, 2008). Next to a JA-induced MYC branch, JA signaling in Arabidopsis contains a JA/ET-induced branch controlled by APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors like ETHYLENE RESPONSE FACTOR1 (ERF1). While the ERF branch has been linked with resistance to necrotrophic pathogens, the MYC branch has been implicated in the wound response and resistance to herbivorous insects (Pieterse et al., 2012).

COI1, JAZ, AP2/ERF and MYC2 orthologs have also been identified in rice, and besides, NAC domain, WRKY and other transcription factors (e.g. *OsJAmyb*) regulate JA signaling in this monocot (Lyons et al., 2013). The NAC transcription factor RIM1 is degraded in response to JA and like MYC2, it controls the expression of early JA-responsive genes (Yoshii et al., 2010). Two other NAC transcription factors (ONAC122 and ONAC131) are also suggested to regulate JA biosynthesis or signaling in rice, seeing their positive effect on *OsLOX* expression and rice resistance to blast (Lyons et al., 2013). Whereas WRKY transcription factors are commonly associated with SA signaling in Arabidopsis, they often show a strong JA-responsiveness in rice (Lyons et al., 2013). This is clearly visible in *OsWRKY30*-overexpression lines that exhibit higher endogenous JA levels and increased resistance to *Rhizoctonia solani*. Aside from their activity against the (hemi-)biotroph *M. oryzae* and necrotroph *R. solani*, JA-dependent immune responses mediate rice defense to the (hemi-)biotroph *Xoo* (De Vleesschauwer et al., 2013). So, contrary to the situation in dicots, JAs represent powerful activators of a broad-spectrum resistance in rice (De Vleesschauwer et al., 2013).

The GA pathway and DELLA growth suppressors

Gibberellins (GAs) are diterpenoid plant growth regulators that are formed in the terpenoid pathway along with terpenes, terpene-derivatives and steroids (Grennan, 2006). Their synthesis in higher plants proceeds in three stages, each completing in different cellular compartments (Fig 2.10; Sakamoto et al., 2004). A first step involves the successive conversion of isoprenoids to geranylgeranyl diphosphate (GGDP), *ent*-copalyl diphosphate (CDP) and *ent*-kaurene, which takes place in the plastids and is catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). Stage 2 is characterized by the synthesis of GA_{12}-aldehyde from *ent*-kaurenoic acid via two membrane-associated cytochrome P450 monooxygenases: *ent*-kaurene oxidase (KO) located on the plastid envelope and *ent*-kaurenoic acid oxidase (KAO) occurring in the endoplasmatic reticulum. The biosynthetic pathway finally splits up in the cytosol, where the parallel early-13-hydroxylation and non-13-hydroxylation branches convert GA_{12} aldehyde to the bioactive GAs, GA_1 and GA_4. These oxidative reactions are catalyzed by GA 13-HYDROXYLASE (GA13ox), GA 20-OXIDASE (GA20ox) and GA 3β-HYDROXYLASE (GA3ox). For effective homeostatic regulation of bioactive GAs levels, GAs are metabolically deactivated by GA 2-OXIDASES (GA2ox) through 2β-hydroxylation (Sakamoto et al., 2004). Feedback regulation is largely restricted to the dioxygenase genes (*OsGA20ox2/SD1*, *OsGA20ox1* and *OsGA3ox2/D18*) and does not occur in the early steps of the GA synthesis pathway (Fan et al., 2007).

GAs promote gene expression and growth by the degradation of DELLA domain proteins (Robert-Seilaniantz et al., 2011). These suppressor proteins are key regulators of GA signaling and repress GA-dependent growth responses (Yang et al., 2012). Whereas Arabidopsis has five DELLA proteins (RGA, GAI, RGL1, RGL2 and RGL3), SLENDER RICE1 (SLR1) is the only DELLA of rice. In presence of bioactive GA, the soluble GA receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1) binds to SLR1, and promotes its SCF^{GID2}-mediated ubiquination and degradation via the proteasome pathway (Eckardt, 2007). Hence, downstream transcription factors like phytochrome interacting factors (PIFs) are released from SLR1-mediated suppression and activate the GA-responsive gene expression (Yang et al., 2012). Next to SLR1, rice SPINDLY and EARLY FLOWERING1 (EL1) negatively control GA signaling. The rice *SPINDLY* gene encodes an O-linked N-acetylglucosamine transferase that controls the suppressive function of SLR1, rather than changing the amount or stability of this protein (Shimada et al., 2006). On the contrary, the casein kinase 1 EL1 phosphorylates and stabilizes the rice DELLA and hence, sustains SLR1 activity (Dai and Xue, 2010).

In addition to their control of GA signaling, DELLAs are also involved in plant defense responses to biotic stresses. However, their immune regulatory activity differs among dicots and monocots. While DELLAs confer resistance to necrotrophs and susceptibility to biotrophs in Arabidopsis by modulating the SA-JA balance, SLR1 mediates rice immunity to (hemi-)biotrophic pathogens like *Xoo* and *M. oryzae* (De Vleesschauwer et al., 2013).

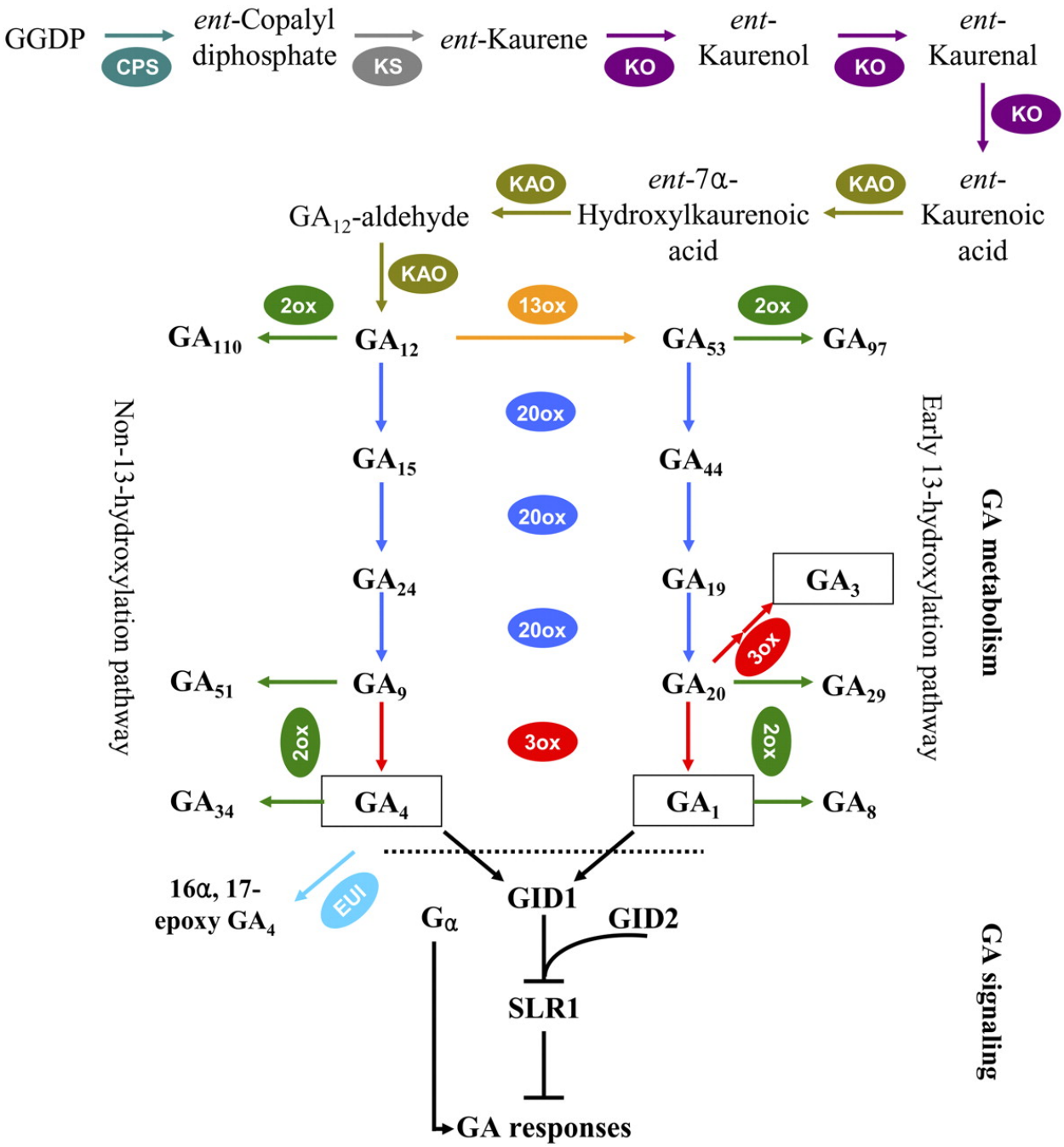

Figure 2.10. The GA biosynthesis and signaling pathway in higher plants (Ma et al., 2011).

The AUX pathway

AUXs regulate almost every aspect of plant growth and development. The biosynthesis of indole-3-acetic acid (IAA), the predominant AUX in plants, proceeds via tryptophan (Trp)-independent or Trp-dependent pathways. In the Trp-dependent IAA biosynthesis, Trp is first produced from chorismate via indole-3-glycerol phosphate in the chloroplasts. Several downstream pathways have been postulated: the indole-3-acetamide (IAM) pathway, the indole-3-pyruvic acid (IPA) pathway, the tryptamine (TAM) pathway and the indole-3-acetaldoxime (IAOx) pathway (Fig 2.11).

In the Trp-independent pathway, indole-3-glycerol phosphate or indole is likely the precursor of IAA. The major IAA biosynthesis pathways in plants are likely the Trp-dependent IAM and/or IPA pathways (Mano and Nemoto, 2012). In Arabidopsis, the primary route is the IPA-cycle, in which both TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) flavin monooxygenase-like proteins are required for the respective conversion of Trp into IPA and finally, IPA into IAA (Fig 2.11; Mashiguchi et al., 2011). In rice, 7 *YUCCA*-like genes have been identified, among which *OsYUCCA1* plays a major role in rice IAA biosynthesis (Yamamoto et al., 2007). In the first two steps of this proposed pathway, Trp is converted to tryptamine (TAM) and then, to N-hydroxytryptamine (NHT) by OsYUCCA1 in the cytoplasm. In the following steps, NHT is subsequently converted to IAOx, indole-3-acetonitrile (IAN) and eventually, indole-3-acetaldehyde (IAAld), from which IAA is synthesized (Fig 2.11; Yamamoto et al., 2007).

The F-box protein TRANSPORT INHIBITOR RESPONSE FACTOR1 (TIR1) functions as the AUX receptor in plants and controls the first step of the signaling downstream of AUXs (Bari and Jones, 2009). When TIR1 perceives AUXs, it forms an Aux/IAA-SCF^{TIR1} complex and induces the degradation of Aux/IAA suppressor proteins. These transcriptional repressors bind auxin response factors (ARFs) and in this way, prevent the interaction of ARFs with the promotor of AUX-responsive genes. During their regulation of plant growth and development, AUXs induce the expression of three gene families: *GRETCHEN HAGEN3 (GH3*), *small auxin-up RNA* (*SAUR*) and *Aux/IAA*. The *GH3* gene family is involved in the regulation of AUX homeostasis and encodes IAA-amido synthetases that conjugate excess of IAA (Bari and Jones, 2009). This gene family is also important for plant defense against fungal pathogens, since it lowers free levels of IAA and suppresses cell wall-loosening expansins (Bari and Jones, 2009). In dicots, AUXs induce susceptibility to biotrophic pathogens by repressing SA levels and blocking SA signaling (Bari and Jones, 2009). In parallel with these findings, rice research revealed the immune suppressive role of AUXs during rice leaf infections with *Xoo* and *M. oryzae*. The AUX-induced susceptibility in rice, however, is independent of SA and JA signaling, and seemed rather due to the activation of expansins that may have potentiated pathogen infection (De Vleesschauwer et al., 2013).

The BR pathway

BRs belong to the isoprenoid-derived class of plant growth regulators. Brassinolide (BL), the most biologically active BR in plants, is a C_{28} complex molecule mainly synthesized from campesterol in the endoplasmatic reticulum (Ashraf et al., 2010). Its biosynthesis in higher plants starts with the conversion of campesterol into campestanol (CN). Then, it splits up into the early and late C-6 oxidation pathways that form BL from castasterone (CS) via 7-oxolactonization.

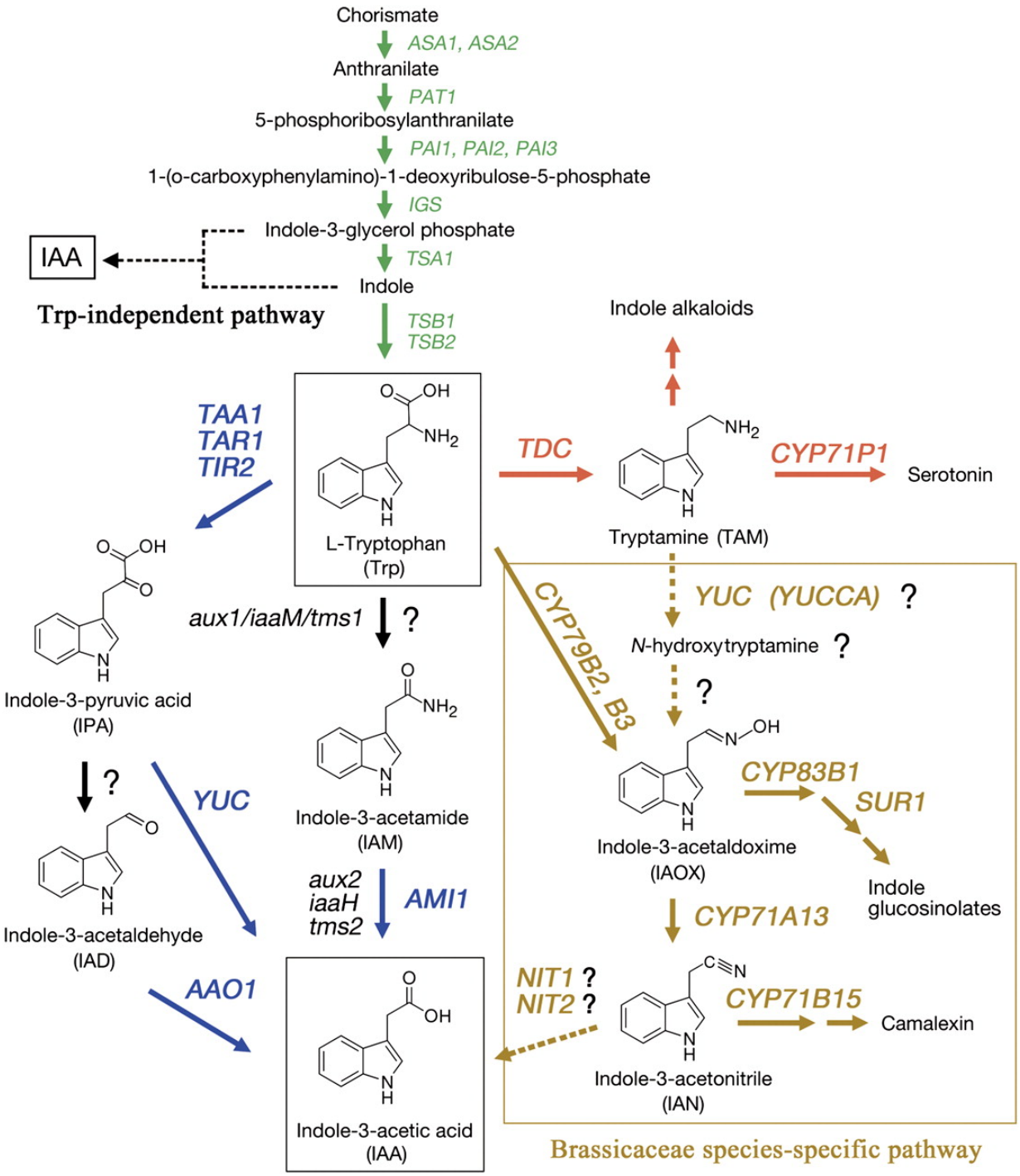

Figure 2.11. Presumptive pathways for IAA biosynthesis in plants (Mano and Nemoto, 2012).

In the early C-6 oxidation pathway, 6-oxoCN is initially formed from CN, which is subsequently converted to cathasterone (CT), teasterone (TE), 3-dehydroteasterone (3DT), typhasterol (TY) and finally, CS. In the late C-6 oxidation pathway, 6-deoxoCT is directly synthesized from CN and afterwards, successively converted to 6-deoxoTE, 3-dehydro-6-DT, 6-deoxoTY, 6-deoxoCS and CS.

These two co-existing oxidation pathways are linked to each other at multiple stages, thereby forming the very intricate BR biosynthetic network as has been observed in both Arabidopsis and rice (Ashraf et al., 2010). In addition, the early C-22 oxidation pathway and a shortcut pathway from campesterol to 6-deoxoTY involving C-23 oxidation, have been reported to participate in the biosynthesis of BRs (Ashraf et al., 2010). The several oxidation steps in these pathways are catalyzed by cytochrome P450 monooxygenases, like DWARF4 (DWF4), CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) and BRASSINOSTEROID-6-OXIDASE (BR6ox) (Divi and Krishna, 2009). Rice orthologues exist and are as well implicated in BR biosynthesis. Like BR6ox in Arabidopsis, OsDWARF links the early and late C-6 oxidation pathways in rice (Fujioka and Yokota, 2003). Other P450 proteins involved in rice BR biosynthesis include DWARF2 (D2) and DWARF 11 (D11). While D2 catalyzes the steps from 6-deoxoTE to 3-dehydro-6-DT and from TE to 3DT (Hong et al., 2003), D11 is suggested to control the supply of 6-deoxoTY and TY (Tanabe et al., 2005).

Molecular studies in both Arabidopsis and rice have provided a detailed model of the signaling downstream of BL (Fig 2.12). BL is recognized at the plasma membrane by BRASSINOSTEROID-INSENSITIVE1 (BRI1). Binding of BL autophosphorylates BRI1 and induces its homodimerization with BRI1-ASSOCIATED KINASE1 (BAK1), a member of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family (Robert-Seilaniantz et al., 2011). BRI1 regulates the phosphorylation level of BAK1 that, in its turn, transphosphorylates BRI1 and increases its kinase activity towards brassinosteroid signaling kinases (BSKs). BRI1 activation also involves the dissociation of BRI1 kinase inhibitor 1 (BKI1) that prevents the interaction between BRI1 and BAK1 under low BR conditions (Ashraf et al., 2010). Downstream of BRI1 and BAK1 acts *bri1* suppressor 1 (BSU1) phosphatase, which suppresses the GLYCOGEN SYNTHASE KINASE-3 (GSK3)-like KINASE BRASSINOSTEROID-INSENSITIVE2 (BIN2) and dephosphorylates the nuclear transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and BRASSINAZOLE-RESISTANT2 (BZR2)/ BRI1-EMS-SUPPRESSOR1 (BES1). BZR1 regulates BR cellular homeostasis by feedback inhibition of BR-biosynthetic genes (Ashraf et al., 2010). It binds to the CGTG(T/C)G motif in the promoters of *CPD* and *DWF4* to suppress their expression, while BES1 binds to the CANNTG motif (E-box) in the *SAUR-AC1* promoter to activate BR-responsive gene expression (Divi and Krishna, 2009). Under low BR conditions, BIN2 phosphorylates BZR1 and BZR2/BES1, and inhibits their transcriptional activity (Ashraf et al., 2010). The phosphorylated BZR1 is then captured by a 14-3-3 protein, and degraded by the proteasome (Nakagawa et al., 2011). In rice, *OsBRI1*, *OsBAK1*, *OsBZR1*, *OsGSK1* and 14-3-3-like genes have been identified (Nagakawa et al., 2011), and negative feedback regulation of biosynthetic genes appears to be conserved (Hong et al., 2003). In addition to the OsBRI1 pathway, the rice heterotrimeric G protein alfa 1 (RGA1) is implicated in BR signaling (Fig 2.12). Unlike OsBRI1, RGA1 is not involved in the feedback regulation of BR biosynthesis. Its role in the BR signal response may rather exist in the modulation of the BR signal response and its coordination with other signaling pathways, such as those associated with ABA, GA and AUX (Nakagawa et al., 2011).

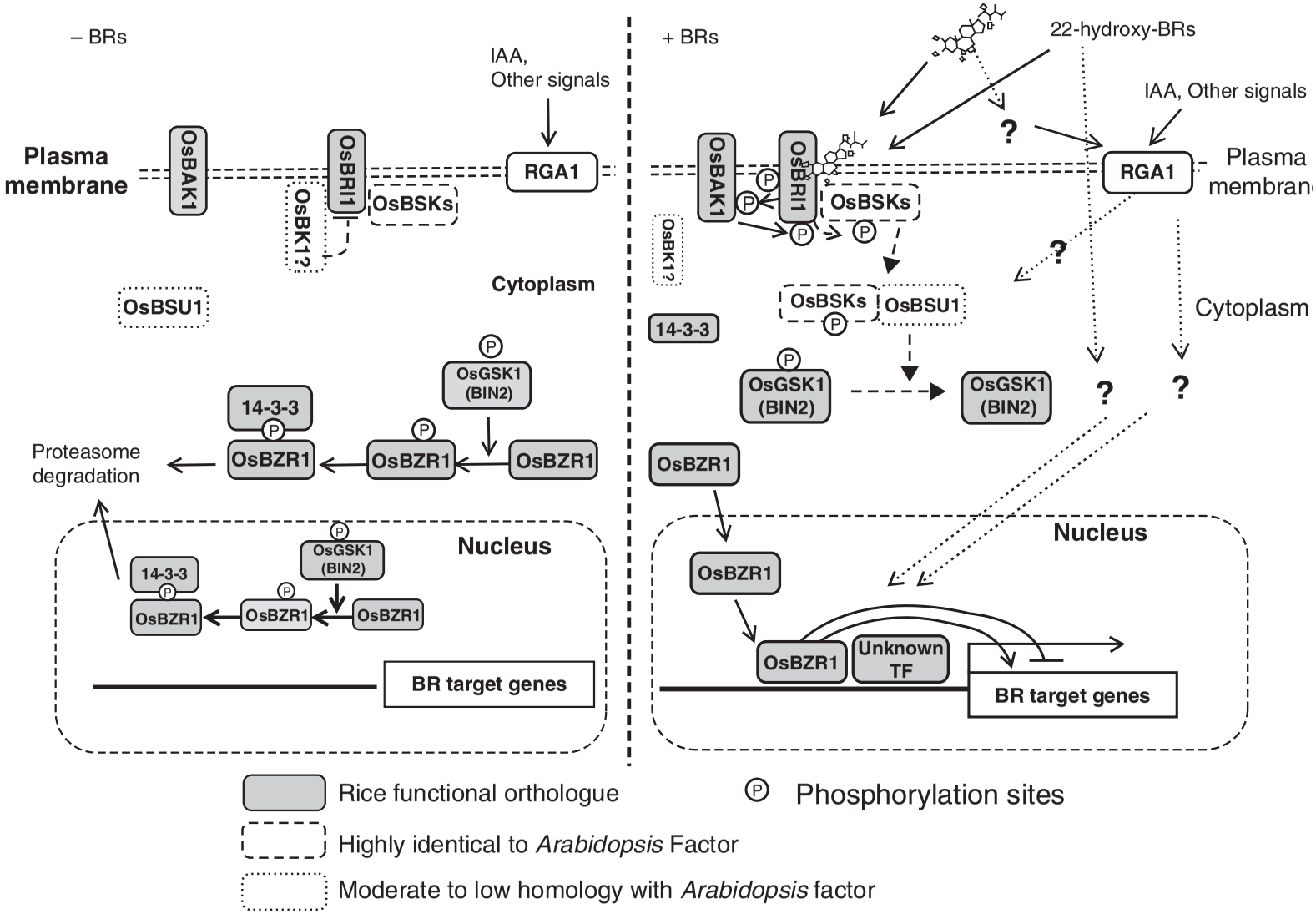

Figure 2.12. Brassinosteroid signal transduction pathway in rice under low (left) and high (right) BR conditions (Nakagawa et al., 2011).

The B3-domain protein RAV-LIKE1 (OsRAVL1) represents another BR signaling factor, which activates both *OsBRI1* and BR-biosynthetic genes and thereby ensures the basal activity of endogenous BRs. Besides, three SVP-group MADS-box genes like *OsMADS55* negatively regulate the BR response (Tong and Chu, 2012). The BU1 protein on the contrary, is a positive regulator that functions in both the OsBRI1 and RGA1 pathway (Tong and Chu, 2012).

Components of the BR response pathway also fulfill important tasks in plant defense. Work in Arabidopsis demonstrated that BAK1 serves as a co-receptor for BRI1 and FLS2, the latter being the receptor for bacterial flagellin (flg22) and activator of microbial-associated molecular pattern (MAMP)-triggered immunity (Wang et al., 2012). BRs can act both antagonistically or synergistically with responses to MAMPs and so, they effectively regulate tradeoffs between growth and immunity (Wang et al., 2012). BRs have also been noted to induce a broad-spectrum disease resistance in tobacco and rice, a process proceeding through an SA-independent pathway (De Vleesschauwer et al., 2013).

Other hormonal signaling pathways involved in rice defense

ET is one of the classical defense hormones and its signaling pathway has often been found to cooperate with JA signaling in the defense of dicots against necrotrophic pathogens (Pieterse et al., 2012). Opposing the SA and JA/ET antagonism in Arabidopsis, rice immune responses to leaf pathogens are mainly mediated by SA and JA, with ET fulfilling either a positive or negative immune regulatory task. A comparable ambiguous role in rice immunity seems to be true for ABA and CKs (De Vleesschauwer et al., 2013). For more details on these hormonal signaling pathways and an overview of the rice defense model, we refer the reader to the reviews of De Vleesschauwer et al. (2013), Yang et al. (2013) and Pieterse et al. (2012).

Chapter 3

Identity and variability of Pythium species associated with yield decline in aerobic rice cultivation in the Philippines

Van Buyten E, Banaay CGB, Vera Cruz C, Höfte M, 2013. *Plant Pathology* **62(1)**, 139-153.

Abstract

The cultivation of aerobic rice in the tropics enables farmers to save water without lowering productivity. Unfortunately, this system suffers from declining yields due to a disease complex, involving nematodes, pathogenic *Pythium* spp. and nutrient deficiencies. Assessing the impact of each underlying factor contributes to efficient disease control measures. This study therefore investigated pathogenic and genotypic variability among *Pythium* species from affected aerobic rice fields in the Philippines using pathogenicity assays and sequence information of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and *β-tubulin* gene. *Pythium* isolates were accurately identified to species level, using the available sequences of *Pythium* reference strains. Three closely related *Pythium* spp., *P. arrhenomanes, P. graminicola* and *P. inflatum*, were recovered from affected aerobic rice fields. All *P. arrhenomanes* isolates reduced rice seedling growth, whereas only few *P. graminicola* isolates and no *P. inflatum* isolates were pathogenic, indicating that *P. arrhenomanes* is probably the most important species affecting rice. Both *P. arrhenomanes* and *P. graminicola* isolates showed little genetic variation, despite the observed pathogenic variation within *P. graminicola*. Intraspecific variation was higher among *P. inflatum* isolates, but again no correlation was observed with phenotype. When screening *P. arrhenomanes* isolates from other hosts like sugarcane, maize and several grasses, a link between pathogenic and genetic variability was detected. However, rice and maize isolates seemed to lack host specificity and therefore, crop rotation with maize might be a risky strategy to manage yield decline in Philippine aerobic rice fields.

3.1. Introduction

The high-yielding rice cultivation in flooded 'paddy' fields is severely threathened by decreasing water supplies and this poses a serious threat to the future food security of billions of people (see Chapter 1). To face these problems, the International Rice Research Institute (IRRI) in the Philippines and the Upland Rice Laboratory of the Chinese Agricultural University (CAU) have developed drought-tolerant rice varieties (Atlin et al., 2006; Bouman et al., 2002) that can be grown, like maize and wheat, in non-flooded fields. An aerobic rice growing system has been engineerd for temperate regions like China, where it enables farmers to save irrigation water and reduce water losses without lowering productivity. Unfortunately, the system fails under tropical conditions (see Chapter 1). Tropical and aerobic growth conditions favor soil-borne pathogens that do not usually harm paddy fields. Continuous monocropping in aerobic rice fields can therefore lead to a build-up of these deleterious organisms, which, along with other unfavorable edaphic changes, may eventually cause progressive yield decline (George et al., 2002; Kreye et al., 2009a; Peng et al. 2006). Beside nematodes, other soil-borne pathogens, such as *Pythium*, *Fusarium* and *Rhizoctonia*-like spp., have been isolated from affected aerobic rice fields in the Philippines (Kreye et al., 2009b). In particular *Pythium* spp. have been implicated as serious threats to rice seedling growth (Furuya et al., 2003) and their occurrence in rice cultivation has been reported around the globe (see Chapter 1). Therefore, our hypothesis was that *Pythium* spp. might significantly affect aerobic rice cultivation in the Philippines.

The current chapter aimed to identify the *Pythium* spp. associated with growth reduction in Philippine aerobic rice fields and to disentangle their relationship with the occurring yield decline. Using DNA sequencing of the ITS region of the rDNA and *β-tubulin* gene and pathogenicity assays, the genetic and phenotypic variability among the collected *Pythium* spp. was assessed and their impact on rice growth was evaluated. In addition, we investigated possible links between intra-specific variation and host-specialization on maize, one of the options for crop rotation in aerobic rice fields.

3.2. Methods

3.2.1. *Pythium* collection

Rice roots were collected on the island of Luzon, the Philippines, from four aerobic rice fields (plots) showing yield decline (PB9-11, PB9-12, PT and PBTP). Soils in plots PB9-11 and PB9-12, situated on the IRRI experimental farm in Los Baños, Laguna, have clay-loam characteristics, whereas plots PT and PBTP, situated in the more northern provinces of Tarlac (Dapdap) and Bulacan, respectively, contain soil with loamy-sand characteristics (Kreye et al., 2009a). Plots PB9-11, PB9-12 and PT were monocropped with drought-tolerant rice variety Apo (NSIC Rc9) and during the wet season, transplanting was preferred to direct seeding. In contrast, plot PBTP was continuously transplanted with drought-tolerant rice variety NSIC Rc192 (IR74371-54-1-1).

Fields at the IRRI site were sampled from equidistant points along an imaginary five-armed zigzag line covering the entire sampling area (50 m × 50 m) at seedling, tillering, panicle initiation and flowering stages during the dry seasons of 2005, 2006, 2008 and 2009 and the wet seasons of 2008 and 2009. This resulted in 10 root samples per arm of the zigzag pattern and a total of 50 samples at each sampling time point. Similarly, a total of 48 samples was collected from 16 bunded subplots (6 m × 6 m) in the experimental plot in Tarlac (PT) during the dry season of 2007 (Kreye et al., 2009b). The transplanted aerobic rice field sampled in Bulacan was not an experimental plot but a farmers' field and therefore, only rice plants showing retarded shoot growth or few tillers were sampled at panicle initiation.

Roots of all plant samples were screened for chocolate brown lesions. These lesions were excised, cut into 0.5-1.0 cm lengths, and subsequently surface-sterilized in 5% NaOCl for 1 min and washed three times in sterile distilled water. Root sections were then blotted dry using sterile paper towels, plated out on potato dextrose agar (PDA) or water agar amended with 200 mg L^{-1} streptomycin sulphate and incubated at 28°C until mycelial growth was observed three days after plating. Single hyphae growing from the plated root pieces were cut from the agar using a binocular. The hyphal tips were subsequently transferred to PDA with 200 mg L^{-1} streptomycin sulphate for purification. *Pythium* isolates were predominantly recovered from rice plants showing reduced growth and sampled at seedling or tillering stage. Ninety-nine isolates out of a total of 115 (Table 3.1) were sent to the Laboratory of Phytopathology, Ghent University in Belgium, for identification. The isolates were divided into eight morphotypes (Figure S3.1). The frequency of each species in a specific field or year was analyzed with the Fisher's exact test (Kirkman, 1996). The correlation between species and sampled field/year was considered statistically significant if the *P*-value of the contingency table was <0.05.

In addition to the Philippine rice isolates, 11 *P. arrhenomanes* and *P. graminicola* isolates from other hosts and geographic origins were retrieved from the CBS Fungal Biodiversity Centre (Utrecht, The Netherlands) or provided by K. Kageyama, M. Tojo and J. Nechwatal (Table 3.2).

3.2.2. Molecular identification

The 110 collected or requested *Pythium* isolates (Tables 3.1, 3.2) were cultured in 50 ml of potato dextrose broth (PDB) at 28°C in the dark for 7 days. Mycelial mats were subsequently harvested by filtration, blotted dry, frozen in liquid nitrogen and crushed using a sterile mortar and pestle. DNA extraction was conducted with a DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions and *Pythium* isolates were identified to species by sequencing the ITS region of the rDNA and the middle part of the *β-tubulin* gene (658 bp out of ±1650 bp).

Table 3.1. Origin, GenBank accession numbers and MUCL collection numbers of *Pythium* isolates collected in affected aerobic rice fields in the Philippines.

						GenBank Accession no.			
Field [a]	Location	Year	*Pythium* spp.	No. of isolates	Isolate no.[b]	Selected isolate	ITS of rDNA [c]	β-tubulin	Collection no.
PB9-11	The Philippines, Los Baños (IRRI)	2008	*P. graminicola*	10	**PB911 120,122,133-140**	PB911 122	HQ877861	HQ877845	MUCL53740
			P. inflatum	1	PB911 119B	PB911 119B	HQ877869	HQ877853	MUCL53746
PB9-12	The Philippines, Los Baños (IRRI)	2005	*P. graminicola*	2	**PB912** 7, **16**	PB912 16	HQ877860	HQ877860	MUCL53739
			P. inflatum	1	**PB912 6**	-	-	-	-
		2006	*P. arrhenomanes*	6	**PB912 75, 76**, 78, 80, **83**, 79B	PB912 75	HQ877857	HQ877846	-
						PB912 80	HQ877859	-	-
			P. inflatum	1	PB912 82	-	-	-	-
		2008	*P. catenulatum*	1	PB912 87	PB912 87	HQ877862	HQ877851	MUCL53733
			P. graminicola	3	**PB912 97, 116, 132**	PB912 116	HQ877865	-	-
						PB912 132	-	-	MUCL53742
			P. inflatum	27	PB912 84, 86, 89-91, 93, **98**,	PB912 90	HQ877868	-	MUCL53743
					99, 101, 102, 104, **105**, 106-109, **110**,	PB912 93	HQ8778673	-	MUCL53744
					111-114, 117, 118-119, 129-131	PB912 102	HQ877866	-	MUCL53804
						PB912 105	HQ877855	-	MUCL53747
						PB912 110	HQ877854	-	MUCL53748
						PB912 117	-	HQ877843	MUCL53745
			Unspecified *Pythium* strains	8	PB912 85, 88, 94-96, 96B, 100, 115	PB912 85	-	HQ877850	MUCL53803
						PB912 88	-	HQ877852	MUCL53734
		2009	*P. arrhenomanes*	5	**PB912 09-3, 09-6**, 09-8, **09-10, 09-15**	PB912 09-6	HQ877864	-	MUCL53735
			P. graminicola	1	PB912 T1E	PB912 T1E	HQ877863	-	MUCL53741
PBTP	The Philippines, Bulacan	2009	*P. inflatum*	2	PBTP 10, 42	PBTP 42	-	HQ877844	MUCL53749
PT	The Philippines, Tarlac (Dapdap)	2007	*P. arrhenomanes*	30	**PT 18, 23, 25**, 26, 28, **32, 36, 37**, 38,	PT 28	HQ877858	-	MUCL53736
					39-41, 43, 44, 46, 50, 53, **54, 57, 58,**	PT 60	-	-	MUCL53737
					60, 62, **64, 68, 70, 74,** T5, T6, T12, T14	PT T12	-	-	MUCL53738
			P. inflatum	1	PT 52	PT 52	HQ877856	HQ877842	MUCL53750

[a]All *Pythium* isolates were obtained from rice cv. Apo except in PBTP where isolates were obtained from cv. IR74371-54-1-1.

[b]PB911, PB912, PBTP and PT represent isolates recovered from plots PB9-11, PB9-12, PBTP and PT in the Philippines, respectively. Isolate no. is marked in bold if screened *in vivo* for pathogenicity towards rice cultivar Apo. *In vitro* pathogenicity trials were conducted with all isolates, except *P. inflatum* isolates PB912 101, 102, 104, 106, 108, 111–114, 118, 119.

[c]ITS sequences of PB912 85 and PB912 88 contained a lot of dimorphic positions and therefore, we only sumitted their β-tubulin sequences to GenBank.

Amplification of the ITS region of the rDNA was performed with forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). For the β-tubulin gene, forward primer BT5 (5'-GTATCATGTGCACGTACTCGG-3') and reverse primer BT6 (5'-CAAGAAAGCCTTACGACGGA-3') were used (Villa et al., 2006). The PCR reaction volume of 50 µl contained 5 µl Q-buffer (QIAGEN), 1 µl dNTPs (10 mM; Fermentas), 10 µl Q-solution (QIAGEN), 3.5 µl of each primer (10 mM), 0.3 µl Taq DNA polymerase (5 U μl^{-1}; Fermentas), 22.7 µl sterile milli-Q water and 4 µl of DNA template. Filled PCR tubes were placed in a thermal cycler (Eppendorf Mastercycle gradient) and the following PCR profile was set: denaturation for 10 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C (ITS1 and ITS4) or 57°C (BT5 and BT6) and 1 min at 72°C, and a final extension step of 10 min at 72°C. Finally, PCR products were purified using the QIAGEN PCR purification kit following the manufacturer's instructions and DNA concentrations were determined with the help of a ND-1000 Spectrophotometer (NanoDrop).

Table 3.2. Additional *Pythium* isolates and GenBank *Pythium* sequences used in this study.

					GenBank Accession no.		
Pythium spp.	Isolate	Host plant	Geographic origin	Year	ITS of rDNA	β-tubulin	Reference
P. angustatum	CBS522.74	Soil	The Netherlands	-	AY598623	-	GenBank
P. aphanidermatum	P36-3	Bent grass	Korea	-	-	DQ071295	GenBank
	CBS118.80	-	-	-	AY598622	-	GenBank
P. aristosporum	PRR115	Bent grass	USA, New York	-	AB160843	DQ071298	GenBank
	CBS263.38	Wheat	Canada	1930	AY598627	-	CBS [a]
P. arrhenomanes	KU9BGP	Bent grass	Japan, Shizuoka	-	AB160840	-	GenBank
	P21-1	Bent grass	Korea	-	AB160841	-	GenBank
	PRR5	Bent grass	USA, New York	-	AB160842	-	GenBank
	ATCC96525	Bermuda grass	USA, Hawaii	-	AB095041	DQ071300	GenBank
	CBS324.62	Maize	USA	1928	AY598628	-	GenBank
	CBS430.86	Maize	The Netherlands	1986	HQ877870	HQ877847	CBS [a]
	Me1b	Soil, maize field	Germany, Hegne	2006	-	-	J.Nechwatal [a]
	Me8a	Soil, maize field	Germany, Wahlwies	2006	-	-	J.Nechwatal [a]
	Me9d	Soil, maize field	Germany, Wahlwies	2006	-	-	J.Nechwatal [a]
	(No name)	Rice	Japan	2004	AB513368	-	GenBank
	(No name)	Rice	Japan	2008	AB513367	-	GenBank
	P54	Reed	Germany, Egg	2003	AY743661	EU152856	GenBank
	(No name)	Soil	Brazil	2009	AB562919	-	GenBank
	ATCC96526	Sugarcane	USA, Louisiana	-	AB095039	DQ071301	GenBank
	ATCC96598	Sugarcane	USA, Louisiana	-	AB160838	DQ071302	GenBank
	CBS293.32	Sugarcane	Mauritius	1932	HQ877871	HQ877848	CBS [a]
	CBS325.62	Sugarcane	USA	1935	HQ877872	HQ877849	CBS [a]
	E-1	Sugarcane	Japan, Kagoshima	-	AB095040	-	GenBank
	TMF	Sugarcane	Japan	-	AJ233444	-	GenBank
	32-1/NBRC100102	Zoysia grass	Japan, Hyogo	-	AB160844	DQ071299	GenBank
	ADO-6-1	Zoysia grass	Japan, Gifu	-	AB160839	-	GenBank
P. catenulatum	CBS842.68	Turf grass	USA	1948	AY598675	-	GenBank
	NBRC100104	Zoysia grass	Japan, Gifu	-	Y18164	DQ071303	GenBank
P. conidiophorum	CBS223.88	Soil	UK	-	AY598629	-	GenBank
P. deliense	CBS314.33	*Nicotiana tabacum*	Sumatra	-	AY598674	-	GenBank
	MAFF305568	Pumpkin	Japan, Okinawa	-	-	DQ071305	GenBank
P. dissimile	CBS155.64	*Pinus radiata*	Australië	-	AY598681	-	GenBank
P. folliculosum	CBS220.94	Soil	Switzerland	-	AY598676	-	GenBank
P. graminicola	IFO31998 [b]	Barley	Japan, Fukui	-	AB217664	DQ071308	GenBank
	OPU480 [c]	*Oryza* spp.	Japan, Shiraoko-cho	2001	AY099310	-	M. Tojo [a]
	Ohmagari [c]	Rice	Japan, Akita	-	-	-	K.Kageyama [a]
	Pma-2 [c]	Rice	Japan, Iwate	-	-	-	K.Kageyama [a]
	MAFF305860	Soil	Japan, Kumamoto	-	AB160837	DQ071309	GenBank
	MAFF425415	Soil, maize field	Japan, Kumamoto	-	AB160836	-	GenBank
	ATCC96234	Soil, maize field	Japan, Kumamoto	-	AB095045	DQ071307	GenBank
	ATCC96600	Sugarcane	USA, Louisiana	-	AB095044	-	GenBank
	CBS327.62	Sugarcane	Jamaica	-	AY598625	-	CBS [a]
	(No name)	Soil	Brazil	2004	AB562908	-	GenBank
P. inflatum	MAFF305863	Soil	Japan	-	AJ233446	DQ071313	GenBank
	CBS168.68	Sugarcane	USA, LA	1966	AY598626	-	GenBank
P. myriotylum	CMR1	Cocoyam	Cameroon	-	DQ222432	-	GenBank
	GF46	Kalanchoe	Japan, Gifu	-	AB095051	DQ071324	GenBank
	ATCC26082	Spinach	Japan, Osaka	-	AB095047	DQ071322	GenBank
	CBS254.70	*Arachis hypogea*	Israël	-	AY598678	-	GenBank
P. periilum	CBS169.68[d]	Soil	USA	-	AY598683	-	GenBank
P. plurisporium	CBS100530	*Agrostis palustris*	USA	-	AY598684	-	GenBank
P. pyrilobum	CBS158.64	*Pinus radiata*	Australië	-	AY598636	-	GenBank
	IFO32560	Bent grass	Japan, Osaka	-	-	DQ071334	GenBank
P. salpingophorum	CBS471.50	*Lupinus angustifolius*	Germany	-	AY598630	-	GenBank
P. scleroteichum	CBS294.37	*Ipomoea batatas*	USA	-	AY598680	-	GenBank
P. sulcatum	CBS603.73	Carot	USA	-	AY598682	-	GenBank
	CTMa7	Carrot	Japan, Gifu	-	-	DQ071337	GenBank

Table 3.2. (Continued)

P. torulosum	CBS316.33	Grass roots	The Netherlands	-	AY598624	-	GenBank
	NBRC100120	Zoysia grass	Japan, Hyogo	-	-	DQ071339	GenBank
	PRR3	Bent grass	USA, New York	-	-	DQ071341	GenBank
P. tracheiphilum	CBS323.65	*Lactuca sativa*	Italy	-	AY598677	-	GenBank
P. vanterpoolii	CBS295.37	*Triticum sativum*	UK	-	AY598685	-	GenBank
	P39-1	Bent grass	USA, New York	-	-	DQ071343	GenBank
	PRR16	Bent grass	Korea	-	-	DQ071344	GenBank
	UOP392	Zoysia grass	Japan	-	-	DQ071345	GenBank
P. volutum	CBS699.83	*Triticum* and *Hordeum*	Japan	-	AY598686	-	GenBank
	IFO31926	Wheat	Japan, Fukui	-	-	DQ071348	GenBank
P. zingiberis	CBS216.82	*Zingiber mioga*	Japan	-	AY598679	-	GenBank

ATCC: American Type Culture Collection; CBS: Centraalbureau voor Schimmelcultures; IFO: Institute for Fermentation (Osaka); of Agriculture: Forestry and Fisheries; NBRC: NITE Biological Resource Center.

[a]Isolates sequenced in the current paper. The isolates were retrieved from the CBS Fungal Biodiversity Centre (Utrecht, The Netherlands) or provided by K. Kageyama, M. Tojo and J. Nechwatal. β-tubulin sequences of CBS327.62 and CBS263.38 were identical to sequences with accesion no. DQ071298 and HQ877860/HQ877845 (see Table 3.1.), respectively, and have not been submitted to GenBank.

[b]Isolates identified as *P. vanterpoolii*-related in the current paper.

[c]Isolates identified as *P. arrhenomanes* in the current paper.

[d]Isolates identified as *P. graminicola* in the current paper.

Sequencing was performed by the Flemish Institute for Biotechnology (VIB; Antwerp, Belgium) and LGC Genomics (Berlin, Germany). After editing the output data with the BioEdit 7 software package, the resulting sequences were compared to GenBank using MEGABLAST for nucleotide queries. In that way, the obtained sequences, consisting of the ITS1 region, 5.8S subunit and the ITS2 region of the rDNA, or 658 bp of the *β-tubulin* gene, were linked with specific *Pythium* species based on their reference strains. To delimit species, the Phylogenetic Species Concept (PSC), Morphological Species Concept (MSC) and Ecological Species Concept (ESC) were taken into account (Giraud et al., 2008). Isolates with a similar morphology, pathogenicity and ITS or β-tubulin sequence, were considered to represent one species. When sequence identity with each of the reference strains was 97% or less and phenotypic differences were observed, isolates were classified as an unknown species. Ex-type strains CBS100530, CBS263.38 and CBS324.62 were used as reference strains to identify *P. plurisporium*, *P. aristosporum* and *P. arrhenomanes* isolates, respectively. Other reference strains were *P. inflatum* CBS168.68, *P. periilum* CBS169.68, *P. myriotylum* CBS254.70 and *P. catenulatum* CBS842.68, and were used for description in the monograph of van der Plaats-Niterink (1981). The β-tubulin sequences of the listed reference strains were not available, except for *P. aristosporum* strain CBS263.38 and *P. graminicola* strain CBS327.62, which were sequenced in the present study.

A direct colony PCR strategy (Calmin et al., 2007) was eventually adopted to expedite our research and eliminate the time-consuming culturing and DNA extraction protocols. Mycelial fragments were immediately added to PCR tubes filled with a 50 µl reaction volume, containing 5 µl Q-buffer (QIAGEN), 1 µl dNTPs (10 mM; Fermentas), 10 µl Q-solution (QIAGEN), 3.5 µl of each primer (10 mM) and 26.7 µl sterile milli-Q water. An initial denaturation step of 10 min at 96°C in a thermal cycler (Eppendorf Mastercycle gradient) enabled the release of DNA from mycelial propagules. Next, 0.3 µl Taq DNA polymerase (5 U μl^{-1}; Fermentas) was added to each tube and the previously described cycling profile was started.

3.2.3. Phylogenetic analysis

Bayesian phylogenetic trees were constructed with the MrBayes software package (Huelsenbeck and Ronquist 2001) using rDNA-ITS and β-tubulin sequence data from the 110 collected or requested *Pythium* isolates (Table 3.1 and 3.2), and 57 additional *Pythium* sequences retrieved from GenBank (Table 3.2). Bayesian inference approaches have been outperforming other methods to reconstruct phylogenetic trees in terms of accuracy and running time (Williams and Moret, 2003). Therefore, MrBayes was preferred for the current phylogenetic study. By means of ClustalW, sequences were compared with each other and afterwards, a nexus file was created in which missing values were defined as 'N' and gaps as '-'. After executing the nexus file in MrBayes the evolutionary model was set to the General Time Reversible model with a proportion of invariable sites and gamma-distributed rate variation across sites (GTR + I + G). This model was selected on the basis of the Akaike's Information Criterion (AIC) using JModeltest 0.1.1. (Posada, 2008). A comparison of different model selection methods with regard to phylogenetic analyses revealed that approaches like AIC and Bayesian methods offer important advantages over hierarchical Likelihood Ratio tests (Posada and Buckley, 2004). Accordingly, AIC was chosen for model evaluation. Next, 20 000 trees were sampled along 2 000 000 generations for the β-tubulin gene. For the ITS region 11 000 trees were sampled in 1 100 000 generations. The average standard deviation of split frequencies at the end of the run was 0.0075 and 0.0083 for the *β-tubulin* gene and ITS region of the rDNA, respectively. The first 25% of the sampled trees were discarded as burn-in and the obtained consensus tree was adjusted with Treeview 1.6 (Page, 1996). The drawn trees are unrooted. To study the relatedness of the aerobic rice isolates to species from another cluster within clade 3 of the *Pythium* phylogeny (Uzuhashi et al., 2010; see Chapter 1), sequences of references strains *P. aphanidermatum* CBS118.80 and *P. deliense* CBS314.33 were included in the analysis. Identical sequences were pooled and listed behind the taxons' name or below the phylogenetic tree. Only credibility values above 0.9 are shown.

3.2.4. *In vitro* screening for pathogenic variability

Seeds of the aerobic rice cultivar Apo were disinfected by agitation in a freshly prepared and buffered bleach solution (2.5% NaOCl in 0.25 M potassium phosphate buffer, pH 5.8 (Chun et al., 1997)) for 2 h.

Subsequently, seeds were thoroughly washed in sterile water, air-dried and six seeds were placed 2 cm apart on square Petri dishes (120 mm × 120 mm) filled with 50 ml of Gamborg B5 basal medium (Gamborg et al., 1968). Seventy-eight *Pythium* isolates collected from affected aerobic rice fields and belonging to the species *P. arrhenomanes* (40), *P. graminicola* (16) and *P. inflatum* (22) (Table 3.1), were screened for their ability to infect rice seeds either before or after germination. When conducting pre-emergence screenings, two mycelial plugs (5 mm diameter) taken from the edge of a four-day-old *Pythium* colony were placed in between seed 2 and 3, and seed 4 and 5 immediately after sowing. Post-emergence screenings were only performed with *P. inflatum* isolates (9) by inoculating Petri dishes 2 days after imbibition, when root radicles were approximately 0.5 cm in length. Petri dishes were incubated at 28°C in the dark for two days and subsequently placed in an upright position (60°C angle) inside a growth chamber with a 12 h day/night cycle and temperature of 28°C (Keijer et al., 1997). The lower parts of the Petri dishes were covered with aluminum foil to protect seedling roots from light. Ten days post inoculation, emergence percentages (i.e. percentage of germinated seeds) were calculated, and root and shoot lengths of the germinating seedlings were measured. The experiment was performed with two replicates for each isolate. Data were relatively expressed to non-inoculated control treatments, and were combined for isolates from the same species and originating from the same field and year. Mean values were compared with the non-inoculated control treatment or with each other to detect statistical significant differences at $P < 0.05$. For statistical analysis, only non-parametric Kruskal-Wallis and Mann-Whitney tests were performed using SPSS 19.0 (SPSS Inc.).

3.2.5. *In vivo* screening for pathogenic variability

Fifty aerobic rice isolates, belonging to the species *P. arrhenomanes* (28), *P. graminicola* (14) and *P. inflatum* (8) (Table 3.1), were inoculated on PDA-filled (22 ml) Petri dishes and incubated at 28°C for one week. Plates that were entirely covered with mycelium were cut into pieces and half of the agar plate was added to a glass jar with 120 g of sterile 1:3 rice grain:rice hull mixture. After 5-7 days of incubation at 28°C, 4.5 g of the inoculated grain:hull substrate was mixed with 75 g of sterile 1:1 sand:garden soil potting mixture and placed in plastic pots (9.5 × 9.5 × 8.5 cm). Twenty seeds of aerobic rice cv. Apo were direct-seeded into each of the pots, which were then placed inside a growth chamber (Percival Scientific) set at 12 h day/night cycle (29°C/26°C) with 70% relative humidity. Soil moisture was maintained at or near field capacity (-15 to -25 kPa) through frequent watering (every two days) until water drained out of the potholes. Three trials with three replicates each were conducted using a Randomized Complete Block Design (RCBD) with three blocks. Fourteen days after sowing, emergence percentages were calculated in each pot by considering seedlings that emerged above the substrate, and seedling heights were measured and averaged per pot. Data were expressed relative to non-inoculated control treatments and combined for isolates from the same species and originating from the same field and year.

Mean values were compared with the non-inoculated control treatment or with each other to reveal statistical significant differences at $P < 0.05$. Statistical analysis was performed using SPSS 19.0 (SPSS Inc.). Non-parametric Kruskal-Wallis and Mann-Whitney tests were performed with emergence percentages. Shoot length data were normally distributed and analyzed using one-way ANOVA when comparing the three species or groups among the *P. arrhenomanes* isolates.

3.2.6. *In vitro* screening for host adaptation

Eleven *P. arrhenomanes* and two *P. graminicola* isolates from different host plants, i.e. maize, rice, sugarcane and wheat, were tested *in vitro* to reveal their pathogenic potential on rice and maize (Table 3.6). Pathogenicity trials on rice cv. Apo were conducted using the previously specified protocol. When using seeds of the cv. Nipponbare, disinfection proceeded by shaking in 70% ethanol for 1 min followed by an agitation step of 15 min in 1% NaOCl. For pathogenicity trials on maize, seeds of cv. LG 34.57 were surface-sterilized in 70% ethanol for 1 min followed by an agitation step of 15 min in 0.5% NaOCl. Subsequently, seeds were thoroughly washed in sterile water, air-dried and three seeds were placed equidistantly on each GB5-filled square Petri dish (120 × 120 mm). Two agar plugs (5 mm diameter) were placed next to the central seed at 1.5 cm distance prior to incubation. Incubation conditions were set as previously described, except for the temperature which was lowered to 25°C. Seven days after inoculation, the isolates were evaluated for their pathogenicity on the basis of maximal shoot lengths of the germinated seedlings, and emergence percentages (i.e. percentage of germinated seeds). The experiment was conducted with four replicates for each isolate. Data were expressed relative to non-inoculated control treatments. Mean values were compared with the non-inoculated control treatment or with each other to detect statistical significant differences ($P < 0.05$). Data were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney tests using SPSS 19.0 (SPSS Inc.).

3.3. Results

3.3.1. Identity and distribution of the isolates

Ninety-nine *Pythium* isolates from affected aerobic rice fields in the Philippines were identified to species based on DNA sequences of the ITS region of the rDNA and the middle part of the *β-tubulin* gene (Table 3.1). Sequence lengths were 658 bp for the *β-tubulin* gene, and varied from 722 bp up to 798 bp for the ITS region. When comparing ITS sequences of the sampled isolates with GenBank entries, the following results were obtained (Table 3.3): 38 isolates belonged to the species *P. aristosporum*, three isolates showed 98-99% identity with both *P. aristosporum* and *P. arrhenomanes*, 33 isolates belonged to *P. inflatum*, 14 isolates showed 99% sequence identity with both *P. graminicola* and *P. periilum*, two isolates were 100% identical to *P. periilum*, eight isolates belonged to an unknown species and one isolate was classified as *P. catenulatum.*

Table 3.3. BLAST search results and reference strains used for identification of the collected aerobic rice isolates.

		ITS region		β-tubulin gene	
Species	Isolate	Reference strain [a]	% Identity	(Reference) strain [a, b]	% Identity
P. arrhenomanes	PB912 80, PT 28	*P. aristo* CBS263.38/ *P. arrh* CBS324.62	99	*P. aristo* CBS263.38/ *P. arrh* strains	98
	PB912 09-6	*P. aristo* CBS263.38/ *P. arrh* CBS324.62	98	*P. aristo* CBS263.38/ *P. arrh* strains	98
	PB912 75,76, 78, 79B, 83, 09-3, 09-8 , 09-10, 09-15 PT 18, 23, 25, 26, 32, 36-41, 43, 44, 46, 50, 53, 54, 57, 58, 60, 62, 64, 68, 70, 74, T5, T6, T12, T14	*P. aristo* CBS263.38	100	*P. aristo* CBS263.38/ *P. arrh* strains	98
P. catenulatum	PB912 87	*P. cate* CBS842.68	99	*P. cate* NBRC100104	99
P. graminicola	PB912 7, 116, 132, T1 PB911 120,122, 133-140	*P. gram* CBS327.62/ *P. perii* CBS169.68	99	*P. gram C*BS327.62	100
	PB912 16, 97	*P. perii* CBS169.68	100	*P. gram* CBS327.62 [c]	100
P. inflatum	PB912 6, 82, 84, 86, 89-91, 93, 98, 99, 101, 102 PB912 104-108, 110-114, 118, 119, 129-131 PB911 119B, PBTP 10, 42	*P. infl* CBS168.68	99	*P. infl* MAFF305863	98
	PB912 109	*P. infl* CBS168.68	96	*P. infl* MAFF305863	98
	PB912 117	*P. infl* CBS168.68	98	*P. infl* MAFF305863	97
	PT 52	*P. infl* CBS168.68	100	*P. infl* MAFF305863	98
Unspecified *Pythium* strains	PB912 85, 94-96, 96B, 100	*P. myri* CBS254.70	93	*P. pyri* IFO31926/ *P. cate* NBRC100104	94
	PB912 88, 115	*P. plur* CBS100530	97	*P. volu/ P. vant/ P. cate/ P. pyri*	93

[a]Acronyms *P. infl, P. aristo, P. arrh, P. gram, P. perii, P. myri, P. cate, P. pyri, P. plur, P. volu* and *P. vant* represent: *P. inflatum, P. aristosporum, P. arrhenomanes, P. graminicola, P. periilum, P. myriotylum, P. catenulatum, P. pyrilobum, P. plurisporium, P. volutum* and *P. vanterpoolii,* respectively.

[b]The β-tubulin gene sequences of the reference strains were not available, except for CBS263.38 and CBS327.62. Therefore, other strains than the references were also used for identification.

[c]The β-tubulin gene of *P. periilum* has not been sequenced.

Despite the lack of good reference strains for identifications based on β-tubulin sequence data, BLAST analysis of β-tubulin sequences appeared to confirm ITS BLAST data or turned out to provide additional information. For instance, isolates classified as *P. periilum* based on ITS sequencing, shared identical β-tubulin sequences with reference strain *P. graminicola C*BS327.62. Along with their similar morphological characteristics and strong ITS sequence identity (> 99%), the distinction of *P. periilum* and *P. graminicola* as two species might be questioned. Moreover, both species only differ in the size of oogonia and the shapes or sizes of their filamentous sporangia (Lévesque and De Cock, 2004). These are, however, questionable delimitation criteria, because it has been reported that the shapes or sizes of filamentous sporangia largely vary among or within *Pythium* species of clade 3 (Uzuhashi et al., 2010). On account of both morphological and genetic findings it is proposed that their classification as one species should be accepted. We selected *P. graminicola* as the new species name, since it was described before *P. periilum* (see Arct.11 of The International Code of Nomenclature for algae, fungi, and plants; McNeill et al., 2012). A comparable situation was observed for *P. aristosporum* and *P. arrhenomanes* isolates. Several reports regarding their high genetic similarity have been published and their distinction as two species has been questioned over time (Nechwatal and Mendgen, 2009; Tambong et al., 2006).

Additionally, the only phenotypic difference between *P. arrhenomanes* and *P. aristosporum* seems to exist in cardinal temperatures and the number of antheridia per oogonium (Lévesque and De Cock, 2004; van der Plaats-Niterink, 1981). It should be noted, however, that the amount of antheridia per oogonium varies considerably among or within *Pythium* species of clade 3 (Uzuhashi et al., 2010). Therefore, consistent with previous reports, we decided to consider them as one species, i.e. *P. arrhenomanes* (McNeill et al., 2012). Consequently, the species distribution shifted as follows: 41 isolates belonged to the species *P. arrhenomanes*, 33 isolates to *P. inflatum*, 16 isolates to *P. graminicola*, eight isolates to an unknown species and one isolate to *P. catenulatum* (Table 3.4).

Table 3.4. Contingency table with observed and expected isolation frequencies of the five *Pythium* species obtained from different aerobic rice plots.

	Frequency of *Pythium* spp.[a]					
	P. arrhenomanes	*P. inflatum*	*P. graminicola*	Unspecified *Pythium* strains	*P. catenulatum*	Total/plot
PT (Tarlac, Dapdap) [b]						
2007 DS	31 (13.30)	1 (10.70)	0 (5.17)	0 (2.59)	0 (0.32)	32
PB9-12 (IRRI, Los Baños) [b]						
2005 DS	0 (1.24)	1 (1.00)	2 (0.49)	0 (0.24)	0 (0.03)	
2006 DS	6 (2.90)	1 (2.33)	0 (1.13)	0 (0.57)	0 (0.07)	
2008 DS	0 (14.50)	24 (11.70)	2 (5.66)	8 (2.83)	1 (0.35)	54
2008 WS	0 (1.66)	3 (1.33)	1 (0.65)	0 (0.32)	0 (0.04)	
2009 DS	4 (1.66)	0 (1.33)	0 (0.65)	0 (0.32)	0 (0.04)	
2009 WS	0 (0.41)	0 (1.33)	1 (0.16)	0 (0.08)	0 (0.01)	
PB9-11 (IRRI, Los Baños) [b]						
2008 WS	0 (4.56)	1 (3.67)	10 (1.78)	0 (0.89)	0 (0.11)	11
PBTP (Bulacan) [b]						
2009 WS	0 (0.83)	2 (0.67)	0 (0.32)	0 (0.16)	0 (0.02)	2
Total/species	41	33	16	8	1	99

[a]Data represent actual numbers of isolates collected among the different plots and years. Between brackets the expected numbers. According to Fisher's exact test, a significant relationship ($P < 0.05$) exists between sampled plot and *Pythium* species. In plot PB9-12, the occurrence of *P. arrhenomanes* was significantly correlated with the year of sampling at $P < 0.05$.

[b]WS and DS are acronyms for wet season and dry season, respectively.

Whereas *P. inflatum* was recovered from each field, *P. graminicola* and *P. arrhenomanes* appeared to be associated with specific fields (Table 3.4). Using the Fisher's exact test, a statistically significant interaction ($P < 0.05$) between sampled field and *Pythium* species could be determined. *P. graminicola* was associated with IRRI experimental Plots PB9-11 and PB9-12, whereas *P. arrhenomanes* isolates mainly originated from Plot PT in Tarlac. The occurrence of *P. arrhenomanes* in Plot PB9-12 was significantly correlated with the year of sampling. In the dry season of 2006, *P. arrhenomanes* isolates were recovered from this plot.

During the wet season of 2006, a strong typhoon inundated the field with soil from an unknown remote site and therefore, the field was left fallow in 2007 and no samples were collected. Sampling was resumed in 2008, but no *P. arrhenomanes* isolates were recovered until 2009. The unspecified *Pythium* strains and *P. catenulatum* were only occasionally isolated in the dry season of 2008 during intensive sampling of Plot PB9-12 (Table 3.4).

3.3.2. Phylogenetic analysis

3.3.2.1. ITS region of rDNA

ITS sequences from 167 *Pythium* isolates were aligned and converted into a dataset for the phylogenetic analysis of the five collected *Pythium* species from affected aerobic rice fields (Tables 3.1 and 3.2). Using the MrBayes software package, a consensus phylogenetic tree was generated as shown in Figure 3.1.

The five identified *Pythium* species aggregated into one major cluster consisting out of four sub clusters. *P. inflatum* and *P. graminicola* isolates grouped together in sub cluster IV, indicating their close relationship. *P. graminicola* isolates from aerobic rice and sugarcane, and the *P. periilum* reference strain from American soil formed a quite uniform group with few nucleotide substitutions, clearly distinguishable from a Brazilian *P. graminicola* soil isolate (accession no. AB5629081). *Pythium i*solate IFO31998 from barley was surprisingly more related with *P. vanterpoolii* than *P. graminicola*, its identity according to GenBank. Genetic variation among aerobic rice isolates of *P. inflatum* was higher than that observed for strains of *P. graminicola* from the same site, since two subgroups could be distinguished. One group contained isolates sharing similar copies of the ITS region of the rDNA with *P. inflatum* reference strain CBS168.68 and isolates PB912 6, 109 and 117 with a high percentage of nucleotide substitutions in their ITS region. The second group only comprised PB912 110, which was clearly more distinct from CBS168.68.

Sub cluster I contained *P. volutum* isolate CBS699.83 and all *P. arrhenomanes* isolates, including three Japanese isolates OPU480, Pma-2 and Ohmagari from rice, and CBS263.38 (wheat) and PRR115 (bent grass), which were formerly described as *P. graminicola* and *P. aristosporum*, respectively. Among these isolates, the Japanese rice strains OPU480 and Pma-2 shared an identical rDNA-ITS region with *P. arrhenomanes* PB912 75 from aerobic rice. These findings suggest the stronger relationship of OPU480, Pma-2 and Ohmagari to *P. arrhenomanes* than *P. graminicola*. The rDNA-ITS region of all *P. aristosporum* isolates was very similar to that of *P. arrhenomanes* isolates. *P. arrhenomanes* isolates from aerobic rice fields were quite similar, since the rDNA-ITS region in 37 out of 41 isolates was identical. When screening isolates from other origins, *P. arrhenomanes* isolates from sugarcane (CBS293.32, E-1, ATCC96598 and ATCC96526), bent grass (P21-1), maize (Me1b and CBS430.86) and rice (Ohmagari, previously identified as *P. graminicola*) seemed to differ more in their ITS region. This host-linked variation was especially pronounced for turf grass isolates NBRC100102 (zoysia grass) and ATCC96525 (Bermuda grass).

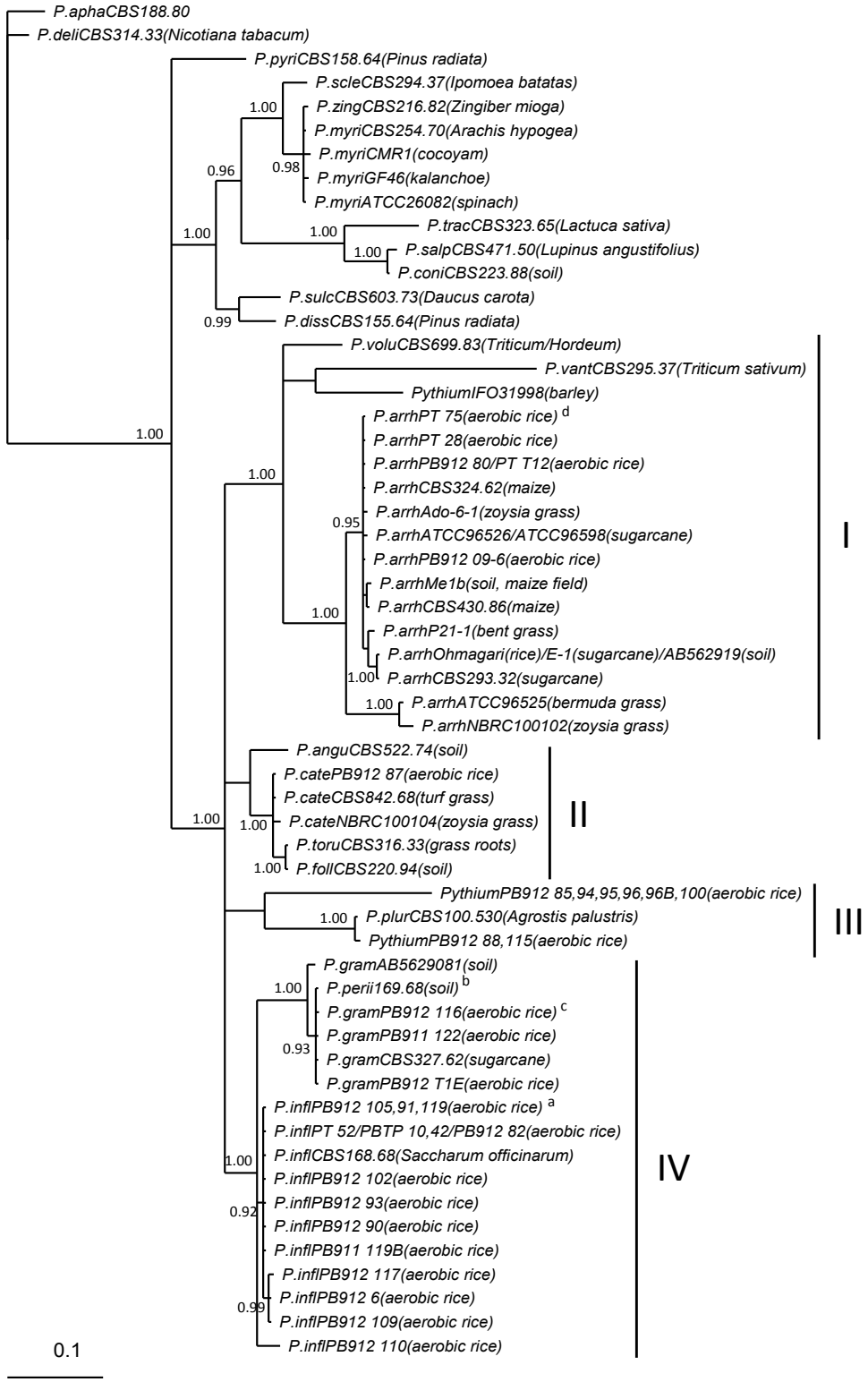

Figure 3.1. Bayesian phylogenetic analysis based on sequences of the Internal Transcribed Spacer (ITS) of the rDNA. The numbers represent credibility values, the scale bar indicates the number of substitutions per site. The acronyms *P. infl, P. arrh, P. gram, P. myri, P. cate, P. pyri, P. plur, P. volu, P. vant, P. sulc*, *P. diss*, *P. angu*, *P. salp*, *P. coni*, *P. trac*, *P. zing*, *P. scle*, *P. toru*, *P. foll*, *P. deli* and *P. apha* represent: *P. inflatum, P. arrhenomanes, P. graminicola, P. myriotylum, P. catenulatum, P. pyrilobum, P. plurisporium, P. volutum, P. vanterpoolii, P. sulcatum, P. dissimile, P. angustatum, P. salpingophorum, P. conidiophorum, P. tracheiphilum, P. zingiberis, P. scleroteichum, P. torulosum, P. folliculosum, P. deliense* and *P. aphanidermatum,* respectively. Lowercase superscript letters after taxons indicate that isolates with identical sequences were pooled.

Figure 3.1. (Continued)

[a]All other *Pythium inflatum* isolates from aerobic rice, [b]PB912 97,16 (aerobic rice)/MAFF305860 (soil)/ATCC96234, MAFF425415 (soil, maize field)/ATCC96600 (sugarcane), [c]all other *P. graminicola* isolates from aerobic rice, [d]CBS263.38 (wheat)/PRR115 (bent grass)/PRR5, KU9BGP (bent grass)/Me8a, Me9d (maize field)/P54 (reed)/AB513368, AB513367, OPU480, Pma-2 (rice)/CBS325.62, TMF (sugarcane) and all other *P. arrhenomanes* isolates from aerobic rice.

All *P. catenulatum* isolates, including aerobic rice isolate PB912 87, were similar and grouped into the same sub cluster II together with *P. torulosum* and *P. folliculosum* isolates. *Pythium* isolates PB912 85 and 88 showed a strong relationship with *P. plurisporium* CBS100530 in sub cluster III, but ITS sequences of both isolates contained a high percentage of dimorphic positions, which probably affected the reliability of their phylogenetic clustering.

3.3.2.2. β-tubulin gene sequences

A slightly different phylogenetic consensus tree was obtained when analyzing a part of the *β-tubulin gene* (Figure 3.2). The five sampled *Pythium* species were spread along two clusters (I and II), supported by a reliability of 100%.

In cluster II, *P. catenulatum* isolate PB912 87 grouped with *P. catenulatum*, *P. torulosum* and *P. pyrilobum* isolates and was similar to *P. catenulatum* NBRC100104 from zoysia grass. In cluster I, two large sub clusters could be differentiated, with sub cluster Ia containing all *P. arrhenomanes* isolates and *P. volutum* IFO31926. The *P. arrhenomanes* isolates from affected aerobic rice fields shared identical partial β-tubulin sequences. When observing isolates from other origins, sequence variation in the *β-tubulin* gene was detected and this heterogeneity was most pronounced in case of turf grass isolates, like NBRC100102 from zoysia grass, as mentioned in the ITS-based analysis. The misidentification of isolates IFO31998, OPU480, Pma-2 and Ohmagari as *P. graminicola* was confirmed. Isolate ATCC96525 (Bermuda grass) unexpectedly clustered far outside of the *P. arrhenomanes* group, which entirely contradicts its ITS-based grouping.

P. graminicola isolates from affected aerobic rice fields grouped in sub cluster Ib together with *P. inflatum* and *P. myriotylum* isolates. The credibility value for this sub cluster was very low. *P. graminicola* isolates had identical partial β-tubulin sequences and minor differences were seen compared with the analyzed GenBank isolates that originated from a maize field (ATCC96234) and Japanse soil (MAFF305860). *P. inflatum* isolates were clearly different from GenBank isolate MAFF305863 (Japanese soil). Contrary to *P. graminicola* and *P. arrhenomanes*, heterogeneity in the *β-tubulin* gene was discovered for Philippine *P. inflatum* isolates. In agreement with the ITS-based phylogenetic study, this might indicate a greater genetic variation among *P. inflatum* isolates from aerobic rice fields compared with the other species.

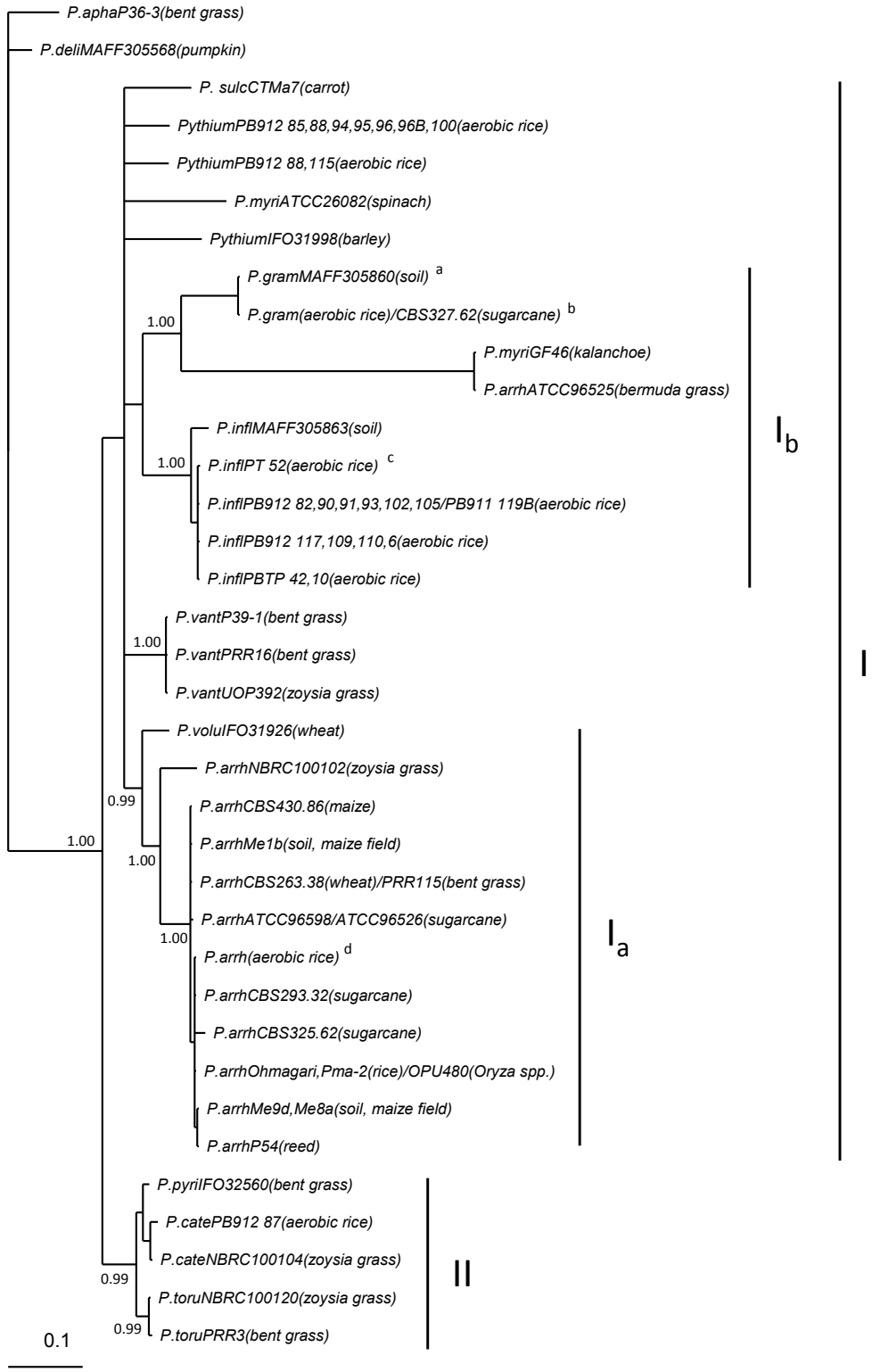

Figure 3.2. Bayesian phylogenetic analysis based sequences of the *β-tubulin* gene. The numbers represent credibility values, the scale bar indicates the number of substitutions per site. The acronyms *P. infl, P. arrh, P. gram, P. myri, P. cate, P. pyri, P. volu, P. vant*, *P. toru*, *P. sulc*, *P. deli* and *P. apha* represent: *P. inflatum, P. arrhenomanes, P. graminicola, P. myriotylum, P. catenulatum, P. pyrilobum, P. volutum, P. vanterpoolii, P. torulosum, P. sulcatum, P. deliense* and *P. aphanidermatum,* respectively. Lowercase superscript letters after taxons indicate that isolates with identical sequences were pooled. [a]*Pythium graminicola* isolate ATCC96234, [b]all *Pythium graminicola* isolates from aerobic rice, , [c]all other *P. inflatum* isolates from aerobic rice and [d]all *P. arrhenomanes* isolates from aerobic rice.

The clustering of *Pythium* isolates PB912 85 and PB912 88 was somewhat different from the ITS-based one, due to a lower degree of dimorphic positions in their β-tubulin sequences. Along with these results, it was also noteworthy that intra-isolate variability was observed in the rDNA ITS and/or β-tubulin sequences of *P. arrhenomanes* isolates from sugarcane (CBS293.32), maize (Me1b and CBS430.86) and rice (all Philippine and Japanese isolates). This intra-isolate variation might be indicative for hybridization or inter-chromosomal variation and has been reported for other *Pythium* spp. as well (Belbahri et al., 2008; Kageyama et al., 2007). Hybridization events result from incomplete intersterility and may generate new pathogens with host ranges and/or virulence levels that differ from the parent's species. Hence, these events are considered as important means for rapid species evolution (Nechwatal and Mendgen, 2008).

3.3.3. *In vitro* screening for pathogenic variability

P. arrhenomanes and *P. graminicola* isolates from affected aerobic rice fields were all pathogenic on aerobic rice cv. Apo (Table 3.5). Mean shoot lengths were significantly smaller ($P < 0.05$) than the average length of non-inoculated control treatments, i.e. 10.49 cm. When inoculated with *P. arrhenomanes*, germinating rice seeds did not develop roots and had very small shoots. In the case of *P. graminicola* inoculation, rice seeds occasionally developed small roots in 50% of all treatments and significantly ($P < 0.05$) less stunting was observed (Figure S3.2). Accordingly, this species was determined to be less aggressive than *P. arrhenomanes*. No significant differences were detected in emergence percentages (Table 3.5). Infection with *P. arrhenomanes* and *P. graminicola* resulted in a high seed emergence of 88% and 87%, respectively, but many of these germinating seedlings quickly died. *P. inflatum* isolates differed in their ability to affect rice seedling growth (Table 3.5). There was no clear correlation between pathogenicity and sampled field, except for isolates PBTP 10 and 42 from a transplanted aerobic rice field in Bulacan (Central Luzon, the Philippines). Together with isolate PB912 130, these were more aggressive compared with other *P. inflatum* isolates and they inflicted a similar percentage of stunting as *P. graminicola*. However, *P. inflatum* isolates were in general significantly ($P < 0.05$) less aggressive than *P. arrhenomanes* and *P. graminicola* isolates (Figure S3.2). This was especially evident when comparing pre-emergence with post-emergence screenings. *P. inflatum* isolates could reduce shoot growth or kill germinating seeds during the first two days after seeding, but their pathogenic potential rapidly decreased afterwards (see Figure S3.3).

3.3.4. *In vivo* screening for pathogenic variability

In vivo pathogenicity assays showed that none of the tested *P. inflatum* isolates were pathogenic on aerobic rice cv. Apo (Table 3.5). Overall averages of shoot length and emergence percentages did not significantly differ from the non-inoculated control treatment ($P \geq 0.05$).

Eighty-eight percent of all *P. graminicola* isolates were tested *in vivo* and only three isolates were pathogenic and significantly reduced shoot length with 23%. In contrast, all *P. arrhenomanes* isolates provoked significant reduction in emergence and shoot length of 29% and 42%, respectively (Table 3.5). Both the degree of stunting and emergence percentages significantly differed between *P. arrhenomanes* and *P. graminicola* treatments ($P < 0.05$).

Table 3.5. Pathogenic potential of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* isolates towards aerobic rice seedlings (cv. Apo).

			In vitro pathogenicity[a]			*In vivo* pathogenicity[b]		
Pythium spp.	Field	Year isolated	No. of isolates tested	Emergence %	Shoot length %	No. of isolates tested	Emergence %	Shoot length %
P. arrhenomanes	PT	2007	30	88 (3.77) a	5 (0.56) a*	20	64 (2.94) a*	55 (2.69) a*
	PB9-12	2006	6	103 (7.97) b	5 (0.38) a*	4	76 (7.03) a	61 (4.00) a*
		2009	4	72 (8.64) a*	5 (0.23) a*	4	74 (7.14) a*	59 (4.81) a*
Overall averages for *P. arrhenomanes* isolates				**88 (3.28) A**	**5 (0.19) A***		**71 (2.50) A***	**58 (2.06) A ***
P. graminicola	PB9-11	2008	10	81 (5.78) cd*	7 (1.30) c*	10	86 (4.92) c	84 (3.22) cd
	PB9-12	2005	2	58 (9.51) c*	18 (10.02) c*	1	91 (3.04) c	100 (6.25) c
		2008	3	98 (6.24) d	11 (2.03) c*	3	97 (6.00) c	77 (3.53) d*
		2009	1	111	10	-	-	-
Overall averages for *P. graminicola* isolates				**87 (4.66) A***	**12 (1.58) B***		**91 (3.78) B**	**87 (2.62) B ***
P. inflatum	PT	2007	1	75	66	-	-	-
	PB9-11	2008	1	83	68	-	-	-
	PB9-12	2005	1	92	69	1	113 (2.03) e	140 (11.30) e
		2006	1	76	48	-	-	-
		2008(1)[c]	6	88 (5.69) e	38 (4.14) e*	4	102 (5.15) e	105 (4.45) f
		2008(2)[c]	10	93 (4.91) e	74 (5.58) f*	3	110 (9.88) e	92 (4.48) f
	PBTP	2009	2	78 (0.14) e*	10 (4.52) g*	-	-	-
Overall averages for *P. inflatum* isolates				**84 (2.42) A***	**53 (4.31) C***		**108 (3.04) C**	**112 (2.39) C**

[a,b]Shoot lengths of germinated seeds and emergence data are expressed relative to non-infected control treatments and represent means for all plants from [a]two replicate plates or [b]three replicate trials per isolate. Data were combined for isolates from the same plot ⁄ year. Statistical analyses were performed by Kruskal-Wallis and Mann-Whitney non-parametric tests for all *in vitro* data and the *in vivo* emergence data. *In vivo* shoot length data were normally distributed and analyzed by one-way ANOVA except for comparisons within the species groups of *P. graminicola* and *P. inflatum*. Within each column and species, values followed by different lowercase letters are significantly distinct at $P < 0.05$. *In vitro* data from groups with one isolate were not included in the analysis. Overall averages followed by the same uppercase letter are not significantly different at $P \geq 0.05$. An * indicates significant differences compared with the non-infected control ($P < 0.05$). Values in parentheses represent standard errors. [c]*P. inflatum* isolates sampled in PB9-12 in 2008 were divided in two groups according to the degree of stunting. 2008(1): isolates with percentage shoot length below 50%; 2008(2): isolates with percentage shoot length above 50%.

3.3.5. *In vitro* screening for host adaptation

When testing the pathogenicity of *P. arrhenomanes* and *P. graminicola* isolates from several hosts on rice cvs. Apo and Nipponbare, and maize cv. LG34.57, isolates from the same host appeared to vary in their pathogenic potential (Table 3.6). Since pathogenicity trials on cvs. Apo and Nipponbare did not generate different data sets, we decided to limit the presented data to the trials with Nipponbare and maize.

Two *P. arrhenomanes* isolates from sugarcane (CBS293.32 and CBS325.62) were screened and isolate CBS293.32 turned out to be weakly aggressive on both rice and maize seedlings ($P \geq 0.05$). Unlike CBS293.32, sugarcane isolate CBS325.62 strongly reduced shoot growth of rice seedlings with 93%. Its negative influence on maize seedlings was even more pronounced, highlighted by the low seed germination of 8%. Four *P. arrhenomanes* isolates sampled from maize (CBS430.86) or a maize field (Me1b, Me8a and Me9d) were moderately to highly aggressive on rice. Isolates Me1b, Me8a and Me9d significantly reduced shoot lengths of germinated rice seeds ($P < 0.05$). CBS430.86 was identified as the least aggressive isolate because infection of rice seeds resulted in 18-26% less stunting, which was statistically significant compared with isolate Me9d ($P = 0.037$).

As expected, exposure of maize seeds to isolates Me8a and Me9d (maize field) resulted in strong disease symptoms, as shown by the very low emergence percentages of 8%. Isolates Me1b and CBS430.86 appeared to be less virulent, with higher emergence percentages of 42 and 50%, respectively, and longer shoots. However, the differences with the other isolates were not statistically significant ($P \geq 0.05$). Similar results were obtained for four isolates originating from different rice cultivars (PT60, OPU480, Pma-2 and Ohmagari). Whereas the least aggressive isolate Pma-2 did not significantly decrease rice seedling shoot length, isolates PT60, OPU480 and Ohmagari significantly reduced shoot length with 88-95% ($P < 0.05$). Most of the rice isolates also seriously affected maize seed germination. Rice isolate PT60, for instance, completely inhibited germination (Figure S3.4). One isolate was detected to be less aggressive on maize (Ohmagari), with both emergence percentages and shoot length data significantly different from PT 60 ($P < 0.05$), but not from the non-inoculated control. Compared to isolate Pma-2, an equally lower degree of rice seedling stunting was inflicted by wheat isolate CBS263.32 ($P = 0.610$) and maize isolate CBS430.86 ($P = 0.778$). Moreover, data of maize trials paralleled these results, suggesting a narrower host range for isolates Pma-2, CBS263.32 and CBS430.86.

Isolate *P. graminicola* CB327.62 from sugarcane was as aggressive on rice as isolate PB912 132 ($P \geq 0.05$), which was obtained from an affected aerobic rice field in the Philippines. Beside rice seedling growth, it also seriously affected maize seedlings, evidenced by the 94% of stunting and low emergence of 17%.

Table 3.6. Pre-emergence pathogenic potential of *P. arrhenomanes* and *P. graminicola* isolates from different hosts towards maize cv. LG 34.57 and rice cv. Nipponbare.

			Pathogenicity on rice (Nipponbare) [a]		Pathogenicity on maize (LG 34.57)[b]	
Pythium spp.	Isolate	Host	Emergence %	Shoot length %	Emergence %	Shoot length %
P. arrhenomanes	CBS430.86	Maize	87 (4.50) a	33 (7.76) bc	50 (16.75) bc	28 (18.18) abcd
	Me1b	Soil, maize field	82 (11.62) a	15 (4.88) abc*	42 (8.50) bc	44 (9.91) bcd
	Me8a	Soil, maize field	100 (5.20) a	8 (2.48) ab*	8 (8.25) ab*	19 (18.78) ab*
	Me9d	Soil, maize field	87 (11.32) a	7 (1.65) a*	8 (8.25) ab*	7 (7.33) ab*
	PT 60	Rice, Apo	73 (7.35) a	5 (1.14) a*	0 a*	0 a*
	OPU480 [c]	Rice	91 (7.35) a	12 (2.70) abc*	17 (9.53) ab*	30 (17.25) abc*
	Ohmagari [c]	Rice	78 (8.62) a	6 (1.35) a*	50 (21.56) bc	59 (23.03) bcd
	Pma-2[d]	Rice	82 (9.00) a	42 (10.55) c	33 (13.68) abc*	44 (16.67) abcd
	CBS293.32	Sugarcane	100 (5.19) a	62 (11.36) c	75 (16.02) c	77 (28.87) cd
	CBS325.62	Sugarcane	96 (4.50) a	7 (1.07) a*	8 (8.25) ab*	18 (17.7) ab*
	CBS263.38 [d]	Wheat	96 (8.62) a	58 (9.75) c	67 (13.68) c	94 (19.73) d
P. graminicola	PB912 132	Rice, Apo	91 (10.39) a'	8 (3.93) a'*	17 (9.61) a'*	4 (2.29) a'*
	CBS327.62	Sugarcane	64 (11.81) a'	27 (8.62) a'*	17 (9.53) a'*	6 (5.10) a'*

[a,b]Shoot length data of germinated seeds and emergence data are expressed relative to non-infected control treatments. For each isolate: emergence (n = 4), shoot length (n = 4). Statistical analyses were performed by Kruskal-Wallis and Mann-Whitney non-parametric tests. An * indicates significant differences compared with the non-infected control ($P < 0.05$). Different lowercase letters mark statistically significant differences between *P. arrhenomanes* or *P. graminicola* (') isolates at $P < 0.05$.

[c]Isolates described in the literature as *P. graminicola*.

[d]Isolates described in the literature as *P. aristosporum*.

Next to the *in vitro* screenings, we also tested the pathogenicity of *P. arrhenomanes* PT60 against maize variety LG34.57 under *in vivo* conditions. The isolate strongly reduced root and shoot lengths of maize seedlings (S3.4), confirming the results of the *in vitro* pathogenicity assays.

3.4. Discussion

This study identified the growth suppression-associated *Pythium* spp. from Philippine aerobic rice fields as three closely related *Pythium* spp. with different pathogenic and morphological characteristics, namely *P. arrhenomanes*, *P. graminicola* and *P. inflatum.* Both *P. arrhenomanes* and *P. graminicola* have been described as serious pathogens of several graminaceous crops including maize (Deep and Lipps, 1996), barley (Olsson and Kadir, 1994), sorghum (Forbes et al., 1987), sugarcane (Lee and Hoy, 1992) and turf grasses (Nelson and Craft, 1991). The effect of *P. arrhenomanes* on the growth of rice seedlings was previously reported (Chun and Schneider, 1998; Furuya et al., 2003). Moreover, this species seems to be present in most of the South-Australian rice fields (Cother and Gilbert, 1993). *P. inflatum* has been reported as a pathogen on maize (Yang et al., 2005a), tomato (Verma, 1987), spruce and beech (Nechwatal and Osswald, 2001).

Data from the *in vivo* pathogenicity trials ranked the sampled *Pythium* spp. according to virulence, with *P. arrhenomanes* being the most aggressive on rice cv. Apo followed by *P. graminicola*, and the non-pathogenic species *P. inflatum.* The collected *P. graminicola* isolates varied from pathogenic to non-pathogenic, whereas all *P. arrhenomanes* isolates were pathogenic. The *in vitro* pathogenicity data confirmed this ranking, but it has to be noted that under the disease-favoring conditions of our *in vitro* system (with high infection pressure and damaged seeds), all *Pythium* isolates were identified as pathogenic. Even the isolates belonging to the species *P. inflatum* significantly affected rice seedling growth under these conditions, but their effect was still significantly less than that of *P. arrhenomanes* and *P. graminicola* isolates.

P. arrhenomanes isolates were mostly recovered from plot PT in Tarlac. The severe growth suppression observed in this field might at least be partly explained by unfavorable soil conditions such as high soil pH, which created major micronutrient deficiencies (Kreye et al., 2009b) and may have enhanced plant susceptibility to *Pythium*. The appearance of *P. arrhenomanes* in plot PB9-12 depended on the year of sampling. Apparently, the inundation of plot PB9-12 by a typhoon in 2006 influenced its microbial population and this may explain the absence of *P. arrhenomanes* in 2008. However, *P. arrhenomanes* was re-isolated in 2009, indicating its competence to re-establish within three years of monocropping. The recover frequency of pathogenic *P. graminicola* isolates from the sampled fields was lower than that of pathogenic *P. arrhenomanes* isolates, pinpointing the greater importance of *P. arrhenomanes* as aerobic rice pathogen. Intriguingly, both species did not occur together, which might be due to competition (Cother and Gilbert, 1993). *P. inflatum* isolates were the most frequently detected, but their importance with regard to the observed yield decline is probably low considering their non-pathogenic features. The unidentified *Pythium* strains and *P. catenulatum* isolate showed a low isolation frequency. Along with their non-pathogenic or weakly virulent characteristics under *in vitro* conditions, this suggests that their occurrence is likely not correlated with yield decline in aerobic rice fields.

The variability and relatedness of the sampled *Pythium* spp. was unraveled by conducting pathogenicity assays and analyzing sequences of the ITS region and the 5.8S gene of the rDNA, and the middle part of the *β-tubulin* gene. *P. arrhenomanes* and *P. graminicola* isolates from aerobic rice fields showed very little intraspecific variation at the genetic level. These results paralleled the low heterogeneity in pathogenic potential for *P. arrhenomanes,* but did not reflect the pathogenic variation among the sampled *P. graminicola* isolates. More genetic variation was detected among *P. inflatum* isolates, but this intraspecific variability was again not related with differences in aggressiveness since they were all non-pathogenic *in vivo*. Including isolates from other origins did reveal a link between genetic and pathogenic variation within the species *P. arrhenomanes*.

For instance, *P. arrhenomanes* isolate CBS293.32 from sugarcane barely affected seedling growth of rice and maize cv. LG34.57. Additionally, its analyzed DNA regions clearly differed from those of other *P. arrhenomanes* isolates. Evaluation of the rDNA-ITS and *β-tubulin* sequences from other sugarcane isolates (E-1, ATCC96598, ATCC96526 and CBS325.62) also revealed unique nucleotide positions. While these findings are not conclusive, they might be indicative for adaptation of *Pythium* isolates to sugarcane. However, one must be careful when evaluating the aggressiveness of culture collection strains since long-term storage may induce loss of pathogenicity. This might be the case for sugarcane strain CBS293.32, which was isolated in 1932. Nevertheless, monocropping of sugarcane is the main cultivation system in tropical and subtropical countries and hence, selection for aggressive and host-specialized *Pythium* spp. is substantially stimulated in sugarcane fields. Pathogenic specialization of *P. arrhenomanes* to particular sugarcane varieties has already been described (Waterhouse and Waterston, 1964), but its effect on certain regions of the genome has not been reported before. Other examples of host-adapting *P. arrhenomanes* isolates have been reported for barley (Olsson and Kadir, 1994). *P. arrhenomanes* isolates pathogenic on turf grasses, such as zoysia grass and bermuda grass, differed genetically from the other isolates. Considering the correlation between pronounced pathogenic and genetic variation within *P. arrhenomanes*, this might once more suggest host specialization.

The phylogenetic study also illustrated that *P. arrhenomanes* or *P. vanterpoolii*-related isolates OPU480, Pma-2, Ohmagari and IFO31998 have been wrongly identified as *P. graminicola*. The sequences of these isolates were significantly different from the sequences of the reference strains that were included in this study. GenBank contains a high number of wrongly labeled sequences and therefore, identifications based on GenBank sequences may lead to misidentifications. Especially *P. arrhenomanes* and *P. graminicola* have often been confused with each other, due to their similar morphological characteristics and intraspecific variation (Chen and Hoy, 1993). This stresses the importance of reference strains, i.e. ex-type, neotype, authentic strains and strains used by Van der Plaats-Niterink (1981), to correctely identify *Pythium* isolates to species level. Analyses based on the ITS region of the rDNA and *β-tubulin* gene also showed that all *P. aristosporum* and *P. perillum* isolates were very similar to *P. arrhenomanes* and *P. graminicola* isolates, respectively, doubting their distinction as different species. Thus, in agreement with other reports, we propose that the four species should be classified as two: *P. arrhenomanes* and *P. graminicola*. These names were selected according to "The International Code of Nomenclature for algae, fungi, and plants" (McNeill et al., 2012), which regulates the scientific naming of algae, fungi and plants, and proposes that the name of the first described species has priority (Art.11).

We also demonstrated that *P. graminicola* isolates from Philippine aerobic rice fields were pathogenic on maize and that *P. arrhenomanes* isolates from maize and rice lacked host specificity. These findings are highly important, since cultivation of rice after two seasons of maize has been reported as a succesfull strategy to overcome yield decline in continuous aerobic rice fields (Nie et al., 2009).

Rice-wheat cropping systems have been described as important food production systems in subtropical regions of Asia, but with regard to tropical Asia and aerobic rice cultivation, maize is considered to be a better candidate crop. Besides its growth in aerobic soils, the cultivation of maize comprises few risks and provides a yield enhancing potential due to its efficient C4-photosynthesis cycle (http://www.knowledgebank.irri.org/ckb/index.php/extras/maize-a-suitable-candidate-for-rice-wheat-systems-diversification). Unfortunately, the present study is questioning the sustainability of this rotation strategy and rather recommends non-host crop rotation or fallow in Philippine aerobic rice fields.

3.5. Conclusions

This chapter provides important information for rice cultivation in the tropics. It is clear that *P. graminicola* and especially *P. arrhenomanes* may act as limiting biotic factors in Philippine aerobic rice fields. To overcome yield decline in these fields, crop rotation and fallow might be successful strategies (Nie et al., 2009). However, rice rotation with maize should be discouraged, because *P. arrhenomanes* isolates of rice and maize showed to lack host specificity and furthermore, *P. graminicola* isolates of rice appeared to be pathogenic on maize.

More knowledge on the *Pythium*-rice pathosystem is necessary to develop efficient disease management strategies. Unfortunately, *Pythium*-rice interactions have been receiving little attention because *Pythium* spp. do not cause great losses in paddy fields where their germination and growth might be inhibited due to high CO_2-concentrations (Johnson, 1988; Voland and Martinson, 1984). Nevertheless, *Pythium* tolerates oxygen-scarce conditions (Voland and Martinson, 1984) and a shift from micro-aerobic to aerobic rice cultivation promotes pathogen growth resulting in rice seedling inhibition (Furuya et al., 2003). Due to the increasing water scarcity, the interest for aerobic rice cultivation in the tropics is growing and hence, *Pythium*-related problems may gain in importance. Consequently, *Pythium* spp. should be considered in rice disease management and breeding programs, and their interaction with rice seedlings should be accurately analyzed.

3.6. Supplementary data

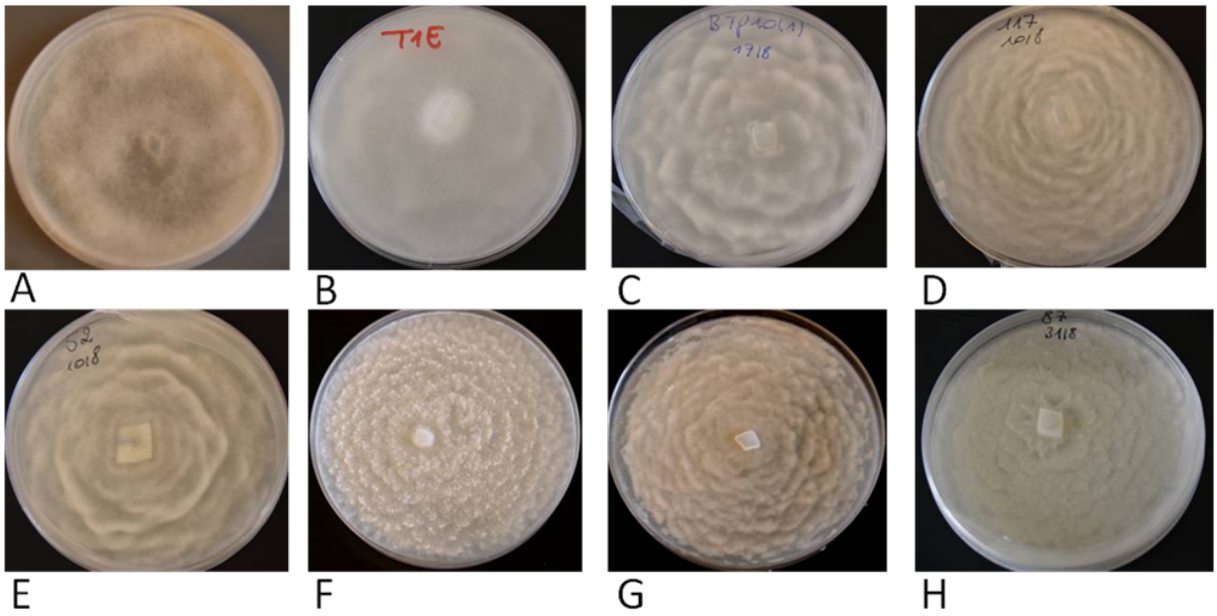

Figure S3.1. The eight morphotypes that were detected among the *Pythium* isolates from Philippine aerobic rice fields. A, colony with massive aerial mycelium and radiate pattern (*P. arrhenomanes* PT 60), **B,** colony with low aerial mycelium and fine radiate pattern (*P. graminicola* PB912 T1E) , **C-H,** colonies with rosette/chrysantemum-like patterns (*P. inflatum* PBTP 10 (C), *P. inflatum* PB912 117 (D), *P. inflatum* PT 52 (E), *Pythium* PB912 115 (F), *Pythium* PB912 100 (G), *P. catenulatum* PB912 87 (H)).

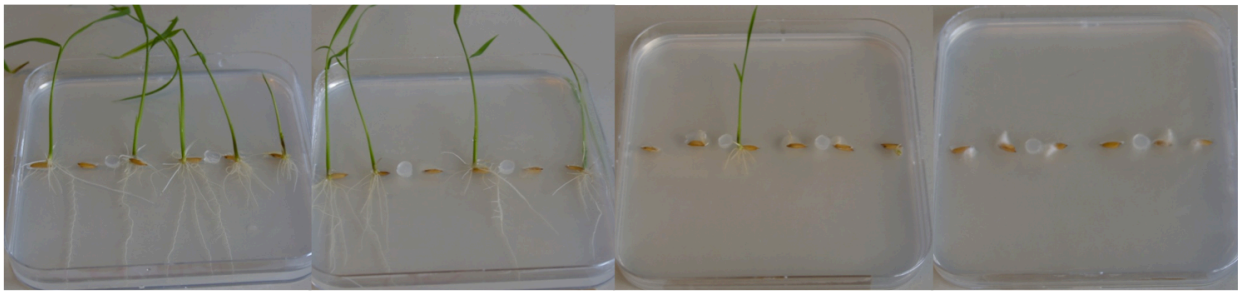

Figure S3.2. Damping-off and stunting of seedling tissues 10 days upon rice seed inoculation with *P. arrhenomanes*, *P. graminicola* and *P. inflatum*.

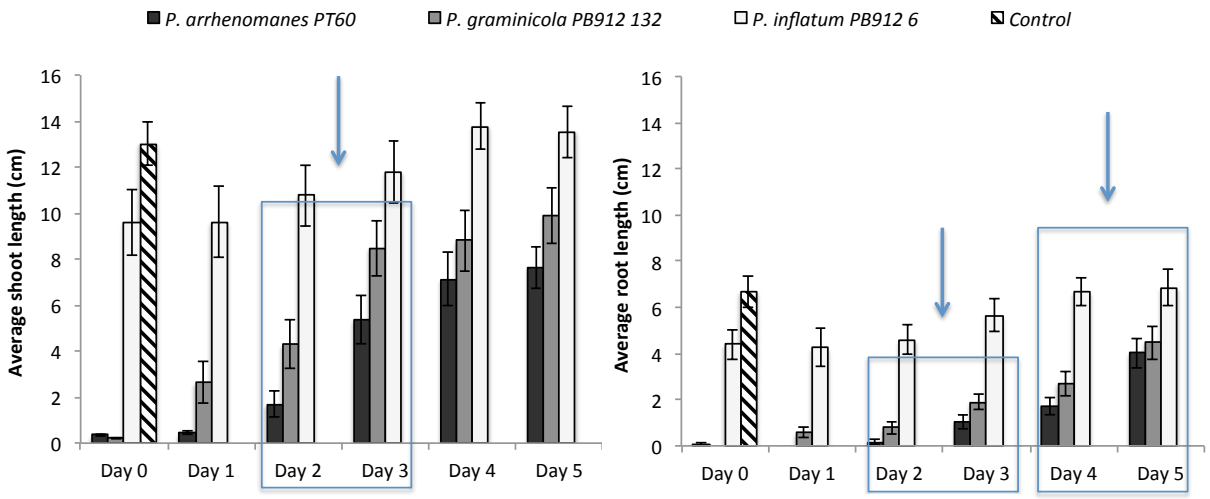

Figure S3.3. The effect of the inoculation day on *Pythium*-induced root and shoot stunting in rice seedlings (cv. Apo). Rice seeds were surface-sterilized and six seeds were placed 2 cm apart on GB5-filled square Petri dishes. Next, plates were inoculated, partly covered and placed in upright position in a 28°C growth chamber (for more details, see methods 3.2.4.). Inoculation happened immediately or was conducted after 1, 2, 3, 4 or 5 days. At 10 days post imbibition, disease symptoms were evaluated by measuring the maximal root and shoot length of the seedlings. Data represent the average lengths from four replicate plates of two different experiments (n = 24). Error bars display standard error values. The blue arrows and frames indicate the sudden increases in root or shoot lengths when inoculation proceeded at 2 versus 3 days and 4 versus 5 days post imbibition. This is consistent with the study of Chun and Schneider (1998), which showed that rice seedlings become more resistant to *Pythium* spp. within 8 days after planting. Based on these results, we decided to inoculate rice seedlings at 3 days post imbibition for histopathological and RNA/DNA-related studies (see chapter 4, 5 and 6).

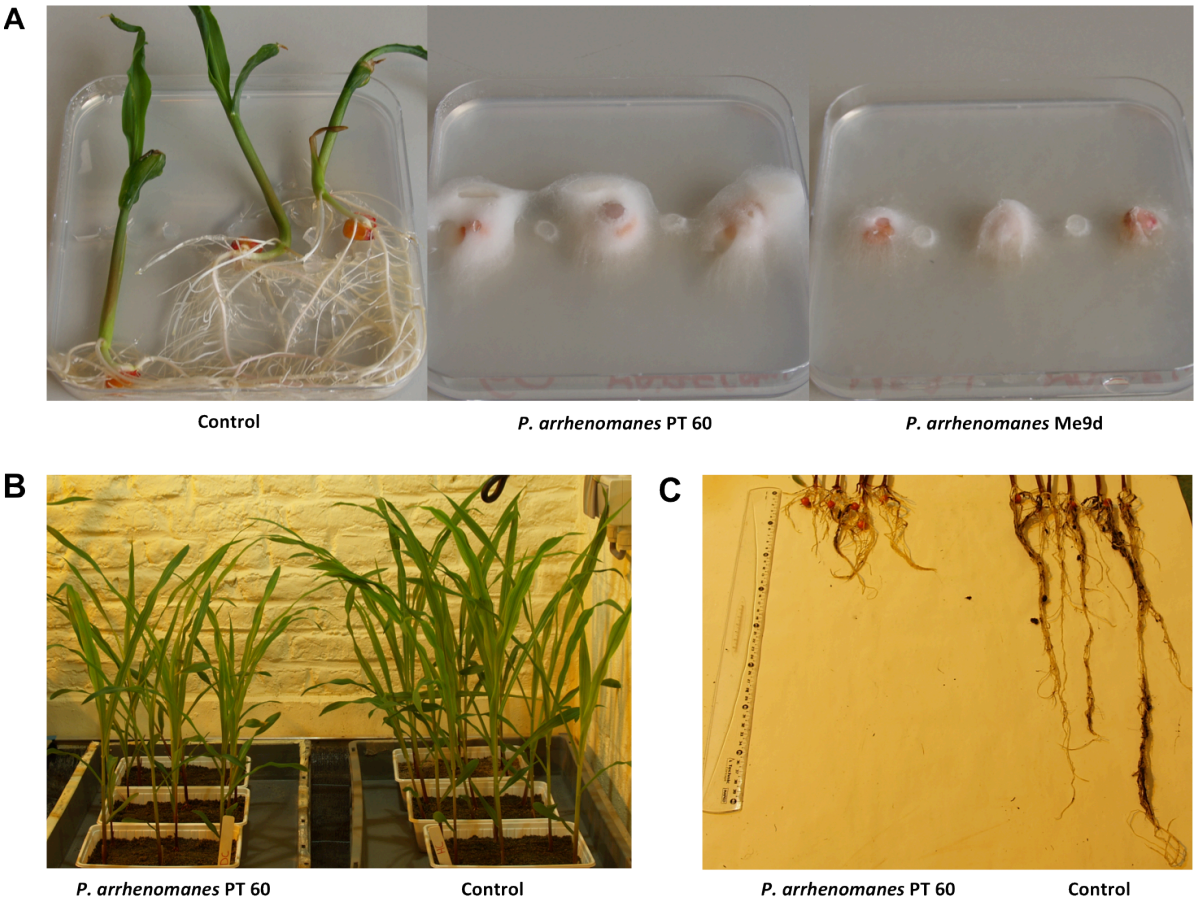

Figure S3.4. Disease symptoms induced by *P. arrhenomanes* on maize seeds and seedlings under *in vitro* (A) and *in vivo* (B, C) conditions. A, Rice isolate (PT 60) showed to be as pathogenic on maize as maize isolate (Me9d). Interestingly, its growth response to maize exudates seemed to be more pronounced than that of Me9d. **B, C,** *In vivo* inoculations resulted in a strong root and shoot stunting compared to the non-inoculated control. For infections, we used an inoculated rice substrate. Rice grains were soaked in water for 3 h, after which they were decanted and autoclaved twice with 24 h in between. Subsequently, the substrate was inoculated with 10 agar plugs of a five-day old *P. arrhenomanes* culture and incubated at 28°C for 7 days. During incubation, the mixture was frequently shaken and humidified if necessary. Maize seeds were sown in a soil mixture (25% potting soil : 75% sand) and four colonized rice-substrate clumps, consisting of more or less six colonized grains, were placed in a square pattern around each maize seed at 1 cm distance and a depth of 2-3 cm. Soil moisture was continuously maintained high and trays were placed in a growth chamber at 24°C and a 16/8 h light cycle. Disease symptoms were scored two weeks after inoculation. The *in vivo* experiments were part of seed-coating trials that have been executed for BASF.

Chapter 4

Pythium species that attack rice roots differ in virulence, host colonization and nutritional profile

Van Buyten E, Höfte M, 2013. Submitted to BMC Plant Biology (under revision)

Abstract

Progressive yield decline in Philippine aerobic rice fields has been recently associated with three closely related *Pythium* spp., *P. arrhenomanes*, *P. graminicola* and *P. inflatum*. To understand their differential aggressiveness toward rice seedlings, we conducted a comparative survey in which host colonization, host responses and carbon utilization profiles were examined using histopathological analyses, phenoarrays, DNA quantifications and gene expression studies. The most aggressive species, *P. arrhenomanes*, quickly colonized the outer and inner root tissues of rice seedlings, including the xylem, by which it possibly blocked the water transport and induced severe stunting, wilting and seedling death. The lower virulence of *P. graminicola* and *P. inflatum* seemed to be reflected in slower colonization processes, limited invasion of the vascular stele and less systemic spread, in which cell wall fortification events appeared to play a role. Progressive hyphal invasions triggered the production of reactive oxygen species (ROS) and phenolic compounds, which was the strongest for *P. arrhenomanes* and was delayed or much weaker upon *P. inflatum* infection. The necrosis marker *OsJAmyb* was faster and more strongly induced by *P. arrhenomanes* than the other two species. Although the least aggressive species was nutritionally the most versatile, *P. arrhenomanes* appeared physiologically more adapted to its host, evidenced by its broad amino acid utilization profile, including D-amino acids, L-threonine and hydroxyl-L-proline. The latter two compounds have been implicated in plant defense and their use by *P. arrhenomanes* could therefore be part of its virulence strategy. This study illustrates that the virulence of rice-pathogenic *Pythium* species is related to their root colonization capacity, the intensity of induced root responses and their ability to utilize amino acids in their colonization niche. Accordingly, this chapter presents important knowledge concerning rice root infections by oomycetes, which could be helpful to further disentangle virulence tactics of soil-borne pathogens.

4.1. Introduction

Pythium species are ubiquitous soil-borne oomycetes that rank from opportunistic up to highly virulent pathogens on many plant species. They mainly infect young plant tissues and cause pre- and post-emergence damping off or reduce the vigor and growth of surviving seedlings. Besides, they infect mature plant roots, resulting in severe necrosis and stunting (Martin and Loper, 1999). Several important graminaceous crops, including maize, wheat, rice, sugarcane, barley, sorghum and turf grasses have been mentioned to suffer from *Pythium* attacks (van der Plaats-Niterink, 1981). Recently, *P. arrhenomanes*, *P. graminicola* and *P. inflatum* were associated with progressive yield decline in Philippine aerobic rice fields (see Chapter 3). These closely related *Pythium* spp. exhibited a varying degree of aggressiveness towards aerobic rice seedlings, among which *P. arrhenomanes* was the most virulent and inflicted a strong pre- and post-emergence damping-off, and stunting of rice shoots, while *P. graminicola* was less virulent and *P. inflatum* was identified as non-pathogenic under *in vivo* growth conditions.

Few histopathological studies have been monitoring the infection process of *Pythium* spp. in monocot roots. Mojdehi et al. (1991) investigated the infection of wheat roots by *P. arrhenomanes* and in this study, the oomycete appeared to penetrate wheat roots via appressoria-like structures. This pathogen also intracellularly invaded the root cortex and, to a lesser extent, the stele, which eventually resulted in severe cortical cell collapse and strong browning of wheat roots. In addition, the interaction between *P. arrhenomanes* and corn has been analyzed (Napi-Acedo and Exconde, 1965), and demonstrated that *P. arrhenomanes* developed two different types of hyphae during its colonization of corn roots. To our knowledge, studies on the interaction of rice roots with *P. graminicola*, *P. arrhenomanes* or *P. inflatum* are currently lacking. Furthermore, comparative histopathological analyses with highly and weakly virulent *Pythium* spp. on monocot roots have never been executed. Therefore, we explored the rice root colonization processes of one isolate each of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* from diseased aerobic rice fields in the Philippines. *In vitro* infection trials allowed the accurate evaluation of rice root and shoot development upon *Pythium* inoculation, and histological and molecular techniques were used to unravel qualitative and quantitative differences among the infection processes of these three species. Using similar techniques, rice root responses to *Pythium* spp. were investigated over time. In addition, phenoarrays were carried out to reveal the nutritional needs of the three oomycetes and to identify carbon-utilization patterns specific to highly virulent *Pythium* species. Our research discovered clear differences in the root colonization capacity and nutritional profiles of rice-attacking *Pythium* spp..

4.2. Methods

4.2.1. *Pythium* isolates

In this study, we selected one isolate each of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* to study the interaction between *Pythium* and rice (Table 4.1). These isolates exhibit different levels of virulence when tested under *in vitro* conditions (see Chapter 3). The isolates were cultured on 22 ml of potato dextrose agar (PDA; Difco Laboratories) at 28 °C in the dark.

Table 4.1. Origin of the *Pythium* strains that were used in this study.

Species	Isolate	Geographic origin	Year of isolation	Collection no.[a]
P. arrhenomanes	PT 60	The Philippines, Tarlac (Dapdap)	2007	MUCL52737
P. graminicola	PB912 132	The Philippines, Los Baños (IRRI)	2008	MUCL53742
P. inflatum	PT 52	The Philippines, Tarlac (Dapdap)	2007	MUCL53750

[a]All isolates were recovered from aerobic rice fields (see Chapter 3).

4.2.2. Plant material and infection trials

The rice cultivar Nipponbare (*O. sativa* subspecies *japonica*) was selected for the *in vitro* monitoring of disease symptoms caused by *P. arrhenomanes*, *P. graminicola* and *P. inflatum* on rice roots and shoots. Seeds were as susceptible to *Pythium* as the aerobic rice cv. 'Apo', the original host. Prior to germination, seeds were disinfected by agitation in 70% EtOH (1 min) and 2% NaOCl (15 min). After three successive rinses in sterile demineralized water and blotting on sterile filter paper, surface-sterilized seeds were plated on square Petri dishes (120 × 120 mm) filled with 50 ml of Gamborg B5 (GB5) medium (Gamborg et al., 1968). Plates were incubated at 28 °C in the dark for three days. Subsequently, seedlings with equal primary root lengths (1.5 cm) were selected and 6 seedlings were transplanted 2 cm apart on fresh GB5 plates. Three mycelial plugs (5 mm in diameter) taken from the edge of a three-day-old *P. arrhenomanes, P. graminicola* or *P. inflatum* colony were placed between the roots of seedlings 1-2, 3-4 and 5-6. Square Petri dishes were partly covered with aluminum foil to shield the roots from light and then, incubated in upright position (60 °C angle) inside a growth chamber with a 12 h day (28 °C) / night (26 °C) cycle. The experiment consisted of three replications per treatment. For disease evaluation, maximal root and shoot lengths were measured ten days post inoculation (dpi) (n = 18).

Disease symptoms on rice shoots were rated on the basis of a disease severity scale (Figure 4.1) (n = 3): score 0, healthy shoots; score 1, shoot length ≥ 50% of the control, green culm and few yellow or brown spotted leaves; score 2, shoot length ≥ 34% of the control, slightly yellowing culm and yellow or brown spotted leaves; score 3, shoot length < 34% of the control, slightly yellowing culm and yellow or brown spotted leaves; score 4, shoot length < 34% of the control, yellow culm and yellow or brown leaves; score 5, shoot length < 34% of the control, brown, dried-out culm and leaves. This scale enabled us to calculate the disease severity index (DSI) for each biological replicate (i.e. Petri dish) using the following equation: (((# x score 0) + (# x score 1) + (# x score 2) + (# x score 3) + (# x score 4) + (# x score 5)) / ((total #) x score 5))) x 100 (with # the no. of seedlings). Most data were not normally distributed and statistically analysed with Kruskal-Wallis and Mann-Whitney non-parametric tests in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$). Data that were normally distributed were evaluated by one-way ANOVA and a Duncan Post-Hoc test ($\alpha = 0.05$, $P \leq \alpha$).

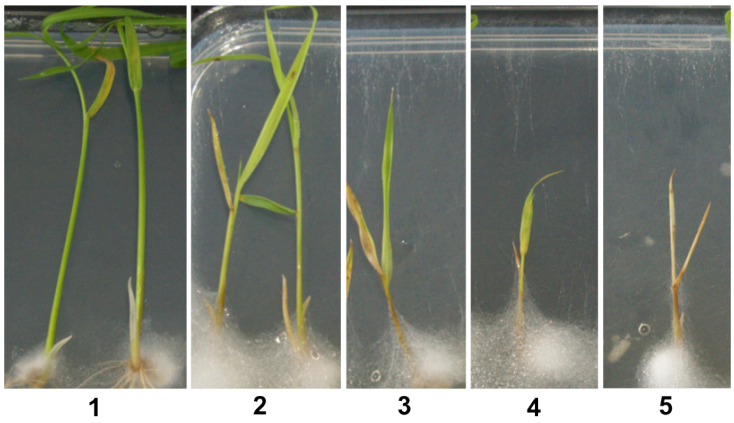

Figure 4.1. The disease severity scale implemented for disease rating on rice shoots. Shoot disease severity scores (1-5) are based on the degree of stunting and wilting.

4.2.3. Microscopic analysis of root samples

Root infection processes were analyzed at various times after *P. arrhenomanes, P. graminicola* and *P. inflatum* inoculation using bright field and epifluorescence microscopy. In this experiment, GB5-culture plates contained four rice seedlings next to which four mycelial plugs were placed at 0.5 cm distance at the right side of each emerging radicle. Parts of the primary roots showing superficial hyphal growth and necrosis were excised from *Pythium*-inoculated rice seedlings. Root samples of ± 0.5 cm were fixated in a 50 mM sodium phosphate buffer (pH 7.2) containing 4% paraformaldehyde and 1% glutaraldehyde, and subsequently dehydrated in a graded series of EtOH and infiltrated with Technovit 7100 solution. The infiltrated root samples were finally embedded in plastic 1 cm^2 - cubes filled with Technovit 7100 histo-embedding medium (Heraeus Kulzer, Wehrheim, Germany).

A Leica RM2265 motorized rotary microtome (Leica Microsystems, Nussloch, Germany) was used to produce 5 µm cross-sections. For each treatment at different time points, three to four root samples of at least two different seedlings were entirely sectioned. This resulted into 50 cuttings per sample, which were randomized on ten microscopic slides. At least two slides were chosen for each staining procedure. *Pythium* hyphae were stained by incubating of sections in 0.1% (w/v) trypan blue in 10% (v/v) acetic acid for 5 min. The accumulation of phenolic compounds was visualized by staining with 0.1% Fluorescent brightener 28 (Calcofluor White M2R) for 1 min. All stained sections were thoroughly rinsed, dried and mounted with neutral mounting medium (DPX, Klinipath, Belgium). The accumulation of hydrogen peroxide (H_2O_2), a marker for plant defense and induced necrosis, was demonstrated by staining of fresh root samples with 3,3'-diaminobenzidine (DAB). Three to four root samples were collected from at least two different seedlings for each treatment at various time points. Immediately after sampling vacuum-infiltration with a 0.1% DAB-solution (pH 4.4) was performed in three successive steps of 5 min in the dark. After an extra incubation step of 4 min, the residual stain was removed and the samples were fixated, sectioned and mounted as described above. The DAB-stained specimens were also treated with trypan blue to visualize *Pythium* hyphae. Digital images were acquired with an Olympus BX51 microscope equipped with an Olympus ColorView III Camera and Xenon light source. A DAPI narrow-band fluorescence cube (BP330-385 nm/DM400/BA420) was selected for analyses with the fluorescent stain. Images were processed with the Olympus analysis cell^F software (Olympus Soft Imaging Solutions, Münster, Germany) and ImageJ 1.44p.

4.2.4. Quantitative detection of *Pythium* spp. in root samples

Primers specific for the multi-copy Internal Transcribed Spacer (ITS) region of the ribosomal DNA (rDNA) of *P. arrhenomanes* PT 60, *P. graminicola* PB912 132 and *P. inflatum* PT 52 were constructed using Primer-BLAST (NCBI) and OligoAnalyzer 2.1. web-software (IDT, Coralville, IA) (Table 4.2). The species specificity of the primers was double-checked with pure DNA of the three *Pythium* spp. and rice root DNA (cv. Nipponbare). Optimal annealing temperatures were assessed by gradient PCR on a thermal cycler (Flexcycler, Analytikjena) and a primer titration was executed on a Mx3005P real-time PCR detection system (Stratagene) using Sybr Green master mix (Fermentas). After setting the optimal reaction conditions, standard curves based on quinquepartite dilution series (10 ng - 1 pg) were run on the real-time PCR to monitor the amplification efficiency and accuracy of the primer pairs. If the curves showed $R^2 \geq 0.985$, slopes between -3.1 à -3.6 and efficiencies between 90-110%, primers were accepted.

Pythium-inoculated rice roots were collected at different time points from rice-seedling culture plates, in two separate experiments (see 4.2.2). In each experiment, samples from two replicate plates were pooled per treatment at each time point. Samples were immediately frozen in liquid nitrogen or prior to freezing surface-sterilized in 1% NaOCl for 1 min and thoroughly washed. Next, DNA was extracted from the finely crushed roots with the DNeasy Plant Mini Kit (QIAGEN).

The quality and concentration of the extracted DNA was determined with a ND-1000 spectrophotometer (NanoDrop). If necessary, ethanol precipitation was applied to concentrate the DNA sample. The extracted DNA (2.5 µl of 1 ng/µl) was added to 96-well plates, filled with 12.5 µl aliquots of Sybr Green master mix (Fermentas) that were supplemented with 2.5 µl of each primer stock solution (Table 4.2), 0.05 µl of ROX solution and 4.95 µl of nuclease-free water. Each DNA sample was analyzed in duplicate with the Mx3005P real-time PCR detection system (Stratagene) using the following thermal profile: an initial denaturation at 95 °C for 10 min, and 40 cycles of 15 s at 95 °C, 30 s at 63 °C and 15 s at 72 °C. To verify amplicon specificity, a default melting-curve analysis (Stratagene) was included. Finally, cycle treshold (Ct) values (n = 4) were implemented in the standard curves' equations to quantify the amount of *Pythium* DNA in the collected root samples.

Table 4.2. Sequences of the species-specific primers that amplify a part of the ITS1 region.of the rDNA.

Pythium isolate	Forward ITS primer (5'-3') [a]	Reverse ITS primer (5'-3') [a]	Size amplicon	GenBank Accession no. [b]
P. arrhenomanes PT 60	ATTCTGTACGCGTGGTCTTCCG (3 µM)	ACCTCACATCTGCCATCTCTCTCC (1 µM)	311 bp	HQ877857
P. graminicola PB912 132	ATGGCTGAACGAAGGTGGGCTG (1 µM)	TCCCGAAAGTGCAATGTGCGTTC (3 µM)	240 bp	HQ877865
P. inflatum PT 52	AGGTGGGCGCATGTATGTGTGTC (500 nM)	ACGTATCGCAGTTCGCAGCG (3 µM)	165 bp	HQ877856

[a]The concentrations for each primer stock are presented between brackets.

[b]The rDNA-ITS regions of *P. arrhenomanes* PB912 75 (HQ877857) and *P. graminicola* PB912 116 (HQ877865) are respectively identical to those of *P. arrhenomanes* PT 60 and *P. graminicola* PB912 132 (see Chapter 3).

4.2.5. Gene expression analysis in *Pythium*-inoculated rice roots

Rice roots were collected from GB5-culture plates at different times upon *Pythium* inoculation (see 4.2.2). In each of three experiments, root samples from two replicate plates were pooled per treatment at each time point. Samples were frozen in liquid nitrogen, finely crushed and afterwards, total RNA was extracted using the spectrum plant total RNA kit (Sigma-Aldrich). A Turbo Dnase treatment (Ambion) was immediately performed and the quality and concentration of the extracted RNA was determined with a ND-1000 spectrophotometer (NanoDrop). Next, complement DNA (cDNA) was synthesised from the total RNA (10 ng/µl) with Multiscribe reverse transcriptase and random primers (Applied Biosystems). This cDNA (2.5 µl of 10 ng/µl) was added to 96-well plates, filled with 12.5 µl aliquots of Sybr Green master mix (Fermentas) that were supplemented with 2.5 µl of each primer stock solution (Table 4.3), 0.05 µl of ROX solution and 4.95 µl of nuclease-free water. Quantitative Real-Time PCR (qPCR) analyses were executed on a Mx3005P real-time PCR detection system (Stratagene) under the following conditions: an initial denaturation step at 95 °C for 10 min, and 40 cycles of 15 s at 95 °C, 30 s at 59 °C and 15 s at 72 °C. After the PCR, a default melting curve (Stratagene) was generated to test amplicon specificity.

The quantity of plant RNA in each sample was normalized using *OsACTIN1* (LOC_Os03g50890) as internal reference (Table 4.3). Ct values (n = 3) were relatively expressed to the non-inoculated control at 1dpi.

Table 4.3. Sequences of the used primers for gene expression analysis.

Gene	Forward primer (5'-3') [a]	Reverse primer (5'-3') [a]	GenBank Accession no.
OsJAmyb	GAGGACCAGAGTGCAAAAGC (3 µM)	CATGGCATCCTTGAACCTCT (3 µM)	AY026332
OsACTIN1	GCGTGGACAAAGTTTTCAACCG (1 µM)	TCTGGTACCCTCATCAGGCATC (3 µM)	X15865

[a]The concentrations for each primer stock are presented between brackets.

4.2.6. Phenoarray

The carbon-utilization patterns of pure *P. arrhenomanes, P. graminicola* and *P. inflatum* cultures were investigated *in vitro* with SF-N2 and SF-P2 MicroPlates (Biolog Inc.) based on the protocol of Chun et al. (2003). In these MicroPlates, each well contains a nutrient base with specific carbon sources, including several carbohydrates, amino acids and carboxylic acids. *Pythium* strains were cultured in 50 ml of potato dextrose broth (PDB; Difco Laboratories) at 28 °C in the dark. After 11 days, liquid cultures were filtered with sterile sieving cloth (1 mm mesh) and the retained mycelial mats were washed in three rinses with sterile demineralized water. The harvested mycelium was subsequently minced in a sterile 1 mM potassium phosphate buffer (pH = 7) and again sieved (1 mm mesh). Optical densities (ODs) of the suspensions were determined at 595 nm in triplicate using a Multiscan EX spectrophotometer (Thermo Labsystems). Next, ODs were adjusted to 0.100 ± 0.006 by dilution in sterile phosphate buffer and three SF-N2 and SF-P2 MicroPlates were filled with 100 µl aliquots of each mycelial suspension. Control plates were filled with 100 µl aliquots of sterile phosphate buffer. Upon an incubation step of 24 h at 28 °C in the dark, carbon source utilization patterns of *P. arrhenomanes* PT 60, *P. graminicola* PB912 132 and *P. inflatum* PT 52 were assessed by turbidity measurements at 595 nm. The experiment was repeated in time to verify the reproducibility. At least six OD-values per carbon source (n = 6) were generated for each treatment. Data were statistically analyzed using Kruskal-Wallis in SPSS 21 ($\alpha = 0.05$, $P \leq \alpha$). When turbidities significantly exceeded the initial OD (= 0.1), *Pythium* spp. were assumed to use the according carbon sources for their growth.

4.3. Results

4.3.1. Macroscopic symptoms on rice seedlings upon *Pythium* infection

Rice seedlings (cv. Nipponbare) were cultured on GB5 agar plates to study the effect of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* on root and shoot development.

Macroscopic evaluation of *Pythium*-inoculated rice seedlings illustrated the intense colonization of rice tissues by *P. arrhenomanes* (Figure 4.2). Both rice seed and root surfaces became massively covered with aerial mycelium by 2 days post inoculation (dpi). When the infection proceeded, the mycelium concentrated primarily on and in the vicinity of the rice seeds. Similarly, a dense white mycelium appeared on the seeds of *P. inflatum*-inoculated cultures by 2 dpi. The superficial colonization was, however, less pronounced in this case. *P. graminicola* hyphae grew well in the medium adjacent to the rice seeds, but rice seedling surfaces were never heavily colonized. When nutrients were eliminated from the medium, we still observed a stimulated hyphal growth near the rice seeds (data not shown). This growth stimulation was probably due to seed exudation. When *Pythium* spp. were cultured in seed exudates alone, an increased *Pythium* growth was indeed observed (Figure S4.1).

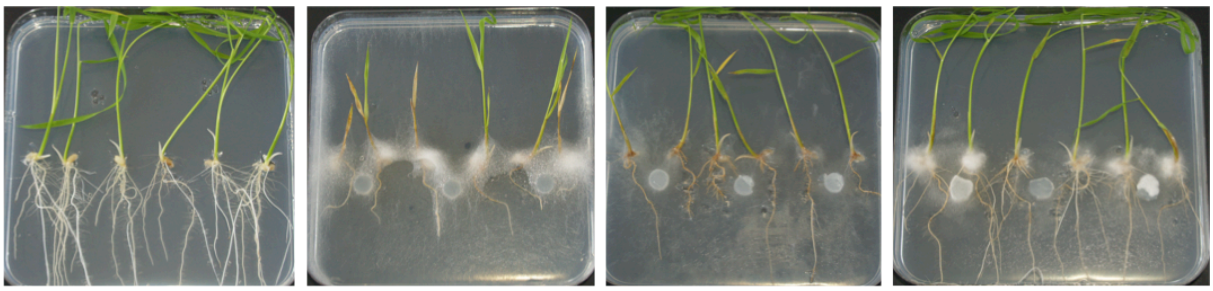

Figure 4.2. Disease symptoms on rice seedlings 10 days after *Pythium* inoculation. Clear differences in stunting and wilting were noticed among *P. arrhenomanes*-, *P. graminicola*- and *P. inflatum*-inoculated rice seedlings. Rice seeds were germinated on Gamborg B5 medium and three days post imbibition, seedlings were inoculated with mycelial plugs. Three replicate plates were evaluated per treatment. Pictures are representative for all replicates.

Evaluation of the *in vitro* cultures at 10 dpi revealed that rice seedlings were very susceptible to *P. arrhenomanes* and *P. graminicola* infection (Figure 4.2, Table 4.4). The most virulent species *P. arrhenomanes* inhibited crown root and lateral root formation. Besides, primary root lengths were significantly reduced by 63% compared to the non-inoculated control ($P \leq 0.05$). Necrosis was visible as typical brown discolorations on the upper part of the primary roots. When shoot growth was monitored upon *P. arrhenomanes*-inoculation, a significant stunting of 61% relative to the control became evident ($P \leq 0.05$). Furthermore, 72% of all inoculated rice seedlings exhibited clear wilting symptoms, visible as yellowing or browning of culms and/or leaves. The high aggressiveness of *P. arrhenomanes* also resulted in a significant seedling death of 28% ($P \leq 0.05$), i.e. the percentage of plantlets with shoot disease severity score 5.

Table 4.4. *Pythium*-induced disease symptoms on rice seedlings at 10 dpi.

Species	Shoot length (cm) [a]	Root length (cm) [a]	DSI % [b]	Dead seedlings % [c]
Control	16.07 (0.34) **a**	9.19 (0.43) **a**	3 (1.92) **a**	0 **a**
P. arrhenomanes	6.24 (0.62) **b**	3.42 (0.15) **b**	59 (7.22) **b**	28 (11.11) **b**
P. graminicola	10.26 (0.57) **c**	4.74 (0.23) **c**	32 (1.00) **c**	0 **a**
P. inflatum	14.97 (0.42) **a**	9.09 (0.50) **a**	23 (0) **d**	0 **a**

[a]Mean shoot and root length data (n = 18).

[b]Average disease severity indices (DSI) (n = 3) were calculated using the disease severity scale (see "Materials and methods"). Indices vary from 0% (healthy shoots) up to 100% (dead shoots) and illustrate the degree of yellowing or browning in culms and leaves in parallel with shoot stunting. Eighteen plantlets were individually scored for each treatment.

[c]The percentage of death seedlings displays the mean number of shoots (n = 3) with disease severity score 5.

[a,b,c]Statistical analyses were mostly performed by Kruskal-Wallis and Mann-Whitney non-parametric tests in SPSS 21 (SPSS Inc.). Only shoot length data were normally distributed and therefore, analysed with one-way ANOVA and the Duncan Post-Hoc test. Distinct lower case letters in each column mark significant differences between the treatments ($\alpha = 0.05$, $P \leq \alpha$). Values between brackets are standard errors.

In contrast to *P. arrhenomanes*, *P. graminicola* never impaired crown root and lateral root formation in rice seedlings. Nonetheless, all root types were highly stunted and exhibited an overall clear brown discoloration. Primary root lengths were significantly shorter ($P \leq 0.05$), with lengths representing 52% of those of the non-inoculated control. Opposing the strong wilting symptoms on *P. arrhenomanes*-inoculated rice seedlings, shoots appeared significantly healthier ($P \leq 0.05$) and survived all upon *P. graminicola* inoculation. Moreover, shoot growth was reduced by 36%, which was significantly less ($P \leq 0.05$) than the stunting evoked by *P. arrhenomanes*. The impact of *P. inflatum* on rice seedling development was rather minor in comparison with *P. arrhenomanes* and *P. graminicola* (Figure 4.2, Table 4.4). Except for a curtailed crown root elongation and necrotic patches on primary, crown and lateral roots, no apparent disease symptoms were observed. Maximal root lengths of *P. inflatum*-inoculated seedlings represented 99% of the control treatment. In addition, shoot stunting was only 7% and appeared not significant ($P > 0.05$). Most shoots were categorized in the lowest disease score, exhibiting only few symptoms of wilting. Disease severity indices were still significantly distinct from the control treatment ($P \leq 0.05$) and consequently, *P. inflatum* was classified as weakly virulent under *in vitro* growth conditions.

4.3.2. Histological study of the *Pythium* infection process in rice roots

To investigate the colonization process of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* inside rice roots, we examined trypan blue-stained root cuttings with bright field microscopy (Fig 4.3). The three *Pythium* spp. invaded primary roots via direct penetration of epidermal cells (Figure 4.3, A).

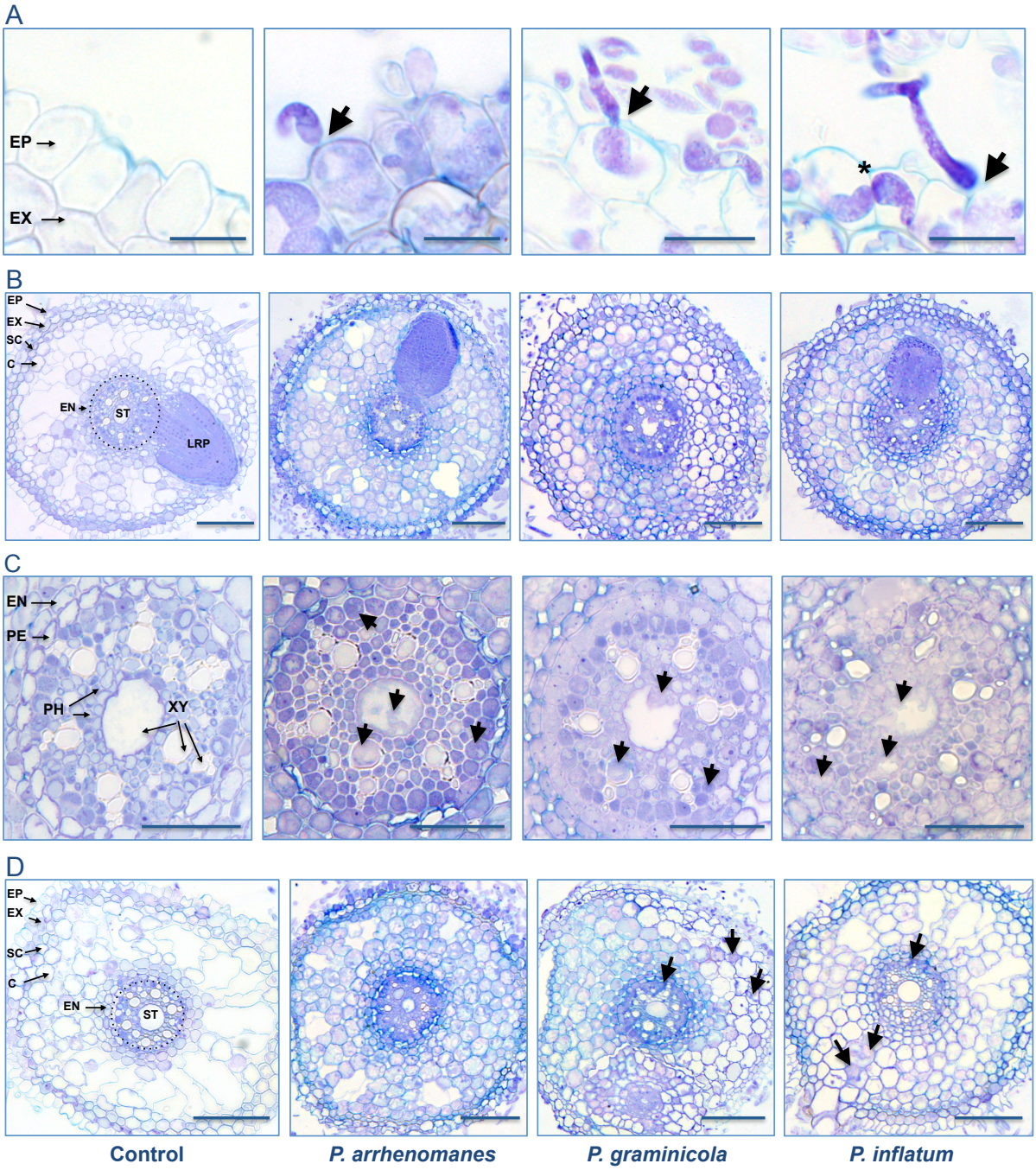

Figure 4.3. Histological study of the *Pythium* infection process in rice roots. A, Direct penetration of the rice rhizodermis by *P. arrhenomanes, P. graminicola* and *P. inflatum*. Arrows indicate sites of hyhal penetration. Bulbous-like *Pythium* hyphae grew intracellularly and became severely constricted right before cell wall passing (*). **B,** Colonization of the inner rice root tissues by *P. arrhenomanes, P. graminicola* and *P. inflatum* in the most heavily infected (upper) parts of the primary root at 27 hpi. **C,** Colonization of the stele by *P. arrhenomanes, P. graminicola* and *P. inflatum* in the most heavily infected parts of the primary root at 27 hpi. Arrows indicate hyphae in the phloem and xylem. **D,** Colonization of the inner root tissues 2 days upon inoculation with *P. arrhenomanes*, *P. graminicola* and *P. inflatum* in the middle part of the primary root. *P. graminicola* and *P. inflatum* hyphae were less abundant in the cortex and vascular tissues (arrows). Abbreviations in the mock pictures designate the epidermis (EP), exodermis (EX), sclerenchyma (SC), cortex (C), endodermis (EN), pericycle (PE), stele (ST), phloem (PH), xylem (XY) and lateral root primordium (LRP). Cross-sections (5 µm) from *Pythium*-infected root tissues were stained with trypan blue. Scale bars in A = 20 µm, in B = 100 µm, in C = 50 µm and in D = 100 µm.

Swollen hyphae rather than specialized appressoria were used as penetration tools. Immediately after *Pythium* ingress, hyphae differentiated in irregularly inflated structures that headed intracellularly to the walls of neighboring cells. Right before cell wall crossing, hyphae became dramatically constricted (Figure 4.3, A). The same strategy was likely adopted during the further spread of *Pythium* hyphae in the cortex and vascular tissue. However, *P. arrhenomanes*, *P. graminicola* and *P. inflatum* differed in the extent to which inner root tissues were colonized. In the most heavily infected parts of the primary root (the upper 1.5 cm), the three *Pythium* spp. strongly colonized the cortex, endodermis and stele within 27 hpi (Figure 4.3, B). A more detailed view onto the stele unraveled the numerous hyphae that were present in the phloem and xylem by that time (Figure 4.3, C). Histological studies on the middle part of the primary root generated similar results for the *P. arrhenomanes* colonization process, while *P. graminicola* hyphae were less abundant in the cortex and vascular tissue (Figure 4.3, D). Besides, the latter pathogen did not invade xylem cells in the majority of the examined cross-sections. In *P. inflatum*-inoculated root samples, the occurrence of a dense hyphal network was less frequent compared to *P. arrhenomanes* and *P. graminicola*-inoculated root samples. In addition, the colonization of the root stele was often limited to the outer cell layers.

4.3.3. Quantification of *Pythium* DNA in rice roots

It has been stated that the combination of qualitative and quantitative techniques allows more accurate time-course monitoring of microbial infection processes in plant roots (Macia-Vicente et al., 2009). Therefore, a DNA-based quantification method was used to further survey the colonization of rice seedling roots by *Pythium* spp.. Real-time PCR on non-surface-sterilized root samples illustrated that *P. arrhenomanes* quickly and massively colonized the entire rice root system (Table 4.5, A). In a first experiment (Exp 1), *P. arrhenomanes* DNA quantities represented 8.42% of the total DNA extract at 1 dpi, which strongly increased up to 49.2% and 59% by 2 and 3 dpi, respectively. Quantities of *P. graminicola* DNA were much lower and maintained more or less the same level over time (i.e. 5-6%). The concentration of *P. inflatum* DNA on and in rice seedlings roots elevated from 1.39% at 1 dpi up to 26.4% at 2 dpi, after which it diminished down to 15.8% by 3dpi. A second experiment (Exp 2) generated similar increasing trends for the *P. arrhenomanes* colonization process. In *P. graminicola*- and *P. inflatum*-inoculated roots, consistent DNA concentrations were measured at 3 dpi (i.e. 5% and 19.6%, respectively), but during the first days, the quantities were respectively higher and lower compared to the first experiment.

qPCR on surface-sterilized root samples enabled the quantification of *Pythium* DNA inside the rice root system. Once more, the concentration of *P. arrhenomanes* DNA clearly elevated over time (Table 4.5, B), with quantities of 0.13% at 20 hpi that multiplied up to 0.84% at 28 hpi and 4.77% at 73 hpi (Exp 1). In contrast, the concentration of *P. graminicola* DNA inside the rice seedling root system was much lower at 20 hpi.

Nonetheless, by 73 hpi, *P. arrhenomanes* and *P. graminicola* DNA quantities attained equal amounts. The share of *P. inflatum* DNA in the total DNA extract was very low during the first 20 h of the infection process. From 28 hpi on, DNA quantities slightly elevated up to approximately 0.1%, but remained much lower than those of the other species. Despite the overall lower infection level in our second experiment, we could observe similar colonization trends for the three *Pythium* spp.. Taken together, these data imply that *P. arrhenomanes* is a better and faster colonizer of rice root surfaces and inner tissues than *P. graminicola* and *P. inflatum*. Furthermore, they demonstrate that the weakly virulent species *P. inflatum* mainly colonizes rice root surfaces, while *P. graminicola* is less efficient in its superficial spread.

Table 4.5. *In planta* quantification of *Pythium* DNA (pg/ng total DNA) using qPCR. A, Concentration of *Pythium* DNA in non-surface sterilized rice seedling roots. Seeds were germinated on Gamborg B5 medium and inoculated at three days post imbibition. Root samples were collected in two different experiments at various times upon inoculation. **B, Concentration of *Pythium* DNA in surface-sterilized rice seedling roots.** Seeds were germinated on plant agar-medium and inoculated at three days post imbibition. Rice roots were collected in two different experiments at various times upon inoculation and were surface-sterilised prior to processing.

	***P. arrhenomanes* ***		***P. graminicola* ***		***P. inflatum* ***	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
A						
1 dpi	84.24 (0.40)	179.26 (17.85)	57.76 (4.70)	179.64 (8.94)	13.88 (0.64)	2.89 (0.27)
2 dpi	492.00 (28.28)	291.42 (19.37)	47.28 (0.85)	142.00 (30.60)	264.10 (03.65)	113.44 (2.09)
3 dpi	590.00 (36.77)	405.32 (82.99)	58.40 (1.58)	51.14 (10.78)	157.70 (25.37)	196.16 (6.79)
B						
20 hpi	1.246 (0.141)	2.162 (2.437)	0.330 (0.027)	0.031 (0.001)	0.010 (0.006)	0.003 (0.001)
28 hpi	8.434 (0.240)	8.782 (0.167)	8.958 (0.325)	1.128 (0.082)	0.966 (0.040)	0.108 (0.006)
73 hpi	47.680 (4.299)	4.564 (0.238)	55.320 (6.505)	0.332 (0.040)	0.857 (0.157)	0.019 (0.001)

*****In each experiment (Exp 1 and 2), 12 rice seedlings were pooled per treatment at various times. The presented data are means of two technical replicates (n = 2). Values between brackets represent standard deviations.

4.3.4. Rice root responses to *Pythium* spp.

4.3.4.1. Reactive oxygen species (ROS)

The accumulation of hydrogen peroxide (H_2O_2) in *Pythium*-inoculated rice seedling roots was visualized with bright field microscopy and an endogenous peroxidase-dependent staining procedure using DAB. When the most heavily infested (upper) parts of the primary roots were analyzed, we detected reddish-brown DAB precipitates in the outer cell layers (Figure 4.4, A; black arrows).

At 1 dpi, H_2O_2 production appeared the strongest in *P. arrhenomanes*-inoculated seedling roots, where they accumulated in the sclerenchyma and some cortical cells. By 2 dpi, DAB precipites became less visible. In *P. graminicola*-inoculated seedlings, DAB concentrated in the epidermis and exodermis of the roots, and this H_2O_2 production also diminished by 2 dpi.

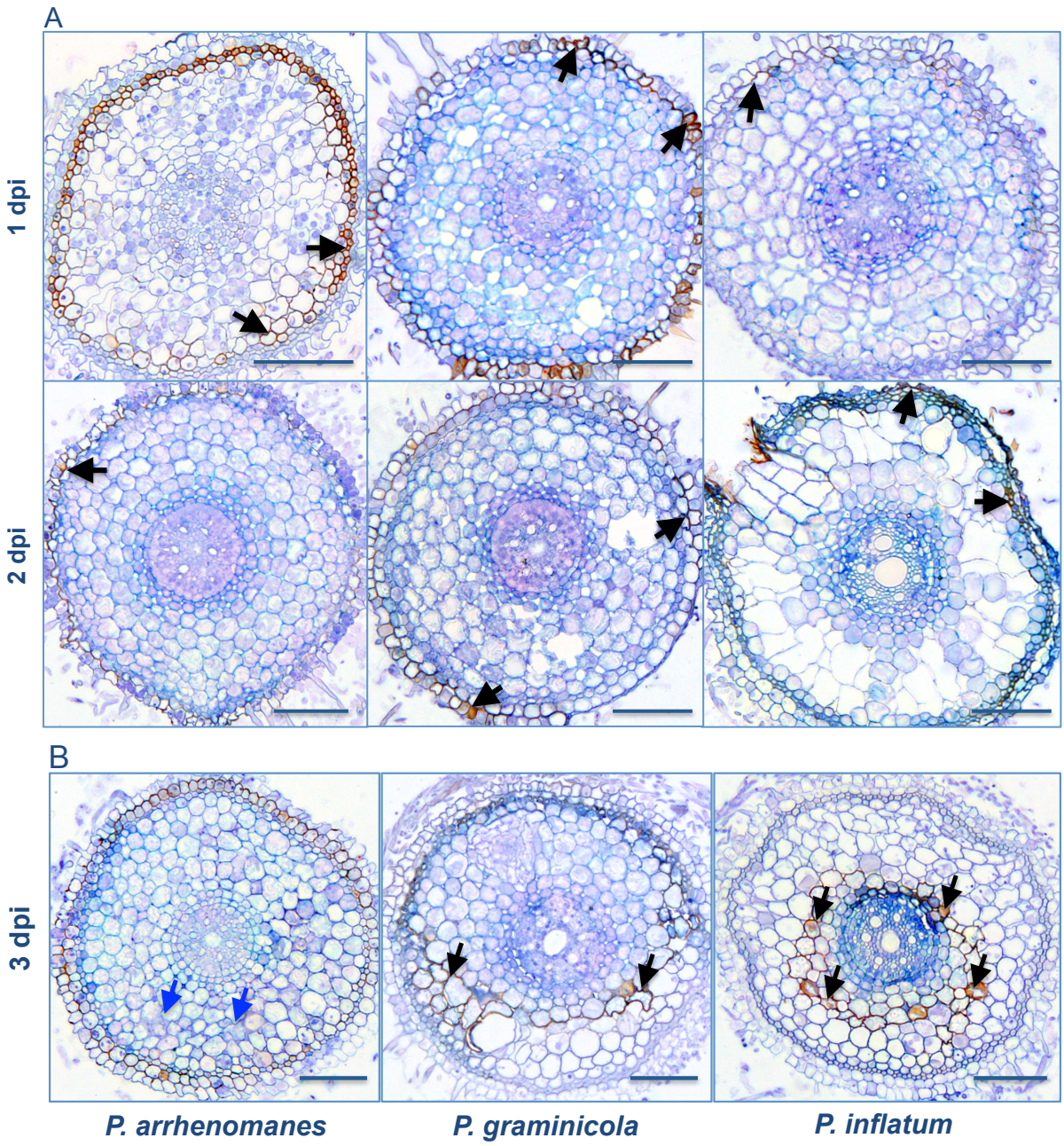

Figure 4.4. H_20_2 production in *Pythium*-inoculated rice roots. A, Black arrows indicate reddish-brown DAB precipitates at 1 and 2 dpi in the most heavily infected parts of the primary root (upper 1.5 cm). **B,** Black arrows indicate defense-related DAB precipitates at 3 dpi in the middle part of the primary root. Blue arrows indicate cell collapse. All cross-sections (5 µm) were stained with trypan blue and DAB. Scale bars in **A** en **B** = 100 µm.

On the contrary, DAB precipitates were barely noticeable during the first 24 h of the *P. inflatum*-rice interaction. By 2 dpi, the H_2O_2 production slightly increased in the epidermis, exodermis and sclerenchyma of seedling roots, but overall a lower response was noted upon inoculation with this species. DAB accumulation did not occur in control roots.

When the middle part of the primary root was examined, we also observed DAB accumulation in the outer and inner cortex of *P. inflatum*- and *P. graminicola*-inoculated seedlings at 3 dpi (Figure 4.4, B; black arrows). In both cases, this H_2O_2 accumulation seemed to hamper hyphal proliferation in a part of the root cortex and slowed down the colonization process. In *P. arrhenomanes*-inoculated seedlings, cortical cells seemed to collapse at this stage of the infection (Figure 4.4, B; blue arrows).

4.3.4.2. Phenolic compounds

The accumulation of phenolic compounds in *Pythium*-inoculated rice seedling roots was visualized as an orange-brown autofluorescence by UV-excitation of calcofluor white M2R-stained root cuttings. The autofluorescence was visible from 2 dpi on (Figure 4.5, white arrows) and was the strongest in *P. arrhenomanes*-inoculated rice seedling roots, where it was emitted from parts of the outer root cortex and the sclerenchyma. In *P. graminicola*-inoculated root cuttings, phenolic compounds were primarily visible in the sclerenchyma and little autofluorescence was emitted from the vascular tissue. Similarly, we detected autofluorescence in the sclerenchyma of *P. inflatum*-inoculated rice roots, but once more, the host responded the weakest to this species. No autofluorescence was detected in control roots.

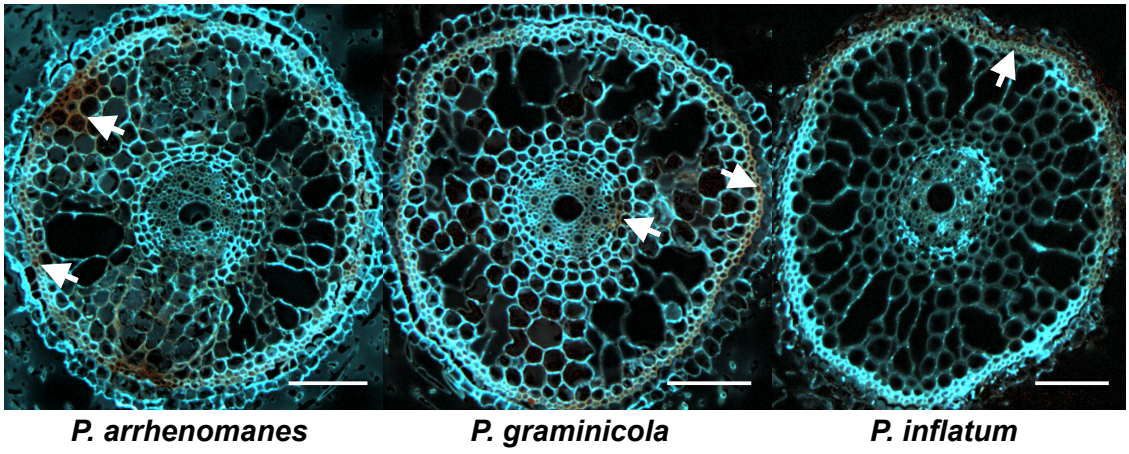

Figure 4.5. Accumulation of phenolic compounds in *Pythium*-inoculated rice roots at 2 dpi. White arrows indicate the orange-coloured autofluorescence upon *Pythium* infection. Cross-sections (5 µm) were stained with Calcofluor white M2R and excited with UV. Scale bars = 100 µm.

4.3.4.3. Expression of the necrosis marker and JA-responsive gene OsJAmyb

To compare the degree of induced necrosis among *P. arrhenomanes*-, *P. graminicola*- and *P. inflatum*-inoculated root systems, the expression of *OsJAmyb* was analyzed by qPCR analysis. This JA- and pathogen-inducible MYB transcription factor has been identified as a necrosis marker and is mainly expressed in plant tissues prior to cell death (Lee et al., 2001). Our gene expression analysis revealed that the three *Pythium* spp. induced necrosis in rice seedling roots (Fig 4.6). *P. arrhenomanes* showed to be the strongest trigger of necrosis at 1, 3 and 4 dpi, when a respective 21.3-fold, 16-fold and 13.4-fold induction in *OsJAmyb* transcription was measured. By 6 dpi, the transcription decreased down to 5.3-fold and approximated the expression level in the control. In *P. graminicola*-inoculated root tissues we noted a lower induction of *OsJAmyb* during the first four days of the infection. By 6 dpi, the expression of the necrosis marker strongly elevated up to 16.2-fold and showed to exceed the level in *P. arrhenomanes*-inoculated roots. Necrosis was barely triggered during the first days upon *P. inflatum* inoculation. Only at 6 dpi, mRNA levels strongly elevated (9.4-fold) and were higher than in *P. arrhenomanes*-inoculated roots.

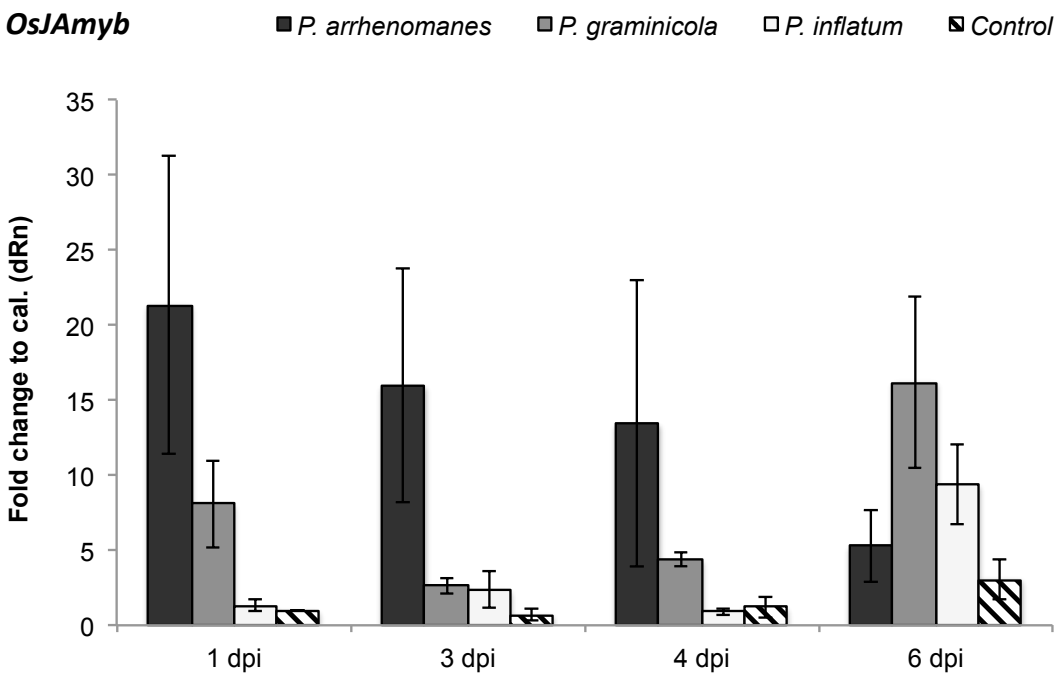

Figure 4.6. Transcription of *OsJAmyb* in *Pythium*-inoculated rice roots at 1, 3, 4 and 6 dpi. Transcript levels were normalized against the internal reference actin and expressed relative to the control at 1dpi. Data are means of three different experiments (n = 3), each experiment representing a pooled sample from at least six rice plantlets. Error bars display the standard error values.

4.3.5. *P. arrhenomanes* and *P. graminicola* are nutritionally less versatile than *P. inflatum*

The growth of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* on various carbon sources was evaluated using phenoarrays. This analysis revealed the ability of *P. inflatum* to use a broad range of carbohydrates, amino acids, carboxylic acids and derivatives (Figure 4.7 and 4.8).

P. inflatum used 80% of all mono-, di-, tri- and polysaccharides, sugar monomers/polymers, glycosides and polyols supplemented to the MicroPlates, while this was only 49% and 47% for *P. arrhenomanes* and *P. graminicola*, respectively (Figure 4.7; Table S4.1 and S4.2). The latter species did not metabolize glycosides of galactose and mannose, while all glycosides stimulated the growth of *P. inflatum*. Furthermore, only 56% and 44% of the tested polyols were utilized by *P. arrhenomanes* and *P. graminicola*, respectively, whereas this was 89% in the case of *P. inflatum*. It was interesting to see that *P. arrhenomanes* and *P. graminicola* grew on different polyols beside glycerol and m-inositol. Compared to *P. inflatum*, they were also less efficient in their use of several saccharides, in particularly monosaccharides. Nevertheless, the overall highest absorbance values (OD > 0.35) were measured in wells pre-filled with the disaccharides sucrose, maltose, gentiobiose and D-cellobiose, and the monosaccharide α-D-glucose. Turbidities in *P. inflatum*-inoculated wells were the highest for these compounds, supporting its better growth on carbohydrates compared to the other species. Besides, *P. graminicola* and *P. arrhenomanes* grew the best on D-fructose (monosaccharide) and D-trehalose (disaccharide), and glycogen (polysaccharide), respectively. *P. arrhenomanes* shared its ability to use D-galactose with *P. inflatum*, while *P. inflatum* and *P. graminicola,* but not *P. arrhenomanes*, were able to use lactulose.

When exploring the growth of the three *Pythium* spp. on amino acids, carboxylic acids and their derivatives, it became clear that *P. inflatum* and *P. arrhenomanes* could use respectively 68% and 61% of these compounds, while this was only 43% for *P. graminicola* (Figure 4.8; Table S4.1 and S4.2). *P. inflatum* exhibited the broadest carboxylic acid-profile and was the only species that could utilize quinic acid, D,L-malic acid, D-saccharic acid, D-glucuronic acid and β-hydroxybutyric acid. Nevertheless, it was not able to metabolize two particular carboxylic acids, i.e. formic acid and α-ketobutyric acid, which intriguingly showed to stimulate the growth of the more virulent species. Aside from these findings, *P. arrhenomanes* appeared unique in its ability to use itaconic acid, while no carboxylic acids or derivatives seemed *P. graminicola*-specific (Table 4.6). We also identified certain carboxylic acids that enhanced the growth of either *P. arrhenomanes* and *P. inflatum* or *P. graminicola* and *P. inflatum* (Table 4.6).

The amino acid-profiles of all tested species included L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-ornithine, L-phenylalanine and L-proline. However, L-threonine, D-serine and D-alanine were exclusively used by *P. arrhenomanes* and hence, it exhibited the broadest amino acid profile among the three species (Fig 4.8).

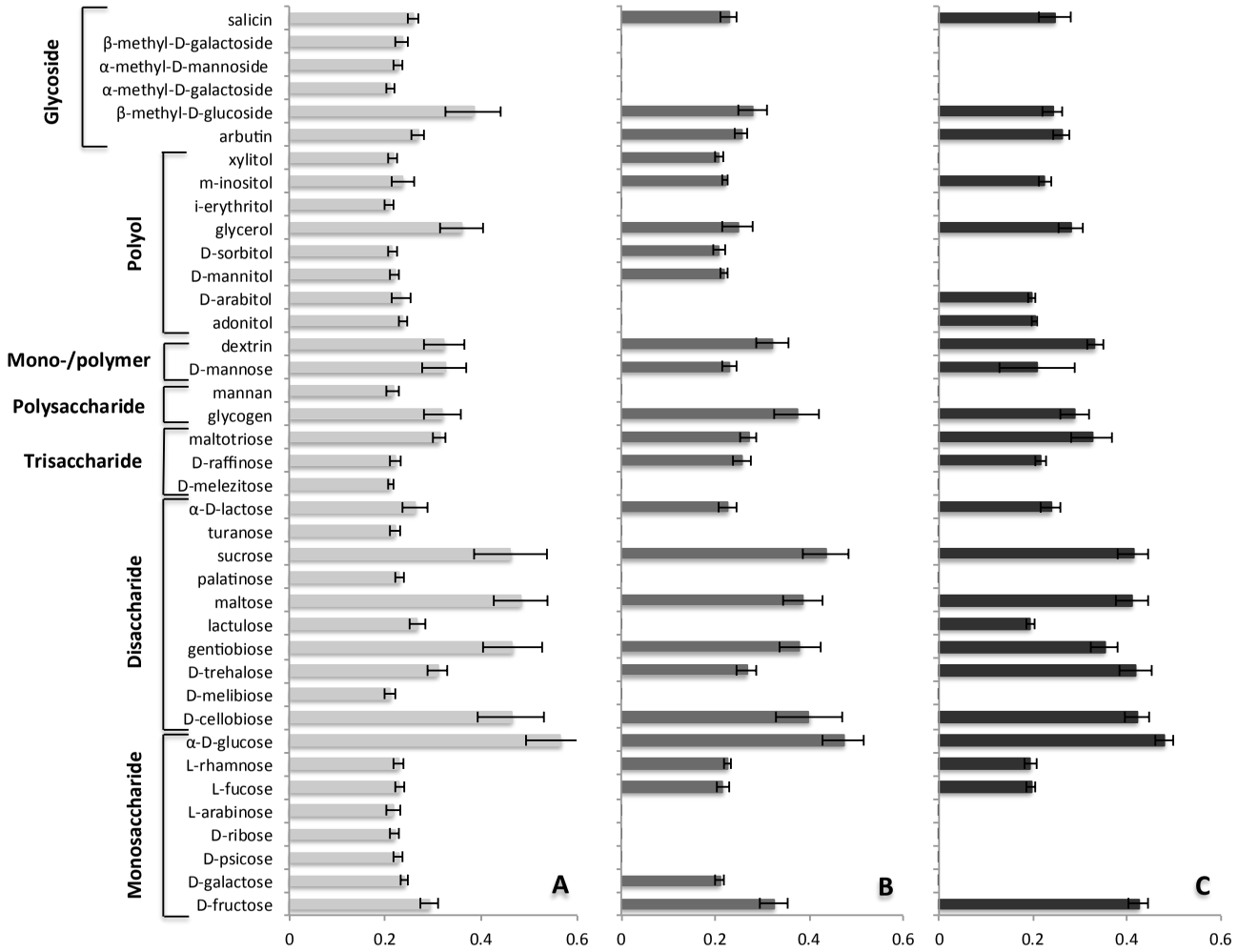

Figure 4.7. Carbohydrates and derivatives metabolized during *in vitro* growth of rice-infecting *Pythium* spp.. A, B and C represent the profiles of *P. inflatum*, *P. arrhenomanes* and *P. graminicola*, respectively. Turbidimetrical measurements were executed 24 hpi. The experiment consisted of three replicate plates per treatment and was repeated in time (n ≥ 6). Only carbon sources with OD-values significantly different from the control-treatment are presented. Statistical analyses were performed using the Kruskal-Wallis non-parametric test in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$).

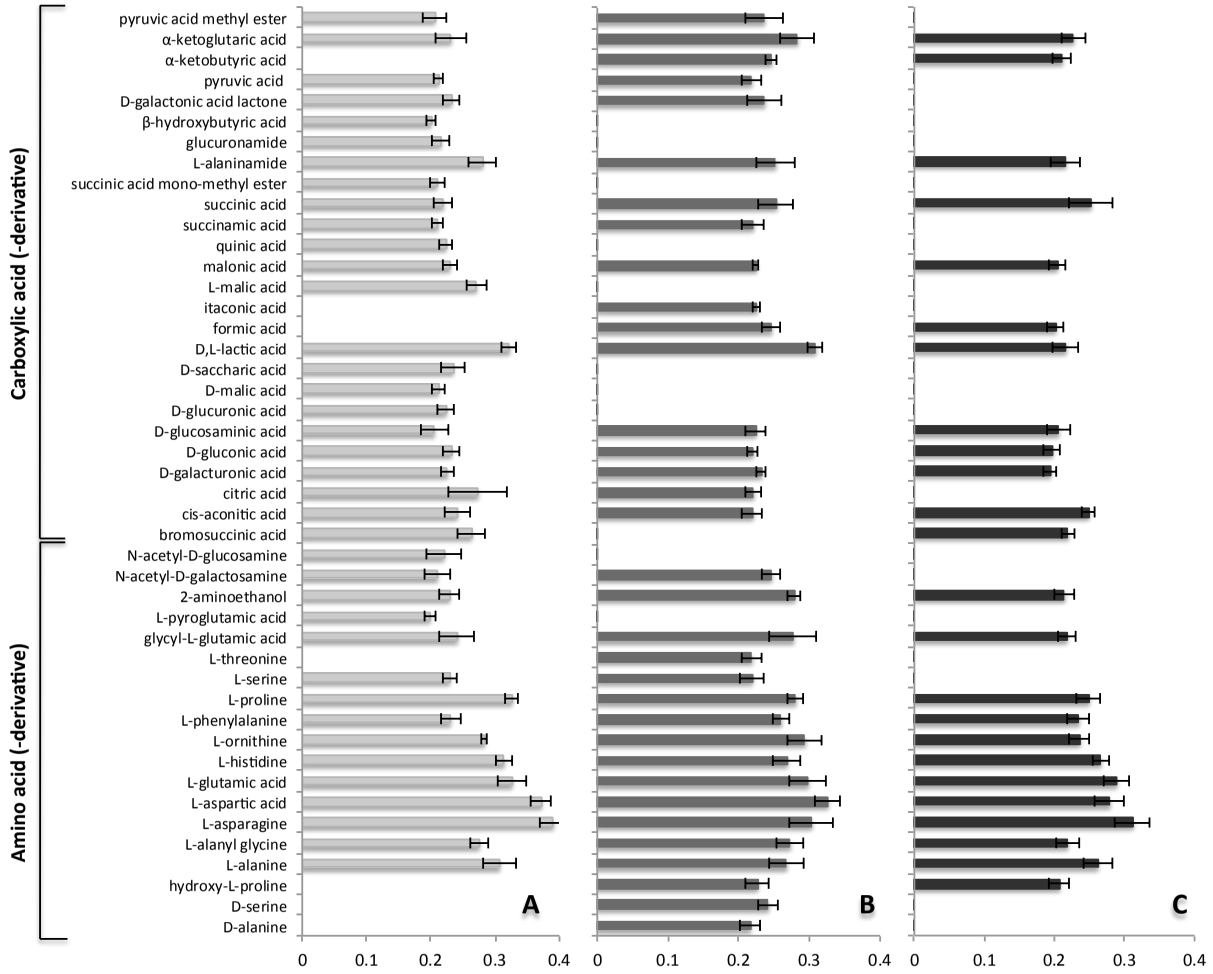

Figure 4.8. Amino acids, carboxylic acids and derivatives metabolized during *in vitro* growth of rice-infecting *Pythium* spp.. A, B and C represent the profiles of *P. inflatum*, *P. arrhenomanes* and *P. graminicola*, respectively. Turbidimetrical measurements were executed 24 hpi. The experiment consisted of three replicate plates per treatment and was repeated in time ($n \geq 6$). Only carbon sources with OD-values significantly different from the control-treatment are presented. Statistical analyses were performed using the Kruskal-Wallis non-parametric test in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$).

P. inflatum and *P. arrhenomanes* could both utilize L-serine, while *P. graminicola* could not. Interestingly, the use of hydroxy-L-proline seemed specific for the more virulent *Pythium* species and none of the three species showed to grow on L-leucine (Table S4.2).

The three *Pythium* species also responded differently to certain other carbon sources (see Table S4.1). The nucleosides thymidine, uridine and inosine, triggered exclusively the growth of *P. arrhenomanes*, while *P. inflatum* was the only species that could grow on putrescine.

Table 4.6. Carbon sources specific to *P. arrhenomanes* and/or *P. graminicola*, and/or shared with *P. inflatum*.

Carbon source	*P.arrhenomanes**	*P.graminicola**	*P.inflatum**
D-alanine	+	-	-
D-serine	+	-	-
itaconic acid	+	-	-
L-threonine	+	-	-
formic acid	+	+	-
hydroxy-L-proline	+	+	-
α-ketobutyric acid	+	+	-
citric acid	+	-	+
D-galactose	+	-	+
D-mannitol	+	-	+
D-sorbitol	+	-	+
L-serine	+	-	+
N-acetyl-D-galactosamine	+	-	+
pyruvic acid	+	-	+
pyruvic acid methyl ester	+	-	+
succinamic acid	+	-	+
xylitol	+	-	+
adonitol	-	+	+
bromosuccinic acid	-	+	+
D-arabitol	-	+	+
lactulose	-	+	+

* + or - indicate if the *Pythium* spp. could respectively grow or not on the according carbon sources.

Statistical analyses were performed using Kruskal-Wallis non-parametric tests in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$).

4.4. Discussion

In this study, three isolates each of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* from diseased aerobic rice fields were selected for a detailed *in vitro* analysis of their host infection process and the consequent disease development. *In vitro* inoculation of rice seedling roots revealed that *P. arrhenomanes* was clearly more virulent than *P. graminicola* and *P. inflatum*. This pathogen inhibited crown and lateral root development, induced severe wilting and caused strong stunting of rice roots and shoots, whereas *P. graminicola*-induced disease symptoms were less pronounced and *P. inflatum* exerted only minor effects on rice seedling development.

Histopathological studies revealed that the three *Pythium* spp. invaded rice seedling roots using simple hyphal structures. Once inside the root tissue, *Pythium* hyphae became irregularly inflated and grew mainly intracellular. Cell wall crossing occurred by means of constricted hyphae, which probably migrated through the plasmodesmata and hence, avoided visible cell wall damage. The invasion of rice roots by *Magnaporthe oryzae* apparently proceeds in a similar way (Marcel et al., 2010). Consistent findings were presented for *Pythium* root infections in other monocots (Mojdehi et al., 1991; McKeen 1977; Napi-Acedo and Exconde, 1965). However, specialized appressoria-like structures instead of simple hyphae enabled rhizodermal penetration in these cases (Mojdehi et al., 1991). After pathogen ingress, we found that *P. arrhenomanes*, *P. graminicola* and *P. inflatum* massively parasitized the inner rice root tissues by hyphal ramification, resulting in the complete filling of cortical and endodermal cells within 27 hpi. Comparably, dense hyphal networks were noted upon infection of *Arabidopsis* plantlets with *P. irregulare* (Adie et al., 2007).

In parallel with their varying aggressiveness, we showed that *P. arrhenomanes*, *P. graminicola* and *P. inflatum* differed in the extent to which systemic tissues were colonized. *P. arrhenomanes* quickly colonized the entire primary root, efficiently spreading from the primary infection site to other parts. Besides, hyphae abundantly invaded the xylem and accordingly, might have blocked the water transport to the shoot, partly explaining the severe wilting and frequent death of rice seedlings upon *P. arrhenomanes* inoculation. Such parasitic features are common for true vascular pathogens like *Fusarium oxysporum* and *Verticillium* spp., where hyphae invade the plant root stele before the endodermis is suberized (Okubara and Paulitz, 2005). In case of *Pythium* infections, however, hyphal blocking of xylem is rather exceptional. Extensive invasion of the vascular stele has only been reported for *P. irregulare* on Arabidopsis (Adie et al., 2007), *P. tracheiphilum* on lettuce (Matta, 1965), *P. sylvaticum* and *P. dissotocum* on strawberry (Nemec, 1972) and *P. ultimum* on cucumber (Chérif et al., 1991). In the latter case, xylem vessels were also occluded by the production of plant defense-related tyloses that attempted to limit hyphal spread. The systemic spread of *P. inflatum*, and to a lesser extent of *P. graminicola*, in the cortex and stele of rice seedling roots, was more limited than that of *P. arrhenomanes*. Especially xylem vessels appeared less colonized by *P. graminicola*, whereas the growth of *P. inflatum* was often limited to few phloem cells, possibly underlying the lower degree of wilting and stunting upon inoculation with these pathogens. Our qPCR-analysis confirmed that *P. arrhenomanes* was the best colonizer of the rice seedling root system, followed by *P. graminicola* and *P. inflatum*, with the latter barely spreading in the inner tissues. Such a positive correlation between *Pythium* root colonization capacity and aggressiveness is not always to be expected. In contrast to our results, Modjehi et al. (1991) reported that in wheat roots, most *P.* arrhenomanes hyphae are blocked at the endodermis. In this study, the stele remained unaffected at distance of the primary infection site until the cells died. Moreover, the colonization process of *P. arrhenomanes* proceeded more slowly in this host than in rice roots. Other contrasting findings have been described for *Pythium* group F-tomato root interactions. *Pythium* group F is a minor pathogen that may cause yield losses without producing visible root symptoms (Rey et al., 1998a).

In tomato roots, it is able to colonize all cell types, including the xylem, within 2-3 dpi. However, it has been discovered that most of the xylem-invading hyphae appear as empty ghost cells. Similar findings have been reported for the biocontrol agent and growth-promoting species *P. oligandrum* in its interaction with tomato roots (Le Floch et al., 2005).

The progressive invasions of *Pythium* hyphae in rice seedling roots triggered the production of reactive oxygen species (ROS) and phenolic compounds, which ultimately evoked cell death. ROS are short-lived molecules that interact with proteins, DNA, lipids and carbohydrates in plant cells, and thereby induce tissue damage and cell death (Heller and Tudzynski et al., 2011). Plants produce scavengers for anti-oxidative protection. However, several biotic and abiotic factors may disturb the balance between ROS and scavengers, and so, evoke an oxidative burst (Apel and Hirt, 2004). Necrotrophic pathogens for instance, may trigger the intracellular production of ROS during the killing of host tissues. On the other hand, ROS-production may as well be part of plant defense responses to biotrophic and hemi-biotrophic pathogens (Heller and Tudzynski, 2011). In the current study, production of hydrogen peroxide (H_2O_2) was observed in the outer cell layers of *Pythium*-inoculated rice tissues and was stronger for the more virulent species at 1 dpi. Oliver et al. (2009) also noticed ROS production upon *P. irregulare* and *P. debaryanum* infection of moss, but in theses cases, ROS accumulated in the *Pythium*-spreading area, where it preceded cell death. The three *Pythium* spp. invaded the stele of rice seedling roots by 1 dpi, while DAB concentrated far behind the infection front, suggesting that the observed H_2O_2 accumulation might be related to immune reactions that arose too late to prevent infection. These likely included cell wall modification events, since *Pythium* spp., like other oomycetes, directly penetrate their host (Oliver et al., 2009) and hydrogen peroxide is involved in the peroxidase-catalyzed cross-linking of cell wall polymers (Brisson et al., 1994). However, it is also possible that the H_2O_2 accumulation in *Pythium*-infected rice roots was indicative for cell death upon nutrient depletion (Able, 2003). Such a trailing necrosis has also been described for *P. irregulare*-Arabidopsis interactions (Adie et al., 2007). Histological analyses of *P. arrhenomanes*-inoculated rice cultures revealed cell collapse in the root cortex at 3 dpi, which confirmed the occurrence of necrosis. Cell collapse resulting from *P. arrhenomanes* infections of wheat seedlings has been previously linked with toxin or enzyme production (Mojdehi et al., 1991). Toxins or substantial amounts of cell wall degrading enzymes are likely not involved in the *Pythium*-rice interaction, because pathogen invasion was not associated with extensive tissue damage and was not preceded by cell death. In *P. inflatum* and *P. graminicola*-inoculated root tissues, we also detected a cortex-related ROS production at 3 dpi that slowed down the *Pythium* colonization process, suggesting that cell wall strengthening occurred and that the interaction between rice roots and *P. inflatum* or *P. graminicola* is less compatible than with *P. arrhenomanes*.

Beside ROS, phenolic compounds fulfill major roles in the response of plants to biotic and abiotic stresses (Hutzler et al., 1998). *Pythium* infections have been mentioned to elicit the accumulation of phenolics (Le Floch et al., 2005; Oliver et al., 2009), which can be incorporated into the cell wall during fortification events or may be liberated during cell death (Oliver et al., 2009). These phenolic accumulations are microscopically visible as autofluorescence upon UV-irradiation (Hutzler et al., 1998) or macroscopically as root browning (Owen-Going et al., 2008). In the present paper, we detected a similar root browning in *Pythium*-inoculated rice cultures. When inner root tissues were studied, we noted the strongest autofluorescence in the cortex of *P. arrhenomanes*-inoculated primary roots, while autofluorescence was mainly emitted from the sclerenchyma in *P. graminicola*-inoculated seedlings and was weakly visible in the outer tissues upon *P. inflatum* inoculation. Taken together, these results illustrate that rice seedlings responded more and/or faster to highly virulent than weakly virulent *Pythium* species.

Elevated expression levels of necrosis marker *OsJAmyb* in *Pythium*-inoculated rice roots provided molecular support for the induced necrosis that accompanied successive accumulation of H_2O_2 and phenolic compounds. Analyses of the transcript levels at various times proved that *P. arrhenomanes* infections induced a higher degree of necrosis, confirming the higher susceptibility of rice seedlings to this pathogen (Lee et al., 2001), while the induction of necrosis was delayed and weaker upon inoculation with *P. inflatum*. The activation of this jasmonic acid (JA)-responsive gene also implied a role for the JA response in the *Pythium*-rice interaction. Many papers have reported on the defense-inducing role of jasmonates (JAs) in *Pythium*-dicot interactions (Adie et al., 2007; Oliver et al., 2009; Staswick et al., 1998), but in monocots, these studies are lacking. It is also known that JAs may fulfill negative regulatory tasks in plant root defense. *Fusarium oxysporum* (*Fox*) for instance has been discovered to hijack CORONATINE INSENSITIVE1 (COI)-mediated JA signaling in Arabidopsis, by which it enhances disease development (Thatcher et al., 2009). More research is required to elucidate the exact role of JA in the *Pythium*-rice pathosystem.

Since the aggressiveness and root colonization capacity of rice-infecting *Pythium* spp. seemed positively linked, we compared the nutritional profiles of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* to elucidate whether highly virulent species were physiologically more adapted to their colonization niche or used specific nutrients as a defense strategy. It was interesting to see that the nutritional profiles of the highly virulent *P. arrhenomanes* and moderately virulent *P. graminicola* were quite similar and clearly different from that of the weakly virulent *P. inflatum*. Carbon-utilization patterns identified *P. inflatum* as nutritionally the most versatile. Similar findings were obtained from comparative analyses between plant pathogenic and non-pathogenic *Pseudomonas* species, in which pathogenic species exhibited reduced nutritional versatility (Rico and Preston, 2008). Nevertheless, the three *Pythium* species grew all very well on sucrose, maltose, gentiobiose, D-cellobiose and α-D-glucose and furthermore, *P. arrhenomanes* and *P. graminicola* strongly multiplied on the storage polysaccharide glycogen, and D-trehalose and D-fructose, respectively.

These carbohydrates were among those previously mentioned as growth-stimulators of *P. aphanidermatum* (Khalil and Alsanius, 2009), *P. myriotylum*, *P. dissotocum*, *P. arrhenomanes* (Chun et al., 2003) and *P. oligandrum* (McQuilken et al., 1992), and which represent, together with other carbohydrates, the largest part of the rice rhizodeposition (Suzuki et al., 2009). In addition, *P. inflatum* and *P. arrhenomanes* were able to grow on D-galactose, which is abundantly exuded by rice roots (Bacilio-Jiménez et al., 2003). Hence, we propose that especially *P. inflatum* and *P. arrhenomanes* and to a lesser extent *P. graminicola* are physiologically adapted to the rice rhizosphere, explaining the stronger superficial colonization of rice tissues by *P. arrhenomanes* and *P. inflatum* during our *in vitro* analysis.

P. inflatum also exhibited the broadest carboxylic acid-utilization pattern. Since the exudation of organic acids is negligible during rice seedling development (Bacilio-Jiménez et al., 2003; Suzuki et al., 2009), carboxylic acids are likely not involved in the initial stages of the *Pythium*-rice interaction. However, malic acid, citric acid and formic acid are present in the phloem sap of rice plants (Hayashi and Chino, 1985) and because our data illustrated that *P. inflatum*, *P. arrhenomanes* and/or *P. graminicola* could grow on these carbon sources, they migt have supported *Pythium* spread inside the rice root stele. The intracellular uptake of these nutrients might have proceeded via haustoria-like structures, which have been described for *P. irregulare* on Arabidopsis (Adie et al., 2007).

In contrast to the *Pseudomonas* study where plant pathogenic species showed to be specialized in the use of the six most abundant amino acids in their primary infection site (Rico and Preston, 2008), we noted that the most virulent species *P. arrhenomanes* exhibited the broadest amino acid-utilization pattern. All *Pythium* spp. were able to grow on a consistent group of nine amino acids that have been proven to attract *Pythium* zoospores (Donaldson and Deacon, 1993), stimulate the zoospore encysting process (Donaldson and Deacon, 1993) and/or promote the growth of various *Pythium* spp. (Chun et al., 2003; Khalil and Alsanius, 2009; McQuilken et al., 1992). Among these, especially histidine, proline and alanine are exuded by rice seedlings during the first weeks after planting (Bacilio-Jiménez et al., 2003), and asparagine and glutamate are present in the rice phloem (Fukumorita and Chino, 1982). Accordingly, these carbon sources could have stimulated the outer and inner root colonization processes of *P. arrhenomanes, P. graminicola* and/or *P. inflatum* in our *in vitro* experiments. It was interesting to see that the amino acid-profile of *P. arrhenomanes* also consisted of D-serine and D-alanine. Up to date, no *Pythium* spp. have been documented to grow on D-enantiomers of amino acids. Since rice seeds contain substantial amounts of D-serine (Gogami et al., 2009), and D-alanine peptides are also present in rice tissues (Manabe, 1992), this exceptional feature possibly contributed to its massive colonization of rice seeds and seedling roots compared to *P. graminicola* and *P. inflatum*, making it more virulent.

The selective utilization of amino acids is not always a nutritional preference. It might also represent a virulence strategy through which pathogens try to lower defense-related compounds (Seifi et al., 2013).

Hydroxyl-L-proline and L-threonine are building blocks of hydroxyproline-rich glycoproteins (also called extensins), which are present in the primary cell wall of monocots and are functionally implicated in cell wall extension and peroxidase-mediated cell wall fortification (Smallwood et al., 1995). *P. arrhenomanes* and *P. graminicola* showed to grow on respectively both amino acids and hydroxyl-L-proline only, revealing the higher ability of *P. arrhenomanes* to block cell wall strengthening in rice roots. However, our histopathological study did not evidence the inhibition of cell wall fortification events during *Pythium*-infections of rice seedlings, but their occurrence could have been delayed. Aside from this, L-threonine represents one of the dominant free amino acids in the rice phloem sap (Fukumorita and Chino, 1982) and may exert growth-suppressive effects during the interaction of plants with obligate biotrophic oomycetes (Stuttmann et al., 2011). Consequently, we speculate that the higher aggressiveness of *P. arrhenomanes* could be linked with the removal of L-threonine from its host tissues. In our phenoarrays, we also noted that the three *Pythium* spp. used the defense-related amino acid L-proline (Cassab, 1998; Hare and Cress, 1997). Additionally, *P. inflatum* seemed to grow on putrescine, a stress-related polyamine. Since the weakest virulent species could grow on both carbon sources, their removal from the environment is probably not determining for the aggressiveness level of rice-pathogenic *Pythium* spp..

4.5. Conclusions

The degree by which *Pythium* spp. can feed on amino acids, and invade rice cortical and stelar cells seems to underlie the intensity of *Pythium*-induced stunting and wilting symptoms in rice seedlings. Highly virulent *Pythium* species quickly and massively colonize rice root tissues, probably by suppressing cell-wall fortification events, removing defense-related compounds from their host's tissues, and growing on D-amino acids of which rice seeds contain substantial amounts. Quick invasion of the vascular stele appears of utmost importance for the virulence level of rice-infecting *Pythium* spp,, since the rice root endodermis and vascular tissues become suberized/lignified during maturation (Clark and Harris, 1981). This might explain why rice seedlings acquire a certain degree of *Pythium*-resistance within 8 days after planting (Chun and Schneider, 1998). Interestingly, gibberellins (GAs) have been noted to mediate lignification in monocot roots (Biemelt et al., 2004) and hence, these plant growth regulators might be involved in rice root defense to *Pythium* spp.. The role of GAs and other plant hormones in the *Pythium*-rice interaction will be discussed in the next chapters.

4.6. Supplementary data

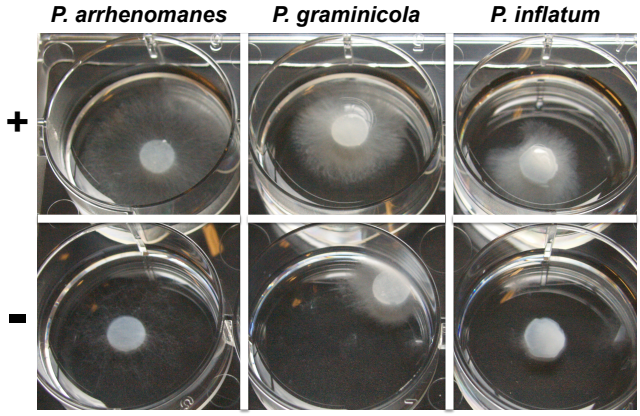

Figure S4.1. The stimulating effect of rice seed exudates on *Pythium* growth. Rice seeds (2.5 g) of the cv. CO-39 (*O. sativa* subspecies *indica*), which is as susceptible to *Pythium* as cv. Apo and Nipponbare, were surface sterilized, washed and incubated in 20 ml of sterile demineralized water at 28 °C. Seed exudates were collected as a watery solution after 24 h of imbibition. Three ml aliquots of water (-) or exudate solutions (+) were added to three 6-well replicate plates and afterwards, inoculated with one PDA plug of a four-day old *P. arrhenomanes, P. graminicola* or *P. inflatum* culture. Plates were incubated at 28 °C and screened after 17 h. A clear stimulation in colony diameter and/or density was visible when *Pythium* spp. were grown in seed exudates. The picture is representative for the three replicate plates.

Table S4.1. Carbon sources that stimulated *Pythium* growth in the phenoarray. Data represent the carbon sources for which the optical density (OD)-values significantly differed from the initial OD (= 0.1) at 24 hpi according to Kruskal-Wallis non-parametric tests in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$). Values between brackets represent standard errors.

C-source	Name	*P .inflatum*	*P. arrhenomanes*	*P. graminicola*
Amino acid	D-alanine	0	0.22 (0.02)	0
	D-serine	0	0.24 (0.01)	0
	L-alanine	0.31 (0.03)	0.27 (0.03)	0.26 (0.02)
	L-asparagine	0.39 (0.02)	0.30 (0.03)	0.31 (0.03)
	L-aspartic acid	0.37 (0.02)	0.32 (0.02)	0.28 (0.02)
	L-glutamic acid	0.33 (0.02)	0.30 (0.03)	0.29 (0.02)
	L-histidine	0.31 (0.01)	0.27 (0.02)	0.27 (0.01)
	L-ornithine	0.28 (0)	0.29 (0.02)	0.24 (0.01)
	L-phenylalanine	0.23 (0.02)	0.26 (0.01)	0.23 (0.02)
	L-proline	0.33 (0.01)	0.28 (0.01)	0.25 (0.02)
	L-serine	0.23 (0.01)	0.22 (0.02)	0
	L-threonine	0	0.22 (0.01)	0
Amino acid derivate	L-alanyl glycine	0.28 (0.01)	0.27 (0.02)	0.22 (0.02)
	glycyl-L-glutamic acid	0.24 (0.03)	0.28 (0.03)	0.22 (0.01)
	hydroxy-L-proline	0	0.23 (0.02)	0.21 (0.01)
	L-pyroglutamic acid	0.20 (0.01)	0	0
	2-aminoethanol	0.23 (0.02)	0.28 (0.01)	0.21 (0.01)
	N-acetyl-D-galactosamine	0.21 (0.02)	0.25 (0.01)	0
	N-acetyl-D-glucosamine	0.22 (0.03)	0	0
Carboxylic acid	bromosuccinic acid	0.26 (0.02)	0	0.22 (0.01)
	cis-aconitic acid	0.24 (0.02)	0.22 (0.01)	0.25 (0.01)
	citric acid	0.27 (0.05)	0.22 (0.01)	0
	D-gluconic acid	0.23 (0.01)	0.22 (0.01)	0.20 (0.01)
	D-glucosaminic acid	0.21 (0.02)	0.22 (0.02)	0.21 (0.02)
	D-glucuronic acid	0.22 (0.01)	0	0
	D-malic acid	0.21 (0.01)	0	0
	D-saccharic acid	0.23 (0.02)	0	0
	D,L-lactic acid	0.32 (0.01)	0.31 (0.01)	0.22 (0.02)
	formic acid	0	0.25 (0.01)	0.20 (0.01)
	itaconic acid	0	0.23 (0.01)	0
	L-malic acid	0.27 (0.02)	0	0
	malonic acid	0.23 (0.01)	0.22 (0)	0.20 (0.01)
	quinic acid	0.22 (0.01)	0	0
	succinamic acid	0.21 (0.01)	0.22 (0.02)	0
	succinic acid	0.22 (0.01)	0.25 (0.03)	0.25 (0.03)
	α-ketobutyric acid	0	0.25 (0.01)	0.21 (0.01)
	D-galacturonic acid	0.23 (0.01)	0.23 (0.01)	0.19 (0.01)
Carboxylic acid derivate	L-alaninamide	0.28 (0.02)	0.25 (0.03)	0.22 (0.02)
	glucuronamide	0.22 (0.01)	0	0
	D-galactonic acid lactone	0.23 (0.01)	0.24 (0.02)	0
	α-ketoglutaric acid	0.23 (0.02)	0.28 (0.02)	0.23 (0.02)
	pyruvic acid	0.21 (0.01)	0.22 (0.01)	0
	pyruvic acid methyl ester	0	0.24 (0.03)	0
	succinic acid mono-methyl ester	0.21 (0.01)	0	0
	β-hydroxybutyric acid	0.20 (0.01)	0	0

Table S4.1. (Continued)

C-source	Name	*P .inflatum*	*P. arrhenomanes*	*P. graminicola*
Carbohydrate	D-cellobiose	0.46 (0.07)	0.40 (0.07)	0.42 (0.03)
	D-fructose	0.29 (0.02)	0.33 (0.03)	0.42 (0.02)
	D-galactose	0.24 (0.01)	0	0
	D-mannose	0.32 (0.05)	0.23 (0.02)	0.21 (0.08)
	D-melezitose	0.21 (0.01)	0	0
	D-melibiose	0.21 (0.01)	0	0
	D-psicose	0.23 (0.01)	0	0
	D-raffinose	0.22 (0.01)	0.26 (0.02)	0.22 (0.01)
	D-ribose	0.22 (0.01)	0	0
	D-trehalose	0.31 (0.02)	0.27 (0.02)	0.41 (0.03)
	dextrin	0.32 (0.04)	0.32 (0.04)	0.33 (0.02)
	gentiobiose	0.46 (0.06)	0.38 (0.05)	0.35 (0.03)
	glycogen	0.32 (0.04)	0.37 (0.05)	0.29 (0.03)
	L-arabinose	0.22 (0.02)	0	0
	L-fucose	0.23 (0.01)	0.22 (0.01)	0.20 (0.01)
	L-rhamnose	0.23 (0.01)	0.23 (0.01)	0.19 (0.01)
	lactulose	0.27 (0.02)	0	0.19 (0.01)
	maltose	0.48 (0.06)	0.39 (0.04)	0.41 (0.04)
	maltotriose	0.31 (0.01)	0.27 (0.02)	0.32 (0.04)
	mannan	0.22 (0.01)	0	0
	palatinose	0.23 (0.01)	0	0
	sucrose	0.46 (0.08)	0.43 (0.05)	0.41 (0.03)
	turanose	0.22 (0.01)	0	0
	α-D-glucose	0.56 (0.07)	0.47 (0.04)	0.48 (0.02)
	α-D-lactose	0.26 (0.03)	0.23 (0.02)	0.24 (0.02)
Carbohydrate derivate	arbutin	0.27 (0.01)	0.26 (0.01)	0.26 (0.02)
	salicin	0.26 (0.01)	0.23 (0.02)	0.25 (0.03)
	α-methyl-D-galactoside	0.21 (0.01)	0	0
	α-methyl-D-mannoside	0.23 (0.01)	0	0
	β-methyl-D-galactoside	0.24 (0.01)	0	0
	β-methyl-D-glucoside	0.38 (0.06)	0.28 (0.03)	0.24 (0.02)
	2,3-butanediol	0	0.22 (0.01)	0
	adonitol	0.24 (0.01)	0	0.20 (0.01)
	D-arabitol	0.23 (0.02)	0	0.20 (0.01)
	D-mannitol	0.22 (0.01)	0.22 (0.01)	0
	D-sorbitol	0.21 (0.01)	0	0
	glycerol	0.36 (0.05)	0.25 (0.03)	0.28 (0.03)
	i-erythritol	0.21 (0.01)	0	0
	m-inositol	0.24 (0.02)	0.22 (0.01)	0.22 (0.01)
	xylitol	0.22 (0.01)	0	0
Amine	phenylethylamine	0.25 (0.02)	0.26 (0.02)	0
	putrescine	0.20 (0.01)	0	0
Nucleoside	inosine	0	0.22 (0.01)	0
	thymidine	0	0.21 (0.01)	0
	uridine	0	0.22 (0.02)	0
Surfactants	tween 40	0.43 (0.03)	0.42 (0.04)	0.43 (0.03)
	tween 80	0.37 (0.05)	0.32 (0.06)	0.38 (0.04)

Table S4.2. Carbon sources that did not stimulate growth of *Pythium* in the phenoarray. The OD-values of the listed carbon sources did not significantly differ from the initial OD (= 0.1) at 24 hpi according to Kruskal-Wallis non-parametric tests in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$).

Carbon source
2'-deoxy adenosine
acetic acid
adenosine
adenosine-5'-Monophosphate
D-fructose-6-phosphate
D-glucose-6-phosphate
D-lactic acid methyl ester
D-tagatose
D-xylose
D,L,α-glycerol phosphate
inulin
L-lactic acid
L-leucine
lactamide
p-hydroxyphenylacetic acid
propionic acid
sedoheptulosan
thymidine-5'-monophosphate
uridine-5'-monophosphate
urocanic acid
α-cyclodextrin
α-D-glucose-1-phosphate
α-hydroxybutyric acid
α-ketovaleric acid
β-cyclodextrin
γ-hydroxybutyric acid

Chapter 5

Brassinosteroids antagonize gibberellin- and salicylate-mediated root immunity in rice

De Vleesschauwer D, Van Buyten E, Satoh K, Balidion J, Mauleon R, Choi IR, Vera-Cruz C, Kikuchi S, Höfte M, 2012. *Plant Physiology* **158(4),** 1833-1846.

Abstract

Brassinosteroids (BRs) are a unique class of plant steroid hormones that orchestrate various growth and developmental processes. Although BRs have long been known to protect plants from a broad range of biotic and abiotic stresses, our understanding of the underlying molecular mechanisms is still rudimentary. Aiming to further decipher the molecular logic of BR-modulated immunity, we have examined the dynamics and impact of BRs during infection of rice (*Oryza sativa*) seedlings with *Pythium graminicola*. Challenging the generalized view that BRs positively regulate plant innate immunity, we show that *P. graminicola* exploits BRs as virulence factors and hijacks the rice BR machinery to inflict disease. Moreover, we demonstrate that this immune-suppressive effect of BRs is due, at least in part, to negative crosstalk with salicylic acid (SA) and gibberellic acid (GA) pathways. BR-mediated suppression of SA-dependent defenses occurred downstream of SA biosynthesis, but upstream of the master defense regulators NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (OsNPR1) and OsWRKY45. In contrast, BR alleviated GA-directed immune responses by interfering at multiple levels with GA metabolism, resulting in indirect stabilization of the DELLA protein and central GA-repressor SLENDER RICE1 (SLR1). Collectively, these data favor a model whereby *P. graminicola* co-opts the plant BR pathway as a decoy to antagonize effectual SA- and GA-mediated defenses. Our results highlight the importance of BRs in modulating plant immunity and uncover pathogen-mediated manipulation of plant steroid homeostasis as a core virulence strategy.

5.1. Introduction

To effectively combat invasion by microbial pathogens, plants have evolved a plethora of sophisticated mechanisms providing several strategic layers of constitutive and inducible defense responses. Many of these responses are regulated by an array of cross-communicating signal transduction pathways in which plant hormones fulfill central roles. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the archetypal defense hormones and their importance in the complex plant innate immune signaling network is well-established (Grant and Jones, 2009; Robert-Seilaniantz et al., 2011). Recently, abscisic acid (ABA), gibberellic acid (GA), cytokinins (CKs), and auxins (AUXs) emerged as critical regulators of plant-microbe interactions as well. Although their significance is less well understood, mounting evidence suggests these hormones influence disease outcomes by positively or negatively interfering with the SA-JA-ET backbone of the immune signaling circuitry (Pieterse et al., 2009). Such interplay or crosstalk between individual hormone conduits is thought to enable plants to flexibly tailor their inducible defense arsenal to the type of invader encountered and to utilize their resources in a cost-efficient manner (Verhage et al., 2010). However, exciting new developments suggest that hormone crosstalk may also be exploited by pathogens to shut down effective defenses through negative network connections (Robert-Seilaniantz et al., 2011). A classic example reflecting this situation is the production by some *Pseudomonas syringae* strains of a phytotoxin called coronatine that structurally resembles JA derivatives. Actively secreted in the host, coronatine is assumed to hyperactivate JA signaling, thereby counteracting SA-dependent defenses and facilitating bacterial invasion (Brooks et al., 2005; Cui et al., 2005; Melotto et al., 2006).

Brassinosteroids (BRs) are one of the latest growth regulators to be implicated in plant immunity. Discovered nearly 40 years ago, BRs are polyhydroxylated steroid hormones with important roles in regulating myriad physiological and developmental processes, including seed germination, skotomorphogenesis, flowering and senescence (Clouse and Sasse, 1998). Over the past decade, molecular genetic studies using Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) as model plants have identified numerous genes involved in BR biosynthesis and gene regulation. Coupled with more recent biochemical approaches, these studies have provided fascinating insights into the various aspects of plant steroid signaling, ranging from BR perception at the cell surface to activation of transcription factors in the nucleus (Kim and Wang, 2010). According to current concepts, BRs directly bind to the extracellular domain of the receptor-like kinase BRASSINOSTEROID INSENSITIVE1 (BRI1; She et al., 2011), thereby inducing a series of biochemical responses, including heterodimerization of BRI1 with, and activation of, another receptor kinase, BRI1-ASSOCIATED KINASE1 (BAK1; Li et al., 2002; Yun et al., 2009), phosphorylation of BRI1-interacting signaling kinases (Tang et al., 2008), and activation of the protein phosphatase BRI1 SUPPRESSOR PROTEIN1 (Kim et al., 2009).

These events eventually culminate in inhibition of the shaggy-like kinase BRI1-INSENSITIVE2 (Vert and Chory, 2006) and resultant activation of the transcription factors BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1)/BRASSINAZOLE-RESISTANT2 (BZR2) that orchestrate downstream gene expression (Sun et al., 2010; Yu et al., 2011). Besides their critical role in growth regulation, BRs are known to protect plants from a broad range of environmental stresses, including low and high temperatures, drought, salinity and insect herbivory (Bajguz and Hayat, 2009). Throughout the past decade, various BR-induced molecular changes that are related to stress tolerance have been identified. These include enhanced expression of stress-responsive genes (Kagale et al., 2007), protection of the translational machinery (Dhaubhadel et al., 2002), potentiated accumulation of osmoprotectants (Divi and Krishna, 2009), NADPH-oxidase-mediated accumulation of hydrogen peroxide (Xia et al., 2009b), and enhanced photosynthesis efficiency (Xia et al., 2009a). Recently, however, Divi et al. (2010) uncovered yet another mode of BR-action during abiotic stress. Using mutant and transgenic Arabidopsis, they demonstrated that 24-epibrassinolide (BL)-induced tolerance to salt and temperature stress is reliant on the SA master regulatory protein NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), implicating a crucial role of the SA signaling pathway in BR-mediated stress responses. Interestingly, BRs may also regulate plant responses to pathogen attack. For instance, exogenous application of BR lowers susceptibility of rice to fungal blast and bacterial blight diseases, and activates resistance of tobacco (*Nicotiana tabacum*) to *Tobacco mosaic virus* (TMV), *P. syringae*, and the powdery mildew fungus *Oidium spp.* (Nakashita et al., 2003). In keeping with these data, there is ample evidence from both field experiments and greenhouse trials demonstrating the protective effects of exogenous BRs against a broad spectrum of fungal, viral and bacterial pathogens (Bajguz and Hayat, 2009). Together with the significantly increased BR levels in TMV-infected tobacco and the immune-suppressive effect of the BR biosynthesis inhibitor brassinazole (BRZ; Nakashita et al., 2003), these findings draw important inferences tagging BRs as powerful activators of broad-spectrum disease resistance in plants. However, in contrast to the relative wealth of information on BRs' function in the plant's developmental program, relatively little is known about the molecular mechanisms underpinning BR-modulated plant immunity. It also remains to be resolved if, and how, BRs adjust and coordinate immune responses to soil-borne pathogens.

Aiming to further decipher the molecular logic of BR-modulated immunity, we have analyzed the role of BRs during progression of rice infection by the root oomycete *Pythium graminicola*. Unlike most other experimentally tractable model plants, rice is a staple food for more than half the world and a model for monocots, which include cereal crops and biofuel grasses (Jung et al., 2008). *P. graminicola*, on the other hand, has recently been earmarked as one of the driving factors behind the progressive yield decline observed in Philippine aerobic rice fields (see Chapter 3). In contrast to the prevailing view that BRs boost plant innate immunity, our results provide compelling evidence that *P. graminicola* exploits endogenous BRs as virulence factors and hijacks the host BR-machinery to inflict disease.

Through genetic, physiological, and pathological analyses, we further show that BRs steer their detrimental effects on *Pythium* resistance, at least in part, through antagonistic crosstalk with SA and GA. While challenging the common assumption that BRs positively influence plant defense responses, these data add substantial breadth to our understanding of the immune-regulatory role of BRs and reveal several heretofore-unknown aspects of BR pathway crosstalk and signal integration.

5.2. Methods

5.2.1. Plant materials and growth conditions

Rice (*Oryza sativa*) lines used in this work included the *japonica* cv. Nipponbare, the corresponding *NahG* (Yang et al., 2004) and *OsWRKY45* RNAi transgenics (Shimono et al., 2007), the BR-deficient mutant alleles *brd1-1* and *brd1-2* (Mori et al., 2002), as well as an *OsNPR1*-silenced line (Yuan et al., 2007) and its wild-type Taipei (cv. *japonica*). The GA-deficient and/or insensitive mutants *d35* (*ent-kaurene oxidase* mutant; Itoh et al., 2004), *Waito-C* (*GA3ox2* mutant; Itoh et al., 2001), gid1-8 (GA-receptor GID1 mutant; Ueguchi-Tanaka et al., 2007), *gid2-2* (F-box protein GID2 mutant; Sasaki et al., 2003), *cps1-1/gid2-2* (ent-copalyl diphosphate synthase mutant crossed with *gid2-2*; Ueguchi-Tanaka et al., 2008), and the SLENDER RICE1 (SLR1) loss- or gain-of-function alleles *slr1-1* (Ikeda et al., 2001), *SLR1-d1*, and *SLR1-d3* (Asano et al., 2009), respectively, all are in the background of *japonica* cultivar T65, as are the BR-insensitive and BR-deficient mutant lines *d61-1*, *d61-2, d2-1*, and *d2-2* (Hong et al., 2003; Yamamuro et al., 2000). For seed multiplication, plants were propagated in the greenhouse (30°C ± 4°C, 16-h photoperiod) and fertilized with 0.5% ammonium sulphate every two weeks until flowering.

5.2.2. *Pythium graminicola* bioassays

Pythium graminicola strain PB912 132, isolated from a diseased aerobic rice field in Los Baños, The Philippines (see Chapter 3), was cultivated on potato dextrose agar at 28°C (PDA; Difco Laboratories). Rice seeds were surface-sterilized by agitation in 2% sodium hypochlorite for 20 min, rinsed three times with sterile demineralized water, plated in square petri dishes (12 × 12 cm) on standard strength Gamborg B5/1% plant agar medium, and subsequently grown in a growth chamber at 28°C (day)/26°C (night) under 12-h photoperiod. Three days after imbibition, i.e. when primary roots were approximately 1 cm in length, germinated seedlings were inoculated by carefully placing a 0.6-cm-diameter agar plug taken from the margin of seven-day-old PDA cultures in between each two plants. Control samples were mock-inoculated with 0.6-cm-diameter PDA plugs. Disease symptoms were scored 7 dpi and disease rates were expressed on the basis of diseased root area and reduction in root length using a 1-to-5 disease severity scale (Fig 5.1, A).

To account for differences in root growth caused by genetic mutations, exogenous hormones or chemical inhibitor compounds per se, all ratings were expressed relative to the respective non-inoculated controls: class I, root length more than 60% of the respective mock treatment, very little necrosis covering less than 10% of total root area; II, root length more than 60% of the respective mock treatment, more than 10% of total root area necrotic; III, root length between 20% and 60% of the respective mock treatment; IV, root length <20% of the respective mock treatment, less than 75% of root area necrotic; V, root length <20% of the respective mock treatment, more than 75% of root area affected. Disease index values were calculated according to the following formula: $[((1 \times a) + (2 \times b) + (3 \times c) + (4 \times d) + (5 \times e))/(a + b + c + d + e)] \times 100/5$, where a, b, c, d, and e are the number of roots with scores I, II, III, IV, and V, respectively.

5.2.3. Chemical treatments

Benzothiadiazole (BTH) (BION 50 WG, Syngenta) and gibberellin A3 (GA3) (Sigma-Aldrich) were dissolved in water at the indicated concentrations. Stock solutions of 24-epibassinolide (BL) (Sigma-Aldrich) and uniconazole (Wako) were in ethanol, whereas brassinazole (BRZ) (Wako) was dissolved in dimethyl sulfoxide (DMSO). Equivalent volumes of both solvents were added to separate control treatments.

5.2.4. Microscopy of root infection

Root infection was monitored at various times after inoculation with *P. graminicola* PB912 132 using a combination of bright-field and epifluorescence microscopy (Olympus BX51 microscope). For detailed information on sample preparation, sectioning, staining, mounting and imaging, see 'methods' chapter 4. *Pythium* structures were visualized by staining of sections with 0.1% (w/v) trypan blue in 10% (v/v) acetic acid. Phenolic compounds were detected by UV-excitation of 0.1% fluorescent brightener 28 (Calcofluor White M2R)-stained sections.

5.2.5. Western-blot analysis

Extraction of total proteins and western blotting were performed as described by Shimada et al. (2006) with minor modifications. Total protein samples were extracted from the roots of seven-day-old rice seedlings grown on Gamborg B5 (GB5) agar plates with 2 x extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCL, 0.5% Tween 20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Following quantification using a Bradford assay (Bio-Rad), protein extracts were mixed with an equal volume of 2× sample buffer (150 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% w/v glycerol, 0.01% w/v bromophenol blue, and 0.1 M w/v dithiothreitol), boiled for 5 min, separated on 7.5% SDS-PAGE gels, and transferred to a Hybond ECL membrane (Amersham Pharmacia Biotech). Polyclonal anti-SLR1 antibody and goat anti-rabbit HRP-conjugated secondary antibody (Dako) were used to quantify SLR1. Peroxidase activity was detected using ECL plus western-blotting substrate (Pierce biotechnology) according to the manufacturer's instructions.

5.2.6. RNA extraction and Quantitative Real-Time PCR (qPCR)

For RNA extraction and qPCR procedures, see chapter 4. The amount of plant RNA in each sample was normalized using *OsACTIN1* (LOC_Os03g50885) as internal control and samples collected from control plants at 1 dpi were selected as calibrator. Nucleotide sequences of all primers used are listed below.

Table 5.1. Primer sequences used for qPCR

Gene	Locus	Forward (5'-3')	Reverse (5'-3')
OsACTIN1	LOC_Os03g50885	GCGTGGACAAAGTTTTCAACCG	TCTGGTACCCTCATCAGGCATC
OsBLE2	LOC_Os07g45570	ACCAGCTGATCATAAGGCGGTCG	GGTGAACATCCTCGTGGCTTCTAG
OsXTH1	LOC_Os04g51460	GGTGGAGGTCCAAGAACACGTACC	TCCGACATGAAGTAGCAGGTGGTG
OsCPD1	LOC_Os11g04710	TTCTTCTCCATCCCCTTTCCTCTCGCCA	CACCCTCCGCCTCAAGAAGCTCCTCAA
OsDWARF2	LOC_Os01g10040	ATTGTCGGCCTCATGTCCCTCC	TCGCCA TCTTCTTCTTGGCCTGG
OsRAVL1	LOC_Os04g49230	TCCTCACCAACTCCACATTACGGT	CAGATCGAGATCCAACGAGGA
OsMADS55	LOC_Os06g11330	TGGAAGAGCTGCAGCAGATG	TCATCACAGATTCAGATGATTG
OsNPR1	LOC_Os01g09800	CACGCCTAAGCCTCGGATTA	TCAGTGAGCAGCATCCTGACT
OsWRKY45	LOC_Os05g25770	GGACGCAGCAATCGTCCGGG	CGGAAGTAGGCCTTTGGGTGC
GA2ox3	LOC_Os01g55240	TTCGGGTACGGCAGCAAGCG	TCAGAGCGGCCCGGAAGACC
GA20ox3	LOC_Os07g07420	CTCGAGTTCACGCAGAGGCACTAC	TGTCGAGGCTTTCATAGCCATTCC
SLR1	LOC_Os06g03710	GCGTTCCGGCGAGTGACGTG	CGCTGCGCGGTGCTCATCTG
SPINDLY	LOC_Os08g44510	TGCGGAGGCATGCAACAACC	AGCAGCATCCATCTTTCCCTGGAC
EL1	LOC_Os03g57940	TTCAGTGGGACAGGCGATGTCTG	GACCTAAGTGGGCATGGACGCTAG

5.2.7. Microarray analysis and data processing

Rice plants (cv. Nipponbare) were grown and inoculated with *P. graminicola* strain PB912 132 as described before. Samples from mock-inoculated and *Pythium*-inoculated plants were taken 1, 2 and 4 dpi, each sample representing a pool from at least 20 individual plants. Three biological replicates were executed, giving a total of 18 RNA samples (two treatments × three time points × three replicates). For microarray analysis, we used a previously described two-dye method allowing direct comparison between two samples on the same microarray (Satoh et al., 2010). In brief, cyanine 3- or cyanine 5-labeled cRNA samples were synthesized from 850 ng of total RNA using a low-input RNA labeling kit (Agilent Technologies) and hybridized to custom-made 60-mer Agilent arrays according to the manufacturer's protocols (Agilent Technologies). Following washing, slide image files were generated using a DNA microarray scanner (G2505B; Agilent Technologies) and signal intensities were extracted and normalized within each array using Feature Extraction version 9.5 (Agilent Technologies). Signal intensities among all arrays were normalized according to the quantile method for standardization (global scaling) using EXPANDER ver 4.1. Significance analysis was performed using a fixed linear model (ANOVA) implemented in R/MAANOVA. Differentially expressed genes were defined as genes with a log2-based signal ratio >0.585 or <−0.585 (i.e. 1.5-fold) and a false discovery rate of less than 0.05.

All microarray data generated in this study are available in the Gene Expression Omnibus (NCBI-GEO; http://www.ncbi.nlm.nih.gov/geo) database under the reference GSE32582. For motif search and cis-element identification, 1-kb promoter regions of all differentially expressed genes were extracted from the Rice Genome Annotation (http://rice.plantbiology.msu.edu) and Orygenes (http://orygenesdb.cirad.fr) databases and analyzed using the RSAT (http://rsat.ulb.ac.be/rsat/), Osiris (http://www.bioinformatics2.wsu.edu/Osiris/), and PLACE programs (http://www.dna.affrc.go.jp/PLACE/).

5.3. Results

5.3.1. BRs suppress basal immunity of rice to the root pathogen *P. graminicola*

In a first attempt to elucidate the role of BRs in root immunity to *P. graminicola*, we tested the effect of exogenously administered BL, a biologically active BR (Nakashita et al., 2003). To this end, rice seeds (cv. Nipponbare) were germinated and grown on agar plates containing various concentrations of BL. Because rice plants are susceptible to *Pythium* infection for only a few days after planting (Chun and Schneider, 1998), three-day-old seedlings were tested for their susceptibility to the virulent strain PB912 132. Within 2 to 3 dpi, roots of solvent-treated control plants developed typical brown necrotic patches, a symptom accompanied by strong reduction of seminal root length compared to non-inoculated controls (Figure 5.1, A). Surprisingly, feeding plants with increasing concentrations of BL favored subsequent infection with *P. graminicola*, resulting in more extensive necrosis and stunting of inoculated roots as compared to the respective mock-inoculated controls (Figure 5.1, B). In contrast, treatment with 1 µM BRZ, a triazole that reversibly and specifically inhibits BR biosynthesis (Asami et al., 2000), led to a substantial reduction in disease severity (Figure 5.1, B). Importantly, neither BL nor BRZ had a significant impact on the in vitro growth of *P. graminicola*, implicating the involvement of plant-mediated responses (data not shown).

To gauge the physiological significance of these findings, we also tested several mutants that are either deficient in or insensitive to BR. As shown in Figure 5.1 (C), disease tests with the BR-deficient mutants *brd1-1, brd1-2, d2-1*, and *d2-2* (Hong et al., 2003; Mori et al., 2002) revealed enhanced resistance as compared to the respective wild-types, while complementing *d2-2* with exogenous BL restored susceptibility to wild-type levels (Figure S5.1). In contrast, no strong differences in overall disease severity could be observed between wild-type T65 plants and the BR-insensitive mutants *d61-1* and *d61-2* (Figure 5.1, C), both of which carry a loss-of-function mutation in the rice BR-receptor gene *OsBRI1* (Yamamuro et al., 2000). This was especially evident in the case of *d61-1*, while in the *d61-2* mutant, statistically significant differences were detected. One possible interpretation of this finding is that BR signaling plays a subordinate role in the *P. graminicola* resistance machinery. However, given the strong negative feedback regulation of BR biosynthesis, it is equally possible that an increase in resistance resulting from BR-insensitivity is masked by the elevated levels of endogenous BRs in *d61* (Yamamuro et al., 2000).

To discriminate between these possibilities, we sought to supply wild-type and signal-defective *d61-1* seedlings with 1 µM BRZ to lower the endogenous BR content and observe any effect on pathogen resistance. Interestingly, despite the higher basal levels of BR in the mutant line, BRZ treatment was equally effective in *d61-1* and the wild-type, suggesting that both BR biosynthesis and BR signaling serve higher susceptibility to *Pythium* attack (Figure 5.1, D).

To further discriminate the immune-regulatory role of BRs, samples of BL- and BRZ-treated Nipponbare roots were collected at various times after inoculation and analyzed for pathogen colonization and cellular reactions using a combination of bright-field and epifluorescence microscopy (Figure 5.1, E). Regardless of BL or BRZ treatment, numerous hyphae were found to be present along the longitudinal axis of the root within 12 h post inoculation. Following penetration (Figure S5.2, A), primary hyphae differentiated rapidly into bulbous invading hyphae, giving rise to a dense network that, by 2-3 dpi, penetrated all inner cell layers of the root, including the cortex, the endodermis, and the vascular tissue (Figure 5.1, E; Figure S5.2, B). Similar to what has been reported for the rice blast pathogen *Magnaporthe oryzae* (Kankanala et al., 2007), invading hyphae became more bulbous prior to crossing the cell wall and constricted dramatically, resulting in a thin invasive peg at the point of passage (Figure S5.2, C). In control plants, early fungal progression, i.e. prior to 2 dpi, was characterized by successive invasions of root cells with no apparent loss of cell viability. In BL-supplemented plants, however, this apparent biotrophic phase was considerably shortened, with necrosis-related autofluorescence being detectable from as early as 24 h post inoculation. BL-treated seedlings were further characterized by dramatically enhanced pathogen growth relative to that seen in non-treated controls (Figure 5.1, E). Comparing control and BRZ-treated seedlings, no marked differences could be observed in pathogen proliferation, despite the substantial difference in overall disease severity between these treatments. However, BRZ treatment caused a significant delay in the onset of tissue necrotization, with the large majority of colonized cells remaining void of autofluorescence until as late as 3 dpi.

5.3.2. Temporal dynamics of BR biosynthesis and signaling in response to *P. graminicola* attack

Recent advances in plant immunity research have provided insights into the ingenious ways by which microbial pathogens modify plant hormone homeostasis to subdue host immune responses and enforce a successful infection (Robert-Seilaniantz et al., 2011). To assess whether *P. graminicola* similarly co-opts the BR pathway to tap into the rice signaling infrastructure, we used qPCR to monitor the steady-state mRNA levels of various BR-responsive, -biosynthetic, and -regulatory genes in roots of inoculated Nipponbare plants grown in the presence or absence of BL. As shown in Figure 5.2, expression of the BL-inducible gene *BRASSINOLIDE ENHANCED2* (*OsBLE2*; Yang et al., 2003) responded strongly to *Pythium* infection, and peaked at 3 dpi at approximately 55-times the levels found in non-inoculated controls.

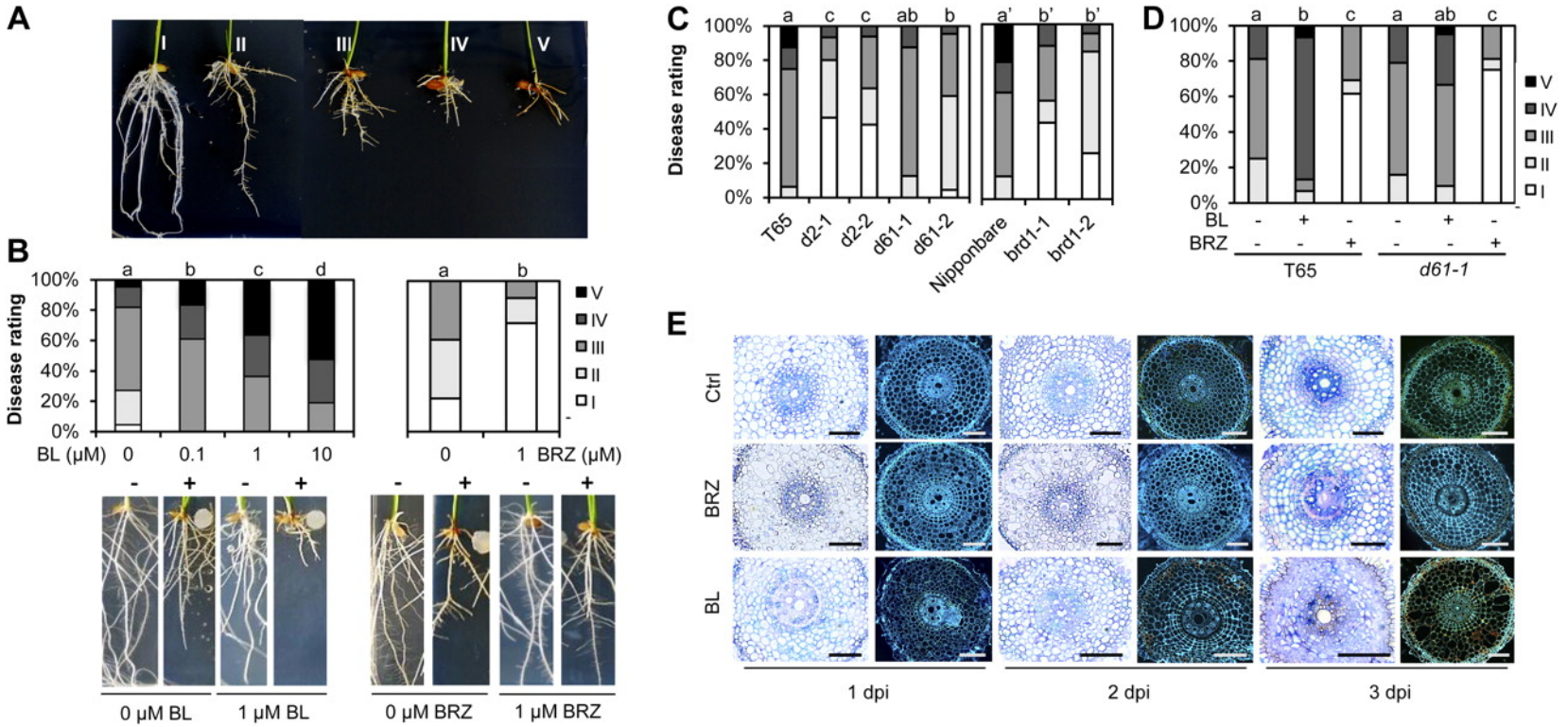

Figure 5.1. BRs suppress root immunity in rice against the ooymycete *P. graminicola*. A, Illustration of the I-to-V disease severity scale used for disease evaluation. For more details on the different disease classes, see 'Methods'. **B,** Disease promoting and reducing effect of exogenously administered BL and BRZ, respectively. Seeds (cv. Nipponbare) were germinated on Gamborg B5 medium containing different concentrations of BL or BRZ, and, 3 days post imbibition, inoculated with 0.6-cm mycelial plugs of virulent *P. graminicola* PB912 132. Representative pictures were taken 7 dpi. Minus sign (−) = mock control, plus sign (+) = inoculated. Data represent one of three experiments with very similar results. Different letters indicate statistically significant differences (Mann Whitney; n ≥ 15; α = 0.05). **C,** Resistance of BR-deficient *d2-1, d2-2, brd1-1*, and *brd1-2* and BR-insensitive *d61-1* and *d61-2*. **D,** Effectiveness of exogenously administered BL and BRZ in wild-type T65 and BR-insensitive *d61-1*. For graphs **C** through **D,** statistical analysis was performed on pooled data from three independent experiments (Mann Whitney; n ≥ 32; α = 0.05). **E,** Microscopic analysis of early infection events in control, BL (1 μM), and BRZ (1 μM) pretreated Nipponbare roots inoculated with *P. graminicola* PB912 132. Left section: colonization of 5 μm root sections as visualized by trypan blue staining and bright-field microscopy. Right section: autofluorescence of representative root sections stained with Calcofluor white M2R (UV light excitation). Scale bars = 100 μM.

At this time point, pathogen-induced transcription of *OsBLE2* was even more pronounced in BL-pretreated plants, as was the expression of the BR-responsive endoglucan transglucosylase OsXTH1 (Duan et al., 2006). Reaching a maximum at 1 dpi in control-inoculated roots, BL-pretreatment caused *OsXTH1* transcript levels to remain high throughout the course of the infection, suggesting a positive correlation between BR-inducible gene expression and overall susceptibility to *P. graminicola*.

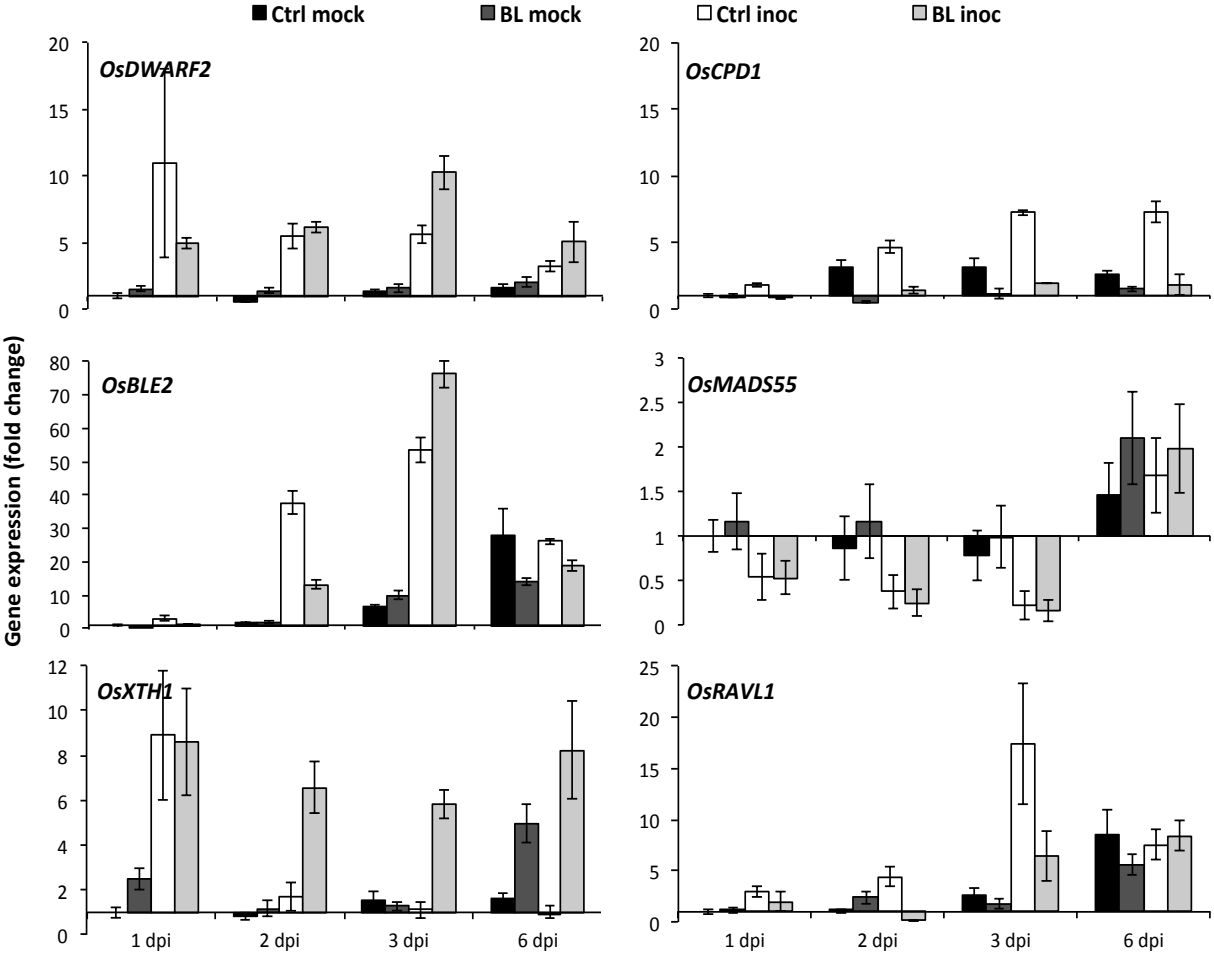

Figure 5.2. Effect of BL-pretreatment on BR-response, -biosynthesis, and -regulatory genes in *P. graminicola*-inoculated rice roots. For details on BL treatments (1 µM) and *Pythium* bioassays see legend to Figure 5.1. Transcript levels were normalized using actin as an internal reference and expressed relative to the mock-inoculated control plants at 1 dpi. Data are means of two technical and two biological replicates from a representative experiment, each biological replicate representing a pooled sample from at least six individual plants. Error bars display standard deviations.

In a similar vein, *Pythium* inoculation entailed a strong up-regulation of the BR-biosynthesis genes *OsDWARF2* and *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM1* (*OsCPD1*), with mRNA levels peaking at 7 to 10 times the levels found in non-inoculated controls.

These results were rather unexpected given the apparent activation of BR signaling in *Pythium*-inoculated roots and the well-established negative feedback regulation of BR biosynthesis (He et al., 2005; Wang et al., 2002). Although not evident in the case of *OsDWARF2*, feedback inhibition was clearly seen for *OsCPD1*, with BL treatment strongly down-regulating expression of the gene relative to that seen in the respective mock controls. These outcomes suggest that *P. graminicola* affects the expression of genes involved in both BR signaling and biosynthesis, thereby bypassing feedback regulatory mechanisms. In line with this notion, pathogen-induced expression of *OsBLE2* and *OsXTH1* was greatly attenuated in signal-defective d61-1, whereas expression of the BR-biosynthesis genes *OsCPD1* and *OsDWARF2* was comparable to or even higher than in the corresponding wild-type T65 (Figure S5.3).

In addition to the increased transcription of BR-biosynthetic and -response genes in *P. graminicola*-inoculated roots, we also found transcriptional alterations of key regulatory genes, including *RAV-LIKE1* (*OsRAVL1*). Consistent with its pivotal role in maintaining basal activity of both BR signaling and biosynthesis (Il Je et al., 2010), *OsRAVL1* mRNA levels responded strongly to pathogen inoculation, showing an approximately 15-fold induction over non-inoculated controls. In the inoculated BL treatment, however, *OsRAVL1* expression was less pronounced, presumably due to the already strong activation of BR signaling in this system. In contrast, both control and BL-pretreated roots exhibited a strong pathogen-specific down-regulation of the BR-negative regulator *MADS-box PROTEIN55* (*OsMADS55*; Lee et al., 2008), suggesting a putative mechanism by which *P. graminicola* elevates BR signal processing.

While demonstrating that successful infection of rice by *P. graminicola* is associated with major transcriptional reprogramming of various BR-associated genes, these data raise the possibility that *P. graminicola* usurps the host BR machinery to induce a state of susceptibility. In line with this hypothesis, genome-wide transcriptome analysis of *P. graminicola* PB912 132-inoculated Nipponbare roots revealed more than one-third of all BR-associated genes (68 out of 192) represented on the array to be significantly altered after *Pythium* attack (data not shown), with examples including the major BR-biosynthetic genes *OsDWARF* (Mori et al., 2002) and *OsDWARF2*, the putative BR-receptor gene *BRI1-LIKE 3* (Nakamura et al., 2006) and the negative signaling regulator *MADS-box PROTEIN1* (Duan et al., 2006). Moreover, analysis of the proximal 1-kb promoter region of all 4381 genes significantly up-regulated by *P. graminicola* identified a significant overrepresentation of various cis-elements known to be responsive to BRs (Table 5.2). These included two E-box elements (CANNTG), various motifs containing the BR-response element (BRRE) and the G-box CACGTG, which contains two inverted repeats of the core BRRE sequence CGTG.

Table 5.2. BR-responsive cis-elements significantly enriched in *P. graminicola*-up-regulated rice genes

Cis-element	Sequence	*P*-value	Reference
BRRE[a]	CGTGCA	0.002	He et al., 2005
	CGTGGC	0.023	He et al., 2005
	ACGTGG	0.012	He et al., 2005
	CCGTGC	0.009	He et al., 2005
G-box	CACGTG	0.006	Sun et al., 2010
E-box[b]	CAGCTG	0.023	Il Je et al., 2010; Yin et al., 2005
	CACATG	0.014	Il Je et al., 2010; Yin et al., 2005

[a]CGTG = BRRE core sequence.

[b]E-box consensus sequence = CANNTG.

5.3.3. BRs antagonize SA-mediated immunity against *P. graminicola*

Mounting evidence indicates that pathogen defense signaling is not a linear single-response event, but a complex network involving multiple effectors and defense signals (Pieterse et al., 2009). Therefore, to further elucidate the molecular machinery underpinning BR-mediated susceptibility to *P. graminicola*, we explored the interaction of BR with other plant defense regulators. Given the high importance of SA in mediating foliar plant immunity (Robert-Seilaniantz et al., 2011), and the recent identification of BR-SA crosstalk in the context of abiotic stress signaling (Divi et al., 2010), we first assessed the involvement of SA-modulated immune responses. Interestingly, we found that application of BL negates resistance conferred by the synthetic SA-analog benzothiadiazole (BTH) (Figure 5.3, A), which is suggestive of negative BR-SA crosstalk. To test this hypothesis, wild-type Nipponbare and SA-deficient *NahG* transgenics (Yang et al., 2004) were routinely treated with 1 µM BRZ and tested for expression of induced resistance. As shown in Figure 5.3, B, *NahG* plants were significantly more sensitive to pathogen attack than corresponding wild-type seedlings, demonstrating the importance of SA biosynthesis in basal resistance to *P. graminicola*. SA accumulation, however, did not appear to be a prerequisite for BRZ-induced resistance, as BRZ application was equally effective in wild-type Nipponbare and *NahG* plants, causing an approximate 30% reduction in basal disease susceptibility in both genotypes.

To further probe the nature of the SA-BR signal interaction, we next monitored the expression of the SA-regulatory genes *OsNPR1* and *OsWRKY45* at various times after inoculation. Both genes encode key regulatory proteins that control distinct branches of the SA signaling cascade in rice (Shimono et al., 2007; Yuan et al., 2007). Following a rapid strong up-regulation upon pathogen infection, transcription of *OsNPR1* and *OsWRKY45* gradually decreased throughout the course of infection, an effect that was greatly accelerated by exogenous BL (Figure 5.3, C). Intriguingly, temporal expression of both genes seemed to be inversely correlated with that of the BR markers *OsBLE2*, *OsCPD1*, and *OsRAVL1* (Figure 5.2), especially within the first 3 dpi.

In a similar vein, BL application also prevented the full age-dependent expression of *OsNPR1* in non-inoculated samples (Figure S5.4, red arrows). Along with the negative impact of exogenous BL on the BTH-inducible resistance (Figure 5.3, A), these results support negative SA-BR crosstalk during *P. graminicola* infection. To assess the functional relevance of this putative antagonism, we quantified the level of BRZ-induced resistance in *OsWRKY45* and *OsNPR1* RNAi lines (Figure 5.3, D). Interestingly, we found that plants silenced for *OsWRKY45* were more susceptible to *P. graminicola* than the wild-type and also failed to develop resistance when treated with BRZ. In contrast, no significant differences in disease susceptibility could be observed between non-treated wild-type (cv. Taipei) and *OsNPR1* RNAi plants. However, similar to the *OsWRKY45* transgenics, *OsNPR1*-silenced plants were unable to develop resistance when treated with BRZ, suggesting that BR antagonizes SA-mediated defenses downstream of SA accumulation, but upstream of *OsNPR1* and *OsWRKY45*.

5.3.4. Repression of GA-mediated defenses is a crucial facet of BR-provoked susceptibility to *P. graminicola*

Gibberellins are a large family of tetracyclic diterpenoid hormones that control nearly every aspect of plant growth and development. Recent work on wild-type tissues of various plants has provided multiple physiological and molecular links to support extensive interplay between the BR and GA signaling pathways (Zhang et al., 2009). Considering these findings and given the emerging role of GA as a bona fide immunity hormone (Robert-Seilaniantz et al., 2011), we sought to extend our analysis of the disease-promoting effect of BRs by scrutinizing potential BR-GA crosstalk.

As shown in Figure S5.5 (A), treatment of wild-type Nipponbare with increasing concentrations of GA3 enhanced resistance to *P. graminicola* in a concentration-dependent manner. Conversely, depletion of endogenous GA levels using the GA-biosynthesis inhibitor uniconazole (Izumi et al., 1984) promoted disease susceptibility. Although care should be taken when interpreting these results since the specificity of uniconazole is not entirely clear (Bidadi et al., 2010), these data strongly suggest that GA acts as a positive player in rice immunity *to P. graminicola* (Supplemental Fig. S5B). Interestingly, supplying plants with both GA3 and BL restored the BL-induced susceptible phenotype (Figure S5.5, A), whereas co-treatment of uniconazole with BL did not confer an additive effect at the level of susceptibility (Figure S5.6, A). Identical results were obtained at the physiological level, with BL and uniconazole inhibiting primary root length to a similar extent (Figure S5.7, A). Moreover, roots of the GA-deficient mutant d35 were less sensitive to exogenous BL than were wild-type roots (Figure S5.7, C). Together, these data infer that: 1) endogenous GA levels represent an important reservoir needed to mount a full innate immune response to *P. graminicola*, 2) that BR inhibits GA responses in roots at least in part by interfering with GA metabolism, and 3) that BR-induced susceptibility may develop coincidently with decreases in endogenous GA content.

Supporting these conclusions, BRZ failed to stimulate root elongation (Figure S5.7, A) and increase disease resistance (Figure S5.6, B) when combined with 10 µM uniconazole.

5.3.5. The DELLA protein and GA-repressor SLR1 is a key player in resistance to *P. graminicola*

The observation that BR-induced susceptibility to *P. graminicola* is steered at least in part through negative crosstalk with GA-controlled responses prompted us to further investigate the role of GA in the rice-*P. graminicola* pathosystem. In contrast to its well-documented role as a plant growth regulator, GA has only recently been implicated in plant immunity. Current concepts suggest that at least in Arabidopsis, GA modulates plant disease resistance by inducing the degradation of DELLAs, a class of nuclear growth-repressing proteins that act as central suppressors of GA signaling (Navarro et al., 2008). Contrary to the five DELLA proteins present in Arabidopsis, rice has only one *DELLA* gene, designated *SLENDER RICE1* (*SLR1*; Ikeda et al., 2001). To elucidate the role and the position of *SLR1* within the signaling network controlling defense and susceptibility against *P. graminicola*, several GA-deficient and/or -insensitive rice mutants that overaccumulate SLR1 (Ueguchi-Tanaka et al., 2008) were screened for their susceptibility to *P. graminicola*.

As shown in Figure 5.4 (A), all mutants tested, either impaired in GA biosynthesis (*Waito-C, d35*), insensitive to GA (*gid1-8, gid2-2*), or both (*cps1-1/gid2-2*), showed increased susceptibility compared with wild-type T65 plants. Enhanced susceptibility to *P. graminicola* was also observed in two independent *SLR1* gain-of-function mutants (Asano et al., 2009; Figure 5.4, B), whereas *slr1-1*, a loss-of-function allele displaying a constitutive GA-response phenotype (Ikeda et al., 2001), exhibited reduced necrosis and increased resistance to pathogen infection compared to inoculated T65 (Figure 5.4, C). Moreover, qPCR analyses revealed *SLR1* to be transiently up-regulated in response to both pathogen inoculation and BL treatment (Figure S5.8), further implicating SLR1 as a core participant in rice responses to *P. graminicola.*

5.3.6. Multi-level interactions mediate BR-GA antagonism in *P. graminicola*-inoculated roots

Theoretically, crosstalk between hormone pathways may occur at the level of biosynthesis regulation, signal transduction, and/or gene expression. To gain further insight into the molecular mechanisms of above-mentioned BR-GA interplay, we measured the transcript levels of multiple GA-biosynthetic and -regulatory genes in roots of inoculated Nipponbare seedlings grown in the presence or absence of 1 µM BL. As shown in Figure 5.4 (D), *Pythium* inoculation strongly induced the *GA-degrading enzyme GIBBERELLIN 2-b-DIOXYGENASE3 (OsGA2ox3*) in both control and BL-treated samples, whereas *the GA-biosynthesis enzyme GIBBERELLIN 20-OXIDASE3* (*OsGA20ox3*) was severely down-regulated following inoculation. Similar results were obtained in our microarray analysis where perception of *P. graminicola*, despite being associated with activation of multiple genes involved in biosynthesis of inactive GA-precursors (i.e. diterpenoids and GA12), led to a general repression of GA20-oxidases but activation of GA2-oxidases (Table S5.1).

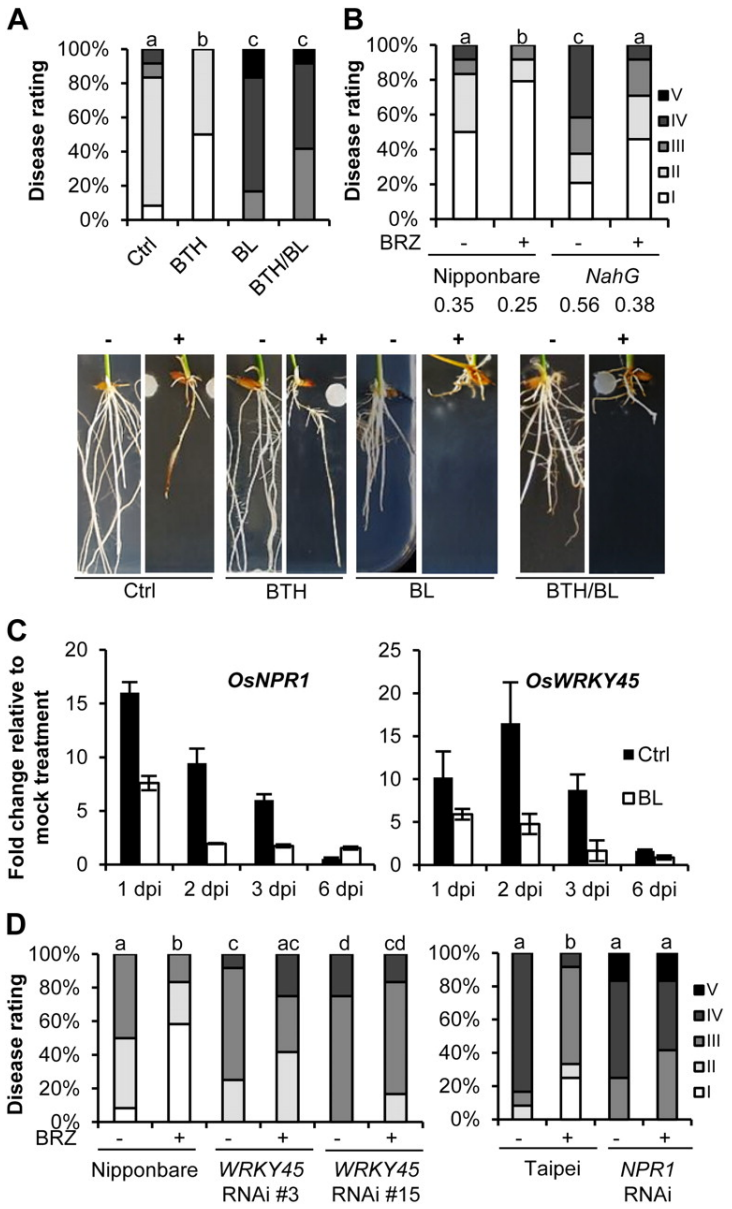

Figure 5.3. BRs antagonize SA-mediated root immunity against *P. graminicola*. A, Exogenous BL (1 µM) attenuates BTH-induced resistance. For chemical induction of resistance, seeds were briefly dipped into a BTH solution (0.5 mM) and subsequently sown on GB5 medium containing 50 mg L^{-1} BTH. BL-treatment, pathogen inoculation, and disease evaluation was performed exactly as described in legend to Figure 5.1. Different letters indicate statistically significant differences (Mann-Whitney; n = 12; α = 0.05). Representative pictures were taken 7 dpi. Minus sign (−) = mock control, plus sign (+) = inoculated**. B,** Effect of BRZ-pretreatment (1 µM) on *Pythium* susceptibility in wild-type Nipponbare and SA-deficient NahG plants. Statistically significant differences between treatments are labeled with different letters (Mann-Whitney; n = 24; α = 0.05). Numbers below graph represent disease index values. **C,** qPCR analysis of the SA-regulatory genes *OsWRKY45* and *OsNPR1* in control and BL-treated Nipponbare roots inoculated with *P. graminicola*. Transcript levels were normalized using actin as an internal reference and for each time point expressed relative to mock-inoculated controls. Data are means of two technical and two biological replicates from a representative experiment, each biological replicate representing a pooled sample from at least six individual plants. Error bars display standard deviations. **D,** BRZ-induced resistance is compromised in *OsWRKY45* and *OsNPR1* RNAi lines. All genotypes were pretreated with 1 µM BRZ and subsequently inoculated with *P. graminicola* PB912 132. Bars labeled with different letters are significantly different (Mann-Whitney; n = 24; α = 0.05). All experiments were repeated at least twice with similar results.

In wild-type rice, expression of GA-biosynthetic genes is feedback inhibited by GA (Itoh et al., 2002). However, on the basis of the strong inhibitory effect of BL on GA-induced *Pythium* resistance as well as the likely role of GA biosynthesis in promoting immunity, we suggest that *P. graminicola* recruits the BR pathway, at least in part, to actively suppress GA biosynthesis in inoculated tissues. In support of this assumption, we found exogenously administered BL to inhibit *GA20ox3* and induce *GA2ox3* within 8 h of incubation (Figure 5.4, E). Intriguingly, treating roots with 50 µM GA3 likewise resulted in a fast and strong down-regulation of the BR-response and BR-biosynthesis genes *OsBLE2* and *OsDWARF2*, respectively, indicating that BR and GA cause cross-inhibitory effects on the reciprocal hormone biosynthesis pathways to interact in a mutually antagonistic manner.

Besides interfering with GA metabolism, BR may also inhibit GA action through transcriptional activation of GA-repressor genes. Over the last few years, several rice proteins with an inhibitory function in GA signaling have been identified. These include the *O*-linked GlcNAc transferase SPINDLY (Shimada et al., 2006), and the casein kinase EARLY FLOWERING1 (EL1; Dai and Xue, 2010). Although expression of *SPINDLY* was transiently up-regulated by BL alone, the most dramatic changes in *SPINDLY* kinetics were seen following pathogen inoculation, with mRNA levels peaking at 2 dpi at approximately six times the levels found in non-inoculated controls (Figure 5.4, D). A similar but slightly more pronounced trend was noticed in BL-pretreated roots where pathogen-induced expression of SPINDLY was evident as late as 6 dpi. Interestingly, transcript accumulation of *EL1* resembled the trend observed for *SPINDLY*, suggesting that *P. graminicola* triggers the expression of GA-repressor proteins, possibly in a BL-dependent manner, as yet another mechanism to antagonize GA.

Given the central role of DELLA proteins in modulating hormonal crosstalk in stress and developmental processes (Robert-Seilaniantz et al., 2011), and the reported ability of SPINDLY and EL1 to fine tune the suppressive function of SLR1 (Dai and Xue, 2010; Shimada et al., 2006), we finally asked whether BR-GA antagonism also is manifested at the level of SLR1 protein stability. To this end, we examined the level of SLR1 (using a monoclonal anti-SLR1 antibody) in wild-type Nipponbare roots grown for seven days in the presence or absence of 1 µM BL. Consistent with previous reports (Sasaki et al., 2003), SLR1 migrated as two bands in SDS-PAGE gels, representing the phosphorylated (top band) and non-phosphorylated (bottom band) form of the protein, respectively. Levels of immunologically detectable SLR1 were obviously higher in BL-treated plants than in non-treated controls before the onset of GA3-treatment (Figure 5.4, F). Following GA3-treatment, however, no clear-cut differences in SLR1-levels were apparent between treatments, except for a slight increase in BL-treated roots at 4 h post GA application. Therefore, our findings propose that BL does not intervene with GA-mediated SLR1 protein turnover per se, but rather indirectly promotes SLR1-stabilization by inactivating GA through the combined repression of GA synthesis and transcriptional activation of GA-repressor genes.

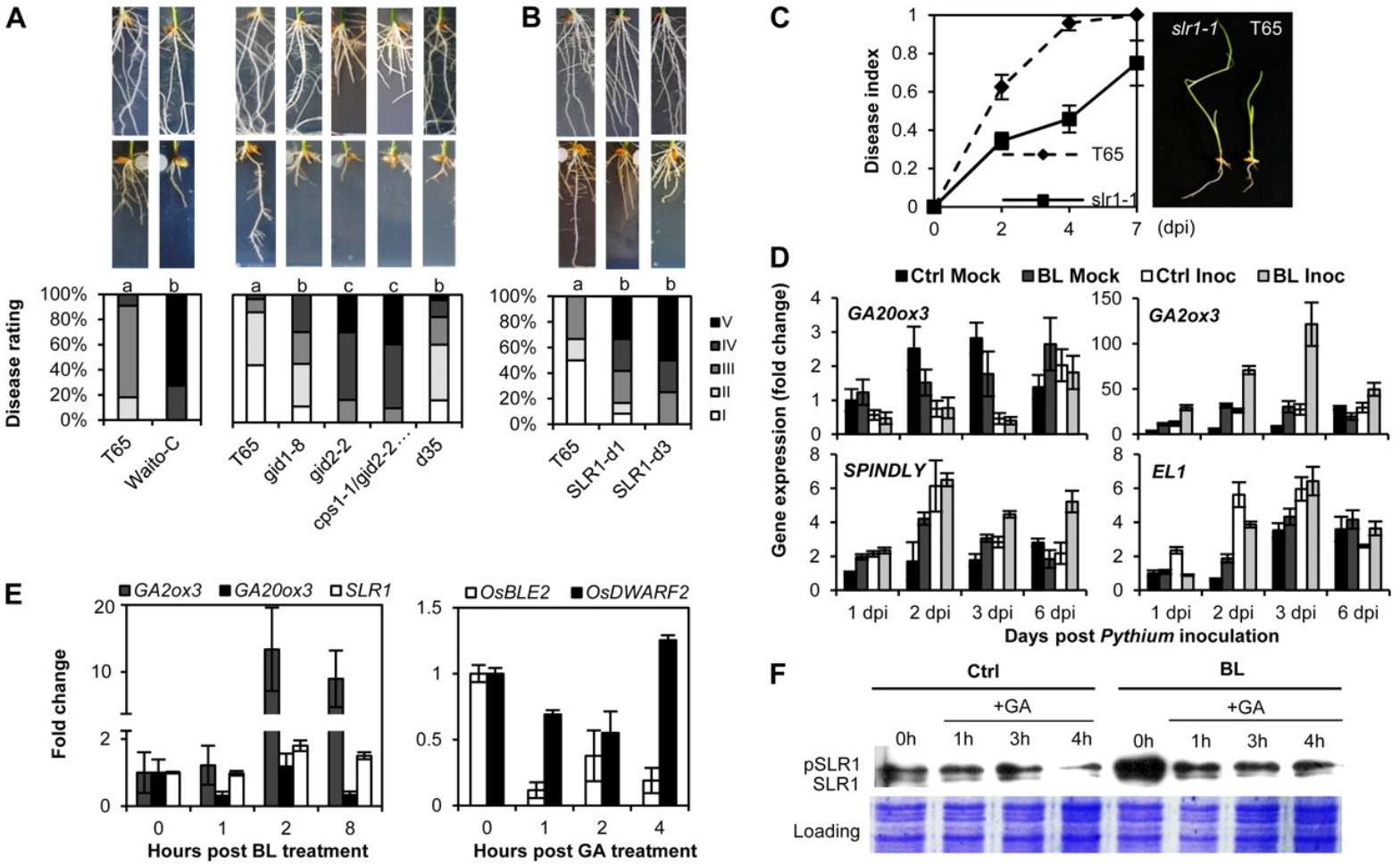

Figure 5.4. BR-induced susceptibility to *P. graminicola* involves repression of GA-mediated defenses. **A,** Bioassays with several GA-deficient and -insensitive mutants revealed a positive role for GA in rice immunity to *P. graminicola*. All genotypes were inoculated as described in legend to Figure 5.1. Different letters indicate statistically significant differences (Mann-Whitney; n ≥ 12; α = 0.05). **B,** Hypersusceptibility of two independent *SLR1* gain-of-function mutants. Bars with different letters are significantly different (Mann-Whitney; n = 12; α = 0.05). Photographs of non-inoculated controls are shown in the top section, representative disease symptoms are depicted in the bottom section. **C,** Growth of *P. graminicola* PB912 132 on wild-type T65 and the constitutive GA-mutant *slr1-1*. Data represent means from three independent biological replicates (n = 15). Error bars display standard errors.

Figure 5.4. (Continued)

D, Effect of BL pretreatment on the transcription of GA-associated genes in roots of cv. Nipponbare inoculated with *P. graminicola*. BL treatments, pathogen inoculation, and qPCR analysis were performed exactly as described in Figures 5.1 and 5.2. **E,** Crosstalk-experiments demonstrating mutual antagonism between BRs and GAs in root tissue of cv. Nipponbare. Six-day-old seedlings grown on GB5 medium were treated with either 1 µM BL or 50 µM GA3 for the indicated times and subjected to qPCR analysis exactly as described in the legend to Figure 5.2. **F,** Immunoblot-analysis of SLR1 protein accumulation in roots of seven-day-old Nipponbare seedlings grown in the presence or absence of 1 µM BL. SLR1-degradation was induced by submerging roots in 500 µM GA3 for the indicated times and protein levels were analyzed by western blotting using a SLR1-specific antibody. A duplicate protein gel was stained with Coomassie Blue (CBB) as loading control.

5.4. Discussion

Originally discovered in the pollen of *Brassica napus* (Grove et al., 1979), BRs encompass a family of over 40 structurally and functionally related steroid compounds. Apart from their well-established function in growth and developmental processes, there is extensive literature demonstrating the remarkable ability of BRs to boost plant responses to several biotic and abiotic stresses. However, very little is known about the mechanisms underlying BR-mediated stress responses. In an attempt to increase our understanding of the immune-regulatory role of BR, we have studied its impact, dynamics and crosstalk with other hormones during the interaction between rice and the soil-borne oomycete *P. graminicola*. Challenging the view of BRs as positive regulators of plant pathogen responses, we show that BRs suppress root immunity to *P. graminicola*, resulting in increased pathogen proliferation and substantially enhanced susceptibility. Moreover, our results support a scenario whereby *P. graminicola* co-opts the plant BR machinery as a decoy strategy to tap into the immune signaling circuitry and interfere with effectual SA- and GA-controlled defenses.

To date, genetic studies aimed at elucidating the role and action mechanisms of BR in plant stress adaptation have been confounded by the often strong and pleiotropic phenotypes of BR biosynthesis and signaling mutants, including extreme dwarfism, sterility, dark-green and epinastic leaves, and delayed development (Bishop, 2003). Moreover, current techniques for BR-measurements require large amounts of plant tissue and often fail to capture stress-induced changes in the levels of the biologically active BRs, castasterone and BL (Shimada et al., 2001; Wu et al., 2008). As a result, most reports currently available are based on exogenous hormone applications. Under our experimental conditions, exogenous BL markedly promoted susceptibility to *P. graminicola*, whereas treatment with BRZ, a highly specific inhibitor of BR biosynthesis, induced substantial levels of resistance.

Along with the extensive transcriptional reprogramming of various BR-biosynthetic, -signaling and -response genes in pathogen-inoculated roots and the overrepresentation of BR-responsive cis-elements in the promoters of *P. graminicola*-dependent rice genes, these findings strongly suggest that *P. graminicola* hijacks the rice BR biosynthesis and signaling machinery to cause disease, thus exploiting BRs as virulence factors. In compliance with this hypothesis, many other microbial pathogens have lately been shown to disarm the plant's weaponry by manipulating host hormone signaling (De Vleesschauwer et al., 2010; Robert-Seilaniantz et al., 2011), highlighting the central importance of hormone homeostasis in molding pathological outcomes. As for many other hormones, BR cellular homeostasis is achieved mainly by end product feedback regulation with activation of BR signaling suppressing BR synthesis (He et al., 2005). The finding that in pathogen-inoculated roots feedback control did not set in upon strong activation of several BR-responsive genes, infers that *P. graminicola* concomitantly affects BR biosynthesis and ensuing signaling. One possible mechanism involves the B3 DNA-binding domain protein RAVL1, which has recently been identified as a transcriptional activator of both BR-biosynthesis and BR-signaling genes (Il Je et al., 2010). Expression of RAVL1 was non-responsive to exogenous BR, but strongly induced following *P. graminicola* inoculation. However, other than attenuating negative feedback inhibition, *P. graminicola* also seems to impinge on downstream signal processing as revealed by the pathogen-specific suppression of the key BR-repressor gene *OsMADS55*. Taken together, these findings strongly argue that *P. graminicola* targets multiple regulatory modules of the BR pathway to disturb BR cellular homeostasis and ensure prolonged activation of this pathway throughout the course of infection.

At least two different scenarios can be hypothesized to explain the negative impact of BRs on *P. graminicola* resistance. First, endogenous BRs may directly benefit the growth and/or virulence of the pathogen. Similar to oomycetes of the genus *Phytophthora*, *Pythium* species lack sterol biosynthetic pathways and thus need to assimilate sterols from their environment or host to support growth and initiate sexual reproduction (Hendrix, 1964). In view of this, it is tempting to speculate that *P. graminicola* uses the plant's BR machinery to secure access to sterols during the infection process and initiate oosporogenesis. However, such a hypothesis is rather unlikely considering the inability of exogenous BL to promote hyphal growth (data not shown) and the fairly similar rates of pathogen colonization in control and BRZ-treated roots. Moreover, despite their strong potential to enhance pathogen growth in culture and in sharp contrast with previous observations in the Arabidopsis-*P. syringae* pathosystem (Griebel and Zeier, 2010), we failed to observe any disease-promoting effect of exogenously administered plant sterols, including sitosterol and stigmasterol (data not shown). Therefore, instead of directly promoting pathogen fitness, host-produced BRs may enhance susceptibility to *P. graminicola* by interfering with specific plant defense signaling pathways. In accordance with this statement, there is ample evidence demonstrating extensive interplay between BR and other small-molecule hormones (Zhang et al., 2009).

For example, BR modulates the biosynthesis of ET (Hansen et al., 2009) and intimately interacts with CKs and AUXs in regulating various developmental processes (Goda et al., 2004; Peleg et al., 2011; Vercruyssen et al., 2011; Vert et al., 2008). Moreover, several microarray experiments and more recent biochemical approaches point to crosstalk between BR and ABA and JA signaling pathways, respectively (Goda et al., 2002; Nemhauser et al., 2006; Zhang et al., 2009).

Recently, BRs were also found to interact with GA (Sun et al., 2010; Zhang et al., 2009). Although most examples of BR-GA signal connections relate to physiological processes, several lines of evidence indicate that BR-GA antagonism also fulfills a pivotal role during the BR-mediated susceptibility to *P. graminicola*. First, disease development was more severe on several GA-deficient and/or -insensitive mutants, implying a positive role of GA in resistance to *P. graminicola*. Second, disruption of endogenous GA levels using the GA-biosynthesis inhibitor, uniconazole, yielded similar susceptibility levels as those observed in BL-treated plantlets, whereas co-application of BL and uniconazole did not entail an additive effect. Third, uniconazole-treatment negated the resistance-inducing effect of BRZ, indicating a requirement of de novo GA-biosynthesis in the BRZ-triggered resistance. Fourth, BL treatment positively regulated the abundance of the DELLA protein and central GA-repressor SLR1, a phenomenon accompanied by BL-suppression of the GA-biosynthetic gene *GA20ox3* and induction of *GA2ox3* involved in GA deactivation. In conjunction with the central importance of SLR1 in *P. graminicola* resistance (Figure S5.8), these findings propose that BR triggers susceptibility to *P. graminicola* at least in part by counteracting pathogen-induced GA synthesis, leading to indirect stabilization of the DELLA protein SLR1. Supporting this hypothesis, various other phytohormones, including ABA, CK, AUX and ET have previously been shown to affect the GA-induced destabilization of DELLA proteins. Thus, AUX promotes GA-induced proteolysis of DELLAs, whereas stress-induced ABA, CK and ET enhance DELLA-stabilization and delay its degradation by GA (Achard et al., 2007; Brenner et al., 2005; Fu and Harberd, 2003; Vriezen et al., 2004). Moreover, recent data have put forth DELLA proteins as pivotal regulators of plant-microbe interactions. In Arabidopsis, DELLAs positively regulate resistance to necrotrophs but impede immunity to biotrophs (Navarro et al., 2008). Accordingly, we found pathogen-induced SLR1 expression to occur predominantly within the first 2 dpi (Figure S5.8), a time frame corresponding to the assumed biotrophic phase of the *P. graminicola* infection cycle.

Intriguingly, the positive effect of BRs on DELLA protein stability could also provide a mechanistic explanation for the observation that BL induces resistance to rice blast and bacterial blight (Nakashita et al., 2003), both of which diseases are known to be favored by high endogenous GA levels (Yang et al., 2008). In a similar vein, BR-modulated DELLA abundance may offer a mechanistic framework for how BRs trigger resistance to abiotic stresses. The study by Achard et al. (2006) with Arabidopsis mutants lacking four of the five DELLA proteins, revealed a positive correlation between DELLA protein levels and tolerance to various abiotic stresses (Achard et al., 2006).

These authors also demonstrated that DELLA proteins elevate the expression of antioxidant enzymes and thereby limit oxidative stress-induced cell death (Achard et al., 2008), two effects which, at least in cucumber (*Cucumis sativus*), have also been attributed to elevated BR-activity (Xia et al., 2009b). In line with our results and given the central role of reactive oxygen species (ROS) as initiating agents in myriad plant stress responses (Miller et al., 2008), BR-conferred abiotic stress tolerance could therefore be explained by an indirect effect based on BR-induction of DELLA-mediated ROS-detoxification.

Besides the negative effect of BR on GA-controlled and SLR1-dependent defenses, our data implicate a crucial role of BR-SA signal interactions in determining rice-*P. graminicola* outcomes. Using a combination of exogenous hormone treatments, qPCR and bioassays with SA-deficient and -insensitive transgenics, we found that BR interferes with SA-dependent defenses downstream of SA biosynthesis, but upstream of the master SA-regulators OsWRKY45 and OsNPR1. Interestingly, the study by Divi et al. (2010) demonstrated that BL application enhances tolerance to salt and thermo stress, two traits that are associated with increased SA signaling and that require functional NPR1 (Divi et al., 2010). Furthermore, in both Arabidopsis and cucumber, BL treatment induces the expression of the classical SA-marker gene *PR1* (Divi et al., 2010; Xia et al., 2009b), while the BR-response regulator BES1 was recently found to physically bind and activate the key defense regulator AtMYB30 (Li et al., 2009). Nevertheless, recent findings suggest that BR may also antagonize plant defense signaling via its transcriptional regulator BZR1. Upon activation by BR, BZR1 binds and represses the promoters of various defense-associated genes, including the flagellin receptor FLS2 and the major R gene *SNC1* (Sun et al., 2010). This apparently ambivalent BR response is also evident in rice. Indeed, whereas our data clearly uncover BR as a negative regulator of SA-mediated immunity against *P. graminicola*, Nakashita et al. (2003) previously demonstrated BL application to enhance resistance against *M. oryzae* and the bacterium *Xanthomonas oryae pv. oryzae*, presumably in an SA-independent manner. Hence, a complex picture is emerging in which steroid hormones, like the other defense hormones SA, JA/ET and ABA, function as global multifaceted regulators of plant innate immunity, with apparently divergent outcomes. Given the widespread role of BRs in various aspects of growth and development, this notion also implies that BRs are positioned at the interface of hormone, developmental and stress signaling, and thus may serve as important regulators of the often-reported trade-off between enhanced disease resistance and plant growth (Bolton, 2009; Purrington, 2000).

A key question arising from our findings is whether the immune-suppressive effect of pathogen-induced BR is unique for the rice-*P. graminicola* pathosystem, or, alternatively, reflects a conserved virulence mechanism based on the manipulation of *in planta* BR signaling and/or biosynthesis. In this respect, it is noteworthy that transgenic Arabidopsis expressing AvrPto, a bacterial effector that targets the BR co-receptor BAK1, not only displays compromised immunity but also exhibits phenotypes similar to these of BR-insensitive mutants (Shan et al., 2008).

Moreover, *AvrPto* expression does not affect the plant response to other growth-promoting hormones, raising the possibility that AvrPto specifically binds BAK1 to modify BR signaling and attenuate host defenses (Shan et al., 2008). Similarly, it was recently reported that enhanced BR signaling resulting from either a gain-of-function mutation in *BAK1* (Jaillais et al., 2011), ectopic expression of *BRI1* (Belkhadir et al., 2012) or application of BL (Albrecht et al., 2012) impedes innate immunity to *P. syringae* conditioned by the conserved microbial signature flg22. These findings are particularly interesting in light of recent reports demonstrating that the obligate biotrophic oomycete *Albugo laibachii* is equipped with a near-complete BR biosynthesis pathway and thus may produce BR itself (Kemen et al., 2011). Also, several fungal pathogens synthesize toxins that closely resemble steroid hormones such as zearalenone (Robert-Seilaniantz et al., 2007). By analogy with coronatine-mediated disease susceptibility (Cui et al., 2005; Melotto et al., 2006), one may suggest that pathogenic microbes employ such BR-mimics as a virulence strategy to break into the plant's signaling infrastructure and suppress host defense responses. Considering the vital role of steroid hormones in coordinating and integrating various cellular, developmental and physiological processes (Sun et al., 2010; Yu et al., 2011), manipulating host BR signaling and hijacking BR hormone crosstalk mechanisms likely represents a powerful virulence strategy controlling the outcome of many plant-pathogen interactions.

5.5. Conclusions

In summary, we have shown that both exogenously administered and endogenous BRs negatively influence root immunity to the rice pathogen *P. graminicola*. Moreover, our results suggest a scenario whereby *P. graminicola* hijacks the rice BR machinery as a decoy to inhibit effective SA- and GA-mediated immune responses (Figure 5.5). In contrast to the common assumption that BRs act as positive players in the plant's defense signaling circuitry, our findings highlight the importance of BRs in modulating immunity during plant growth and uncover pathogen-mediated manipulation of plant steroid homeostasis as a core virulence strategy.

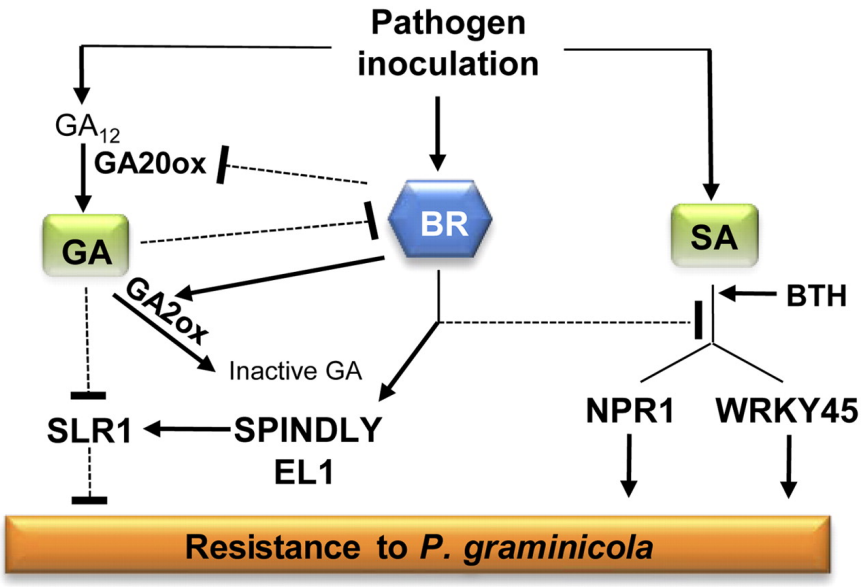

Figure 5.5. Model illustrating how BR, SA, and GA and defense-related crosstalk among this triad of stress hormones molds rice immunity to *P. graminicola*. Perception of *P. graminicola* leads to substantial increases in endogenous BR levels and activation of BR signal transduction. This pathogen-triggered BR action, which likely results from combined induction of de novo BR biosynthesis, attenuation of negative feedback regulation and potentiated derepression of BR signaling, plays a dual role in promoting susceptibility. On the one hand, BR antagonizes effective SA-mediated defenses by interfering downstream of SA accumulation, but upstream of *OsNPR1* and *OsWRKY45*. On the other hand, BR dampens effectual immune responses directed by GA. Operating at both the level of biosynthesis regulation and signal transduction, this BR-GA antagonism leads to indirect stabilization of the DELLA protein SLR1. Sharp, black lines represent positive effects; blunted, dotted lines mark antagonistic interactions.

5.6. Supplementary data

Table S5.1. GA metabolism genes significantly induced or repressed following inoculation of rice roots with *P. graminicola*. Numerical values for the green-to-red gradient represent log2-fold changes relative to the non- inoculated controls. Significant expression changes (threshold of 1.5-fold change and false discovery rate [FDR] P<0.05) are listed in bold. dpi = days post inoculation.

Biological process	Locus	1 dpi	2 dpi	4 dpi	Description
	LOC_Os04g10060	**3.79**	**3.04**	**3.54**	1-deoxy-D-xylulose-5-phosphate reductoisomerase
	LOC_Os04g10010	**2.88**	**2.43**	**2.81**	acetyl-CoA C-acetyltransferase
	LOC_Os12g30824	**5.53**	**3.57**	**2.81**	farnesyl diphosphate synthase
	LOC_Os07g09190	**3.27**	**2.92**	**2.69**	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
	LOC_Os04g09900	**2.78**	**2.27**	**2.37**	diphosphomevalonate decarboxylase
	LOC_Os02g39160	**2.74**	**2.02**	**2.34**	ent-copalyl diphosphate synthase
	LOC_Os07g39270	**3.05**	**1.99**	**2.25**	ent-cassa-12,15-diene synthase
	LOC_Os01g01710	**2.60**	**1.89**	**2.18**	(E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase
	LOC_Os02g36140	**3.06**	**2.68**	**2.14**	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
	LOC_Os03g52170	**2.48**	**1.93**	**1.97**	hydroxymethylglutaryl-CoA synthase
	LOC_Os11g28530	**2.04**	**1.93**	**1.97**	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
Diterpenoid	LOC_Os02g45660	**2.10**	**1.79**	**1.84**	syn-copalyl-diphosphate synthase
biosynthesis	LOC_Os01g58790	**1.92**	**1.33**	**1.47**	momilactone-A synthase
	LOC_Os05g34180	**0.96**	**0.76**	**1.02**	syn-pimara-7,15-diene synthase
	LOC_Os01g02020	**0.61**	0.02	0.38	1-deoxy-D-xylulose-5-phosphate synthase
	LOC_Os03g02710	-0.01	**-0.91**	-0.40	isopentenyl-diphosphate delta-isomerase
	LOC_Os09g34960	-0.50	**-0.61**	-0.49	1-deoxy-D-xylulose-5-phosphate synthase
	LOC_Os02g17780	-0.95	**-2.04**	-0.59	geranylgeranyl diphosphate synthase, type II
	LOC_Os08g40180	-0.26	**-0.77**	**-0.72**	hydroxymethylglutaryl-CoA reductase (NADPH)
	LOC_Os02g01760	-0.91	**-0.71**	**-0.75**	hydroxymethylglutaryl-CoA synthase
	LOC_Os05g33840	0.40	**-1.42**	**-1.30**	stemar-13-ene synthase
	LOC_Os01g50760	**-1.13**	**-1.39**	**-2.04**	ent-sandaracopimaradiene synthase
GA12 biosynthesis	LOC_Os06g37224	**2.03**	**2.30**	**1.96**	ent-kaurene oxidase
	LOC_Os06g37300	**3.90**	**3.13**	**3.24**	D35, ent-kaurene oxidase
GA biosynthesis	LOC_Os05g34854	**-1.03**	0.34	0.69	gibberellin 20-oxidase
	LOC_Os07g01340	**-0.54**	**-1.03**	0.52	gibberellin 20-oxidase
GA inactivation	LOC_Os01g55240	**2.16**	**1.78**	**2.00**	gibberellin 2-beta-dioxygenase (GA2ox3)
	LOC_Os05g43880	**2.02**	**1.79**	**1.25**	gibberellin 2-beta-dioxygenase

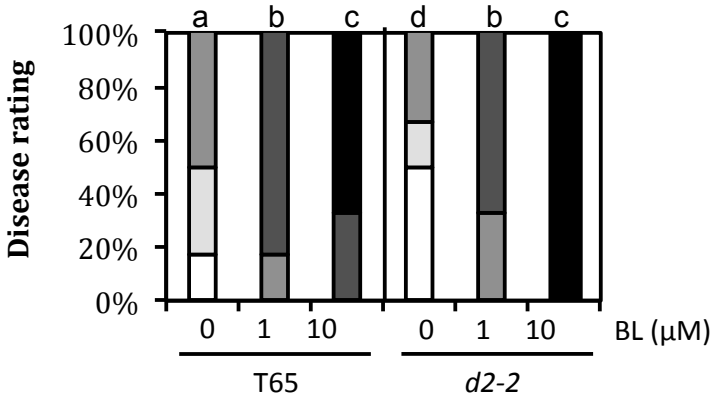

Figure S5.1. Effect of BL pretreatment on resistance to *P. graminicola* in the BR-deficient mutant d2-2. Seeds (cv. Nipponbare) were germinated on Gamborg B5 medium containing different concentrations of BL and, three days post imbibition, inoculated with 0.6-cm mycelial plugs of the virulent strain *P. graminicola* PB912 132. Bars with different letters are significantly different (Mann-Whitney; $n \geq 12$; $\alpha = 0.05$). Chemical treatments, inoculations with *P. graminicola* 132, and disease evaluation were performed exactly as described in Figure 5.1.

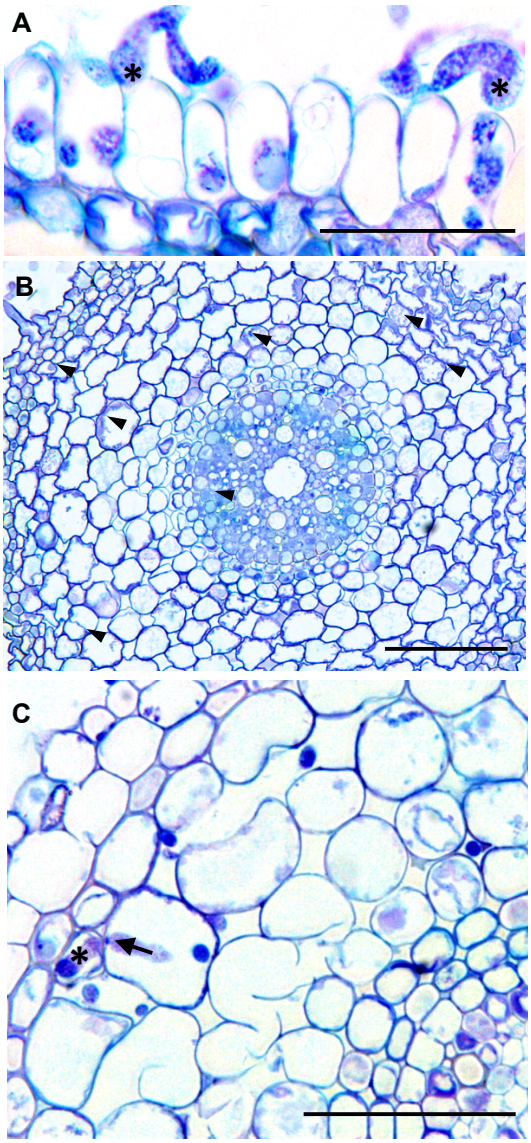

Figure S5.2. Morphology and dynamics of *P. graminicola* colonization of rice roots. A, Penetration of root epidermal cells by appressoria-like structures (*). **B,** Vigorous invasion of epidermis, cortex, endodermis and vascular tissue by intracellular hyphae (arrowheads) in roots of cultivar Nipponbare at two days post inoculation. Scale bars = 100 µm. **C,** Bulbous-like invading hyphae (*) show a dramatic constriction (black arrow) prior to spreading to neighboring cells. Scale bars = 50 µm. Pathogen mycelium was stained with Trypan blue and visualized using bright field microscopy.

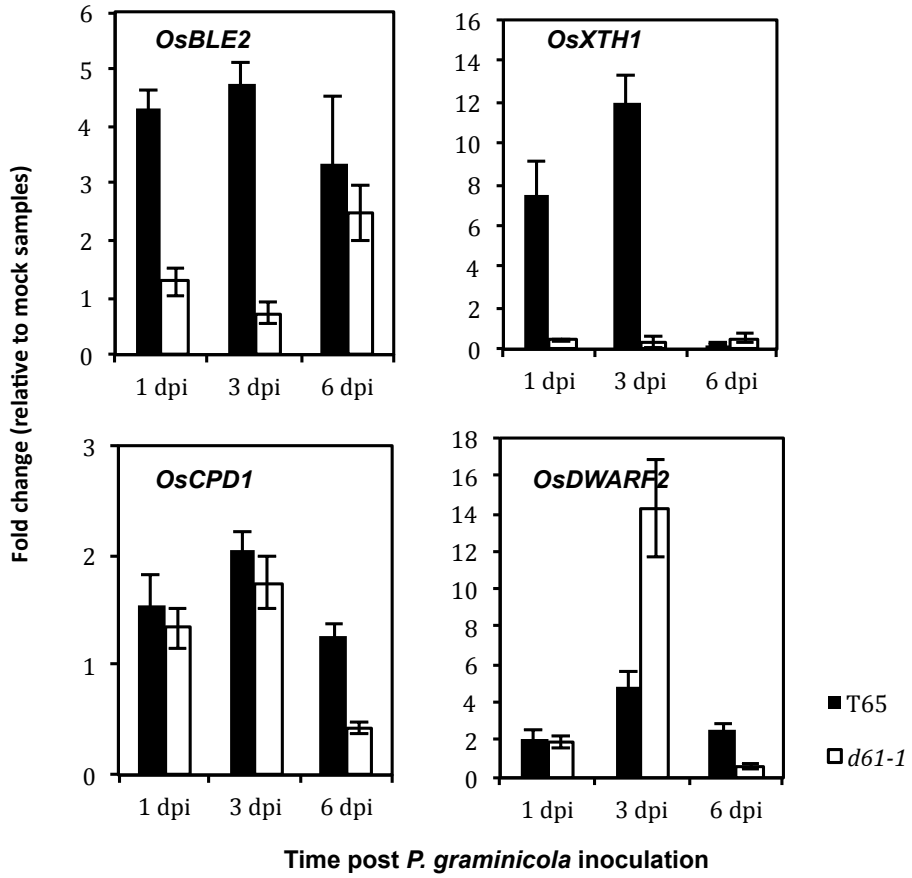

Figure S5.3. **Expression of BR-responsive (*OsBLE2*, *OsXTH1*) and BR-biosynthetic (*OsCPD1*, *OsDWARF2*) genes in wild-type T65 and signal-defective *d61-1* seedlings inoculated with *P. graminicola*.** Transcript levels were normalized using actin as an internal reference and for each time point expressed relative to the normalized expression levels in the respective mock-inoculated control. dpi = days post inoculation.

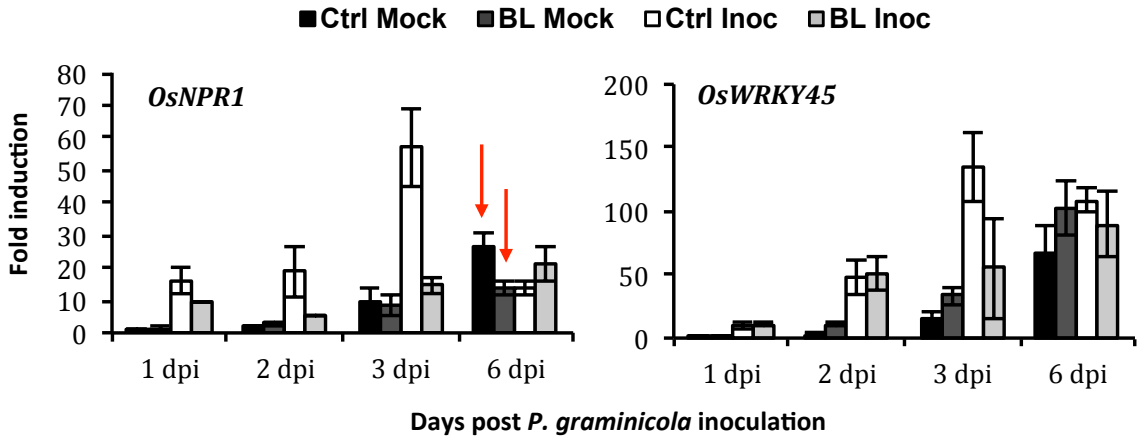

Figure S5.4. Effect of BL pretreatment on transcript accumulation of the SA marker genes *OsNPR1* and *OsWRKY45* in roots of cv. Nipponbare inoculated with *P. graminicola*. RNA was extracted at the indicated times after inoculation, converted to cDNA and subjected to quantitative PCR analyses. Data are the same as those shown in Figure 5.3 (C), but expressed relative to the normalized expression levels in mock-treated control plants at 1 dpi. dpi = days post inoculation.

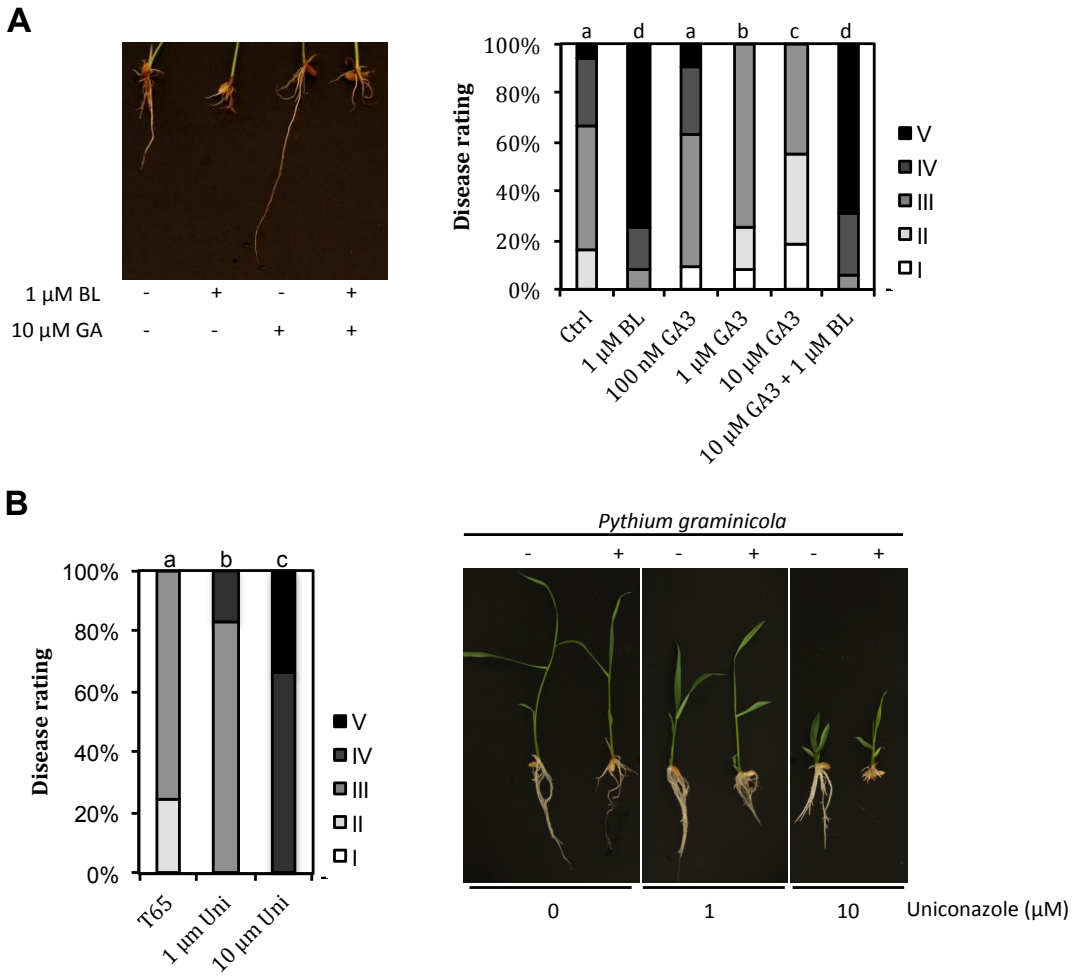

Figure S5.5. **Repression of GA biosynthesis is an integral part of BR-mediated susceptibility to *P. graminicola*. A,** Exogenous BL application suppresses GA_3-induced resistance. Different letters indicate statistically significant differences (Mann-Whitney; n = 12 ; α = 0.05). **B,** Effect of Uniconazole, a GA-biosynthesis inhibitor, on basal resistance to *P. graminicola*. Bars with different letters are significantly different (Mann-Whitney; n ≥ 12 ; α = 0.05). Chemical treatments, inoculations with *P. graminicola* 132, and disease evaluation were performed exactly as described in Figure 5.1. Pictures were taken either 7 (panel B) or 10 (panel A) days post inoculation. Data represent one of three experiments with similar results.

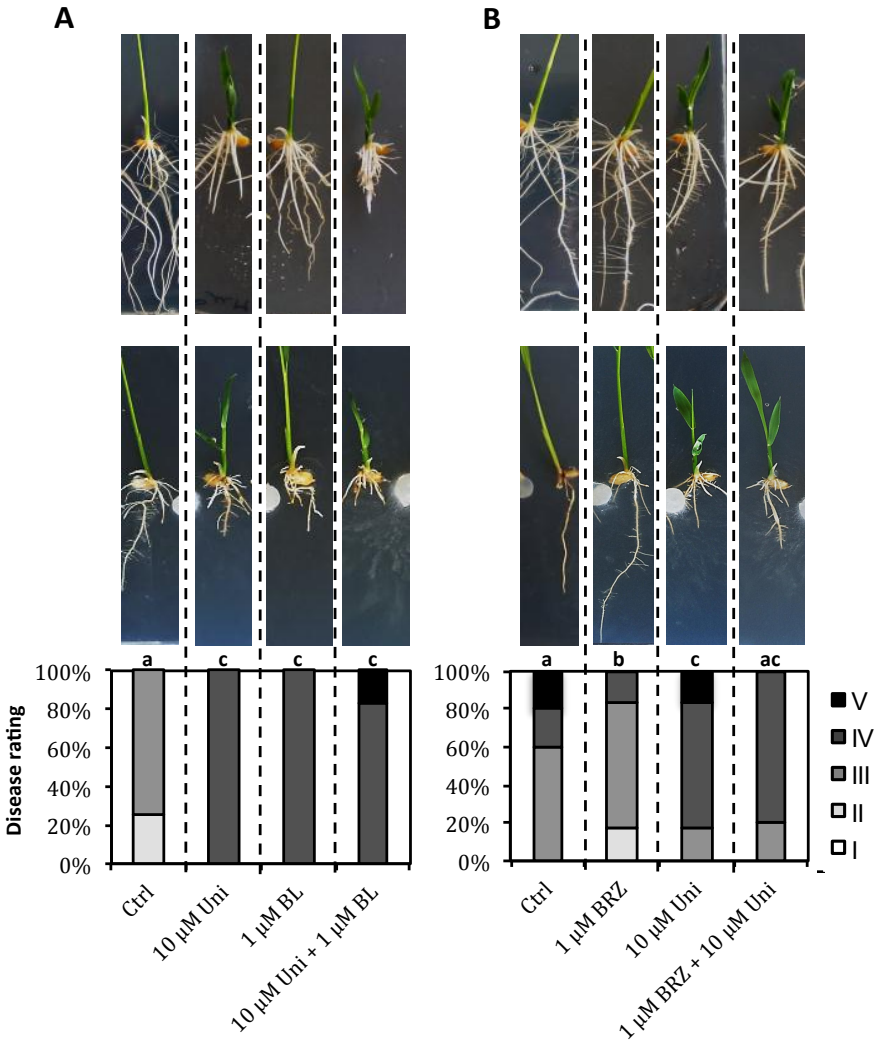

Figure S5.6. Uniconazole treatment mimics BL-induced susceptibility but attenuates BRZ-triggered resistance. Different letters indicate statistically significant differences (Mann-Whitney; $n \geq 21$; $\alpha = 0.05$). Chemical treatments, inoculations with *P. graminicola* 132, and disease evaluation were performed exactly as described in Figure 5.1. Pictures were taken 7 days post inoculation; upper panels show non-inoculated controls, lower panels depict representative disease symptoms. Experiments were repeated at least twice with nearly identical results.

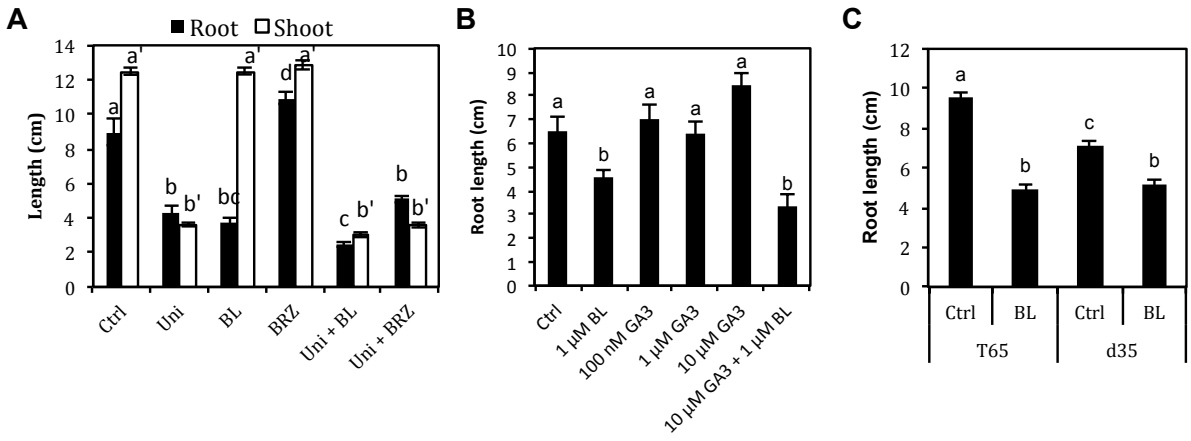

Figure S5.7. Physiological read-outs of GA-BL interactions in rice leaf and root tissue. A, Root and shoot length of 10-day-old seedlings grown on GB5 medium with or without 10 µM uniconazole, 1 µM BL and/or 1 µM BRZ. Each data point represents the mean ± SE of at least 10 seedlings. Different letters indicate statistically significant differences (Bonferroni; α = 0.05). **B,** Measurement of root lengths of 10-day-old seedlings grown in the presence or absence of different concentrations of BL and/or GA3. The results are presented as mean values ± SE from six to 12 plants. Bars with different letters are significantly different (Mann-Whitney; α = 0.05). **C,** Root elongation of 10-day-old wild-type T65 and GA-deficient *d35* seedlings grown in the presence of 1 µM BL. Values are mean ± SE of at least 27 seedlings. Different letters indicate statistically significant differences (Bonferroni; α = 0.05).

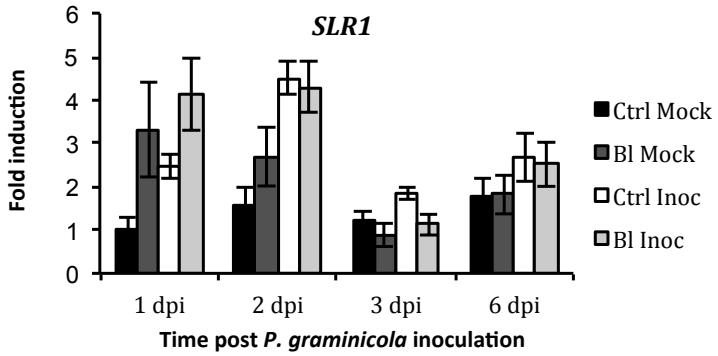

Figure S5.8. **Effect of BL pretreatment on transcript accumulation of the DELLA gene *SLR1* in roots of cv. Nipponbare inoculated with *P. graminicola*.** RNA was extracted at the indicated times after inoculation, converted to cDNA and subjected to quantitative PCR analyses. Transcript levels were normalized using actin as an internal reference and expressed relative to the normalized expression levels in mock-treated control plants at 1 dpi. Data are means ± SD of two technical and two biological replicates from a representative experiment, each replicate representing a pooled sample of six individual roots. dpi = days post inoculation.

Chapter 6

A comparative survey on the role of brassinosteroids, jasmonates and auxins in the interaction of P. arrhenomanes, P. inflatum and P. graminicola with rice

Abstract

Compared to the large amount of information on stress responses in Arabidopsis roots, defense signaling in rice roots and its manipulation by soil-borne pathogens has not been studied intensively. The previous chapter demonstrated that brassinosteroids (BRs) suppress root immunity to the rice-attacking oomycete *P. graminicola*. However, it is not known whether the disease-enhancing effects of BRs depend on the pathogen or are similar among different *Pythium* species. In contrast to their well-documented role in Arabidopsis roots, little knowledge is available on the role of jasmonates (JAs) in rice-root interactions. In addition, research on the regulatory activity of auxins (AUXs) in rice root immunity has been lagging behind. To broaden our knowledge on this matter, we studied and compared the roles of BRs, JAs and AUXs during the interaction of rice seedling roots with three *Pythium* spp. that strongly varied in aggressiveness. Experiments with exogenously applied hormones and hormone biosynthesis inhibitors, and gene expression analyses revealed that BRs might fulfill a conserved negative role in rice root immunity to *Pythium* spp.. Especially the activation of BR biosynthesis and the suppression of the antagonistic and resistance-mediating gibberellic acid (GA) pathway appeared essential for the aggressiveness of the *Pythium* spp.. It was interesting to see that JAs may not be prominently involved in rice responses to these oomycetes, which contrasts with findings in Arabidopsis. We also elucidated the ability of rice-infecting *Pythium* spp. to produce indole-3-acetic acid (IAA), which likely enabled the hijacking of the AUX signaling cascade in their host and consequently, contributed to disease development. Moreover, the most virulent species seemed to degrade IAA, unraveling another mechanism by which *Pythium* spp. may adjust AUX levels in their surroundings and hence, interfere with host responses.

6.1. Introduction

During their entire life cycle, plants are continuously challenged by a changing environment. To survive biotic and abiotic stress conditions, plants depend on their finely regulated immune system (Pieterse et al., 2009). Salicylic acid (SA), jasmonates (JAs) and ethylene (ET) represent the archetypal defense hormones, whose signaling pathways cross-communicate through antagonistic and synergistic interactions, and thereby, allow the plant to finely regulate its defense response to the encountered invader (Chapter 1; Pieterse et al., 2012). In plant leaves, SA-mediated responses are mainly involved in the resistance to (hemi-)biotrophs, while JA/ET-dependent responses rather ward off necrotrophs (Glazebrook, 2005). In contrast, it seems that SA, JAs and ET are involved in plant root defense to soil-borne pathogens, irrespective of the pathogen's lifestyle (Adie et al., 2007; Ali et al., 2013; Attard et al., 2010; Berrocal-Lobo and Molina, 2008; Johansson et al., 2006; Wubben et al., 2008). Jasmonates for instance, have been identified as key players in the defense of dicot roots to pathogenic *Pythium* spp. (Adie et al., 2007; Staswick et al., 2002; Vijayan et al., 1998), other oomycetes (Attard et al., 2010) and fungi (Anderson et al., 2004; Johansson et al., 2006). Intriguingly, Thatcher et al. (2009) proved that the fungus *Fusarium oxysporum* (*Fox*) may hijack CORONATINE INSENSITIVE1 (COI1)-mediated JA signaling in Arabidopsis roots to induce susceptibility (Thatcher et al., 2009). In the previous chapter (5), we showed that SA and GAs confer rice root immunity to *Pythium* spp.. Howevere, the role of JAs in the *Pythium*-rice interaction still remains to be resolved.

Both dicot- and monocot-related studies have also demonstrated the major importance of BRs in modulating biotic stress tolerance (Belkhadir et al., 2012; Nakashita et al., 2003). In contrast to their main resistance-inducing role in aerial tissues, BRs appear to confer disease susceptibility in plant roots. In a dose-dependent manner, they either induce susceptibility or resistance upon root knot nematode (RKN) attack, a process involving negative JA-BR crosstalk (Nahar et al., 2013). Besides, we also illustrated that the aerobic rice pathogen *P. graminicola* co-opts the BR pathway in rice seedlings as a virulence strategy to counteract SA- and GA-dependent immune responses (Chapter 5). So by targeting the BR pathway, soil-borne pathogens seem to disturb steroid homeostasis in their host, which has been defined as a critical step in the establishment of plant immunity (Belkhadir et al., 2012). It is still unclear if other rice-attacking *Pythium* spp., like the highly virulent *P. arrhenomanes* and weakly virulent *P. inflatum*, similarly hijack the BR pathway to promote pathogenesis, and whether higher virulence levels of *Pythium* spp. involve stronger effects on both the BR biosynthesis and signaling.

Auxins, the key regulators of plant growth and development (Teale et al., 2006), also participate in plant responses to biotic challenges (Fu and Wang, 2011). They promote disease susceptibility in various plant-pathogen interactions, by increasing the extensibility of the cell wall, regulating the opening of stomata and suppressing SA-mediated immune responses (Fu and Wang, 2011). Microorganisms may produce indole-3-acetic acid (IAA), the predominant AUX in plants, and other effectors, by which they evoke endogenous AUX imbalances, stimulate AUX signaling or disturb AUX distribution in plants, and accordingly, interfere with plant development, growth and defense (Fu and Wang, 2011). In line with this, *Pythium* spp. have been discovered to deliver IAA in the tomato rhizosphere to promote root hair formation and facilitate their invasion (Mojdehi et al., 1990; Rey et al., 2001). It is unknown whether rice-infecting *Pythium* spp. produce IAA as a virulence strategy. Furthermore, no studies have been analyzing the effect of these oomycetes on the AUX pathway in their host.

In this chapter, we therefore attempted to decipher the role of BRs, JAs and AUXs in the susceptibility of rice roots to three *Pythium* spp. that exhibited varying virulence levels towards rice seedlings. We examined the effect of exogenous hormones and hormone-biosynthesis inhibitors on the disease development upon *Pythium* infections. In addition, defense-related gene transcription was analyzed upon inoculation with the three *Pythium* spp. to elucidate the reason behind their distinct levels of virulence. Moreover, we verified whether these oomycetes may produce IAA and accordingly, might be able to hijack the AUX pathway in rice roots.

6.2. Methods

6.2.1. *Pythium* strains and infection trials

For details on the *Pythium* strains and infection trials see 'Methods' chapter 4. Disease evaluation was executed by determining maximal root and shoot lengths, and the percentage of dead seedlings at 10 days post inoculation (dpi) (n = 18). Data were statistically analyzed with Kruskal-Wallis and Mann-Whitney non-parametric tests in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$).

6.2.2. Chemical treatments

Different chemicals were added to the Gamborg B5 (GB5) medium to evaluate the effect of hormones and hormone-biosynthesis inhibitors on the *Pythium*-induced disease symptoms. Methyl jasmonate (MeJA; Sigma-Aldrich), 24-epibrassinolide (BL; Sigma-Aldrich), indole-3-acetic acid (IAA; Sigma-Aldrich), the JA biosynthesis inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA; Sigma-Aldrich) and the BR biosynthesis inhibitor brassinazole (BRZ; Wako) were used. All products were dissolved in water, except BL, which was dissolved in ethanol (EtOH), ETYA, which was dissolved in a few drops of ethanol (EtOH) before diluting in water, and BRZ, which was dissolved in dimethyl sulfoxide (DMSO).

The chemicals were applied in the following concentrations: 10 µM of ETYA, 25 µM of MeJA, 1 µM of BL, 1 µM of BRZ, and 1 or 10 µM of IAA. Only for BRZ and ETYA treatments, seeds were also pre-germinated on the chemical-containing GB5 medium.

6.2.3. Gene expression analysis in *Pythium*-inoculated rice roots

Samples were collected and processed like previously described and a standard Quantitative Real-Time PCR (qPCR) protocol was executed (see 'Methods' chapter 4). Forward and reverse primers that were used are presented in Table 6.1. The quantity of plant RNA in each sample was normalized using *OsACTIN1* (LOC_Os03g50890) as internal reference. Cycle threshold (Ct) values (n = 3) were expressed relative to the non-inoculated control at each time point.

Table 6.1. Sequences of the used primers for gene expression analysis

Gene	Forward primer (5'-3') [a]	Reverse primer (5'-3') [a]	Locus	GenBank Accession no.
OsBLE2	ACCAGCTGATCATAAGGCGGTCG	GGTGAACATCCTCGTGGCTTCTAG	LOC_Os07g45570	AB072978.1
OsDWARF2	ATTGTCGGCCTCATGTCCCTCC	TCGCCATCTTCTTCTTGGCCTGG	LOC_Os01g10040	-
OsGA20ox3	CTCGAGTTCACGCAGAGGCACTAC	TGTCGAGGCTTTCATAGCCATTCC	LOC_Os07g07420	-
OsEL1	TTCAGTGGGACAGGCGATGTCTG	GACCTAAGTGGGCATGGACGCTAG	LOC_Os03g57940	-
JiOsPR10	CGGACGCTTACAACTAAATCG	AAACAAAACCATTCTCCGACAG	LOC_0s03g18850	AF395880
OsAOS2	TGCGCGACCGCCTCGATTTC	GGCCAGGCGGGACGTTGATG	-	NM_001055971.1
OsIAA9	CAACGACCACCAAGGCGAGAAG	CCAGGCAACCAAAACCGAGCTG	LOC_Os02g0805100	NM_001054972.1
OsEXPB3	CTTTGAGTGGTTGGAGTGGTGG	GCAGCCTTCTTGGAGATGGAA	LOC_Os10g0555900	NM_001071883.1
OsACTIN1	GCGTGGACAAAGTTTTCAACCG	TCTGGTACCCTCATCAGGCATC	LOC_Os03g57940	X15865

[a]The concentration of each primer stock was 3µM except for the *OsACTIN1* forward primer (1 µM).

6.2.4. *In vitro* IAA production by *Pythium* spp.

The *in vitro* IAA production by *P. arrhenomanes*, *P. graminicola* and *P. inflatum* was investigated using a colorimetric quantification method (Glickmann and Dessaux, 1995; Gravel et al., 2007). Ten mycelial plugs (5 mm in diameter) of an actively-growing *Pythium*-colony were added to 100 ml of half-strength Tryptic Soy Broth (TSB; Difco Laboratories), either supplemented with 1 mM L-tryptophane (Trp) or not, and shaken at 28°C and 120 rpm in the dark for seven days. Afterwards, the mycelium was discarded and the filtrate was filter-sterilized (0.22 µm mesh). Aliquots of 1 ml were subsequently mixed with 1 ml Salkowski reagens (12 g $FeCl_3$ in 7.9M H_2S0_4; Glickmann and Dessaux, 1995) and incubated at 21 °C in the dark. After 30 min, optical density (OD)-values were determined at 530 nm and converted into IAA concentrations using an IAA-based standard curve in half-strength TSB. The experiment consisted of five replicates per treatment.

6.3. Results

6.3.1. The role of BRs in *Pythium*-rice interactions

6.3.1.1. Effect of exogenous BL and BRZ on P. arrhenomanes- and P. inflatum-induced disease symptoms

To evaluate the impact of an altered BR homeostasis on seedling susceptibility to *P. arrhenomanes* and *P. inflatum*, 24-epibrassinolide (BL), the most biologically active BR in plants, and brassinazole (BRZ), a highly specific BR biosynthesis inhibitor (Asami et al., 2000), were exogenously applied to rice roots. The results show that 1 µM of BL severely increased the susceptibility of rice seedlings to *P. inflatum*, whereas 1 µM of BRZ increased their resistance to this oomycete. Seven days post inoculation a strong root stunting was observed in *P. inflatum*-inoculated rice seedlings when BL was exogenously applied to the roots (Figure 6.1). BL treatment did not affect root necrosis or shoot stunting upon inoculation with this pathogen. On the contrary, when the medium was supplemented with BRZ, root development was enhanced compared to the DMSO-inoculated control plates. More and longer crown roots were detected in this case. Brassinolide did not stimulate the disease development upon *P. arrhenomanes* inoculation (Figure 6.1). Shoot stunting and the percentage of dead seedlings seemed to be reduced after BL treatment, but wilting symptoms and necrotic spots were still visible on the shoots and the percentage of root stunting did not alter. On the other hand, BRZ treatments promoted shoot growth and shoot health compared to the DMSO control, evidenced by a strongly reduced seedling death (0%) and the absence of necrotic spots on the shoots.

6.3.1.2. Expression of BR-associated genes in Pythium-inoculated rice seedling roots

The potential of *P. arrhenomanes* and *P. inflatum* to stimulate the BR pathway in rice seedling roots, in a way similar to *P. graminicola*, was verified by determining the relative expression levels of the BR-response gene *BRASSINOLIDE ENHANCED2* (*OsBLE2)* and the BR-biosynthesis gene *OsDWARF2* in *Pythium*-inoculated rice roots using qPCR (Figure 6.2). This analysis showed that *P. arrhenomanes* strongly triggered the transcription of *OsDWARF2* in rice seedling roots compared to the non-inoculated control. The averaged gene expression levels elevated 9.7-fold at 1 dpi, after which they increased up to 16.3-fold at 3 dpi and decreased down to 4.3-fold at 4 dpi. Similar to *P. arrhenomanes* infections, *P. graminicola* infection induced the transcription of the BR-biosynthesis gene during the first days of its interaction with rice seedling roots, with a slightly lower induction of 3.1-fold at 1 dpi, 12.6-fold at 3 dpi and 1.7-fold at the latest time point. In *P. inflatum*-inoculated tissues, the expression of *OsDWARF2* fluctuated around basal levels, except on 3 dpi, when it was 5.1 times the level found in control roots. Since the data were not statistically analyzed, we could not draw conclusions about the significance of these differences.

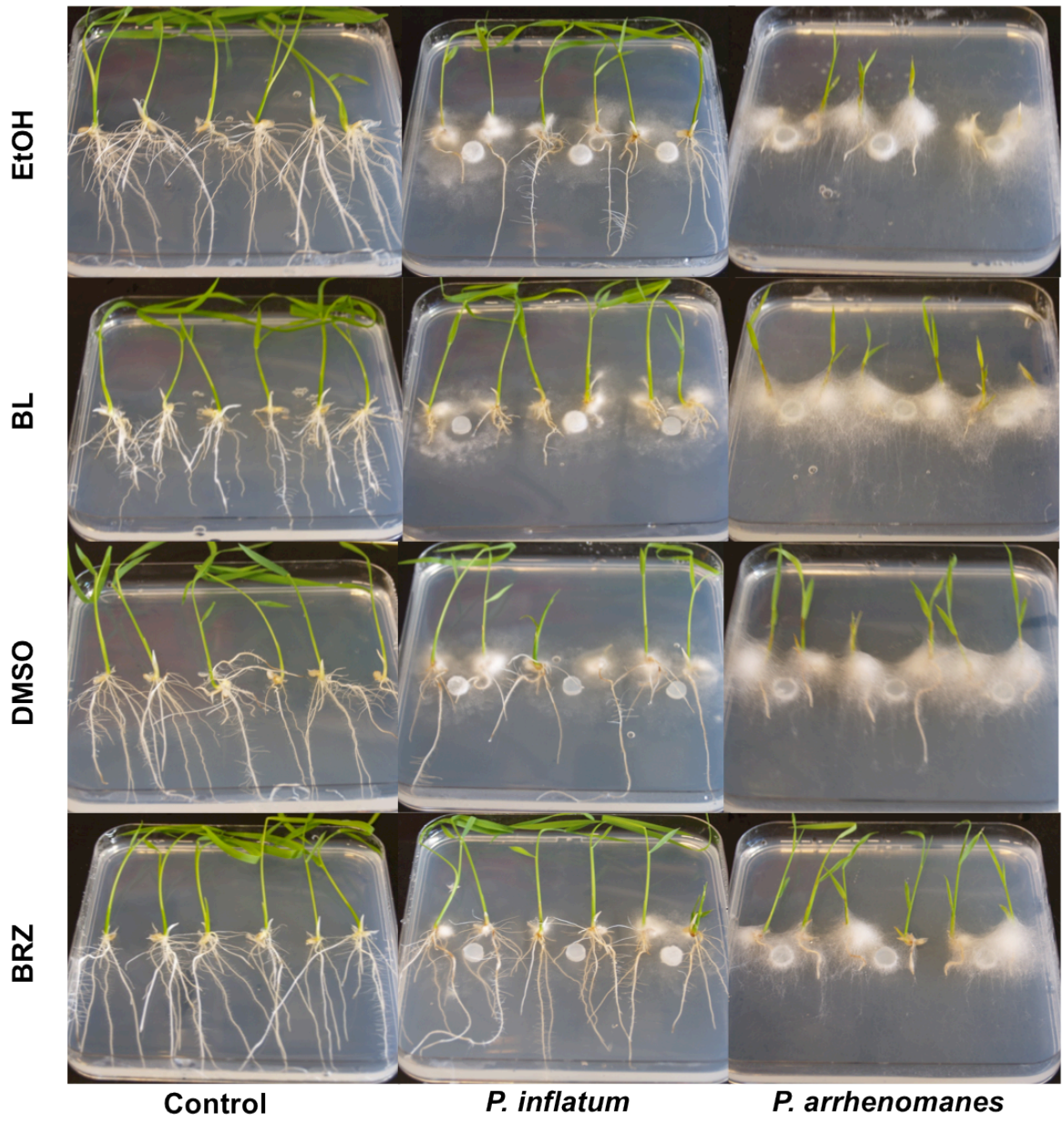

Figure 6.1. The effect of exogenous BL and BRZ on *P. inflatum*- and *P. arrhenomanes*-induced disease symptoms at 7 dpi. EtOH and DMSO are the respective control treatments for BL and BRZ applications. Pictures are representative for the replicate plates.

In contrast to *OsDWARF2*, the expression of *OsBLE2* was first slightly down-regulated in *P. arrhenomanes*-inoculated rice roots (-2.3-fold on average). Subsequently, transcript levels elevated with 4.7-fold at 3 dpi and normalized again by 4 dpi. In *P. graminicola*- and *P. inflatum*-inoculated tissues, *OsBLE2* transcription was barely influenced at 1 dpi, but afterwards, it followed a similar trend as that seen in *P. arrhenomanes*-inoculated tissues. However, the upregulation at 3 dpi seemed much stronger upon *P. inflatum* and *P. graminicola* inoculation, but the statistical significance of these differences was not evaluated.

The expression patterns of *OsBLE2* and *OsDWARF2* were also presented per biological repeat (i.e. Exp A, B and C) to link the transcript levels of both BR-related genes at each time point (Figure 6.2). Hence, we tried to unravel if *P. arrhenomanes* and *P. inflatum* might as well bypass negative feedback regulation in the BR pathway of rice seedling roots, a characteristic that has been proposed for *P. graminicola* (see Chapter 5). The gene expression profiles from the three biological repeats revealed that trends were more similar in *P. graminicola* and *P. inflatum*-inoculated rice roots, where an induced transcription of *OsDWARF2* frequently accompanied the activation of *OsBLE2* and vice versa. In *P. arrhenomanes*-inoculated tissues on the contrary, the expression levels of both genes frequently followed opposite trends.

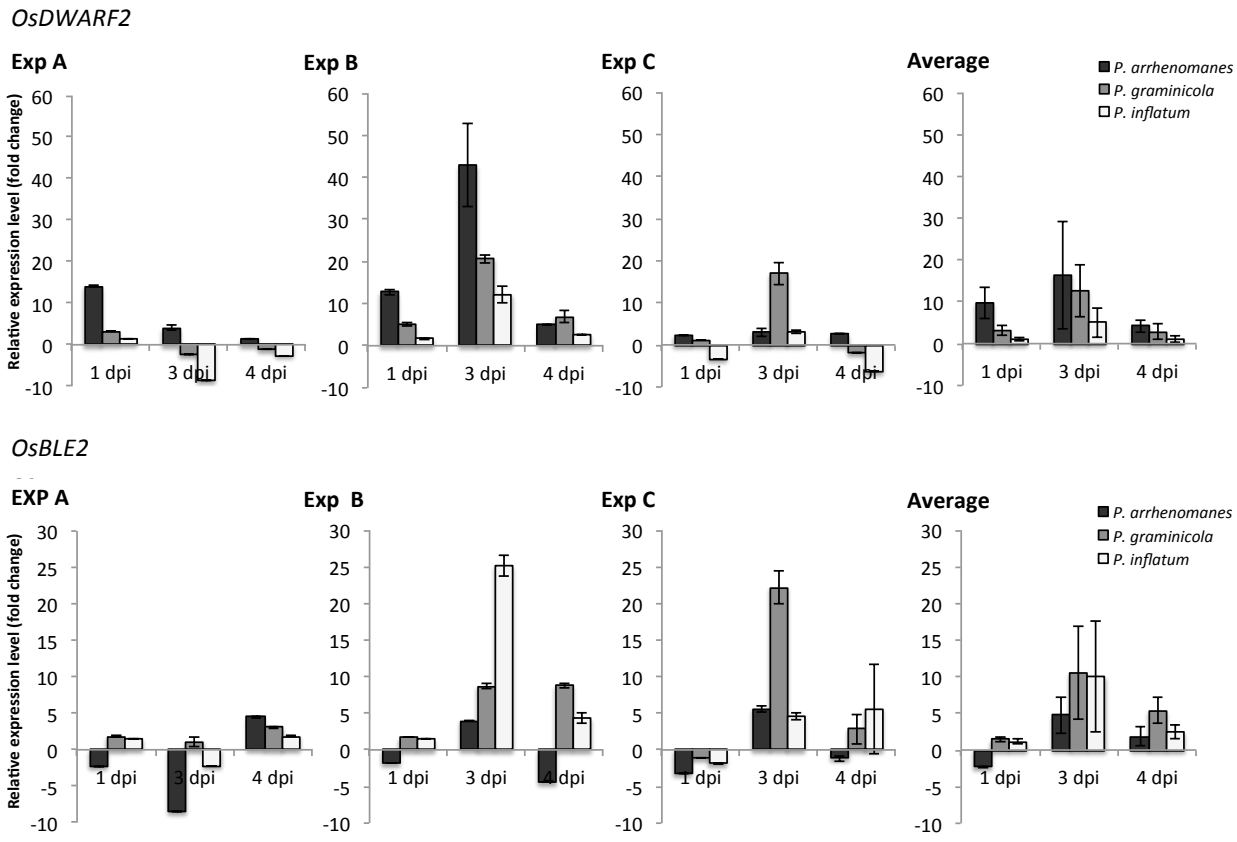

Figure 6.2. Transcript accumulation of BR-response gene *OsBLE2* and BR-biosynthetic gene *OsDWARF2* in *Pythium*-inoculated rice roots on 1, 3 and 4 dpi, with Exp A, B and C representing three biological replicates. Data are means of two technical repeats, except in the utmost right graphs where data represent the averages of the three biological replicates. Data were normalized with the internal reference actin and expressed relative to the non-inoculated control at each time point. Error bars display standard deviations, except in the averaged graphs were error bars display standard errors.

6.3.1.3. Expression of GA-associated genes in Pythium-inoculated rice seedling roots

In an attempt to elucidate the putative involvement of the GA pathway in rice seedling resistance to *P. arrhenomanes* and *P. inflatum*, we analyzed the relative expression of the GA biosynthesis gene *GIBBERELLIN 20-OXIDASE3* (*OsGA20oxidase3*) and the GA signaling repressor *EARLY FLOWERING1* (*OsEL1*) in *Pythium*-inoculated rice seedling roots by qPCR. The expression of *OsGA20ox3* and *OsEL1* highly varied among the biological repetitions (i.e. Exp A, B and C) and therefore, we did not average the obtained data. In the three experiments, *P. arrhenomanes* severely attenuated the mRNA levels of the GA biosynthesis gene (Figure 6.3, blue arrows), evidenced by Ct-values that consistently exceeded 40 cycles from 3 dpi on.

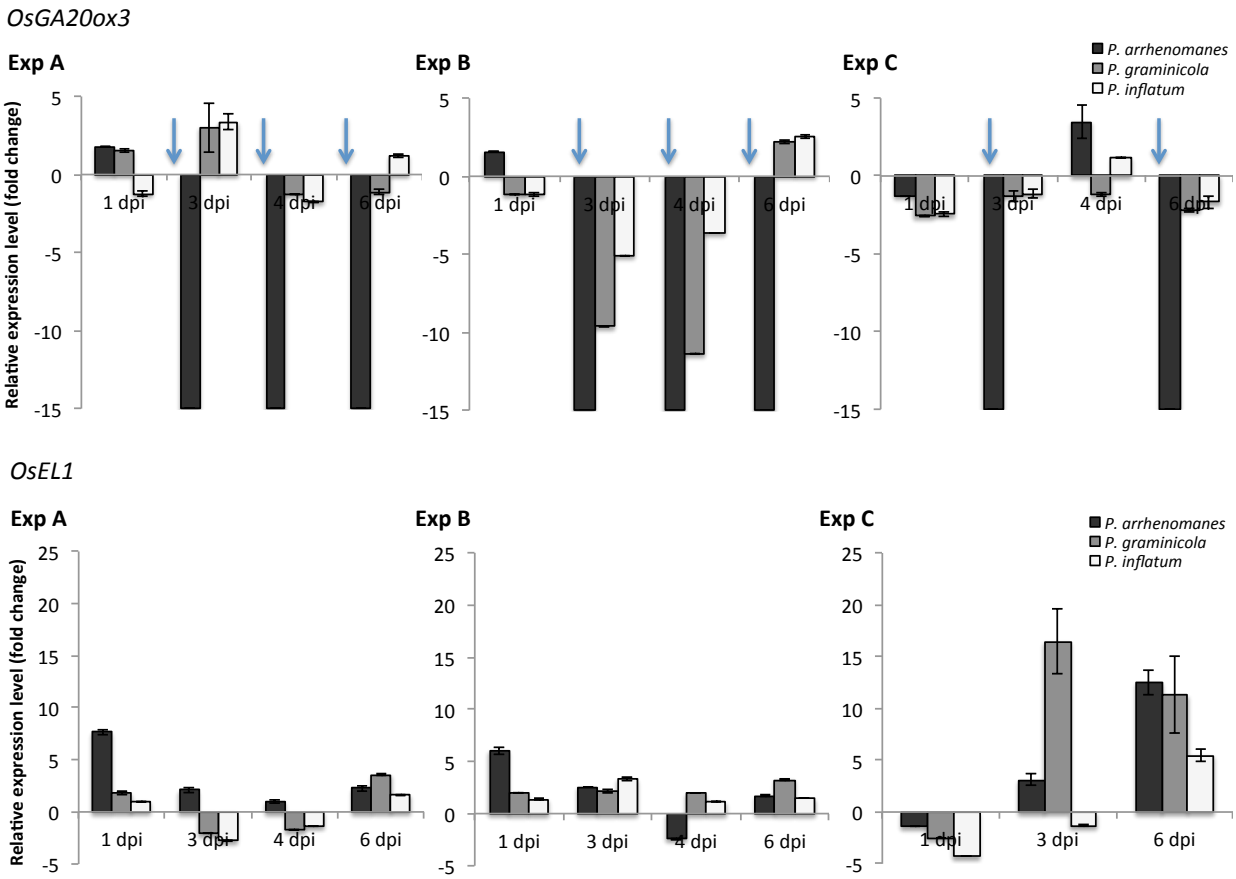

Figure 6.3. Transcript levels of GA biosynthesis gene *OsGA20oxidase3* and GA signaling repressor *OsEL1* in *Pythium*-inoculated rice roots on 1, 3, 4 and 6 dpi, with Exp A, B and C representing three biological replicates. Data are means of two technical repetitions and were normalized with the internal reference actin and expressed relative to the non-inoculated control at each time point. Blue arrows depict the treatments with *P. arrhenomanes* for which the expression was extremely down regulated (Ct > 40 cycles). Error bars display standard deviations.

In *P. graminicola* and *P. inflatum*-inoculated tissues, transcript levels were either (strongly) down-regulated or slightly induced compared to the non-inoculated control. In Exp A, *P. graminicola* and *P. inflatum* barely affected GA biosynthesis. Basal expression levels were noticed on all time points, except on 3 dpi, when the transcription was slightly induced with 3-fold and 3.4-fold in *P. graminicola*- and *P. inflatum*-inoculated roots, respectively. On the contrary, in Exp B, *P. graminicola* strongly repressed the transcription of *OsGA20ox3* with -9.6-fold and -11.4-fold at 3 and 4 dpi, respectively, while this was -5.1-fold and -3.6-fold upon inoculation with *P. inflatum*. In the third experiment, both pathogens slightly down-regulated *OsGA20ox3* mRNA levels (around -2.5-fold) at 1 dpi, after which the expression returned to basal levels and reached -2.2-fold and -1.7-fold by 6 dpi with *P. graminicola* and *P. inflatum*, respectively.

Opposing its serious and long inhibition of GA biosynthesis, the impact of *P. arrhenomanes* on GA signaling in rice seedling roots was less pronounced. The GA signaling repressor *OsEL1* was strongly induced by *P. arrhenomanes* at 1 dpi, with expression levels that were 8 times (Exp A) and 6 times (Exp B) higher than those in the non-inoculated control. Afterwards, transcript levels decreased and approximated 2-fold by 6 dpi in both experiments (Figure 6.3). In *P. graminicola*-inoculated tissues, we observed an opposite trend, with elevated expression levels of maximum 3.6-fold by the end of the time course analysis. In *P. inflatum*-inoculated roots, *OsEL1* expression only altered at 3 dpi. Contrary to Exp A and B, totally different data were obtained in Exp C, where *OsEL1* expression followed a strong increasing trend up to 6 dpi for the three species. Experiment A and B were set up simultaneously, whereas experiment C was a repetition in time. This may clarify the high variability among the observations.

6.3.2. The role of JAs in the *Pythium*-rice interaction

6.3.2.1. Effect of exogenous MeJA and ETYA on disease development in Pythium-inoculated rice seedlings

The role of JA in *Pythium*-rice root interactions needs to be clarified. Therefore, we surveyed the effect of exogenous MeJA and the lipoxygenase (LOX)-inhibitor ETYA on the disease symptoms inflicted by rice root-infecting *Pythium* spp.. When the agar medium was supplemented with MeJA (25 µM), seedling mortality lowered with 86% (Table 6.2; $P \leq 0.05$) in *P. arrhenomanes*-inoculated rice seedlings. Application of ETYA (10 µM) resulted in a reduction of 41%, but this decrease was not statistically significant ($P > 0.05$). In *P. graminicola*-inoculated seedlings, seedling mortality was significantly induced with 13% after MeJA (Table 2; $P \leq 0.05$). A similar increase was also observed for ETYA treatments, but this appeared once more not statistically significant ($P > 0.05$). The overall outcome of MeJA and ETYA treatments on seedling death seemed negligible in the case of *P. inflatum* inoculations (Table 6.2; $P > 0.05$).

Table 6.2. Effect of exogenous MeJA and JA biosynthesis inhibitor ETYA on *Pythium*-induced seedling death at 10 dpi.

	Non-treated control [a]	25 µM MeJA [a]	10 µM ETYA [a]
P. arrhenomanes	29 (7.98) * a	4 (4.17) b	17 (6.80) * ab
P. graminicola	0 a	13 (4.17) * b	13 (12.50) ab
P. inflatum	0 a	4 (4.17) a	0 a

[a]Data represent mean percentages of dead seedlings in four replicate plates from two different experiments (n = 4). Values between brackets display standard errors. Statistical analyses were performed by Kruskal-Wallis and Mann-Whitney non-parametric tests in SPSS 21 (SPSS Inc.) ($\alpha = 0.05$, $P \leq \alpha$). Distinct lower case letters in each row mark significant differences between the treatments. Data followed by * are significantly different from the control, where no dead seedlings were detected. Values between brackets display standard errors.

Exogenous MeJA significantly shortened rice seedling roots with 47% in non-inoculated control plates (Figure 6.4, 6.5). When rice seedlings were inoculated with *P. arrhenomanes* or *P. graminicola*, root lengths increased with respectively 107% and 62% in presence of MeJA (Figure 6.4; $P \leq 0.05$). It was interesting to see that shoot stunting also significantly diminished upon MeJA application in *P. arrhenomanes*-inoculated rice cultures ($P \leq 0.05$), suggesting a resistance-mediating role for this hormone.

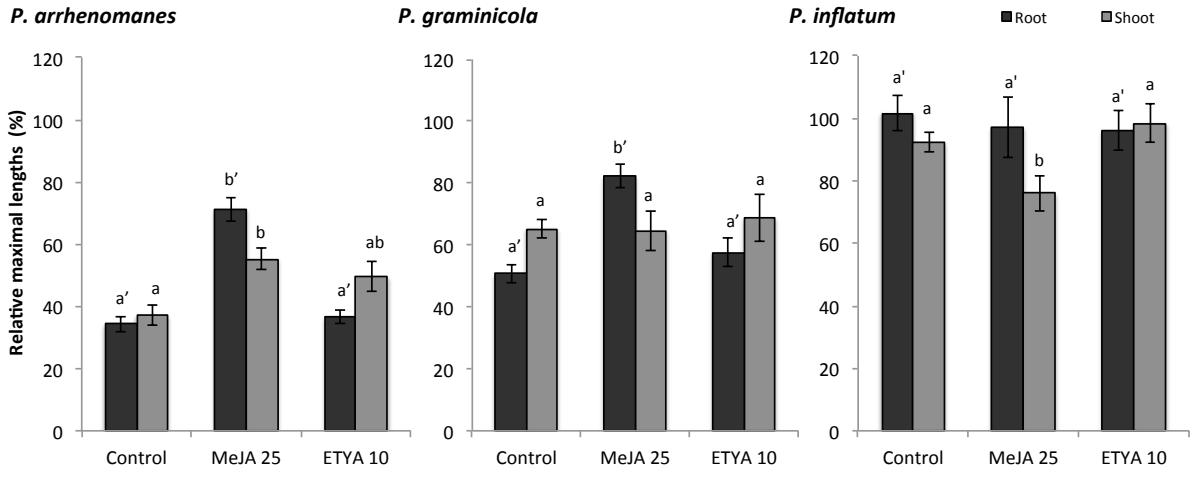

Figure 6.4. Effect of exogenous MeJA and JA biosynthesis inhibitor ETYA on *Pythium*-induced disease symptoms at 10 dpi. Data represent mean maximal shoot and root length (%) expressed relative to the non-treated control, from four biological replicate plates (n = 24). Treatments included: 25 µM of MeJA and 10 µM of ETYA. Error bars display the standard error values. Different lower case letters represent statistically significant differences among the treatments at $\alpha = 0.05$, $P \leq \alpha$. Statistical analyses were performed by Kruskal-Wallis and Mann-Whitney non-parametric tests in SPSS 21 (SPPS Inc.).

On the contrary, in *P. graminicola*-inoculated seedlings, the development of crown and lateral roots appeared frequently inhibited by MeJA treatment and besides, root necrosis was enhanced (Figure 6.5). Exogenous MeJA also exerted a rather negative effect on rice seedling resistance to *P. inflatum*, revealed by the significantly decrease in shoot lengths with 18% (Figure 6.4; $P \leq 0.05$), more root necrosis and shorter crown roots upon treatment (Figure 6.5). Compared to the activity of MeJA, ETYA did not significantly affect the *Pythium*-induced disease symptoms in rice seedlings (Figure 6.4; $P > 0.05$).

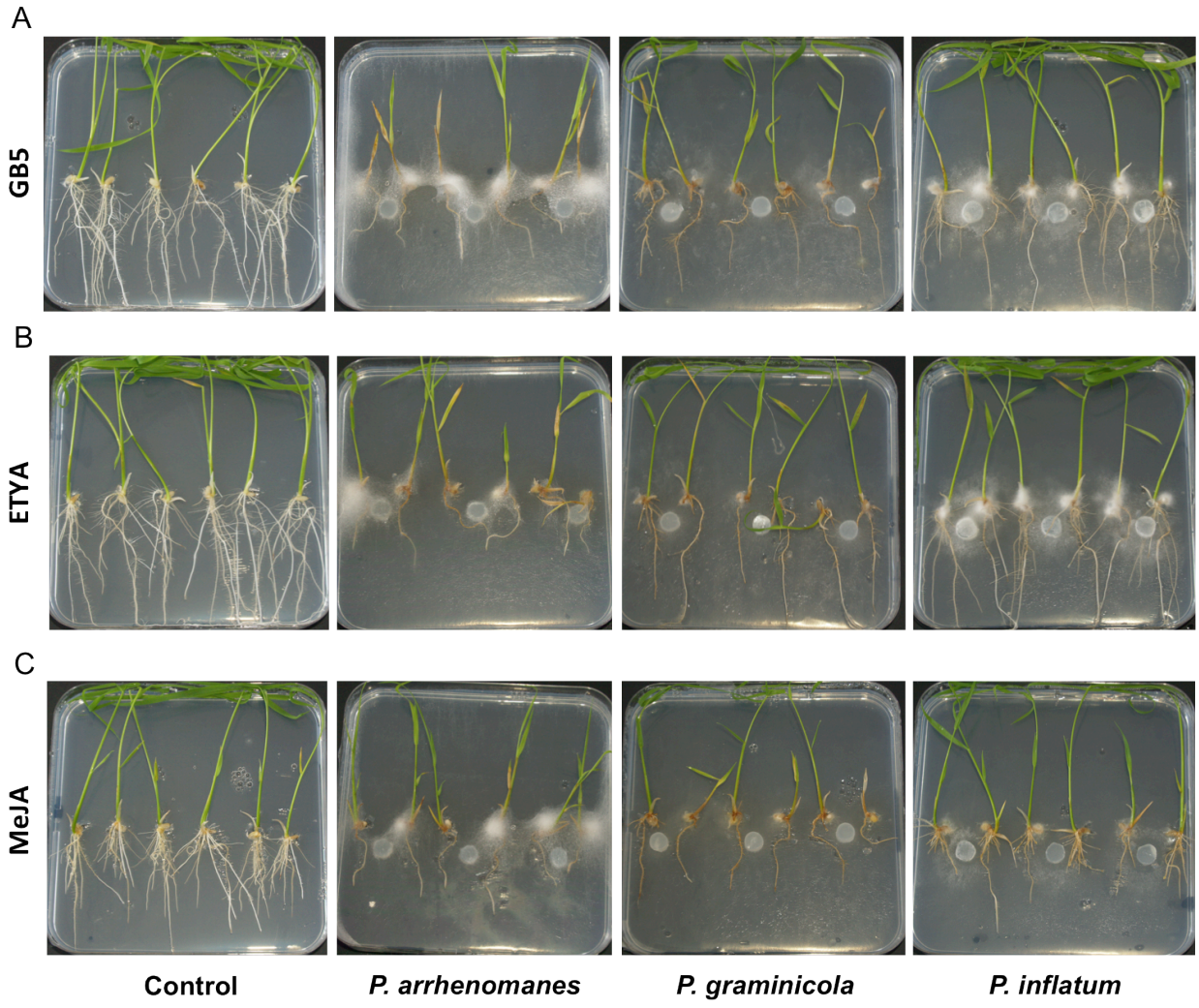

Figure 6.5. Effect of exogenous MeJA and JA biosynthesis inhibitor ETYA on *Pythium*-induced disease symptoms at 10 dpi. A, non-treated rice cultures. Roots of the control treatment were long and grew horizontal on the bottom of the Petri dish. **B** and **C**, cultures treated with respectively 10 µM of ETYA and 25 µM of MeJA. Pictures are representative for the three replicate plates.

6.3.2.2. Analysis of JA-associated gene expression in Pythium-inoculated rice seedling roots

In order to obtain a more detailed view on the role of the JA pathway in the *Pythium*-rice interaction, we investigated the relative expression of two JA-associated genes, i.e. *OsAOS2*, which encodes the biosynthetic enzyme allene oxide synthase (AOS), and *JIOsPR10*, a JA-responsive and stress related gene, at 1, 3 and 6 days post inoculation using qPCR (Figure 6.6). The three *Pythium* spp. drastically triggered the expression of the JA biosynthesis gene *OsAOS2* in rice seedling roots upon inoculation, with the greatest effect exerted by *P. arrhenomanes*. In *P. arrhenomanes*-inoculated roots, the transcription of *OsAOS2* peaked at 1 dpi at approximately 13.1 times (Log base 2) the level in non-inoculated control roots. Afterwards, the expression decreased, but at 6 dpi, it was still 5.6 times higher than in control roots. In *P. graminicola*-inoculated tissues, the gene was also significantly induced with 9.2-fold at 1 dpi, after which the expression similarly decreased by 6 dpi. The induction of *OsAOS2* was less pronounced in *P. inflatum*-inoculated tissues, where mRNA levels elevated with 5-fold at 1 dpi and remained relatively constant throughout the infection process.

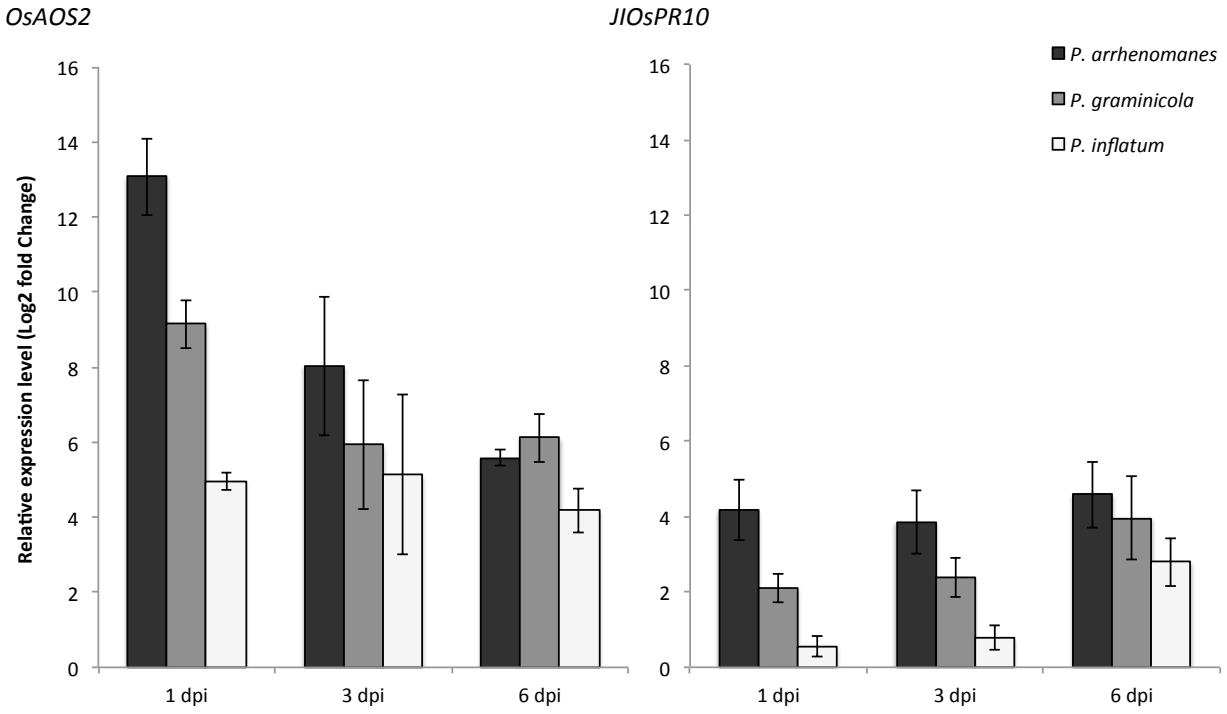

Figure 6.6. Transcript accumulation of JA response gene *JIOsPR10* and JA biosynthesis gene *OsAOS2* in *Pythium*-inoculated rice roots on 1, 3 and 6 days post inoculation. Data are means of three biological repetitions. Data were normalized with the internal reference actin and expressed relative to the non-inoculated control at each time point. Error bars display the standard error values.

Comparable to *OsAOS2*, the transcription of *JIOsPR10* was the strongest activated by *P. arrhenomanes*, with relative transcript levels of 4.2-fold (Log base 2) at 1 dpi, which remained more or less constant during the following days (Figure 6.6). In *P. graminicola*-inoculated root tissues, the expression of the PATHOGENESIS-RELATED (PR)-protein followed an increasing trend, starting from 2.1-fold at 1 dpi and reaching 4-fold by 6 dpi. Inoculations with *P. inflatum* barely triggered the expression of *JIOsPR10* during the first days of the interaction, but from 6 dpi on, transcript levels were strongly elevated with 2.8-fold compared to the non-inoculated control.

6.3.3. The role of AUXs in the *Pythium*-rice interaction

6.3.3.1. IAA production by rice-attacking Pythium spp.

Since diverse microorganisms produce indole-3-acetic acid (IAA) to interfere with plant development and defense-related pathways (Fu and Wang, 2011), we analyzed the ability of rice-pathogenic *Pythium* spp. to produce IAA as a virulence strategy. After one week of incubation, IAA (and -analogues) was detected in the filtrates of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* cultures using a colorimetric quantification method (Volckaert, 2009). As shown in table 6.3, this IAA synthesis was enhanced by supplementation of the culture medium with L-tryptophane (Trp) (Volckaert, 2009). Unfortunately, mycelial biomasses were not quantified at the time of measurement and hence, it would be inaccurate to compare IAA quantities among the three *Pythium* species or to compare their efficiency by which they convert tryptophane. However, *P. arrhenomanes* and *P. graminicola* exhibit a similar growth speed, while the growth of *P. inflatum* is significantly slower (data not shown). From this we might conclude that the measured IAA quantity for *P. inflatum* is probably underestimated, whereas *P. graminicola* may indeed be a better producer of IAA than *P. arrhenomanes*.

Table 6.3. The *in vitro* production of IAA by *P. arrhenomanes*, *P. graminicola* and *P. inflatum* after seven days of growth and its response to 1 mM L-tryptophane (TrP) (Volckaert, 2009).

	µM IAA (-TrP) [a]	µM IAA (+TrP) [a]
P. arrhenomanes	7.32 (0.85)	11.96 (0.94)
P. graminicola	20.70 (2.71)	40.91 (4.80)
P. inflatum	1.87 (0.77)	5.18 (2.06)

[a]Data represent average IAA concentrations in five replicate culture flasks (n = 5). Values between brackets represent standard errors.

6.3.3.2. Effect of exogenous IAA on disease development in Pythium-inoculated rice seedlings

To analyze the role of AUXs in the disease development upon *Pythium*-rice interactions, rice seedlings were treated with 1 µM or 10 µM of IAA, and the percentages of dead seedlings and seedling stunting were determined. The lowest IAA concentration significantly induced seedling mortality with 13% in *P. graminicola*-inoculated rice seedling (Table 6.4; $P \leq 0.05$), while it had no significant effect on seedling death in *P. inflatum*-inoculated or *P. arrhenomanes*-inoculated seedlings ($P > 0.05$). At a concentration of 10 µM, IAA did not significantly affect *Pythium*-induced seedling death (Table 6.4; $P > 0.05$).

Table 6.4. Effect of exogenous IAA on *Pythium*-induced seedling death at 10 dpi.

	Non-treated control [a]	1 µM IAA [a]	10 µM IAA [a]
P. arrhenomanes	29 (7.98) * a	13 (7.98) a	38 (14.23) * a
P. graminicola	0 a	13 (4.17) * b	4 (4.17) ab
P. inflatum	0 a	0 a	4 (4.17) a

[a]Data represent average percentages of dead seedlings in four replicate plates from two different experiments (n = 4). Values between brackets display standard errors. Statistical analyses were performed by Mann-Whitney non-parametric tests in SPSS 21 (SPPS Inc.) ($\alpha = 0.05$, $P \leq \alpha$). Distinct lower case letters in each row mark significant differences between the treatments. Data followed by * are significantly different from the non-inoculated control, where no dead seedlings were detected. Values between brackets are standard errors.

When determining rice seedling root and shoot lengths upon IAA treatment, it became apparent that treatments with 1 µM of IAA shortened rice root systems in non-inoculated control plates (Figure 6.7, 6.8). In rice cultures inoculated with *P. inflatum*, the hormone seemed to significantly reduce root lengths with 33% and shoot lengths with 14% (Figure 6.7; $P \leq 0.05$) in a concentration of 1 µM, while it did not significantly alter seedling stunting upon *P. arrhenomanes* or *P. graminicola* infections ($P > 0.05$). Nevertheless, we could notice more necrosis on the roots in *P. graminicola*-inoculated seedlings when the medium was supplemented with 1 µM of IAA (Figure 6.8). Ten µM of IAA induced a more severe root stunting in non-inoculated control plates (Figure 6.8). Nonetheless, this high concentration did not affect root or shoot stunting upon *P. inflatum* inoculation (Figure 6.7; $P > 0.05$). In the case of *P. arrhenomanes* and *P. graminicola*, 10 µM of IAA did not stimulate shoot stunting and even increased root lengths with 114% in *P. arrhenomanes*-inoculated seedlings and 44% in *P. graminicola*-inoculated seedlings ($P \leq 0.05$).

It should, however, be noted that this high IAA concentration resulted in more *P. inflatum*- and *P. graminicola*-induced root necrosis, revealing that both 1 and 10 µM of IAA stimulate the susceptibility of rice seedlings to these oomycetes.

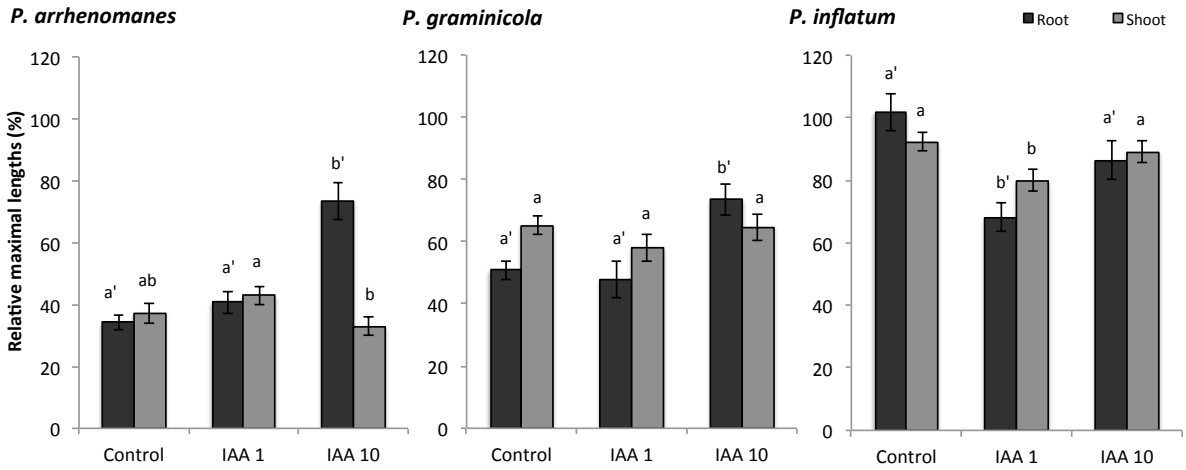

Figure 6.7. Effect of exogenous IAA on *Pythium*-induced disease symptoms at 10 dpi. Data represent mean shoot and root length percentages expressed relative to the non-treated control, from four biological replicate plates (n = 24). Treatments included: 1 µM and 10 µM of IAA. Error bars display the standard error values. Different lower case letters represent statistically significant differences among the treatments at $\alpha = 0.05$, $P \leq \alpha$. Statistical analyses were performed by Kruskal-Wallis and Mann-Whitney non-parametric tests in SPSS 21 (SPPS Inc.).

6.3.3.3. Analysis of AUX-responsive gene expression in Pythium-inoculated rice seedling roots

To verify if *Pythium* spp. may interfere with the AUX pathway in their host, we examined the relative expression of two AUX-responsive genes, i.e. *OsIAA9* and *OsEXPB3*, in rice seedling roots at 1, 3, 4 and 6 dpi with *Pythium* using qPCR. Transcription of *OsIAA9*, a member of the *Aux/IAA* gene family that is highly responsive to AUX (Jain et al., 2006), was strongly elevated after inoculation with *P. arrhenomanes* (Figure 6.9). Gene expression levels ranged from 45.2-fold and 31.3-fold at 1 and 3 dpi, respectively, which lowered down to 2-fold by 6 dpi. The induction of *OsIAA9* was less strong at the beginning of the *P. graminicola*-rice interaction (4.3-fold) and remained constant throughout the entire time course analysis. Intriguingly, despite the *in vitro* production of IAA by *P. inflatum*, expression levels of *OsIAA9* in rice seedling roots barely altered following inoculation with this weakly virulent pathogen.

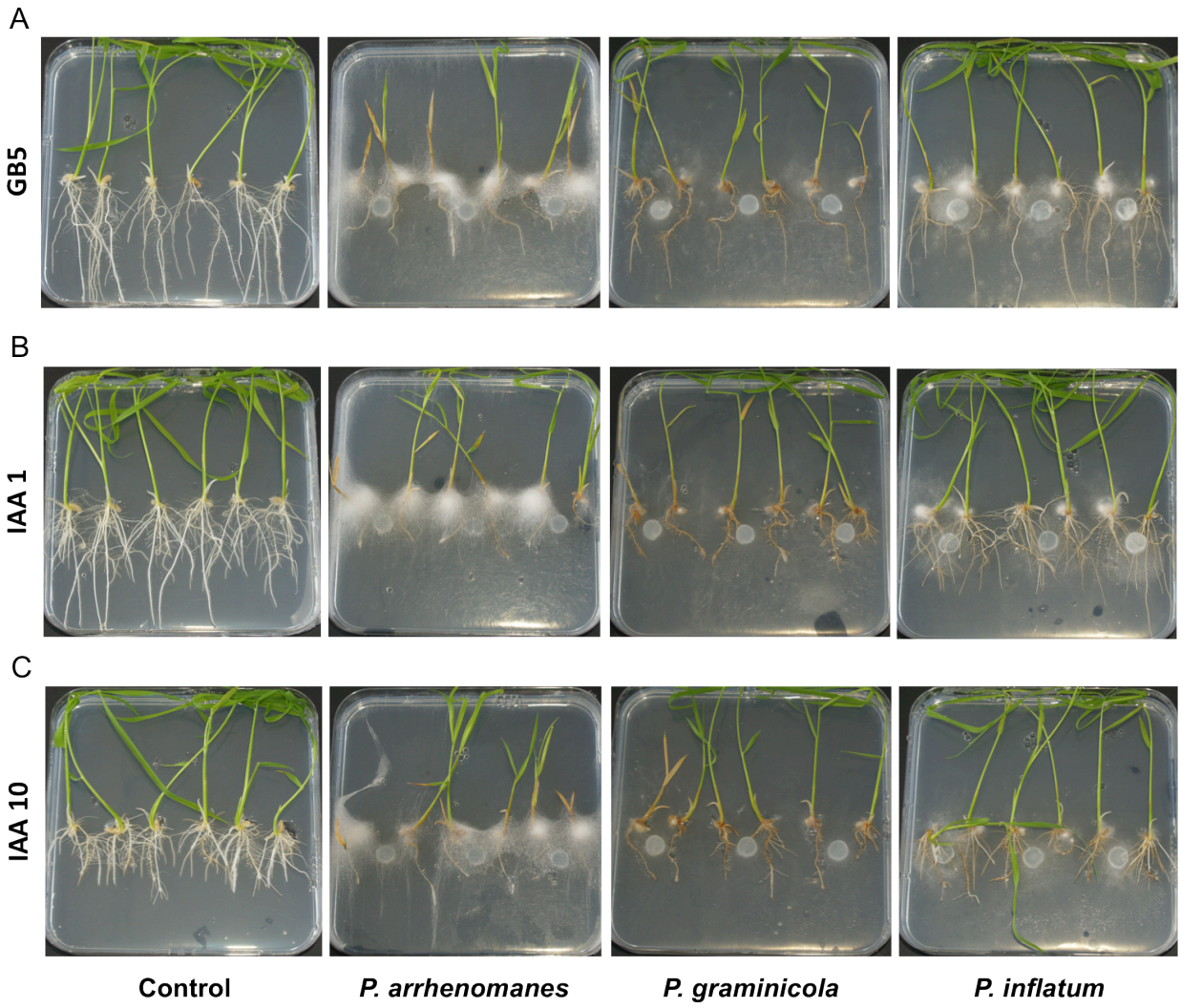

Figure 6.8. Effect of exogenous IAA on *Pythium*-induced disease symptoms at 10 dpi. A, non-treated rice cultures**, B, and C,** medium supplemented with respectively 1 and 10 µM of IAA**.** Pictures are representative for the three replicate plates.

The AUX-responsive gene *OsEXPB3* belongs to the family of cell wall-loosening genes, namely expansins, which function in AUX-regulated growth and can enhance plant vulnerability to pathogenic invaders (Ding et al, 2008). One day post *Pythium* inoculation, the expression of *OsEXPB3* was not altered compared to the non-inoculated control (Figure 6.9). From 3 dpi, we noted strongly elevated mRNA levels of *OsEXPB3* in *P. arrhenomanes*-inoculated root tissues, with levels that were 14.3 times higher at 3 dpi and 6.3 times higher at 4 dpi compared to the control. Transcript levels in *P. graminicola*-inoculated tissues were higher and fluctuated from 19-fold at 3 dpi to 10-fold at 4 dpi. On the contrary, *P. inflatum* only weakly triggered the expression of *OsEXPB3* in rice seedling roots, with a maximum fold change of 5.8 at 3 dpi that decreased down to 2.8-fold by the latest time point.

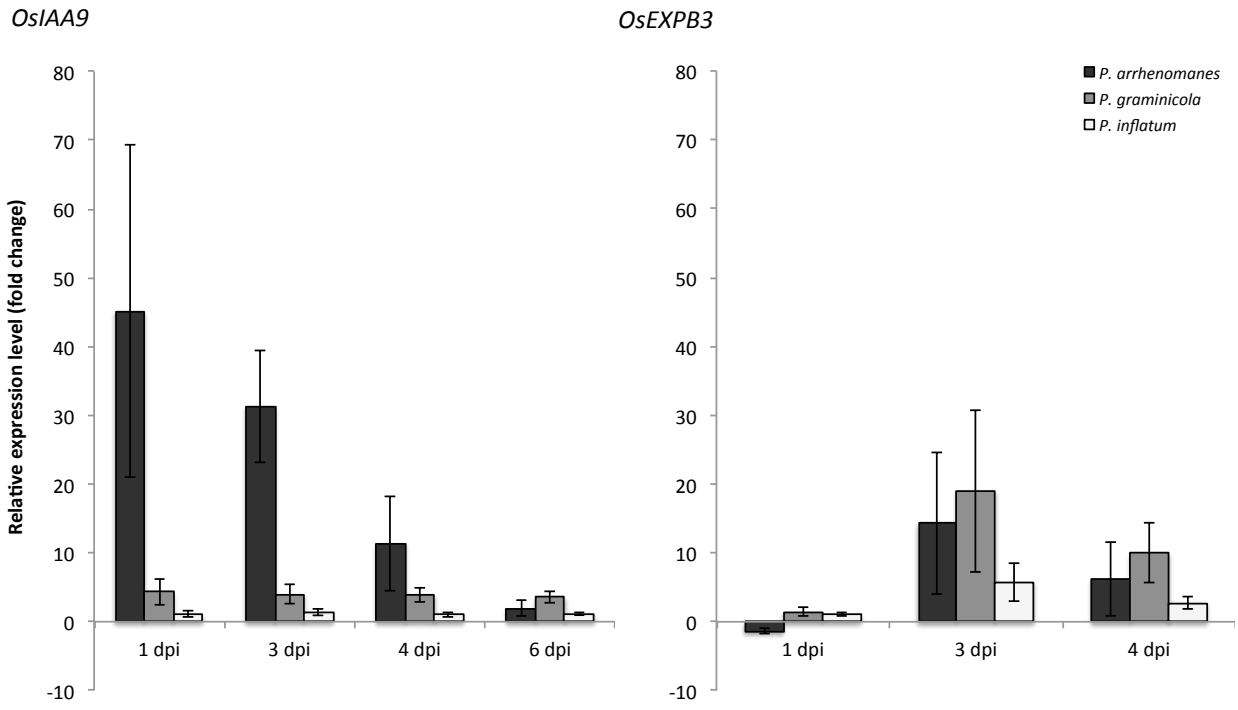

Figure 9. Transcript accumulation of AUX-responsive genes *OsIAA9* and *OsEXPB3* in *Pythium*-inoculated rice roots on 1, 3, 4 (and 6) days post inoculation. Data are means of three biological repetitions. Data were normalized with the internal reference actin and expressed relative to the non-inoculated control at each time point. Error bars display the standard error values.

6.4. Discussion

6.4.1. Brassinosteroids fulfill a conserved disease-mediating role in *Pythium*-rice interactions

In chapter 5 we discussed the role of BRs in the *P. graminicola*-rice interaction and revealed that *P. graminicola* disturbs the steroid homeostasis in rice seedling roots to induce host susceptibility (De Vleesschauwer et al., 2012). To elucidate whether this virulence strategy is conserved among other rice-root infecting *Pythium* spp., we examined the effect of BRs on root immunity to *P. arrhenomanes* and *P. inflatum*, and analyzed the pathogen-induced transcriptional reprogramming of BR-associated genes. In line with the research on *P. graminicola*, the depletion of endogenous BRs in *P. inflatum*- and *P. arrhenomanes*-inoculated rice seedlings induced substantial levels of resistance, evidenced by less root stunting, shoot stunting, seedling mortality and/or wilting symptoms upon infection, while feeding rice seedling roots with BRs strongly enhanced susceptibility, at least in the case of *P. inflatum*. Brassinolide did not enhance *P. arrhenomanes*-induced disease symptoms, which was possibly related to the already high virulence level of this pathogen. In *P. arrhenomanes*-inoculated seedlings we also noticed longer shoots upon BL application, which is likely due to the shoot growth-promoting effects of BL (Ashraf et al., 2010). Contrary to *P. graminicola* infections, BL did not promote root necrosis upon *P. inflatum* or *P. arrhenomanes* infections, marking once more the strong differences among the three pathogens.

Data from the present study also confirmed the reprogramming of BR-biosynthetic and BR-response genes during the infection process of *P. graminicola* in rice seedling roots (see Chapter 5). Furthermore, we showed that BR biosynthesis was mainly triggered by *P. arrhenomanes* and *P. graminicola* in rice seedling roots, while the BR response was especially induced by *P. graminicola* and *P. inflatum*. This confirms the simultaneous induction of both BR biosynthesis and ensuing signaling by *P. graminicola*, which possibly reflects the bypass of negative feedback control and serves a higher susceptibility to this pathogen (see Chapter 5). Interestingly, gene expression patterns of BR-biosynthetic and -responsive genes were more comparable for *P. graminicola*- and *P. inflatum*-inoculated roots. Collectively, these findings suggest that highly virulent and less virulent *Pythium* spp. affect different regulatory modules in the BR pathway to disturb BR homeostasis in rice seedling roots.

Since BRs have been shown to antagonize GA-mediated immunity of rice seedlings to *P. graminicola* (see Chapter 5), we investigated if the molecular machinery underpinning BR-mediated susceptibility in *P. inflatum*- and *P. arrhenomanes*-inoculated rice seedlings also acted at least in part through negative crosstalk with GA-controlled responses. It became evident that the three *Pythium* spp. suppressed the GA biosynthesis and GA signaling in rice seedling roots according to their virulence level. The most aggressive species *P. arrhenomanes* posed a strong and long inhibitory effect on the GA biosynthesis, which might explain why BRZ treatments failed in elongating seedling roots upon *P. arrhenomanes* inoculation. We observed a similar phenomenon in rice seedlings treated with both the GA biosynthesis-inhibitor unicazole and BRZ (see Chapter 5). The rice dwarf virus also represses GA biosynthesis to cause the typical dwarfed growth phenotype (Zhu et al., 2005). In compliance with what has recently been stated by Qin et al. (2013), these observations argue the pivotal roles of GA biosynthesis enzymes in the regulation of rice immunity. By triggering the expression of *EL1*, *P. arrhenomanes* also strongly inhibited GA signaling. Seeing that EL1 regulates the activity and stability of the rice DELLA protein, we assume that, comparable to *P. graminicola*-rice interactions, SLENDER RICE1 (SLR1) might represent a core participant in *P. arrhenomanes*-rice interactions. In general, *P. inflatum* and, to a lesser extent, *P. graminicola* exerted weaker suppressive effects on the GA pathway. Coupled with the fact that GAs positively regulate lignification in monocot roots (Biemelt et al., 2004), this probably underlied the lower efficiency by which these species invaded the rice root vascular stele (see Chapter 4). All together, these findings imply that BRs play a conserved disease-mediating role in *Pythium*-rice interactions, and that the virulence level of *Pythium* spp. depends on their activation of BR biosynthesis and their ability to counterbalance the GA pathway in rice seedling roots.

6.4.2. JAs are not prominently involved in *Pythium*-rice interactions

Dicot-related studies demonstrated that JA-controlled immune responses participate in the protection of plant roots to several pathogens including *Pythium spp.* (Adie et al., 2007; Staswick et al., 2002; Vijayan et al., 1998).

Remarkably, in the case of root pathogenic fungi, JA signaling appeared to mediate both disease susceptibility and resistance (Thatcher et al., 2009). To resolve which immune regulatory tasks JAs fulfill in *Pythium*-rice interactions, we first investigated the effect of exogenous MeJA on *Pythium*-induced disease symptoms in rice seedlings. MeJA slightly induced resistance in rice seedlings to *P. arrhenomanes*, evidenced by less stunted seedlings and seedling mortality, while it slightly promoted susceptibility in *P. graminicola*- and *P. inflatum*-inoculated rice seedlings. In the latter cases, shoots were more stunted, root development was reduced and roots became more necrotic. So, the effect of exogenous MeJA appeared related to the virulence level of the three *Pythium* spp.. Because blocking of JA biosynthesis *in planta* did not exert any significant effect, we speculate that JA signaling, rather than de novo JA biosynthesis, might affect the outcome of *Pythium*-rice interactions. This would be consistent with the research of Thatcher et al. (2009), where JA signaling, but not JA biosynthesis, appeared involved in the Arabidopsis response to *Fusarium oxysporum*.

Gene expression studies proved that the three *Pythium* spp. strongly triggered JA signaling and JA biosynthesis in rice seedling roots, according to their virulence level. Transcript levels of *ALLENE OXIDE SYNTHASE2* (*OsAOS2*) were extremely high in *P. arrhenomanes*-inoculated tissues. The activation of this JA-biosynthetic gene may have reflected endogenous JA accumulation in response to this pathogen (Hause et al., 2007). Comparable dramatic increases in *OsAOS2*-expression have also been noted upon *M. grisea* infections of rice leaves, where JA seemed to be involved in defense (Mei et al., 2006). Nonetheless, we have no indications that this gene would regulate rice defense responses to *Pythium* spp.. Pathological analyses with an *OsAOS2*-overexpression (ox) rice line, which is known to contain higher basal JA levels (Mei et al., 2006), exhibited the same susceptible phenotype upon *P. graminicola* infection as its wild-type (Verbeeck, 2013). Coupled with the results from ETYA treatments, these data propose that host-derived JAs are not critical for the regulation of rice immune responses to *Pythium* spp. and that *Pythium* spp. rather indirectly elevate endogenous JA levels in their host. The high *OsAOS2* mRNA levels in *Pythium*-inoculated rice roots might be correlated with the glucose-inducible features of this gene. Like mycorrhizae, *Pythium* spp. may stimulate a shoot-to-root carbohydrate flux upon infection and thereby, activate JA biosynthesis in the rice root system (Hause et al., 2007). On the other hand, it is tempting to speculate that the effect of *Pythium* infection on other hormonal pathways elevates JA biosynthesis in rice roots.

When the transcription of *JIOsPR10* was evaluated, mRNA levels were also higher at the onset of the most virulent interaction, while the induction was lower in *P. graminicola*-inoculated tissues and significantly delayed upon inoculation with *P. inflatum*. Since the interaction of *P. inflatum* and *P. graminicola* with rice has been determined as less compatible (see Chapter 4), one should expect a stronger and faster induction of *JIOsPR10* if this gene would be involved in disease resistance (Lee et al., 2001). As a result, it is hypothesized that JA-dependent transcription of PR-genes does not confer rice immunity to *Pythium* spp..

The experiments with the *OsAOS2*-ox lines support this concept, considering the significantly activated PR-expression in these transgenics (Mei et al., 2006). In chapter 4, we also presented the results of a gene expression analysis with *OsJAmyb*, which revealed a higher up-regulation of JA signaling in more compatible *Pythium*-rice interactions. Challenging most studies on root-pathogen interactions, we propose that JAs are not prominently involved in rice defense to *Pythium* spp..

Through crosstalk with other hormonal pathways, JA signaling seems to slightly alter the response of rice roots to *Pythium* spp.. Some elegant work by Yang et al. (2012) recently revealed mutual antagonistic interactions between the JA and GA signaling pathways in rice and proved that MeJA treatments exhibit stabilizing effects on SLENDER RICE1 (SLR1) levels by delaying GA-induced degradation. So, beside BRs, JAs may counterbalance the GA pathway in rice roots and contribute to the stabilization of SLR1, which ultimately affects the susceptibility of rice seedlings to *Pythium* spp. (see Chapter 5). In addition, it has been discovered that JAs also cross-communicate with BRs in rice roots (Nahar et al., 2013) and with SA and AUXs in rice leaves (reviewed by Yang et al., 2013). So, the outcome of a stimulated JA pathway may therefore depend on the balance between these interacting pathways and might clarify the opposing effect of MeJA treatments on the *P. arrhenomanes*- versus *P. graminicola*- and *P. inflatum*-induced disease symptoms.

6.4.3. IAA production by *Pythium spp.* as a possible virulence strategy

Seeing that roots are the most sensitive plant organs to IAA fluctuations (Leveau and Lindow, 2005), manipulation of the AUX pool in plants represents a highly interesting virulence tactic for root pathogenic microorganisms. Several *Pythium* species that affect plant roots produce IAA or derivatives, which have been suggested to play important roles in pathogenesis (Gravel et al., 2007; Rey et al., 2001). The results from the present study prove that *P. arrhenomanes*, *P. graminicola* and *P. inflatum* are also able to synthesize IAA, at least *in vitro*. This IAA production was enhanced in the presence of L-tryptophan (Trp), indicating that rice-pathogenic *Pythium* spp. can synthesize IAA in a Trp-dependent manner. A similar feature has been demonstrated for the major and minor tomato pathogens *P. ultimum* and *Pythium* group F, respectively (Rey et al., 2001) and the rice blast pathogen *Magnaporthe oryzae* (Tanaka et al., 2011). The Trp-induced production of IAA by *Pythium* spp. is also plausible under *in vivo* circumstances, considering the presence of Trp in plant root exudates (Lugtenberg and Kamilova, 2009) and rice seeds (Wakasa et al., 2006).

To elucidate whether rice-attacking *Pythium* spp. use AUX biosynthesis as a virulence strategy, we examined if exogenous IAA could enhance *Pythium*-induced disease symptoms. At 1 µM, IAA promoted stunting, root necrosis and/or seedling death in *P. inflatum*- and *P. graminicola*-inoculated seedlings, while it did not affect disease development in *P. arrhenomanes*-inoculated tissues.

A tentimes higher concentration engraved root necrosis upon *P. inflatum* and *P. graminicola* inoculation. Strikingly, it also reduced root stunting in *P. arrhenomanes*- and *P. graminicola*-inoculated cultures. Similar concentration-dependent effects have been previously noted for exogenous IAA in the *P. ultimum*-tomato interaction, where low concentrations promoted disease development and high concentrations generated healthier plantlets (Gravel et al., 2007). However, instead of being indicative for induced resistance, the root-elongating effects of high IAA concentrations might as well be related to IAA consumption by the *Pythium* spp.. IAA-producing *Pseudomonas putida* strains for instance have been reported to utilize IAA as an additional growth substrate or as a manner to tightly control the IAA levels in their environment. Interestingly, when exogenous IAA was applied to radish roots, *P. putida* strain 1290 completely abolished the inhibitory effect of IAA on root elongation (Leveau and Lindow, 2005). So the degradation of IAA might clarify why *Pythium*-inoculated rice roots elongated after treatment with 10 µM IAA.

When exploring the effect of the three *Pythium* spp. on the AUX pathway in rice seedling roots, it became clear that endogenous AUX levels rose during *P. arrhenomanes* and *P. graminicola* infections. The transcription of *OsIAA9*, an *Aux/IAA* gene family member that is highly responsive to AUX (Jain et al., 2006), was strongly triggered upon inoculation with *P. arrhenomanes,* while this was less in the case of *P. graminicola* and negligible in the case of *P. inflatum*. These observations are suggestive for the *in vivo* secretion of IAA by *P. arrhenomanes* and *P. graminicola*. Nevertheless, pathogen-delivered effectors could also have activated the endogenous IAA biosynthesis pathway or may have suppressed the IAA-amido synthethase gene family *Gretchen Hagen3* (*GH3*) *in planta* (Ding et al., 2008). Furthermore, components of the polar AUX transport system could have been targeted to modulate AUX levels in rice roots (Kidd et al., 2011). Since *P. inflatum* barely altered the transcription of *OsIAA9* in rice roots, we might conclude that it did not elevate endogenous IAA levels or that its induction of the AUX signaling pathway was totally repressed by plant defense responses.

The expression of expansins was also significantly induced by *P. arrhenomanes* and *P. graminicola* infections, whereas transcript levels were much lower in *P. inflatum*-inoculated tissues. Expansins may serve several functions in *Pythium*-rice interactions. First of all, they might facilitate the intra- and intercellular spread of *Pythium* spp. from the primary infection site to the systemic tissues by increasing the flexibility of the plant cell wall. For instance, *Magnaporthe oryzae* was described to produce low amounts of free IAA during its biotrophic life stage in rice leaves, by which it locally affected the AUX response and probably induced cell-wall loosening expansins to promote its ingression (Tanaka et al., 2011). A comparable role for these cell wall modifying proteins has been proposed in the *Xanthomonas oryzae*-rice interaction (Ding et al., 2008). Since *P. inflatum* only weakly induced expansin expression upon inoculation, this might elucidate why its systemic spread in rice roots was more limited compared to *P. graminicola* and *P. arrhenomanes* (see Chapter 4). Secondly, expansins may generate extra nutrients for *Pythium* spp. by elevating nutrient leakage from the cell (Ding et al., 2008; Huckelhoven, 2007).

Finally, they could as well be implicated in *Pythium*-directed formation and elongation of root hairs (Suzuki et al., 2003). Root hairs are highly active in root exudation (Datta et al., 2011) and represent attractive invasion sites for these oomycetes (Mojdehi et al., 1990; Rey et al., 2001).

In conclusion, the above-presented results suggest that rice-infecting *Pythium* spp. produce IAA (and derivatives) by which they, depending on their virulence level, may elevate endogenous AUX levels and induce AUX signaling to increase the vulnerability of rice seedlings. Similar to dicots, AUX signaling counteracts SA-mediated plant immunity in rice (Fu and Wang, 2011). Since SA was recently identified as a resistance-inducing player in the *P. graminicola*-rice interaction (see Chapter 5), and crosstalk-related studies revealed interplay between BRs-AUXs (Song et al., 2009), *Pythium* spp. may hijack the AUX signaling cascade in rice roots to stimulate the BR pathway and suppress root immunity.

6.5. Conclusions

The hijacking of the BR machinery in rice roots could represent a conserved virulence strategy among rice-attacking *Pythium* spp.. Our results spark the idea that the aggressiveness level of *Pythium* spp. towards rice is mainly linked with their ability to induce BR biosynthesis and suppress the GA pathway in their host. So, consistent with what has been proposed by Belkhadir et al. (2012), oomycetes seem to induce optimal endogenous BR concentrations by modifying BR biosynthesis and/or BR signaling and accordingly create an ideal environment for growth and reproduction. Furthermore, in contradiction with its pivotal role in plant root immunity, we discovered that JA probably does not function as a core player in *Pythium*-rice interactions. Nevertheless, JA signaling appeared to slightly alter the response of rice roots to *Pythium* spp., with the outcome possibly relying on its interplay with GA, BR, AUX (Figure 6.10) and other signaling cascades. Our findings also suggest that *Pythium*-derived IAA may hijack the AUX signaling cascade in rice roots to induce susceptibility and facilitate ingress. Furthermore, when abundantly present, *Pythium* spp. seemed able to use IAA as a growth substrate. This dual IAA-producing/IAA-degrading function of *Pythium* spp. might provide another strategy by which they can control IAA levels in their host and increase their fitness under nutrient-scarce conditions.

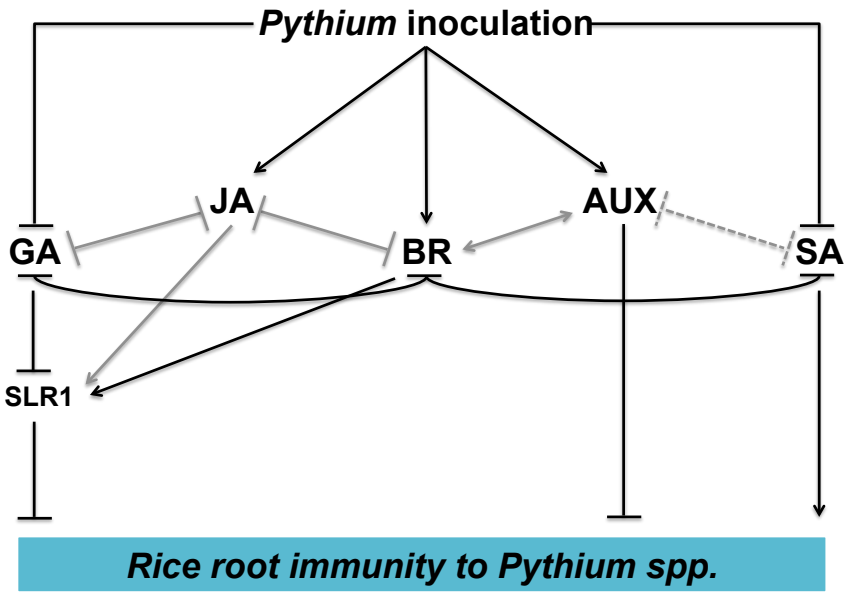

Figure 6.10. Crosstalk among plant hormones in *Pythium*-inoculated rice seedling roots. Arrows depict positive interactions, while blunts depict antagonistic interactions. Black lines are based on the results of chapter 5 and 6, grey lines are based on the results of other studies (Nahar et al., 2013, Yang et al., 2012) and grey, dotted lines are hypothetical.

Chapter 7

Soil-borne pathogens manipulate hormone pathways involved in monocot root development to suppress defense

Review in progress

This chapter was written together with dr.ir.T.Kyndt (Ghent University).

Abstract

Since plant defense-related studies have been focusing on dicots and leaf pathogens, our understanding of root immunity, particularly for monocots, is still rudimentary. Therefore, we overviewed the existing literature on root-pathogen interactions in rice and compared it with the available knowledge on Arabidopsis. Our analysis suggests that the classical defense hormones salicylic acid (SA) and jasmonic acid (JA) are the key players in root defense against soil-borne pathogens. In both rice and Arabidopsis, they mediate a general root resistance against nematodes, fungi or oomycetes, irrespective of their lifestyle. ET-mediated immune responses also occur, depending on the plant group or the encountered microorganism. Other phytohormones, auxins (AUXs), cytokinins (CKs), brassinosteroids (BRs), abscisic acid (ABA) and gibberellins (GAs), which are regulating root development, are linked to this immunity backbone and can effectively fine-tune root defense responses. By exploring how soil-borne microorganisms interact with the immune system in plant roots, we discovered that the typical development-controlling hormones are recruited by pathogens to suppress immunity and facilitate infection. Intriguingly, beneficial microbes seem to adopt a similar strategy in order to establish a long-term and intimate relationship with their host.

7.1. Introduction

Plant roots fulfill several crucial tasks, including water and nutrient uptake, storage, anchoring and responses to environmental stresses. In the complex soil environment, they continuously interact with various microbes, most of them unharmfull or even beneficial, but also pathogenic fungi, oomycetes, bacteria, nematodes and viruses (Okubara and Paulitz, 2005). Fortunately, disease resistance rules in most plant-pathogen encounters. Immunity evolves from biochemical dialogues at the plant-pathogen interface, leading to molecular changes in the plant tissue, among which alterations in hormone concentration and perception. These events trigger hormonal signal transduction pathways that eventually result in effective defense responses (see Chapter 2). Research on *Arabidopsis thaliana* revealed that SA- and JA-dependent signaling pathways build up the backbone of the plant immune system (Pieterse et al., 2012). Crosstalk between the signaling pathways allows plants to prioritize one pathway over the other and to survive simultaneous or sequential challenges with various pathogens (Pieterse et al., 2012). Next to these key hormones, several other phytohormones, including ET, BRs, ABA, GAs, CKs and AUXs, direct specific defense strategies depending on the nature and life style of the attacker (see Chapter 2; Bari and Jones, 2009; Pieterse et al., 2012).

Despite the complexity of this plant defense-signaling network, pathogens have developed strategies to manipulate the plant immune system for their own benefit (Pieterse et al., 2012). By delivering effector molecules, pathogenic microorganisms may perturb hormone homeostasis and induce inappropriate defense responses or alter plant growth. This renders plants more susceptible, and potentiates pathogen infection and the development of specialized feeding structures. Such microbe-mediated changes have been frequently studied in dicots. Unfortunately, monocot-related research lags behind. Distinct endogenous hormone levels, however, spark the idea that defense strategies are tissue and plant group-related. Unlike dicots, monocots have higher endogenous SA levels in their shoots (Kogel and Langen, 2005) and at least in case of rice, lower endogenous SA levels in their roots (Chen et al., 1997; Silverman et al., 1995). Besides, the endogenous JA level in rice plants is much lower than that of Arabidopsis (5 to 10 ng/g of fresh weight (FW) versus 10 to 100ng/g of FW for the basal level (Mei et al., 2006; Schweizer et al., 1998), and 10 to 40 ng/g of FW versus 1,000 to 2,000 ng/g of FW after wounding (Kubigsteltig and Weiler 2003)). Therefore, it is assumed that immune systems are differentially regulated in roots and shoots of dicots and monocots, and hence, data cannot be extrapolated. For more information on the aboveground hormonal defense network involved in rice innate immunity, we refer the reader to a number of excellent recent reviews (Chen & Ronald, 2011; De Vleesschauwer et al., 2013; Sharma et al., 2013).

This literature review aims to summarize the existing knowledge on the role of phytohormones in monocot root defense. Before we dive into the rice (*Oryza sativa* L.) root system, an overview of existing knowledge from Arabidopsis root-pathogen interactions is given. To help the reader in grasping our key messages, we shortly discuss the hormonal regulation of root development in both model plants. Then, this review puts forth the root developmental pathways that soil-borne pathogens target to suppress immunity and facilitate infection. Finally, an overview is provided of the known or hypothetical effectors that root parasites adopt to hijack hormone biosynthesis and signaling machineries. By analyzing how beneficial microbes colonize monocot roots, this paper also reveals interesting similarities between parasitic and mutualistic root-microbe interactions.

7.2. Phytohormones implicated in Arabidopsis root defense

With plenty of molecular/genetic tools available, *Arabidopsis thaliana* is an attractive model for dissection of the cellular and molecular events underlying dicot root responses to pathogens. Hence, scientific knowledge on immune responses in diverse root-pathogen interactions is most elaborate for this plant. Several studies suggest that JA/ET and SA pathways cooperate in the defense of *A. thaliana* roots to oomycetes (*Phytophthora* and *Pythium* spp.) (Table 7.1). For instance, Attard et al. (2010) revealed the contribution of SA, JA and ET signaling to early root defense against the hemi-biotrophic oomycete *Phytophthora nicotianae*. Infection trials with another hemi-biotroph, *Pythium irregulare*, unraveled the crucial role of ABA biosynthesis in preceding JA-dependent defense in Arabidopsis, and the smaller but significant function of SA and ET (Adie et al., 2007; Staswick et al., 1998). Furthermore, ET-insensitive Arabidopsis mutants showed a higher susceptibility than their wild-type (WT) to *Pythium sylvaticum*, *P. aphanidermatum* and "*P. jasmonium"* (Geraats et al., 2002). The latter *Pythium* species also became more virulent on JA-insensitive or JA-biosynthesis mutants (Vijayan et al., 1998).

Immune responses upon dicot infections with root-pathogenic fungi also rely on SA, JA and ET pathways (Table 7.1). Analyses with *nonexpressor pathogen-related genes1* (*npr1)*-mutants illustrated the importance of the SA pathway in root defense against the hemi-biotrophic fungi *Verticillium longisporum* (Johansson et al., 2006) and *Fusarium oxysporum* (*Fox*) (Berrocal-Lobo and Molina, 2008). Moreover, exogenous ET and JA have been noted to trigger resistance against both fungi. Anderson et al. (2004) confirmed the importance of the JA/ET pathways in defense against *Fox* and revealed that (1) exogenous ABA could suppress the JA/ET–activated transcription of defense genes while (2) ABA deficiency results in lower root susceptibility for *Fox* (Anderson et al., 2004). Their results suggested a mutual antagonistic interaction between ABA and the JA/ET pathway in Arabidopsis root defense, with ABA as a negative regulator of root immunity.

However, this is in contradiction with the above-described observations of Adie et al. (2007) where ABA synthesis was shown to be required for JA production and the activation of plant defenses against *P. irregulare*. These apparent contradictions might be explained by a different role played by the regulatory molecules involved in the hormone biosynthesis and subsequent signal transduction pathways. It is for instance not unlikely that although accumulation of JAs and ABA can lead to defense, some of their signaling components could also be manipulated by the pathogen to play a part in susceptibility. Demonstrating this possibility, the paper of Thatcher et al. (2009) proposed that *Fox* hijacks the CORONATINE INSENSITIVE1 (COI1)-mediated JA signaling in Arabidopsis roots to confer susceptibility.

The regulatory network including SA, ET and JAs also inhibits root-gall inducing pathogens, e.g. protozoa and sedentary nematodes, albeit with varying levels of success (Table 7.1). *Plasmodiophora brassicae* is an obligate biotroph that induces clubroot galls in Arabidopsis roots. In contrast to the clear disease-limiting effects of exogenous SA treatments (Agarwal et al., 2011), the role of JA and ET in root defense to *P. brassicae* is rather obscure. JA-insensitivity (*JA conjugate synthase* (*jar1)* and *coi1* mutants) did not influence the outcome of the *P. brassicae-Arabidopsis* interaction according to Agarwal et al. (2011). Intriguingly, Siemens et al. (2002) noticed more clubroot symptoms on the methyl jasmonate (MeJA)-insensitive *jar1-1* Arabidopsis mutant than on its WT. Besides, overproduction of ET or a defective ET signaling did not alter the susceptibility to this pathogen in the study of Alix et al. (2007), whereas in other analyses identical mutations resulted in respectively more and less tolerance to clubroot (Knaust and Ludwig-Müller, 2013). These contrasting results are probably due to different infection pressures. It seems that at low spore concentrations ET signaling slightly impairs *P. brassicae*-induced gall growth in Arabidopsis (Knaust and Ludwig-Müller, 2013), while it fails in doing so when the infection pressure is high. Based on the above observations, the authors concluded that SA, and not ET or JA, acts as a major component in the root defense of dicots against *P. brassicae.*

Sedentary nematodes induce giant cells (root knot nematodes) or syncytia (cyst nematodes) on plant roots. With regard to cyst nematodes, research by Wubben et al. (2008) revealed that SA signaling restricts *Heterodera schachtii* in its parasitism of Arabidopsis roots. Consistent with *Verticillium* and *Fusarium* root infections, *AtNPR1* appeared indispensable in this SA-mediated root protection (Cao et al., 1997; Wubben et al., 2008). The ET pathway on the contrary, has been proposed as a negative regulator of root resistance to *H. schachtii,* since root exudates from ET-overproducing mutants were found to be more attractive to cyst nematodes (Wubben et al. 2001), and these plants are hyper-susceptible (Goverse et al. 2000; Wubben et al. 2001). Contradicting this view, a very recent paper by Ali et al. (2013) confirmed the role of ET-responses in defense against cyst nematodes, and this seemed related to JA activation. Interestingly, Fudali et al. (2013) similarly illustrated the positive regulatory role of ET in the resistance to root knot nematode (RKN) *Meloidogyne hapla,* and proved that high endogenous levels of ET and a constitutive ET response make Arabidopsis roots less attractive for second-stage nematode juveniles.

These contrasting results might be due to a distinct role played by ET at different stages of the infection process. Whereas ethylene accumulation in plant roots seems to repel nematodes, this hormone appears to have a positive role during later phases, once the nematode is making a feeding site in the plant root. In this respect, the positive interaction between ET and AUX has for instance been shown to be important for gall maturation by RKN in tomato (Glazer et al. 1986). Despite the lack of literature discussing the role of JAs in Arabidopsis-nematode interactions, unpublished results (Kyndt and Goverse, unpublished data) also suggest the implication of JA biosynthesis in root immunity against the RKN *Meloidogyne incognita*.

In general, we can conclude that both SA and JA control the defense of dicot roots to soil-borne pathogens, irrespective of their lifestyle. Remarkably, in the case of root pathogenic fungi, JA signaling appeared to mediate both disease susceptibility and resistance. The mechanism behind this duality seemed to be linked with the different branches in the JA signal transduction pathway that result in either senescence and plant growth or induction of JA-dependent defense responses. Hence, the JA pathway is an interesting target for pathogens like *Fox* that induce wilting (Thatcher et al., 2009). Contradicting results concerning ABA-mediated responses, impede us from drawing clear conclusions with regard to its role in dicot root immunity. This urges the need for further in-depth analyses. Nonetheless, we presume that the susceptibility- or resistance-inducing features of ABA might be associated with crosstalk with the JA pathway, and the broad function that JA may fulfill in dicot roots. Consistent with the view of Okubara and Paulitz (2005), we also assume that the regulatory task of ET in the dicot's root immune system depends on pathosystem-related factors. Moreover, where SA and JA/ET pathways generally antagonize each other in plant shoots (Robert-Seilaniantz et al., 2011; Yang et al., 2013), there is no evidence for such dichotomy in the Arabidopsis root defense network.

7.3. Phytohormones implicated in rice root defense

Research regarding biotic stress responses in monocot roots has been lagging behind. Nonetheless, various soil-borne pathogens can seriously arrest monocot root growth and restrict yields. The oomycetes *Pythium arrhenomanes, P. graminicola* and *P. inflatum* have been recently associated with progressive yield decline in Philippine aerobic rice fields (see Chapter 3). Exogenous root treatments with the SA analogue benzothiadiazole (BTH) and gibberellin A3 (GA3) showed to protect rice seedlings from *P. graminicola* infections (see Chapter 5). Additionally, SA-deficient, GA-deficient and GA-signaling rice mutants exhibited highly diseased phenotypes upon infections with this pathogen, implying the pivotal roles of SA and GAs in rice root immunity. In contrast to shoot defense-related studies, BRs rather operated as disease enhancing compounds, which was partly addressed to their antagonistic interplay with SA and GA activity (see Chapter 5).

Aerobic rice fields are also extremely susceptible to nematode infestations. The sedentary RKN *Meloidogyne graminicola* is one of the most aggressive nematodes attacking aerobically grown rice roots. However, rice cyst nematodes *Heterodera oryzae* and *Heterodera sacchari*, lesion nematodes *Pratylenchus zeae* and *Pratylenchus indicus*, and the rice root rot nematode (RRN) *Hirschmanniella oryzae*, commonly occur in other rice production systems (Bridge et al., 2005). It was recently discovered that rice immune responses to the RKN *M. graminicola* (Nahar et al., 2011) and RRN *H. oryzae* (Nahar et al., 2012) rely on the JA pathway. These studies also revealed the resistance-inducing effects of ET, and proved that ET activated and likely modulated JA-dependent defense reactions against *M. graminicola* (Nahar et al., 2011). While the role of SA seemed rather minor in the rice-*M. graminicola* interaction (Nahar et al., 2011), BTH strongly induced root defense against *H. oryzae* (Nahar et al., 2012). Collectively, these data highlight the crucial role of JA in rice defense to nematodes and illustrate that the significance of SA depends on the nematode type (Table 7.1). Similar to *Pythium*-rice interactions, BRs promote rice root susceptibility to RKN. Enhancing endogenous ABA levels also promotes root susceptibility for RKN and RRN (Nahar et al., 2012, 2013; unpublished results). The immune suppressive effect of BR and ABA is suggested to evolve from their negative crosstalk with JA-, ET- and SA-based immune responses (Nahar et al., 2013, 2012; unpublished results).

Little knowledge is available concerning rice immune responses to root-pathogenic fungi. One paper discussed the disease-reducing effect of BTH in *Magnaporthe oryzae*-inoculated rice roots (Jansen et al., 2006). However, this effect appeared to be linked with the direct antibiotic activity that BTH might exert rather than the activation of immune responses (Wu et al., 2008).

In line with dicot-based investigations, we can conclude that MeJA and/or BTH seem potent inducers of rice immune responses to nematodes and oomycetes. This stresses the conserved role of SA and JA in innate immunity against soil-borne pathogens. Next to these hormones, ET and GA appeared to guide biotic stress reactions in rice roots. Their contribution was, however, less frequent or of lower significance. Comparable with Arabidopsis, the pathogen's lifestyle had no major influence on the regulatory activity of each hormone and accordingly, we may assume that in both monocots and dicots, SA, JA and ET pathways cooperate in a general root protection towards biotic stresses (Table 7.1). This opposes the situation in dicot shoots, where antagonistic SA- and ET/JA-dependent responses generally regulate the resistance against (hemi-)biotrophic and necrotrophic pathogens, respectively (Robert-Seilaniantz et al., 2011; Yang et al., 2013). But exceptions to this rule have been noted as well (Gutjahr and Paszkowski, 2009) and in rice, it is well established that SA- and JA-based immune responses operate either synchronously or antagonistically (Yang et al., 2013). This emphasizes once more that the extrapolation of findings from one plant to another might cause misleading generalizations.

ABA showed to counterbalance the SA-, JA- and ET-induced immune reactions in rice roots after pathogen challenge. A similar immune-suppressive function was elucidated for BRs, despite their commonly accepted role in conferring disease resistance against several biotic and abiotic stresses (Divi et al., 2010; Nakashita et al., 2003).

Table 7.1. Plant hormones stimulating or suppressing root defense to soil-borne pathogens in dicots and monocots. The table summarizes the available data on the role of the classical defense hormones SA, JAs and ET, as well as some (fragmentary) information on ABA and BRs, in monocot and dicot root defense.

				Hormones[b]					
	Pathogen	Classification	Lifestyle[a]	SA	JA	ET	ABA	BR	References
Arabidopsis	*Phytophthora nicotianae*	Oomycete	h	+	+	+	nd	nd	Attard et al., 2010
	Pythium irregulare	Oomycete	h	+	++	+	++	nd	Staswick et al., 2002; Adie et al., 2007; Geraats et al., 2002
	"Pythium jasmonium"	Oomycete	n	nd	++	++	nd	nd	Vijayan et al., 1998; Geraats et al., 2002
	Pythium sylvaticum, aphanidermatum	Oomycete	n	nd	nd	++	nd	nd	Geraats et al., 2002
	Plasmodiophora brassicae	Protist	b	++	+/-	+/-	nd	nd	Alix et al., 2007; Agarwal et al., 2011; Siemens et al., 2002; Siemens et al., 2006
	Heterodera schachtii	Nematode	b	++	++	+/-	nd	nd	Goverse et al., 2000; Wubben et al.,2001, 2008; Ali et al., 2013; Kyndt and Goverse, unpublished data
	Meloidogyne incognita/hapla	Nematode	b	++	++	++	nd	nd	Fudali et al., 2013; Kyndt and Goverse, unpublished data
	Verticillium longisporum	Fungus	h	+	++	++	nd	nd	Johansson et al., 2006
	Fusarium oxysporum	Fungus	h	++	+/-	++	+/-	nd	Berrocal-Lobo and Molina, 2004; Anderson et al., 2004; Thatcher et al., 2009
Rice	*Pythium graminicola*	Oomycete	h	++	nd	nd	nd	-	De Vleesschauwer et al., 2012
	Hirschmanniella oryzae	Nematode	m	++	++	++	-	nd	Nahar et al., 2012b
	Meloidogyne graminicola	Nematode	b	+	++	++	-	-	Nahar et al., 2011; Nahar et al., 2012a; Nahar et al., 2012b

* b= biotroph, h= hemibiotroph, n=necrotroph , m=migratory nematode

[a]The abbreviations represent: b= biotroph, h= hemibiotroph, n=necrotroph , m=migratory nematode.

[b]The symbols +, - and +/- refer to resistance inducing, resistance repressing and both resistance inducing and repressing effects, respectively. 'nd' stands for not determined.

Interestingly, research by Albrecht et al. (2012) and Belkhadir et al. (2012) recently proposed a general mechanism in which BR-induced growth directly antagonized pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) signaling in Arabidopsis, revealing a trade-off between plant growth and innate immunity. This antagonism seemed to proceed in a BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED KINASE1 (BAK1)-dependent and -independent manner, and relied on appropriate endogenous BR levels (Albrecht et al., 2012; Belkhadir et al., 2012). BAK1 is a leucine rich repeat-receptor kinase (LRR-RK) that interacts with both the BR-receptor BRI1 and the flg22-receptor FLS2 (Wang, 2012). BRI1 overexpression antagonizes BAK1-mediated PAMP-signaling (Belkhadir et al., 2012). The BR-activation of BRI1 may, however, also exert a stimulating effect on BAK1-mediated FLS2-signaling (Belkhadir et al., 2012). Wang (2012) presumed that the growth-related BR-activation of BRI1 has an antagonistic effect on BAK1-mediated PTI signaling in young tissues, whereas it increases BAK1-mediated PTI signaling in mature leaves. This might resolve the contrasting roles that BRs fulfill in defense of rice and tobacco leaves (Nakashita et al., 2003), versus its immune-suppressive effects in rice seedling roots.

All together, these arguments spark the idea that the outcome of the BR response depends on tissue- and plant age-related factors. It has to be noted that at high concentrations external BR applications induced defense against RKN (Nahar et al., 2013). This was however accompanied by a strong feedback inhibition on the internal BR biosynthesis, which demonstrated that data from exogenous hormone applications should always be interpreted cautiously since feedback effects are commonly present in hormone biosynthesis pathways to control their internal homeostasis.

7.4. Hormonal pathways involved in root development

A coordinated root organogenesis requires an efficient communication among various plant tissues and organs. This signaling task is accomplished by phytohormones, which are delivered by hormone biosynthesis pools located in the roots and/or aerial plant parts. From these pools, hormones are diverted to and concentrated in different root tissues, which triggers specific cells to undergo division or expansion and directs root formation and growth (Smith and De Smet, 2012).

In dicotyledonous plant species, a single primary root and a branching network of lateral roots develop during the embryonic growth stage and remain dominant during the entire life cycle of the plant. Conversely, in monocots, embryonic and mature root systems exhibit clear differences (Fig 7.1). AUXs fulfill fundamental roles in the proper shaping of these embryonic and mature root systems (Hodge et al., 2009). AUX biosynthesis, signaling and transport for instance, are required for the crown root formation in rice, and lateral root formation and root elongation in both rice and Arabidopsis (Hodge et al., 2009; McSteen, 2010). Although AUXs play key regulatory tasks in root development and growth, their activity is modulated by other hormones (Hodge et al., 2009), among which BRs, ET and CKs are the most prominent. While AUXs consistently antagonizes CKs, they either stimulate or counteract ET and BRs depending on the root type or plant group.

Crown root development in rice is coordinated by synergistic AUX-ET (Liu et al., 2011) and antagonistic AUX-CK (Coudert et al., 2010; Kitomi et al., 2011) interactions. Since BRs are crucial factors for this process (Hong et al., 2004), and AUX promotes BR biosynthesis in Arabidopsis (Chung et al., 2011), AUXs and BRs might also cooperate in the crown root organogenesis of monocots.

Antagonistic AUX-CK interactions play as well at the level of lateral branching in monocots (Debi et al., 2005) and dicots (Smith and De Smet, 2012). By interfering with AUX signaling and AUX distribution, CKs pose the strongest inhibitory effect on lateral root organogenesis (Bielach et al., 2012). ET and BRs are also thought to affect this process by manipulating the AUX transport (Smith and De Smet, 2012). While AUXs and ET act antagonistically on lateral root branching in Arabidopsis (Muday et al., 2012), AUXs and BRs rather cooperate (Bao et al., 2004; Yoshimitsu et al., 2011). Unlike dicots, monocots do not depend on endogenous BRs for lateral root formation (Hong et al., 2004).

Root elongation in both plant groups seems coordinated by the interplay between AUXs and ET/BRs. Exogenous AUX promotes the number of lateral roots in Arabidopsis and the number of lateral and crown roots in rice, while it inhibits root elongation in both model plants (Hodge et al., 2009; McSteen, 2010). This negative growth response seems to be due to antagonistic AUX-BRs (Yoshimitsu et al., 2011) and synergistic AUX-ET interactions (Muday et al., 2012) in Arabidopsis. Exogenous ET also restricts root growth in this dicot, a phenomenon that depends on the intracellular AUX level (Rahman et al., 2001) and a functional AUX signaling (Muday et al., 2012). In rice, on the other hand, endogenous ET may antagonize AUX-induced responses and negate the inhibiting effect of exogenous AUX on root elongation (Yin et al., 2011).

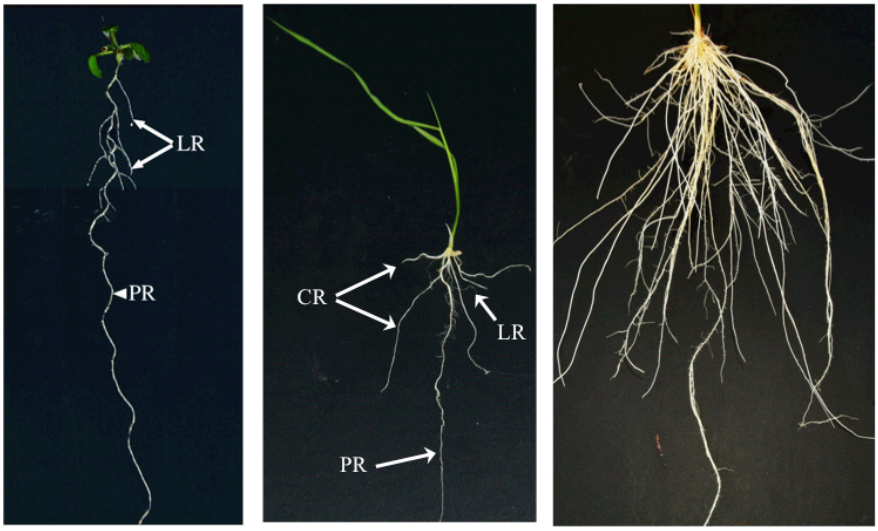

Figure 7.1. Morphology of the *Arabidopsis* root system (left; Pacheco-Villalobos and Hardtke, 2012) compared with embryonic (centre) and mature (right) rice root systems. The early root system of rice seedlings consists out of one primary root (PR) beside five crown (adventitious) roots (CR) that emerge from the node of the coleoptile (Coudert et al., 2010). The compactness of the mature rice root system is due to the abundant crown and lateral roots (LR), which functionally substitute the embryonic root system when the life cycle propagates. We refer to the reviews of Hochholdinger et al. (2004) and Smith and De Smet (2012) for more details on this subject.

Aside from these pivotal players, ABA fulfills an important role in the establishment of the monocot root system. Research by Yao et al. (2002) identified endogenous ABA as a critical factor for the changes in hormone homeostasis during early rice root organogenesis. This was evidenced by the phenotype of ABA-mutants, which exhibited a strongly impaired primary, crown and lateral root growth.

Other studies described the importance of endogenous ABA in the drought response of rice roots (De Smet et al., 2006), and reported on the root growth suppressing effect of exogenous ABA in rice (Konishi et al., 2005) and Arabidopsis (De Smet et al., 2003). ABA treatments also stimulated lateral root development in rice (Chen et al., 2006), whereas they rather blocked branching in Arabidopsis (De Smet et al., 2003).

The tasks of JAs and GAs in light of root developmental processes have been less studied. Dicot-related research demonstrated the root elongating effect of exogenous GA (Konishi et al., 2005). Besides, shoot apex-derived AUX showed to promote the GA-driven destabilization of DELLA growth suppressors in Arabidopsis (Fu and Harberd, 2003), while GAs and CKs rather interact antagonistically. These findings stress the importance of GA as an integrator of the AUX-CK antagonism involved in root growth of dicots (Petricka et al., 2012). More research is necessary to further resolve the function of GAs in dicot and especially monocot root development.

JA-related studies demonstrated the negative effect of exogenous JA on primary root growth in Arabidopsis and rice (Yang et al., 2012). Furthermore, JA treatments showed to promote lateral rooting in both model plants, which, at least in case of Arabidopsis, seemed to proceed via interplay with AUXs (Sun et al., 2009; Wang et al., 2002). Despite its regulating activity in root development, JAs seem to prioritize defense over growth (Yang et al., 2012). Based on this information, we propose that GA, ABA and the AUX-CK/BR/ET network, encompass the key intrinsic regulatory pathways that control root development in monocots and dicots.

7.5. Root developmental pathways as susceptibility factors for soil-borne pathogens

7.5.1. Targeting BR and ABA pathways to induce susceptibility

7.5.1.1. Exploiting BR-GA/SA/JA antagonistic crosstalk

Recent research has demonstrated that BRs are activators of disease development upon rice root infections with the RKN *M. graminicola* and oomycete *P. graminicola* (De Vleesschauwer et al., 2012; Nahar et al., 2013). Transcriptome analyses on *Pythium*-inoculated (De Vleesschauwer et al., 2012) and nematode-inoculated rice roots (Kyndt et al., 2012) elucidated a strongly induced BR pathway, which negated the immunity-mediating GA and SA pathways (De Vleesschauwer et al., 2012), and JA pathway (Nahar et al., 2013), respectively. The antagonistic BR-GA crosstalk is controlled by key regulator and rice DELLA protein SLENDER RICE1 (SLR1), a repressor of the GA response (Ikeda et al., 2001). Since BRs negatively interact with the key defense hormones SA and JA in rice roots (De Vleesschauwer et al., 2012; Nahar et al., 2013), it is suggested that induction of the BR pathway represents a core virulence strategy to overcome root immunity in monocots.

It remains to be investigated how root pathogens stimulate the BR pathway to confer susceptibility. Toxic cell death-inducing compounds might be involved, since they showed to interact with BR signaling in dicot plants (Masuda et al., 2007; Qutob et al., 2002). Besides, *Pythium* spp. have been reported to synthesize unidentified necrosis-inducing and root growth-inhibiting components (Mojdehi et al., 1990). Based on these arguments, we propose that *P. graminicola* might produce toxins to stimulate BR signaling and BR biosynthesis in rice seedling roots.

7.5.1.2. Exploiting ABA-SA/JA/ET antagonistic crosstalk

ABA-related root responses may also be affected by soil-borne pathogens. Although contrasting reports have been disputing the exact role of ABA in the *Fox*-Arabidopsis interaction (Berrocal-Lobo and Molina, 2008), there are indications that *Fox* might hijack the ABA pathway to inflict disease. Anderson et al. (2004) revealed the disease-promoting features of ABA in *Fox*-inoculated Arabidopsis roots. ABA likely exerted its negative effect on JA/ET-dependent signaling via the basic-helix-loop-helix transcription factor AtMYC2 at the ETHYLENE RESPONSE FACTOR1 (ERF1)-branch (Anderson et al., 2004; Berrocal-Lobo and Molina, 2004). Considering the key role of ERF1 in the protection of dicot roots to soil-borne pathogens (Berrocal-Lobo and Molina, 2004; Okubara and Paulitz, 2005), it seems appealing that the ABA pathway participates in the virulence strategy of *Fox*. Dörffling et al. (1984) proved that *Fusarium* spp. are able to synthesize ABA and accordingly, *Fox* might be able to activate ABA signaling in Arabidopsis and render its roots susceptible.

ABA also accomplishes a negative role in the interaction of rice roots with nematodes. The rice RKN and RRN were recently found to stimulate ABA biosynthesis in their host and hence, they could antagonize SA, JA and ET-responsive defense gene expression (Nahar et al., in preparation, Nahar et al. 2012). Unfortunately, knowledge on how pathogens interfere with this ABA-JA/ET/SA antagonism in monocot roots is currently lacking.

7.5.2. Targeting AUX, CK and GA pathways to promote ingress and induce hyperthrophic feeding sites

7.5.2.1. Exploiting antagonistic AUX-CK crosstalk

Both sedentary nematodes and *P. brassica*e rely on the induction of cell elongation and division for the formation of hypertrophic feeding sites in plant roots. The importance of AUXs and CKs seems evident in this respect (Dello Ioio et al., 2008). In the interaction of dicots with cyst and root knot nematodes, AUXs and AUX-dependent programs are crucial for the development of root galls (Grunewald et al., 2009a). By hijacking the AUX transport network in Arabidopsis, cyst nematodes disturb the AUX distribution *in planta* and so, promote infection (Grunewald et al., 2009b). Lee et al. (2011) showed that the cyst nematode *H. schachtii* synthesizes the Hs19C07 effector, which targets the AUX influx transporter LAX3.

In this way, it is assumed to initiate AUX accumulation in its primary syncytial cell (Grunewald et al., 2009b; Mazarei et al., 2003). Interestingly, AUX accumulation upon cyst and RKN nematode infections is only transient. Two to five days post inoculation, the AUX response shifts to neighboring cells (Grunewald et al., 2008; Hutangura et al., 1999; Karczmarek et al., 2004), which stimulates the feeding site's radial expansion and coincides with increased CK levels (Lohar et al., 2004). For rice RKN, AUXs seem equally important targets during feeding site establishment. Kyndt et al. (2012) demonstrated that giant cells of *M. graminicola* exhibit an enhanced AUX response in the early stages of the infection (Kyndt et al., 2012), which probably triggers gall initiation and reflects AUX accumulation. Beside their indirect effect, RKN and cyst nematodes may also directly perturb endogenous hormone levels through local injections of AUXs (De Meuttter et al., 2005) and CKs (De Meutter et al., 2003). This is probably of utmost importance for the generation of highly expanding multinuclear cells upon plant-nematode encounters. In addition, as AUXs and SA are generally mutually antagonistic, (Yang et al., 2013) inducing AUX signaling might be an effective strategy to counterbalance SA-based defense reactions (Nahar et al., 2011; Wubben et al., 2001).

Similarly, CKs and AUXs seem also important for clubroot gall development in Arabidopsis. The induction of clubroot galls has been correlated with increasing levels of indole-3-acetic acid (IAA) (Ludwig-Müller et al. 1999), elevated expression levels of AUX homeostasis genes (Siemens et al., 2006) and decreased expression levels of CK homeostasis genes in Arabidopsis (Agarwal et al., 2011; Siemens et al., 2006). In addition, both a disrupted AUX signaling (Alix et al., 2007; Siemens et al., 2002) as well as lowered endogenous CK levels (Siemens et al., 2006) have been demonstrated to increase clubroot tolerance. It is unknown how *P. brassicae* affects the AUX and CK pathways in its host, but *Plasmodiophora*-derived hormones might be involved. Like sedentary nematodes, the pathogen is assumed to produce CKs (Müller and Hilgenberg, 1986). However, its ability to synthesize IAA remains elusive. Taken together, these data emphasize the importance of CK and AUX imbalances for the establishment of pathogen-induced hypertrophy.

7.5.2.2. Increasing AUX levels and AUX signaling to promote pathogen ingress

Although oomycetes do not form galls like nematodes or protozoa, *Pythium* spp. have also been reported to synthesize AUX compounds in the rhizosphere of plant roots. Along with concentration-dependent factors, the established relationship with the plant seems to determine the role of AUXs in the interaction of *Pythium* spp. with their host (Gravel et al., 2007; Le Floch et al., 2003). AUXs derived from pathogenic *Pythium* group F isolates have been noted to reduce root elongation, cause root swellings and stimulate root hair formation in tomato (Rey et al., 2001). Since root hairs represent the preferred invasion sites for *Pythium* spp. (Mojdehi et al., 1990) and AUXs play fundamental roles in the formation of these structures (Suzuki et al., 2003), they may enhance the colonization of tomato roots by *Pythium* group F isolates and accordingly, stimulate disease development.

In contrast to *Pythium* infections, *Fox* infections do not respond to exogenous IAA and do not alter endogenous IAA levels in their host. The fungus rather targets AUX signaling and transport in Arabidopsis to colonize its root tissues more effectively (Kidd et al., 2011).

7.5.2.3. Exploiting antagonistic GA-JA crosstalk

Gibberellins promote plant growth by opposing the effect of DELLA/SLR1 growth suppressors (Achard and Genschik, 2009). Galls formed by RKN in rice (Kyndt et al., 2012), as well as tomato (Bar-Or et al., 2005) and soybean root syncytia (Klink et al. 2007), are characterized by strongly activated GA pathways. Therefore, it is hypothesized that nematodes up-regulate the GA pathway and inactivate SLR1 in their feeding site to promote cell elongation and accordingly, potentiate the infection process. In addition, DELLA proteins have been noted to enhance JA signaling through physical interaction with JASMONATE ZIM-domain PROTEIN1 (JAZ1) (Hou et al. 2010). This implies a mechanism by which GAs may down-regulate JA-dependent responses to block nematode resistance in rice roots.

7.5.3. Direct effect on SA-mediated root immunity

Nematodes are also able to directly suppress SA-based defense reactions in plant roots. The cyst nematode *H. schachtii* secretes the effector 10A06, by which it interferes with polyamine biosynthesis and disrupts SA-responsive defense gene expression (Hewezi et al., 2010). Another effector that potentially manipulates the SA pathway upon nematode infection is chorismate mutase (CM). This enzyme is a key regulator of the shikimate-pathway in plants and bacteria, and converts chorismate to prephenate, which can be transformed into a range of metabolites, SA included (Wildermuth et al., 2001). Despite lacking a functional shikimate-pathway, different plant nematode groups, such as cyst nematodes, RKNs and migratory endoparasites, produce CM (Doyle and Lambert, 2003; Haegeman et al., 2011; Huang et al., 2005; Jones et al., 2003; Lambert et al., 1999; Long et al., 2006; Lu et al., 2008; Vanholme et al., 2009). Although not yet experimentally proven, nematodes may adopt this effector to reduce the pool of chorismate in their host and consequently, repress SA-mediated root immunity.

7.6. How do mutualists colonize monocot roots? Parallels with pathogenic interactions.

Beneficial microbes, like mycorrhizal fungi and *Rhizobia* bacteria, have also evolved efficient strategies to short-circuit induced defense responses in monocots and eventually, retrieve a compatible status in their host (Pieterse et al., 2012). When comparing parasitic with mutualistic interactions, remarkable parallels become evident.

Like pathogens, beneficial microbes induce JA- and SA-dependent immune responses upon recognition, which verifies the existence of an ancient pattern of responses to microbial colonization (Güimil et al. 2005). *Piriformospora indica*, *Glomus mosseae* and probably other mycorrhizae initially elicit weak SA-mediated defense responses that are afterwards suppressed to avoid the synthesis of defense-related gene products and to allow colonization of the host's root system (Blilou et al., 2000; Schäfer et al., 2009). The effect of JA accumulation in mutualistic interactions is less clear. Hause et al. (2002) proved that in *Glomus intraradices*-infested barley roots, JA levels especially rise when the symbiosis is fully established. This appeals the idea that JAs rather assist in arbuscule formation than in immunity. Only small increases in endogenous JA appear at the onset of the interaction (Hause et al., 2002). At this stage, JAs, along with ET, have been speculated to direct the extent of fungal colonization (Gutjahr and Paszkowski, 2009; Zamioudis and Pieterse, 2012). These arguments support the intricate role that JAs might play during the establishment of beneficial associations.

Also apparent is the need for changes in ABA, AUX and/or GA homeostasis to successfully colonize monocot roots, re-program monocot root defense and manipulate monocot root development by both mutualistic (Chi et al., 2005, 2010; Schäfer et al., 2009) and pathogenic microorganisms. A detailed transcriptomic investigation of the *P. indica*-barley interaction revealed that AUXs, GAs, ABA and JAs levels are altered upon the basidiomycete's infestation (Schäfer et al., 2009). By stimulating the ABA pathway, *P. indica* likely antagonizes SA-dependent immune responses and prepares for cell penetration and further colonization. The mycorrhizal fungus also causes GA accumulation in barley roots by enhancing the transcription of GA biosynthesis genes. Since mutants impaired in GA synthesis exhibit reduced fungal colonization, GAs are thought to suppress basal root defense in this monocot. Besides, both AUX signaling and AUX synthesis are strongly up-regulated during *P. indica*-barley interactions. These changes in AUX and GA pathways/homeostasis probably underlie the enhanced lateral root formation and general growth promotion upon *P. indica* colonization, and may clarify the impaired plant defense.

7.7. Conclusions

In both rice and Arabidopsis, SA and JAs seem to be the key resistance factors in root defense, while the significance of ET depends on the pathosystem. The induction of root immunity often parallels with a trade-off towards growth. This is especially evident in the case of the root growth-repressing hormone JA, which stabilizes DELLA/SLR1 and as a result, prioritizes defense over growth (Yang et al., 2012). BRs are also pivotal players in the regulation of this growth-immunity balance (Albrecht et al., 2012, Belkhadir et al., 2012). Interestingly, recent studies showed that rice root pathogens are able to exploit BRs as virulence factors. Aside from BRs, AUXs, CKs, GAs and ABA seem core targets for soil-borne microorganisms to suppress immunity, potentiate root colonization and/or modify root systems for their own benefit.

We believe that both beneficial and detrimental soil-borne microorganisms stimulate root developmental pathways in an attempt to defeat basal JA- and SA-mediated root immunity in monocots (Figure 7.2).

Yang et al. (2013) recently provided an overview of the immune system in rice shoots and revealed intriguing similarities. They showed that SA and JA are either synchronously or antagonistically activated during stress responses in rice leaves, and indicated the growth-promoting hormones AUX and GA as susceptibility factors for pathogen infection in rice shoots (Yang et al., 2013). Accordingly, it seems that both rice root and leaf pathogens target growth-mediating hormonal pathways in their host to antagonize plant immune responses and inflict disease.

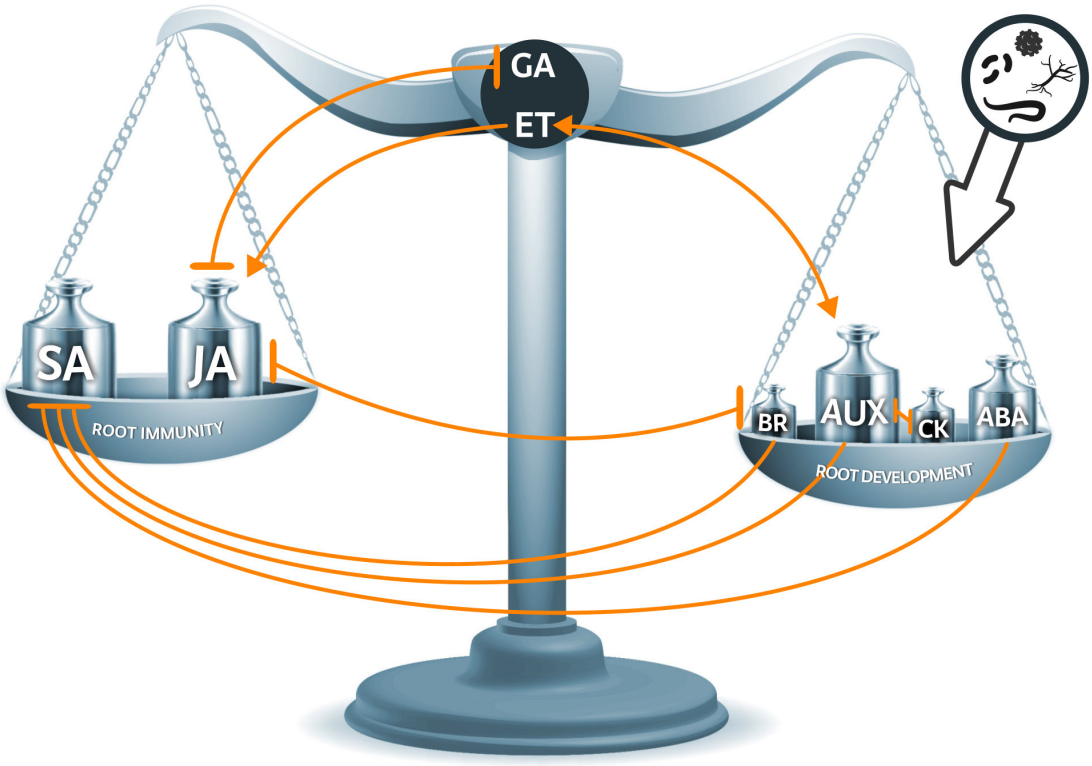

Figure 7.2. Balance between root developmental and defense-related pathways in monocot roots. Based on above literature analyses we have created a generalized scheme in which plant hormones are categorized into two major groups: hormones mainly involved in root immunity and hormones mainly regulating root development. GA and ET showed to conduct crucial tasks in both processes, and hence, we centered them on top of the scale. Depending on the encountered stress factor, a plant chooses to invest energy in either root immunity or root development. Making this choice will automatically push the balance towards one side and negatively affect the other. Soil-borne pathogens, including nematodes, oomycetes, fungi and bacteria, are suggested to interfere with root development, by which they suppress immunity and facilitate infection.

Future studies should focus on the roles of strigolactones (SLs) in root development and defense. This relatively new group of plant hormones has recently been found to affect root elongation (Koltai et al., 2010) and adventitious rooting (Rasmussen et al., 2012) in dicots and furthermore, it plays an important role in root-microbe interactions. Investigations revealed that an unique composition of SLs triggers the pre-symbiotic phase of arbuscular mycorrhizal fungi, including the germination of spores and branching of hyphae prior to root colonization (Akiyama et al., 2005, 2010). Remarkably, synthetic SLs exert an overall inhibiting effect on the growth of phytopathogenic fungi (Dor et al., 2011). Additional research should resolve which position SLs hold in the growth-immunity balance.

SUMMARY POINTS

1) SA and JAs are the key players in the immune system of monocot roots.
2) SA, JAs and ET cooperate in the protection of monocot roots against biotic stresses.
3) GAs, AUXs and ABA are the key players in root development of monocots. Besides, CKs, ET or BRs modulate the regulatory activity of AUXs through antagonistic and/or synergistic interactions.
4) Parasitic and beneficial microbes that interact with monocot roots manipulate AUXs, CKs, GAs, BRs and/or ABA pathways.
5) By targeting root developmental pathways, soil-borne microorganisms attempt to defeat root defense, facilitate root colonization, induce hypertrophic feeding sites or establish a long intimate relationship with monocots.

Chapter 8
General discussion and future perspectives

8.1. Main conclusions

8.1.1. *P. arrhenomanes* is the dominant *Pythium* species associated with yield decline in rice fields

Since 2005, the IRRI in the Philippines has been intensively studying the incidence of progressive yield decline in aerobic rice fields. Several abiotic and biotic factors, including nutrient deficiencies and root knot nematodes (RKN), have been proposed to underlie these yield losses (Kreye et al., 2009a; Kreye et al., 2009b; Nie et al., 2009a) and in this dissertation, we identified three closely related *Pythium* spp., *P. arrhenomanes, P. graminicola* and *P. inflatum*, which also seemed to be associated with the problems in these rice fields (see Chapter 3). All *P. arrhenomanes* isolates were characterized as highly virulent towards aerobic rice seedlings (cv. Apo), evoking pre- and post-emergence damping-off, stunting of rice shoots and roots, root necrosis and shoot wilting. On the contrary, only few *P. graminicola* isolates were pathogenic under *in vivo* conditions and their virulence was lower compared to *P. arrhenomanes* isolates, which stresses the importance of the latter species. *P. inflatum* isolates were identified as non-pathogenic in *in vivo* pathogenicity trials. However, under the disease-favoring conditions of our *in vitro* system, where infection pressures were high, rice seeds were damaged and seedling roots could be invaded within 2-4 days post imbibition, all *Pythium* isolates were shown to be pathogenic. Even *P. inflatum* isolates significantly affected rice seedling shoot lengths *in vitro*, but their virulence level was still significantly less than that of *P. arrhenomanes* and *P. graminicola* isolates. Consequently, we considered *P. inflatum* as a minor pathogen on aerobic rice.

P. arrhenomanes was the most frequently recovered species from affected aerobic fields in the Philippines, followed by *P. inflatum* and *P. graminicola*. By analyzing *Pythium* communities in paddy fields (Table 8.1), we discovered that *P. arrhenomanes* was consistently the most aggressive and dominant species, next to others like *P. myriotylum*, *P. vanterpoolii* and *Pythium* group F. It seems that the *Pythium* complex in rice fields consists worldwide of *P. arrhenomanes* and an additional group of less virulent and/or non-pathogenic species, of which the composition may be shaped by the geographic localization, agricultural system and/or rice cultivar.

Table 8.1. The *Pythium* communities associated with seedling diseases in rice fields

Main species	Other frequently isolated species	Origin	Country	Reference
P. arrhenomanes	*P. graminicola*, *P. inflatum*	Aerobic rice field	Philippines	Van Buyten et al., 2013
P. arrhenomanes	*Pythium* group F, *P.vanterpoolii, P.myriotylum*	Paddy field	South-Australia	Cother and Gilbert, 1992 and 1993
*P. arrhenomanes**	-	Paddy nursery	Japan	Furaya et al., 2003; Kobori et al., 2004
P. tolurosum	-	Wild rice	California	Marcum and Davis, 2006

*Wrongly identified as *P. aristosporum* and *P. graminicola* by Furaya et al. (2003) and Kobori et al. (2004), respectively.

However, one exception has been found in wild rice, where *P. tolurosum* appeared to be the most important causal agent of damping-off (Marcum and Davis, 2006). This is probably linked with the genetic differences between wild rice (*Zizania* spp.) and the dominant cultivated rice species, *Oryza sativa* L.. Currently, aerobic rice is also cropped in several tropical and temperate countries other than the Philippines (Bouman et al., 2006; Humphreys et al., 2005). It would be of particular interest to study the resident *Pythium* communities in these rice fields and to verify our hypothesis.

8.1.2. Rice-infecting *P. arrhenomanes* isolates are genetically similar and not adapted to their host

Intraspecific variability at the genetic level has frequently been reported for plant-pathogenic *Pythium* spp. (Belbahri et al., 2008; Kageyama et al., 2005, 2007; Matsumoto et al., 2000; Perneel et al., 2006). This type of variation has been shown to correlate with differences in pathogenicity and hence, might be indicative for host adaptation (Perneel et al., 2006). In chapter 3, we demonstrated that *P. arrhenomanes* and *P. graminicola* isolates from aerobic rice exhibited little genetic variation, despite the pathogenic variability among *P. graminicola* isolates. The intraspecific genetic variation was higher for *P. inflatum*, but also in this case, no correlation with the phenotype could be detected. This opposed the results of a study on the cocoyam root rotter *P. myriotylum*, where pathogenic isolates were genetically distinct from non-pathogenic isolates, indicating a certain degree of host adaptation (Perneel et al., 2006). Consequently, we suggested that the pathogenicity of rice-attacking *Pythium* isolates is not correlated with specific base mutations in the rDNA-ITS or *β-tubulin* gene sequences. On the contrary, when *P. arrhenomanes* isolates from other hosts were included in our study, we could reveal a link between genetic and pathogenic variation (see Chapter 3). So host adaptation seems to occur within *P. arrhenomanes*, but not for maize- and rice-infecting isolates. Sequencing of other genes in combination with Amplified Fragment Length Polymorphism (AFLP) should be used to verify this hypothesis.

When *P. arrhenomanes* isolates from other rice varieties were screened for their pathogenicity towards aerobic rice (cv. Apo) and vice versa, we noticed that the cultivar did not determine the pathogenicity or virulence of the isolates. Therefore, we speculated that fewer problems with *Pythium* spp. in paddy fields are not due to the cropped variety, but rather rely on environmental factors like water level, temperature, light, or soil chemical and physical properties. For instance, it is known that a reduced seedling establishment in water-seeded rice fields is more common during cold and cloudy weather conditions (Cother and Gilbert, 1993). Besides, changes in nutrient levels, field capacity, pH and percentages of clay or organic material may strongly influence the composition of *Pythium* communities in agricultural soils (Broders et al., 2009). Oxygen levels are also important, since micro-aerobic conditions, like seen in rice paddies, may inhibit the germination of *Pythium* propagules (Johnson, 1998; Voland and Martinson, 1984).

To control *Pythium* spp. in aerobic rice fields, crop rotation strategies may allow effective disease management. In China, aerobic rice is rotated with winter crops (e.g. wheat) and legumes (e.g. soybean) (Nie et al., 2012), and no problems with yield decline have been reported. Unfortunately, the cultivation of wheat is less productive in tropical Asia. Maize has been presented as a more suitable crop for rice-wheat diversifications in the tropics (http://www.knowledgebank.irri.org/ckb/index.php/extras/maize-a-suitable-candidate-for-rice- wheat-systems-diversification) and the study of Nie et al. (2009b) supported this statement, by demonstrating that the yield of aerobic rice after two seasons of maize is significantly enhanced. Strikingly, our study proved that *P. arrhenomanes* isolates from rice and maize lack host specificity and are equally virulent on both crops (see Chapter 3). Rotation strategies with maize would accordingly stimulate and/or assure the persistence of *P. arrhenomanes* in aerobic rice fields, and therefore, the sustainability of this rotation strategy is questioned. Non-graminaceous crops, such as sweet potato and soybean, or seasons of fallow, would be better options for crop rotation in the Philippines. This should be combined with a good weed-management, since weeds may represent alternative hosts for *P. arrhenomanes* (Dissanayeke et al., 1997). Breeding for resistance might also be successful considering the low intraspecific variability within *P. arrhenomanes* isolates from rice. Some promising tolerant varieties, like cv. IR81413-BB-75-4, have already been discovered (Kreye et al., unpublished).

8.1.3. The differential aggressiveness of rice-infecting *Pythium* spp. is linked with their root colonization capacity and growth on amino acids

In chapter 4, 5 and 6 we focused on the interaction between *Pythium* and rice seedlings. A comparative analysis with *P. arrhenomanes*, *P. graminicola* and *P. inflatum* was executed, to unravel the driving factors behind their varying aggressiveness. Our histopathological analysis revealed that the highly virulent species *P. arrhenomanes* quickly and massively colonized the outer and inner root tissues of rice seedlings, including the root stele (see Chapter 4). Like a true vascular pathogen it extensively invaded the xylem, which is rather exceptional for plant-colonizing *Pythium* spp., since abundant invasion of vascular tissues has only been reported for five other species (Adie et al., 2007; Chérif et al., 1991; Matta, 1965; Nemec, 1972). Most of the time, hyphal growth remains limited to the outer root tissues and is arrested at the endodermis. Those hyphae that ingress the vascular tissue are often restricted in spread or quickly die (Desilets et al., 1994; Le Floch et al., 2005; Mojdehi et al., 1991; Rey et al., 1998a&b). Also in the case of rice-colonizing *P. graminicola* and *P. inflatum* isolates, a more limited invasion of the vascular stele was detected beside slower colonization processes and a weaker or delayed production of reactive oxygen species (ROS) (see Chapter 4). Hence they probably induced a lower degree of wilting and seedling death. In addition, cell wall fortification events slowed down the systemic spread of *Pythium* hyphae in the root cortex of *P. graminicola*- and *P. inflatum*-inoculated rice seedlings. The suppression of such inducible defence responses is probably of utmost importance for the virulence of rice-attacking *Pythium* spp..

Along with its putative inhibition or postponement of cell wall strengthening processes, the broad amino acid profile of *P. arrhenomanes* likely facilitated its quick occupation of rice seeds and root tissues upon inoculation. Plant pathogens need carbon sources for their growth and are often specialized in the use of the most abundant nutrients in their ecological niche (Rico and Preston, 2008). Our study demonstrated that, although *P. inflatum* was nutritionally the most versatile species, *P. arrhenomanes* seemed more adapted to its host by its strong growth on the most common amino acids and carbohydrates in rice exudates. Furthermore, this pathogen exhibited the unique ability to utilize D-amino acids, such as D-serine, of which rice seeds contain substantial amounts (Gogami et al, 2009). Biocontrol agents that reduce the availability of these carbon sources and inhibit the exudate-induced germination of *P. arrhenomanes* propagules, would be highly effective in controlling this pathogen (Kageyama and Nelson, 2003). Our phenoarray also illustrated that *P. arrhenomanes* could use L-threonine for its growth and aside from this, *P. graminicola* and *P. arrhenomanes* were both able to proliferate on the cell wall-associated amino acid hydroxyl-L-proline. These findings imply the potential of rice-pathogenic *Pythium* spp. to seize defense-related compounds in their host. A similar phenomenon was seen during the parasitic interaction of *P. syringae* pv. tomato with Arabidopsis (Park et al., 2010). The utilization of specific amino acids by these microorganisms may be part of their virulence strategy (Seifi et al., 2013).

8.1.4. GAs and SA, rather than JAs, orchestrate rice immunity to *Pythium* spp..

Jasmonates have been identified as pivotal players in the protection of dicot roots to pathogenic *Pythium* spp., nematodes and several fungi (see Chapter 7). Unfortunately, in monocots, root-related research has been lagging behind. A recent paper showed that root immune responses to rice nematodes also largely rely on the JA pathway (Nahar et al., 2011). In contrast with these results, we have strong indications that GAs and SA, rather than JAs, represent key regulators in the resistance of rice seedling roots to *Pythium* spp.. In chapter 5, we presented the immunity-mediating roles of GAs and SA in the interaction of rice with *P. graminicola*. By comparing the effect of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* on the GA pathway in rice roots, it became apparent that the highly aggressive *P. arrhenomanes* strongly suppressed GA biosynthesis in rice seedlings during the course of the infection and severely attenuated GA signaling at the onset of the interaction (see Chapter 6). Since cell wall fortification events are the most important defense responses against oomycetes (Oliver et al., 2009) and exogenous GA mediates lignin deposition in monocots (Biemelt et al., 2004), we suggest that the suppression of the GA pathway is indispensable for the successful colonization of rice seedlings by *Pythium* spp..

Interestingly, GA biosynthesis and signalling in plants have repeatedly been considered as valuable targets for crop improvement. During the green revolution, semi-dwarf high-yielding rice cultivars were developed with an improved lodging-resistance and greater harvest index, allowing for the increased use of nitrogen fertilizers (Spielmeyer et al., 2002).

The phenotype of these varieties reflected a partial block in GA biosynthesis and appeared to evolve from a defective GA biosynthetic gene *GIBBERELLIN 20-OXIDASE2* (*OsGA20ox2)* (Spielmeyer et al., 2002). Also in sugar beet and maize, manipulation of the GA pathway seems a promising strategy to ameliorate crop performances. Mutasa-Göttgens et al. (2009) discovered that the inactivation of GA and repression of the GA signal transduction cascade in sugar beet delays the transition to its reproductive growth stage and hence, this study revealed a manner to alter the architecture and yields in this crop. In maize, the overexpression of the GA-biosynthetic gene *GIBBERELLIN 20-OXIDASE1 (GA20ox1)* has been resulting in more biomass production and taller plants (http://www.vib.be/nl/nieuws/Pages/VIB-maïs-veldproef-Wetteren-Genetisch-gewijzigde-maïs-ook-in-het-veld-groter.aspx.), and current research at the VIB (Ghent, Belgium) is evaluating to which extent this genetic modification might contribute to higher yields in the field and might effect mycorrhizal colonization (Hilde Nelissen, personal communication). Considering the key roles of GAs in *Pythium*-rice interactions and the severe inhibition of *OsGA20ox3* transcription by *P. arrhenomanes*, it is clear that manipulation of the GA pathway would entail major effects on *Pythium*-induced disease symptoms and therefore, researchers should consider *Pythium* epidemiology during crop improvement.

8.1.5. Rice-infecting *Pythium* spp. affect AUX and BR pathways to antagonize root resistance and inflict disease

Plants are protected against the majority of microorganisms they encounter (Abramavotich and Martin, 2004). Successful pathogens depend on the reprogramming of the plant's hormone signalling circuitry and the evasion or attenuation of plant immune responses (Pieterse et al., 2012). To this end, intruders deliver pathogenicity factors like phytohormones or their functional mimics, by which they perturb hormone homeostasis and alter ensuing signalling cascades (Robert-Seilaniantz et al., 2011). Especially for root pathogens, the plant's AUX pool represents an interesting target, since roots strongly respond to IAA fluctuations (Leveau and Lindow, 2005).

Various *Pythium* spp. have been documented to produce indole-3-acetic acid (IAA) as a putative pathogenesis mechanism (Gravel et al., 2007; Rey et al., 2001) and in chapter 6, we illustrated a similar feature for *P. arrhenomanes*, *P. graminicola* and *P. inflatum* isolates from aerobic rice. Furthermore, we demonstrated the disease-enhancing effect of exogenous IAA and hypothesised that *Pythium*-derived IAA (or other effectors) elevates endogenous AUX levels and AUX signalling in rice to facilitate infection and undermine immunity. Next to IAA synthesis, *P. arrhenomanes* and *P. graminicola* isolates from aerobic rice were assumed to degrade IAA, revealing another strategy by which they may control IAA levels in their environment. For rice root knot nematodes (RKN), AUXs seem an equally important target.

Kyndt et al. (2012) demonstrated that giant cells of *M. graminicola* exhibit enhanced AUX responses in the early stages of the infection, which probably trigger gall initiation and reflect AUX accumulations. Comparably, *Xanthomonas oryzae pv oryzae*, *Xanthomonas oryzae pv oryzicola* and *Magnaporthe grisea* induce AUX accumulations in rice leaves as part of their virulence strategy (Fu et al., 2011). These leaf pathogens could be controlled by the expression of the IAA-amido synthetase *OsGH3-2* under a strong pathogen-inducible promoter, which inhibits the localized concentration of IAA (Fu et al., 2011). Seeing that *Pythium* spp. and RKN similarly alter endogenous AUX levels in rice roots, this gene possesses strong potential to induce broad-spectrum disease resistance in rice (Fu et al., 2011).

Beside the regulation of AUX levels, rice root pathogens try to induce optimal BR concentrations in their host. In chapter 5, we obtained strong evidence that *P. graminicola* exploits BRs as virulence factors to tap into the rice immune-signalling machinery, and inhibit SA- and GA-dependent defense responses. Besides, in chapter 6, we showed that hijacking of the BR pathway appeared to be conserved among other rice-pathogenic *Pythium* spp. and that a changed steroid homeostasis seemed crucial for the virulence of these species. Rice RKN have also been noted to trigger the BR pathway in rice and hence, negate JA-dependent defense responses (Nahar et al., 2013). These results are in contrast with shoot-related studies (Nakashita et al., 2003) and call into question the value of BRs for crop improvement (Dive and Krishna, 2009). It remains to be elucidated how rice root pathogens affect the BR machinery. It is known that phytotoxins, like trichothecenes of *Fusarium* spp., may interfere with the BR pathway in plants (Masuda et al., 2007) and that *P. arrhenomanes* produces toxic substances during its interaction with wheat roots. However, we have no indications that toxins are involved in the infection process of *P. arrhenomanes* in rice seedling roots (see Chapter 4). Because BRs and AUXs cross-communicate in rice (Song et al., 2009), it may also be possible that rice root pathogens produce IAA to interfere with BR homeostasis.

8.2. Future prospects

Although this work largely answered our initial research questions (see Chapter 1), it urges further investigation. Besides, it sparked new ideas for future studies and these are listed in the following paragraphs.

- Within *P. arrhenomanes*, *P. graminicola* and *P. inflatum* isolates from aerobic rice, we detected consistent dimorphic positions (double bases) in the electropherograms of their Internal Transcribed Spacer (ITS) region of the rDNA and/or *β-tubulin* gene. Such intra-isolate variation might be indicative for hybridization, inter-chromosomal variation or heterokaryosis, and has been reported for other *Pythium* spp. as well (Belbahri et al., 2008; Kageyama et al., 2007).

Within three *P. inflatum* isolates double peaks were abundant and one triple peak was observed at consistent positions in the electropherogram of the *β-tubulin* gene. The latter finding is very important since the *β-tubulin* gene is supposed to be single copy in *Pythium* spp. (Mu et al., 1999) and therefore, polymorphic peaks may evidence an alloploid microorganism (Nechwatal and Mendgen, 2009). Moreover, the colony morphologies of these *P. inflatum* isolates appeared to be unstable, which is suggestive for aneuploidy (Vercauteren et al., 2009). Future research should clarify if *Pythium* isolates from aerobic rice fields could indeed be aneu- or polyploid and/or whether sexual outcrossing could have occurred within these homothallic species. It has to be noted that hyphal tip or single spore cultures should be generated before performing an in-depth study on the intra-isolate variation of these *Pythium* isolates.

- *P. arrhenomanes* and *P. graminicola* were never simultaneously recovered from aerobic rice fields (see Chapter 3). Therefore, we proposed that the two pathogens strongly compete with each other during the colonization of rice seed and root tissues. Preliminary experiments in which rice seedlings were co-inoculated with *P. arrhenomanes* and *P. graminicola* illustrated that when both pathogens were present in the inoculum, *P. arrhenomanes* was more limited in its colonization, while *P. graminicola* was rather stimulated (Table 8.2). This promoting effect could be associated with the strong influence of *P. arrhenomanes* on the rice immune system, which may have facilitated the infection process of *P. graminicola*. Gene expression studies on co-inoculated rice seedling roots would allow clarifying the underlying physiological mechanism. Besides, quantification assays upon sequential inoculations, in combination with re-isolations, would elucidate whether these species can ban each other from rice tissues. A paper by Paulitz and Baker (1988) reported on this matter and discovered that *P. nunn* could displace *P. ultimum* from bean leaf fragments. Aside from competition, it is possible that certain environmental conditions like soil substrate, pH, humidity, nutrients or the residing soil microbial population could have advantaged one *Pythium* species over the other (Broders et al., 2009). Analysis of these possible determinants would help to understand the observations in aerobic rice fields.

Table 8.2. DNA quantities (pg/ ng total DNA) of *P. arrhenomanes* and *P. graminicola* in rice seedling roots, after single and co-inoculations.

	***P. arrhenomanes* in mix**		***P. arrhenomanes***		***P. graminicola* in mix**		***P. graminicola***	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
20 hpi	5.4 (0.18)	44.38 (2.52)	67.54 (3.54)	121.34 (10.94)	96.28 (5.20)	25.17 (0.12)	134.62 (0.59)	71.22 (0.65)
28 hpi	5.75 (3.02)	111.72 (1.58)	152.76 (31.00)	130.88 (4.98)	109.02 (4.44)	143.06 (1.28)	96.74 (9.19)	75.88 (7.86)
73 hpi	48.76 (2.32)	107.34 (3.59)	140.36 (0.62)	126.64 (5.18)	171.76 (2.43)	107.26 (1.95)	81.87 (10.32)	64.52 (3.22)

'Mix 'refers to co-inoculations. The values between brackets display the standard deviations.

- *Pythium* spp. may also compete with other root pathogens in agricultural fields. A mutual antagonism has been described for *P. arrhenomanes* and nematodes in sugarcane fields (Bond et al., 2004). Similarly, *Pythium* spp. and the RKN *M. graminicola* seem to antagonize each other in aerobic rice fields. Research executed by the IRRI showed that *Pythium* spp. were especially recovered from aerobic rice seedlings up to tillering stage, while nematodes were more abundant in mature plants (Banaay et al., 2013). In addition, co-inoculations with nematodes and *Pythium* spp. resulted in less disease symptoms on rice varieties that had some resistance or tolerance to *Pythium*, while in *P. arrhenomanes*-susceptible cultivars more damage was noted (Kreye et al., unpublished). Together with dr. ir. T. Kyndt, an FWO-project was written concerning the mechanisms by which RKN and *Pythium* spp. counteract each other in aerobic rice fields. This project will examine direct antagonistic interactions between *Pythium* spp. and RKN, by evaluating the nematicidal effect of *Pythium* toxins, the parasitism of RKN by *Pythium*, and the effect of RKN-derived endoglucanases on the *Pythium* cell wall. Additionally, we suggested that *Pythium* spp. may indirectly enhance the protection of rice seedlings to nematodes by their strong effect on the JA pathway (see Chapter 6) or the induction of other defence reactions. Using transcriptional and metabolic profiling, the major plant pathways influenced by single and dual infections will be identified and their role in the observed antagonism will be functionally analysed.

- Recently, dr. ir. D. De Vleesschauwer and the Kikuchi Lab (NIAS, Japan) executed a genome-wide transcriptome analysis on *P. graminicola*-inoculated rice roots at 1, 2 and 4 dpi using micro arrays. By analysing the differentially expressed genes (DEGs) at 1 dpi, we unravelled the transcriptional reprogramming of many genes involved in amino acid metabolism and transport (Figure 8.1). Among these amino acids, several showed to serve *P. graminicola* growth (see Chapter 4) and therefore, we assumed that *P. graminicola* might disturb the amino acid homeostasis in rice roots to enhance its fitness. By leaking more amino acids or containing more free amino acids, rice roots might be stimulated to attract *P. graminicola* and/or support its colonization process. In addition, the micro-array data proved that the synthesis of L-tryptophane was up-regulated ($Log_2 > 1$) at 1 dpi, which could be related to the important role of IAA in the *Pythium*-rice pathosystem (see Chapter 6). Besides, the synthesis of L-arginine and L-ornithine was induced at 1 dpi ($Log_2 > 1$), whereas the degradation of L-proline was repressed ($Log_2 < -1$). Seifi et al. (2013) reviewed the role of glutamate metabolism in plant disease and defense, and showed that arginine, ornithine and proline participate in the 'evasion' pathway in plants, entailing a metabolic state in which the process of cell death is facilitated and infections by necrotrophs are stimulated. Moreover, 'evasion' is accompanied by the remobilization of nitrogen, away from the challenged plant cell by which ammonia accumulation is prevented. Consistent with the report of Howell et al. (1988) these findings suggest that ammonia inhibits *Pythium* spp., including *P. graminicola*.

Consequently, we speculated that the yield increases in aerobic rice fields following ammonium-sulphate fertilization (Nie et al., 2009a) might also be due to the disease-suppressive effect of these compounds. Intriguingly, our micro-array analysis also revealed a possible accumulation of L-threonine at 1 dpi. Considering the defense-related characteristics of L-threonine, this likely reflected rice immune responses to *P. graminicola* (Stuttmann et al., 2011; see Chapter 4). Currently, micro-arrays on *P. arrhenomanes*- and *P. inflatum*-inoculated rice seedling roots are being analysed at the Phytopathology lab (Ghent University). Together with the implementation of metabolomics, this will enable to verify our interesting assumptions and compare the effect of different *Pythium* spp. on the rice root metabolome.

- It has been proven that rice seedlings become more resistant to *Pythium* spp. within eight days after planting (Chun and Schneider, 1998). The underlying mechanisms of this increased resistance have, however, not been elucidated. In chapter 4 and 6, we put forth the role of a GA-mediated lignification of the rice root stele, but other physiological mechanisms could as well be involved. By comparing root responses to *Pythium* spp. in the susceptible versus resistant stage of rice seedlings (see Chapter 3) and in susceptible versus more tolerant rice cultivars (Kreye et al., unpublished), we might broaden our knowledge concerning the hormonal regulatory network governing rice immunity to *Pythium* spp..

- To fully understand the role of JAs, AUXs and BRs in the interaction of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* with rice seedlings, an in depth-study using several mutant and transgenic rice lines affected in the perception, biosynthesis or transport of these hormones, and proteomics, is recommended. Furthermore, additional efforts should be undertaken to unravel hormonal crosstalk during *Pythium*-rice interactions. In chapter 5, it was demonstrated that BRs antagonize GA- and SA-mediated root immunity in *P. graminicola*-rice interactions. It should be confirmed whether this negative crosstalk also plays during interactions of rice seedlings with *P. arrhenomanes* and *P. graminicola*, and if a higher virulence level is indeed correlated with a stronger suppressive effect on the GA pathway. In chapter 6, we proposed that the outcome of an activated JA pathway depends on its balance with the GA and BR pathway, or other hormone pathways. Although nematode-related research recently proved that BRs antagonize the JA pathway in rice roots (Nahar et al., 2013), we did not obtain evidence of such antagonism in *Pythium*-rice interactions (see Chapter 6). Moreover, JAs and BRs both stabilize the rice DELLA protein SLENDER RICE1 (SLR1) and counterbalance the GA pathway in rice roots (De Vleeschauwer et al., 2012; Yang et al., 2012), and therefore, it is proposed that they rather cooperate during *Pythium*-rice interactions. Next to JAs, AUXs may interact with BRs in rice tissues (Song et al., 2009). It should be clarified if these hormonal pathways also interact during rice root responses to *Pythium* infections and if the activation of the BR pathway could be addressed to the pathogen's effect on the AUX pathway. In addition, it is not clear if AUXs may directly negate the GA- and SA-dependent immune responses in rice roots and whether *Pythium* spp. might exploit these potential virulence paths.

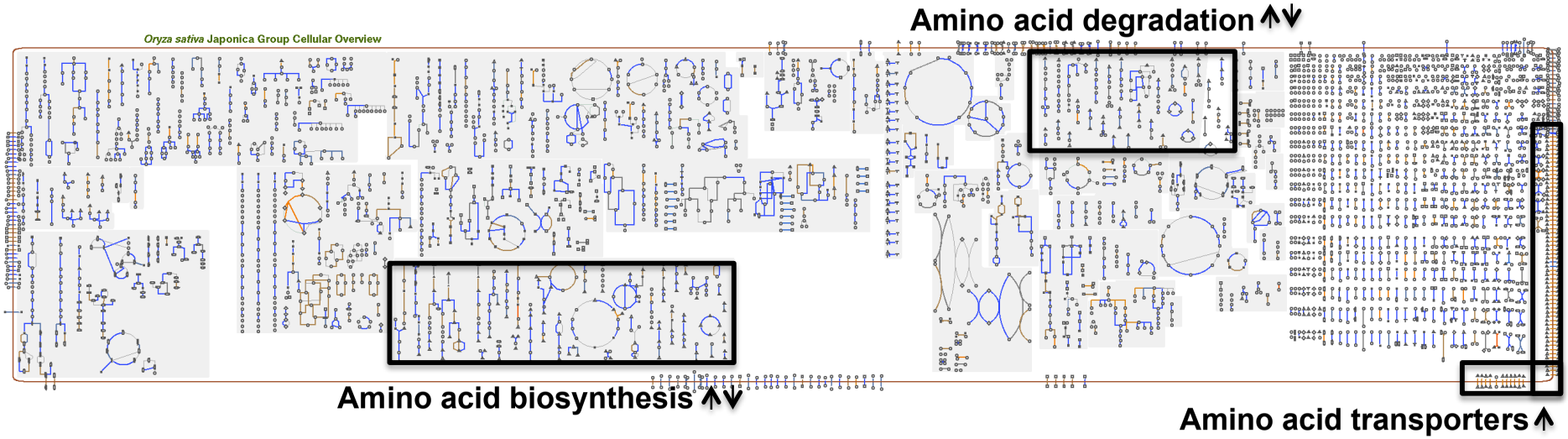

Figure 8.1. RiceCyc overview of the differentially expressed genes in rice root cells one day after *P.graminicola* infection. The reaction lines are colour-coded according to the relative expression of a gene (x) involved in a particular reaction step (orange= +2.5<x< +5.83, brown= +0.83<x<+2.5, grey= -0.83<x<+0.83, light blue= -0.83<x<-2.5, blue= -2.5<x<-5.83). Each node represents a metabolite and the triangles stand for amino acids. MetaCyc version 15.0. was used.

- The pathogenicity mechanisms involved in *Pythium*-plant interactions are still poorly understood. Compared to *Phytopthora* spp., less genomic information is available for *Pythium* spp., which has been delaying the identification and functional analysis of their effector repertoire. Lévesque et al. (2010) sequenced the genome of the necrotroph *P. ultimum* and predicted its secretion of protease inhibitors, toxins, cell wall degrading enzymes (CWDEs), lipases, phospholipases, elicitors and compared to *Phytophthora* spp. a unique family of cadherins, a highly expanded family of proteases, no cutinases or pectin esterases, few Crinkler genes and no RXLR effectors. While it was not mentioned in the paper of Lévesque et al. (2010), *Pythium ultimum* and other *Pythium* species may also synthesize IAA to facilitate infection and cause disease (Gravel et al., 2007; Le Floch et al., 2003; Modjehi et al., 1990; Rey et al., 2001). In Chapter 6, we demonstrated a similar feature for rice-pathogenic *Pythium* spp.. Since exogenous IAA exerted a negative effect on rice resistance to *Pythium* spp., these oomycetes are suggested to deliver IAA in their host to trigger the AUX pathway and hence, interfere with root immunity. To confirm this hypothesis, the production of IAA during *Pythium*-rice interactions should be demonstrated. Immunohistochemical analyses and transformed rice lines may allow the localization of IAA and AUX responses in *Pythium*-inoculated rice roots (Tanaka et al., 2011). Aside from its synthesis, rice-infecting *Pythium* spp. are also suggested to degrade IAA under specific circumstances (see Chapter 6), providing another strategy by which they may control IAA levels in their surroundings (Leveau and Lindow, 2005). It would be interesting to verify this assumption, since it might be the first report on IAA degradation by these oomycetes.

- A recent paper reported on a novel biofungicide KNB422, which is based on *Talaromyces* spp. and might entail broad-spectrum resistance against soil-borne pathogens in paddy fields, including *Pythium* spp. (Miyake et al., 2012). It is not known whether this biocontrol agent could as well be effective against *Pythium* spp. in aerobic rice fields, where soils are not maintained under saturated conditions. Banaay et al. (2012) recently described the biocontrol and growth-promoting activity of *Trichoderma ghanese* in its interaction with aerobic rice seedlings (cv. Apo). This fungus diminished *P. arrhenomanes*-induced shoot and root stunting with 21% and 100%, respectively, and therefore, it may represent an interesting BCA for use in Philippine aerobic rice fields. Besides, we have indications that *Pseudomonas* spp. might effectively control rice-infecting *Pythium spp.*. The fluorescent pseudomonad CMR12a, which was isolated from cocoyam roots in Cameroon and controls an array of soil-borne plant diseases (D'aes et al., 2011; Perneel et al., 2006), significantly enhanced the root and shoot lengths of rice seedlings (cv. Apo) during preliminary infection trials with *P. arrhenomanes* (Van Buyten, unpublished results). The above-listed biocontrol agents might contribute to the sustainability of aerobic rice cultivation in the Philippines. Nevertheless, their efficacy needs to be evaluated under field conditions, where interactions with other microorganisms, like RKN and several environmental factors might exert great effects.

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Summary

Global warming and a continuously rising world population are causing water scarcity for agricultural usage. The traditional rice cultivation in flooded paddy fields consumes tremendous amounts of fresh water and therefore, its sustainability is severely threatened. Since rice paddies provide 75% of the total rice production, this poses a huge problem on the future food security of billions of people mainly living in Asia. Several strategies have been developed to save water in paddy fields, but the breeding for improved upland-adapted rice cultivars seemed the most promising. The International Rice Research Institute (IRRI) in the Philippines and the Upland Rice Laboratory of the Chinese Agricultural University (CAU) in China have developed rice cultivars that, like wheat and maize, can be direct-seeded and grown in non-flooded and non-puddled fields, allowing water-savings up to 50% compared to rice paddies. Being responsive to extra fertilization and irrigation, aerobic rice cultivars yield significantly more than traditional upland cultivars and enable farmers to lower the amounts of irrigation water without affecting productivity. Unfortunately, the aerobic rice system has been suffering from declining yields due to autotoxicity, nematodes and/or nutrient deficiencies in Brazil, Japan and the Philippines. The IRRI has also reported on *Pythium* spp. in Philippine aerobic rice fields, which seemed to contribute to the observed yield decline upon monocultivation. Since *Pythium* spp. cause worldwide significant losses in the cultivation of economically important crops and their role in rice seedling disease has been frequently documented, we decided to characterize the *Pythium* spp. present in Philippine aerobic rice fields and to accurately investigate their interaction with rice seedlings.

Ninety-nine *Pythium* isolates were recovered from four different rice fields on the main island Luzon of the Philippine Archipelago. Using pathogenicity assays and sequence information of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and *β-tubulin* gene, five closely related *Pythium* species could be identified among which three appeared to be associated with progressive yield decline in monocropped aerobic rice fields, namely *P. arrhenomanes, P. graminicola* and *P. inflatum*. *P. arrhenomanes* isolates were the most virulent and reduced rice seedling growth drastically. Moreover, they were frequently recovered from aerobic rice fields, stressing the importance of this species with regard to the occurring yield losses. *P. graminicola* was less virulent and exhibited intraspecific pathogenic variation that was not linked with variation at the genetic level. *P. inflatum* isolates were non-pathogenic, except when the infection pressure was high and the infection could proceed within four days post rice seed imbibition. This species showed a higher level of intraspecific genetic variability, but this was again not correlated with the phenotype. Therefore, we suggested that rice-attacking *Pythium* spp. are probably not host-specific. In compliance with this hypothesis, we discovered that *P. arrhenomanes* isolates from rice and maize seemed to lack host specificity, questioning the suitability of maize as a candidate for crop rotation in aerobic rice fields.

To understand the differential aggressiveness of *P. arrhenomanes*, *P. graminicola* and *P. inflatum* towards rice seedlings, we selected one isolate of each species for a detailed comparative survey on their interaction with rice roots. Histopathological, molecular and phenoarray analyses illustrated that *P. arrhenomanes* could colonize the entire rice root system, including the vascular stele, more quickly and massively compared to *P. graminicola* and *P. inflatum*. Its highly efficient colonization process seemed to be driven by its potential to utilize the most abundant amino acids and sugars in rice exudates and rice tissues, to feed on defense-related compounds in its host and to inhibit or postpone cell wall fortification. This, in combination with its stronger and faster induction of reactive oxygen species (ROS), phenolics and ultimately, necrosis, is probably the reason why *P. arrhenomanes* is able to induce more pronounced disease symptoms than the other two species.

In the second part of this dissertation, we focused on the hormonal network involved in the regulation of rice root responses to *Pythium* spp.. We first summarized the existing knowledge on the rice and Arabidopsis root immune system, to improve our understanding on biotic stress responses in plant roots. Salicylic acid (SA)- and jasmonic acid (JA)-controlled immune responses appeared to participate in the defense of both monocot and dicots to soil-borne pathogens, irrespective of the pathogen's lifestyle. ET-mediated immune responses also occur, depending on the plant group or the encountered microorganism. It is well-established that successful pathogens may evade effective immune responses by delivering effectors and hence, perturb hormone homeostasis and induce inappropriate defense responses. Based on ample evidence, we discovered that especially auxins (AUXs), cytokinins (CKs), brassinosteroids (BRs), abscisic acid (ABA) or gibberellins (GAs) might be recruited by soil-borne pathogens to suppress plant immunity and facilitate root invasion, colonization and the development of hypertrophic feeding sites.
Through genetic, physiological and pathological analyses we challenged the common assumption that BRs confer resistance against a broad-spectrum of pathogenic microorganisms and elucidated that *P. graminicola* employs BRs as virulence factors and interferes with the rice BR machinery to antagonize effectual SA- and GA-mediated defenses. Moreover, we proved that the immune-suppressive effect of BRs played downstream of SA biosynthesis, and upstream of the master defense regulators NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 OsNPR1 and OsWRKY45. GA-directed immune responses appeared counteracted at multiple levels resulting in the indirect stabilization of the rice DELLA protein SLENDER RICE1 (SLR1). Additional experiments with *P. graminicola* and *P. inflatum* suggest that manipulation of the steroid homeostasis in rice represents a conserved virulence strategy among rice-pathogenic *Pythium* spp..
Moreover, our results imply that more aggressive *Pythium* spp. exert stronger effects on the BR biosynthesis, by which they completely inhibit GA biosynthesis and possibly delay or repress cell wall fortification processes.

Aiming to further uncover the hormonal pathways recruited by rice-pathogenic *Pythium* spp., we explored the effect of exogenous JA and indole-3-acetic acid (IAA) on the disease development upon *Pythium* inoculation and examined the potential transcriptional reprogramming of several JA- and AUX-responsive genes in *Pythium*-inoculated rice roots. In contrast to many other root-pathogen interactions, JAs appeared not prominently involved in rice responses to *Pythium* spp.. Exogenous methyl jasmonate (MeJA) weakly induced resistance upon *P. arrhenomanes* inoculation, while it slightly promoted disease development in the case of *P. graminicola* and *P. inflatum*. The balance between JA signaling and other hormonal cascades is suggested to underpin the different outcomes of an activated JA pathway. Like several other soil-borne pathogens, *P. arrhenomanes, P. graminicola* and *P. inflatum* turned out to produce IAA and contrasting results from pathological analyses also unraveled the putative potential of the most virulent species to degrade IAA when it is abundantly present. Along with the strong influence of *P. arrhenomanes* on AUX signaling, we proposed that *Pythium* spp. try to maintain optimal IAA levels in their ecological niche to interfere with the AUX signaling cascade in their host and consequently, inflict disease.

In conclusion, this dissertation broadened the knowledge concerning rice root infections by oomycetes and helped to further disentangle virulence tactics of soil-borne pathogens.

Samenvatting

De opwarming van de aarde en de voortdurend stijgende wereldbevolking verminderen de hoeveelheid water dat beschikbaar is voor de landbouw. De traditionele rijstteelt in zogenaamde 'rijstpaddies', waarbij rijstvelden voortdurend onder water staan, verbruikt enorme hoeveelheden vers water waardoor de duurzaamheid ervan ernstig bedreigd wordt. Gezien deze rijstvelden voor 75% bijdragen tot de totale rijstproductie, vormt dit een groot probleem voor de toekomstige voedselveiligheid van miljarden mensen, voornamelijk in Azië. Verscheidene strategieën werden reeds ontwikkeld om het waterverbruik in rijstpaddies te verlagen, maar het ontwikkelingen van droogte-tolerante rijstcultivars bleek het meest veelbelovend. Het International Rice Research Institute (IRRI) in de Filippijnen en het Upland Rice Laboratory van de Chinese Agricultural University (CAU) in China hebben rijstcultivars ontwikkeld die, net zoals tarwe en mais, rechtstreeks gezaaid en gekweekt kunnen worden in aerobe gronden en een waterbesparing tot 50% toelaten in vergelijking met rijstpaddies. Omdat ze goed reageren op extra bemesting en irrigatie brengen aërobe rijstcultivars aanzienlijk meer op dan traditionele 'upland' cultivars en laten ze landbouwers ook toe de hoeveelheid irrigatiewater te verminderen zonder in productiviteit in te boeten. Helaas lijdt het aërobe rijstteeltsysteem aan opbrengstverliezen ten gevolge van autotoxiciteit, nematoden en/of nutriëntentekorten in Brazilië, Japan en de Filippijnen. Het IRRI heeft ook *Pythium* soorten gedetecteerd in Filipijnse aerobe rijstvelden, die worden verondersteld bij te dragen tot de oogstdalingen na monoteelt. Omdat *Pythium* soorten wereldwijd aanzienlijke verliezen veroorzaken in de teelt van economisch belangrijke gewassen en ze ook pathogeen zijn ten opzichte van rijstzaailingen, hebben we besloten om de *Pythium* soorten in Filipijnse aerobe rijstvelden te karakteriseren en hun interactie met rijstzaailingen grondig te bestuderen.

Negenennegentig *Pythium* isolaten werden verzameld in vier verschillende rijstvelden op het hoofdeiland Luzon van de Filipijnse eilandengroep. Gebruikmakend van pathogeniteitstesten en sequentie-informatie van de Internal Transcribed Spacer (ITS) region van het ribosomale DNA (rDNA) en het *β*-tubuline gen, konden vijf nauw verwante *Pythium* soorten geïdentificeerd worden waarvan er drie geassocieerd leken met de oogstverliezen in monogecultiveerde aërobe rijstvelden, met name *P. arrhenomanes* , *P. graminicola* en *P. inflatum*. *P. arrhenomanes* isolaten waren het meeste virulent en verminderden de groei van rijstzaailingen drastisch. Bovendien werden ze vaak aangetroffen in aërobe rijstvelden, wat het belang van deze soort met betrekking tot de optredende oogstverliezen sterk benadrukt. *P. graminicola* was minder schadelijk en vertoonde een intraspecifieke pathogene variabiliteit die niet gekoppeld bleek te zijn met variatie op het genetische niveau. Geen van de *P. inflatum* isolaten waren pathogeen, uitgezonderd bij hoge infectiedruk en wanneer infectie binnen de vier dagen na imbibitie tot stand kon komen. *P. inflatum* toonde een hogere intraspecifieke genetische variabiliteit, maar weerom bleek deze niet gecorreleerd te zijn met het fenotype. Daarom veronderstellen we dat rijst-pathogene *Pythium* soorten waarschijnlijk niet gastheer-specifiek zijn.

In overeenstemming met deze hypothese toonden we aan dat *P. arrhenomanes* isolaten van rijst en mais ziektesymptomen kunnen veroorzaken op beide gewassen, wat de duurzaamheid van een rijst-maïs vruchtwisseling in äerobe rijstvelden in vraag stelt.

Om de verschillende aggresiviteit van *P. arrhenomanes*, *P. graminicola* en *P. inflatum* beter te kunnen begrijpen, hebben we drie isolaten van elke soort geselecteerd voor een gedetailleerd, vergelijkend onderzoek naar hun interactie met rijstwortels. Uit histopathologische, moleculaire en phenoarray analyses leerden we dat *P. arrhenomanes* het hele rijstwortelstelsel, inclusief het vaatweefsel, veel sneller en massaler kon koloniseren dan *P. graminicola* en *P. inflatum*. Dit efficiënte kolonisatieproces leek aangedreven door het vermogen van *P. arrhenomanes* om de meest voorkomende aminozuren en suikers in rijstexudaten en rijstweefsels te gebruiken, om zich te voeden met afweer-gerelateerde componenten in zijn gastheer en om celwandversterking te onderdrukken of uit te stellen. Dit, in combinatie met de sterke en snelle inductie van reactieve zuurstof species, fenolen en, uiteindelijk, necrose, verklaart waarschijnlijk waarom *P. arrhenomanes* sterkere ziekteverschijnselen kan induceren dan de andere twee *Pythium* soorten.

In het tweede deel van dit proefschrift hebben we ons toegespitst op het hormonale netwerk dat betrokken is in de regulatie van rijstafweerreacties tegen *Pythium* soorten. We hebben eerst een samenvatting gemaakt van de literatuur rond het immuunsysteem in de wortels van rijst en Arabidopsis, om zo onze kennis over biotische stressreacties te verruimen. Salicylzuur- (SA) en jasmijnzuur (JA)-gestuurde afweerreacties bleken zowel in monocotylen als dicotylen bescherming te bieden tegen tal van bodempathogenen, ongeacht de levensstijl van de pathogeen. ET-gestuurde imuunresponsen bleken ook actief in plantafweer, afhankelijk van de plant-pathogeen interactie. Talrijke studies hebben aangetoond dat succesvolle pathogenen effectieve immuunreacties kunnen ontwijken door het produceren van effectoren die de hormoonhomeostase in de waardplant verstoren en inefficiënte verdedigingsreacties opwekken. Onze literatuurstudie toonde aan dat vooral auxines (AUXs), cytokinines (CK), brassinosteroïden (BRS), abscissinezuur (ABA) en/of gibberellines (GAs) doelwitten zijn van bodempathogenen om plantafweer te onderdrukken en wortelinvasie, wortelkolonisatie en de ontwikkeling van gespecialiseerde voedingsstructuren te vergemakkelijken.

Door middel van genetische, physiologische en pathologische studies hebben we bewezen dat *P. graminicola* BRs gebruikt als virulentiefactoren en interfereert met de BR-biosynthese en -signalisatie in rijst om doeltreffende SA- en GA-gestuurde verdedigingsmechanismen te neutraliseren. Dit druist in tegen de algemene veronderstelling dat BRs een breed-spectrum resistentie induceren. Bovendien hebben we gedemonstreerd dat het ziekte-verhogende effect van BRs zich downstream van de SA biosynthese afspeelt, en upstream van NONEXPRESSOR VAN PATHOGENESE-VERWANTE GENES1 OsNPR1 en OsWRKY45.

GA-gerichte immuunreacties leken geïnhibeerd op meerdere niveaus, wat onrechtreeks leidde tot de stabilisatie van het rijst DELLA eiwit, SLENDER RICE1 (SLR1). Op basis van aanvullende experimenten met *P. graminicola* en *P. inflatum* kunnen we speculeren dat de manipulatie van de steroïdenhomeostase in rijst waarschijnlijk een geconserveerde virulentiestrategie is binnen rijst-pathogene *Pythium* soorten. Bovendien suggereren onze resultaten dat agressievere *Pythium* soorten sterkere effecten uitoefenen op de BR biosynthese, waardoor ze de GA biosynthese volledig onderdrukken en celwandversterkingsprocessen mogelijks vertragen of inhiberen.

Met het oog op het verder ontrafelen van het hormonale netwerk dat betrokken is in de gevoeligheid van rijstzaailingen tegen *Pythium* soorten, werd het effect van exogeen JA en indool-3-azijnzuur (IAA) op de ziekteontwikkeling na *Pythium* inoculatie onderzocht en werd de vermoedelijke transcriptionele herprogrammering van verscheidene JA- en AUX-responsieve genen in *Pythium*-geïnoculeerde rijstwortels bestudeerd. In tegenstelling tot vele andere wortel-pathogeen interacties, bleken jasmonaten (JAs) niet prominent betrokken in rijstafweer tegen *Pythium* soorten. Exogeen methyljasmonaat (MeJA) bood slechts een zwakke weerstand tegen *P. arrhenomanes*, terwijl het de ziekteontwikkeling in het geval van *P. graminicola* en *P. inflatum* lichtjes bevorderde. Er wordt verondersteld dat het evenwicht tussen JA signalisatie en andere hormonale signaaltransductiewegen van belang is voor het effect van MeJA. Net als vele andere bodempathogenen, bleken *P. arrhenomanes*, *P. graminicola* en *P. inflatum* ook IAA te produceren. Contrasterende resultaten uit pathologische analyses laten vermoeden dat de meest virulente *Pythium* soorten in staat zijn om IAA te degraderen. Naast de vaststelling dat *P. arrhenomanes* een sterke invloed uitoefent op de AUX-signalisatie, doet dit alles vermoeden dat *Pythium* soorten optimale IAA-concentraties in hun omgeving proberen te handhaven om ziekte te veroorzaken.

Dit proefschrift heeft onze kennis verruimd betreffende interacties tussen rijstwortels en oomyceten. Daarnaast heeft het ook bijgedragen tot het ontrafelen van de virulentiestrategieën die bodempathogenen kunnen hanteren.

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RESEARCH EXPERIENCE

PhD at Ghent University (2008-present) (public defence planned in September-October 2013)
Subject: "*Pythium* spp. affecting aerobic rice cultivation in the Philippines: characterization, intraspecific variability, virulence strategies and plant defense"
Field: Phytopathology
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Scientific researcher at Ghent University (2007-2008)
Project: "Integrated disease management based on the life cycle of *Phytophthora porri*"
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Supervisor: Prof. Dr. ir. M. Höfte

EDUCATION

Doctoral training program, Ghent University (2007-2013)
VRTC summer school on advanced light microscopy (VIB), Personal efficiency, Academic English: writing skills, Effective scientific communication, …

Master in Bioscience engineering, cell and gene biotechnology, Ghent University (2004-2007)
Thesis: "Isolation and screening of *Trichoderma* spp. used for the biological control of *Pythium myriotylum* root rot on cocoyam"
Promotor: Prof. Dr. ir. M. Höfte

Bachelor in Bioscience engineering, University of Antwerp (2001-2004)

NATIONAL & INTERNATIONAL COLLABORATIONS

Research for Fair-Fruit (2013)
Diagnostic analysis on sugar snaps from Guatemala.

Research for BASF (2010-2012)
Screening of maize seed coatings active against root-infecting *Pythium* spp..

Research at the International Rice Research Institute (IRRI), The Philippines (September 2009)

Research at the Institute of Agricultural Research for Development (IRAD), Cameroun (July 2006)

TEACHING EXPERIENCE

Practical courses on Crop Protection and Molecular Phytopathology (2007-2012)

SUPERVISION OF UNDERGRADUATE STUDENTS

Arne Steelandt, "Onderzoek naar nieuwe bodemgebonden pathogenen bij rijst in Zuidoost-Azië" (2008-2009)

Liton Chandra Sen, "Role of Pythium spp. in yield decline in aerobic rice" (2008-2009)

Anneleen Volckaert, "*Pythium* spp. in de tropische aerobe rijstteelt in Zuidoost-Azië: rol van indool-3-aijnzuur in groeibevordering en pathogeniteit" (2009-2010)

PUBLICATION LIST

Peer reviewed

1. Declercq B, Van Buyten E, Claeys S, Cap N, De Nies J, Pollet S, Höfte M (2010). **"Molecular characterization of *Phytophthora porri* and closely related species and their pathogenicity on leek".** European Journal of Phytopathology, 127(3), 341-350.

2. Van Buyten E, Banaay CGB, Vera-Cruz C, Höfte M (2013). **"Identity and variability of *Pythium* spp. associated with yield decline in aerobic rice cultivation in the Philippines**". Plant Pathology, 62(1), 139-153.

3. De Vleesschauwer D, Van Buyten E, Satoh K, Balidion J, Mauleon R, Choi I, Vera-Cruz C, Kikuchi S, Höfte M (2012). **"Brassinosteroids antagonize gibberellin- and salicylate-mediated root immunity in rice".** Plant Physiology, 158(4), 1833-1846.

Submitted (under revision)

1. Van Buyten E, Höfte M (2013). **"*Pythium* spp. that affect rice roots differ in host colonization and nutritional profile.** Submitted to BMC Plant Biology.

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1. Van Buyten E, Steelandt A, Höfte M, 2009. ***Pythium* spp. in de tropsiche aerobe rijstteelt.** Gewasbescherming, 40(5), 254-255.

2. Van Buyten E, De Vleesschauwer D, Höfte M, 2013. **Virulentiestrategieën van *Pythium* soorten om rijstwortels aan te tasten.** Gewasbescherming, 44(2), 53.

CONFERENCE CONTRIBUTIONS, ORAL PRESENTATIONS

KNPV workshop on *Pythium & Phytophthora* (The Netherlands, March 2009)
Van Buyten E, Höfte M. Pythium *spp*. in de tropische aerobe rijstteelt: identificatie, intraspecifieke variabiliteit, pathogeniteit en biologische bestrijding.

62th International Symposium on Crop Protection (Belgium, May 2010)
Van Buyten E, De Vleesschauwer D, Vera Cruz CM, Höfte M. *Pythium* spp. in the tropical aerobic rice cultivation: identification, intraspecific variability, pathogenicity and disease resistance.

5th meeting of the Belgian Plant Biotechnology Association (Belgium, Nov 2011)
Van Buyten E, De Vleesschauwer D, Vera Cruz CM, Höfte M. Brassinosteroids shorthen the biotrophic phase of *Pythium* spp. affecting aerobic rice roots.

KNPV workshop on *Pythium & Phytophthora* (The Netherlands, April 2012)
Van Buyten E, Höfte M. Strategieën van *Pythium* soorten om het wortelstelsel van rijstzaailingen aan te tasten.

1st International Conference on Brassinosteroids (Barcelona, June 2012)
De Vleesschauwer D, Van Buyten E, Satoh K, Balidion J, Mauleon R, Choi I, Vera-Cruz C, Kikuchi S, Höfte M. Brassinosteroids antagonize gibberellin- and salicylate-mediated root immunity in rice.

CONFERENCE CONTRIBUTIONS, POSTER PRESENTATIONS

61th International Symposium on Crop Protection (Belgium, May 2009)**:**
Van Buyten E, Steelandt A and Höfte M. *Pythium* spp. in the tropical aerobic rice cultivation: identification, pathogenicity, intraspecific variability and biological control.

62th International Symposium on Crop Protection (Belgium, May 2010)
De Maeyer K, D'aes J, Khuong HG, Van Buyten E, Höfte M. Involvement of phenazines and cyclic lipopeptides in biocontrol of soil-borne pathogens by *Pseudomonas* sp. CMR12a depends on substrate characteristics and pathogen type.

5th meeting of the Belgian Plant Biotechnology Association (Belgium, Nov 2011)
Van Buyten E, De Vleesschauwer D, Vera Cruz CM, Höfte M. Brassinosteroids shorthen the biotrophic phase of *Pythium* spp. affecting aerobic rice roots.
1st International Conference on Brassinosteroids (Barcelona, June 2012)
Van Buyten E, De Vleesschauwer D, Vera Cruz CM, Höfte M. Brassinosteroids are involved in the susceptibility of rice roots against *Pythium* spp.

AWARDS

Second price in the Image J-contest of the VRTC summer school on advanced light microscopy (2011)
Place: Vlaams Instituut voor Biotechnology (VIB), Ghent
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