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The analysis of *Pythium arrhenomanes-Meloidogyne graminicola* infection on rice

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The analysis of *Pythium arrhenomanes-Meloidogyne graminicola* infection on rice

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**Summary:** The root knot nematode-*Meloidogyne graminicola* and Oomycetes-*Pythium arrhenomanes* have been known as main biotic factors behind yield decline in aerobic rice production. But, improved rice grain yield have also been reported on rice cultivar-Apo and IR81413-BB-75-4 line in the presence of both pathogens together compared to single infection. Present studies were carried out to evaluate the interaction of *P. arrhenomanes* and *M. graminicola* on rice. Initially, a fluorescent labeling technique of *M. graminicola* juveniles was tested to use for infection experiments, in order to be able to score the infection under *in vivo* conditions, but this approach did not work in the rice root system. Infection experiments were conducted on different rice cultivars, mutant (Jasmonic acid biosynthesis), overexpression transgenic line (less auxin content) and chemical inhibitor treated plants. Based on developmental stages of root knot nematodes, there was a synergistic interaction between *M. graminicola* and *P. arrhenomanes* on rice cultivar-Nipponbare while in Palawan slightly antagonistic interaction was observed during double infection. There was no interaction observed between both pathogens in cultivar-Apo and IR81413-BB-75-3 line. The role of the Jasmonic acid (JA) signalling pathway in the interaction was unclear based on infection experiments on Nihonmasari & JA-biosynthesis mutant (*hebiba*). However, in Nipponbare, the JA biosynthesis gene was upregulated after *P. arrhenomanes* infection but a significant suppression of this gene was found in root knot nematode infected plants. Auxin seems to be an important factor for nematode development as a *OsGH3.1* overexpression line (which contains less free auxin) was less susceptible to *M. graminicola* infection. There was a significant reduction in the number of females in the *OsGH3.1* overexpression line after single *M. graminicola* infection. However, female numbers in this line were significantly increased in the presence of *P. arrhenomanes* in double infected plants, which suggests that *P. arrhenomanes* complements the usually low auxin levels in the root of the *OsGH3.1* overexpression line. The basal expression level of *OsGH3.1* was significantly higher (exemplifying lower free auxin content) in the IR81413-BB-75-3 line compared to Nipponbare, which might explain the generally lower number of galls as well as nematodes in the IR81413-BB-75-3 line. There was a very low amount of *in planta P. arrhenomanes* DNA quantified in all of the samples, which indicates that the Synthetic Absorbent Polymer (SAP) substrate system used in these here-described experiments might not be optimal for *P. arrhenomanes* penetration and colonization of the root tissue, although disease symptoms were observed.

**Keywords:** Root knot nematodes, Oomycetes, Interaction, Synergism, Antagonism, Gene expression.
1 Background information

Rice (*Oryza sativa* L.) is the major food grain which meet the diets of over half of the world population, mainly grown in tropical and subtropical regions (Han *et al.*, 2007). Now, rice is cultivated in 114 countries (Nicol *et al.*, 2011) and estimated that 346 million acre of rice is being grown throughout the world, in which 263 million acres are infested with damaging levels of plant parasitic nematodes (Hollis Jr *et al.*, 1984). Root-knot nematodes are the most destructive amongst all plant parasitic nematodes (Karssen *et al.*, 2006; Padgham & Sikora, 2007; Perry *et al.*, 2009).

Among root knot nematodes, *Meloidogyne graminicola* is one the most damaging type of nematode attacking rice roots (Bridge *et al.*, 2005; Kyndt *et al.*, 2014b). Introduction of water saving rice production systems and the cultivation of aerobic rice varieties favors the buildup of large populations of *M. graminicola* in rice fields (De Waele & Elsen, 2007). They cause substantial damage on rice, depending on which cropping system is being used (Netscher & Erlan, 1993). They are responsible for great yield losses (20% to 70%) in many countries (Chantanao, 1962; Roy, 1973; Soriano *et al.*, 2000; Soriano & Reversat, 2003; Padgham *et al.*, 2004; Pokharel *et al.*, 2007). On the other hand, Oomycetes-*Pythium* spp. are one of the most common group of seedling disease pathogens in rice-production areas. It is responsible for pre- and post-emergence death, and causes 70% and 48% of average growth reductions of root and shoot respectively in surviving seedlings (Cother & Gilbert, 1993; Eberle *et al.*, 2007). It was found in 47% of soil samples, sampled across rice fields of the Riverine Plain in New South Wales, Australia (Cother & Gilbert, 1992). A number of isolates belonging to *Pythium arrhenomanes*, *P. graminicola* and *P. inflatum* were recovered from affected aerobic rice fields in the Philippines in which all of the isolates of *P. arrhenomanes* were found pathogenic to rice (*Van Buyten et al.*, 2013; this species was also found as pathogenic to rice seedlings in northern Japanese rice fields recently (Toda *et al.*, 2015). Root knot nematode-*M. graminicola* and Oomycetes-*Pythium* spp. have been recognized as main biotic causes to yield decline in the aerobic rice production system (Kreye *et al.*, 2010a; Kreye *et al.*, 2010b).

Combined nematode and fungal/or oomycetes infection (so called double infection) showed increased damage and higher yield losses of plants compared to the single infection (Atkinson, 1892; Johnson & Littrell, 1970; Powell, 1971; Roberts *et al.*, 1995; Bhattacharai *et al.*, 2009). Possible explanation is that nematode infection influences gene expression in plants, aimed at suppressing
host defense pathways. Such manipulation of plant gene expression by the nematode might lead to subsequent pathogen infections (Conrath et al., 2002; Bezemer & van Dam, 2005; Wurst & van der Putten, 2007). Surprisingly, recent IRRI study found a reverse effect of double infection on cultivar-Apo and IR81413-BB-75-4, where double infection of *P. arrhenomanes* and *M. graminicola* was found to cause increased rice grain yield, compared to solely *M. graminicola* infection on rice (Kreye et al., 2010b). These findings inspired to elucidate the mechanism of interaction of *P. arrhenomanes* and *M. graminicola* on rice roots.

The expression of pathogenesis-related (PR) proteins in rice is mainly regulated by key phytohormones such as Salicylic acid (SA), Jasmonic acid (JA) and Ethylene (ET) (Nahar et al., 2011; De Vleesschauwer et al., 2012; Kyndt et al., 2012; Riemann et al., 2013; Tamaoki et al., 2013; Uenoa et al., 2013; De Vleesschauwer et al., 2014; Kyndt et al., 2014a; Taniguchi et al., 2014). Others phytohormones such as Abscisic acid (ABA), Auxin (AUX), Gibberellic acid (GA), and Brassinosteroids (BR) either act as a virulence factor for pathogens to manipulate defense signaling pathways or play a positive role in defense in rice to some extents (Ding et al., 2008; Domingo et al., 2009; De Vleesschauwer et al., 2010; De Vleesschauwer et al., 2012; Nahar et al., 2012; Jiang et al., 2013; Nahar et al., 2013; Qin et al., 2013; Van Buyten, 2013). The JA hormonal pathway plays a key role in rice defense against biotrophic root pathogen *M. graminicola* (Nahar et al., 2011; Kyndt et al., 2012; Kyndt et al., 2014a); while JA and ET pathway play a major role in defense against necrotrophic pathogens (Geraats et al., 2002; Bari & Jones, 2009; De Vleesschauwer et al., 2012). It is known that auxin is essential for giant cell initiation and development by root knot nematodes (Karczmarek et al., 2004). On the other hand, *Pythium* spp. are capable to produce IAA by themselves, have potential to induce endogenous AUX level and have ability to regulate auxin signaling (Rey et al., 2001; Van Buyten, 2013). Unfortunately, the molecular mechanism of hormonal pathways in rice underlying the interaction of *M. graminicola* and *P. arrhenomanes* is unclear.

Present study was undertaken with following objectives- I) to evaluate gall and nematode development in rice roots under *in vivo* condition, II) to study the interaction of *P. arrhenomanes* and *M. graminicola* on different rice cultivars, mutant and transgenic line, III) to investigate the role of hormone signaling pathways (more specifically: JA, SA, ET and Auxin) during single and double infection on rice roots, IV) to quantify *in planta* *Pythium arrhenomanes* DNA, and V) to
search for genes coding for necrosis and ethylene inducing peptide 1 (Nep1) like proteins (NLPs) in *P. arrhenomanes*.

2 Materials and Methods

2.1 Plant Material and Growth Conditions

Different rice (*Oryza sativa* L.) cultivars-Nipponbare (provided by USDA), Palawan 1 & 2 (traditional upland rice cultivar, originated in IRRI; Palawan-2, GID 48535), Apo and IR81413-BB-75-3 (provided by IRRI; called IR line in this thesis), were used in interaction experiments. In addition, *hehiba* (JA biosynthesis mutant line) and its corresponding wild-type CV Nihonmasari, *OsGH3.1* -overexpression line (which accumulates less auxin) and its corresponding wild-type cv. Bomba (obtained from USDA) were also used in interaction experiment. Rice seeds were surface sterilized (4% NaOCl), subsequently rinsed with sterile water three times, germinated on wet paper towel for 4 days at 30°C in an incubator and transplanted into Synthetic Absorbent Polymer (SAP) substrate medium in a PVC tube at 1 plant/SAP tube (Reversat *et al.*, 1999). Plants were grown at 26°C temperature under 12h/12h light regime with 70% to 75% relative humidity in the growth chamber; and were fertilized with 10mL of Hoagland solution (an artificial nutrient source) per plant twice per week.

2.2 Maintenance of Pathogens

The *Meloidogyne graminicola* culture (originally isolated in the Philippines) was maintained on rice cultivar-Nipponbare and tropical Asian wild type grasses-*Echinocloa crus-galli*. Root systems of 3 month old infected plants were cut into pieces and nematodes were extracted using the modified Baermann method (Luc *et al.*, 2005). Second stage juveniles were collected after 48 hours of incubation. The pure culture of *Pythium arrhenomanes* (P60, isolated from rice field in Philippines) was given by Phytopathology laboratory, Department of Crop Protection, Ghent University, Belgium; and culture was maintained on PDA (Potato Dextrose Agar) medium and 3-4 days old culture was used to inoculate rice seedlings.

2.3 Infection Assay and susceptibility scoring

In each infection assay, there were four different treatments performed with the above mentioned rice cultivars: single infection of both pathogens, a double infection (*M. graminicola* plus *P. arrhenomanes*) and an uninfected control (mock). Four days old rice seedlings were transplanted
on SAP media and a single plug of *P. arrhenomanes* (Appendix, Figure 1) was used to infect rice seedlings on day 9 (single infection) after making hole in the SAP media near roots, about 1 ml of nematode suspension containing 250 second-stage juveniles of *M. graminicola* were used to infect rice seedlings on day 11 (single infection), double infection with both pathogens (*P. arrhenomanes* on day 9 and *M. graminicola* on day 11), and mock infection was done on control plants with agar block/water.

To evaluate susceptibility to root knot nematode infection, root samples were harvested on day 12. Root samples were boiled in 0.8% acetic acid solution containing 0.013% acid fuchsin for 3 minutes, followed by rinsing with running tap water, de-stained in acid glycerol (1.5 ml of 37% HCl plus 500 ml of Glycerol). Effect of pathogens were evaluated by measuring the shoot and root length of the plants, counting the number of galls, total number of nematodes per root system, and the number of nematode developmental stages (J2, J3/J4, female and eggmass) per plant (Figure 1).

![Images of developmental stages](image1)

Figure 1. Developmental stages of *M. graminicola* in rice roots

### 2.4 Fluorescent labelling of nematodes

Nematodes were purified on a 100% sucrose solution followed by washing with sterile water. For PKH26 labeling, a total of 45,000 second stage juveniles (J2) of *M. graminicola* were resuspended in 1.5 ml of sterile water prior to addition of 2 µl PKH26 (stock solution $1 \times 10^{-3}$ M) from the MINI26 PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich). The suspension was mixed by inverting several times, then incubated for 15 minutes in the dark at room temperature. Nematodes were rinsed three times by repeated transfers to 30 ml of sterile water to remove excess dye, followed by centrifuging ($375 \times g$ for 3 min). Stained and non-stained nematodes were observed on standard microscopy glass slides using confocal microscope. PKH26 stained nematodes and unstained nematodes were immediately used to inoculate rice roots. After 12 days, roots were put
on glass slides and observed under the confocal microscope. In parallel, roots of similarly treated plants were boiled in acid fuchsin to check the infectivity and nematode development of the stained nematodes inside the rice roots.

2.5 Chemical Treatments

JA & SA biosynthesis inhibitor SHAM (2-Hydroxybenzenecarboxyhydroxamic acid) and JA biosynthesis inhibitor DIECA (Diethylidithiocarbamic acid diethylammonium salt), were used on rice cultivar Nihonmasari. These chemical inhibitors were dissolved in 1mL of pure ethanol prior to diluting in water containing 0.02% (v/v) Tween 20 in which the concentrations of SHAM (200 mM) and DIECA (100 mM) were prepared. Infection was similar as described above, with the addition of the chemical treatment on day 10, in which rice shoots were sprayed with chemical inhibitors (SHAM and DIECA) using vaporizers; SHAM was also used as root drench in a separate trial.

2.6 RNA Extraction, cDNA Synthesis, and qRT-PCR

On day 12 of the infection assay, root samples were harvested for gene expression studies on rice cv. Nipponbare and IR line. Root RNA was extracted using RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions and RNA concentration was measured using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific). Extracted RNA was treated with DNaseI to eliminate residual DNA. A total of 3 microgram of RNA was treated with 2 µl of DNaseI (1 unit per µl; Fermentas), 1 µl of RiboLock RNase Inhibitor (40 unit per µl; Fermentas), and 1.8 µl of 10x DNaseI buffer with MgCl2 (Fermentas) in a total volume of 18 µl (including water). The mixture was incubated at 37°C for 30 min followed by addition of 2 µl of 25 mM EDTA (Fermentas) and incubated at 65°C for 10 min to terminate the reaction.

First strand cDNA synthesis was carried out in three steps: (1) addition of 4µl of RNAse free water, 1 µl of oligo(dT) (700 ng/µl), and 2 µl of 10 mM deoxyribonucleotide triphosphates (dNTPs, Invitrogen), in a total volume of 27 µl including 20 µl of DNase treated RNA which was incubated at 65°C temperature for 5 minutes; (2) addition of 8 µl of 5x first strand buffer (Invitrogen) and 4 µl of 0.1 M DDT (dithiothreitol) which was incubated at 42°C for 2 minutes followed by put on ice; and (3) addition of 1 µl of SuperScript II Reverse Transcriptase (200 unit per µl; Invitrogen) which was incubated at 42°C for 2 hours. The solution was diluted to 100 µl by adding 60 µl of water, and the cDNA quality was checked using 5 µl of 5x MyTaq reaction buffer (preoptimized
conc. of dNTPs and MgCl₂, BIOLINE), 5 µl of cDNA template, 1 µl of forward and reverse primers (10µM) each for reference gene OsExpnarsai, 0.15 µl of Taq-polymerase (5u/µl, BIOLINE), 12.85 µl of water in a total volume of 25 µl. The programme was 95°C for 5 minutes, 95°C for 45s, 55°C for 45s, 72°C for 30s (repeated 40X), 72°C for 5 minutes. Products were checked on a 1.5% agarose gel (0.5x TAE).

For qRT-PCR analysis, each reaction contained 1 µl of cDNA, 1 µl of forward and reverse primer (10 µM) each, 7 µl sterile water and 10µl of 2xSensi Mix™ SYBR No-ROX Mastermix (BIOLINE) in a total volume of 20µl. A list of primers used for qRT-PCR is presented in Table 1. Each treatment was evaluated in two biological replicates (4 plants in each biological replicate) and three technical replicates. The PCR reactions were conducted in the Rotor-Gene 3000 (Corbett Life Science) using Rotor gene Disc (Qiagen), and results were generated using Rotor Gene software. All qRT-PCR were conducted under the following conditions: hold temperature at 95°C for 10 min, and 40 cycles at 95°C for 25s, 58°C for 25s, and 72°C for 20s. A melting curve was generated to test the amplicon specificity.

Table 1. An overview of the selected target and reference genes with their Gene Bank Locus number and the primer pairs used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene studied</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (3′-5′)</th>
<th>Locus number</th>
</tr>
</thead>
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<tr>
<td>Reference</td>
<td>OsExpnarsai</td>
<td>AGGAAACATGGAGAAGAACAAGG</td>
<td>CAGAGGTTGGTGCAGATGAA</td>
</tr>
<tr>
<td>Reference</td>
<td>OsEXP</td>
<td>TGTGAGCAGCTTCTGTGTTG</td>
<td>TGGTTGGCCTGTGAGATCG</td>
</tr>
<tr>
<td>Reference</td>
<td>OsEIF5C</td>
<td>CACGTTACGGTGACACCTTTT</td>
<td>GAGCCTCCTCTTCCTCTCAG</td>
</tr>
<tr>
<td>PR-10</td>
<td>OsPR10</td>
<td>CCTCGCGAATACGCCTAA</td>
<td>CTCAACGCGGAGAATTTC</td>
</tr>
<tr>
<td>JA biosynthesis</td>
<td>OsAO52</td>
<td>CAATACGTGTACTGGCTGAATGG</td>
<td>AAGGTGTGTAACGGGAGGA</td>
</tr>
<tr>
<td>SA biosynthesis</td>
<td>OsICS1</td>
<td>TGTCACCACAAAGGACATCTGG</td>
<td>TGGCCCTCAACCTTTAAACATGCC</td>
</tr>
<tr>
<td>Auxin Biosynthesis</td>
<td>OsYUCCA-1</td>
<td>GCACAACCTCGAGTGCTACC</td>
<td>CGTCCACTTCCTTGTCAC</td>
</tr>
<tr>
<td>Auxin response</td>
<td>OsGH3.1</td>
<td>GCAATGGAAACAAAGGCAAGGA</td>
<td>CAGATCATACCCCTAGCTTCA</td>
</tr>
<tr>
<td>ET biosynthesis</td>
<td>OsACS1</td>
<td>GATGGTCTCGGATGATCCA</td>
<td>GTGGGGGGAAAACCTGAAAT</td>
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<tr>
<td>ET response</td>
<td>OsEIN2b</td>
<td>TAGGGGGAACCTTGACATTTG</td>
<td>TGGAGGGCAGAAGTGTGTT</td>
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</table>

2.7 Quantification of P. arrhenomanes DNA in rice roots by qPCR

Root samples were collected from single P. arrhenomanes and double (M. graminicola and P. arrhenomanes) infection in both Nipponbare and IR line. In addition, some root samples were included from a raised bed interaction study performed by Ruben Verbeek at IRRI in the period
January – May, 2015 (samples given by Ruben Verbeek, PhD. Student, Ghent University, Belgium) to compare infection pressure between SAP system and soil system. Root tissue was finely crushed in liquid nitrogen. DNA was extracted using DNeasy Plant Mini Kit (Qiagen) according to manufacturer’s instruction. Primers specific for the Internal Transcribed Spacer (ITS)-region of the ribosomal DNA of *P. arrhenomanes* PT 60 (ITS60 F(5′-3′): ATTCTGTACGTCGTGCTTTCCG; ITS60 R(3′-5′): ACCTCATCTGCCCATCTCCTC were used to quantify *in planta* *P. arrhenomanes* DNA (Van Buyten & Hofte, 2013). Rice specific primers 400F (5′-3′): TTCTCCCAATCGTCTCG and 401R (3′-5′): TTAGGTGGAGGGAGCAATC were used to normalize the *Pythium* DNA. Each reaction contained 1 µl of DNA, 1 µl of forward and reverse primer (10 µM) each, 7 µl sterile water and 10µl of 2xSensi Mix™ SYBR No-ROX Mastermix (BIOLINE) in a total volume of 20µl. Three technical replicates were used for all samples. Standard curve based on dilution series (1 ng, 0.1 ng, 0.01ng, 0.001ng) were run to quantify plant and *Pythium* DNA. The standard curve with R² value of 0.9981 and a slope (y=-0.3081x+4.9047) were generated. The reactions were conducted in CFX Connect™ Real-Time PCR Detection System using 96-Well PCR Plates (BIO-RAD). qPCR were conducted using following thermal cycles: 10 min at 95°C, and 45 cycles of 25s at 95°C, 58°C for 25s, and 72°C for 20s. To test the amplicon specificity, a melting curve was generated by gradually increasing the temperature to 95°C. The qPCR data were analyzed using the CFX Manager™ software.

**2.8 Statistical analysis**

Data generated during the experiment were checked for normality and homogeneity of variance. Data on shoot and root length of rice cultivars were found to be normal and were analyzed using one-way ANOVA with Duncan’s Post-Hoc test in SPSS. Number of galls and total nematodes per root system were also normally distributed which was analyzed using independent T-test in SPSS. Data on developmental stages of *M. graminicola* were not normally distributed and homogeneity of variance was not uniform, so Mann–Whitney (for 2 variables) non-parametric tests, SPSS were performed. qRT-PCR data were analyzed using the REST 9 software (Pfaffl *et al*., 2002); this software uses a permutation analysis between control group and sample to compare the relative expression levels and to determine the statistical significance.
3 Results

3.1 Experiment on fluorescent labelling of nematodes

A fluorescent nematode staining technique as reported recently (Dinh et al., 2014), was tested out in order to be able to quantify nematode development in living root tissues. The staining of the nematodes was successful (Figure 2) and there was no adverse effect of staining on their penetration (Figure 3 A) and similar pattern of the number of female in the rice roots (Figure 3 B). Living infected roots were checked to see the fluorescence of the nematodes inside the root (Figure 4, A-B), but it was not possible to get a clear picture of the vascular bundles, in which nematode development was expected (Figure 5).

![Figure 2](image)

**Figure 2.** (A) Non-stained J2 and (B) Fluorescent labeling (stained) second stage juveniles of *M. graminicola* on glass slide under confocal microscope
**Figure 3.** (A) Number galls/root system. Independent sample T-test were done on number of galls per root system between stained and non-stained of *M. graminicola* at Nipponbare. (B) Developmental stages of *M. graminicola* between non-stain and stained nematodes at Nipponbare. Mann–Whitney non-parametric tests were done on number of J2s, J3/J4 and females between stained and non-stained of *M. graminicola* at Nipponbare. Bar represent Mean±Standard error of 4 replicates.

**Figure 4.** (A) Confocal microscopic view on the surface of the rice root; (B) Confocal microscopic view little beneath to the surface of the rice root.
### 3.2 Interaction experiments on Nipponbare, Palawan, Apo and IR line

To evaluate the interaction between *M. graminicola* and *P. arrhenomanes* on different rice cultivars, we compared the nematode development and gall formation in the roots infected only with nematodes versus double-infected root systems. When the number of galls or development was lower in the double infection versus single infection, the interaction was considered to be antagonistic. When the number of galls or development was higher in the double infection versus single infection, the interaction was considered to be synergistic.

In the first trial of the experiment, unusually slow nematode development was observed irrespective of rice cultivars tested (Appendix, Figure 2). As the development of J2s of the nematodes might be affected by an external influence, it was decided to leave this trial out of the analysis.

In two other independent trials, shoot lengths were reduced significantly after single *P. arrhenomanes* infection and after double infection compared to mock infected rice varieties (Figure 6 & 9). There were no clear trends found based on the root length among treatments in the rice cultivars tested, although root lengths were generally smaller in infected plants (Figure 7). In Nipponbare, based on nematode development, there was synergistic effect between both pathogens, i.e., the higher number of galls, total nematode counts and mean developmental stages were

![Figure 5. Confocal microscopic view in the middle of the rice root](image)
observed in double infected \((M. graminicola \text{ plus } P. arrhenomanes)\) plants as compared to single \(M. graminicola\) infected plants (Figure 8 & 10). In Palawan-2, there was a similar number of galls found between single and double infection but total nematode counts and number of females were lower in double infected plants compared to single \(M. graminicola\) infected plants, although this effect was not spectacular (Figure 8B & 10). The interaction seemed to be slightly antagonistic in Palawan-2. There was no difference observed between single \(M. graminicola\) infected and double infected plants in cultivar-Apo and IR line rice cultivars in terms of number of galls (Figure 8A), total nematodes counts per root system (Figure 8B) as well as developmental stages of RKN (Figure 10). Interestingly, cultivar-Apo and IR line seemed to be less susceptible to the nematodes as compared to Nipponbare and Palawan-2 (Figure 8B).

![Figure 6](image_url)

**Figure 6.** Shoot length of different rice cultivars after 12 days post infection (dpi) of \(M. graminicola\) (single infection), 14 dpi of \(P. arrhenomanes\) (single infection) and double infection (12 dpi of \(M. graminicola\) plus 14 dpi of \(P. arrhenomanes\)). Bar represent Mean ± Standard error of 6 replicates (2nd Trial) and 8 replicates (3rd Trial). Letter(s) on error bars are based on statistical analysis (Duncan’s multiple range test) after one way ANOVA. Means followed by the same letter(s) do not differ significantly (\(P > 0.05\)).
**Figure 7.** Root length of different rice cultivars after 12 dpi of *M. graminicola* (single infection), 14 dpi of *P. arrhenomanes* (single infection) and double infection (12 dpi of *M. graminicola* plus 14 dpi of *P. arrhenomanes*). Bar represent Mean ± Standard error of 6 replicates (2nd Trial) and 8 replicates (3rd Trial). Letter(s) on error bars are based on statistical analysis (Duncan’s multiple range test) after one way ANOVA. Means followed by the same letter(s) do not differ significantly (P > 0.05).

**Figure 8.** (A) Number of galls and (B) Number of nematodes/root system on different rice cultivars at 12 dpi of *M. graminicola*. Bar represent Mean ± Standard error of 6 replicates (2nd Trial) and 8 replicates (3rd Trial). Independent sample T-test were done on number of galls and total nematode count per root system between single *M. graminicola* infected and double infected (*M. graminicola* plus *P. arrhenomanes*) plants in each cultivar. Asterisks indicate statistically significant different between Mg and Mg+Py (P < 0.05).
Figure 9. Response of different rice cultivar to root knot nematode- *M. graminicola* and Oomycetes- *P. arrhenomanes*. Mock-without any pathogen inoculation; Mg-single *M. graminicola* infection; Py-single *P. arrhenomanes* infection; Mg+Py-both *M. graminicola* and *P. arrhenomanes* infection.
Figure 10. Developmental stages of *M. graminicola* at 12 dpi on different rice cultivars. Bar represent as Mean ± Standard error 6 replicates (2nd Trial) and 8 replicates (3rd Trial). Mean value of number of female is displayed in the bar. Mann–Whitney non-parametric tests were done on number of females between single *M. graminicola* infected and double infected (*M. graminicola* plus *P. arrhenomanes*) plants in each trial.

### 3.3 Role of JA on interaction of *M. graminicola* and *P. arrhenomanes*

In order to know the role of defense hormone-Jasmonic acid on interaction between biotrophic-*M. graminicola* and Oomycetes-*P. arrhenomanes*, experiments were conducted on Nihonmasari (wild-type), mutant line-*hebiba* (JA biosynthesis mutant) and JA pathway inhibitors (SHAM and DIECA) treated Nihonmasari. In Nihonmasari and chemical inhibitor treated Nihonmasari, there was significant reduction of shoot length in single *P. arrhenomanes* infected and double infected (*M. graminicola* and *P. arrhenomanes*) plants compared to the control and single *M. graminicola* infected plants (Figure 11 & 15). In most of the cases, reduction of root length were observed in pathogen infected plants as compared to control plants (Figure 12). Based on number of galls per root system, there was a synergistic effect in double infected roots in 2nd trial of Nihonmasari and first trial of DIECA treated Nihonmasari (Figure 13). There was no statistical difference in the number of total nematodes as well as the number of females per root system between single *M. graminicola* infected and double infected plants (Figure 14 & 16). But, there was a lower mean number of females observed in double infected plants compared to single *M. graminicola* infected plants in first trial of Nihonmasari, *hebiba* (both trials), foliar applied DIECA, foliar sprayed and root drenched SHAM. In case of the 2nd trail, Nihonmasari gave a synergistic effect (Figure 16).
Since the interaction between both pathogens in Nihonmasari was not consistent (once synergism, once slight antagonism) it is hard to draw conclusions from these experiments.

**Figure 11.** Shoot length of different rice cultivars after 12 dpi of *M. graminicola* (single infection), 14 dpi of *P. arrhenomanes* (single infection) and double infection (12 dpi of *M. graminicola* plus 14 dpi of *P. arrhenomanes*). Bar represent as mean ± standard error of 8 replicates. Letter(s) on error bars are based on statistical analysis (Duncan’s multiple range test) after one way ANOVA. Means followed by the same letter(s) do not differ significantly (*P* > 0.05).

**Figure 12.** Root length of different rice cultivars after 12 dpi of *M. graminicola* (single infection), 14 dpi of *P. arrhenomanes* (single infection) and double infection (12 dpi of *M. graminicola* plus 14 dpi of *P. arrhenomanes*). Bar represent Mean ± Standard error of 8 replicates. Letter(s) on error bars are based on statistical analysis (Duncan’s multiple range test) after one way ANOVA. Means followed by the same letter do not differ significantly (*P* > 0.05).
**Figure 13.** Number of galls/root system at 12 dpi of *M. graminicola* at Nihonmasari, *hebiba* and chemical inhibitor treated Nihonmasari. Bar represent Mean ± Standard error of 8 replicates. Independent sample T-test were done on number of galls per root system between single *M. graminicola* infected and double infected (*M. graminicola* plus *P. arrhenomanes*) plants in each trial under each cultivar. Asterisks indicate statistically significant different between Mg and Mg+Py (P < 0.05).

**Figure 14.** Number of nematodes/root system at 12 dpi of *M. graminicola* at Nihonmasari, *hebiba* and chemical inhibitor treated Nihonmasari. Bar represent as Mean ± Standard error of 8 replicates. Independent sample T-test were done on total nematode count per root system between single *M. graminicola* infected and double infected (*M. graminicola* plus *P. arrhenomanes*) plants in each trial under each cultivar.
Figure 15. Response of Nihonmasari, hebiba and chemical inhibitor treated Nihonmasari to root knot nematode- *M. graminicola* and Oomycetes- *P. arrhenomanes*. Mock—without any pathogen inoculation; Mg—single *M. graminicola* infection; Py—single *P. arrhenomanes* infection; Mg+Py—both *M. graminicola* and *P. arrhenomanes* infection.
Figure 16. Developmental stages of *M. graminicola* at 12 dpi at Nihonmasari, *hebiba* and chemical inhibitor treated Nihonmasari. Bar represent as Mean ± Standard error of 8 replicates. Mean value of number of female is displayed in the bar. Mann–Whitney non-parametric tests were done on number of females between single *M. graminicola* infected and double infected (*M. graminicola* plus *P. arrhenomanes*) plants in each trial for each cultivar.

3.4 Role of Auxin on interaction of *M. graminicola* and *P. arrhenomanes*

To evaluate the role of auxin, infection assay was done on overexpression *OsGH3.1*, a transgenic line in which plants have less free auxin, and its wild-type Bomba. Both in wild type and the *OsGH3.1* overexpression line, there was significant reduction of shoot length in single *P. arrhenomanes* infected and double infected (*M. graminicola* and *P. arrhenomanes*) plants compared to control and single *M. graminicola* infected (Figure 17 & 21). But, both in wild type and the *OsGH3.1* overexpression line, there was significant reduction of root length in single *M. graminicola* infected and double infected (*M. graminicola* and *P. arrhenomanes*) plants compared to single *P. arrhenomanes* infected and control plants (Figure 18). There was no significant difference found in the number of galls and total nematodes counts between single *M. graminicola* infected and double infected (*M. graminicola* and *P. arrhenomanes*) plants (Figure 19 A-D). However, there was a significantly higher number of females observed in double infected plants compared to single *M. graminicola* infected plants in the *OsGH3.1* overexpression line, while this
was not observed in wild type Bomba (Figure 20). Bomba showed a slight antagonism between the pathogens while a synergistic interaction was seen in the OsGH3.1 overexpression line (Figure 20).

**Figure 17.** Shoot length of Bomba and overexpression OsGH3.1 line after 12 dpi of *M. graminicola* (single infection), 14 dpi of *P. arrhenomanes* (single infection) and double infection (12 dpi of *M. graminicola* plus 14 dpi of *P. arrhenomanes*). Bar represent Mean ± Standard error of 8 replicates. Letter(s) on error bars are based on statistical analysis (Duncan’s multiple range test) after one way ANOVA. Means followed by the same letter(s) do not differ significantly (P > 0.05).

**Figure 18.** Root length of Bomba and overexpression OsGH3.1 line after 12 dpi of *M. graminicola* (single infection), 14 dpi of *P. arrhenomanes* (single infection) and double infection (12 dpi of *M. graminicola* plus 14 dpi of *P. arrhenomanes*). Bar represent Mean ± Standard error of 8 replicates. Letter(s) on error bars are based on statistical analysis (Duncan’s multiple range test) after one way ANOVA. Means followed by the same letter(s) do not differ significantly (P > 0.05).
Figure 19. (A) Gall/root system in Bomba, (B) Gall/root system in overexpression OsGH3.1 line, (C) Total nematodes counts/root system in Bomba, (D) Total nematodes counts/root system in overexpression OsGH3.1 line at 12 dpi of *M. graminicola*. Bar represent Mean ± Standard error of 8 replicates. Independent sample T-test were done between single *M. graminicola* infected and double infected (*M. graminicola* plus *P. arrhenomanes*) plants in each cultivar/transgenic line.

Figure 20. Developmental stages of *M. graminicola* at 12 dpi at Bomba and overexpression OsGH3.1 line. Bar represent mean ± standard error of 8 replicates. Mean value of the number of female is displayed in the bar. Mann–Whitney non-parametric tests were done on number of females between single *M. graminicola* infected and double infected (*M. graminicola* plus *P. arrhenomanes*) plants in each cultivar/transgenic line. Asterisks indicate statistically significant different between Mg and Mg+Py (P <0.05).
Gene expression studies on Nipponbare and IR line

The gene expression was studied to elucidate the molecular mechanisms behind the interaction. In Nipponbare (Figure 22), the transcript of OsPR10, a general pathogenesis-related gene, was slightly down-regulated in M. graminicola infected roots while significantly up-regulated in P. arrhenomanes infected roots. In double infected (P. arrhenomanes plus M. graminicola) roots, this gene was up-regulated although less pronounced compared to single P. arrhenomanes infected roots. The JA biosynthesis gene, OsAOS2 was downregulated in single M. graminicola infected roots, but significantly upregulated in the presence of necrotrophic pathogen-P. arrhenomanes; interestingly this gene was slightly suppressed in double infected roots. Transcripts of SA-biosynthesis gene, OsICS1 was slightly down-regulated irrespective of the treatments. Expression of auxin biosynthesis gene, OsYUCCA1 and auxin responsive gene, OsGH3.1 was not strongly changed after infection. ET-biosynthesis gene, OsACS1 was down-regulated in all the treatments but more pronounced in single P. arrhenomanes infected roots. Ethylene responsive gene,
OsEIN2b was significantly up-regulated in all of the treatments in which more pronounced in single *M. graminicola* infected roots.

**Figure 22.** Relative expression levels of seven different genes in root samples of Nipponbare with single nematode infection (1 dpi of *M. graminicola; Mg*); single *P. arrhenomanes* infection (3 dpi of *P. arrhenomanes; Py*); double infection (1 dpi of *M. graminicola* plus 3 dpi of *P. arrhenomanes; Mg+Py*). Bars represent the mean of two biological replicates, each containing root samples of a pool of four plants. Asterisks indicate statistically significant differential expression in comparison with uninfected plants.

In IR line (Figure 23), transcripts of both general pathogenesis gene, *OsPR10* and JA-biosynthesis gene, *OsAOS2* were found to be down-regulated both in single *M. graminicola* infected and single *P. arrhenomanes* infected roots while in double infected roots, *OsPR10* was upregulated and *OsAOS2* expression did not change. Transcripts of SA biosynthesis gene, *OsICS1* and auxin biosynthesis gene, *OsYUCCA1* were down-regulated after all treatments. Transcripts of auxin responsive gene, *OsGH3.1* was upregulated in single *M. graminicola* infected as well as in double infected roots but slightly down-regulated in single *P. arrhenomanes* infected roots. Interestingly, both ethylene biosynthesis gene, *OsACS1* and ethylene responsive gene, *OsEIN2b* were upregulated in single *P. arrhenomanes* infected roots as well as in double infected roots, but the expression of *OsACS1* was not altered and *OsEIN2b* was slightly down-regulated after single infection of *M. graminicola*. 
23. Relative expression levels of seven different genes in root samples of IR line with single nematode infection (1 dpi of *M. graminicola*; Mg); single *P. arrhenomanes* infection (3 dpi of *P. arrhenomanes*; Py); double infection (1 dpi of *M. graminicola* plus 3 dpi of *P. arrhenomanes*; Mg+Py). Bars represent the mean of two biological replicates, each containing root samples of a pool of four plants. Asterisks indicate statistically significant differential expression in comparison with uninfected plants.

![Graph showing relative expression levels of seven different genes in root samples of IR line with single or double nematode infection.](image)

**Figure 24.** Basal expression levels of seven different genes in root samples of uninfected IR line compared to uninfected Nipponbare (expression set at 1 for all genes). Bars represent Mean±Standard error of two biological replicates (three technical replicates), each containing root samples of a pool of four plants. Asterisks indicate statistically significant differential expression.
IR line was observed to support the lower number of nematodes and gene expression pattern was different as compared to Nipponbare. That was the reason behind to compare the basal gene expression level between uninfected root samples of Nipponbare and IR line. Interestingly, basal level of auxin responsive gene- OsGH3.1 was very higher in IR line compared to Nipponbare (Figure 24). Basal expression of OsPR10 and OsEIN2b were also higher while expression of OsAOS2, OsICS1 and OsACS1 were lower in IR line compared to Nipponbare roots (Figure 24).

3.6 In planta P. arrhenomanes DNA quantification in rice root samples

To evaluate infection pressure, rice root samples obtained from SAP system was use to quantify P. arrhenomanes based on its DNA amplification. IRRI samples were included in this experiment as positive control. Amplification of plant DNA and melting peak was similar in both samples obtained from SAP system and IRRI raised bed which suggests DNA extraction seems to be fine (Figure 25). However, Cq-values for the P. arrhenomanes target gene were very higher (more than 15 cycles different) for the SAP samples as compared to the IRRI samples (Figure 26). Moreover, there was an unusual melting peak in samples obtained from the SAP system (Figure 26) while IRRI samples gave as usual melting peak. After quantification using the standard curve, it was confirmed that there was only a very low amount of in planta P. arrhenomanes DNA found in all of the samples obtained from the SAP system, which is very low as compared to IRRI samples (Figure 27).

![Figure 25](image_url).

*Figure 25.* Plant DNA amplification and melting peak of different sample obtained from IRRI raised bed and SAP system.
Figure 26. *Pythium arrhenomanes* DNA amplification and melting peak of different sample obtained from IRRI raised bed and SAP system.

Figure 27. *In planta* quantification of *Pythium arrhenomanes* DNA (ng/µl) using q-PCR.

4 Discussion

The infective second-stage juveniles (J2s) of *M. graminicola* move through the soil amongst a complex of repellent and attractant chemicals to find their host. After invasion of the young root of the plant, J2s migrate to the root tip where they take a U-turn around in the meristem before they can enter the stele. Subsequently, they move upwards in the vascular cylinder towards root vascular cells which are transformed into large multinucleate feeding cells due to secretion of
effectors in the host cell, the so-called giant cells (Gheysen & Mitchum, 2011; Kyndt et al., 2013). J2s become sedentary after establishing feeding sites and molt three times within the gall to become either adult females or males through J3 and J4 stages (Karssen et al., 2013).

In present study, fluorescently labelled J2s of RKN with PKH26 were generated and infected on rice roots, in order to try to see *in vivo* nematode development (J2, J3/J4, Female and Eggmass) in rice roots. This experimental approach did not work due to thickness of the root and its non-visibility inside the root by confocal microscope. Fluorescently labelled nematodes were used successfully in the Arabidopsis root system, in which *Meloidogyne chitwoodi, Heterodera schachtii, Pratylenchus penetrans* were used to infect roots of Arabidopsis to check nematode behavior and development (Dinh et al., 2014). The positive result obtained with Arabidopsis could be due to very thin cortex layer and transparent nature of Arabidopsis root system.

### 4.1 Interaction experiment

Present study was focused on the interaction of the Oomycetes-*P. arrhenomanes* and root knot nematode-*M. graminicola* in different rice cultivars. In some cases, it has been reported that attack by one pathogen make plants more vulnerable to another pathogen. On the other hand, plants might react differently in which one pathogen could trigger defense in plants and as a result the plant would become less suitable for the second pathogen.

Rice cultivar Nipponbare has been known as susceptible to *M. graminicola* (Nahar et al., 2011; Kyndt et al., 2012; Nahar et al., 2012; Kyndt et al., 2014a) and to necrotrophic pathogen-*P. arrhenomanes* (Van Buyten & Hofte, 2013). Similarly, in present study, this cultivar was found to support a substantial number of galls and total nematodes counts. Shoot length was stunted significantly due to *P. arrhenomanes* infection. After double infection, plants showed synergistic interaction resulting in lower shoot length, significantly higher number of galls and total nematodes compared to single *M. graminicola* infected roots. Similarly, shoot and root length of sugarcane was significantly reduced due to single as well as combined effects of *P. arrhenomanes* and nematodes (*Mesocriconema xenoplax, Paratrichodorus minor* and *Tylenchorhynchus annulatus*) (Bond et al., 2004). In current study, a slight antagonistic interaction in Palawan-2 after double infection in rice roots, which is contradictory with earlier reports (Kreye et al., 2010b) but found as consistent with a recent study performed at IRRI under greenhouse conditions (Verbeek et al., 2015). There are 12 different genotypes under the common name Palawan available in the
Philippines, this could be the reason to have different outcomes. In this study, *Oryza sativa* L. cultivar ‘Palawan’ (GID 48535, IRRI) was used in 2nd trial. In present study, IR81413-BB-75-3 line has been used for interaction experiments, which is considered as a sister line of IR81413-BB-75-4. In current study, nematode infection was not affected by *P. arrhenomanes* infection in both Apo and IR line, indicating there is no interaction that affects the nematode population. Interestingly, in our study, both of the cultivars were found to support a lower number of nematodes compared to Nipponbare and Palawan-2. Rice cultivar-Apo has been reported as tolerant (Kreye et al., 2010b) to both *P. arrhenomanes* and *M. graminicola* (especially when infection done with J2s, but not with chopped roots) while IR81413-BB-75-4 line was categorized as resistant to *P. arrhenomanes* and susceptible to *M. graminicola* (Kreye et al., 2010b).

To evaluate the role of Jasmonate (JA) on infection process, interaction experiment were also conducted on rice cultivar-Nihonmasari and JA biosynthesis mutant-*hebiba*. Wild-type (First trial) and mutant plants (*hebiba*), both foliar sprayed and root drenched SHAM on Nihonmasari showed similar patterns based on galls and total nematode counts per root system. As wild-type (Nihonmasari) gave opposite results between first and second trial, it is difficult to conclude the role of JA on interaction based on experiment on Nihonmasari.

In current study, wild type rice cultivar-Bomba and a transgenic *OsGH3.1* overexpression line were also used to know the role of auxin in interaction of *M. graminicola* and *P. arrhenomanes* on rice roots. It has been reported by several authors that levels of free IAA content are decreased in overexpression *OsGH3.1* transgenic line (Domingo et al., 2009), and in *OsGH3-2* overexpression line (Du et al., 2012). Interestingly, there was a faster nematode development and the higher eggmass in wild-type compared to the transgenic line which indicates the role of auxin in the nematode developmental process. There was a similar number of total nematode counts between single *M. graminicola* infected and double infected roots both in wild type and mutant line which suggest auxin does not play role in nematode penetration. However, in the transgenic line, significantly more females were observed in double infected roots compared to single *M. graminicola* infected roots which suggest that the presence of *P. arrhenomanes* might complement the lower levels of auxin in this line, consequently leading to faster nematode development in the double-infected plants compared to single nematode infected roots.
4.2 Gene expression studies

The gene expression levels have been studied on rice cultivar-Nipponbare and IR81413-BB-75-3 line after single and double infections to elucidate the role of hormonal pathways in the interaction. Biosynthesis and/or responsive genes of JA, SA, Auxin, and ET pathway were studied together with general pathogenesis-related gene OsPR10. In current study, there was a down regulation of the transcripts of general pathogenesis gene, OsPR10 in single M. graminicola infected roots, both in Nipponbare and IR line, which is consistent with previous findings (Nahar et al., 2011). There was a strong up-regulation of OsPR10 gene observed in single P. arrhenomanes infected roots in Nipponbare which is also consistent with recent findings (Van Buyten, 2013). Interestingly, in Nipponbare, OsPR10 was up-regulated after double infection but less pronounced than after single P. arrhenomanes infection which suggests in double infection, P. arrhenomanes triggered the expression of OsPR10 which is suppressed by M. graminicola. It was observed that the basal level of OsPR10 was higher in IR-mock compared to Nipponbare-Mock, which could explain the lower susceptibility of IR line to root knot nematode-M. graminicola.

In Nipponbare, there was a significant up-regulation of OsAOS2 (JA biosynthesis gene) transcripts in single P. arrhenomanes infected roots. This results is consistent with recent findings in which OsAOS2 gene was found to be strongly upregulated in rice seedlings after P. arrhenomanes infection at one day post infection (Van Buyten, 2013). There was a down-regulation of OsAOS2 gene in single M. graminicola infected roots. Suppression of the JA pathway by root knot nematodes has been reported in several studies (Nahar et al., 2011; Kyndt et al., 2012; Kyndt et al., 2014a). In double infection, there was a down-regulation of this gene but less pronounced compared to single M. graminicola infected roots which suggests P. arrhenomanes triggers JA signaling in roots but M. graminicola suppresses the JA pathway in double infection. Expression of OsAOS2 gene was different in IR line. This gene was suppressed in both single M. graminicola infected and single P. arrhenomanes infected roots; there was no change in expression level in double infection which suggest that there could be other signaling pathway or crosstalk with other hormones involved in IR line.

In the current study, SA biosynthesis gene, OsICS1 was suppressed in all the treatments, i.e., in single infection by P. arrhenomanes, single infected M. graminicola and double infection in both Nipponbare and IR line suggest that necrotrophic pathogen-P. arrhenomanes and biotrophic
pathogen- *M. graminicola* might be able to suppress SA biosynthesis pathway in rice roots and SA signaling does not play role in the interaction. Present findings on suppression of SA pathway by *M. graminicola* is consistent with a previous study (Nahar *et al.*, 2011) while suppression of SA pathway due to *P. arrhenomanes* was found unclear in rice roots (Van Buyten, 2013).

Higher expression of auxin responsive gene, *OsGH3.1* results in the degradation of free auxin content. Basal level of *OsGH3.1* was significantly higher in IR-mock compared to Nipponbare-mock which suggest a lower free auxin content in roots of the IR line, which might result in the lower number of galls, total nematode counts and slower nematode development in this line. Similarly, overexpression of *OsGH3.1* in rice led to lower auxin content consequently enhanced resistant to fungal pathogens (Domingo *et al.*, 2009). In the interaction experiment, a significantly slower nematode development was also observed in single *M. graminicola* infected roots in overexpression *OsGH3.1* line compared to wild type (Bomba). On the other hand, *Pythium* spp. are known to secrete IAA in the tomato rhizosphere to promote their invasion (Mojdehi *et al.*, 1990; Rey *et al.*, 2001). The *P. arrhenomanes* isolate was shown to produce auxin under *in vitro* conditions (Van Buyten, 2013). In present study, there was a significant higher number of females observed in the double infection than single infection with *M. graminicola* in the *OsGH3.1* overexpression line (which have lower auxin content) suggesting that *P. arrhenomanes* might trigger IAA in double infected rice roots and as a result faster nematode development was observed.

Ethylene production is one of the earliest response to a wide variety of biotic or abiotic stresses by plants (Abeles *et al.*, 1992). *Pythium sylvaticum, P. jasmonium,* and *P. irregulare* did cause significantly more disease symptoms in ethylene mutants (*ein2-1*) than in wild-type plants suggesting the role of ET in plant defense (Geraats *et al.*, 2002). Ethylene signaling and biosynthesis mutants (*OsEin2b RNAi*) supported a higher number of root knot nematodes (RKN) in the rice roots (Nahar *et al.*, 2011). In Nipponbare, ET-biosynthesis gene, *OsACS1* was down-regulated while ethylene response gene, *OsEIN2b* was up-regulated in all of the treatments. On the other hand, in the IR line both *OsACS1* and *OsEIN2b* gene were up-regulated in single *P. arrhenomanes* infected and double infected roots but suppressed in single *M. graminicola* infected roots. Interestingly, basal level of *OsEIN2b* was significantly higher which might explain the role of ethylene in immunity in IR line consequently the lower number of nematodes present in the root system.
4.3 *In planta* *Pythium arrhenomanes* DNA quantification

In order to know infection pressure in SAP system, root samples harvested from Nipponbare and IR line were used to quantify *P. arrhenomanes* based on DNA amplification. DNA extraction seemed to be fine as plant DNA was amplified properly in all samples. However, for *Pythium* target gene, there were very late Cq-values and an unusual melting peak, which indicates a very low amount *P. arrhenomanes* in the root samples. This could be confirmed when samples were compared to IRRI samples. This suggests that *P. arrhenomanes* might not enter into the root tissue properly in SAP system. In contrast, the higher amount of *in planta* *P. arrhenomanes* DNA was quantified in rice roots when infection was done on rice seedlings grown in Gamborg B5 medium (Van Buyten & Hofte, 2013). It seems that the SAP system is not an optimal environment for *P. arrhenomanes* to colonize inside the rice root. As only a single plug of *P. arrhenomanes* was added in each SAP tube, the inoculum might be too low. Furthermore, the inoculum might not be distributed properly in the whole SAP substrate. However, *P. arrhenomanes* did have an effect on the plants, as observed by the significant reduction of shoot length on all of the cultivars tested. In addition, influence on the nematode development in double infection in Nipponbare, Palawan-2, and overexpression *OsGH3.1* transgenic line indicates the presence of *P. arrhenomanes* in the substrate.

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**Is NLP present in *Pythium arrhenomanes***?

Many fungi, bacteria and oomycetes secrete necrosis and ethylene inducing peptide 1 (Nep1) like proteins (NLPs) which induce necrosis and immunity related responses (Pemberton & Salmond, 2004; Gijzen & Nurnberger, 2006; Feng *et al.*, 2014; Oome & Van den Ackerveken, 2014). A large NLP gene families have been found in genome sequence of Oomycetes genus-*Phytophthora* (Tyler *et al.*, 2006) which is highly diverse and expanded (Qutob *et al.*, 2002; Gijzen & Nurnberger, 2006; Kanneganti *et al.*, 2006). NLP homologues have also been reported in *Pythium monospermum* (Worle & Seitz, 2003; Tyler *et al.*, 2006); *P. aphanidermatum* (Veit *et al.*, 2001); and *P. ultimum* (Levesque *et al.*, 2010).

There was four primers designed, based on known nucleotide sequences encoding NLP (Necrosis and ethylene inducing protein) in *P. aphanidermatum*. The primers are presented in Table 2. A PCR gradient (55°C to 60°C) was performed to amplify the NLP homologues using *P.
*arrhenomanes* genomic DNA. PCR products were run on 1.5% agarose gel having 2.5µl of Midori Green Advance DNA stain (Nippon Genetics Europe GmbH) per 100 ml of agarose gel (Figure 28, I). Desired bands were cut out and purified using PCR purification kit (Qiagen). PCR products were ligated to pGEM-T vector and heat-shocked in competent *E. coli* cells. Colony PCR was carried out to find out positive colonies (Figure 28, II & III). Plasmids were isolated from positive colonies using the plasmid purification kit (Fermentas). Plasmids were sent for sequencing after addition of plasmid specific primers T7 and SP6.

There was no match with known NLP homologues after blast search in NCBI database. For instance, one shorter sequence obtained from C2 colonies had similarity with *Phytophthora capsici* (pectate lyse gene) and *Phytophthora cinnamomi* (alpha tubulin gene) which is not the expected gene (Figure 29). This could be due to bad primer design or pick up of wrong bands from gel or simply because NLP might not be present in *P. arrhenomanes*.

Table 2. List primers used to search for NLP gene in *Pythium arrhenomanes*

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>401</td>
<td>Py_aphNLP1F GATCAACCATGATGCTGTGC</td>
</tr>
<tr>
<td>402</td>
<td>Py_aphNLP1R ACGAGATCTTGGCGGTAATG</td>
</tr>
<tr>
<td>403</td>
<td>Py_aphNLP2F GGTACATGCCAAGGACTCG</td>
</tr>
<tr>
<td>404</td>
<td>Py_aphNLP2R TTTTCGAGGAAGCCAGATGT</td>
</tr>
</tbody>
</table>
Figure 28. (I) PCR products of two primer pair combinations (401+402 and 403+402), desired bands (A, B, and C) were cut out; (II) colony PCR of product C. Colonies 1 and 2 (C1 and C2) were used to purify plasmid and sent for sequencing; (III) colony PCR of products A and B, in which colonies (A3, A4, B2 and B3) were used to purify plasmid and sent for sequencing.
Conclusion

The rice cultivars behaved different after single and double infection with biotrophic-*M. graminicola* and necrotrophic-*P. arrhenomanes*. Based on nematode development, a synergistic interaction found in Nipponbare and slightly antagonistic interaction observed in Palawan-2 while there was no interaction found in Apo and IR line. In Nipponbare, *P. arrhenomanes* triggered JA signaling in roots which seems to be suppressed by *M. graminicola*. Auxin seems to play an important role in nematode development and *P. arrhenomanes* might be able to complement auxin level in roots. *OsGH3.1* was highly expressed, probably leading to less auxin level in IR line, and consequently the lower number of nematode infection. In SAP system, *P. arrhenomanes* infection pressure was very low and might not be optimal. The gene encoding NLPs was not detected in *P. arrhenomanes*.

Future works

- SAP system needs to be optimized for *P. arrhenomanes* colonization inside the roots.
- Experiment related to auxin transgenic line (*Ox-OsGH3.1*) with corresponding wild type need to be replicated independently.
qPCR need to be done on ethylene response genes (*OsERF70* and *OsEBP89*) to know the role of ethylene in immunity along with interaction experiment on ethylene transgenic line (*OsEin2b* RNAi line), its corresponding wild-type rice cultivar, ET-biosynthesis inhibitors (aminoethoxyvinyl-glycine, AVG; aminooxyacetic acid, AOA) treated plants.

More experiment needed to confirm whether gene encoding NLPs is present or not in *P. arrhenomanes*.

Histopathological studies needed to know details about infection in the root system.

It would be interesting to investigate the role of reactive oxygen species (ROS), phenolic compounds and callose deposition in rice roots on the interaction of both pathogens.

### 7 Acknowledgement

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### 8 References


Appendix

**Figure 1.** The pure culture of *P. arrhenomanes*, cork borer of size 4 used to make agar plugs of pathogen to infect rice seedlings.

**Figure 2.** Developmental stages of *M. graminicola* at 12 days post infection (dpi) at different rice cultivars (First trial). Bar represent as Mean ± Standard error of 6 replicates.