Promoters:  
**Prof. Dr. Godelieve Gheysen**  
Department of Molecular Biotechnology  
Ghent University, Belgium  

**Prof. Dr. ir. Tina Kyndt**  
Department of Molecular Biotechnology  
Ghent University, Belgium  

**Prof. Dr. Abdul Mannan Akanda**  
Department of Plant Pathology  
Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh  

Dean:  
**Prof. dr. ir. Marc Van Meirvenne**  

Rector:  
**Prof. Dr. Anne De Paepe**
Characterisation of the interaction between rice and the parasitic nematode *Ditylenchus angustus*

Shakhina Khanam

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) of Applied Biological Sciences
Karakterisatie van de interactie tussen rijst en de parasitaire nematode *Ditylenchus angustus*

Cover illustration: The picture of the front cover represents the susceptibility symptoms in rice caused by the nematode *Ditylenchus angustus* which is characterized by white patches at the leaf base.


ISBN-number: 978-90-5989-927-8

The author and the promotors give the authorization to consult and to copy parts of this work for personal use only. Any other use is limited by the laws of Copyright. Permission to reproduce any material contained in this work should be obtained from the author.

Promoters

Prof. dr. Godelieve Gheysen
Prof. dr. ir. Tina Kyndt
Prof. dr. Abdul Mannan Akanda

Author

Shakhina Khanam
Members of the examination committee

Prof. dr. ir. Patrick Van Damme (chairman)
Department of Plant Production
Tropical and Subtropical Agricultural and Ethnobotany Unit
Faculty of Bioscience Engineering, Ghent University

Prof. dr. ir. Monica Höfte (secretary)
Department of Crop protection
Faculty of BioScience Engineering, Ghent University

Prof. dr. Godelieve Gheysen (promoter)
Department of Molecular Biotechnology
Ghent University, Belgium

Prof. dr. ir. Tina Kyndt (promoter)
Department of Molecular Biotechnology
Ghent University, Belgium

Prof. dr. Abdul Mannan Akanda (promoter)
Department of Plant Pathology
Bangabandhu Sheikh Mujibur Rahman Agricultural University,
Bangladesh

Prof. dr. Sofie Goormachtig
Department of Plant Systems Biology,
Flanders Interuniversity Institute for Biotechnology (VIB)
Faculty of Science, Ghent University

Prof. dr. Dirk De Waele
Department of Biosystems,
Faculty of Bioscience Engineering, K. U. Leuven

Prof. dr. ir. Wim Wesemael
Crop Protection
Institute for Agricultural and Fisheries Research (ILVO) Merelbeke
Acknowledgements

First and foremost I would like to express my special appreciation and thanks to my promoter, Professor dr. Godelieve Gheysen, for giving me the oppurtunity to start my PhD research in her lab. She has been very kind and supportive since the days I began working the project, never losing her belief in me to finish this project successfully. I will remain ever thankful to her. My another promoter, Professor dr. ir. Tina Kyndt deserves a very special thanks. Her scientific knowledge, inspiring ideas, and enthusiasm about my research project was a real support to me over the last few years. I thank my local promoter, Professor dr. Abdul Mannan Akanda for his help and support, I received during the research period in Bangladesh.

I thank the members of the examination committee, Prof. dr. ir. Patrick Van Damme, Prof. dr. ir. Monica Höfte, Prof. dr. Sofie Goormachtig, Prof. dr. Dirk De Waele and Prof. dr. ir. Wim Wesemael.

I thank my lovely (ex-) colleagues of NEMA research group of the Department of Molecular Biotechnology for supporting and inspiring me in various ways. Thanks for the nice moments we spent together. Special thanks to Sofie and Fien for your kind help and cooperation.

The financial and administrative support rendered by VLIR-UOS, Belgium is highly acknowledged.

Lastly, I would like to thank my family for all their love and encouragement. For my parents who raised me with a love of science and supported me in all my pursuits. And most of all for my loving, supportive, encouraging, and patient husband whose faithful support during the final stages of this PhD is so appreciated. I express my heavenly love to my son Rahmi. My Bangladeshi friends live in and out of Belgium also deserve my sincerest thanks.

Shakhina Khanam
# Contents

List of abbreviations ........................................................................................................... 1

Chapter 1. General Introduction ......................................................................................... 3

1.1 Rice (*Oryza sativa* L.): the host .................................................................................. 4

1.1.1 Origin and geographical distribution ......................................................................... 4

1.1.2 Morphology and plant growth stages ......................................................................... 5

1.1.3 Types of rice ............................................................................................................... 6

1.1.4 Rice agro-ecosystem .................................................................................................. 6

1.1.5 Rice production in Bangladesh ................................................................................. 7

1.2 Nematodes: the pathogens ............................................................................................ 9

1.2.1 Nematodes of rice ..................................................................................................... 9

1.2.2 Nematode under study: *Ditylenchus angustus* ........................................................ 14

1.2.3 Management and control of plant parasitic nematodes ........................................... 19

1.3 The Plant immunity system .......................................................................................... 22

1.4 Induced resistance ......................................................................................................... 24

1.4.1 Systemic acquired resistance ..................................................................................... 25

1.4.2 Induced systemic defense .......................................................................................... 26

1.4.3. Priming defence ....................................................................................................... 28

1.4.4 Induced resistance signaling molecules against plant parasitic nematodes .......... 29

1.5 Mechanisms of resistance against plant parasitic nematodes ..................................... 31

1.6 Plant hormone signaling in defence .............................................................................. 32

1.6.1 Salicylate signaling pathway ....................................................................................... 32

1.6.2 Jasmonate signaling pathway .................................................................................... 34

1.6.3 Ethylene signaling pathway ....................................................................................... 35

1.6.4 Cross talk between hormones ................................................................................... 36

1.6.5 Role of hormones in rice-nematode interactions ..................................................... 39

1.7 Pathogenesis-related (PR) proteins ................................................................................ 41

1.8 Secondary metabolites .................................................................................................. 42

1.9 Biocontrol activity of *Bacillus* species ......................................................................... 43

2.0 Scope and outline of the thesis ....................................................................................... 46
Chapter 2. Identification of Bangladeshi rice varieties resistant to ufra disease caused by the nematode *Ditylenchus angustus* .......................................................... 48

2.1 Abstract ....................................................................................................................... 49
2.2 Introduction .................................................................................................................. 49
2.3 Results ........................................................................................................................ 51
   2.3.1 Relationship between the early scoring system and the number of nematodes inside the plant 51
   2.3.2 Response of rice genotypes to *D. angustus* in pot experiments: early scoring ............. 53
   2.3.3 Response of rice genotypes to *D. angustus* in pot experiments: late scoring ............ 54
   2.3.4 Confirmation of the resistant genotypes under field conditions in rainfed conditions ....... 58
2.4. Discussion .................................................................................................................. 59
2.5. Conclusion .................................................................................................................. 61
2.6 Materials and methods............................................................................................ 62
   2.6.1 Rice germplasm collection .................................................................................... 62
   2.6.2 Pot experiment ...................................................................................................... 62
   2.6.3 Field experiment .................................................................................................. 63
   2.6.4 Nematode culture ................................................................................................ 63
   2.6.5 Inoculum preparation and inoculation ..................................................................... 63
   2.6.6 Relationship between the early scoring system and the actual number of nematodes inside the plant ....................................................................................... 64
   2.6.7 Susceptibility/Resistance scoring methods ........................................................... 64
   2.6.8 Data analysis ........................................................................................................ 65

Chapter 3. The rice cultivar Manikpukha is resistant to the stem nematode *Ditylenchus angustus* due to post-infection mechanisms .............................................................. 66

3.2 Introduction .................................................................................................................. 67
3.3 Results ........................................................................................................................ 69
   3.3.1 *Ditylenchus angustus* invaded equally in resistant and susceptible rice genotypes ......... 69
   3.3.2 The Development of *D. angustus* is delayed in the resistant genotype compared to the susceptible genotypes ................................................................. 70
   3.3.3 *D. angustus* completely failed to reproduce within the resistant genotype ............... 72
3.4 Discussion .................................................................................................................... 73
3.5 Conclusion .................................................................................................................... 76
3.6. Materials and methods ............................................................................................ 76
   3.6.1 Rice genotypes ..................................................................................................... 76
Chapter 4. The resistant Manikpukha shows a rapid defense response to *Ditylenchus angustus* infection, with higher *PAL* expression and lignin accumulation .......................................................................................................................... 78

4.1 Abstract .................................................................................................................................................................................. 79

4.2 Introduction ................................................................................................................................................................................. 79

4.3 Results ....................................................................................................................................................................................... 81

4.3.1 Mutants or transgenic rice plants deficient in SA/JA/ET are more susceptible to *Ditylenchus angustus* infection ........................................................................................................................................................................... 81

4.3.2 Hormones and hormone inhibitors treatment confirm a positive role of the SA/JA/ET pathways in the control of *D. angustus* infection ................................................................................................................................................................. 82

4.3.3 Innate defence response in rice shoot tissues upon *D. angustus* infection ................................................................. 84

4.3.4. SA and JA accumulation upon nematode infection in the ufra susceptible Nipponbare and the resistant Manikpukha ................................................................................................................................................. 87

4.3.5. Lignin accumulation upon nematode infection in the ufra susceptible Nipponbare and the resistant Manikpukha ................................................................................................................................................. 88

4.4 Discussion .................................................................................................................................................................................... 89

4.5 Conclusion .................................................................................................................................................................................... 93

4.6 Materials and methods ................................................................................................................................................................. 93

4.6.1 Plant materials and growth condition ........................................................................................................................................ 93

4.6.2 Infection experiments ............................................................................................................................................................ 94

4.6.3 Chemical treatments ............................................................................................................................................................ 94

4.6.4 Data collection and statistical analysis .................................................................................................................................... 95

4.6.5 RNA extraction, and cDNA synthesis ...................................................................................................................................... 95

4.6.6 qRT-PCR ................................................................................................................................................................................ 96

4.6.7 SA and JA quantification ........................................................................................................................................................ 96

4.6.8 Lignin measurement ............................................................................................................................................................ 97

Chapter 5: Effect of *Bacillus velezensis* strain BSK isolated from Bangladesh on rice growth and defence against *Ditylenchus angustus* .................................................................................................................. 98

5.1 Abstract ....................................................................................................................................................................................... 99

5.2 Introduction .................................................................................................................................................................................... 99

5.3 Results ......................................................................................................................................................................................... 102
5.3.1 Effect of *Bacillus velezensis* strain BSK on rice growth ........................................ 102
5.3.2 Determination of *Ditylenchus angustus* infection in *Bacillus velezensis* strain BSK treated rice plants .......................................................... 103
5.3.2 Gene expression in systemic rice shoots upon *Bacillus velezensis* strain BSK inoculation.... 104
5.4 Discussion .......................................................................................................................... 105
5.5 Conclusion .......................................................................................................................... 108
5.6 Materials and methods ....................................................................................................... 109
  5.6.1 Plant materials and bacterial culture conditions ............................................................. 109
  5.6.2 Nematode culture ......................................................................................................... 109
  5.6.3 Growth analysis of rice treated with *Bacillus velezensis* strain BSK ............................. 109
  5.6.4 Infection experiments with *Ditylenchus angustus* ....................................................... 110
  5.6.5 Data collection and statistical analysis ......................................................................... 110
  5.6.6 RNA extraction, and cDNA synthesis ......................................................................... 111
  5.6.7 qRT-PCR ..................................................................................................................... 111

Chapter 6. General conclusion and perspectives ....................................................................... 113
  6.1 ‘Manikpukha’, a promising ufra resistant variety ............................................................... 114
  6.2 SA, JA and ET play a positive role in rice basal defence against the rice stem nematode *D. angustus* ........................................................................................................ 117
  6.3 *Bacillus velezensis* strain BSK, a potential bio-control agent against *D. angustus* .............. 118

Summary .................................................................................................................................. 121
Nederlandse samenvatting ......................................................................................................... 124
References ................................................................................................................................. 126
Curriculum Vitae ....................................................................................................................... 178
Appendix .................................................................................................................................. 179
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>amino cyclopropane carboxylate</td>
</tr>
<tr>
<td>ACO</td>
<td>aminocyclopropane-1-carboxylate oxidase gene</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOA</td>
<td>aminooxyacetic acid</td>
</tr>
<tr>
<td>AOS</td>
<td>allene oxide synthase</td>
</tr>
<tr>
<td>Avr</td>
<td>avirulence</td>
</tr>
<tr>
<td>BR</td>
<td>brassinosteroids</td>
</tr>
<tr>
<td>BTH</td>
<td>benzo(1,2,3)thiadiazole-7-carbothloic acid 5–methyl ester</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>dai</td>
<td>days after-inoculation</td>
</tr>
<tr>
<td>DIECA</td>
<td>ammonium diethylthiocarbamic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>dpi</td>
<td>days post inoculation</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiotreitol</td>
</tr>
<tr>
<td>Eth</td>
<td>ethephon</td>
</tr>
<tr>
<td>ERF</td>
<td>ethylene response factor</td>
</tr>
<tr>
<td>ET</td>
<td>ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>effector-triggered immunity</td>
</tr>
<tr>
<td>ETS</td>
<td>effector-triggered susceptibility</td>
</tr>
<tr>
<td>FLS2</td>
<td>FLAGELLIN-SENSING 2</td>
</tr>
<tr>
<td>F-primer</td>
<td>forward primer</td>
</tr>
<tr>
<td>HR</td>
<td>hypersensitive response</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>ICS</td>
<td>isochorismate synthase</td>
</tr>
<tr>
<td>ISR</td>
<td>induced systemic resistance</td>
</tr>
<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LOX</td>
<td>lipoxygenase</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe Associated Molecular Pattern</td>
</tr>
<tr>
<td>M(A)PK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MeJA</td>
<td>methyl jasmonate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NahG</td>
<td>salicylate hydroxylase</td>
</tr>
<tr>
<td>NB-LRR</td>
<td>Nucleotide binding-leucine rich repeat</td>
</tr>
<tr>
<td>NH1</td>
<td>NPR1 homolog 1</td>
</tr>
<tr>
<td>NPR1</td>
<td>non-expressor of PR proteins 1</td>
</tr>
<tr>
<td>OPR</td>
<td>Phytodienoic acid reductase</td>
</tr>
<tr>
<td>PAL</td>
<td>phenylalanine ammonia lyase</td>
</tr>
<tr>
<td>PGPR</td>
<td>plant growth promoting rhizobacteria</td>
</tr>
<tr>
<td>PGPF</td>
<td>Plant growth promoting fungi</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDF1.2</td>
<td>plant defensing 1.2</td>
</tr>
<tr>
<td>PPN</td>
<td>plant parasitic nematode</td>
</tr>
<tr>
<td>PR</td>
<td>pathogenesis-related</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP-triggered immunity</td>
</tr>
<tr>
<td>RKN</td>
<td>root-knot nematode</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>R-primer</td>
<td>reverse primer</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>systematic acquired resistance</td>
</tr>
<tr>
<td>TFs</td>
<td>Transcription factors</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Chapter 1. General Introduction
1.1 Rice (*Oryza sativa* L.): the host

1.1.1 Origin and geographical distribution

Rice (*Oryza sativa* L.) was domesticated in South and Southeast Asia but its origin and domestication process are contentious and have long been argued. One genetic evidence indicated that the two main subspecies of Asian rice, indica and japonica originate from a single domestication of wild rice *O. rufipogon* about 8,200–13,500 years ago, in the region of Yangtze Valley of China (Molina *et al.*, 2011). Another genetic study showed that *O. sativa* subsp. *japonica* was first domesticated from *O. rufipogon* around the central area of the Pearl River in southern China and *O. sativa* subsp. *indica* rice was later developed from crosses between japonica rice and local wild rice which was spread into South East and South Asia (Huang *et al.*, 2012). The monsoonal rain, the warm and humid environment and the specific physiography of the Yangtze Valley of China offered a perfect environment for this rice domestication. Now rice is cultivated throughout the tropical and subtropical regions of the world between 55 N and 36 S latitudes (Fig 1.1). Rice can inhabit a variety of ecosystems, and tolerate extremes in sun exposure and moisture levels (Vaughan, 1994). The most favourable temperature for rice growth, development and reproduction is 30°C during daytime and 20°C during night time. Heavy soils such as clay, clay loam and loam that have a high water holding capacity with pH ranging from 5 to 8 are suitable for rice cultivation (Warrier *et al.*, 2011).

![Fig 1.1 Map showing rice (*Oryza sativa* L.) cultivation area around the world. The area in red shows the centre of origin of rice. (Source of the figure: http://www.targetmap.com/viewer.aspx?reportId=17614)](image-url)
1.1.2 Morphology and plant growth stages

The rice plant is an annual grass consisting of roots, round hollow jointed culms, leaves and a terminal panicle. The root system consists of two main types: crown roots (mat roots and ordinary roots) and nodal roots (Fig 1.2). After seed germination, small roots called seminal roots or embryonic roots are observed for a short period of time and they are later replaced by the crown roots. Crown roots develop below the soil surface, they are fibrous and produce root hairs to absorb water and nutrients. Nodal roots develop from the nodes above the soil surface and can often be found in deep water rice. The rice stem or culm is made up of a series of nodes and internodes in alternating order. The internode is hollow and the length of internode generally increases from the lower to the upper part of the stem. The node is the solid portion that bears a leaf and a bud which can grow into a tiller. An inflorescence or panicle is the terminal component of the rice tiller. The panicle bears rice spikelets, which develop into grains. A spikelet consists of the pedicel and the floret. A floret includes the lemma, palea and the flower. A flower consists of a pistil (female organ) and six stamens (male organs). The rice grain is the ripened ovary, with the lemma and palea firmly attached to it (IRRI, 2007a).

![Fig 1.2 The morphology of a rice plant (Source of the figure: http://www.rkmp.co.in/content/morphology-of-rice-plant-copy).](image)

The growth of the rice plant can be divided into three main stages: 1. vegetative (germination to panicle initiation), 2. reproductive (panicle initiation to flowering) and 3. ripening (flowering to mature grain). The growth duration of a rice plant depends on the variety and growing environment.
Traditional rice varieties require about 150 days whereas the modern, high yielding and early maturing varieties require 90 days to reach the mature grain stage.

### 1.1.3 Types of rice

The genus *Oryza* contains two cultivated and twenty-one wild species. The two cultivated species of rice are: *O. sativa*, the Asian rice which is widely grown and *O. glaberrima*, the African rice, which is grown in West Africa. Most rice cultivars can be positioned within two major subspecies of *O. sativa*: subsp. *japonica* and subsp. *indica*, which differ in physiology and morphological traits (Londo *et al.*, 2006). Indica rice is usually found in the lowlands of tropical Asia, whereas japonica rice is typically found in the upland hills of southern China, northeast Asia, Southeast Asia and Indonesia, as well as in regions outside Asia such as, Africa, North America, Europe and South America (Khush, 1997). Indica plants have broad to narrow, light green leaves with tall to intermediate plant stature, grains are long to short, slender and spikelets are awnless. Indica grains shatter easily and the amylose content in the seed is 23-31%. On the other hand, Japonica has narrow, dark green leaves, medium-height tillers. Grains are short, roundish with awnless to long awned spikelets. Japonica grains do not shatter easily and amylose content in the grain is 0-20% (IRRI, 2007b). New promising African upland rice varieties called NERICA have been developed through crossing African rice varieties and Asian rice varieties.

### 1.1.4 Rice agro-ecosystem

Rice agro-ecosystems can be divided into four major ecosystems, based on hydrological characteristics: 1. irrigated, 2. rainfed upland, 3. rainfed lowland and 4. Flood-prone rice (Khush, 1997). Irrigated rice is divided into irrigated wet season area and irrigated dry season area. Irrigated rice is grown in bunded fields with irrigation at regular intervals to maintain a layer of 5-10 cm standing water in the field. This system is the most important rice production system in Asia. The irrigated ecosystem accounts for 50% of the harvested rice area (Fig 1.3) which provides 75% of the world’s rice production. The rice productivity is higher in the irrigated system compared to other ecosystems, with an average yield vary from about 3 t/ha to 10 t/ha (FAO, 2016).

Rainfed lowland rice is grown in river deltas and coastal areas mainly in South Asia, parts of Southeast Asia, and Africa. This rice is grown in bunded fields. This ecosystem is more subject
to flooding and drought compared to the irrigated rice ecosystems because of lack of water supply and/or water control for irrigation. Approximately 34% of rice is grown in this ecosystem (Fig 1.3), which covers 20% of the world’s rice production. The rice productivity is very low due to the lack of improved management practices, with an average yield of about 1.5–2.5 t/ha (FAO, 2016).

Rainfed upland rice is grown in un-bunded field in Asia, Latin America and Africa. In this ecosystem, water supply relies only on rainfall resulting in mostly aerobic soil conditions. This ecosystem accounts for 9% of the harvested rice area (Fig 1.3) which provides 4% of the world’s total rice production. Rice yields in upland systems are generally low, averaging 1 to 2 t/ha (FAO, 2016).

The flood prone ecosystem is divided into the deep water rice and the intermediate rainfed lowland. Special rice types in this ecosystem are deep water rice, tidal rice, floating rice and submergence tolerant rice. Flooding occurs during part of the growing season and the water depth varies from 50 cm to more than 3m. This flood prone rice grown in low-lying lands in river deltas of South and Southeast Asia accounts for 7% of the harvested rice area from which 3% area is covered with deep water rice.

Fig 1.3 The percentage of rice area under the different ecosystems (IRRI, 2007c).

1.1.5 Rice production in Bangladesh

Rice is the second most important cereal crop in the world. The total annual rice production in the world is 741.3 million tonnes (494.4 million tonnes, milled basis) (FAO, 2015) where over 90% of
the world’s rice is being produced in Asia (Fig 1.4). In Bangladesh, rice is a staple food for about 162 million people. The rice sector in Bangladesh contributes one-half of the agricultural gross domestic product (GDP) and one-sixth of the national income.

In Bangladesh, rice is planted in about 75% of the total cropped area and over 80% of the total irrigated area. All rice varieties in Bangladesh are grouped into five distinct ecotypes 1. Boro, 2. Transplanted Aus (T. Aus), 3. Transplanted Aman (T. Aman), 4. Upland Aus (direct-seeded Aus) and 5. Deepwater rice (floating rice). Boro rice is grown completely under the irrigated ecosystem during the dry period (November to July) while T. Aman (during July to December), T. Aus (during April to August) and Upland rice (during March to July) are grown under the rainfed ecosystem (Sattar, 2005). In 2015, the rice harvested area in Bangladesh reached 12 million ha from which 52 million ton of rice is produced (34 million tonnes, milled basis). The production of Aman and Boro was 19.8 million and 29.0 million tonnes (13.2 million and 19.3 million tonnes, milled basis) respectively. The average yield of rice is 4.3 t/ha (USDA, 2015). Bangladesh is one of the top ten rice producing countries of the world (Fig 1.4, FAO, 2015). The rice production in Bangladesh has risen steadily from 10.59 million tons in the year 1971 to 52 million tons in 2015. This increasing rate of rice production is mainly due to the adoption of modern high yielding rice varieties. The Bangladesh Rice Research Institute (BRRI) has developed 72 high yielding modern rice varieties and four hybrid rice varieties so far. These BRRI released high yielding varieties cover 56% of the total rice area which contributes about 74% of the total annual rice production of the country.
1.2 Nematodes: the pathogens

The nematodes or roundworms under the phylum Nematoda (De Ley & Blaxter, 2002) are pseudocoelomate, unsegmented worm-like animals. Nematodes are the most numerous Metazoa on earth, occurring in almost every habitat (Cobb, 1915). Till now over 25,000 nematode species have been described (Hodda, 2011; Zhang, 2013). They undergo four molts from juvenile to the adult phase. Based on small subunit ribosomal RNA phylogenetics (Blaxter et al., 1998), the phylum nematoda was divided into five different clades. In a recent update, the phylum was divided over twelve clades (Van Megan et al., 2009). This molecular phylogenetic analysis also indicated that the nematodes have evolved several times the ability to parasitize animals and plants during their evolution (Blaxter et al., 1998). Nematodes are either free living, feeding on bacteria, fungi, protozoa or parasites of animals (44% of the described species) and plants (17% of the described species).

Plant parasitic nematodes are recognized as major agricultural pathogens and cause crop losses throughout the world. Damage caused by plant plant nematodes has been estimated at $US80 billion per year (Nicol et al., 2011). The first described plant parasitic nematode was the wheat seed gall nematode *Anguina tritici*, discovered by Needham in 1743 in wheat seed. Till now about 4100 plant parasitic nematode species have been described (Decraemer & Hunt, 2006). Plant parasitic nematode feed on all part of the plant mostly by piercing the plant cell wall with a hollow, retractable, needle-like mouth spear called stylet. Plant parasitic nematodes cause significant yield losses in rice, ranging from 10% to 25% worldwide (Bridge et al., 2005).

Based on the parasitic strategies, plant parasitic nematodes can be classified into two major groups: ectoparasitic nematodes and endoparasitic nematodes. Each group can be further divided into migratory and (semi) sedentary plant parasitic nematodes according to their feeding strategies. Endoparasitic nematodes can be further divided into migratory or sedentary nematodes.

1.2.1 Nematodes of rice

Here, the focus will be on plant parasitic nematodes that are able to infect rice. More than 200 species of plant parasitic nematodes have been reported that parasitize rice (Prot, 1994). The distribution of some nematodes is geographically restricted, while others occur throughout the rice
growing areas of the world. Nematode parasites on rice may be divided into foliar parasites and root parasites.

**Root parasites**

**a) Ectoparasitic nematodes**

Ectoparasitic nematodes remain outside of the plant tissue throughout their life cycle and feed on the cytoplasm by using the stylet to puncture plant cells. The longer the stylet the deeper they can feed within plant tissues. Several species of *Tylenchorhynchus* (*T. marlini, T. mashhoodi, T. brassicae, T. annulatus*), *Criconemoides* (*C. onoensis, C. rustica, C. komanaensis*), *Helicotylenchus* (*H. pseudorobustus, H. exallus*) have been reported in rice. This type of nematodes feeds on root hairs and/or epidermal cells and the damage is limited to necrosis of the cells.

**b) Endoparasitic nematodes**

**Migratory endoparasitic nematodes**

In this type of parasitism, the entire nematode penetrates the root tissues. All life stages of the nematodes can be found in the soil or in the plant tissues. They have no fixed feeding site within plant tissues but feed while migrating through plant cells (Fig. 1.5A). Aboveground symptoms include stunting, leaf chlorosis, reduction of tillering and wilting. In the root, they cause cell necrosis and cavities, and as a result infected roots turn brown and rot. The most important migratory endoparasitic nematodes of rice belong to the genus *Hirschmanniella*, known as the rice root nematodes. Several species of *Hirschmanniella* (*H. belli, H. oryzae, H. gracilis, H. imamuri, H. mucronata*) have been reported from irrigated, lowland and deep water rice with the most commonly reported species being *H. oryzae*. Another type of migratory nematode that parasitizes rice is *Pratylenchus* spp. known as root-lesion nematode. *P. zeae* and *P. brachyurus* are the most common *Pratylenchus* species recorded on rice.
Sedentary endoparasitic nematodes

Sedentary nematodes establish a permanent feeding site within the host plant where they reside for almost their whole life cycle. Among sedentary endoparasites, the two most damaging nematodes are the root-knot (*Meloidogyne* spp.) and cyst nematode (*Heterodera* spp.).

**Root-knot nematodes (RKN)**

A number of *Meloidogyne* spp. (*M. graminicola*, *M. oryzae*, *M. incognita*, *M. javanica*, *M. arenaria*, *M. salas*) have been recorded that parasitize rice throughout the rice growing areas. Among these, *M. graminicola* and *M. incognita* are the most damaging, causing up to 70% yield losses in rice at the field level (Bridge *et al.*, 2005). The infective second stage juveniles (J2s) of root-knot nematodes are attracted to plant roots and penetrate the root at the cell elongation region just behind the root tip. After penetration, J2s migrate intercellularly until they have found a suitable cell to initiate feeding site formation. The feeding sites formed by root-knot nematodes are called giant cells, formed by nuclear divisions without cell division. Hyperplasy and hypertrophy of the surrounding cells leads to the formation of a gall or root-knot (Jones & Payne, 1978; Gheysen & Jones, 2006). J2s feed on the giant cells, and undergo three moults to become pyriform shaped females, which lay eggs inside the root tissues. The life cycle of a root knot nematode is presented in fig 1.5B. The galls produced by *M. graminicola* at the root tip of young plant are hook shaped. The above ground symptoms caused by the nematodes are chlorosis, stunting, reduction of tillering, unfilled spikelets and poor yield. *M. graminicola* can survive in waterlogged soil whereas *M. incognita* cannot survive long periods under flooded conditions (Bridge & Page, 1982). *M. graminicola* can survive in waterlogged soil as eggs in egg masses or juveniles for a long period; juveniles can survive for at least 5 months (Bridge & Page, 1982) and some egg masses can remain viable for at least 14 months (Roy, 1982). Juveniles cannot invade the rice roots in flooded conditions but immediately invade the roots when the water is drained (Bridge & Page, 1982). In contrast to *M. graminicola*-galls, the galls produced by *M. incognita* are not hook-shaped and females lay their egg masses on the root surface.
Cyst nematodes

Four species of cyst nematodes have been reported from upland and irrigated rice fields. *Heterodera sacchari* is commonly found in West Africa and Trinidad, *H. elachista* in Japan, *H. oryzae* in West Africa and Bangladesh and *H. oryzicola* in India (Fortuner & Merny, 1979). The life cycle of cyst nematodes is similar to root-knot nematode. J2s of cyst nematodes penetrate the root tissue and migrate to the vascular cylinder to induce a feeding site called a syncytium. A syncytium is a large multinucleate cell formed by widening of plasmodesmata and cell wall degradation by plant enzymes, resulting in the fusion of adjacent protoplasts of over hundred cells (Grundler, 1998). The J2s feed from the syncytium and undergo three molts to become adults, the adult female is fertilized by the male. Some of the eggs are deposited outside the female’s body in a gelatinous matrix, and the remaining eggs are retained within the female’s body. The female body of cyst nematodes becomes tanned and hard to form the typical cyst. The life cycle of a cyst nematode is presented in fig 1.5C. Symptoms caused by cyst nematode infection on rice include stunting, chorosis, reduction of tillers, root necrosis, early flowering, and partial filling of kernels (Coyne & Plowright, 2000). Yield losses caused by *H. elachista* and *H. oryzicola* have been reported to be 20% and 40% respectively (Bridge *et al*., 2005).
C) Semi-endoparasitic nematodes

The reniform nematode, *Rotylenchulus reniformis*, a sedentary semi-endoparasite, was found to be associated with upland rice (Villanueva *et al.*, 1992) but it is not a common parasite of rice (Bridge *et al.*, 2005). The young females (infective stage) penetrate the plant roots, inserting about one-third of the anterior body, and they establish feeding sites on endodermal and pericycle cells which are similar to the syncytia of cyst nematodes. After feeding, the posterior part of the female swells near the vulval region and the female body outside the root takes a bean or kidney shape. The uterine glands produce a gelatinous matrix into which eggs are laid.

Foliar parasite

The foliar parasitic nematodes on rice include two important species: *Aphelenchoides besseyi* and *Ditylenchus angustus*.

*Aphelenchoides besseyi*

*A. besseyi* is globally distributed and causes white tip disease in rice. It is one of the few seed borne nematodes and it is restricted to deep water, irrigated and flooded rice systems. The nematodes feed ectoparasitically on the growing point of the plant. With the development of the plant, the nematodes move to the developing panicles, spikelets, and feed on the plant’s reproductive structures, eventually settle in the rice seeds (Huang & Huang, 1972; Fig 1.6). They remain coiled and desiccated inside the grains until they become again active when the crop is sown. The nematodes complete their lifecycle within 8–12 days at 30°C. The nematode causes characteristic whitening of the top 3 to 5 cm of the leaf tips (Bridge *et al.*, 2005). The flag leaf becomes crinkled and distorted, infected seeds stay small and distorted with necrotic lesions. The yield losses due to these nematodes vary with country, locality and rice environment (Bridge *et al.*, 2005).

*Ditylenchus angustus*

The other major foliar nematode in rice is *Ditylenchus angustus*, which is mostly limited to the south and southeast of Asia (Butler, 1919; Bridge *et al.*, 2005). Because this nematode was studied in this research, it is described in more detail in the subsequent paragraph.
1.2.2 Nematode under study: *Ditylenchus angustus*

**Scientific classification**

Phylum: Nematoda  
Class: Tylenchoidea  
Order: Tylenchida  
Family: Anguinidae  
Genus: *Ditylenchus*  
Species: *Ditylenchus angustus* (Butler, 1913) Filipjev, 1936

*Ditylenchus angustus* was first reported by Butler (1913) in Bangladesh (former East Bengal) and was named *Tylenchus angustus*. In 1932, Goodey transferred the genus to *Anguillulina* which was finally transferred to the genus *Ditylenchus* by Filipjev (1936).

**Common name**

The preferred common name for *D. angustus* is the rice stem nematode. The disease caused by this nematode in rice is known as ufra disease, hence this nematode is also referred to as the ufra nematode. In Bangladesh, locally this disease is called as “dak pora”. In Myanmar this nematode is known as “akhet-pet”, in Vietnam as “tiem dot san”, in Thailand as “yadngo” (CABI/EPPO, 1999).
Chapter 1                                                                                                         General introduction

Morphological features

Measurements of the critical morphological features were reported by many investigators (Butler, 1913; Goodey, 1932; Mian & Latif, 1994; Ibrahim & Perry, 1994; Das & Bajaj, 2008). Mian & Latif (1994) provided measurements of Bangladeshi isolates of *D. angustus* and according to them the length of the female is 1.0–1.2mm and the male is 0.9–1.1mm. General body morphology in case of female and male nematodes is similar. Males are as numerous as females. The nematode body is slender with fine transverse striated cuticle. The lip region is flat, unstriated, the set off from the body is not distinct. The cephalic region is lightly sclerotized, hexaradiate with six equal lips. The nematode stylet is moderately developed, the conus is attenuated, about 45% of total stylet length, the knob is small but distinct. Lateral fields are one-fourth of body width or slightly less, with 4 incisures, extending almost to the tail tip. Deirids are immediately posterior to the level of excretory pore. Phasmids are close behind the mid-part of the tail, pore-like, difficult to see. The procorpus is cylindrical, the median oesophageal bulb oval, with a distinct valvular apparatus, the isthmus narrow, cylindrical, slightly overlapping the intestine mainly on the ventral side, cardia are absent. The nerve ring is conspicuous, behind the median oesophageal bulb (Fig 1.7).

In case of female, the vulva is a transverse slit, the vaginal tube oblique, reaching more than halfway across the body. The spermathecal is very elongated, filled with large rounded sperms. The anterior ovary is outstretched with oocytes in a single row. The post-uterine sac is collapsed, without sperms. The tail is conoid, tapering to a sharply pointed terminus resembling a mucro (Fig 1.7).

In case of male, spicules is ventrally curved, simple; the gubernaculum is short and simple. A bursa is present, extending almost to the tail tip (Fig 1.7).

Juveniles are similar to adults in gross morphology, with the oesophagus proportionally longer than in adults. The size range of second stage juveniles (J2) is 0.28-0.43 mm, the third stage juveniles (J3) is 0.43-0.63 mm and fourth stage juveniles (J4) is from 0.63 to 0.87 mm.
Fig 1.7 *Ditylenchus angustus* (Butler, 1913) Filipjev, 1936. A. Female B. Female oesophageal region C. En-face view D. Cephalic framework E. Female cross section at mid-body F. Female tail G. Lateral field at mid-body H. Juvenile tail I. Male tail, lateral view J. Male tail, ventral view K. Female vulval region. Taken from Seshadri & Dasgupta (1975).

**Biology and Life cycle**

All life stages of *D. angustus* are infective although the J4 stage shows the highest infectivity (Plowright & Gill, 1994). The nematode feeds mainly as ectoparasite but endoparasitic behaviour has also been observed (Singh *et al*., 2013 and personal observations: Fig 1.8). The nematode enters the plant mainly at the collar region, migrates upward with the growth of the shoot and feeds on newly forming tissues in the rolled leaf sheath (Fig 1.6). At rice harvest, the nematodes remain in a coiled, quiescent state, mainly in the dried glumes of the lower panicle spikelets (Latif *et al*., 2006), in crop residues (Cox & Rahman, 1979; Kinh, 1981), soil (Cuc, 1982a), seeds (Ibrahim & Perry, 1993; Prasad & Varaprasad, 2002) and weed host (Latif *et al*., 2006). Although the presence of living nematodes in the grains has been reported (Prasad & Varaprasad, 2002), the chance of transmission by seed is very unlikely when the seeds are properly sun dried to a moisture content between 12 and 14% (Bridge & Starr, 2007; Ibrahim & Perry, 1993). It has been reported that the
nematode can survive in desiccated conditions for several months in infested plant materials (Butler, 1913; Cox & Rahman, 1979; Ibrahim & Perry, 1993). The J4 has superior survival attributes compared to all other stages (Ibrahim & Perry, 1993). The activity and infectivity of the nematode resumes as the water content of the field increases. Dissemination of nematodes from field to field is mainly through irrigation water (Sein & Zan, 1977; Rahman & Evans, 1987) and tidal fresh water inundation in deep water rice. Stem or leaf contact under high humidity also causes nematode spreading (Rahman & Evans, 1987). The nematodes can invade a host within one hour (Rahman, 2003).

Reproduction is amphimictic and the nematode completes its life cycle within 10-20 days at 27-30°C (Bridge & Starr, 2007). The nematodes may complete at least three generations within one growing season (Rahman, 2003). The females start to oviposit 1 day after reaching adulthood (Ali & Ishibashi, 1996). The females lay 50 to 100 eggs in their lifetime (Rahman, 2003). At 24–26°C, the eggs are deposited at the two-celled stage. The J1 molts into J2 within the egg and hatches spontaneously in water without host stimuli (Ali & Ishibashi, 1996; Ali et al., 1995). The J2 and J3 require one day each, while the J4 stage requires two days to become adults (Ali et al., 1997).

![Fig 1.8 Ditylenchus angustus and eggs inside a rice leaf stained with acid fuchsin, revealing endoparasitic behaviour.](image)

**Signs and symptoms**

In the vegetative stage of the crop, *D. angustus* infestation is characterized by malformations and white patches, or speckles in a splash pattern at the leaf base (Fig 1.9A). Brownish stains may develop on leaves and leaf sheaths, and the area of the upper internodes of the stem turn dark brown (Rahman, 2003). Infected leaf bases and sheath become twisted and distorted (Fig 1.9B). Sometimes, lower nodes become swollen with irregular branching giving the appearance of a bushy plant (Rahman, 2003).
At the reproductive stage, the nematodes move upwards to feed on the ear primordia and the developing ear heads. The panicle’s head and flag leaves become twisted and distorted. Depending on the time and degree of infestation, panicles often remain completely enclosed within a swollen sheath or partially emerge or without filled grains (Bridge et al., 2005; Rahman, 2003). Based on the extent of panicle emergence, Cox & Rahman (1980) identified three types of ufra disease: 1. Ufra I (panicle fails to emerge and completely enclosed by the leaf sheath), 2. Ufra II (panicles partially emerge with or without filled grains), 3. Ufra III (panicles fully emerge without filled grains) (Fig 1.10). In the field, the disease looks as dark brown patches (Bridge et al., 2005; Cox & Rahman, 1980). Diseased plants can be differentiated from healthy plants by their erect posture.

Fig 1.9 Rice plant infected by *Ditylenchus angustus* A. white patches at the leaf base B. twisted and distorted panicles and flag leaf.

Fig 1.10 Rice plants infected by *Ditylenchus angustus* A. Ufra I (panicle fails to emerge and completely enclosed by the leaf sheath), B. Ufra II (panicles partially emerge with or without filled grains) and C. Ufra III (panicles fully emerge without filled grains). Source of images: http://www.knowledgebank-brri.org/rice_disease.php
Known hosts

*D. angustus* is an obligate parasite that infects cultivated and wild rice species (*Oryza sativa*, *O. alta*, *O. cubensis*, *O. eichingeri*, *O. glaberrima*, *O. latifolia*, *O. meyeriana*, *O. minuta*, *O. nivara*, *O. perennis*, *O. officinalis*, *O. rufipogon*) (McGeachie & Rahman, 1983; Bridge et al., 1990) and a few weed species (*Leersia hexandra*, *Echinocloa colona*, *Sacciolepsis interrupta*) (Vuong & Rabarijoela, 1968; Cuc & Giang, 1982b).

Economic importance

The disease mainly occurs in deep water rice (Butler, 1919). However together with the shrinkage of deep water rice cultivation and increased production of irrigated and rainfed rice, infections caused by *D. angustus* also appear in these conditions (Prasad et al., 2000; Latif et al., 2004). Nevertheless, the economic impact caused by this nematode in the world is relatively low (Rahman, 2003) because of its restricted distribution (Bridge et al., 2005). But if the nematode occurs, yield loss can reach 100% in deepwater, irrigated and lowland rice (Cuc & Kinh, 1981). Yield loss studies in different countries have demonstrated the devastation caused by the nematode (Rao et al., 1986; Hashioka, 1963; Cuc & Kinh, 1981). For instance, in Bangladesh, 40 to 49% or sporadically 90% yield losses have been reported (Latif et al., 2011a; Latif et al., 2011b). Even a low infection percentage in transplanted seedlings can cause substantial yield losses (Bridge & Starr, 2007).

1.2.3 Management and control of plant parasitic nematodes

Preventive

Phytosanitary measures are of major importance to reduce the economic loss caused by plant-parasitic nematodes. According to the International Plant Protection Convention (IPPC), phytosanitary measures include any regulation, legislation or procedure to prevent the introduction and/or spread of a plant pest. Certification of plant propagating materials, clean machinery, and controlled irrigation water can reduce the nematode dissemination. Phytosanitary approaches appear to be effective in managing *D. angustus*. Burning of infested stubble, straw, ratoon crops, and control of water flow are effective to reduce ufra disease (Rahman, 2003).
Cultural control

Cultural control practices can play an important role for PPN management. Cultural control of PPN encompasses crop rotation, hot water treatment, dry heating, multiple cropping, weed control, soil amendment, cover crops and flooding. The success of optimum use of cultural practices depends on the understanding of the target nematode including its identification, hosts, and environmental preferences. Lengthening the overwinter period of *D. angustus* can decrease the nematodes as well as the nematode infectivity (Cox & Rahman, 1980; Das & Bhagawati, 1992). As *D. angustus* has a relatively narrow host range compared to other root knot nematodes, removal of wild rice and other weed hosts can reduce the chances of nematode survival between crops. Late sowing, earlier harvesting by using short duration cultivars can prolong the time that the nematodes should survive in the absence of a host plant (Rahman, 2003). Crop rotation is also a useful approach in the management of ufra disease. Rotation of rice with non-host crops such as jute or mustard can reduce ufra incidence (Chakraborti, 2000; Miah & Rahman, 1985; Rahman, 2003). Application of different organic amendments such as neem (*Azadirachta indica*), leaf dust, neem cake, neem seed dust, mustard (*Brassica nigra*) cake, sesame (*Sesamum indicum*) cake, jute (*Corchorus olitorius*) seed dust, biskatali (*Polygonum hydropiper*) leaf dust, bankalmi (*Ipomoea sepiaria*) leaf dust can significantly reduce the ufra severity (Latif *et al*., 2006).

Chemical control

Nematicides are applied to reduce plant damage by reducing nematode invasion in the plant root or to reduce the transmission of nematode-borne viruses to the plant. It is used as part of integrated management approach or as the sole component. Nematicides can be classified according to their chemical group (e.g. carbamates, organophosphates), mode of application (e.g. fumigant, non-fumigant) and mode of action (e.g. acetylcholinesterase). Several nematicides have been used effectively to manage ufra nematode. The use of carbofuran, benomyl (Miah & Rahman, 1985), Miral (isazofos) and Tecto 60FL (thiabendazole) (Mondal & Miah, 1987), fenamiphos and disulfoton (Rahman & Miah, 1985), carbosulfan and triazophos (Das, 1997) resulted in significant control of ufra disease. However, the use of nematicides generates public concern due to the adverse impact of chemicals on the environment, soil microorganisms, ground water and human
health. So there is increased pressure to reduce or abandon the use of chemicals (Haydock et al., 2013).

**Biological control**

According to Eilenberg et al. (2001), biological control can be defined as the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be. Biological control is an environmentally friendly method for pest and disease management. A number of organisms including fungi, bacteria, viruses, nematodes and other invertebrates showed antagonistic activity against plant parasitic nematodes (Stirling, 1991; Viaene et al., 2006; Lee & Kim, 2016; Zhang et al., 2016; Vos et al., 2012).

**Host resistance**

With increasing restrictions on chemical control, the use of nematode-resistant cultivars offers an effective and environmentally safe alternative to chemicals for plant parasitic nematodes control. Both plant resistance and tolerance to nematodes are important as control strategies. Plants carry inherent disease resistance in both natural and cultivated populations. A large number of rice genotypes have been screened against *D. angustus* and some resistance to *D. angustus* has been found in different rice genotypes. The wild rice species *O. subulata* and the cultivar Rayada (RDA)-16-06 (Miah & Bakr, 1977), RDA B3, RDA 14, RDA 4, RDA 2, RDA B4, RDA B8, RDA 3, Bazail 65 and RDA B5 (Bora & Medhi, 1992; Das et al., 2000; Das & Sarmah, 1995), Fukuhonami, Hayakikari, Akiyutaka, Matshonami, Aokazi, Koshinishini, Kinonishiki, Akinishiki, Shinanokogane, Hunenwase, Rayeda 4849 and Rayeda 4851 (Latif et al., 2011a; Latif et al., 2011b) showed resistance against *D. angustus*. The Burmese cultivar, B-69-1 showed tolerance towards ufra nematode infection (Sein, 1977). The current cultivation of these identified resistant varieties in the field is limited because of their low yield potential. Although nematode resistance genes have been characterized in several crop plants (Veremis et al., 1996; Cai et al., 1997; Kaloshian et al., 1998; Milligan et al., 1998), no resistance gene has been identified in rice against *D. angustus* so far. To develop high yielding ufra resistant variety by breeding or genetic engineering, it is prerequisite to identify and characterize the resistance gene(s).
1.3 The Plant immunity system

Plants come across a wide range of pathogenic microorganisms in their natural environment. To protect themselves against microbes and diseases, plants have developed an extraordinary array of immune and defence mechanisms in their cells. The initial barrier for any kind of invader is plant preformed defence including the plant cell walls and their reinforcements (Underwood, 2015) and toxic phytochemicals (Broekaert et al., 1997). Beside these, plants induce a sophisticated system of responses upon infection which is triggered by two-tiered microbial recognition (Jones & Dangl, 2006; Dodds & Rathjen, 2010).

The first tier of plant innate immunity is governed by pattern recognition receptors (PRRs) that are activated by recognition of evolutionary conserved pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) such as fungal chitin, bacterial flagellin or bacterial lipopolysaccharides, peptidoglycan. Activation of PRRs leads to multiple downstream defence signalling events such as production of ion fluxes, callose, phenolics, reactive oxygen species that limit colonization. This immunity is known as PAMP-triggered immunity (PTI) (Fig 1.11, Jones & Dangl, 2006). PTI also leads to produce phytoalexins, pathogenesis-related proteins (PR proteins), the activation of the mitogen activated protein kinase (MAPK) cascade and transcriptional changes (Spoel & Dong, 2012). PAMPs/MAMPs are molecules that are important for survival and pathogenicity and are conserved among diverse pathogens species or strains. Mutation of these PAMPs/MAMPs limits the ability of a pathogen to spread in populations by impairing the infection processes (Lee et al., 2006). In plants, the receptors to recognize PAMPs/MAMPs are typically receptor kinases (Ronald & Beutler, 2010). The bacterial elicitor flg22, a peptide representing the elicitor-active epitope of flagellin triggers immunity in Arabidopsis seedlings carrying the receptor-like kinase FLS2 (Gomez-Gomez & Boller, 2000). In rice, flg22 triggered immunity is mediated through OsFLS2, which is the rice ortholog of FLS2. Flagellin induces the expression of numerous defence related genes, H$_2$O$_2$ generation and hypersensitive cell death in various plants (Tanaka et al., 2003; Zipfel et al., 2004). In rice, a variety of different PAMPs have been found to be active, for instance, fungal chitin, bacterial lipopolysaccharides (Desaki et al., 2006; Kaku et al., 2006). Recently ascarosides (Ascr#18) were identified as an evolutionarily conserved family of nematode pheromones that can be recognized by plants. After ascaroside recognition plants induce hallmark defense responses including the
expression of genes associated with PTI, such as activation of MAPKs, as well as salicylic acid- and jasmonic acid-mediated defense signaling pathways (Manosalva et al., 2015). Teixeira et al. (2016) showed that root-knot nematode induces PTI in Arabidopsis root. Nematode recognition leading to PTI response involves camalexin and glucosinolate biosynthesis. They have also showed that root-knot nematode-induced glucosinolate biosynthesis pathway was BRASSINOSTEROID INSENSITIVE-ASSOCIATED KINASE 1 (BAK1, a common partner of distinct receptors of PAMPs/MAMPs) dependent and the camalexin biosynthesis pathway was only partially dependent on BAK1 suggesting the existence of diverse nematode recognition mechanisms.

Virulent pathogens are able to circumvent PTI by secreting so-called effectors into the host, thereby leading to effector-triggered susceptibility (ETS). However, pathogen effectors (avirulence proteins, Avr) can be recognised by nucleotide binding-leucine rich repeat (NB-LRR) proteins of plant, encoded by most R (resistance) genes. This second tier of immunity called effector-triggered immunity (ETI), acts largely inside the cell (Fig 1.11). ETI is a stronger and more intense defence response, often associated with programmed cell death of the infected cells, known as hypersensitive response (HR). NB-LRR proteins activate defence responses including oxidative burst, changes in cellular redox and in gene expression. LRR-mediated disease resistance is effective against obligate biotrophs, or hemibiotrophic pathogens but not against necrotrophic pathogens (Glazebrook, 2005). Recognition of specific pathogen effectors by R genes is either through direct binding or by recognition of the effector’s alteration of a host protein (Jones & Dangl, 2006). Among different rice NB-LRR proteins, PITA is the best characterized one (Bryan et al., 2000). This protein directly recognizes the Magnaporthe grisea avirulence protein (AvrPita), inducing localized cell death to prevent M. grisea to spread to adjoining rice cells (Jia et al., 2000). To date approximately 100 rice R genes conferring resistance to Magnaporthe oryzae have been identified and more than 20 of them have been cloned (Guo et al., 2016; Liu et al., 2014; Zhai et al., 2014; Devanna et al., 2014).

1.4 Induced resistance

Induced resistance can be defined as a state of enhanced defensive capacity of plants that results in resistance to subsequent attackers. The induced state of resistance in plants elicited by certain biological or chemical inducers can be effective against a broad range of pathogens including fungi, bacteria, viruses, nematodes and insect herbivores. This type of resistance is expressed not only locally at the site of induction but also systemically in different plant parts that are separated from the inducer. The induced resistance can be divided broadly into systemic acquired resistance (SAR), induced systemic resistance (ISR), β-aminobutyric acid -induced resistance (BABA-IR), and wound induced resistance (WIR). These resistance signaling pathways that are elicited by pathogens, beneficial microbes, insects or any kind of defence-inducing molecule somewhat overlap and share common signalling components (Pieterse et al., 2014). The induced resistance is often related with an enhanced capacity to mobilize cellular defence responses – a process called ‘priming’ (Conrath et al., 2006). The primed plants are able to show more rapid and enhanced/better defence responses to subsequent biotic and abiotic stress.

In this section, we provide an overview of two best-studied induced resistance mechanisms: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR and ISR can be distinguished on the basis of the nature of the elicitor and the regulatory pathways associated.
Typically, SAR is mediated by the salicylic acid (SA) pathway with the activation of a large set of genes that encode pathogenesis-related proteins (PRs), on the other hand ISR is generally regulated by jasmonic acid (JA) - and ethylene (ET)-dependent signaling pathways and is typically not related with the direct activation of PR genes (Fig 1.12, Van Wees et al., 1997; Vallad & Goodman, 2004).

Fig 1.12 a. Schematic representation of biologically induced disease resistance triggered by pathogen infection (SAR; red arrow) and colonization of the roots by beneficial microbes (ISR; purple arrow) b. Schematic representation of molecular components and mechanisms involved in pathogen-induced SAR and rhizobacteria-mediated ISR. Solid black lines indicate established interactions; dashed black lines indicate hypothetical interactions. Colored arrows indicate systemic translocation of long-distance signals (indicated in the same color at the base of the arrows). Ac acetylation, ET ethylene, ETI effector-triggered immunity, Fe iron, ISR induced systemic resistance, JA jasmonic acid, MAMP microbe-associated molecular pattern, Me methylation, PAMP pathogen-associated molecular pattern, PRR pattern-recognition receptor, PTI PAMP-triggered immunity, R protein Resistance protein, SA salicylic acid, SAR systemic acquired resistance, TF transcription factor. From Pieterse & Van Wees (2015).

1.4.1 Systemic acquired resistance

First evidence of systemic acquired resistance came from Ross in 1961, who observed that inoculation of tobacco mosaic virus (TMV) in half-leaves of tobacco plant induced a high level of resistance to TMV in the opposite half-leaves. SAR can be induced by virulent, avirulent, and non-pathogenic microbes or by application of various synthetic chemical compounds such as SA or its
functional analogs INA (2,6-dichloroisonicotinic acid) and BTH (S-methyl benzo-1, 2,3-thiadiazole-7-carbothioate), probenazole (3-allyloxy-1,2-benziso-thiazole-1,1-dioxide), BIT (1,2-benzisothiazole-1,1-dioxide) NCI (N-cyanomethyl-2 chloroisonicotinamide) or tiadinil (3’-chloro-4,4’-dimethyl-1,2,3-thiadiazole-5-carboxanilide).

Activation of SAR involves the generation and transport of signals from locally infested leaves to the uninfected distal tissues (Guedes et al., 1980; Tuzun & Kuc, 1985). Past years, many translocated signals contributing SAR have been proposed (Park et al., 2007; Chaturvedi et al., 2012; Yu et al., 2013; Jung et al., 2009; Navarova et al., 2012; El-Shetehy et al., 2015). The plant hormone SA and several components of the SA pathway including the methylated derivative of SA (methyl SA, MeSA, Park et al., 2007) are major regulators of SAR. Upon SAR induction, a biphasic change in cellular reduction potential occurs, resulting in reduction of NONEXPRESSER OF PATHOGENESIS-RELATED GENES1 (NPR1) from oligomers to a monomers (Mou et al., 2003). The SA signal is transduced by monomeric NPR1 into the nucleus. In the nucleus, NPR1 interacts with TGA transcription factors along with WRKY transcription factors to activate SA-responsive PR gene. Recent findings reveals that SA-independent systemic signals induce a gene encoding SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8), which phosphorylates NPR1 during SAR (Lee et al., 2015). Together with the SA-mediated monomerization of NPR1, their observations indicate that SA signals and SnRK2.8-mediated phosphorylation function coordinately to activate NPR1 through a dual-step process in developing systemic immunity in Arabidopsis thaliana. Recent evidence shows that NPR1 and its two paralogues, NPR3 and NPR4 are SA receptors that bind to SA with different affinity to regulate NPR1 stability (Fu et al., 2012; Wu et al., 2012). PR−1 is the best characterized PR gene that can be used as a marker for SAR in many plant species (Van Loon et al., 2006; Fu & Dong, 2013).

1.4.2 Induced systemic defense

In 1991, ISR was described by several researchers who demonstrated that plant roots colonized by several strains of plant growth-promoting rhizobacteria (PGPR) can excite the plant immune system in above-ground plant parts resulting in resistance against a broad spectrum of pathogens (Van Peer et al., 1991; Wei et al. 1991; Alstrom 1991). ISR can be triggered by various PGPR including different strains of Bacillus, Pseudomonas, Serratia, non-pathogenic plant growth-promoting fungi (PGPF) like Fusarium oxysporum, Trichoderma spp., Piriformospora indica
strains and also symbiotic arbuscular mycorrhizal fungi (Van Wees et al., 2008; Pieterse et al., 2014). A number of beneficial microorganisms are known to induce ISR in rice (De Vleesschauwer et al., 2006; 2009; Chithrashree et al., 2011; Radja Commarea et al., 2002).

Beneficial microbes produce elicitors that are responsible for ISR. A number of elicitors produced by beneficial microbes have been demonstrated to elicit ISR including lipopolysaccharides (LPS), iron-regulated metabolites pyoverdin, SA, 2,4 diacylphloroglucinol (DAPG), pyocyanin, N-acyl homoserine lactones, specific volatile organic compounds (Lee et al., 2012). Different microbial molecules have been recognized as ISR elicitors in monocots. For instance, Pseudomonas strains produce siderophores and antibiotics such as pseudobactins and pyocyanin that are defence elicitors in rice against Magnaporthe oryzae (De Vleesschauwer et al., 2008; De Vleesschauwer & Hofte, 2009).

Studies with defence signaling pathways involved in ISR revealed that pathogen-induced SAR and rhizobacteria-mediated ISR are regulated by distinct signaling pathways. Analysis of a large number of ISR-triggering plant-beneficial microbes demonstrated that not SA, but JA and ET are often the central regulators of ISR (Van Loon & Bakker 2005; Van Wees et al., 2008; Pieterse et al., 2014). For example, in Arabidopsis, the nonpathogenic, root-colonizing strain WCS417r of Pseudomonas fluorescens triggers an ISR response against infection by the bacterial leaf pathogen P. syringae pv. tomato. It has been shown that the transgenic Arabidopsis NahG plants that are unable to accumulate SA and wild-type plants were equally responsive to P. fluorescens WCS417r-mediated induction of resistance (Pieterse et al., 1996). On the other hand, the jasmonate response mutant jar1 and the ethylene response mutant etr1 were incapable to develop ISR against P. syringae pv. tomato (Pieterse et al., 1998). In rice, ISR triggered by P. fluorescens WCS374r against the leaf blast pathogen M. oryzae is regulated by an SA-independent but jasmonic acid/ethylene-modulated signal transduction pathway (De Vleesschauwer et al., 2008). Although ISR triggered by beneficial microbes is usually regulated through SA-independent resistance mechanisms, several examples of PGPR and PGPF have been reported to trigger ISR in an SA-dependent manner (De Vleesschauwer & Hofte 2009; Van de Mortel et al., 2012; Molitor et al., 2011).

The regulatory protein NPR1 is not only important for SAR but also plays an essential role for rhizobacteria-ISR. The role of NPR1 in ISR appears to be different from SAR because in SAR,
NPR1 functions as a transcriptional co-activator of SA-responsive *PR* genes whereas, ISR generally functions without *PR* gene activation. However, the molecular mechanism by which NPR1 functions in JA/ET-dependent ISR is unknown. Few studies have investigated to identify the signaling components involved in ISR related long distance signaling. Several studies indicating that the R2R3 type transcription factor MYB72 plays an essential role in the generation and/or translocation of a long-distance ISR signal (Verhagen *et al*., 2004; Zamioudis *et al*., 2014; Van der Ent *et al*., 2008).

### 1.4.3. Priming defence

Priming is the phenomenon by which cells are enabled to react to very low levels of a stimulus in a more rapid and robust manner than non-primed cells. Hence, primed plants show faster and/or stronger, activation of defence responses when challenged by biotic and abiotic stress (Conrath, 2009, 2011; Conrath *et al*., 2015). Many molecular and genetic studies reveal that defence priming is one of the important key process in various types of systemic plant immunity such as, SAR (Kohler *et al*., 2002; Jung *et al*., 2009), ISR (Conrath *et al*., 2002; Pieterse *et al*., 2014), resistance conferred by arbuscular mycorrhizal fungi (Pozo *et al*., 2009), β-aminobutyric acid–induced resistance (BABA-IR, Jakab *et al*., 2002) and wound-induced resistance (Chassot *et al*., 2008). Priming can be elicited by beneficial microorganisms, herivores, necrotizing attackers, MAMPs, pathogen-derived effectors and various natural and synthetic compounds (Conrath *et al*., 2006; Heil & Bueno, 2007; Frost *et al*., 2008).

Studies with molecular aspects of defence priming found for different molecules in the priming mechanism: accumulation of dormant MAPKs, enhanced *PAL1* and *PRI1* defence gene expression (Beckers *et al*., 2009), elevated levels of pattern recognition receptors (Tateda *et al*., 2014), activation of defence related transcription factors, chromatin modifications (Bruce *et al*., 2007) alterations of primary metabolism (Bolton, 2009), and accumulation of the azelaic acid (Jung *et al*., 2009) are all potentially crucial in the priming mechanism. However, the exact molecular mechanism of priming still remains elusive.

The benefits of priming are that it is cost-effective, since it does not involve direct activation of defence pathways, and it can provide resistance against a broad range of attackers. Therefore,
priming offers an environmentally safe, effective, economically cheap, and ecologically long lasting option for sustainable crop production.

1.4.4 Induced resistance signaling molecules against plant parasitic nematodes

Induced resistance has been shown to be a possible strategy for the control of plant parasitic nematodes. A number of PGPR and PGPF were found to be effective against plant parasitic nematodes in different plant species (Lee & Kim, 2016; Zhang et al., 2016; Vos et al., 2012). Among PGPR, *Bacillus* spp. and *Pseudomonas* spp. are dominant populations in the rhizosphere that can affect plant parasitic nematode (Rovira & Sands, 1977; Racke & Sikora 1992; Sikora, 1992). Rhizobacteria can reduce nematode populations by different mechanisms such as by affecting nematode behaviour (Sikora & Hoffmann-Hergarten, 1992), competing for essential nutrients (Oostendorp & Sikora, 1990), promoting plant growth (El-Nagdi & Youssef, 2004), interfering with plant–nematode recognition (Oostendorp & Sikora, 1990), antagonising by means of the production of toxins, enzymes and other metabolic products (Siddiqui & Mahmood, 1999) and inducing systemic resistance in the plant (Hasky-Gunther & Sikora, 1995). Rhizobacteria-mediated ISR against PPN has been studied by many researchers (Hasky-Gunther & Sikora, 1995; Hasky-Gunther et al., 1998; Mahdi et al., 2001a; 2001b, Hauschild et al., 2000; Anita et al., 2004). Siddiqui & Shaukat (2004) reported that ISR in tomato induced by *P. aeruginosa* 7NSK2 and *P. fluorescens* CHA0 against *M. javanica* works through an SA independent signal transduction pathway. Biocontrol activity of *P. fluorescens* isolate Pf1 against *M. incognita* in tomato is associated with enhanced PR-protein activity, the involvement of enzymes in the phenylpropanoid pathway and with an accumulation of phenolics (Anita et al., 2004). Schafer (2007) demonstrated that the gene coding for the phenylalanine ammonia-lyase enzyme (PAL5) was upregulated *Bacillus sphaericus* B43- mediated ISR against *M. incognita* in tomato.

Arbuscular mycorrhizal fungi (AMF) are well known PGPF that can successfully control PPN. In addition to direct competition or inhibition, altered plant growth and altered rhizosphere interactions, AMF can also trigger biochemical changes associated with plant defense mechanisms and induced resistance in controlling plant parasitic nematodes (Elsen et al., 2008; Vos et al., 2012; Hao et al., 2012). Elsen et al. (2008) first demonstrated that the AMF *Glomus intraradices* has the ability to induce systemic resistance in banana plants towards *Radopholus similis* and *Pratylenchus coffeae* using a split-root compartmental set-up.
SAR against PPN with different chemicals has been studied in many plant-nematode interactions. Fujimoto et al. (2011) investigated the effect on RKN, *M. incognita* infection and gene expression in tomato after foliar application with methyl jasmonate (MeJA). They found that foliar treatment with MeJA significantly reduced RKN infection and the expression level of proteinase inhibitors and multicystatin may be effective as marker genes for estimating the induced resistance response against RKN. Foliar application with BTH, MeJA and ET are also effective against *M. graminicola* and *H. oryza* in rice (Nahar et al., 2011; Nahar et al., 2012). Molinari et al. (2014) tested SA and its synthetic functional analogues BTH and INA in tomato, eggplant and pepper against *M. incognita* and found that soil drench with SA and INA and root dipping with SA and BTH can inhibit nematode reproduction and reduce root galling.

Primbing of rice with β-aminobutyric acid (BABA) treatment inhibited *M. graminicola* penetration, and delayed nematode and giant cell development. It has been demonstrated that BABA-induced resistance against this RKN likely occurs independently of JA and ET, mainly through the activation of basal defense mechanisms of the plant, such as reactive oxygen species accumulation, lignin formation, and callose deposition (Ji et al., 2015a). Huang et al. (2015a) demonstrated that the priming effect of biochar amendments (Biochar is a product of biomass pyrolysis) in rice (*Oryza sativa* cv. Nipponbare) against *M. graminicola* partially depends on the ET signaling pathway and enhanced H₂O₂ accumulation. Same authors also showed that priming effect of thiamine (Vitamin B1, VB1) against *M. graminicola* in rice involves H₂O₂ and phenylpropanoid-mediated lignin production (Huang et al., 2015b).

Light quality can also modify the resistance of tomato plants to *M. incognita*. The exposure of tomato shoots to red light at night (20 µmol m⁻² s⁻¹) induces systemic resistance against RKN in the roots and this effect is partly dependent on the JA and SA defence pathways (Yang et al., 2014). Both positive and negative photo-orientation of the nematodes have been measured in vitro (Burr et al., 1989; Burr et al., 2000; Riga, 2004). However, information regarding the mechanism of photo-orientation in nematodes is limited. Generally, root plant parasitic nematodes remain in the soil and inside the root tissues. So the light induced plant resistance towards these nematodes is most likely caused by a priming effect.
1.5 Mechanisms of resistance against plant parasitic nematodes

In nematology, resistance is defined as the ability of a host to block or reduce nematode reproduction (Cook & Evan, 1987; Trudgill, 1991). Resistant plants can interfere at several levels of the nematode infection cycle such as, root attraction, penetration or invasion, migration, feeding site establishment (in case of sedentary nematodes), development and reproduction of nematodes. Resistance occurring before nematodes enter into the root or shoot tissues is referred to as pre-infectional resistance. If the resistance occurs after the nematodes penetrate or invade the root or shoot surface it is termed post-infectional resistance (Cook & Evans, 1987). Investigation of resistance mechanisms in many incompatible plant-nematode interactions indicates that both types of resistance (pre and post infectional) occur depending on specific plant-nematode interactions.

Recognition of and attraction to host plants are important steps that precede infection to the host. In case of root parasitic nematodes, host recognition involves signals from the root that can stimulate egg hatch and attraction toward the roots (Perry, 1997). It is generally accepted that nematodes get oriented to the roots over gradients of chemicals such as CO\(_2\) (Perry, 1997; Prot, 1980), root exudates (Green, 1971), pH, electrical potentials (Bird, 1959). Auxin-oriented migration and multiplication has been reported in case of rice foliar nematode *Aphelenchoides besseyi* (Feng et al., 2014). However, to the best of our knowledge, there is no report about attraction of *D. angustus* towards the plant shoot.

Cabasan *et al.* (2012) found that resistant rice genotypes expressed a mechanism of pre-infectional resistance to *M. graminicola* infection. J2 penetration was significantly lower in the resistant rice genotypes compared with the susceptible rice genotypes. This type of pre-infectional mechanism was also observed in other incompatible host-nematode interactions (Bendezu & Starr, 2003; Das *et al.*, 2008; Moon *et al.*, 2010). Differences in root morphology or biochemical defence compounds could contribute to this type of resistance (Huang, 1985; Diomande, 1984; Valette *et al.*, 1998).

Attraction, penetration, motility, and reproduction of *Pratylenchus thornei* in wheat was studied by Linsell *et al.* (2014). They observed no significant difference in the rate of attraction toward or penetration of *P. thornei* in resistant or susceptible roots. However, the nematode migration, juvenile maturation and reproduction was significantly lower in and near resistant roots suggesting
that resistance acts post-infectional. Other studies have also reported suppression of migration, development and reproduction in resistant plants associated with accumulation of phytoalexins such as the isoflavonoid glyceolin (Huang & Barker, 1986; Kaplan et al., 1980). Differences in invasion and reproduction of D. angustus between rice cultivars have been reported by Plowright et al. (1996).

1.6 Plant hormone signaling in defence

Plant hormones are a group of structurally unrelated small molecules that act at low concentrations to regulate many aspects of plant growth, development and defence. In this section, we report on current knowledge on the role of the classical defence hormones salicylate (SA), jasmonate (JA), and ethylene (ET) in plant-pathogen interactions. At the end of this section, we provide an overview of their role in rice-nematode interactions. Studies with mutants and transgenic plants affected in biosynthesis, perception and signal transduction of these hormones have been helpful to understand the role of each component of hormone signaling pathways in plant defence. A number of excellent recent reviews describes the hormone signaling pathways in rice (De Vleesschauwer et al., 2013, 2014; Sharma et al., 2013; Yang et al., 2013).

1.6.1 Salicylate signaling pathway

The possible role of SA in defence signaling disease resistance was first demonstrated by White and his co-workers who showed increased PR proteins accumulation and enhanced resistance to tobacco mosaic virus (TMV) upon injection of tobacco leaves with SA or aspirin (Raskin, 1992; Klessig & Malamy, 1994). In plants, SA can be synthesized via two distinct enzymatic pathways: the phenylpropanoid pathway and the isochorismate pathway. Both pathways require the primary metabolite chorismate. In the phenylpropanoid pathway, chorismate-derived L-phenylalanine is converted into SA via a series of reactions initially catalyzed by phenylalanine ammonia lyase (PAL, Lee et al., 1995). In the isochorismate pathway, chorismate is converted to SA via isochorismate catalysed by isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Wildermuth et al., 2001; Verberne et al., 2000).

The defence signaling pathway mediated by SA plays an essential role in SAR. NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) is the central positive regulator
of SAR in Arabidopsis. Among NPR1-like genes in rice, OsNH1 is the closest homolog sharing 60% similarity with Arabidopsis NPR1 (Chern et al., 2005; Yuan et al., 2007). WRKY family of TFs has been suggested to play an important role through the W-box motif in their promoter regions (Rushton et al., 1996; Eulgem et al., 1999; Maleck et al., 2000). In Arabidopsis, more than 99% of BTH response genes are regulated by NPR1 and several WRKY TFs are regulated downstream of NPR1.

Rice has a large WRKY transcription family where OsWRKY45 is the key regulator of BTH-induced and SA-mediated defence responses, conferring broad spectrum resistance to rice (Shimono et al., 2007; 2012; Takatsuji et al., 2010). OsWRKY45 is also involved in the regulation of diterpenoid phytoalexin production in the SA/cytokinin signaling pathway as well as BTH mediated priming in rice leaves (Akagi et al., 2014). In rice OsWRKY45 is independent of OsNPR1 and it has been proven that the SA signaling pathway in rice branches into two subpathways: OsNPR1- and OsWRKY45- dependent pathway (Shimono et al., 2007).

After pathogen infection, accumulation of SA and its conjugates induces multiple PR proteins and the onset of local and systemic acquired resistance (SAR) in the plant (Durrant & Dong 2004). Transgenic plants expressing the bacterial salicylate hydroxylase gene (NahG) that prevents SA accumulation by degrading SA to catechol (Gaffney et al., 1993; Delaney et al., 1994) or plants defective in SA biosynthesis (with suppressed PAL expression or mutations in SID2/EDS16 that encode ICS) failed to develop SAR or PR gene expression and showed enhanced pathogen susceptibility (Pallas et al., 1996; Nawarth et al., 2002). On the other hand, application of SA or its synthetic functional analog BTH activates PR-gene expression and enhances resistance to pathogens. A significant reduction in mature females and egg deposition of root-knot nematodes was observed after foliar application of BTH on grapevine (Owen et al., 2002). Resistant tomato plants carrying the Mi-1 gene partially lost the resistance against root knot nematodes (Branch et al., 2004) and aphids (Li et al., 2006) when also expressing the NahG gene and the resistance was restored when BTH was supplied to the tomato plant (Branch et al., 2004). A. thaliana mutants perturbed in SA biosynthesis or signal transduction were more susceptible whereas SA-treated wild type plants showed decreased susceptibility towards H. schachtii infection (Wubben et al., 2008).

Rice seedlings accumulate high levels of free endogenous SA (up to 37 µg g⁻¹ fresh weight) compared to healthy tobacco or Arabidopsis (< 0.1 µg g⁻¹ fresh weight) (Silverman et al., 1995;
Chen et al., 1997). Despite such high endogenous SA levels, application of more SA or BTH to rice plants induces PR-gene expression, activates local and systemic acquired resistance and enhances resistance to pathogens. In addition, it is shown that endogenous SA plays an essential role to modulate redox potential and protect rice plants from oxidative damage caused by aging as well as biotic and abiotic stress (Yang et al., 2004). It was observed that the response against M. grisea after SA application on rice was plant age dependent; the adult rice plants showed resistance to M. grisea but not the young plants (Iwai et al., 2007).

1.6.2 Jasmonate signaling pathway

JA and its metabolites, collectively called jasmonates, play a key role in plant defence responses to pathogen infection and insect herbivory (Pieterse et al., 2012; Farmer et al., 2003; Blee, 2002). Jasmonates, lipid-derived signal molecules are initially generated in the chloroplast involving the conversion of the LOX product to the unstable allene oxide 12, 13 (S)-epoxy octadecatrienoic acid by Allene oxide synthase (AOS). This unstable epoxide is then converted by allene oxide cyclase (AOC) to the cyclopentenone ring-containing 12-oxo-phytodienoic acid (OPDA). OPDA then translocates into peroxisomes, where JA is synthesized from OPDA through reduction by 12-oxo-phytodienoic acid reductase (OPR) and three steps of β-oxidation. Jasmonic acid carboxyl methyltransferase (JMT) converts JA to methyl jasmonate (MeJA). In JA signaling, WRKY TFs and jasmonoyl-L-isoleucine (JA-Ile) play important roles in modulating plant defence against pathogen attack (Schluttenhofer et al., 2014). In the rice genome there are more than 100 WRKY genes identified; many genes of them associated with plant defence (Pandey & Somssich, 2009; Tao et al., 2009). In rice, the expression of OsJAR1 (encoding JA-Ile synthase) after blast attack indicates its involvement in pathogen defence (Wakuta et al., 2011)

Studies with transgenic rice lines overexpressing JA biosynthesis and response genes revealed that JA plays a significant role in defence gene activation against fungi and bacteria (Mei et al., 2006; Agrawal et al., 2003; Deng et al., 2012). Riemann et al. (2013) found that the JA deficient mutants cpm2 and hebiba were more susceptible against Magnaporthe oryza. JA also plays an important role in regulating the biosynthesis of different secondary metabolites for instance, terpenes, terpene indole alkaloids, flavonoids, and phenylpropanoids (Wasternack & Hause, 2013). In rice, flavonoid and diterpenoid phytoalexins (such as momilactones, sakuranetin) have been found (Miyamoto et
Chapter 1

General introduction

(2014) and some of these are induced in rice leaves after pathogen infections (Wakuta et al., 2011; Riemann et al., 2013; Shimizu et al., 2013).

JA biosynthesis and signaling pathways are important in plant defence against plant parasitic nematodes infection. Arabidopsis infected with H. schachtii showed significant upregulation of JA biosynthesis genes in early parasitism which was paralleled by increased endogenous JA concentrations (Kammerhofer et al., 2015). Later stages of H. schachtii infection showed no changes in JA gene expression (Hamamouch et al., 2011). Kammerhofer et al. (2015) confirmed the importance of the JA biosynthesis pathway by showing that JA biosynthesis mutants, dde and lox6 are more susceptible to H. schachtii compared to wild type. However, both the JA biosynthesis and signaling pathways were suppressed during syncytium formation and nematode development in the compatible interaction of H. glycines and soybean at 5 and 10 dpi (Ithal et al., 2007). On the other hand, root knot nematode susceptibility of tomato depends on intact JA signaling pathway through COII, not on JA biosynthesis (Bhattarai et al., 2008). It has been shown that exogenous MeJA application enhanced resistance against different nematode species in oat, spinach, and tomato plant probably by uplifting the level of different compounds for instance phytoectosteroids, flavonoids, proteinase inhibitors (Thurau et al., 2003; Soriano et al., 2004; Cooper et al., 2005).

1.6.3 Ethylene signaling pathway

ET is a gaseous phytohormone that plays important roles in plant’s growth and development as well as biotic and abiotic stress responses of plants (Morgan & Drew, 1997; van Loon et al., 2006). ET is generated from S-adenosyl L-methionine. At first S-adenosyl L-methionine is converted to ACC (S-adenosyl-L-methionine methylthioadenosine-lyase) by the enzyme ACC synthase (ACS) and then ACC is oxidized by ACC oxidase (ACO) to form ethylene, CO₂ and cyanide (Adams & Yang, 1979). In rice, six putative OsACS and seven putative OsACO and their respective genes were reported (Iwai et al., 2006; Rzewuski & Sauter, 2008). In the ET signaling pathway, EIN2 (ETHYLENE INSENSITIVE2) is the key signaling component which is cleaved off (the C-terminal part of EIN2) after activation and moves to the nucleus to mediate the ET signaling through the key transcription factor EIN3 (ETHYLENE INSENSITIVE3) and EIL1 (EIN3 LIKE1). EIN3 and EIL1 activate expression of ERF1 (ETHYLENE RESPONSE FACTOR1) (Merchant et al., 2013; Solano et al., 1998; Zander et al., 2012). The rice homologs of other genes
in ET signaling have been isolated, characterized and reviewed (Watanabe et al., 2004; Yau et al., 2004; Mao et al., 2006; Nakano et al., 2006).

ET plays a prominent role in mediating rice disease resistance against rice blast (M. oryzae) and sheath blight disease (R. solani). Transgenic rice lines overexpressing OsACS2 showed increased ET levels and defence gene expression upon pathogen infection compared to wild type plants (Helliwell et al., 2013). Iwai et al. (2006) also showed the importance of ET and the coproduct, cyanide for hypersensitive reaction (HR) accompanying resistance to blast (M. grisea) in young rice plants. On the other hand, application of aminooxyacetic acid, an ACS inhibitor, resulted in expanding lesions instead of HR lesions (Iwai et al., 2006). However, the importance of ET biosynthesis and signaling pathways in disease resistance are sometimes controversial. For example, ET application on soybean roots increased their attractivity towards the soybean cyst nematode (SCN), while ET inhibitors reduced penetration (Tucker et al., 2010). Arabidopsis mutants overproducing ET (eto1, eto2 and eto3) showed hyper-susceptibility to H. schachtii (Wubben et al., 2001). In contrast, the ET biosynthesis and signaling pathways positively contribute to RKN (M. hapla) resistance in tomato, and elevated levels of ethylene were shown to be correlated with decreased host attraction by RKN (Fudali et al., 2013). Such differences in the role of ET biosynthesis and signaling pathways in plant defence could be due to differences in the specific plant-pathogen interactions (Glazebrook, 2005; Pieterse et al., 2012).

1.6.4 Cross talk between hormones

Defence signaling pathways interact with each other in regulating defence responses against pathogens. Cross talk between hormone signaling pathways are important for effective systemic immunity.

Cross-talk between SA and other hormone pathways

In general, SA is associated with plant defence against biotrophic and hemibiotrophic pathogens and JA and ET play important role in defence against necrotrophic pathogens and insect herbivory (Glazebrook, 2005). Arabidopsis plants infected with biotrophic Pseudomonas syringae show an activated SA pathway and suppressed JA signaling, rendering the plants more susceptible to the necrotrophic pathogen Alternaria brassicicola (Spoel et al., 2009). This cross-communication is
mediated through a novel function of NPR1 in the cytosol. Spoel et al. (2003) showed that treatment of 35S::NPR1-HBD (the mutant npr1 plants engineered to constitutively express a fusion protein of NPR1 and the hormone binding domain of the rat glucocorticoid receptor) with both SA and MeJA, suppressed the MeJA-induced expression of PDF1.2, not only in the presence of DEX (steroid hormone dexamethasone that allow the NPR1-HBD fusion protein to translocate into the nucleus) but also in its absence, when NPR1 was retained in the cytosol indicating that nuclear localization of NPR1 is not required to suppress the MeJA induced expression of PDF1.2 by SA. The antagonistic interaction between SA and JA has also been observed in rice and OsNH1, the closest rice homolog of NPR1 also represses JA signaling pathway (Yuan et al., 2007). Rice defence mediated by OsWRKY13 activates the SA-dependent signaling pathway and suppresses the JA-dependent signaling pathway against bacterial blight and fungal blast (Qiu et al., 2007).

Although the interaction between SA- and JA-dependent signaling is mostly antagonistic, synergistic interactions have been described as well (Schenk et al., 2000; Kunkel & Brooks, 2002; Beckers & Spoel, 2006; Mur et al., 2006). Rice microarray analysis showed that a common defence system is activated by both JA and SA. More than half of all the BTH-induced genes are up-regulated by JA application proposing that a major portion of the SA-upregulated genes are regulated by JA-dependent signaling in rice (Tamaoki et al., 2013).

Many studies have demonstrated that SA and ET interact negatively in relation to defence (O’Donnell et al., 2001; Shen et al., 2011) however also synergistic interactions have been reported. In tobacco plants, ET is essential for SA-dependent SAR that is triggered upon infection by tobacco mosaic virus (Verberne et al., 2003). ET enhances the expression of the the SA-responsive marker gene PR-1 in Arabidopsis through EIN2 dependent ET signaling (De Vos et al., 2006). Glazebrook et al. (2003) showed that the ein2 mutation affects the expression of many SA-responsive genes in Arabidopsis. Moreover, it has been demonstrated that ET shapes the final outcome of the SA-JA signal interaction (Leon-Reyes et al., 2009). Synergistic interactions among SA, JA and ET have been reported in several plant species in response to RKN (Fujimoto et al., 2011; Fudali et al., 2013).

With few exceptions, abscisic acid (ABA) interacts negatively with SA dependent defence. ABA induces susceptibility in Arabidopsis to an avirulent strain of P. syringae pv. tomato via suppression of the accumulation of components crucial for a resistance response. Microarray
analysis in *Arabidopsis* revealed that ABA treatment suppressed many defence-related genes, including those important for the biosynthesis of phenylpropanoids, such as lignin and salicylic acid (Mohr & Cahill, 2007). In rice, ABA suppresses the SA-mediated defence and renders the plants more susceptible to *Magnaporthe oryzae* (Jiang et al., 2010) and *Xanthomonas oryzae* pv. *oryzae* (Xu et al., 2013). It has been reported that auxin promotes disease symptoms caused by *P. syringae* pv. *tomato* strain DC3000 in *Arabidopsis* (Chen et al., 2007). Wang et al. (2007) demonstrated that SA represses auxin-related genes, including the *TIR1* receptor gene to promote resistance. In rice, brassinosteroids antagonize gibberellin- and salicylate-mediated root immunity against *Pythium graminicola* (De Vleesschauwer et al., 2012).

**Cross-talk between JA and other hormone pathways**

Generally JA and ET act synergistically against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Howe & Jander, 2008). But there is also evidence of synergistic interactions between JA and ET against biotrophic pathogens (Nahar et al., 2011). In Arabidopsis, regulation of the plant defensin gene *PDF1.2* requires concomitant activation of the JA and ET response pathways (Leon-Reyes et al., 2009). Two transcription factors, ERF1 and ORA59 are also induced and expressed synergistically by these two hormones (Lorenzo et al., 2003; Pre et al., 2008). However, an antagonistic interaction between JA and ET may also occur. Knocking-out OsEDR1 in rice resulted in reduced expression levels of the ACC synthase gene family and decreased production of ET. However, the rice plants showed activated JA- and SA-associated pathways and enhanced resistance to bacteria *Xanthomonas oryzae* pv. *oryzae*. This phenomenon suggest that OsEDR1 regulates the antagonistic interaction between the JA or SA pathway and the ET pathway (Shen et al., 2011).

A complex interplay between JA and ABA signaling pathways to regulate plant defence gene expression in *Arabidopsis* has been reported. Adie et al. (2007) showed that treatment with ABA induced JA accumulation, indicating synergism. In contrast, disruption of a positive regulator of ABA signaling *AtMYC2*, increased transcription of JA-responsive defense genes in *Arabidopsis* which suggest an antagonism between JA and ABA signaling pathways (Anderson et al., 2004). The cross talk between JA and auxin has been well studied in *Arabidopsis*. It has been reported that auxin downregulates the genes involved in JA biosynthesis (Liu & Wang, 2006; Rojo et al., 1998). In contrast, positive interaction between JA and auxin was also demonstrated (Grunewald
et al., 2009). Also gibberellins can play a role in plant defence (Navarro et al., 2008; Beneventi et al., 2013). The GA-responsive DELLLA proteins promote susceptibility to biotrophic pathogens and resistance to necrotrophic pathogens by modulating the relative strength of the SA and JA signaling pathways (Navarro et al., 2008).

**Cross-talk between ET and ABA**

ET not only interacts with SA and JA that are mentioned above, but also with other hormone signaling pathways. For example, ET and ABA function antagonistically during rice defence responses. Bailey et al. (2009) observed that exogenous ABA application reduced ET generation in rice resulting in blast disease susceptibility. On the other hand, exogenous ABA enhances resistance against the brown spot pathogen *Cochliobolus miyabeanus* in rice through antagonistically cross-talking with the ET pathway in an *OsMPK5*-dependent manner (De Vleesschauwer et al., 2010). RNAi suppression of the ABA-inducible *OsMPK5* gene resulted in increased levels of ET, constitutive expression of PR genes and enhanced disease resistance (Xiong & Yang, 2003; Bailey et al., 2009).

**1.6.5 Role of hormones in rice-nematode interactions**

The hormone signaling network involved in plant defence against nematodes has been studied mostly on dicotyledonous plants against fungal and bacterial pathogens, but there are limited studies on hormone-regulated processes in the interaction between monocots and nematodes. However, progress has been made in recent years concerning the role of hormone signaling pathways in rice-nematode interactions.

The role of the three classical hormones SA, JA and ET was investigated in rice defence against RKN, *M. graminicola* and migratory root nematode *Hirschmanniella oryzae* infection by Nahar et al. (2011) and Nahar et al. (2012). They found that exogenous hormone application in the rice shoot activated the SA, JA or ET pathway in the root resulting in lower susceptibility of rice against these nematodes. JA biosynthesis is of major importance against RKN, whereas an intact SA, JA and ET pathway is a prerequisite for defence against *H. oryzae*. The SA, JA and ET biosynthesis and signaling pathways also play important roles in resistant responses of rice. Kumari et al. (2016) reported that the RKN resistant variety Vandana significantly upregulated the SA biosynthesis
Chapter 1

General introduction

genes, JA and ET pathway genes at early (2 days post inoculation) and later (6 days post inoculation) stages of nematode infection whereas in susceptible variety Pusa 1121, these genes are suppressed at later stage (6 dpi) of nematode infection.

Other hormones, such as ABA, gibberellins (GA), auxin, cytokinins (CKs) and brassinosteroids (BRs) also play important roles in regulation of the immune signaling network in rice against nematodes often through crosstalk with the SA, JA/ET pathways. ABA plays a negative role in rice defence against the migratory root nematode, *H. oryzae* by antagonizing JA biosynthesis and signaling (Nahar *et al.*, 2012). BRs interact negatively with JA in rice roots against RKN (Nahar *et al.*, 2013). MeJA application on the shoots of rice plants activates JA-dependent rice innate immunity and strongly suppresses the BR pathway in the root. Moreover, the involvement of auxin, GA, BR in feeding site formation caused by RKN has been documented (Kyndt *et al.*, 2012; Ji *et al.*, 2013). The positive and negative role of different hormones and their interaction in rice defence against the RKN and the migratory nematode is represented in fig 1.13 (Kyndt *et al.*, 2014).

In addition, there is also evidence that plant pathogens can produce phytohormones or their functional mimics to manipulate hormone biosynthesis and signaling resulting in hormonal imbalances and alterations in plant defence. PPN secrete so-called effectors into their host plant in order to facilitate infection. Increasing evidence indicates that effectors are able to suppress the plant defence responses through deregulation of hormone biosynthesis (Hewezi & Baum, 2013; Kikuchi *et al.*, 2014; Bauters *et al.*, 2014; Haegeman *et al.*, 2013).

However, the role of phytohormones has only been investigated so far for defence against rice root nematodes, but almost no research has been conducted on their role in defence against rice foliar nematodes, like *D. angustus*. 
Fig 1.13 Model showing the interactions between hormones in the root system and their effect in defence against the root-knot nematode *Meloidogyne graminicola* and the migratory nematode *Hirschmanniella oryzae* in rice. Red lines indicate nematodes interfering with the hormonal defense network to achieve root susceptibility. Perpendicular lines indicate antagonism, and arrows indicate activation. From Kyndt et al. (2014). Abbreviations: ABA, abscisic acid; BR, brassinosteroid; ET, ethylene; JA, jasmonic acid; SA, salicylic acid.

### 1.7 Pathogenesis-related (PR) proteins

PR proteins can be defined as proteins induced in plants under pathological or related conditions. PR proteins were first discovered in the early 1970s in tobacco leaves that showed a hypersensitive reaction to tobacco mosaic virus. Like other plant species, the structure, function and evolutionary relationships of a number of PR gene families have been studied in rice (Mitsuhara et al., 2008; Nakazaki et al., 2006; van Loon et al., 2006). In rice, PR genes are induced in various tissues by diverse biotic stresses (Zhao et al., 2008; Mitsuhara et al., 2008; Wu et al., 2011), environmental stresses (drought, cold stress, salt, wounding; Kim et al., 2008; Hashimoto et al., 2004), and chemical treatments (jasmonic acid, salicylic acid, ethylene, H$_2$O$_2$) (Kim et al., 2008; Nakashita et al., 2001). Some PR proteins have been found in different parts of healthy tissues induced by internal plant development stimuli and thus play a role in rice growth and development (Mitsuhara et al., 2008; Nakazaki et al., 2006; van Loon et al., 2006). PR proteins are very stable at low pH and relatively resistant to the action of proteolytic enzymes. They are monomers with low molecular mass and they are localized in different compartments such as the vacuole, the cell wall and/or the apoplast. Basic PR proteins are mostly located in the vacuole and most acidic PR proteins are found in the intercellular space (Van Loon et al., 2006).
Plants induce distinct sets of PR proteins in response to different pathogens. In *Arabidopsis thaliana*, SA induces the expression of PR1, PR2, and PR5 in response to biotrophic pathogen, whereas JA induces PR3, PR4 and PR12 in the defence reaction against necrotrophic pathogens (Thomma et al., 1998). Many reports have demonstrated that PR proteins play important roles in resistance responses. Susceptible tomato plants infected with *Meloidogyne incognita* downregulate the *PR* genes in both roots and shoots whereas resistant tomato infected plants induced higher levels of *PR-1* gene expression in shoots (Molinari et al., 2014). Overexpression of *PR5* in rice reduced infection of rice caused by *Rhizoctonia solani* (Grover & Growthaman, 2003). Wu et al. (2011) observed higher levels of OsPR1A, OsPR1B, and OsPR10A proteins in *Xa21*-mediated resistance response in rice to *Xanthomonas oryzae* pv. *oryzae* (Xoo). There is also evidence that the abundance of some PR proteins is greater in susceptible interactions compared to resistant reactions and different PR proteins have different functions in susceptible and resistant interactions (Hou et al., 2012). Ji et al. (2015b) showed the important role of rice thionin genes (PR13 family, also known as thionins, a group of antimicrobial peptides) in rice defence against two damaging root pathogens of rice, *M. graminicola* and *Pythium graminicola*. They found that transgenic lines of *Oryza sativa* cv. Nipponbare overproducing *OsTHI7* decreased susceptibility to *M. graminicola* infection and *Pythium graminicola* colonization.

### 1.8 Secondary metabolites

Secondary metabolites are organic compounds that play an important role in defence against herbivores, pests and pathogens (Wink, 2003; Verpoorte, 2000). More than 100000 diverse secondary metabolites are produced by plants (Dixon, 2001). Based on their biosynthetic origins, secondary metabolites can be divided into three major groups: the terpenoids, the alkaloids, and the phenylpropanoids and allied phenolic compounds.

Elicitors such as, chitosan (CHT), salicylic acid (SA), methyl salicylate (MeSA) and methyl jasmonate (MeJA) trigger induction of defence response in plants by altering phenolic compounds of the plant. For instance, these elicitors increased total phenolic content of eggplant roots. The lignin deposition in the cell wall and PAL activity were significantly higher in eggplant roots after elicitation and this increased resistance against *Ralstonia solanacearum* (Mondal, 2010). Ji et al.
(2015a) observed that BABA induces PAL gene expression and lignin deposition in the gall produced by RKN, *M. graminicola* in rice.

Secondary metabolites play important roles in plant defence against PPN. Sclareol, a natural diterpene increased resistance in tomato and *Arabidopsis* roots against RKN by inhibiting root penetration. Sclareol induced phenylpropanoid metabolism and ET biosynthesis and signaling genes by activating MPK3 and MPK6, mitogen activated protein kinase, ET dependent lignin accumulation in *Arabidopsis* roots (Fujimoto *et al*., 2015). The elevated level of phenolics and lignin deposition has been found to be important in tomato resistance to the root-knot nematode *M. incognita* (Paulson & Webster, 1972; Melillo *et al*., 1989). Phytoalexin medicarpin play a role in resistance of alfalfa (*Medicago sativa*) to the root-lesion nematode, *P. penetrans* (Baldridge *et al*., 1998).

Phenolics play an essential role for nematode resistance in *Musa* spp. towards the burrowing nematode *Radopholus similis* infection. Tannins, flavan-3, 4-diols and anigorufone phenylphenalenone-type phytoalexins (Collingborn *et al*., 2000; Holscher *et al*., 2014) were detected in the resistant Musa cultivars. In addition, extensive secondary cell wall lignification of vascular bundles was observed in *R. similis*-infected plants compared to non-infected plants (Dhakshinanmoorthy *et al*., 2014). Plowright *et al*. (1996) observed an increased production of phenolic compounds such as, chlorogenic acid and the rice phytoalexin sakuranetin in the ufra resistant cultivar Rayada 16-06 at 5 days after *D. angustus* infection.

1.9 Biocontrol activity of *Bacillus* species

*Bacillus* species are widely used for biological control of many plant diseases in different hosts including *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* (Kloepper *et al*., 2004; McSpadeen Gardener, 2004). Several *Bacillus* species have been developed as commercial bio-pesticides, because *Bacillus* species can produce endospores and persist successfully in natural environments for a long period after treatment (Hu *et al*., 2011). Several *Bacillus* spp. have been used in rice cultivation for the promotion of plant growth and control of rice insects (Kandibane *et al*., 2010) and diseases (Jetiyanon & Plianbangchang, 2010; Maketon 2004). New species are continually being described and evaluated to control different plant pathogens (Nascimento *et al*., 2016; Bach *et al*., 2016; Zhou *et al*., 2016).
Rice plants treated with *B. amyloliquefaciens* FZB42 suspensions showed significant improvement in resistance to bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* and bacterial leaf streak caused by *X. oryzae* pv. *oryzicola* over untreated plants by producing the antibiotic compounds difficidin and bacilysin (Wu et al., 2015). Wu et al. (2015) observed that difficidin and bacilysin caused downregulated expression of genes involved in *Xanthomonas* virulence, cell division, and protein and cell wall synthesis. Elshakh et al. (2016) investigated the *in vitro* and *in vivo* bactericidal mode of action of Bacillus strains including *B. subtilis* A15, *B. amyloliquefaciens* D29 and *B. methylotrophicus* H8 against bacterial leaf blight caused by *X. oryzae* pv. *oryzae*. They found that the antibacterial mechanisms of the three strains may be at least partly associated with the ability to secrete lipopeptides (bacillomycin, fengycin, iturin and surfactin). Controlling the bacterial blight disease in greenhouse conditions was achieved through through activation of inducing systemic resistance mechanisms (Elshakh et al., 2016). A novel endophytic strain, *B. oryzicola* YC7007 has been reported to suppress bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae*, panicle blight caused by *Burkholderia glumae* via induced systemic resistance and antibiotic production (Chung et al., 2015). This strain can also control bakanae disease of rice, caused by *Fusarium fujikuroi* (Hossain et al., 2016) by direct inhibition, and was also capable of inducing systemic resistance against the pathogen through primed induction of the jasmonic acid pathway (Hossain et al., 2016). Chandler et al. (2015) showed that soil application of *B. subtilis* BBG111 trigger induced systemic resistance in rice against rice sheath blight caused by *Rhizoctonia solani*. They also revealed that *B. subtilis* BBG111 cyclic lipopeptides, fengycin and surfactin target the JA, ET and/or auxin pathways indicating the role of fengycin and surfactin, in the induced defence state.

Numerous *Bacillus* strains can also suppress plant parasitic nematode infection. In addition to direct antagonism by *Bacillus* spp. towards plant parasitic nematode species (Kloepfer et al., 1992, Siddiqui & Mahmood, 1999; Li et al., 2005), *Bacillus* spp. also induce systemic resistance to suppress the nematode population in different crop species (Hasky-Gunther & Sikora, 1995; Schafer et al. 2006; Hauschild et al. 2000; Almaghrabi et al., 2013). Treatment of tomato plants with an isolate of *B. thuringiensis*, designated CR-371 resulted in a significant reduction in galls caused by the root-knot nematode, *M. incognita* in both greenhouse and field trials. The same strain also reduced the *Pratylenchus penetrans* population in the root of strawberry plants under greenhouse conditions (Zuckerman et al., 1993). Li et al. (2007) and Li et al. (2008) identified
nematicidal cry genes such as cry5B and cry6A from *B. thuringiensis* and integrated them by genetic modification into crops to control *Meloidogyne* spp. *B. firmus* is an important nematophagous bacterium that effectively controls several different plant parasitic nematodes (Mendoza *et al.*, 2008; Mendoza & Sikora, 2009; Schrimsher *et al.*, 2011; Terefe *et al.*, 2009; Xiong *et al.*, 2015). A *B. firmus* strain isolated from soil in Israel has been developed as biological nematicidal agent by the Agro-Green Company and registered under the trade name of BioNem-WP in Israel. A peptidase S8 superfamily protein called Sep1 was identified in *B. firmus* strain DS-1 that had toxicity against the nematodes *Caenorhabditis elegans* and *M. incognita*. The Sep1 protein degrade multiple cuticle-associated proteins and destroys host physical barriers due to its serine protease activity (Geng *et al.*, 2016). Treatment of tomato seeds with *B. subtilis* isolates Sb4-23, Mc5-Re2, and Mc2-Re2 significantly reduced the numbers of galls and egg masses produced by *M. incognita* on tomato compared with the untreated control by induced systemic resistance (Adam *et al.*, 2014). *B. amyloliquefaciens* FZB42 has been shown to reduce the numbers of galls and egg masses in tomato caused by *M. incognita* (Burkett-Cadena *et al.*, 2008). Soil drenches or seed treatments of tomato with the *B. methylotrophicus* strain R2-2 can suppress disease caused by root-knot nematode *M. incognita* in plate, greenhouse and field conditions (Zhou *et al.*, 2016). Although ISR in plants against plant parasitic nematodes mediated by different *Bacillus* spp. has been documented, no studies thus far have evaluated ISR in rice against the rice foliar nematode *D. angustus*.

Even though a great amount of research focused on species of the genus, the taxonomic organization of the group remains disordered and often confusing. Accurate taxonomic information is necessary because it enables scientists to understand the biodiversity and relationships among living organisms from different ecosystems (Gevers *et al.*, 2005). To better understand the phylogenetics of a number of *Bacillus* species, recently Dunlap *et al.* (2016) showed that there are minor differences between the strains *B. oryzicola* KACC 18228, *B. velezensis* NRRL B-41580, *B. methylotrophicus* KACC 13015 and *B. amyloliquefaciens* subsp *plantarum* FZB42 through morphological, physiological, chemotaxonomic and comparative genome analysis. The pairwise *in silico* DNA-DNA hybridization values calculated in comparisons between the strains were all greater than 84%, which is well above the standard species threshold of 70%. Hence, Dunlap *et al.* (2016) propose that *B. methylotrophicus* KACC 13015, *B. amyloliquefaciens* subsp *plantarum*
FZB42, and *B. oryzae* KACC 18228 should be reclassified as later heterotypic synonyms of *B. velezensis* NRRL B-41580.

## 2.0 Scope and outline of the thesis

Environmentally friendly approaches to control the nematode *D. angustus* are limited. Whether plant resistance and biological control are environmentally sound and economically viable for nematode control remains to be further studied for this nematode. Moreover, in contrast to root nematodes of rice, there is no knowledge concerning signaling pathways involved in rice defence against foliar nematodes, such as *Ditylenchus angustus*. Studies involving rice-*D. angustus* interactions will provide valuable information for the development of sustainable ufra management strategies.

In chapter 2, we evaluated the susceptibility of different rice genotypes (irrigated, rainfed, deep water and landraces) for the Bangladeshi population of *D. angustus* to identify resistant varieties. The experiment was initially conducted in plastic pots using artificial inoculation, in both rainfed and irrigated ecosystems, and the promising varieties were analysed further in field conditions. The rice varieties were first scored at two different time points: at 28 days post inoculation (dpi), based on the postinfectional reactions and at 55 dpi, based on the percentage of tiller infections. Out of the 87 varieties, one landrace named ‘Manikpukha’ was found to be highly resistant in both pot and field conditions.

Chapter 3 focuses on the resistance mechanism of rice cultivar Manikpukha to *D. angustus*. We investigated invasion, post-infectional development, and reproduction of *D. angustus* in the resistant rice genotype Manikpukha and in the susceptible rice genotypes BR26 and Nipponbare to identify the stage (s) at which resistance occurs.

In chapter 4, we examined the role of SA, JA, and ET in rice defence against *D. angustus*. Nematode infection experiments with rice lines that are mutants or transgenics deficient in SA, JA and ET biosynthesis or signaling pathways, exogenous hormone applications, biosynthesis inhibition were performed. A gene expression analysis was done to observe the expression pattern of some SA/JA/ET-marker genes between a compatible and incompatible rice-*D. angustus* interaction. Furthermore, level of SA, JA and lignin was measured in resistant versus susceptible cultivars.
Chapter 5 describes the potential of a rhizobacterium *B. velezensis* strain BSK, isolated from Bangladesh, in promoting plant growth of rice and inducing systemic resistance to *D. angustus*. We also studied the relative expression of some SA/JA/ET-marker genes involved in rice-*D. angustus*-bacteria interactions.

Finally, in chapter 6, we briefly recapitulate the results and discuss the applications of this work and future prospects.
Chapter 2. Identification of Bangladeshi rice varieties resistant to ufra disease caused by the nematode *Ditylenchus angustus*

2.1 Abstract

The rice stem nematode *Ditylenchus angustus* causes “Ufra” disease in rice resulting in substantial yield losses. Although it is predominant in deep water rice in South and Southeast Asia, this nematode also infects irrigated and rainfed low land rice. This study evaluates the susceptibility of different rice genotypes (irrigated, rainfed, deep water and landraces) for the Bangladeshi population of *D. angustus* to identify resistant varieties. The experiment was executed using artificial inoculation, in both rainfed and irrigated ecosystems. The rice varieties were first scored at 28 days post inoculation (dpi), and ranked based on the postinfectional reactions and severity of symptoms on a 0-16 rating scale. The susceptibility of the varieties was also evaluated at a later time point, i.e. 55 dpi, based on the percentage of tiller infections, using a disease index scoring system ranging from 0 to 9. Both screening methods showed a similar ranking of the varieties for susceptibility/resistance against this nematode. The experiment was initially conducted in plastic pots, and the promising varieties were analysed further in field conditions. Out of the 87 varieties, one landrace named ‘Manikpukha’ proved to be highly resistant, while 6 other varieties showed resistance and 13 varieties showed moderately resistant responses under both pot and field conditions. The promising varieties found in the present investigation can be used in rice breeding programs as well as for further detailed studies to develop a sustainable ufra management strategy.

2.2. Introduction

Ufra disease caused by the rice stem nematode, *Ditylenchus angustus* (Butler, 1913), is known as one of the most devastating rice diseases in some South and South-East Asian countries (Bridge *et al*., 1990). Ufra disease was first reported by Butler (1913) in Bangladesh (former East Bengal). The disease mainly occurs in deep water rice (Butler, 1919). However together with the shrinkage of deep water rice cultivation and increased production of irrigated and rainfed rice, infections caused by *D. angustus* also appear in these conditions (Prasad *et al*., 2000; Latif *et al*., 2004).

All life stages of the nematode are infective although the J4 stage shows the highest infectivity (Plowright & Gill, 1994). The nematode enters the plant mainly at the collar region, migrates upward with the growth of the shoot and feeds on newly forming tissues in the rolled leaf sheath causing malformation. In the vegetative stage, white patches, or speckles in a splash pattern are
observed at the leaf base. Brownish stains may develop on leaves and leaf sheaths. Upon severe infection, the panicle heads and flag leaves become twisted and distorted. Yield loss studies in different countries have demonstrated the devastation caused by the nematode. For instance, in Bangladesh, 40 to 49% or sporadically 90% yield losses have been reported (Latif et al., 2011a; Latif et al., 2011b). Even a low infection percentage in transplanted seedlings can cause substantial yield losses (Bridge & Starr, 2007).

Reproduction is amphimictic and the nematode completes its life cycle within 10-20 days at 27-30°C (Bridge & Starr, 2007). At rice harvest, the nematodes remain in a coiled, quiescent state, mainly in the dried glumes of the lower panicle spikelets (Latif et al., 2006). Although the presence of living nematodes in the grains has been reported (Prasad & Varaprasad, 2002), the chance of transmission by seed is very unlikely when the seeds are properly sun dried (Bridge & Starr, 2007). Survival of nematodes from one season to another takes place in crop residues, soil and alternative hosts like weeds (Cox & Rahman, 1979). Dissemination of nematodes from field to field is mainly through irrigation water (Rahman & Evans, 1987).

For nematode management, different practices like burning of stubble and straw, destruction of ratoons, wild rice, regulation of irrigation water (Sein & Zan, 1977), delayed sowing and transplanting (McGeachie & Rahman, 1983), use of organic amendments, and crop rotation (Chakraborti, 2000; Latif et al., 2006) are recommended. But implementation of these cultural and physical practices are constricted due to socioeconomic reasons. Highly persistent nematicides, principally carbofuran, are the main basis of nematode management in Bangladesh. However, negative effects of nematicides on the environment and non-target organisms (Haydock, 2006) underline the urgent need for alternative environment-friendly strategies.

Host plant resistance to *D. angustus* is a promising and sustainable option to limit yield losses caused by this nematode. A large number of rice genotypes, wild rice, and breeding lines have been screened for resistance against the nematode in different countries. Some sources of resistance were identified in previous studies (Miah & Bakr, 1977; Rahman, 1987; Latif et al., 2011a; Latif et al., 2011b), but there is no high-yielding ufra-resistant variety available. Currently, some high-yielding varieties released by the Bangladesh Rice Research Institute (BRRI) and different landraces are being cultivated in different part of Bangladesh. However, except for a few varieties, the information on the host response of most of these high yielding varieties and several Bangladesh
landraces to *D. angustus* remains unknown. Therefore the present study was performed to evaluate the host response to *D. angustus* infection of Bangladesh Rice Research Institute (BRRI) released high-yielding varieties, some local varieties and deep water rice varieties.

### 2.3. Results

#### 2.3.1. Relationship between the early scoring system and the number of nematodes inside the plant

Before screening the rice genotypes for ufra susceptibility/resistance the early scoring method was evaluated using 11 rice genotypes (Table 2.1) known to have different levels of susceptibility from previous studies (Latif *et al*., 2011a; Latif *et al*., 2011b). These rice genotypes were inoculated with 100 nematodes per seedling and evaluated using the early scoring system i.e., based on the intensity of chlorotic discolorations at the leaf base (Fig. 2.1), represented in Table 2.1. Counting of nematodes at 28 dpi revealed that there was a strong linear correlation \( r = 0.953, P <0.001 \) between symptom scoring and the total number of *D. angustus* per variety (Fig. 2.2).

Fig 2.1. Susceptibility symptoms to *Ditylenchus angustus* with severity rated 0 to 16.
Table 2.1

Symptom rating of rice susceptibility to *D. angustus* at 28 dpi based on chlorotic discoloration at the leaf base.

<table>
<thead>
<tr>
<th>Rice genotypes</th>
<th>Symptom type</th>
<th>Rice genotypes</th>
<th>Symptom type</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-16-06-3</td>
<td>S0</td>
<td>Gabura</td>
<td>S4</td>
</tr>
<tr>
<td>Kartik sail</td>
<td>S1</td>
<td>IR56</td>
<td>S8</td>
</tr>
<tr>
<td>Aokazi</td>
<td>S1</td>
<td>Bazail 249</td>
<td>S8</td>
</tr>
<tr>
<td>Bazail 252</td>
<td>S2</td>
<td>BR11</td>
<td>S16</td>
</tr>
<tr>
<td>IR30</td>
<td>S2</td>
<td>BR3</td>
<td>S16</td>
</tr>
<tr>
<td>Indra sail</td>
<td>S4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S0, S1, S2, S4, S8, S16 represent different intensities of ufra symptoms.

![Graph](image)

Fig. 2.2. Relationship between the early scoring system (at 28 dpi) and the actual number of nematodes inside the plant. Black dots represent the mean and standard error of 15 plants and different letters on the dot indicate significant differences (Duncan Multiple Range Test with $P = 0.05$). The graph is the representation of one of two independent experiments with similar results and $r$ represents the correlation co-efficient value with p value. Data was not pooled for repeated experiments due to the large variation in nematode numbers and virulence in control plants between experiments. The data from another replicate is shown in appendix Fig A1.
2.3.2. Response of rice genotypes to *D. angustus* in pot experiments: early scoring

The main symptom of *D. angustus* infection in vegetative rice plants is leaf chlorosis. White patches or white splash patterns are observed at the basis of young leaves (Fig. 2.3A). Based on the intensity of this leaf chlorosis (Fig. 2.1) all tested rice genotypes were scored at 28 dpi. This evaluation was executed under both rainfed and irrigated conditions. Results were similar in both ecosystems (Table 2.2). One landrace named Manikpukha showed a resistant reaction (Fig. 2.3: B-D). Six BRRI-released high-yielding varieties showed susceptibility with score 1. Thirteen varieties received score 2 and fourteen varieties were scored as 4 (Table 2.2). Twenty six varieties, mostly landraces and some BRRI-released varieties showed symptoms with severity rating score 8. The rest of the varieties, mainly including the deep water varieties, was scored as 16 with maximum susceptibility (Table 2.2).

![Fig. 2.3. Reactions in rice to *Ditylenchus angustus* infection. A. Susceptibility symptom: white splash pattern at the leaf base, B-D. Resistant responses in Manikpukha: pale yellowish green patches on the leaf blade (B), whitish discoloration over the entire length of the mid-rib (C), and yellowish white rectangular pattern in the leaf blade which gradually turns into necrosis (D).](image-url)
Table 2.2

Response of rice genotypes to *D. angustus* at 28 dpi based on chlorotic discoloration at the leaf base under rain-fed and irrigated conditions in pot experiments.

<table>
<thead>
<tr>
<th>Symptom types</th>
<th>Response of rice genotypes under rain-fed and irrigated ecosystem</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0=R</td>
<td>Manikpukha, R-16-6-3 (Resistant control)</td>
</tr>
<tr>
<td>S1</td>
<td>BR7, BR18, BRRI Dhan 35, BRRI Dhan 37, BRRI Dhan 40, BRRI Dhan 45</td>
</tr>
<tr>
<td>S2</td>
<td>BR5, BR9, BR10, BR17, BR23, BRRI Dhan 31, BRRI Dhan 32, BRRI Dhan 34, BRRI Dhan 44, BRRI Dhan 46, BRRI Dhan 52, BRRI Dhan 54, BRRI Dhan 56</td>
</tr>
<tr>
<td>S4</td>
<td>Sadamota, Mowlata, BR 1, BR4, BR20, BR24, BR25, BRRI Dhan 27, BRRI Dhan 30, BRRI Dhan 38, BRRI Dhan 39, BRRI Dhan 42, BRRI Dhan 49, BRRI Dhan 51</td>
</tr>
<tr>
<td>S8</td>
<td>Maloti, Kalomota, Dudkolom, Khey, Sakkhor khora, Nakhuchimota, DWR9, DWR12, DWR14, DWR17, DWR20, BR2, BR6, BR8, BR15, BR16, BR19, BR22, BRRI Dhan 28, BRRI Dhan 29, BRRI Dhan 33, BRRI Dhan 36, BRRI Dhan 43, BRRI Dhan 47, BRRI Dhan 48, BRRI Dhan 55</td>
</tr>
<tr>
<td>S16</td>
<td>Munar, Chikon, DWR1, DWR2, DWR3, DWR4, DWR5, DWR6, DWR7, DWR8, DWR10, DWR11, DWR13, DWR15, DWR16, DWR18, DWR19, BR3, BR11 (Susceptible control), BR12, BR14, BR21, BR26, BRRI Dhan 41, BRRI Dhan 50, BRRI Dhan 53</td>
</tr>
</tbody>
</table>

S0, S1, S2, S4, S8, S16 represent different intensities of ufra symptoms and R represents resistance

2.3.3. Response of rice genotypes to *D. angustus* in pot experiments: late scoring

With further development and depending upon the severity of infection, the symptoms appeared in different parts of the plant such as tillers, panicles, spikelets and seeds. All genotypes were analysed at 55 dpi under both irrigated and rainfed conditions based on percentage of tiller infection (Table 2.4). From figure 2.4, 2.5 and 2.6 it can be observed that each variety showed a similar reaction in both ecosystems, although the tiller infections were generally less severe in irrigated conditions compared to rainfed conditions. As expected the resistant control variety R-16-6-3 showed a resistant response while the susceptible control BR11 showed a highly susceptible response. No tiller infection (0%) was observed in ‘Manikpukha’, resulting in disease index 0 in both ecosystems. Six varieties showed a resistant reaction (disease index 1) with 6.6 to 17.5% tiller infection in rain-fed conditions and 5 to 15% in irrigated conditions. Thirteen varieties showed a moderately resistant reaction in both ecosystems (disease index 3). A moderately susceptible response (disease index 5) was shown by fourteen varieties. Most of the deep water and local varieties showed a susceptible to highly susceptible reaction (disease index 7 and 9 respectively) in both ecosystems.
There was a clear correlation between the response of the tested rice genotypes at 28 dpi and at 55 dpi (Table 2.2, Fig. 2.4-2.6). Genotypes that scored lower in terms of symptom scoring at 28 dpi (Table 2.2) also showed less tiller infections at 55 dpi (Fig. 2.4-2.6). For instance, Manikpukha, which showed no susceptibility symptoms at 28 dpi, also did not show any tiller infections at 55 dpi. The varieties with the strongest symptoms at 28 dpi also showed the highest percentage of tiller infections, and were hence categorized as highly susceptible.

Fig. 2.4. Percentage of tiller infections of the local varieties under irrigated and rainfed condition at 55 dpi in pot experiments. The code above the bar represents the reaction of the varieties towards *D. angustus* based on the percentage of tiller infections (HR= Highly Resistant, HS= Highly Susceptible, S= Susceptible, MS= Moderately Susceptible). The number at the bottom of the bar represents the disease index (DI) depending upon percentage tiller infections.
Fig. 2.5. Percentage of tiller infections of the deep water rice (DWR) varieties under irrigated and rainfed condition at 55 dpi in pot experiments. The code above the bar represents the reaction of the varieties towards *D. angustus* based on the percentage of tiller infections (HS= Highly Susceptible, S= Susceptible). The number at the bottom of the bar represents the disease index (DI) depending upon percentage tiller infection.
Fig. 2.6. Percentage of tiller infections of the BRRI released high yielding varieties under irrigated and rainfed condition at 55 dpi in pot experiments including resistant and susceptible control varieties A) from BR1 to BRRI dhan 30 and B) from BRRI dhan 31 to BRRI dhan 56. The code above the bar represents the reaction of the varieties towards *D. angustus* based on the percentage of tiller infections (HR= Highly Resistant, R=Resistant, MR=Moderately Resistant, MS= Moderately Susceptible, S= Susceptible, HS= Highly Susceptible). The number at the bottom of the bar represents the disease index (DI) depending upon percentage tiller infections.
2.3.4. Confirmation of the resistant genotypes under field conditions in rainfed conditions

The varieties that were identified to be highly resistant, resistant and moderately resistant in the pot experiments were further evaluated in the field. The results in the field (Table 2.3 and fig. 2.7) confirmed the results of the pot experiments. Tiller infections of most varieties were slightly higher in the field in comparison with pot experiments although the percentage of tiller infections was within the range of the reaction categories (Table 2.4).

Table 2.3

Response of rice genotypes to *D. angustus* at 28 dpi based on chlorotic discoloration at the leaf base under rain-fed conditions in field experiment.

<table>
<thead>
<tr>
<th>Symptom types</th>
<th>Response of rice genotypes under rain-fed conditions in the field</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0 = R</td>
<td>Manikpukha, R-16-06-03 (Resistant control)</td>
</tr>
<tr>
<td>S1</td>
<td>BR7, BR18, BRRI Dhan 35, BRRI Dhan 37, BRRI Dhan 40, BRRI Dhan 45</td>
</tr>
<tr>
<td>S2</td>
<td>BR5, BR9, BR10, BR17, BR23, BRRI Dhan 31, BRRI Dhan 32, BRRI Dhan 34, BRRI Dhan 44, BRRI Dhan 46, BRRI Dhan 52, BRRI Dhan 54, BRRI Dhan 56</td>
</tr>
<tr>
<td>S16</td>
<td>BR11 (Susceptible control)</td>
</tr>
</tbody>
</table>

S0, S1, S2, S16 represent different intensities of susceptible ufra symptoms and R represents the resistance reactions.

Fig. 2.7. Percentage of tiller infections of the highly resistant, resistant and moderately resistant varieties in the field under rainfed condition at 55 dpi. The code above the bar represents the reaction of the varieties towards *D. angustus* based on the percentage of tiller infections (HR= Highly Resistant, HS= Highly Susceptible, R= Resistant, MR= Moderately Resistant). The number at the bottom of the bar represents the disease index (DI) depending upon percentage tiller infections.
2.4. Discussion

To identify good sources of resistance against *D. angustus*, 85 rice genotypes were evaluated at two different time points after infection, an earlier time point based on symptom development (28 dpi) and at a later time point based on the percentage of tiller infections (55 dpi). Screening for disease resistance largely depends on accurate and precise screening methods. These methods must also be rapid, enabling the screening of a large number of materials with reproducible results. Plowright *et al.* (1992) described the so-called ‘seedling based technique’ to assess rice resistance and susceptibility to *D. angustus* at early rice growth stage. This technique, which we followed to score our genotypes at the early time point, is based on symptom development on the youngest leaf on a scale of 0-16. Rice screening against ufra resistance has also been executed based on the percentage of tiller infections by many investigators (Miah & Bakr, 1977; Rahman, 1987; Latif *et al.*, 2011a; Latif *et al.*, 2011b). In our studies we combined both methods for the evaluation of rice genotypes. It has to be noted that these early and late screening methods gave similar results in all evaluated genotypes and under all growth conditions. This suggests that both screening methods are equally effective to screen a large number of rice genotypes for ufra resistance.

Plowright *et al.* (1992) also counted the number of nematodes per plant at 28 dpi. The number of nematodes per genotype was not counted in the here-executed experiment. However we have demonstrated for a small set of genotypes that there is a strong correlation (*r* = 0.953, *P* <0.001) between the number of nematodes and the used symptom scoring system at the early time point. Plowright & Gill (1994) also demonstrated a good correlation (*r* = 0.735, *P* < 0.001) between symptom rating and number of nematodes. Studies with other stem nematodes also showed a close relationship between symptoms and reproduction of nematodes in infected seedlings (Williams, 1972; Whitehead *et al.*, 1987). Several experiments have showed the relationship between the symptoms and yield: a higher percentage of ufra symptoms resulted in significant yield losses compared to healthy panicles (Latif *et al.*, 2011a; Latif *et al.*, 2011b).

(Latif *et al.*, 2011a; Latif *et al.*, 2011b). It has been found that higher percentage of total ufra (damage tiller+ufra1+ufra2+ufra3) resulted significant yield losses compared to healthy panicles. Inoculum density and inoculation technique are important factors to screen for genotype resistance. A seedling inoculation method was implemented in the current study as it mimics natural
conditions and this method has been proven to be the easiest and most effective method for nematode inoculation (Rahman & Evans, 1987). The water level was maintained at the collar region of the top leaf during inoculation, because shallow water level or submergence of the leaf sheath delays infection and symptom development (Plowright & Gill, 1994). In our experiment, 100 nematodes per seedling were used as inoculum, as it was observed in a previous study that 100 or more nematodes per seedling are able to establish and spread infection to new tillers (Rahman & Evans, 1987). Although different environmental conditions may lead to differences in nematode population density in the same rice variety (Ramakrishnan et al., 1984; Dash et al., 2008), our tested rice genotypes showed more or less similar responses in the two tested ecosystems (irrigated and rainfed condition), in both pot and field experiments. Nevertheless, the percentage of tiller infections was generally slightly lower in irrigated conditions compared to rain-fed conditions. This difference could be due to a slower reproduction of the nematodes at lower temperatures (Dao, 1970; Vrain et al., 1978).

A wide range of susceptibility was observed among BRRI released high yielding varieties, deep water rice varieties and landraces. Out of 85 varieties, one landrace was found to be highly resistant, 6 varieties were classified as resistant and 13 varieties were moderately resistant, 14 varieties were moderately susceptible and the rest of the varieties in our study were scored as susceptible to highly susceptible to *D. angustus* in the pot experiment. Some varieties such as BR3, BR11, BRRI dhan 28, BRRI dhan 48, Kartik sail, Aokazi, Bazail 252, IR50, Indra sail, Gabura, IR 56, Bazail 249 that were evaluated in previous studies (Latif et al., 2011a; Latif et al., 2011b) responded similarly in our experiment. This type of genotypic variation in susceptibility of rice genotypes to *D. angustus* was also observed in previous investigations (Latif et al., 2011a; Latif et al., 2011b; Miah & Bakr, 1977; Bora & Medhi, 1992; Rahman, 1987). Rahman (1987) identified some sources of resistance in deep water rice, for example, Bazail 65, CNL 319 and the Rayada group of lines. A large number of irrigated and rainfed rice genotypes were screened by Latif et al. (2011a) and Latif et al. (2011b) and some sources of resistance were identified. But the use of those varieties in the farmers’ field are limited because of the lower yield potentials. In Bangladesh, farmers mainly cultivate the BRRI released high yielding varieties (see Appendix Table A1), or in some cases high yielding local rice varieties. The results of our experiment provide information about the status of ufra susceptibility of the currently used varieties in Bangladesh. Confirmation of the highly resistant, resistant and moderately resistant varieties in the field demonstrates the
potential of these varieties to be used in the farmer fields. Remarkably, most of the tested local varieties showed a susceptible to highly susceptible reaction. This observation underlines the high risk of ufra related yield losses in Bangladeshi rice fields.

In the current study, a hypersensitive response (HR) was observed in the highly resistant variety Manikpukha. This HR includes pale yellowish green patches on the leaf blade, whitish discoloration at the entire length of the mid-rib and yellowish white rectangular patterns in the leaf blade gradually turning into necrosis. Plowright & Gill (1994) observed similar types of reactions in resistant rice genotypes. A hypersensitive response towards *D. angustus* might be due to the presence of one or more resistance genes. Nematode resistance genes have been identified in several crop plants, mapped to chromosomal locations, characterized and cloned (Veremis *et al*., 1996; Cai *et al*., 1997; Kaloshian *et al*., 1998; Milligan *et al*., 1998; Rouppe van der Voort *et al*., 1999). One of the best-characterized nematode resistance genes is the *Mi* gene of tomato, which confers resistance to several root knot nematode species, some isolates of potato aphids and white flies (Williamson, 1998; Vos *et al*., 1998; Rossi *et al*., 1998; Nombela *et al*., 2003). Also for migratory nematodes loci conferring resistance have been identified in some crops (Williams *et al*., 2002; Zwart *et al*., 2005; Nicol and Ortiz-Monasterio, 2004; Sharma *et al*., 2011; Galal *et al*., 2014). To the best of our knowledge, no resistance gene has been identified in rice against *D. angustus*. Plowright *et al*. (1996) reported differences of phenolic compounds in ufra resistant and susceptible varieties and showed that chlorogenic acid and the phytoalexin sakuranetin could be involved in rice resistance against *D. angustus*. The here-identified highly resistant variety Manikpukha or other promising BRRI released high yielding resistant varieties can be used for further investigation of resistance mechanisms, identification of resistance gene(s) and analysis of rice-*D. angustus* interaction at the molecular level that will provide valuable information to develop ufra resistant varieties.

### 2.5. Conclusion

From the current study of BRRI released high yielding varieties, local varieties and deep water rice varieties, we conclude that a wide range of disease reactions in response to *D. angustus* was observed. Among the genotypes evaluated, one variety ‘Manikpukha’ showed a highly resistant reaction, 6 varieties were classified resistant, 13 varieties moderately resistant, 14 moderately
susceptible, 26 susceptible and 26 varieties showed a highly susceptible reaction. The highly resistant local variety ‘Manikpukha’ can be used as a parent to develop ufra resistant high yielding varieties by hybridization programmes, as well as for further detailed studies. The results of our study also recommend the cultivation of BRRI released high yielding resistant varieties that were identified in our experiment such as BR7, BR18, BRRI Dhan 35, BRRI Dhan 37, BRRI Dhan 40, and BRRI Dhan 45 in ufra infected areas as a cost-effective, environmentally friendly method to reduce the level of *D. angustus* infections.

### 2.6 Materials and methods

#### 2.6.1. Rice germplasm collection

The experiment was conducted in 2012 and 2013 at BRRI, Gazipur-1701. In this experiment, a total of 87 rice genotypes (see Appendix table A1), including one resistant and one susceptible control, were used for screening. Out of the 87 rice genotypes, 55 were BRRI released high yielding varieties, 21 varieties were deep water varieties and 11 landraces. All the varieties except the landraces were collected from the Genetic Resource and Seed (GRS) division of BRRI, Gazipur-1701. The landraces were collected from farmers’ fields of the Barisal district, located in the southern part of Bangladesh.

#### 2.6.2. Pot experiment

All varieties were first screened in plastic pots (30 cm diameter and 40 cm height) under either irrigated (November to March, average temperature for each month in 2012 was 24.5, 20, 17.8, 22.8, 26.8°C respectively and in 2013 average temperature was 24, 20, 17, 23, 26°C respectively) or rain-fed conditions (April to October, average temperature for each month in 2012 was 28.4, 29, 29.4, 29.5, 28.8, 29.4, 28.7°C respectively and in 2013 was 28, 28, 30, 29, 28, 29, 27°C respectively) (Bangladesh Meterological Department, 2013). Two-third of each pot was filled with sandy loam soil. Seeds were germinated on wet filter paper for 5 days at 30°C before sowing. Twenty germinated seeds were sown by forceps approximately 0.5 cm deep in each pot. The plants were watered and fertilized at regular intervals. The experiment was laid out in a completely randomized design with 5 replications in both ecosystems. Taken together, for each replication 100 individual seedlings were grown and evaluated.
2.6.3. Field experiment

The field experiment was conducted in the research field of BRRI under rain-fed conditions, in 2013. The plot size used for the field study was 2x3 m. The distance from hill to hill and row to row was 20 cm. The soil type of the experimental field belongs to Salna Series of Madhupur Tract of Agro Ecological Zone (AEZ) 28 which is characterized by silty clay with pH value of 6-6.5. The experimental site is subtropical in nature with heavy rainfall during June-September and scanty in winter. Earthen levee 25 cm height was made around each plot to maintain the water level and to prevent the spread of nematodes. The experiment was laid out in a randomized block design with 3 plots per variety. Fertilization and weeding were done at regular intervals.

2.6.4. Nematode culture

The nematode, *Ditylenchus angustus* obtained from Plant Pathology Division of BRRI was originally isolated from an infested farmer’s field in the Gazipur district, Bangladesh. The nematode culture was maintained on the susceptible rice cultivar BR11 in controlled greenhouse conditions (25 to 28°C; RH 80%).

2.6.5. Inoculum preparation and inoculation

The nematodes were extracted from ufra infected rice stems of greenhouse grown rice plants using the modified Baermann method (Luc *et al.*, 2005). The stems were longitudinally divided, cut into 5mm pieces and placed over a sieve to let the nematodes migrate out of the plant tissues overnight. The average number of nematodes per stem was estimated and each plant was then infected with a number of stem pieces accounting for ca. 100 nematodes for both pot and field experiments.

The inoculation method was done following the method described by Rahman (1993). This means that first the water was raised up to the upper most node of the 15-day old seedlings (Fig. 2.8). Then, infected stems, cut into small pieces, were spread evenly on top of the water. The water level was maintained to the uppermost seedling node for the following two weeks.
2.6.6. Relationship between the early scoring system and the actual number of nematodes inside the plant

To check the correlation between the early scoring method (according to Plowright et al., 1992; described below) and the number of infecting nematodes, an experiment was conducted with 11 rice genotypes that showed different susceptibility to ufra in previous studies (Latif et al., 2011a; Latif et al., 2011b). The genotypes were collected from the Genetic Resource and Seed (GRS) division of BRRI. The experiment was conducted in plastic pots (7.5 cm diameter and 12 cm height) filled with sandy loam. Seeds were germinated on wet filter paper for 5 days at 30°C before sowing. One germinated seed was sown in each plastic pot and 15 seedlings for each genotype were maintained per replication. Two replications were performed. Fifteen days after sowing, 100 nematodes were inoculated per plastic pot as described above. The plants were scored at 28 dpi based on the early scoring system. After scoring, the plants were cut into small pieces and soaked overnight in water, to release the nematodes. The total number of nematodes per plant was then counted using a binocular microscope.

2.6.7. Susceptibility/Resistance scoring methods

Screening of rice genotypes for ufra susceptibility was done based on scoring at two different time points: 28 days post inoculation (dpi; which we refer to as ‘early’) and 55 dpi (which we refer to
as ‘late’). The early scoring was done according to Plowright et al. (1992) and is based on the intensity of chlorotic discolorations at the leaf base, a characteristic symptom of ufra infection at the vegetative stage (Fig. 2.1). Different reactions like no symptom, necrosis in the mid-rib, pale green white patches with rectangular shape etc. were taken into consideration to categorize the test entries.

The late scoring method was based on the percentage of tiller infections and the test entries were categorized into six groups as proposed by Rahman (1987) and IRRI (1996) (Table 2.4).

Table 2.4

<table>
<thead>
<tr>
<th>Disease Index (DI)</th>
<th>% tiller infection</th>
<th>Symptoms</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>No symptoms</td>
<td>Highly resistant</td>
</tr>
<tr>
<td>1</td>
<td>1-20</td>
<td>May or may not be visible</td>
<td>Resistant</td>
</tr>
<tr>
<td>3</td>
<td>21-40</td>
<td>Visible</td>
<td>Moderately resistant</td>
</tr>
<tr>
<td>5</td>
<td>41-60</td>
<td>Visible</td>
<td>Moderately susceptible</td>
</tr>
<tr>
<td>7</td>
<td>61-80</td>
<td>Visible</td>
<td>Susceptible</td>
</tr>
<tr>
<td>9</td>
<td>81-100</td>
<td>Visible</td>
<td>Highly susceptible</td>
</tr>
</tbody>
</table>

2.6.8. Data analysis

The data were analysed using IBM SPSS version 22 (IBM SPSS, Inc., Chicago, IL, USA). The data of the relationship between the early scoring system and the actual number of nematodes were analysed using ANOVA. Normality and homogeneity of the data were tested with Kolmogorov-Smirnov Test of Composite Normality \((P = 0.05)\) and Levene test \((P = 0.05)\) respectively. Mean nematode numbers of each rice genotype with different symptom types were compared by Duncan’s multiple mean comparison test. Correlation between the scoring system and the number of nematodes was determined by Pearson product-moment correlation coefficient with significance test \((P = 0.05)\).
Chapter 3. The rice cultivar Manikpukha is resistant to the stem nematode *Ditylenchus angustus* due to post-infection mechanisms
3.1 Abstract

Invasion, post-infectional development, and reproduction of *Ditylenchus angustus* in the resistant rice genotype Manikpukha and in the susceptible rice genotypes BR26 and Nipponbare were compared to identify the stage(s) at which resistance occurs. There was no significant difference in nematode invasion in the resistant and susceptible genotypes at 1 and 3 days after nematode inoculation (dai). The post-infectional development of *D. angustus* was assessed at 7, 14, 21, and 28 dai and it was found that nematode development was slower in the resistant genotype Manikpukha compared to the susceptible genotypes BR26 and Nipponbare. Nematode reproduction completely failed in the resistant rice genotype, whereas the two susceptible genotypes supported high nematode reproduction as determined by the number of eggs per plant at 14, 21, and 28 dai. All the observations indicate that the resistance in Manikpukha is associated with reduced development and reproduction of *D. angustus* implying that resistance acts post-invasion.

3.2 Introduction

Plant resistance has been considered as the most promising component in parasitic nematodes management programmes. Resistance to nematodes has been defined as the ability of plants to restrict or prevent nematode reproduction (Cook & Evan, 1987; Trudgill, 1991). Resistance can interfere at several stages of the nematode-host plant infection cycle; either pre-infection (host recognition, host penetration or invasion) or post-infection (migration, feeding site formation in case of sedentary nematodes, development, and reproduction of nematodes). Basic understanding of resistance mechanisms is essential to acquire insights of nature, timing and action of resistance genes in order to efficiently use resistant cultivars in breeding programmes as well as to advance phenotypic screening methods.

The process by which *Ditylenchus angustus* invades and develops inside the rice plants can be separated into several steps: host recognition, invasion, development, and reproduction. It is well accepted that root parasitic nematodes are attracted to roots over gradients of chemicals and probe the root surface with their stylet to puncture the cells. However, there is no literature how *D. angustus* is attracted to its host plant before invasion of the shoots. *D. angustus* is an obligate parasite that causes “Ufra” disease in rice. All life stages of the nematode can invade the plant with
infectivity ranking juvenile stage 4 (J4) > adult > J3 > J2 (Plowright & Gill, 1994). The nematode enters the plant mainly at the collar region and feeds mostly ectoparasitically on young foliar tissues. The nematode is carried or migrates upward with the growth of the shoot and feeds on newly forming tissues in the rolled leaf sheath, the panicles, and the seeds. Reproduction is amphimictic and the eggs are deposited at the two-celled stage. The J1 molts into J2 within the egg and hatches spontaneously in water without the need for host stimuli (Ali & Ishibashi, 1996; Ali et al., 1995). The nematode completes its life cycle within 10-20 days at 27-30°C (Bridge & Starr, 2007).

Resistance to *D. angustus* has been found in different *O. sativa* cultivars (Bora & Medhi, 1992; Das et al., 2000; Das & Sarmah, 1995; Latif et al. 2011a, 2011b) and in the wild rice species *O. subulata* (Miah & Bakr, 1977). A rice cultivar, ‘Manikpukha’ showed a very high degree of resistance against *D. angustus* both in pot and field experiments (Khanam et al., 2016, chapter 2). The mechanisms of resistance to nematodes have been investigated in many plant-nematode interactions (Linsell et al., 2014; Cabasan et al. 2012; Pegard et al., 2005; Pedrosa et al., 1996), however the knowledge on resistance to *D. angustus* is limited. Plowright et al. (1996) examined population dynamics of *D. angustus* in several resistant and susceptible rice cultivars. They observed that the invasion rate varied between cultivars at 2 days after inoculation; more number of nematodes invaded in susceptible cv. NC492 and resistant cv. R 16-06 (B) compared to other susceptible or resistant cultivars. The number of nematodes at 28 days after inoculation was higher in susceptible cultivars than the resistant cultivars. Their observation indicated that the mechanism of resistance varies between different resistant cultivars. However, they only focused on invasion and reproduction of *D. angustus*. The information on nematode development inside resistant and susceptible genotypes remains unknown. Thus, this study aimed to investigate invasion, development and reproduction of *D. angustus* in our identified resistant genotype Manikpukha in comparison with two susceptible genotypes, BR26 and Nipponbare to determine the stage(s) at which the resistance is acting.
Chapter 3

Resistance mechanism

3.3 Results

3.3.1 *Ditylenchus angustus* invaded equally in resistant and susceptible rice genotypes

The number of *D. angustus* invading the plants was measured at 1 and 3 days after inoculation (dai) in one resistant rice genotype, Manikpukha and two susceptible genotypes, Nipponbare and BR26. The number of nematodes recovered per plant was not significantly ($\alpha = 0.05$) different in Manikpukha and Nipponbare but was significantly higher in BR26 than the other two genotypes at 1 dai (Fig 3.1). At 3 dai, no significant differences were observed among these three rice genotypes. There was a significant increase in nematode invasion over time in all genotypes (Fig 3.1). The invasion of each developmental nematode stage was also recorded by counting the number of second- or third-stage juveniles (J2/J3s), fourth-stage juveniles (J4s) and adults at 1 and 3 dai (Fig 3.2). Both at 1 and 3 dai, J4s were the highest in number invaded in the plant compared to J2/J3s and adults (Fig 3.2). At 1 and 3 dai, there was no significant difference in J2/J3s and J4s invasion among the genotypes but in case of adults, the invasion was significantly lower in the resistant Manikpukha compared with the two susceptible rice genotypes at 1 dai.

![Fig 3.1](image)

**Fig 3.1.** Number of *Ditylenchus angustus* in shoots of resistant and susceptible rice genotypes at 1 and 3 days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. Bars represent the mean and standard error of the number of nematodes per plant recorded on 8 plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). Data represent one of three independent experiments with similar results. $^R$Resistant genotype; $^S$Susceptible genotype. The data from another replicate is shown in appendix Fig A2.
Fig 3.2 Shoot invasion of D. angustus second- or third-stage juveniles (J2/J3), fourth-stage juveniles (J4) and adults in resistant and susceptible rice genotypes at 1 (A) and 3 (B) days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of D. angustus. Bars represent the mean number of nematodes per plant recorded on 8 plants. Different letters indicate statistically significant differences (non-parametric Kruskal-Wallis test with $\alpha = 0.05$). Data represent one of three independent experiments with similar results. 8Resistant genotype; 5Susceptible genotype. The data from another replicate is shown in appendix Fig A3.

### 3.3.2 The Development of D. angustus is delayed in the resistant genotype compared to the susceptible genotypes

The development of D. angustus within resistant and susceptible shoots was assessed by counting the number of each developmental stage of the nematode life cycle at 7, 14, 21, and 28 dai. From figure 3.3, it is observed that juveniles had started to develop into females (40%) at 7 dai in the susceptible genotype BR26. In contrast, the resistant Manikpukha has only 14% females, which is significantly lower compared to Nipponbare and BR26. At 14 dai, both susceptible genotypes Nipponbare and BR26 have second generation J2s (75% and 74% respectively). At 21 and 28 dai, second generation J2/J3s, J4s, and adults were observed in both susceptible genotypes, whereas no second generation nematodes were observed in the resistant genotype. A higher percentage of J4 (57%) and males (29%) compared to females (14%) was observed in the resistant genotype. When comparing the total number of nematodes between resistant and susceptible genotypes at 28 dai, there was a significantly higher number of D. angustus in Nipponbare and BR26 than in the resistant Manikpukha (Fig 3.4).
Fig 3.3. Percentage of *Ditylenchus angustus* developmental stages feeding on resistant and susceptible rice shoots at different times of the nematode life cycle (A) 7, (B) 14, (C) 21 and (D) 28 days after inoculation (dai). Bars represent the mean percentage of each stage of nematodes recorded on 8 plants. Nematode development in Manikpukha was compared to the susceptible rice genotypes Nipponbare and BR26. Single asterisks indicate significant differences of developmental stages of Manikpukha from BR26 and double asterisks indicate a significant difference between Manikpukha and both Nipponbare and BR26 (Independent Samples *t* test with $\alpha = 0.05$). Data represent one of two independent experiments with similar results. *R*Resistant genotype; *S*Susceptible genotype; Sg second generation. The data from another replicate is shown in appendix Fig A4.
Fig 3.4. Number of *Ditylenchus angustus* in shoots of resistant and susceptible rice genotypes at 7, 14, 21 and 28 days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. Lines represent the mean and standard error of the number of nematodes per plant recorded on 8 plants. Data represent one of two independent experiments with similar results. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). §Resistant genotype; ¶Susceptible genotype. The data from another replicate is shown in appendix Fig A5.

### 3.3.3 *D. angustus* completely failed to reproduce within the resistant genotype

The number of eggs laid by *D. angustus* females was counted at 14, 21, and 28 dai in the resistant and susceptible genotypes (Fig 3.5). We observed that nematode reproduction completely failed in the resistant rice, whereas up to 800 eggs per plant were collected from the susceptible genotypes (Fig. 3.5). Between two susceptible genotypes, there was no difference in number of eggs per plant at 14 and 21 dai, but BR26 had significantly more eggs at 28 dai.
Fig 3.5. Number of *Ditylenchus angustus* eggs per plant in resistant and susceptible rice genotypes at 14, 21, and 28 days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. Bars represent the mean and standard error of the number of eggs per plant recorded on 8 plants. Data represent one of two independent experiments with similar results. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). $^R$Resistant genotype; $^S$Susceptible genotype. The data from another replicate is shown in appendix Fig A6.

### 3.4 Discussion

To gain insights in the resistance mechanism of rice cultivar Manipukha to *D. angustus*, this study investigated the invasion, post-infectional development, and reproduction of the nematode in the resistant and two susceptible genotypes. Before host invasion, hatching, recognition of, and attraction to host plants are essential steps for host-nematode interactions. Plant signals such as root diffusates and temperature are regarded as important stimuli for hatching and attraction of many parasitic nematodes toward the host roots (Green, 1971; Schmitt & Riggs, 1991; Baxter & Blake, 1967). However, it is known that hatching of *D. angustus* is not dependent on host stimuli; it spontaneously occurs in water (Ali & Ishibashi, 1996; Ali *et al.*, 1995). Hatching of root-knot nematodes is dependent solely on suitable temperature and moisture conditions, without host stimulus. However, in some cases, root diffusates and generation number within a season can influence the hatching response (Curtis *et al.*, 2009).

The ability of plant parasitic nematodes to find host roots or to respond to a chemical stimulus has been studied with a number of diverse experimental conditions and some of them found to be effective to assess host-finding behaviour of root parasitic nematodes in the laboratory (Dalzell *et al.*, 2011; Wang *et al.*, 2009; Wuyts *et al.*, 2006). For examples, gradients of chemicals such as
CO₂ (Perry, 1997; Prot, 1980), pH, temperature, and electrical potentials (Bird, 1959), auxin (Feng et al., 2014) play a role for attracting the nematodes. However, in many cases, activity of root diffusates does not seem to be associated to host resistance or susceptibility (William & Beane, 1979; Turner & Stone, 1984; Rawsthorne & Brodie, 1986). Our results show that the rate of invasion of *D. angustus* in the resistant and susceptible rice genotypes was not significantly different, suggesting that host attraction is not important for the resistance reaction of Manikpukha. In several other cases as well, resistance does not interfere with nematode root attraction (Linsell et al., 2014; Balhadere & Evans, 1994; Cabasan et al., 2012).

Our results also demonstrate that all vermiform stages of *D. angustus* invaded the rice genotypes (Fig 3.2). The rate of invasion by J4 was significantly higher than the other stages, which is similar with previous observations (Plowright & Gill, 1994; Ibrahim & Perry, 1994). Plowright et al. (1996) examined population dynamics of *D. angustus* in several resistant and susceptible rice cultivars and observed that the number of nematodes that invaded the plant varied between cultivars; a higher number of nematodes was invading susceptible cv. NC492 and resistant cv. R 16-06 (B) compared to other susceptible or resistant cultivars. Studies of resistance mechanisms in several crop species to root knot, cyst and root lesion nematodes indicate that suppression of invasion/penetration rarely plays a role in resistance (Kim et al., 1987; Creech et al., 1995; Williams & Fisher, 1993; Linsell et al., 2014). As plant parasitic nematodes possess a stylet and generally secrete cell wall degrading enzymes, most nematodes seem able to overcome the physical barrier of plant tissues. However, in some other studies resistant plants expressed pre-infectional resistance because penetration and/or invasion of nematodes were significantly lower in resistant plants compared to susceptible plants (Cabasan et al. 2012; Pegard et al., 2005; Fogain & Gowen, 1998; Valette et al., 1998) which might be due to extra cell wall barriers in the root or biochemical defence compounds (Huang, 1985; Diomande, 1984; Valette et al., 1998).

The analysis of nematode development over time in the resistant and susceptible rice genotypes performed in the current study showed that nematodes progress to the next developmental stage at a faster and higher rate in the shoots of susceptible genotypes compared to the resistant rice genotype. In the resistant variety Manikpukha more nematodes remained in the juvenile stage resulting in less adult nematodes. The higher percentage of male nematodes compared to females at 21 and 28 dai in the resistant genotype indicates that the nematodes had undergone stress conditions in the resistant genotypes (Bird 1971; Triantaphyllou 1973). In our study, we observed
that resistance to *D. angustus* in Manikpukha may not only be due to delayed development but also to suppressed fecundity of the female. The reproduction of the nematodes completely failed in the resistant genotype. Such delayed nematode development and hence reduced egg production have also been reported in previous studies on resistant cultivars (McClure *et al.* 1974; Pedrosa *et al.*, 1996, Linsell *et al.*, 2014).

All the observations indicate that the resistance in Manikpukha is associated with reduced development and reproduction, which means that the resistant plant expressed post-infectional resistance to *D. angustus*. This incompatible interaction in Manikpukha with *D. angustus* is characterized by a hypersensitive reaction (HR) that leads to necrosis (chapter 2). Cell necrosis in the cortex and vascular tissues has been reported to halt or delay the nematode growth, development, reproduction, and/or establishment of nematode feeding sites in various crop species (Jena & Rao, 1977; Anwar & McKenry, 2000). HR responses in plants, following the recognition of nematodes, are accompanied by changes in transcriptional and defence signaling pathways such as production or release of reactive oxygen species (ROS, Davies *et al*., 2015), H$_2$O$_2$ accumulation (Melillo *et al*., 2006), salicylic acid, lipoxygenase enzymes (Bhattarai *et al*., 2008; Klink *et al*., 2009), and phenolic compounds (Paulson & Webster, 1972; Pegard *et al*., 2005).

Plowright *et al.* (1996) observed an increased level of chlorogenic acid in response to *D. angustus* infection in resistant varieties, but not in *D. angustus* infected susceptible plants. Another phenolic compound in rice, the phytoalexin sakuranetin was identified in the resistant variety Rayada 16-06 and this compound could have a functional role in resistance against the ufra nematode. Histological observations of a *Radopholus similis* infected, partially resistant, banana cultivar ‘Yangambi Km 5’ revealed the constitutive presence of phenolics such as high levels of lignin and flavonoids limiting host penetration and colonization by *R. similis* (Valette *et al*., 1998). Recently, Holscher *et al.* (2014) isolated, identified and located a banana specific group of phytoalexins, the phenylphenalenones, in *R. similis*-caused lesions in roots. An in-depth investigation of the rice–*D. angustus* interaction at the cellular and molecular level could lead to the development of more efficient control strategies against this nematode.
3.5 Conclusion

The resistance in Manikpukha acts post invasion with the suppression of female development and, thus, reproduction. There was no significant difference in the rate of invasion in the resistant and susceptible shoots. Development of *D. angustus* is suppressed in resistant plants suggesting that the resistant genotype could produce compounds that inhibit juvenile development or constrain nematode feeding. Further biochemical and molecular investigations into the *D. angustus*-rice response can enhance the development of Ufra resistant high yielding rice varieties.

3.6. Materials and methods

3.6.1 Rice genotypes

The study investigated three rice genotypes that showed a different response to *D. angustus*: one *D. angustus* resistant genotype Manikpukha (Khanam *et al*., 2015, chapter 2) and two susceptible genotypes: BR26 (Latif *et al*., 2011, chapter 2) and Nipponbare. The seeds of Manikpukha (indica type) and BR26 (indica type) were collected from Bangladesh Rice Research Institute (BRRI) and the seeds of Nipponbare (japonica type) were provided by USDA, GSOR-100.

3.6.2 Nematode culture

The nematode, *D. angustus* obtained from the Plant Pathology Division of BRRI was originally isolated from an infested farmer's field in the Gazipur district, Bangladesh. The nematode culture was maintained in vivo on a susceptible rice cultivar, BR11 (indica type; provided by BRRI) at 26°C under a 12h/12h light-regime (150 μmol/m²/s) and 70-75% Relative Humidity. The nematodes were extracted from ufra infected rice stems using the modified Baermann method (Luc *et al*., 2005). The stems were longitudinally divided, cut into 5mm pieces and placed over a sieve to let the nematodes migrate out of the plant tissues overnight.

3.6.3 Analysis of invasion, development, and reproduction

The experiments to assess the invasion, development, and reproduction of *D. angustus* within resistant and susceptible rice genotypes were conducted under controlled room conditions (26°C, 12h/12h light-regime, 70-75% Relative Humidity). The seeds were pre-germinated on wet filter
paper for 5 days at 30°C before transfer to SAP-substrate (Reversat et al., 1999) in a glass tube (15×2.5 cm). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. The inoculation method was done as described by Rahman (1993). This means that first the water was raised up to the upper most node of the 15-day old seedlings, then ca. 100 nematodes were spread evenly on top of the water. The water level was maintained to the uppermost seedling node for one week. Afterwards, the water was removed from the tube.

For the invasion experiment, the plants were collected at 1 and 3 days after nematode inoculation (dai) and for the development and reproduction experiments, the plants were collected at 7, 14, 21, and 28 dai. The stems were longitudinally divided, cut into 5mm pieces and placed over a sieve to let the nematodes migrate out of the plant tissues overnight. The number of *D. angustus* that invaded plants was determined by counting the J2/J3s, J4s, and adults per plant using a stereomicroscope. Different nematode developmental stages were identified on the basis of visual separation of stages using total body length (Ibrahim & Perry, 1994). Reproduction was assessed by calculating the number of eggs per plant.

### 3.6.4 Data analysis

The data from each experiment were analysed using IBM SPSS version 22 (IBM SPSS, Inc., Chicago, IL, USA). Normality and homogeneity of the data were tested with the Kolmogorov-Smirnov Test of Composite Normality ($\alpha = 0.05$) and the Levene test ($\alpha = 0.05$) respectively. The number of nematode developmental stages at 1 and 3 dai were analysed using the non-parametric Kruskal-Wallis test with $\alpha = 0.05$. Percentage of *D. angustus* developmental stages at 7, 14, 21, and 28 dai within Manikpukha was compared to the susceptible rice genotype Nipponbare and BR26 and the data were analysed using Independent Samples *t* test. All other data were subjected to analysis of variance (ANOVA). When a significant difference was identified, individual comparisons were done by Duncan’s multiple range test ($\alpha = 0.05$).
Chapter 4. The resistant Manikpukha shows a rapid defense response to *Ditylenchus angustus* infection, with higher *PAL* expression and lignin accumulation
4.1 Abstract

Next to their well-documented function in plant growth and development, plant hormones regulate the signaling network in plant defence responses against different biotic and abiotic stresses. In this chapter, we studied the role of three classical defence hormones salicylate (SA), jasmonate (JA) and ethylene (ET) in rice defence against *Ditylenchus angustus*. Nematode infection experiments with rice lines that are mutants or transgenics deficient in SA, JA and ET biosynthesis or signaling pathways showed that these lines are more susceptible to the nematodes compared to the control plants. Exogenous foliar application of methyl jasmonate, BTH (Benzothiadiazole, an analog of SA) and ethephon makes the plants more resistant towards the nematodes, whereas pharmacological inhibition of biosynthesis of these three hormones increases nematode susceptibility. Collectively, our results demonstrate that the SA, JA and ET pathways are important in basal defence of rice against *D. angustus*. A gene expression analysis showed a remarkable contrast in the expression pattern of some SA/JA/ET-marker genes between a compatible and incompatible rice-*D. angustus* interaction. Our data reveal that *OsPAL1* might be triggering the defence response in the resistant cultivar, but this is not correlated with increased SA production. Lignin measurement showed that, although their basal levels are similar, the resistant cultivar had a significantly higher lignin content upon nematode infection, while this content was decreased upon nematode infection in the susceptible cultivar.

4.2 Introduction

Plants have developed an extraordinary array of immune and defence mechanisms to protect themselves against a wide range of pathogenic microorganisms. Besides preformed defence (Underwood, 2015; Broekaert *et al.*, 1997), plants induce a sophisticated system of responses upon pathogen infection which is triggered by two tiered microbial recognition, termed PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006; Dodds & Rathjen, 2010). Timely recognition of a pathogen combined with fast and effective induction of defence responses ultimately determines the resistance/susceptibility of a plant.

PTI is triggered by pattern recognition receptors (PRRs) that are activated by recognition of evolutionary conserved pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs).
Chapter 4  Role of SA, JA, and ET in rice defence against *D. angustus*

Activation of PRRs at the cell surface leads to a battery of host defence responses ensuing a basal level of resistance to prevent pathogen growth. Virulent pathogens are able to circumvent PTI by secreting so-called effectors into the host. In turn, plants acquired resistance (*R*) genes to recognize specific pathogen effectors resulting in ETI which is often associated with programmed cell death of the infected cells, known as hypersensitive response (HR). PTI as well as ETI activation are correlated, among others, with the activation of complex phytohormone-based signaling networks, in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play central roles.

The importance of SA, JA and ET signaling pathways and their interaction in the regulation of the plant’s immune response to microbial pathogens, nematodes and insect pests is well documented (Pieterse *et al.*, 2012; Nahar *et al.*, 2011; 2012; Howe & Jander, 2008). In general, SA is associated with plant defence against biotrophic and hemibiotrophic pathogens and JA and ET play important roles in defence against necrotrophic pathogens, insect herbivory and root-knot nematodes (Glazebrook, 2005; Howe & Jander, 2008; Nahar *et al.*, 2011). In addition to well established antagonistic interactions between SA and JA/ET defence pathways, a number of synergistic interactions have also been reported (Beckers & Spoel 2006; Mur *et al.*, 2006; Makandar *et al.*, 2010; Zhu *et al.*, 2014). Although most information on the molecular interplay between plants and pathogens were generated on dicotyledonous plants, tremendous progress has been made into the immune-regulatory role of phytohormones in monocots such as rice. Rice is one of the most important food crops in the world and a promising model system for studying monocots because of its amount of genetic and molecular resources, the small and fully sequenced genome and its ease of transformation (Jung *et al.*, 2008).

The production of secondary metabolites play important in constitutive or induced defence plays an important role in plant defence against PPN (Fujimoto *et al.*, 2015; Dhakshinamoorthy *et al.*, 2014; Holscher *et al.*, 2014; Ji *et al.*, 2015; Kumari *et al.*, 2016). One of the most well-known secondary metabolic pathways in plants involves the biosynthesis of phenylpropanoids through the shikimic acid pathway. In the starting point of the pathway, phenylalanine ammonia-lyase (PAL) catalyzes the deamination of phenylalanine to cinnamate. Phenylpropanoid metabolism involves a complex series of branching biochemical pathways that provide plants with many different compounds, which are widely used as structural cell components (lignin, suberin and other cell wall-associated phenolics), pigments (flavonoids, anthocyanins), immunity signals (SA) and toxins (coumarins and furanocoumarins; Dixon *et al.*, 2002; Vog, 2010).
Mounting evidence suggests the significant role of SA, JA and ET in defence of rice in response to plant-parasitic nematodes. For instance, the JA pathway is of central importance in root defence against the root knot nematode *Meloidogyne graminicola*, whereas SA, JA and ET are all important for defence against the migratory root nematode *Hirschmanniella oryzae* (Nahar *et al.*, 2011; Nahar *et al.*, 2012). A gene expression analysis of rice upon root knot nematode infection reveals significant differential expression patterns of a number of well-identified SA/JA/ET-marker genes when comparing susceptible and resistant interactions (Kumari *et al.*, 2016). However, in contrast to root nematodes of rice, there is no knowledge concerning signaling pathways involved in rice defence against foliar nematodes, such as *Ditylenchus angustus*.

*D. angustus* is an obligate parasite of rice that causes substantial yield losses in South and Southeast Asia. The nematode invades the plant mainly at the collar region where it feeds mostly ectoparasitically on newly forming tissues in the rolled leaf sheath, the panicles, and the seeds. Reproduction is amphimictic and the nematode completes its life cycle within 10-20 days at 27-30°C (Bridge & Starr, 2007). For nematode management through environment friendly approaches, it is pre-requisite to investigate the plant-nematode interaction at a molecular level, in order to design effective strategies for nematode resistance in crops. In this chapter, we present an in-depth characterization of the role of SA, JA and ET in plant susceptibility/resistance to *D. angustus*. By using exogenous hormone and hormone inhibitor application, mutants and transgenic lines, gene expression analyses and metabolite measurements, we provide several lines of evidence that SA, JA and ET play a positive role in rice basal defence against *D. angustus*.

### 4.3 Results

#### 4.3.1 Mutants or transgenic rice plants deficient in SA/JA/ET are more susceptible to *Ditylenchus angustus* infection

The role of SA, JA and ET in rice defence against *D. angustus* was evaluated by studying different rice mutants or transgenic plants that are impaired in one of these three pathways. Fig 4.1 shows that mutant and transgenic rice plants affected in either the SA, JA or ET hormone pathway had a significantly higher number of nematodes per plant compared to control plants. The SA-signaling deficient *WRKY45*-RNAi line contained 36% more nematodes, the SA-deficient transgenic *NahG* had 42% more nematodes, the ethylene insensitive *EIN2b* RNAi line had 46% more nematodes
and the JA insensitive Coi-RNAi line had 56% more nematodes compared to control Nipponbare (Fig 4A). Fig 4B shows that the JA biosynthesis mutant hebiba also had a significantly higher (50% increase) number of nematodes per plant compared to the control Nihonmasari.

Fig 4.1. (A). Susceptibility towards *Ditylenchus angustus* in SA signalling deficient *WRKY45* RNAi, SA deficient transgenic *NahG*, ET insensitive *Ein2b*, JA insensitive *Coi* RNAi, and the corresponding wild type Nipponbare plants and (B) Susceptibility for *D. angustus* in JA biosynthesis mutant *hebiba* and the corresponding wild type Nihonmasari. Bars represent the mean and standard error of the number of nematodes per plant 20 days after nematode inoculation, recorded on 8 plants. Different letters in the picture A and B indicate statistically significant differences (Duncan Multiple Range Test with $\alpha = 0.05$). Data represent one of three independent experiments with similar results. The data from another replicate is shown in appendix Fig A7.

**4.3.2 Hormones and hormone inhibitors treatment confirm a positive role of the SA/JA/ET pathways in the control of *D. angustus* infection**

Given the observed *D. angustus* susceptibility of SA, JA or ET hormone pathway mutant and transgenic rice plants, we studied the effect of application of these hormones and corresponding hormone inhibitors on *D. angustus* infection in rice. The data are presented in Fig 4.2.

From fig 4.2, it is observed that the exogenous application of Benzo-1, 2, 3-Thiadiazole-7-Carbothioic Acid S-Methyl Ester (BTH, a synthetic analog of SA), methyl jasmonate (MeJA) or
2-chloroethyl phosphonic acid (Ethephon, Eth) 24h before inoculation significantly reduces the number of nematodes per plant. BTH-, Eth- and MeJA-treated plants had a 51%, 46%, and 39% reduction in number of nematodes per plant in comparison with control plants, respectively. In contrast, blocking of SA, JA and ET biosynthesis yielded a significant increase in the number of nematodes per plant compared to the untreated control plants. Inhibition of ET production through aminooxyacetic acid application (AOA; Mao et al., 2006; Iwai et al., 2006) resulted in a 22% increase, blocking of JA biosynthesis with diethyldithiocarbamic acid (DIECA; Farmer et al., 1994) resulted in 24% increase and blocking of the phenylpropanoid pathway with L-2-Aminooxy-3-phenylpropinoic acid (AOPP; Amrhein & Godeke, 1977) resulted in 31% increase in number of nematodes per plant compared to the control plants. The root and shoot growth of the plants were monitored throughout the experiment but no observable phenotypic differences in plant growth and development were detected.

Fig 4.2 Effect of foliar application of plant hormones and corresponding hormone inhibitors on rice defence against *D. angustus* infection. Shoots of fifteen-day-old plants were sprayed until runoff with 100 μM MeJA, 250 μM BTH, 500 μM ethephon, 25 mM AOA, 100 μM DIECA, 100 μM AOPP or the corresponding control solution. After 24 h of treatment, plants were inoculated with 100 nematodes of *D. angustus*. Bars represent the mean and standard error of the number of nematodes per plant 20 days after nematode inoculation recorded on 8 plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with \( a = 0.05 \)). Data represent one of three independent experiments with similar results. MeJA, Methyl jasmonate; Eth, Ethephone; BTH, Benzathidiazole; AOA, Aminooxyacetic acid; DIECA, Diethyldithiocarbamic acid; AOPP, L-2-Aminooxy-3-phenylpropinoic acid. The data from other replicate is shown in appendix Fig A8.
4.3.3 Innate defence response in rice shoot tissues upon *D. angustus* infection

To investigate the response of a susceptible and a resistant rice genotype upon *D. angustus* infection, expression of 12 SA/JA/ET- marker genes was evaluated. Shoot tissues of ufra susceptible Nipponbare and ufra resistant Manikpukha were evaluated at 1, 5, 10, and 21 dpi, using quantitative real-time PCR (qRT-PCR). This gene expression profile was compared to uninfected control plants of the same age. The results are presented in fig 4.3.

4.3.3.1 SA-dependent responses upon *D. angustus* infection in rice shoot tissues

Two SA biosynthesis genes, *OsICS1* (Chen et al., 2009) and *OsPAL1* (Lee et al., 1995) and one SA response gene *OsWRKY45* (Qiu et al., 2004) were evaluated for studying the importance of SA-dependent response in rice upon nematode infection. In plants, SA can be synthesized via two distinct enzymatic pathways: the phenylpropanoid pathway and the isochorismate pathway. *OsPAL1* is a key enzyme of the phenylpropanoid pathway starting from phenylalanine, while *OsICS1* is the first catalytic enzyme of the isochorismate pathway starting from chorismate.

mRNA levels of *OsPAL1* were significantly and consistently down-regulated at all the time points (1, 5, 10, and 21 dpi) in the shoots of Nipponbare in response to *D. angustus* infection. On the contrary, the *OsPAL1* gene was strongly up-regulated in the infected shoots of Manikpukha at all time points. *OsICS1*, on the other hand did not show differential expression compared to non-infected control plants in Nipponbare, while this gene was slightly induced in Manikpukha at all time points compared to corresponding non-infected shoot tissues. The transcript level of the SA responsive gene *OsWRKY45* was only slightly changed in Nipponbare shoot tissues compared to non-infected control plants at 1 and 5 dpi, but significantly up-regulated at 10 and 20 dpi. However, in the shoots of infected Manikpukha, *OsWRKY45* was only significantly upregulated at 1 dpi, but not significantly altered at other time points.

4.3.3.2 JA-dependent responses upon *D. angustus* infection in rice shoot tissues

To investigate the importance of the JA-dependent response upon *D. angustus* infection, *OsAOS2* (a key enzyme in JA biosynthesis; Agrawal et al., 2004; Mei et al., 2006), *OsJMT1* (enzyme which converts JA to the volatile component MeJA; Seo et al., 2001) and *OsJAmyb* (JA-inducible Myb transcription factor; Lee et al., 2001) were evaluated as JA-marker genes.
Early upon infection (at 1 and 5 dpi), \textit{OsAOS2} was slightly downregulated in the shoots of Nipponbare but this gene was significantly up-regulated at 10 dpi. On the other hand, the mRNA level of \textit{OsAOS2} was slightly higher in the shoot tissues of infected Manikpukha in comparison with the control tissues. \textit{OsJMT1} was strongly up-regulated in Manikpukha at 1 dpi, but not at other time points or in Nipponbare. \textit{OsJAm} was significantly up-regulated at 1 dpi in Nipponbare, but not at other time points. In the shoots of infected Manikpukha, \textit{OsJAm} was slightly induced at all time points.

### 4.3.3.3 ET-dependent responses upon \textit{D. angustus} infection in rice shoot tissues

Two ET biosynthesis genes, \textit{OsACS1} and \textit{OsACO7} (Iwai \textit{et al.}, 2006), one ET signaling gene, \textit{OsEin2b} (\textit{EIN2} is a central signal transducer in the ET signaling pathway; Jun \textit{et al.}, 2004) and one ET-responsive gene, \textit{OsERF1} (Hu \textit{et al.}, 2008) were evaluated to investigate the ET-dependent response upon \textit{D. angustus} infection.

The mRNA level of \textit{OsACO7} did not show differential levels in the shoots of Nipponbare or Manikpukha at any time point upon nematode infection. A near baseline expression or downregulation of \textit{OsACS1} was detected in the infected shoots of Nipponbare at all time points, with significant downregulation at 21 dpi. On the other hand, in the incompatible interaction \textit{OsACS1} was slightly induced with significant induction at 10 dpi. Almost no transcription alteration of \textit{OsEin2b} was detected in the infected shoots of Nipponbare at 1, 5, and 10 dpi but this gene was significantly up-regulated at 21 dpi compared to corresponding non-infected plants. On the contrary, in Manikpukha \textit{OsEin2b} was consistently upregulated at all time points, with significant up-regulation at 5 and 10 dpi. The ET-inducible gene \textit{OsERF1} was consistently attenuated in the infected shoots of Nipponbare, although only significant at 5 dpi. In the shoots of Manikpukha upon nematode infection, this \textit{OsERF1} gene was slightly upregulated at 1, 10 and 21 dpi and slightly down-regulated at 5 dpi.

### 4.3.3.2 General defence responses upon \textit{D. angustus} infection in rice shoot tissues

To further elucidate the general defence response of rice triggered upon nematode infection, the differential expression of two PR genes, \textit{OsPR1a} (Mitsuhara \textit{et al.}, 2008) and \textit{OsPR5} (Anzlovar & Dermastia, 2003) was assessed. mRNA level of \textit{OsPR1a} was not differentially expressed in the infected shoots of both Nipponbare and Manikpukha. However, this gene was slightly down-regulated or near baseline in Nipponbare, while slightly up-regulated in Manikpukha at all time
points except at 5 dpi. OsPR5 was up-regulated in the infected shoots of Nipponbare at all 4 time points, with significant induction at 1, 5, and 10 dpi. On contrary, OsPR5 was not differentially expressed in the shoots of Manikpukha upon *D. angustus* infection.

Fig 4.3 Analysis of the expression levels (using qRT-PCR) of some selected defense-related genes in the shoot tissues of the ufra susceptible genotype, Nipponbare A) and the ufra resistant genotype Manikpukha B) at 1, 5, 10, and 21 days after infection (dpi) with *Ditylenchus angustus*. Bars represent the mean and standard error of the relative amount of transcripts of these genes from two biological replicates (n=2), each containing a pool of shoot tissue from three infected plants in comparison with uninfected control plants grown under the same conditions. Gene expression levels were normalized using two internal reference genes, *OsEXP* and *OEXPnarsai*. Asterisks indicate statistically significant (P=0.001) differential expression in comparison with untreated uninfected plants, performed by 2000 randomisations using REST2009.
4.3.4. SA and JA accumulation upon nematode infection in the ufra susceptible Nipponbare and the resistant Manikpukha

Based on the observed expression profiles, we decided to quantify the SA and JA accumulation in the collar region of infected and non-infected shoots of Nipponbare and Manikpukha at 1 dpi, using high performance liquid chromatography followed by mass spectrometry analysis. Results (Fig. 4.4) indicated that the endogenous SA level is very high in rice shoots compared to JA level, which is consistent with the fact that rice accumulates very high levels of SA in young shoots and leaves (Yang et al., 2004). From fig 4.4A it is observed that there was no significant difference in SA accumulation among uninfected Nipponbare and Manikpukha plants. Upon infection, SA levels significantly decreased in Nipponbare, but not in Manikphukha. In case of JA, the level of JA significantly increased upon infection in the resistant Manikpukha plants compared to uninfected Manikpukha, but did not change upon infection in Nipponbare (Fig. 4.4B). The basal level of JA was not different when comparing the susceptible with the resistant genotype.

Fig 4.4. Results of SA (A) and JA (B) accumulation in infected and non-infected collar region of shoots of the ufra susceptible Nipponbare and the resistant Manikpukha at 1 dpi. SA and JA were quantified in shoot tissues using mass spectrophotometry, and is expressed as ng hormone per g of tissue. Bars represent the mean and standard error of six replicates, each containing a pool of shoot tissues from three plants. Different letters represent statistically significant differences according to the non-parametric Kruskal-Wallis test in A) and according to Duncan’s multiple range test B) with \( P \leq 0.05 \). Nipp ctrl, Nipponbare uninfected control; Nipp 1 dpi, Nipponbare 1 days post inoculation; Manik ctrl, Manikpukha uninfected control; Manik 1 dpi, Manikpukha 1 days post inoculation.
4.3.5. Lignin accumulation upon nematode infection in the ufra susceptible Nipponbare and the resistant Manikpukha

The remarkable contrasting expression pattern of OsPAL1 gene in the compatible versus the incompatible rice-\textit{D. angustus} interaction, together with the fact that PAL-inhibition leads to a significant increase in nematode numbers in the susceptible genotype (Fig. 4.2) prompted us to assess lignin accumulation in the shoot tissues of both cultivars. The results showed that there are no significant differences in lignin content in non-infected Nipponbare versus Manikpukha shoots. However, upon nematode infection, the resistant plants accumulated a significantly higher amount of lignin. Remarkably, the lignin content was significantly decreased in the susceptible genotype upon infection.

![Graph](image)

Fig 4.5. Lignin content in infected and non-infected rice shoots of the ufra susceptible Nipponbare and the resistant Manikpukha at 5 dpi. Lignin accumulation is expressed as percentage of cell wall residues. Bars represent the mean and standard error of five replicates, each containing 100 mg of dry cell walls, isolated from a pool of shoot tissues from six plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with \( P \leq 0.05 \)). Nipp ctrl, Nipponbare uninfected control; Nipp 5 dpi, Nipponbare 5 days post inoculation; Manik ctrl, Manikpukha uninfected control; Manik 5 dpi, Manikpukha 5 days post inoculation.
4.4 Discussion

SA, JA and ET are the three main defence hormones that play key roles in regulating responses to a wide variety of internal and external stimuli. A number of studies have been done to characterize the role of these three classical hormones against PPN mostly on dicotyledonous plants (e.g. Wubben et al., 2001; 2008; Bhattarai et al., 2008; Kammerhofer et al., 2015), also some on monocotyledonous plants. To our knowledge, no research has been done regarding defence signaling pathways in rice upon infection with foliar nematodes, among which *D. angustus*. This nematode is an obligate biotroph that feeds on newly forming tissues in the rolled leaf sheath, the panicles, and the seeds.

Our investigation on the susceptibility level of *D. angustus* in rice mutants or transgenic plants impaired in the SA, JA or ET pathways showed that the all these lines were more susceptible compared to control plants. It has already been reported that the SA-deficient transgenic *NahG* plants (Yang et al., 2004), the JA biosynthesis mutant *hebiba* (Riemann et al., 2003) and ET insensitive *EIN2b* (Bailey et al., 2009) are more susceptible towards *M. graminicola* and migratory nematode, *H. oryzae* (Nahar et al., 2011; Nahar et al., 2012). The rice mutant *hebiba* is due to a genomic deletion of 169 kb, which comprises 26 annotated genes (Riemann et al., 2013; Nordström et al., 2013). One of the genes encodes allene oxide cyclase (AOC), part of the jasmonate biosynthetic pathway, loss of which leads to jasmonate deficiency. Another gene contained within the deleted interval gene encodes the alpha/beta-fold hydrolase D14L, which is homologous to *Arabidopsis thaliana* KARRIKIN INSENSITIVE2/HYPOSENSITIVE TO LIGHT (KAI2/HTL; Gutjahr et al., 2015). This hydrolase acts together with the F-box protein D3/MAX2 in the perception of karrikins. Karrikins is a plant growth regulator first identified in smoke that induce seed germination in fire-chasing plants (Waters et al., 2012; Guo et al., 2013). Karrikins is also a necessary signaling component for the establishment of arbuscular mycorrhizal (AM) symbiosis (Gutjahr et al., 2015). Therefore, the enhanced susceptibility of *hebiba* to *D. angustus* could also be casued by other factors than the lower JA level.

For further confirmation, we sprayed MeJA, Eth and BTH and corresponding hormone inhibitors on the shoots of ufra susceptible Nipponbare and we found that exogenous MeJA, Eth and BTH application are equally important for resistance against *D. angustus*, while the hormone inhibitors
make the plants more susceptible to this nematode. It is important to note that AOPP not only inhibits PAL but also auxin biosynthesis (Soeno et al., 2010). Hormone application has been shown to induce defence against many fungal, bacterial and nematode pathogens (De Vleesschauwer et al., 2008; Nakashita et al., 2003; Nahar et al., 2011; Nahar et al., 2012). BTH/SA application on roots of Arabidopsis or tomato, and shoots of okra, cowpea or grapevines induce resistance to cyst and RKN (Owen et al., 2002; Nandi et al., 2003; Branch et al., 2004; Wubben et al., 2008), possibly by elevated expression of defence related genes. Nahar et al. (2011) observed that foliar application with MeJA and ethephon strongly activated defence pathways in systemic rice roots, exemplified by the strong upregulation of pathogenesis-related genes OsPR1a and OsPR1b and make the rice plants more resistant towards RKN, M. graminicola. BTH, MeJA, and Eth application on rice shoot also induced resistance against migratory nematode, H. oryzae (Nahar et al., 2012).

Analysis of gene expression of SA, JA and ET defence signaling pathway in both compatible and incompatible rice-D. angustus interaction revealed that OsICS1 and OsWRKY45-based SA-signaling is induced at 1 dpi in resistant plants, while the OsPAL1 seems important throughout the time points. Based on its contrasting expression profile between the compatible and incompatible interaction, we hypothesize that OsPAL1 is involved in triggering the defence responses in the resistant Manikpukha. Moreover, the slight but consistent upregulation of OsICS1 probably contributes to the defence in the resistant cultivar. SA responsive OsWRKY45 might play a role during the early defence response (1 dpi) of Manikpukha, but is only later induced (10 and 20 dpi) in Nipponbare upon D. angustus infection.

The result also showed that JA biosynthesis and signaling at 1 dpi and OsACS1, OsERF1 at 5 and 10 dpi might a have role in defence in resistant plants to the nematode. However, downregulation (OsPAL1, OsACS1 and OsERF1) or base line expression of most of the genes of SA, JA and ET pathway in the susceptible cultivar during the time course of nematode infection indicates that D. angustus can suppress the defence response triggered by the plants. Taken together, these data indicate that the JA pathway might be temporarily activated during the early defence response (1 dpi) of Manikpukha upon D. angustus infection and a consistent upregulation of OsACS1 and OsEin2b upon nematode infection in the incompatible interaction suggests a positive correlation between ET-inducible gene expression in rice and defence to D. angustus. Previous data from our lab on other rice-nematode interactions showed that SA (except OsPAL1) and ET pathway genes were attenuated at 1-3 days after RKN inoculation in locally infected tissues (Nahar et al., 2011)
as well as in systemic shoot tissues (Kyndt et al., 2012). On the other hand, OsACS1 and OsAOS2 was upregulated in the root of H. oryzae infected plants (Nahar et al., 2011), whereas the phenylpropanoid pathway and ethylene pathway genes were upregulated in systemic shoot tissues at 3 days after H. oryzae inoculation (Kyndt et al., 2012). Kumari et al. (2016) found that the RKN resistant variety Vandana significantly upregulated the SA biosynthesis genes, JA and ET pathway genes at early (2 days post inoculation) and later (6 days post inoculation) stages of nematode infection whereas in susceptible variety Pusa 1121, these genes are suppressed at later stages of nematode infection.

Activation of rice OsPRI genes (OsPRIa and OsPRIb) is an important part of rice defence which can be regulated by different hormone signaling molecules such as JA, SA, ET and ABA (Agrawal et al., 2000, 2001a, 2001b; Reymond & Farmer, 1998). The rice OsPR5 gene also has been shown to be effective against pathogens (Datta et al., 1999; Grover & Growthaman, 2003) and is regulated by SA and JA (Rakwal et al., 2001; Fu et al., 2013). The importance of PR genes in rice defence against PPN has been reported in previous investigations (Nahar et al., 2011; 2012; Ji et al., 2015; Kyndt et al., 2012; Kumari et al., 2016). PR genes such as, OsPRI1b, OsPRI10 and OsPRI1a are strongly upregulated in rice roots upon M. graminicola infection at 3 and 7 dpi suggesting activation of defence pathways in the roots (Kyndt et al., 2012). Kumari et al. (2016) demonstrated that OsPRI1a and OsPRI10 were consistently upregulated in the roots of both susceptible and resistant cultivars during RKN infection at 2 and 6 dpi whereas, OsPRI1b was significantly attenuated in the susceptible root at both time points. We have investigated two PR genes, OsPRI1a and OsPR5 in both compatible and incompatible interactions in the current study. Intriguingly, our result showed that only OsPR5 was significantly upregulated in susceptible shoot tissues at all time points (1, 5, 10 and 21 dpi) upon D. angustus infection. In the incompatible interactions, these two PR genes did not show significant differential expression upon nematode infection. The explanation could be that OsPR5 may have particular function in susceptible response compared to resistant reactions and different PR proteins have different functions in susceptible and resistant interactions (Hou et al., 2012) or the nematodes can influence the expression pattern of our studied PR genes. Nevertheless, our experiments showed interesting phenomena awaiting further interpretation. However, differential expression of PR genes has also been reported in systemic shoot tissues of different crops upon RKN and H. oryzae infection (Sanz-Alferez et al. 2008; Hamamouch et al., 2011; Kyndt et al., 2012; Molinari et al., 2014; Kumari et al., 2016).
Measurement of SA in susceptible and resistant cultivars of rice at 1 dpi showed no significant changes in hormone accumulation after nematode infection in the resistant cultivar but the SA accumulation was significantly reduced in the infected susceptible Nipponbare plants at 1 day after nematode infection. However, our gene expression analysis with the resistant cultivar showed that the phenylpropanoid pathway (involving *OsPAL1*) was strongly activated in the shoots of the resistant variety, but this is not paralleled with SA level at 1 dpi (Fig 4.3 and Fig. 4.4A). We therefore hypothesize that PAL, which is the entry-point enzyme of the general phenylpropanoid pathway could contribute to the biosynthesis of phenolics, lignins, stilbenes and many other compounds (Winkel-Shirley, 2001; Vogt, 2010; Fraser & Chapple, 2011), rather than SA production. It has been reported that phytoalexins, isoflavonoids, phenylpropanoids, lignins play important roles in plant defence (Hölscher *et al.* 2014; Collingborn *et al.* 2000; Plowright *et al.*, 1996). It has been reported that the phenylpropanoid pathway is known to be positively regulated by JA and its derivate methyl jasmonate (MeJA), which have been found to induce the accumulation of PAL (Kazan & Manners, 2008; Galis *et al.*, 2006; Pauwels *et al.*, 2008; Taheri *et al.*, 2010). In case of JA measurements, a significant increase in JA accumulation was observed in the resistant Manikpukha plants at 1 dpi. The results of gene expression analysis of JA pathways show strong similarity with the observed JA accumulation at 1 dpi (Fig 4.3 and Fig. 4.4B).

The contribution of lignin to the plant basal defence against PPN has been well documented (Fogain & Gowen, 1995; Wuts *et al.*, 2006; Wuts *et al.*, 2007). We found a significantly higher lignin content in the resistant cultivar upon nematode infection compared to the susceptible cultivar. This high accumulation of lignin in the shoot tissues of the resistant cultivar probably strengthens the plant cell wall, which can inhibit nematode penetration and migration inside the resistant plants (Gheysen & Jones, 2006). The higher lignin content in the resistant cultivar is most likely due to the high upregulation of the *OsPAL1* gene upon infection. PAL catalyzes the deamination of phenylalanine to trans-cinnamic acid, also a precursor for the lignin biosynthesis pathway (Dixon & Pavia, 1995). The results also showed that the lignin content and the *OsPAL1*-expression decreases in the susceptible plant after nematode infection. Similarly, PAL genes were significantly suppressed in giant cells induced by *M. graminicola* in rice (Ji *et al.*, 2013). β-aminobutyric acid (BABA) application, which makes the plants more resistant towards RKN, also strongly induces the genes involved in lignin biosynthesis in rice plants (Ji *et al.*, 2015). Again similar to our results,
Kumari et al. (2016) recorded a greater induction of lignin biosynthesis genes in the roots of a resistant cultivar versus a susceptible cultivar at 2 days after RKN inoculation.

The OsPAL1 gene was significantly upregulated in the resistant cultivar Manikpukha which is comparable with the result of fig. 4.2 where we found that application of PAL inhibitor makes the plants more susceptible for nematode infection resulting in a higher number of nematodes per plant. We have also found that this increased upregulation OsPAL1 is not correlated with nematodes per plant. Interestingly higher lignin content in the resistant cultivar which might be partly due to high upregulation of OsPAL1 upon nematode infection indicates that the general phenylpropanoid pathway might have an important role in rice resistance against D. angustus. Therefore, it will be particularly interesting to investigate other secondary metabolites such as phenolics, flavonoids in the resistant cultivar upon nematode infection. Moreover, the application of PAL inhibitor in Manikpukha will provide valuable insights about the important of the phenylpropanoid pathway.

4.5 Conclusion

In summary, SA, JA and ET play a positive role in rice basal defence against D. angustus. We have also shown that OsPAL1 is upregulated in the resistant plant upon nematode infection, but its induction is not correlated with increased SA production. Our data rather demonstrate that lignin accumulation contributes to rice resistance against the nematode, D. angustus.

4.6 Materials and methods

4.6.1 Plant materials and growth condition

The SA-deficient NahG lines (Yang et al., 2004), ET transgenic OsEin2b RNAi line (Bailey et al., 2009) were kindly provided by Yinong Yang (Pennsylvania State University) and the corresponding wild type ‘Nipponbare’ (japonica type) was provided by USDA; GSOR-100. The JA biosynthesis mutant hebiba (Riemann et al., 2003) and it background ‘Nihonmasari’ were generously provided by P. Nick (Karlsruhe University, Germany). OsWRKY45 RNAi line was a gift from Hiroshi Takatsuji (Plant Disease Resistant Research Unit, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan). Manikpukha (indica type), an ufra resistant variety (Khanam et al., 2015) was collected from Bangladesh Rice Research Institute (BRRI),
Gazipur-1706. Before transferring to SAP-substrate (Reversat et al., 1999) in glass tube (15×2.5 cm), the seeds were pre-germinated on wet filter paper for 5 days at 30°C. The glass tubes were kept at 26°C under a 12h/12h light-regime (150 μmol/m²/s) and 70-75% Relative Humidity.

4.6.2 Infection experiments

The nematode, *D. angustus* obtained from Plant Pathology Division of BRRI was originally isolated from an infested farmer's field in the Gazipur district, Bangladesh. The nematode culture was maintained *in vivo* on a susceptible rice cultivar, BR3 (indica type; provided by BRRI) at 26°C under a 12h/12h light-regime (150 μmol/m²/s) and 70-75% Relative Humidity. The nematodes were extracted from *D. angustus* infected rice stems using the modified Baermann method (Luc et al., 2005). The stems were longitudinally divided, cut into 5mm pieces and placed over a sieve to let the nematode migrate out of the plant tissues overnight. The nematode suspension was collected and counted using a stereomicroscope. Fifteen days old rice seedlings were inoculated with approximately 100 nematodes of *D. angustus* per plant or mock-inoculated with water. The inoculation method was done as described by Rahman (1993). At 15 days after sowing, the water was raised up to the upper most node of the seedling. The infection level of the plant was evaluated at 20 days post inoculation (dpi) by counting the number of nematodes per plant.

4.6.3 Chemical treatments

The chemicals methyl jasmonate (MeJA), and ethephon and BTH (benzo 1, 2, 3 thiadiazole-7-carbothioic acid S-methyl ester were purchased from Sigma (Bornem, Belgium). For the pharmacological experiment, JA biosynthesis inhibitor, diethylthiocarbamic acid (DIECA), ET biosynthesis inhibitor aminoxyacetic acid (AOA) and SA pathway inhibitor, L-2-Aminooxy-3-phenylpropinoic acid (PALi) were also purchased from Sigma (Bornem, Belgium). The chemicals were used in the following concentrations: MeJA (100 μM), BTH (250 μM), ethephon (500 μM), DIECA (100 μM), AOA (25-50mM) and PALi (100 μM). All hormone and hormone inhibitor solutions were prepared in separate vaporizers; the solutions were dissolved in water containing 0.02% (v/v) Tween 20. Before diluting into water, the chemicals were first dissolved in ethanol except ethephon and BTH. The prepared chemicals with indicated concentration were sprayed with vaporizers as a fine mist of either compound on to the leaves of 15 days old seedlings until runoff. In the control treatment, only distilled water containing 0.02% (v/v) Tween 20 was sprayed. In the
infection experiment, the nematode inoculation was done 24 hours after the chemical spray. All experiments were repeated three times.

4.6.4 Data collection and statistical analysis

Gene expression analysis was done by analysing the qPCR obtained data using the REST software 2009 (Corbett Research; Pfaffl et al., 2002). This software compares the relative expression between control and sample group with statistical significance. All other data were analysed in statistical software, IBM SPSS version 22 (IBM SPSS, Inc., Chicago, IL, USA). Collected data from infection experiments were analysed using analysis of variance (ANOVA) and the mean differences of the control and treated group were analysed by Duncan’s multiple range test ($\alpha = 0.05$). Before analysis, normality of the data were tested with Kolmogorov-Smirnov Test of Composite Normality ($\alpha = 0.05$) by boxplot visualization. Homogeneity of variance was checked by applying the Levene test ($\alpha = 0.05$). The assumptions of normality and homoscedasticity of the data were found to be fulfilled. Data from hormone measurement experiments were analysed using the non-parametric Kruskal-Wallis test ($\alpha =0.05$).

4.6.5 RNA extraction, and cDNA synthesis

RNA extraction was done by using the RNeasy Plant Mini kit (Qiagen, Venlo, The Netherlands). After addition of RTL buffer, an extra sonication step was done. The concentration and purity of RNA was measured by a NanoVueTM spectrophotometer (GE Healthcare). Afterwards, the extracted RNA was treated with DNaseI to remove all contaminating DNA. One microgram of RNA was treated with 1 µl DNaseI (1 U· µl$^{-1}$; Fermentas), 1 µl RiboLock™ RNase Inhibitor (40 U· µl$^{-1}$; Fermentas) and 1.8 µl DNaseI buffer (10x, Fermentas) in a total volume of 18 µl. The mixture was incubated at 37°C for 30 min. Then 2 µl 25mm EDTA (Fermentas) were added followed by another incubation at 65°C for 10 min to stop the reaction.

There were three steps in synthesizing first strand cDNA. The first step was the addition of 1 µl oligo dT (700 ng·µl$^{-1}$), 2 µl 10 mm dNTPs (Invitrogen, Gent, Belgium) and 4 µl RNAse-free water to the DNAse-treated RNA and incubation at 65°C for 10 min to remove secondary structures. The second step was the addition of 8 µl 5x first strand buffer (Invitrogen) and 4 µl 0.1 M DTT (Invitrogen) to the incubated mixture from the first step. The mixtures were then incubated for 2
min at 42°C. The third step was addition of 1 µl SuperScript II Reverse Transcriptase (200 U· µl⁻¹; Invitrogen), with incubation for 2 h at 42°C. After completing the final step of cDNA synthesis, 60 µl water was added to the solution to dilute. The cDNA quality was examined by standard PCR with some reference genes and the PCR products were checked on a 1.5% agarose gel.

4.6.6 qRT-PCR

The SensiMix SYBR No-ROX Kit (Bioline, London, UK) was used to perform qRT-PCR. Each reaction contained 10µl of 2×SensiMix, 500 nM of each primer and 1 µl of cDNA in a total volume of 20 µl. All reactions were performed in three technical replicates on a Rotor-Gene 3000 (Corbett Life Science, Hilden, Germany) and analyzed with Rotor-Gene 6000 software version 1.7. The conditions for performing the PCR reactions were: 10 min at 95°C and 45 cycles of (25 s at 95°C, 60s at 58°C and 20 s at 72°C). A melting curve was generated after the PCR reaction by gradually increasing the temperature to 95°C to test for amplicon specificity. The data were analyzed by REST 2009 software to determine statistically significant differences (Pfaffl et al., 2002).

Table 4.1: Summary of the reference and target genes used in the study, with their GenBank accession number or locus number, and the primer pair used for qRT-PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>GenBank Accession/Locus no.</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsEXP</td>
<td>LOC_Os03g27010</td>
<td>TGTGAGCAGCTTCTCGTTTG</td>
<td>TGGTGTTGCTCTGAGATCG</td>
</tr>
<tr>
<td>OsEXPناسر</td>
<td>LOC_Os07g02340.1</td>
<td>CACGTTACGGTGACACCTTTTT</td>
<td>GAGCCTCTCTCTCTCTCTCAG</td>
</tr>
<tr>
<td>OsPAL1</td>
<td>LOC_Os02g14630</td>
<td>TGTGGGTGCTCTGCGTCG</td>
<td>AAGGGTGTTGCTGCGCACGAG</td>
</tr>
<tr>
<td>OsJCS1</td>
<td>LOC_Os09g19734</td>
<td>TGTCCTCCACAAAGGGCTCTG</td>
<td>TGGCTTCCAACCTTTAAACTGCG</td>
</tr>
<tr>
<td>OsWRKY45</td>
<td>Os05g0323900</td>
<td>AATTGCTTGGTCGTCAGAAGA</td>
<td>AAGTGGCCTTTTGCTGCTT</td>
</tr>
<tr>
<td>OsAOS2</td>
<td>NM_001055971.1</td>
<td>TGGCCGACAGCTCTGAGTTC</td>
<td>GGGCAGCGGGAGCTGAGTG</td>
</tr>
<tr>
<td>OsJMT1</td>
<td>LOC_Os06g0214600</td>
<td>CACGCTCAGTCCAAAGATGA</td>
<td>CTCAACCGTTTGGGCAAC</td>
</tr>
<tr>
<td>OsJnyc</td>
<td>AY026332</td>
<td>GAGGACGAGATGCAAAAGC</td>
<td>CATGGCCATCTTTAAGGTAAC</td>
</tr>
<tr>
<td>OsACS1</td>
<td>LOC_Os03g51740</td>
<td>GATGGGTGCTCGTGATGACACA</td>
<td>GTCGGGGGAAAATGAAAAAT</td>
</tr>
<tr>
<td>OsACO7</td>
<td>LOC_Os01g39860</td>
<td>GACTACTACCGAGCGCAACA</td>
<td>GATTAGCGACGCCAGTTT</td>
</tr>
<tr>
<td>OsEin2b</td>
<td>Os07g06190</td>
<td>GCGCATTGTGGGAGAGAG</td>
<td>CAGGGCGGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>OsERF1</td>
<td>LOC_Os04g62220</td>
<td>AAGGGTCAATAATGCCCCGTTCA</td>
<td>TCCACACACACACATCAG</td>
</tr>
<tr>
<td>OsPR1a</td>
<td>Os07g0418500</td>
<td>TGCTATGCTATGCTATGCTT</td>
<td>CACTAACGGCAATAACGGCTGACA</td>
</tr>
<tr>
<td>OsPRS</td>
<td>X68197</td>
<td>ACCCTCTACCGCTGACCTC</td>
<td>GAAGACGACTTGGTAGT</td>
</tr>
</tbody>
</table>

4.6.7 SA and JA quantification

The phytohormone SA and JA were quantified in the shoots of D. angustus infected and non-infected Nipponbare and Manikpukha at 5 dpi. Collected shoot materials were homogenized by grinding in liquid N₂ and extracted at -80°C using the modified Bieleski solvent. After filtration
and evaporation, chromatographic separation was performed on a U-HPLC system (Thermo Fisher Scientific) equipped with a Nucleodur C18 column (50 x 2 mm; 1.8 µm dp) and using a mobile phase gradient consisting of acidified methanol and water. Mass spectrometric analysis was carried out in selected-ion monitoring (SIM) mode with a Q Exactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific), operating in both positive and negative electrospray ionization mode at a resolution of 70,000 full width at half maximum.

### 4.6.8 Lignin measurement

Lignin content was measured in the shoots of *D. angustus* infected and non-infected Nipponbare and Manikpukha at 5 dpi. Lignin content was measured using a modified Klason method (Bosch *et al.*, 2015), where the 4 hour incubation with a soxhlet extractor was replaced by a 1 hour incubation at 121 °C in an autoclave.
Chapter 5: Effect of *Bacillus velezensis* strain BSK isolated from Bangladesh on rice growth and defence against *Ditylenchus angustus*

Shakhina Khanam, Abdul Mannan Akanda, Tina Kyndt and Godelieve Gheysen
5.1 Abstract

The potential of a rhizobacterium, *Bacillus velezensis* strain BSK isolated from Bangladesh in promoting plant growth of rice and inducing systemic resistance to the rice stem nematode, *Ditylenchus angustus* was evaluated in this study. The effect of the bacterium on plant growth was tested at three different concentrations (10^4, 10^6 and 10^8 colony forming units per ml; cfu ml^{-1}) and with two different methods of inoculation (seed and soil inoculation). Our results showed that soil inoculation with 10^4 cfu ml^{-1} appeared to increase root and shoot length with a significant increase of root and shoot weight. Soil drenching with bacteria prior to *D. angustus* inoculation in rice resulted in a significantly lower number of nematodes per plant as compared with non-treated control plants. The plant response upon soil inoculation of bacteria with or without *D. angustus* infection was studied at the transcriptional level in systemic shoot tissues by qRT-PCR. The relative expression of some SA/JA/ET-marker genes was investigated. The result reveals that the bacteria can influence the gene expression in the systemic shoot tissues and this might affect the outcome of the rice-*D. angustus* interaction.

5.2 Introduction

Biological control is considered as an ecologically and economically friendly approach to reduce plant diseases. Biological control can be defined as the use of living organisms or their metabolites to reduce the population density or disease impact of a specific pathogen or pest organism (Eilenberg et al., 2001). Bacteria that colonize the rhizosphere and exert beneficial effects on plant growth and development are generally termed as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980; Lugtenberg and Kamilova, 2009). PGPR are one of the important group of soil microorganisms that are capable of suppressing a wide range of pathogens including plant-parasitic nematodes (PPN). Among PGPR, *Bacillus* spp. and *Pseudomonas* spp. are dominant populations in the rhizosphere that can affect PPN (Rovira & Sands, 1977; Rack & Sikora 1992; Sikora, 1992). Rhizobacteria can reduce nematode populations by different mechanisms such as by affecting nematode behaviour (Sikora & Hoffmann-Hergarten, 1992), competing for essential nutrients (Oostendorp & Sikora, 1990), promoting plant growth (El-Nagdi & Youssef, 2004), interfering with plant–nematode recognition (Oostendorp & Sikora, 1990), antagonising the
nematodes by the production of toxins, enzymes and other metabolites (Siddiqui & Mahmood, 1999) and inducing systemic resistance (Hasky-Gunther & Sikora, 1995).

The bacterial strain used in the current study was isolated from a field of Bangbandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh. The isolated bacteria was identified based on partial gyrase B (gyrB) gene sequence analysis using BLAST. The isolated bacteria showed closest similarity to *Bacillus methylo trophicus* strain B25, *B. amyloliquefaciens* subsp. *plantarum* strain NJN-6, *B. amyloliquefaciens* subsp. *plantarum* strain CAU B946, and *B. amyloliquefaciens* strain BGP20, all with 100% sequence identity. However, Dunlap *et al.* (2016) recently showed that *B. oryzae* KACC 18228, *B. velezensis* NRRL B-41580, *B. methylo trophicus* KACC 13015 and *B. amyloliquefaciens* subsp *plantarum* FZB42 are closely related and they propose to reclassify these strains as later heterotypic synonyms of *B. velezensis*. Hence, we named our isolate *B. velezensis* strain BSK. Bacterial strains belonging to the clade *B. velezensis* are all plant-associated microorganisms that have been reported as potential bio-control agents against a number of pathogens including nematodes (Borriss, 2011; Chowdhury *et al.*, 2013, 2015; Burkett-Cadena *et al.*, 2008; Almaghrabi *et al.*, 2013).

Besides production of antimicrobial, antiviral and nematicidal compounds, PGPR also can induce systemic resistance (ISR; Van Wees *et al.*, 2008; Pieterse *et al.*, 2014; De Vleesschauwer *et al.*, 2006; 2009; Doornbos *et al.*, 2012) which contributes to pathogen suppression. A number of bacterial elicitors contributing to ISR such as flagella, lipopolysaccharides, quorum-sensing molecules, volatile organic compounds and cyclic lipopeptides (Jourdan *et al.*, 2009; Pieterse *et al.*, 2014) has been identified. Cyclic lipopeptides namely surfactin, iturin and fengycin family CLPs can be produced by multiple *Bacillus* spp. with a number of biological activities (Raaijmakers *et al.*, 2010; Wu *et al.*, 2015; Elshak *et al.*, 2016). Cawoy *et al.* (2014) showed that lipopeptides are the main ingredients for inhibition of fungal phytopathogens by *B. subtilis/amyloliquefaciens*. Stimulation of ISR by bacterial metabolites is likely the main mechanism responsible for biocontrol action of *B. amyloliquefaciens* strains FZB42 (Wu *et al.*, 2015; Chowdhury *et al.*, 2015; Cawoy *et al.*, 2015). Soil application of *B. subtilis* BBG111 triggers induced systemic resistance in rice against rice sheath blight caused by *Rhizoctonia solani*. Cyclic lipopeptides, fengycin and surfactin produced by *B. subtilis* BBG111 target JA, ET and/or auxin pathways indicating the role of fengycin and surfactin in the induced defence state (Chandler *et al.*
Geng et al. (2016) identified a protein from *B. firmus* DS-1 called Sep 1 that exhibited serine protease activity and degraded the intestinal tissues of nematodes.

Studies with defence signaling pathways revealed that ISR triggered by PGPR is generally regulated by jasmonic acid (JA) - and ethylene (ET)-dependent signaling pathways and is typically not related with the direct activation of PR genes (Van Wees et al., 1997; Vallad & Goodman, 2004). However, several examples of PGPR have been reported to trigger ISR in an SA-dependent manner (De Vleesschauwer & Hofte 2009; Van de Mortel et al., 2012). Treatment with *B. amyloliquefaciens* Lx-11 that was found to possess biocontrol activity against rice bacterial leaf streak (BLS) caused by *Xanthomonas oryzae* pv. *oryzicola* concurrently induced the defence-related genes *PR1a, PR1b, NPR1* and *PAL* in the leaves of rice (Zhang et al., 2012). Siddiqui & Shaukat (2004) reported that ISR in tomato induced by *P. aeruginosa* 7NSK2 and *P. fluorescens* CHA0 against *Meloidogyne javanica* works through an SA independent signal transduction pathway.

The rice stem nematode, *Ditylenchus angustus* (Butler, 1913) is one of the most important plant parasitic nematode of rice in South and Southeast Asia which is responsible for “Ufra” disease in rice. It is an obligate parasite that causes considerable yield losses in deep water, irrigated and rainfed rice (Prasad et al., 2000; Latif et al., 2004). Farmers are mostly dependent on nematicides as a relatively reliable method of nematode control. However, increasing use of chemical inputs causes numerous negative effects, i.e., development of nematode resistance and non-target environmental impacts (Weger et al., 1995; Haydock et al., 2013), emphasizing the need for an environmentally friendly, alternative management strategy to control this nematode. The use of beneficial microbes, in association with host resistance, holds great prospective as an environmentally friendly approach for plant disease management (Pérez-García et al., 2011).

In this chapter, we evaluated (1) the effect of *B. velezensis* strain BSK on rice growth at different bacterial concentrations, (2) the potential of strain BSK to induce systemic resistance against *D. angustus* at different bacterial concentrations and (3) the involvement of SA, JA and ET defence signaling pathways involved in rice-*D. angustus*- *B. velezensis* strain BSK interactions.
5.3 Results

5.3.1 Effect of *Bacillus velezensis* strain BSK on rice growth

From Fig. 5.1 A it is observed that in case of shoot and root length, there was no significant difference between bacteria treated and control rice plants. Among three concentrations tested as seed and soil treatment, soil treatment with $10^4$ cfu ml$^{-1}$ bacterial concentration performed best. In case of fresh shoot and root weight (Fig. 5.1 B), a significant increase was observed in plants treated with the lowest concentration of bacteria compared to the control plants. Soil treatment with $10^4$ cfu ml$^{-1}$ produced significantly higher shoot and root weight than control plants respectively.
Fig 5.1. Effect of Bacillus velezensis strain BSK on rice growth at $10^4$, $10^6$ and $10^8$ cfu ml$^{-1}$ upon seed and soil treatment, at 28 days after sowing. (A) Shoot and root length, (B) Fresh shoot and root weight. The bars represent the mean and standard error recorded on 8 plants. Different letters indicate statistically significant differences (Dunca’s multiple range test with $\alpha=0.05$). Data represent one of three independent experiments with similar results. The data from the other replicates is shown in appendix fig A9.

5.3.2 Determination of Ditylenchus angustus infection in Bacillus velezensis strain BSK treated rice plants

Based on the positive effect on rice growth after soil treatment with the bacteria, we further studied the potential of strain BSK as an antagonistic organism against the rice stem nematode, *D. angustus*. The bacteria treated rice plants had a significantly reduced number of nematodes per plant compared to the control plants. The plants treated with $10^4$ cfu ml$^{-1}$ had 69%, $10^6$ cfu ml$^{-1}$ had
68% and $10^8$ cfu ml$^{-1}$ had 12% reduction in number of nematodes per plant compared to the control plants.

Fig 5.2. Effect of soil treatment with *Bacillus velezensis* strain BSK, at different concentrations, on the number of nematodes per plant. The bacteria at $10^4$, $10^6$ and $10^8$ cfu ml$^{-1}$ were drenched over SAP-substrate during seedling sowing and 12 days after sowing. Plants were inoculated with 100 nematodes of *D. angustus* at 1 day after bacteria inoculation. The bars represent the mean and standard error of the number of nematodes per plant 20 days after nematode inoculation recorded on 8 plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). Data represent one of three independent experiments with similar results. The data from other replicates is in appendix Fig A10.

### 5.3.2 Gene expression in systemic rice shoots upon *Bacillus velezensis* strain BSK inoculation

The plant response in systemic shoot tissues upon soil inoculation of bacteria with or without *D. angustus* infection was studied at the transcriptional level by qRT-PCR. The relative expression of 6 defence related genes of SA/JA/ET-marker genes was investigated (Fig 5.3). The expression of most tested genes was not significantly different between treatment and control (Fig. 5.3). The SA pathway genes *OsICS1* and *OsWRKY45* tend to be downregulated in shoot tissues from infected plants, except for *OsICS1* being slightly upregulated at 6 days after nematode inoculation in the bacteria treated plants. The JA pathway genes, *OsAOS2* and *OsJAmyb* tend to be upregulated upon nematode infection with or without bacteria treatment. The *OsEIN2* gene from the ET pathway showed a downregulation trend at 6 dai upon nematode infection with or without bacteria.
treatment. The pathogenesis gene \textit{OsPR10} was upregulated in all cases with a significant difference at 6 dai in nematode-infected plants which were treated with \textit{B. velezensis} strain BSK.

![Graph showing expression levels of defence-related genes](image)

**Fig 5.3.** Analysis of the expression levels (using qRT-PCR) of some selected defence-related genes in rice shoots upon \textit{Bacillus velezensis} strain BSK with or without \textit{Ditylenchus angustus} infection at 1 and 6 days after bacterial inoculation (dai). Bars represent the mean and standard error of the relative amount of transcripts of these genes from two biological replicates (n=2), each containing a pool of shoot tissue from three infected plants in comparison with the corresponding control plants grown under the same conditions. Gene expression levels were normalized using two internal reference genes, \textit{OsEXP} and \textit{OsEXPnarsai}. Asterisks indicate statistically significant (\(P \leq 0.001\)) differential expression in comparison with untreated uninfected plants, performed by 2000 randomisations using REST 2009.

### 5.4 Discussion

Biological control using antagonistic bacteria is considered to be environmentally friendly and can be applied as an integrated disease management tool. In line with this view, in this study we evaluated the efficacy of a rhizobacterium, \textit{B. velezensis} strain BSK on rice growth and possible ISR activity against \textit{D. angustus}. Moreover, we have investigated the underlying gene expression pattern with regards to the phytohormone pathways of SA, JA and ET in the rice- strain BSK -\textit{D. angustus} interaction.

The study of strain BSK effect on rice growth showed that soil treatment with \(10^4\) cfu ml\(^{-1}\) promotes a significant increase of root and shoot weight compared to control plants. However, the higher bacterial concentrations (\(10^6\) and \(10^8\) cfu ml\(^{-1}\)) showed no difference in shoot, root length and weight compared with the non-inoculated control rice plants. The growth promoting effect at low
bacterial concentration supports the findings of Ramírez & Joseph (2010), who have shown that plant growth promotion by *B. amyloliquefaciens* FZB45 depends on inoculum rate and P-related soil properties; the lower rate (10⁶ spores per seedling) was superior compared to the higher (10⁸ spores per seedling) for shoot fresh weight and plant inorganic P content. They observed that strain FZB45 exerts a direct mechanism on plant growth, probably by indole-3-acetic acid (IAA) production, which creates a concentration-dependent response to inoculation and interacts with phytase-mediated effects (Ramírez & Joseph, 2010).

*B. methylotrophicus* sp. nov. from rice rhizosphere soil was found to promote plant growth (Madhaiyan *et al*., 2010). A number of rhizobacteria has been identified as plant growth promoting in rice (Adhikari *et al*., 2001; Ashrafuzzaman *et al*., 2009; Suprapta *et al*., 2016). PGPR promote plant growth and development through a variety of mechanisms such as the production of phytohormones, the suppression of deleterious organisms, the activation of phosphate solubilisation and promotion of the mineral nutrient uptake. It has been reported that the presence of IAA and phytase activity of *B. amyloliquefaciens* FZB45 are important for plant growth-promotion under phosphate limitation (Idris *et al*., 2007; Idriss *et al*., 2002). Ryu *et al*. (2003) found that *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a release a blend of volatile components, in particular, the volatile components 2, 3-butanediol and acetoin that promote growth of *Arabidopsis thaliana*. Nothing is know about *B. velezensis* strain BSK. Further studies are needed to reveal the mechanism of growth promotion by strain BSK.

A number of *Bacillus* species has been shown to induce systemic resistance reducing penetration and reproduction of RKN or cyst nematodes in different crop species (Hasky-Gunther & Sikora, 1995; Schafer *et al*., 2006; Hauschild *et al*., 2000; Almaghrabi *et al*., 2013). Using the strain BSK we evaluated for the first time the effect of ISR against the rice stem nematode, *D. angustus*. We observed that drenching of soil with strain BSK significantly reduced the number of *D. angustus* on rice by up to 69% compared to the control without BSK. Our results also showed that treatment with a low concentration of strain BSK (10⁴ and 10⁶ cfu ml⁻¹) performed better for controlling nematodes compared to a higher concentration (10⁸ cfu ml⁻¹). Soil drenches or seed treatments of tomato with a new *Bacillus* species, *B. methylotrophicus* strain R2-2 has been found to suppress disease caused by the root-knot nematode *M. incognita* in petridish, greenhouse and field conditions (Zhou *et al*., 2016). Another *B. methylotrophicus* strain BC79 was found to reduce the
rice blast caused by *Magnaporthe oryzae* (Shan et al., 2013). A novel endophytic strain, *B. oryzicola* YC7007 isolated from rice roots has been reported to suppress bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae*, panicle blight caused by *Burkholderia glumae* and bakanae disease of rice caused by *Fusarium fujikuroi* (Hossain et al., 2016) via direct inhibition, induced systemic resistance through primed induction of the jasmonic acid pathway (Hossain et al., 2016) and antibiotic production (Chung et al., 2015). It has been reported that volatile compounds consisting of 2, 3- butanediol and acetoin (3-hydroxy-2-butanone) from *B. amyloliquefaciens* activates ISR in *Arabidopsis* seedlings (Ryu et al., 2003). Chowdhury et al. (2015) demonstrated that cyclic lipopolysaccharide contribute to the ISR plant response triggered by FZB42. However, almost nothing is known about strain BSK mediated ISR against pathogens and hence the mechanisms remains to be further studied.

Typically, rhizobacteria mediated ISR is mediated by JA and/or ET signaling pathways. By contrast, SA seems to be an important messenger in SAR (Pieterse et al., 2014). However, analysis using the *B. subtilis* strain FB17 on defense-compromised mutants of *A. thaliana* indicated that ISR against *Pseudomonas syringae* pv. tomato DC3000 occurs via NPR1 and requires SA/ET whereas jasmonic acid (JA) is not essential (Rudrappa et al., 2010). Chowdhury et al. (2015) observed that *B. amyloliquefaciens* subsp. *plantarum* FZB42 increased expression of *PR1* (pathogenesis protein 1, an SA marker gene), and plant defensin factor 1.2 (*PDF1.2*; defensin, JA/ET marker gene) in lettuce in absence of the pathogen, suggesting that SA and ET pathways are involved in upregulating the defense response. However, simultaneous presence of these rhizobacteria and the pathogen *R. solani* enhanced *PDF1.2* expression and decreased *PR-1* expression suggesting a synergistic activation of the JA/ET pathway and suppression of the SA pathway in presence of both micro-organisms. We studied SA, JA and ET signaling pathway to elucidate the BSK mediated ISR against *D. angustus* in rice (Fig 5.3). In our observation, no significant differential expression of any of the SA, JA and ET pathway genes was detected. However, we observed a trend of downregulation of the SA pathway genes and upregulation of *OsAOS2* and *OsPR10* genes at 1 day after BSK inoculation in absence of the nematodes. In presence of both BSK and the nematodes, a trend of upregulation was observed for *OsICS1, OsAOS2*, and *OsJamyb* and a significant upregulation was observed for the *OsPR10* genes. Although further experiments are clearly needed, our results provide an indication that SA biosynthesis and the JA pathway and *OsPR10* activation might be involved in strain BSK induced priming for resistance to *D. angustus*. *OsPR10* is a
defence marker gene in rice which is positively regulated by different hormone signaling molecules such as SA, JA and ABA (Rakwal et al., 2001; Hwang et al., 2008; Choi et al., 2015). A strong upregulation of the JA inducible PR marker genes JiOISR10 and PR10b was observed in rice cell cultures treated with cyclic lipopeptides (fengycin and mycosubtilin) produced by B. subtilis (Chandler et al., 2015).

A number of studies revealed that ISR mediated by rhizobacteria involved cell wall reinforcement and accumulation of H$_2$O$_2$ and defence-related phenolics, following pathogen infection (Ahn et al., 2007; De Vleesschauwer et al., 2008). Root-drench application of surfactin and live cells of mutant B. amyloliquefaciens strain FZB42-AK3 (produces surfactin, but not bacillomycin D and fengycin) significantly reduced disease incidence caused by Magnaporthe oryzae on perennial ryegrass through activation of multi-layered induced systemic defence responses including the rapid accumulation of H$_2$O$_2$, phenolic/polyphenolic compounds and callose at the sites of attempted pathogen entry, together with the timely oxidative burst driving single or multicellular HR-type reaction and enhanced expression of defence related genes (Rahman et al., 2015). Biocontrol activity of P. fluorescens isolate Pf1 against M. incognita in tomato is associated with enhanced PR-protein activity, the involvement of enzymes in the phenylpropanoid pathway and with an accumulation of phenolics (Anita et al., 2004). Ji et al. (2015) showed that BABA-induced resistance against RKN likely occurs independently of JA and ET, mainly through the activation of basal defense mechanisms of the plant, such as reactive oxygen species accumulation, lignin formation, and callose deposition. The non-significant expression of SA, JA and ET signaling pathways in BSK-mediated ISR in rice to D. angustus indicates that probably other mechanisms are involved in this tripartite interaction. Thus, further molecular, biochemical and histochemical analysis in the rice-strain BSK-D. angustus interaction will provide valuable information for efficient use of this biocontrol agent against nematodes.

5.5 Conclusion

In summary, our results showed that a strain similar to B. velezensis strain BSK is a potential plant growth-promoting rhizobacterium and biocontrol agent against D. angustus. Our results showed that soil inoculation with a low bacterial density (10$^4$ cfu ml$^{-1}$) promotes growth in rice and controls the nematode at a significant level, suggesting B. velezensis strain BSK as an economical and ecological alternative of chemical control for nematode management. Our results also reveals that
the OsPR10 gene is strongly induced in the rice-BSK-D. angustus interaction while the SA biosynthesis and JA pathway are not strongly involved in B. velezensis strain BSK induced systemic resistance.

5.6 Materials and methods

5.6.1 Plant materials and bacterial culture conditions

All experiments were conducted in controlled conditions (26°C under a 12h/12h light-regime, 150 μmol/m²/s and 70-75% Relative Humidity) with Nipponbare rice (japonica type, provided by USDA; GSOR-100). B. velezensis strain BSK was isolated from roots of healthy wheat (Triticum aestivum) plants from the research field of the Bangbandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, Bangladesh. The bacteria were grown on solid Luria-Bertani broth (LB) medium at 25 °C for 2 days. After scraping off the plates, the bacterial cells were suspended in sterile saline (0.85% NaCl). Desired concentrations of the bacterial suspensions were adjusted based on their optical density at 620 nm.

5.6.2 Nematode culture

The nematode D. angustus was obtained from the plant pathology division of Bangladesh Rice Research Institute (BRRI), Gazipur-1706. The nematode was originally isolated from an infested rice field in Gazipur district, Bangladesh. The nematode culture was maintained in vivo on the susceptible rice cultivar BR3 (indica type; provided by BRRI) at 26°C under a 12h/12h light-regime (150 μmol/m²/s) and 70-75% Relative Humidity. Nematodes were extracted from D. angustus infected stem using the modified Baermann method (Luc et al., 2005). The stems were longitudinally divided, cut into 5mm pieces and placed over a sieve overnight to release nematodes from the plant tissues. The nematode suspension was collected and nematodes were counted under a light microscope.

5.6.3 Growth analysis of rice treated with Bacillus velezensis strain BSK

The effect of B. velezensis strain BSK on rice growth was tested at a concentration of 10⁴, 10⁶, and 10⁸ cfu ml⁻¹ with two different methods: seed and soil treatments. Rice seeds were surface-sterilized with 70% ethanol for 1 min and shaken in 1.2% (w/v) NaClO solution for 15 mins. The seeds were
then washed three times with sterile distilled water. The seeds were pre-germinated on wet filter paper for 5 days at 30°C before transferring to SAP-substrate (Reversat et al., 1999) in a glass tube (15×2.5 cm). In case of seed treatment, one gram of the surface sterilized seeds were soaked in 10 ml bacterial suspension (10^4, 10^6, and 10^8 cfu ml^-1) and water as control for 30 mins and then dried under laminar air flow. For soil treatment, the bacteria was drenched over SAP-substrate at a concentration of 10^4, 10^6, and 10^8 cfu ml^-1 during seedling transferring to SAP-substrate and 12 days after sowing. In control treatment, only distilled water was drenched over SAP-substrate. Root, shoot length and weight were measured 28 days after sowing. Bacterial association on the root surface of the plant was confirmed by the time the bioassays were discontinued. Roots of three plants of each treatment were rinsed with sterile water and serial dilutions of the water were plated on LB medium and analysed after incubation at 25 °C for 2 days.

5.6.4 Infection experiments with *Ditylenchus angustus*

Surface sterilization of rice seeds (Nipponbare) and bacteria culture were done as described before. The bacteria was soil drenched at a concentration of 10^4, 10^6, and 10^8 cfu ml^-1 during seedling transferring to SAP-substrate and 12 days after sowing. In control treatment, only distilled water was drenched over SAP-substrate. At 13 days after sowing, the bacterial and mock treated plants were inoculated with approximately 100 nematodes of *D. angustus* per plant (as described by Rahman, 1993). The water level was raised up to the upper most node of the 13-days old seedlings and the nematodes were inoculated into the water. The infection level of the plants was evaluated at 20 days post inoculation (dpi) by counting the number of nematodes per plant.

5.6.5 Data collection and statistical analysis

Gene expression analysis was done by analysing the qPCR obtained data using the REST software 2009 (Corbett Research; Pfaffl et al., 2002). This software compares the relative expression between control and sample group with statistical significance. All other data were analysed in statistical software, IBM SPSS version 22 (IBM SPSS, Inc., Chicago, IL, USA). Collected data were analysed using analysis of variance (ANOVA) and the mean differences of the control and treated group were analysed by Duncan’s multiple range test ($\alpha = 0.05$). Before analysis, normality of the data were tested with Kolmogorov-Smirnov Test of Composite Normality ($\alpha = 0.05$) by
boxplot visualization. Homogeneity of variance was checked by applying the Levene test ($\alpha = 0.05$). The assumptions of normality and homoscedasticity of the data were found to be fulfilled.

5.6.6 RNA extraction, and cDNA synthesis

RNA extraction was done by using the RNeasy Plant Mini kit (Qiagen, Venlo, The Netherlands). After addition of RTL buffer, an extra sonication step was done. The concentration and purity of RNA was measured by a NanoVueTM spectrophotometer (GE Healthcare). Afterwards, the extracted RNA was treated with DNaseI to remove all contaminating DNA. One microgram of RNA was treated with 1 µl DNaseI (1 U· µl$^{-1}$; Fermentas), 1 µl RiboLock™ RNase Inhibitor (40 U· µl$^{-1}$; Fermentas) and 1.8 µl DNaseI buffer (10×, Fermentas) in a total volume of 18 µl. The mixture was incubated at 37°C for 30 min. Then 2 µl 25mm EDTA (Fermentas) were added followed by another incubation at 65°C for 10 min to stop the reaction.

There were three steps in synthesizing first strand cDNA. The first step was the addition of 1 µl oligo dT (700 ng·µl$^{-1}$), 2 µl 10 mm dNTPs (Invitrogen, Gent, Belgium) and 4 µl RNAse-free water to the DNase-treated RNA and incubation at 65°C for 10 min to remove secondary structures. The second step was the addition of 8 µl 5× first strand buffer (Invitrogen) and 4 µl 0.1 M DTT (Invitrogen) to the incubated mixture from the first step. The mixtures were then incubated for 2 min at 42°C. The third step was addition of 1 µl SuperScript II Reverse Transcriptase (200 U· µl$^{-1}$; Invitrogen), with incubation for 2 h at 42°C. After completing the final step of cDNA synthesis, 60 µl water was added to the solution to dilute. The cDNA quality was examined by standard PCR with some reference genes and the PCR products were checked on a 1.5% agarose gel.

5.6.7 qRT-PCR

The SensiMix SYBR No-ROX Kit (Bioline, London, UK) was used to perform qRT-PCR. Each reaction contained 10µl of 2×SensiMix, 500 nM of each primer and 1 µl of cDNA in a total volume of 20 µl. All reactions were performed in three technical replicates on a Rotor-Gene 3000 (Corbett Life Science, Hilden, Germany) and analyzed with Rotor-Gene 6000 software version 1.7. The conditions for performing the PCR reactions were: 10 min at 95°C and 45 cycles of (25 s at 95°C, 60s at 58°C and 20 s at 72°C). A melting curve was generated after the PCR reaction by gradually increasing the temperature to 95°C to test for amplicon specificity. The data were analyzed by REST 2009 software to determine statistically significant differences (Pfaffl et al., 2002).
Table: Summary of the reference and target genes used in the study, with their GenBank accession number or locus number, and the primer pair used for qRT-PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>GenBank Accession/Locus no.</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsEXP</td>
<td>LOC_Os03g27010</td>
<td>TGTGAGCAGCTTCGTTTG</td>
<td>TGTGTTGCTCTGTAGATCG</td>
</tr>
<tr>
<td>OsEXP/Narsai</td>
<td>LOC_Os07g02340.1</td>
<td>CACGTTACGTTGACACCTTTT</td>
<td>GACGCTTCCTCTTCCTTCAG</td>
</tr>
<tr>
<td>OsICS1</td>
<td>LOC_Os09g19734</td>
<td>TGITCCCCACAAAGGCATCTCG</td>
<td>TGGGCCCTCAAACCTTTAAAACATGCC</td>
</tr>
<tr>
<td>OsWRKY45</td>
<td>Os05g0323900</td>
<td>AATTTCGTAGTCGTAAGAAA</td>
<td>AAGTAGGCTTTTGGGACTCTT</td>
</tr>
<tr>
<td>OsAOS2</td>
<td>NM_001055971.1</td>
<td>TGCACGACCCCTCTGGATTTC</td>
<td>GCCACGCGGGGACCTTGATG</td>
</tr>
<tr>
<td>OsAmyb</td>
<td>AY026332</td>
<td>GAGGACCAGATGGAAAAGC</td>
<td>CATGGCATCTCTGAACCTCT</td>
</tr>
<tr>
<td>OsEin2</td>
<td>Os07g06190</td>
<td>GCGCATGTGTAGAAGACGA</td>
<td>CAGGCAGCTCGAATCAAGT</td>
</tr>
<tr>
<td>OsPR10</td>
<td>AF395880</td>
<td>CGGACGTTAACAATTCATCG</td>
<td>AAACAAACCAGCTTCCGACAG</td>
</tr>
</tbody>
</table>
Chapter 6. General conclusion and perspectives
6.1 ‘Manikpukha’, a promising ufra resistant variety

The rice stem nematode, *Ditylenchus angustus* (Butler, 1913), is one of the most devastating rice nematodes in some South and South-East Asian countries (Bridge *et al*., 1990). The nematode causes ‘Ufra’ disease in rice resulting in substantial yield losses in deep water, irrigated and rainfed ecosystems. With increasing restrictions on the use of chemical nematicides, the use of plant resistance for nematode control has grown in importance. Although some rice genotypes have been identified as ufra resistant in previous investigations (Miah & Bakr, 1977; Latif *et al*. 2011a; 2011b), the current cultivation of these identified resistant varieties in the field is limited because of their low yield potential. No high-yielding ufra-resistant variety is currently available.

We screened 85 rice genotypes for resistance to *D. angustus* (Chapter 2), from which 55 were BRRI released high yielding varieties and 11 were local varieties that are currently cultivated in different regions of Bangladesh. Our results showed that one local variety Manikpukha was highly resistant and six high yielding varieties (BR7, BR18, BRRI Dhan 35, BRRI Dhan 37, BRRI Dhan 40, and BRRI Dhan 45) were resistant against *D. angustus* based on two phenotypic screening methods. In addition, we have proved that there is a strong correlation between the number of nematodes and the used phenotypic scoring systems. Our study provides a view about the status of ufra susceptibility of the currently used varieties in Bangladesh. Remarkably, most of the tested local varieties showed a susceptible to highly susceptible reaction, which provides a concern about the high risk of using those varieties in ufra infected areas. Based on this study we can also recommend the farmers to cultivate BRRI released high yielding resistant varieties in ufra infected areas. Our identified highly resistant variety ‘Manikpukha’ has some advantages compared to the resistant reference variety R-16-06-3. R-16-06-3 is a deep water rice variety, which is not cultivated in Bangladesh nowadays because of the declining cultivation of deep water rice varieties. In contrast, Manikpukha is a local aman variety that is still cultivated in some parts of Bangladesh. In Manikpukha, a comparatively lower number of nematodes was observed compared to R-16-06-3 at 28 dpi. However, it will be interesting to test wether Manikphukha is also resistant against other nematodes and other populations of *D. angustus* to evaluate the durability of the observed resistance.

A more detailed investigation of the resistance in relation to the nematode’s life cycle (Chapter 3) showed that the resistance in Manikpukha is associated with reduced development and
reproduction of the nematodes implying that resistance acts post-invasion. Plowright et al. (1996) observed that invasion rate of *D. angustus* varied among resistant rice cultivars and the number of nematodes at 28 days after inoculation was higher in the susceptible cultivars compared to resistant cultivars. However, the information on nematode development inside resistant cultivars was lacking in previous investigation. In this thesis, we showed that the development of nematode was slower in the resistant cultivar Manikpukha compared to the susceptible cultivars (Chapter 3). We also found that the reproduction of the nematodes were completely failed in the resistant genotype. In case of RKN, it has been reported that *M. incognita* juveniles were smaller in resistant tomatoes compared to susceptible tomatoes (Dropkin et al., 1969) and females of *M. graminicola* were significantly smaller inside the resistant rice genotypes in comparison with susceptible rice genotypes (Cabasan et al., 2012). Further investigations on the morphological differences of *D. angustus* in resistant versus susceptible rice genotypes will be interesting.

The incompatible interaction between Manikpukha and *D. angustus* is characterized by a hypersensitive reaction (HR) that leads to necrosis (chapter 2). HR responses in plants following the recognition of nematodes are accompanied by changes in transcriptional and defence signaling pathways such as, production or release of reactive oxygen species (ROS, Davies et al., 2015), H$_2$O$_2$ accumulation (Melillo et al., 2006), salicylic acid, lipoxygenase enzymes (Bhattarai et al., 2008; Klink et al., 2009), Ca$^{2+}$ (Davies et al., 2014) and secondary metabolites (Paulson & Webster, 1972; Pegard et al., 2005). In chapter 4, we have showed that lignin accumulation was significantly increased in the resistant Manikphukha upon *D. angustus* infection. It will be interesting to study the ROS, H$_2$O$_2$ and Ca$^{2+}$ production in the resistant cultivar Manikpukha in comparison with the susceptible cultivar upon nematode infection. Furthermore, isolation, identification and localization of different secondary metabolites that are present in the resistant cultivars and could trigger defence against *D. angustus* should be investigated using analytical, biochemical and/or histochemical analyses. The examination of anti-nematode properties of these metabolites will provide valuable information for engineering nematode resistant varieties (Hölscher et al., 2014).

A comparative study of the gene expression changes of the SA, JA and ET pathways upon *D. angustus* infection in the resistant cultivar Manikpunkha and the susceptible cultivar Nipponbare demonstrates that some genes remarkably changed in resistant plants compared to susceptible plants. These genes could at least partly govern resistance/susceptibility of plants to nematodes (Chapter 4). At the early stage of the rice-nematode interaction (1 dpi), all of the 12 SA/JA/ET
marker genes were upregulated in the resistant variety. We observed a significant upregulation of OsPAL1, OsWRKY45 and OsJMT1, whereas most of those genes were downregulated in the susceptible cultivar. Most remarkably, OsPAL1 was significantly upregulated in the resistant variety while it was downregulated in the susceptible Nipponbare upon nematode infection at 1, 5, 10 and 21 dpi. It has been reported that PAL, which is the entry-point enzyme of the general phenylpropanoid pathway could contribute in the biosynthesis of phenolics, SA, lignins, stilbenes and many other compounds (Winkel-Shirley, 2001; Vogt, 2010; Fraser & Chapple, 2011). Our results showed that the upregulation of OsPAL1 is not correlated with increased SA production in the resistant cultivar. However, the increased lignin content in the resistant cultivar is most likely due to the high expression of OsPAL1 upon infection. Moreover, the inhibition of the phenylpropanoid pathway through application of the PAL inhibitor yielded significantly higher numbers of nematodes in the susceptible cultivar. In this susceptible cultivar, a decreased level of lignin was observed upon infection, indicating again the importance of the phenylpropanoid pathway in rice resistance to D. angustus. Therefore, it will be particularly interesting to investigate the phenylpropanoid pathway in more detail, to get insights of the underlying mechanism involved in resistance against D. angustus. Moreover, application of the PAL inhibitor in Manikpukha before nematode infection will indicate how important the phenylpropanoid pathway is in the resistance of that cultivar and how does D. angustus suppress this pathways upon infection in Nipponbare.

Studies on the resistance of Manikpukha to D. angustus (Chapter 3), expression of defence-related genes of the SA, JA and ET pathway and lignin measurements (Chapter 4) reveal that Manikpukha is a good candidate for the development of an ufra resistant high yielding variety. However, before breeding for high-yielding ufra resistant varieties can be started, more knowledge on the inheritance of the resistance (monogenic or polygenic, race-specific or race-nonspecific) is a pre-requisite. Later on, molecular markers (for marker-assisted selection) can facilitate the introduction of the resistance into breeding materials and for positional cloning of the resistance gene(s) (Rouppe van der Voort et al., 1999; Milligan et al., 199; Rossi et al., 1998; Nombela et al., 2003; Galal et al., 2014).
6.2 SA, JA and ET play a positive role in rice basal defence against the rice stem nematode *D. angustus*

Even in susceptible rice plants, there is some basal defense against *D. angustus* infection. Three classical defence hormones SA, JA and ET are known to play a major role in regulating this plant basal defence against various pathogens and pests (Lorenzo and Solano 2005; Broekaert *et al*., 2006; Glazebrook, 2005). In general, SA is associated with plant defence against biotrophic and hemibiotrophic pathogens and JA and ET play important roles in defence against necrotrophic pathogens and insect herbivory (Glazebrook, 2005). Although most information on the molecular interplay between plants and nematodes were focused on dicotyledonous plants, progress has been made in recent years concerning the role of hormone signalling pathways in rice-nematode interactions. However, nothing was known about the interaction between rice and rice stem nematode, *D. angustus* with regards to phytohormones.

In this thesis for the first time we provided an in-depth characterization of the role of SA, JA and ET in rice basal defence against *D. angustus* (Chapter 4). Based on hormone treatment, biosynthesis inhibition, analysis of mutant and transgenic plants it was shown that SA, JA and ET play a positive role in basal defence of rice against *D. angustus*. Interestingly, similar results were obtained in rice defence against the root rot nematode, *Hirschmanniella oryzae* by Nahar *et al.* (2012) who observed that intact SA, JA and ET biosynthesis pathways are prerequisite. But in the case of the RKN *Meloidogyne graminicola* the same authors found that the JA pathway plays a pivotal role and the ET pathway works through activation of the JA defence pathway in rice defence against RKN (Nahar *et al*., 2011). Such similarities of *D. angustus* with the root rot nematode can be explained by the fact that both are migratory nematodes who have no fixed feeding site within plant tissues but they feed while migrating through plant cells. On the other hand, RKN induce a specialised feeding structure from which they feed during several weeks.

Hormone signalling crosstalk plays an important role in plant response to a wide range of biotic and abiotic stresses. Through cross talk between defence pathways, the plant is able to fine tune its defence reaction against a specific pathogen (Pieterse *et al*., 2001) which eventually determines disease resistance or susceptibility. In addition to SA, JA and ET, other hormones, such as ABA, gibberellins (GA), auxin, cytokinins (CKs) and brassinosteroids (BRs) also play important roles in regulation of the immune signaling network in rice against nematodes often through crosstalk with
the SA, JA/ET pathways (Nahar et al., 2012; 2013; Kyndt et al., 2012; Ji et al., 2013). Deciphering the crosstalk among SA, JA, ET and other hormone dependent pathways in rice defence against the foliar nematode, *D. angustus* is a future challenge.

Increasing evidence indicates that nematode proteins are being actively introduced into the plant throughout the nematode life cycle and some of the effector proteins are able to suppress the plant defence responses through interfering with the hormone homeostasis (Hewezi & Baum, 2013; Kikuchi et al., 2014; Bauters et al., 2014; Haegeman et al., 2013). High-throughput expressed sequence tag (EST) sequencing on mixed stages of *Hirschmanniella oryzae* (Bauters et al., 2014) and second-stage juveniles of *M. graminicola* (Haegeman et al., 2013) illustrated that the rice nematodes contain an arsenal of plant-cell-wall-modifying proteins to facilitate migration through the host roots. The presence of chorismate mutase and isochorismatase in both *M. graminicola* and *Hirschmanniella oryzae* has been reported to potentially deregulate the SA biosynthesis pathway in the host. Transcriptome sequencing of the rice white-tip nematode *Aphelenchoides besseyi* has also been performed (Kikuchi et al., 2014). However, almost nothing is known about *D. angustus* effector proteins. Further study is necessary to gain insight into the role of nematode effectors in suppressing the basal defence of rice.

### 6.3 *Bacillus velezensis* strain BSK, a potential bio-control agent against *D. angustus*

Biological control using microbial agents against plant-parasitic nematodes has received immense attention in the last decades as an alternative to chemical control. We evaluated the potential of a rhizobacterium, *B. velezensis* strain BSK isolated from Bangladesh in promoting plant growth of rice and inducing systemic resistance to *D. angustus* (Chapter 5).

Our result showed that strain BSK treated plants tend to have increased shoot, root length and weight compared to the control plants. We found significantly higher fresh root and shoot weight in case of soil treatment with $10^4$ cfu ml$^{-1}$ whereas higher bacterial concentrations ($10^6$ and $10^8$ cfu ml$^{-1}$) were less effective to increase the growth of rice shoots and roots. Studies with *B. amyloliquefaciens* strains FZB42 (Chen et al., 2007) showed that strain FZB45 exerts a direct mechanism on plant growth, probably by indole-3-acetic acid (IAA) production, which creates a concentration-dependent response to inoculation and interacts with phytase-mediated effects (Ramírez & Joseph, 2010). Our data also demonstrate that strain BSK mediated ISR significantly
reduces the number of *D. angustus* on rice, especially with lower inoculum concentrations (10^4 and 10^6 cfu ml^-1).

Recent studies have demonstrated that PGPR can regulate plant growth and elicit disease resistance without physical contact by releasing volatiles (Ryu *et al*., 2003; Ryu *et al*., 2004; Fernando *et al*., 2005; Bailly *et al*., 2012). Identification of volatiles produced by strain BSK, and investigation of the plant response to potential bacterial volatiles can be analysed using different techniques such as ultra-high performance liquid chromatography, mass spectrometry and gene expression profiling. This will provide valuable insights about the mechanisms of biocontrol activity of *Bacillus velezensis* strain BSK.

*Bacillus* spp. are known to produce a wide array of antimicrobial compounds among which lytic enzymes, antibiotics and a range of non-ribosomally synthesized (lipo)peptides and polyketides (Chen *et al*., 2009; Rückert *et al*., 2011). Cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin families are of high interest because of the role of the different LP families for the inhibition of different pathogens (Wu *et al*., 2015; Chowdhury *et al*., 2015; Cawoy *et al*., 2015). Cyclic lipopeptides, fengycin and surfactin produced by *B. subtilis* BBG111 trigger JA, ET and/or auxin pathways in rice against rice sheath blight caused by *Rhizoctonia solani* (Chandler *et al*., 2015). However, nothing is known about cyclic lipopeptides produced by strain BSK and their functional role against pathogens. Further studies on bacterial lipopeptides will be an interesting topic.

A number of mechanisms have been described to be involved in PGPR mediated ISR against pathogen including plant parasitic nematodes such as involvement of SA, JA, ET and other hormone signaling pathways (Pieterse *et al*., 2014; Chowdhury *et al*., 2015), PR proteins, cell wall reinforcement, accumulation of H_2O_2, defence-related phenolics (Ahn *et al*., 2007; De Vleesschauwer *et al*., 2008; Rahman *et al*., 2015) and the involvement of enzymes in the phenylpropanoid pathway (Anita *et al*., 2004; Schafer 2007). We have investigated the SA, JA and ET signaling pathway within the tripartite system consisting of *Bacillus velezensis* strain BSK, the nematode *D. angustus* and the rice plant. Our results showed that *OsPR10* gene is upregulated in the rice-BSK-*D. angustus* interaction while the SA biosynthesis and JA pathway are only marginally affected in the same interaction. It will be interesting to study the role of other hormones.
or defence-related phenolics, to decipher the mode of action of strain BSK mediated induced systemic resistance against *D. angustus*.

*Bacillus* spp. can produce various molecules that are toxic to nematodes (Li *et al.*, 2005, 2007, 2008; Siddiqui, 2002). Liu *et al.* (2013) identified a gene within the *pzn* gene cluster involved in nematicidal activity using a random transposon insertion library of FZB42. Two nematicidal compounds were identified from *B. cereus* strain S2, called C16 sphingosine and phytosphingosine by polarity gradient extraction, silica gel column chromatography and HPLC. It will also be interesting to study the biocontrol potential of strain BSK against root parasitic nematodes such as root knot nematodes, cyst nematodes and migratory ecto- and endoparasitic nematodes. In the case of field application, it would be prudent to select a PGPR having a broad spectrum of activity involving plant growth promotion and induction of resistance against multiple diseases and pests. Comparison of the control potential of *B. velezensis* strain BSK with commercially available bacterial products against plant parasitic nematodes will provide knowledge on its commercial value.

Although *B. velezensis* strain BSK showing great promise for antagonizing *D. angustus* in the lab, before application as a biocontrol agent, it requires a long way of greenhouse experiments with different rice cultivars and rotational crops, type of soils and different climatic conditions and finally, field experiments to find out the optimum bacterial formulations.

In conclusion, the knowledge gathered from the applied and fundamental research of this PhD will benefit rice cultivation in Bangladesh. Based on the result of ufra susceptibility of currently used high yielding BRRI varieties, farmers from ufra infected areas can choose the right rice variety for cultivation to avoid yield losses caused by *D. angustus*. Moreover, the information about the underlying mechanisms of resistance in the resistant cultivar towards this nematode provide valuable insights to develop ufra resistant high yielding varieties. With this thesis, biocontrol potential of a rhizobacteria towards *D. angustus* provides an alternative environmentally friendly approach to control this pest.
Summary

Rice is the second most important cereal crop in the world as well as staple food for over half of the world’s population. The dramatic growth of global human population requires an increase in rice production. However, high rice productivity is impaired with a variety of biotic and abiotic factors. Plant parasitic nematodes cause significant yield losses in rice, ranging from 10% to 25% worldwide. The rice stem nematode, *Ditylenchus angustus* is an obligate parasite causing ufra disease in rice. *D. angustus* infection results in substantial yield losses in South and Southeast Asia. For instance in Bangladesh, yield losses by this nematode have been reported ranging from 40-90%. The nematode enters the plant mainly at the collar region, migrates upward with shoot growth and feeds on newly forming tissues in the rolled leaf sheath, causing malformation. In the vegetative stage, white patches, or speckles in a splash pattern are observed at the leaf base. At the reproductive stage, the panicle heads and flag leaves become twisted and distorted, leading to significantly lower rice production. The control of this nematode has relied heavily on chemical nematicides in the past. However, the notorious toxicity of chemical nematicides to wildlife and human health emphasizes the urgent need for an environmentally friendly, alternative management strategy to control this nematode. In this thesis, we focused on strategies that are environmentally sound and economically viable for nematode control, such as plant resistance and biological control.

In the first part of this thesis, a number of rice genotypes (BRRI released high yielding varieties, local varieties and deep water rice varieties) were screened for resistance to the Bangladeshi population of *D. angustus* using different nematode inoculation assays, in both rainfed and irrigated ecosystems (Chapter 2). Out of the 85 genotypes, one landrace named ‘Manikpukha’ proved to be highly resistant, while 6 other varieties showed resistance and 13 varieties showed moderately resistant responses under both pot and field conditions. The highly resistant local variety ‘Manikpukha’ found in our study will be a potential candidate to develop ufra resistant high yielding varieties. BRRI released high yielding varieties that were identified as resistant against the nematode can be used directly in ufra infected areas as a cost-effective, environmentally friendly method to reduce the level of *D. angustus* infections.
We aimed further to explore the resistance mechanism in the identified highly resistant genotype Manikpukha. Invasion, post-infectional development, and reproduction of *D. angustus* in Manikpukha and in the susceptible rice genotypes BR26 and Nipponbare were compared to identify the stage(s) at which resistance occurs (Chapter 3). All the observations indicate that the resistance in Manikpukha is associated with reduced development and reproduction of *D. angustus* implying that resistance acts post-invasion. This valuable information of resistance mechanisms in Manikpukha is adding further insights into the nature, timing and action of resistance genes which will help to efficiently use resistant cultivars in breeding programmes as well as to advance phenotypic screening methods.

Timely recognition of a pathogen combined with fast and effective induction of defence responses ultimate determine the resistance/susceptibility of a plant. The activation of an effective defence response profoundly depends on the action of phytohormones, in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play central roles. The role of SA, JA and ET was investigated in rice defence against *D. angustus* (Chapter 4). Experiments with hormone biosynthesis inhibition, mutants and transgenic lines showed that SA, JA and ET pathways play a positive role in basal defence against the nematode. Gene expression analysis using qRT-PCR demonstrate that many defence-related genes are attenuated in shoot tissues of *D. angustus* infected susceptible plants while those genes are up-regulated in shoot tissues of nematode infected resistant plants. Our data reveal a significantly higher expression of *OsPAL1* in the infected resistant plants, but this is not correlated with enhanced SA production. We have also observed that lignin accumulation was significantly higher in the resistant plants upon nematode infection compared to susceptible plants.

In the last part of the thesis, the potential of a rhizobacterium *B. velezensis* strain BSK in promoting plant growth of rice and induced systemic resistance to *D. angustus* was reported. Our results showed that soil inoculation with low bacterial density ($10^4$ cfu ml$^{-1}$) promotes growth in rice and controls the nematode at a significant level, suggesting *B. velezensis* strain BSK as an economical and ecological alternative of chemical control for nematode management. qRT-PCR data shows that the *OsPR10* gene is highly induced in the rice-BSK-*D. angustus* interaction while genes involved in the SA biosynthesis and JA pathway only show a slight response. Further studies on elucidation of the underlying mechanisms of strain BSK induced systemic resistance will provide valuable insights for potential application of strain BSK in PPN management.
In this thesis, we investigated several environmentally sound strategies for *D. angustus* management. Further molecular analysis should be focused on the rice-nematode interaction regarding plant resistance gene/genes, hormone signaling pathways, mode-of-action of *B. velezensis* strain BSK induced systemic resistance to enhance the development of a sustainable ufra management strategy.
Nederlandse samenvatting

Karakterisatie van de interactie tussen rijst en de parasitaire nematode *Ditylenchus angustus*


In het eerste deel van de thesis werden een aantal rijstgenotypes, waaronder BRRI-variëteiten en lokale cultivars, getest voor resistentie tegen *D. angustus* in verschillende rijstcultuursystemen (Hoofdstuk 2). Uit 85 geteste genotypes werd Manikpukha geïdentificeerd als zeer resistent en verschillende andere variëteiten bleken ook enige resistentie te vertonen zowel in pot- als in veldcondities. Manikpukha is een interessante kandidaat om ufra-resistente variëteiten te ontwikkelen met hoge opbrengst. BRRI-hoge opbrengst-variëteiten die als resistent uit de testen kwamen kunnen onmiddellijk ingezet worden in ufra-besmette gebieden als een kostenefficiënte milieuvriendelijke methode om het infectieniveau van *D. angustus* terug te dringen.

In hoofdstuk 3 hebben we de resistentie van Manikpukha aan verder onderzoek onderworpen. De penetratie, ontwikkeling en reproductie van *D. angustus* werd vergeleken tussen de resistente Manikpukha en de gevoelige Nipponbare om te weten op welke stap de resistentie zijn effect heeft. Alle observaties wijzen er op dat de resistentie pas optreedt na infectie, de nematoden dringen de planten wel binnen maar ontwikkelen en vermeerderen zich minder goed in Manikpukha dan in
Samenvatting

Nipponbare. Deze informatie is belangrijk voor het gebruik van resistentie in veredelingsprogramma’s bv. om verschillende resistenties te combineren.

In plantenaafweer is het essentieel dat de pathogeen snel herkend wordt zodat de plant tijdig kan reageren. De activatie van afweerresponsen hangt sterk af van de actie van plantenhormonen, waarbij salicylzuur (SA), jasmonaat (JA) en ethyleen (ET) een centrale rol spelen. Daarom werd de rol van deze hormonen bij de interactie tussen rijst en *D. angustus* geanalyseerd. Experimenten met hormooninhibitoren, mutanten en transgene lijnen toonden aan dat de SA-, JA- en ET-pathways een rol spelen in de basale plantenaafweer tegen de nematode. Analyse van genexpressie via qRT-PCR toonde aan dat veel afweer-gerelateerde genen neergereguleerd werden bij infectie van een gevoelige plant, terwijl ze opgereguleerd worden in de resiste Manikpukha. Vooral de hogere expressie van *OsPAL1* na infectie van de resiste plant was opvallend. Deze hogere expressie is niet gerelateerd met een hogere SA-productie, maar wel met een hogere lignine-accumulatie.

In het laatste deel van dit onderzoek werd het potentieel van de rhizobacterium *B. velezensis* strain BSK onderzocht voor groeibevordering van rijst en voor de inductie van systemische resistentie tegen *D. angustus*. Inoculatie van deze bacterie met een lage densiteit (104 cfu/ml) gaf daarbij een uitstekend resultaat, zodat het de moeite loont om deze stam verder te onderzoeken als een economisch en ecologisch aanvaardbaar alternatief voor de chemische controle van de nematode *D. angustus*. qRT-PCR toonde aan dat *OsPR10* mogelijk een rol speelt in de rijst-BSK-*D. angustus*-interactie terwijl de SA- en JA-pathway minder belangrijk lijken. Verder onderzoek is echter nodig om de onderliggende mechanismen beter te begrijpen en bruikbare methoden te ontwikkelen voor de toepassing van *B. velezensis* strain BSK in biocontrole van nematodeninfectie bij rijst.

Samengevat hebben we in dit werk onderzoek gedaan naar mogelijke alternatieve strategieën voor controle van *D. angustus*-infecties in rijst. Verder moleculair onderzoek inzake de plantenresistentie tegen *D. angustus* en de onderliggende genen, de hormoonsignalisatie en de systemische inductie van resistentie door *B. velezensis* strain BSK kunnen bijdragen tot de optimalisatie van controlemethoden van ufra in rijstvelden.
References


Anderson JP, Badruzaufari E, Schenk PM, Manners JM, Desmond OJ, Ebert PR, Kazan K. 2004. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways


References


Branch C, Hwang CF, Navarre DA, Williamson VM. 2004. Salicylic acid is part of the Mi-1-mediated defence response to root knot nematode in tomato. Molecular Plant-Microbe Interactions 17, 351-35.


Butler EJ. 1919. The rice worm (Tylenchus angustus) and its control. Memorial Department of Agriculture India 10, 1-37.


---

References


Guedes MEM, Richmond S, Kuc J. 1980. Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with Colletotrichum lagenarium and the onset of flowering and fruiting. Physiological Plant Pathology 17, 229–233.


Hasky-Gunther K, Hoffmann-Hergarten S, Sikora RA. 1998. Resistance against the potato cyst nematode *Globodera pallida* systemically induced by the rhizobacteria *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43). Fundamental and Applied Nematology 21, 511–517.


IRRI, International Rice Research Institute, 1996. Standard Evaluation System for rice. 4th Ed., P. O. Box 933, 1099 Manila, the Philippines, p. 43.


Mao CZ, Wang SM, Jia QJ, Wu P. 2006. OsEIL1, a rice homolog of the Arabidopsis EIN3 regulates the ethylene response as a positive component. Plant Molecular Biology 61, 141–152.


Mendoza AR, Sikora RA. 2009. Biological control of *Radopholus similis* in banana by combined application of the mutualistic endophyte *Fusarium oxysporum* strain 162, the egg pathogen*Paecilomyces lilacinus* strain 251 and the antagonistic bacteria *Bacillus firmus*. Biocontrol 54, 263–272.


Raaijmakers JM., de Bruijn I, Nybroe O. Ongena M. 2010. Natural functions of lipopeptides from Bacillus and Pseudomonas: more than surfactants and antibiotics. Fems Microbiology Reviews 34, 1037–1062.


Yang YN, Qi M, Mei C. 2004. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. The Plant Journal 40, 909-919.


Curriculum Vitae

Personal information

Full name: Shakhina Khanam
Date of birth: 01 July 1984
Nationality: Bangladeshi
Personal e-mail: Shakhina.khanam@hotmail.com
Work address: Department of Molecular Biotechnology, Coupure links 653, Gent 9000, Belgium
Tel: +3292645967
Work e-mail: shakhina.khanam@ugent.be

Education

2011 – 2016: PhD training
The Doctoral School of Bioscience Engineering,
Ghent University, Belgium
2009 – 2011: Master of Science in Nematology
Ghent University, Belgium
2004 – 2008: Bachelor of Science in Agriculture
Sher-e-Bangla Agricultural University, Bangladesh

Professional Career

VLIR-UOS ICP Sandwich PhD Scholarship (2011-2015): The Flemish competitive scholarship for doing PhD at the Ghent University of Belgium.
VLIR-UOS Masters scholarship (2009-2011): The Flemish competitive scholarship for doing International Masters in Nematology at the Ghent University of Belgium.

Publications with peer reviewing


Conference and symposia contributions


# Appendix

Table A1. Characteristics of the rice varieties used to evaluate the response against *Ditylenchus angustus*

<table>
<thead>
<tr>
<th>Rice variety</th>
<th>BRRI Accession no.</th>
<th>Crop cycle (days)</th>
<th>plant height (cm)</th>
<th>Grain yield (t/ha)</th>
<th>Ecosystem</th>
<th>Ecotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maloti</td>
<td>3660</td>
<td>136</td>
<td>135-143</td>
<td>2.18-2.20</td>
<td>Rainfed</td>
<td>Aus, Aman</td>
</tr>
<tr>
<td>Munar</td>
<td>3889</td>
<td>146</td>
<td>135</td>
<td>2.10</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>Kalomota</td>
<td>5991</td>
<td>120</td>
<td>127</td>
<td>2.53</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Sadamota</td>
<td>1576</td>
<td>134</td>
<td>134</td>
<td>2.37</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Mowlata</td>
<td>7922</td>
<td>140</td>
<td>130</td>
<td>2.55</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Dudkolom</td>
<td>4068</td>
<td>109</td>
<td>110</td>
<td>2.27</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Kheyya</td>
<td>1709</td>
<td>149</td>
<td>120</td>
<td>2.25</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Chikon</td>
<td>1227</td>
<td>142</td>
<td>100</td>
<td>2.26</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Nakhuchimota</td>
<td>1606</td>
<td>147</td>
<td>140</td>
<td>2.51</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Sakkhor khora</td>
<td>1605</td>
<td>134</td>
<td>140</td>
<td>2.27</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Manikpukha</td>
<td>--</td>
<td>155</td>
<td>145</td>
<td>1.85</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>DWR1</td>
<td>6351</td>
<td>127</td>
<td>130</td>
<td>1.20</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR2</td>
<td>6352</td>
<td>127</td>
<td>130</td>
<td>1.05</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR3</td>
<td>6353</td>
<td>133</td>
<td>140</td>
<td>0.98</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR4</td>
<td>6354</td>
<td>134</td>
<td>140</td>
<td>1.20</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR5</td>
<td>6355</td>
<td>125</td>
<td>165</td>
<td>1.08</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR6</td>
<td>6356</td>
<td>125</td>
<td>165</td>
<td>1.30</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR7</td>
<td>6357</td>
<td>127</td>
<td>130</td>
<td>1.50</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR8</td>
<td>6358</td>
<td>127</td>
<td>140</td>
<td>1.38</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR9</td>
<td>6359</td>
<td>127</td>
<td>130</td>
<td>1.40</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR10</td>
<td>6360</td>
<td>119</td>
<td>160</td>
<td>1.02</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR11</td>
<td>6361</td>
<td>141</td>
<td>162</td>
<td>1.12</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR12</td>
<td>6362</td>
<td>153</td>
<td>145</td>
<td>1.25</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR13</td>
<td>6363</td>
<td>141</td>
<td>135</td>
<td>1.40</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR14</td>
<td>6364</td>
<td>133</td>
<td>140</td>
<td>1.40</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR15</td>
<td>6365</td>
<td>125</td>
<td>120</td>
<td>1.38</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR16</td>
<td>6366</td>
<td>125</td>
<td>130</td>
<td>1.02</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR17</td>
<td>6367</td>
<td>127</td>
<td>160</td>
<td>1.24</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR18</td>
<td>6368</td>
<td>125</td>
<td>140</td>
<td>1.23</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR19</td>
<td>6369</td>
<td>131</td>
<td>135</td>
<td>1.50</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>DWR20</td>
<td>6370</td>
<td>131</td>
<td>127</td>
<td>1.60</td>
<td>DWR</td>
<td>DWR</td>
</tr>
<tr>
<td>BR1</td>
<td>6865</td>
<td>120-150</td>
<td>88</td>
<td>4.0-5.5</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR2</td>
<td>6866</td>
<td>160-125</td>
<td>120</td>
<td>5.0-4.0</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR3</td>
<td>4759</td>
<td>130-170</td>
<td>95-100</td>
<td>4.0-6.5</td>
<td>Rainfed</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR4</td>
<td>7088</td>
<td>145</td>
<td>125</td>
<td>5.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BR5</td>
<td>4343</td>
<td>150</td>
<td>120</td>
<td>3.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BR6</td>
<td>6867</td>
<td>110-150</td>
<td>100-113</td>
<td>3.5-4.5</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR7</td>
<td>6868</td>
<td>130-155</td>
<td>125</td>
<td>3.5-4.5</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR8</td>
<td>6869</td>
<td>125-160</td>
<td>125</td>
<td>5.0-6.0</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR9</td>
<td>6870</td>
<td>120-155</td>
<td>125</td>
<td>5.0-6.0</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR10</td>
<td>7089</td>
<td>150</td>
<td>115</td>
<td>5.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BR11</td>
<td>7318</td>
<td>145</td>
<td>115</td>
<td>5.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BR12</td>
<td>6871</td>
<td>130-170</td>
<td>105</td>
<td>4.5-5.5</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
</tbody>
</table>
Table A1. Continue

<table>
<thead>
<tr>
<th>Rice variety</th>
<th>BRRI Accession no.</th>
<th>Crop cycle (days)</th>
<th>plant height (cm)</th>
<th>Grain yield (t/ha)</th>
<th>Ecosystem</th>
<th>Ecotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR14</td>
<td>6872</td>
<td>120-160</td>
<td>120</td>
<td>5.0-6.0</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR15</td>
<td>6873</td>
<td>125-165</td>
<td>90-100</td>
<td>5.0-5.5</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR16</td>
<td>6874</td>
<td>165-130</td>
<td>90-110</td>
<td>6.0-5.0</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BR17</td>
<td>6875</td>
<td>155</td>
<td>125</td>
<td>6.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BR18</td>
<td>6876</td>
<td>170</td>
<td>115</td>
<td>6.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BR19</td>
<td>6877</td>
<td>170</td>
<td>110</td>
<td>6.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BR18</td>
<td>3687</td>
<td>115</td>
<td>120</td>
<td>3.5</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>BR21</td>
<td>6199</td>
<td>110</td>
<td>100</td>
<td>3.0</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>BR22</td>
<td>7090</td>
<td>150</td>
<td>125</td>
<td>5.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BR23</td>
<td>7091</td>
<td>150</td>
<td>120</td>
<td>5.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BR24</td>
<td>4276</td>
<td>105</td>
<td>105</td>
<td>3.5</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>BR25</td>
<td>4277</td>
<td>135</td>
<td>138</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BR26</td>
<td>4278</td>
<td>115</td>
<td>115</td>
<td>4.0</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>BRRI Dhan 27</td>
<td>4408</td>
<td>115</td>
<td>140</td>
<td>4.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 28</td>
<td>4409</td>
<td>140</td>
<td>90</td>
<td>6.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BRRI Dhan 29</td>
<td>4410</td>
<td>160</td>
<td>95</td>
<td>7.5</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BRRI Dhan 30</td>
<td>4411</td>
<td>145</td>
<td>120</td>
<td>5.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 31</td>
<td>4412</td>
<td>141</td>
<td>115</td>
<td>5.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 32</td>
<td>4413</td>
<td>130</td>
<td>120</td>
<td>5.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 33</td>
<td>7092</td>
<td>118</td>
<td>100</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 34</td>
<td>7093</td>
<td>135</td>
<td>117</td>
<td>3.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 35</td>
<td>6878</td>
<td>155</td>
<td>105</td>
<td>5.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BRRI Dhan 36</td>
<td>6879</td>
<td>140</td>
<td>90</td>
<td>5.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BRRI Dhan 37</td>
<td>7094</td>
<td>140</td>
<td>125</td>
<td>3.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 38</td>
<td>7095</td>
<td>140</td>
<td>125</td>
<td>3.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 39</td>
<td>7096</td>
<td>122</td>
<td>106</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 41</td>
<td>7098</td>
<td>148</td>
<td>115</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 42</td>
<td>6214</td>
<td>100</td>
<td>100</td>
<td>3.5</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>BRRI Dhan 43</td>
<td>6215</td>
<td>100</td>
<td>100</td>
<td>3.5</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>BRRI Dhan 44</td>
<td>7099</td>
<td>145</td>
<td>130</td>
<td>5.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 45</td>
<td>6880</td>
<td>145</td>
<td>100</td>
<td>6.5</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BRRI Dhan 46</td>
<td>7100</td>
<td>150</td>
<td>105</td>
<td>4.7</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 47</td>
<td>6881</td>
<td>152</td>
<td>105</td>
<td>6.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BRRI Dhan 48</td>
<td>7980</td>
<td>110</td>
<td>105</td>
<td>5.5</td>
<td>Rainfed</td>
<td>Aus</td>
</tr>
<tr>
<td>BRRI Dhan 49</td>
<td>7101</td>
<td>135</td>
<td>100</td>
<td>5.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 50</td>
<td>6882</td>
<td>155</td>
<td>82</td>
<td>6.0</td>
<td>Irrigated</td>
<td>Boro</td>
</tr>
<tr>
<td>BRRI Dhan 51</td>
<td>7319</td>
<td>140-145</td>
<td>90</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 52</td>
<td>7320</td>
<td>145</td>
<td>116</td>
<td>5.0</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 53</td>
<td>7321</td>
<td>125</td>
<td>105</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 54</td>
<td>7322</td>
<td>135</td>
<td>115</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>BRRI Dhan 55</td>
<td>7323</td>
<td>145-105</td>
<td>100</td>
<td>7.0-5.0</td>
<td>Irrigated</td>
<td>Boro, Aus</td>
</tr>
<tr>
<td>BRRI Dhan 56</td>
<td>--</td>
<td>105-110</td>
<td>115</td>
<td>4.5</td>
<td>Rainfed</td>
<td>Aman</td>
</tr>
<tr>
<td>Rayeda 16-06-3</td>
<td>--</td>
<td>135</td>
<td>160</td>
<td>4.5</td>
<td>DWR</td>
<td>DWR</td>
</tr>
</tbody>
</table>

All rice varieties belong to the indica type.

Source: Genetic Resource and Seed (GRS) division of Bangladesh Rice research Institute (BRRI), Gazipur-1701 and Hossain et al. (2013)
Fig. A1. Relationship between the early scoring system (at 28 dpi) and the actual number of nematodes inside the plant. Black dots represent the mean and standard error of 15 plants and different letters on the dot indicate significant differences (Duncan Multiple Range Test with $P = 0.05$). The graph is the representation of one of two independent experiments with similar results and $r$ represents the correlation co-efficient value with $p$ value. Data represent one of another replicate than the one in Fig 2.2.
Fig A2. Number of *Ditylenchus angustus* in shoots of resistant and susceptible rice genotypes at 1 and 3 days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. Bars represent the mean and standard error of the number of nematodes per plant recorded on 8 plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with α = 0.05). Data represent two of three independent experiments with similar results. *R*Resistant genotype; *S*Susceptible genotype. Data represent another two replications (A & B) other than the one in Fig 3.1
Fig A3. Shoot invasion of *Ditylenchus angustus* second-or third-stage juveniles (J2/J3), fourth-stage juveniles (J4) and adults in resistant and susceptible rice genotypes at 1 (A) and 3 (B) days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. Bars represent the mean number of nematodes per plant recorded on 8 plants. Different letters indicate statistically significant differences (non-parametric Kruskal-Wallis test with $\alpha = 0.05$). Data represent two of three independent experiments with similar results (1 and 2) other than the one in Fig 3.2. RResistant genotype; S Susceptible genotype.
Fig A4. Percentage of *Ditylenchus angustus* developmental stages feeding on resistant and susceptible rice shoots at different times of the nematode life cycle (A) 7, (B) 14, (C) 21 and (D) 28 days after inoculation (dai). Bars represent the mean percentage of each stage of nematodes recorded on 8 plants. Nematode development in Manikpukha was compared to the susceptible rice genotypes Nipponbare and BR26. Single asterisks indicate significant differences of developmental stages of Manikpukha from BR26 and double asterisks indicate a significant difference between Manikpukha and both Nipponbare and BR26 (Independent Samples *t* test with *α* = 0.05). Data represent one of two independent experiments with similar results. *R*Resistant genotype; *S* Susceptible genotype; *Sg* second generation. Data represent one of another replicate than the one in Fig 3.3.
Fig A5. Number of *Ditylenchus angustus* in shoots of resistant and susceptible rice genotypes at 7, 14, 21 and 28 days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. Lines represent the mean and standard error of the number of nematodes per plant recorded on 8 plants. Data represent one of two independent experiments with similar results. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). $^R$Resistant genotype; $^S$Susceptible genotype. Data represent one of another replicate than the one in Fig 3.4.

Fig A6. Number of *Ditylenchus angustus* eggs per plant in resistant and susceptible rice genotypes at 14, 21, and 28 days after inoculation (dai). 15 days old rice plants were inoculated with 100 nematodes of *D. angustus*. Bars represent the mean and standard error of the number of eggs per plant recorded on 8 plants. Data represent one of two independent experiments with similar results. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). $^R$Resistant genotype; $^S$Susceptible genotype. Data represent one of another replicate than the one in Fig 3.5.
Fig A7. (A). Susceptibility towards *Ditylenchus angustus* in SA signalling deficient *WRKY45* RNAi, SA deficient transgenic *NahG*, ET insensitive *Ein2b*, JA insensitive *Coi* RNAi, and the corresponding wild type Nipponbare plants and (B) Susceptibility for *D. angustus* in JA biosynthesis mutant *hebiba* and the corresponding wild type Nihonmasari. Bars represent the mean and standard error of the number of nematodes per plant 20 days after nematode inoculation, recorded on 8 plants. Different letters in the picture A and B indicate statistically significant differences (Duncan Multiple Range Test with $\alpha = 0.05$). Data represent two (1 & 2) of three independent experiments with similar results other than the one in Fig 4.1.
Fig A8. Effect of foliar application of plant hormones and corresponding hormone inhibitors on rice defence against *D. angustus* infection. Shoots of fifteen-day-old plants were sprayed until runoff with 100 µM MeJA, 250 µM BTH, 500 µM ethephon, 25 mM AOA, 100 µM DIECA, 100 µM PALi or the corresponding control solution. After 24 h of treatment, plants were inoculated with 100 nematodes of *D. angustus*. Bars represent the mean and standard error of the number of nematodes per plant 20 days after nematode inoculation recorded on 8 plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). Data represent two (1 & 2) of three independent experiments with similar results than the one in Fig 4.2. MeJA, Methyl jasmonate; Eth, Ethephone; BTH, Benzathiadiazole; AOA, Aminooxyacetic acid; DIECA, Diethyldithiocarbamic acid; PALi, L-2-Aminooxy-3-phenylpropinoic acid.
Fig A9. Effect of *Bacillus velezensis* strain BSK on rice growth at $10^4$, $10^6$ and $10^8$ cfu ml$^{-1}$ upon seed and soil treatment, at 28 days after sowing. (A) Shoot and root length, (B) Shoot and root weight. The bars represent the mean and standard error recorded on 8 plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha=0.05$). Data represent one of three independent experiments with similar results. Data represent one of another replicate than the one in fig 5.1.
Fig A10. Effect of soil treatment with *Bacillus velezensis* strain BSK, at different concentrations, on the number of nematodes per plant. The bacteria at $10^4$, $10^6$ and $10^8$ cfu ml$^{-1}$ were drenched over SAP-substrate during seedling sowing and 12 days after sowing. Plants were inoculated with 100 nematodes of *D. angustus* at 1 day after bacteria inoculation. The bars represent the mean and standard error of the number of nematodes per plant 20 days after nematode inoculation recorded on 8 plants. Different letters indicate statistically significant differences (Duncan’s multiple range test with $\alpha = 0.05$). Data represent two of three independent experiments (1 & 2) with similar results. Data represent other than the one in fig 5.2.