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Original Article

Nematode development and changes in enzymatic defensive activity in rice plants upon *Meloidogyne graminicola* infection for preliminary screening of resistant cultivars♣

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Abstract

Root-knot nematode (RKN) has been reported to damage various rice cultivars in many countries. This study was conducted to evaluate the resistant levels of rice to *M. graminicola* infection. Each plant was inoculated with 100 second stage juveniles of RKN and their resistance was checked 15 days post inoculation. The result showed that among all six evaluated rice cultivars, RD6 exhibited the highest resistance against RKN by showing the lowest of gall numbers and gall index. Moreover, it is demonstrated that high resistance of RD6 cultivar was correlated with increasing defensive enzyme activities of PAL, PPO and POD during early inoculation. In contrast, KDML105 was the most susceptible cultivar, showing the greatest gall numbers and percentage of females inside rice roots. In addition, defensive enzyme activities were not significantly different from control (un-inoculation). Therefore, RD6 cultivar might be useful for the breeding program to control RKN in further studies.

Keywords: *Oryza sativa* L., plant defense-related enzymes, rice resistance, RD6, root-knot nematodes

1. Introduction

Rice root-knot nematodes, *Meloidogyne graminicola*, are considered as serious problems both upland (rainfed) and lowland (irrigated) rice production systems in several countries (Centre for Agriculture and Biosciences International, 2019; Ravindra, Sehgal, Narasimhamurthy, Jayalakshmi, & Khan, 2017). The main symptom on rice roots caused by *M. graminicola* is hook shaped galls (root swellings), mainly formed at the root tips of infected plants. The plants are disrupted of water and nutrient transport in the root vascular system leading to above ground symptoms such as yellowing, stunting, chlorosis and vigor depletion and resulting in poor growth and substantial yield losses in crops (Mantelin, Bellafiore, & Kyndt, 2017). The severity of disease varies with inoculums load, time of infection, age of the plants etc. (Ravindra *et al*., 2017). The rice yield losses caused by *M. graminicola* have been reported up to 8-70% in India, Nepal, Bangladesh and Indonesia (Jain, Mathur, & Singh, 2007; Padgham, 2003; Pokharel *et al*., 2010). In addition, the report in Thailand presented 25% yield loss of jasmine rice caused

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by *M. graminicola* in Nakhon Nayok and Pathum Thani provinces (Chongkid & Taengthong, 2014). Prevention and eradication of these nematodes are difficult to effectively practice. Although, the chemical control is considered to be one of the most popular methods due to its convenience and ease to apply, this method is harmful to farmers and the environment (Aktar, Sengupta, & Chowdhury, 2009). Another interesting method being employed to decrease the population of nematodes is to develop plant resistance. This method does not only protect plants from nematode penetration but also is effective and environmentally friendly (Duan, Yu, Bai, Zhu, & Wang, 2014).

Plant defense responses are divided into two groups including systemic acquired resistance; SAR and induced systemic resistance; ISR (Choudhary, Prakash, & Johri, 2007). For SAR, this defensive mechanism can be activated throughout a plant after being exposed to elicitors from pathogen infection or nonpathogenic microbes, or artificial chemical trigger (such as 2,6-dichloroisonicotinic acid, chitosan or salicylic acid), which is effective against a wide range of pathogens and is mediated by a SA-dependent process (Duan *et al*., 2014; Hartman, Pawlowski, Chang, & Hill, 2016). Several plant enzymes are activated upon pathogen infection such as phenylalanine ammonia lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), lipoxygenase (LOX), superoxide dismutase (SOD), and β-1,3 glucanase (Ngadze, Icishahayo, Coutinho, & van der Waals, 2012). PAL is the primary enzyme in the phenylpropanoid pathway, which is related to biosynthesis of several defense-related secondary compounds e.g. phenolics, phytoalexins, and lignins (Duan *et al*., 2014; Ngadze *et al*., 2012; Pellegrini, Rohfritsch, Fritig, & Legrand, 1994). PPO and POD are oxidases that catalyze the formation of lignin and other oxidative phenols that can contribute reinforcing the cell wall structure and restrict development of herbivorous insects and plant-parasitic nematodes (Chawla, Choudhary, Kaur, & Jindal, 2013; Duan *et al*., 2014; Han *et al*., 2016). This information indicated that PAL, PPO and POD are associated with the resistant mechanism of several plants.

So far, there has been no complete report on rice resistance against root-knot nematodes and its mechanisms in Thailand. Therefore, the objectives of this study were to evaluate the resistant levels of rice cultivars against *M. graminicola* and to determine the defensive enzymes, including PAL, PPO and POD, induced in rice in response to *M. graminicola* infection.

2. Materials and Methods

2.1 Nematode culture and inoculation

Second stage juveniles (J2s) of *M. graminicola* were obtained from infected Khao Dawk Mali 105 rice cultivar, cultured in the greenhouse of the Department of Plant Pathology, Kasetsart University, Thailand. The infected rice was uprooted and washed free of soil by tap water. Eggs were extracted from the roots using a hypochlorite extraction method (Jindapunnapat *et al*., 2019). Briefly, the root systems were cut into small pieces (<1 mm), placed in 0.6% hypochlorite and then shaken by hands for 2.30 min. Egg suspensions were poured through nested sieves (250 and 25μm aperture) and rinsed by tap water until they were completely cleaned. Eggs retained on the 25-μm pore sieve were rinsed into glass beakers using tap water, incubated for seven days according to Baermann funnel method and then J2s were collected for use in the experiments.

Six rice cultivars (*Oryza sativa* L. ssp. *indica*) including Khao Dawk Mali 105 (KDML105), Pathum Thani 1 (PT1), San-Pah-Tawng 1 (SPT1), Rice Department No. 6 (RD6), Rice Department No. 43 (RD43), and Rice Department No. 57 (RD57) were evaluated for their resistance to *M. graminicola* infection. One seven-day-old rice plant was transferred into a 50 mL falcon tube (with a hole made at the bottom) containing 50 grams of sterilized sand wetted with hydroponic solution (Phonpho, Nuntagij, & Saetiew, 2017). Three days later, each plant was inoculated with approximately one-hundred J2s of RKN. The experiment was arranged by randomized completely block design (RCBD) with five replications and the experiment was repeated twice. A 10 mL of tap water was given for three times per week until the results were determined.

2.2 Resistance/susceptibility scoring methods

At 15th day post inoculation (DPI), nematode-inoculated plants were uprooted and washed free of soil by tap water. Numbers of galls were observed and counted under a stereo microscope (Olympus SZ, Japan). The root galling scores were rated in accordance with Zhan *et al.* (2018) as follows: 0 = no gall; 1= 1-2 galls; 2= 3-10 galls; 3=11-20 galls; 4= 21-30 galls; 5= >30 galls. Subsequently, obtained scores were used to calculate gall index (GI) using the following formula:

$$\mathrm{GI} = \frac{\sum(\mathrm{Si} \times \mathrm{Ni})}{\mathrm{N} \times 5} \times 100$$

where, Si was root galling scale of 0, 1, 2, 3, 4, 5. Ni was the number of plants in each root galling scale. N was the total number of evaluated plants in each plot. GI was translated into resistance/susceptibility as follows: immune (I) means GI= 0; highly resistant (HR) 0.1≤GI≤5.0; resistant (R) 5.1≤GI≤25.0; moderately susceptible (MS) 25.1≤GI≤50.0; susceptible (S) 50.1≤GI≤75.0; highly susceptible (HS) GI>75.0. In addition, the reproductive factor (RF) of nematode was calculated according to the following ratio: RF = Pf/(Pi × root weight), where Pi = initial population, Pf = final population (the sum of eggs + J2s) (Phan, Waele, Lorieux, Xiong, & Bellafiore, 2018).

2.3 Nematode development in rice cultivars

To investigate RKN development in each rice cultivar, rice root systems were collected and washed thoroughly at 2 and 14 DPI. Plants were checked for the number of egg masses and the development of nematode growth stages by staining the roots with acid fuchsin dye (Bhagat *et al.*, 2019).

2.4 Defensive enzyme analyses

Rice cultivars with the greatest and the lowest levels of resistance were chosen from previous experiments to determine defensive enzyme activities of phenylalanine ammonia lyase (PAL), peroxidase (POD) and polyphenol

oxidase (PPO) in rice at 1, 2, 3, 4 and 7 DPI, with no nematode inoculation served as control. 0.1 g of root tissues was crushed in a cooled mortar with pestle in 1.5 mL of 50 mM potassium phosphate buffer (pH 8.8). Then, the root extracts were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 10,000 rpm for 5 min. The obtained supernatant (crude enzyme) was stored at 2-4 °C until use in enzyme activity determination (Kittimorakul, Eksomtramage, Sunpapao, & Chairin, 2020).

PAL activity*:* A total of 2 mL reaction mixture, consisting 100 µL of crude enzyme, 900 µL of distilled water and 1 mL of substrate solution (3 mM L-phenylalanin solution dissolved in 150 mM Tris-HCl buffer pH 8.6), was rapidly mixed in a quartz cuvette. The reaction mixture was incubated at room temperature (25±3 °C) for 3 min and the activity was determined by measuring the absorbance at 270 nm in a UV spectrophotometer (UV 5300 METASH, China). Enzyme activity (U mL^{-1}) was calculated according to Havir and Hanson (1970).

PPO activity*:* the PPO activity was measured using catechol as a substrate. A total of 1.25 mL reaction mixture, consisting 100 µL of crude extract, 100 µL of distilled water, 1 mL of 0.1 M potassium phosphate buffer (KPB) and 50 µL of substrate solution (0.3 M catechol solution dissolved in distilled water), was rapidly mixed in a glass cuvette. The reaction mixture was incubated at room temperature (25±3 °C) for 3 min and immediately measured under the absorbance at 495 nm in a UV spectrophotometer. Enzyme activity (U mL^{-1}) was calculated according to Mayer, Harel, and Ben-Shaul (1966) description.

POD activity*:* the POD activity was measured using guaiacol as a substrate. A total of 3 mL reaction mixture, consisting 20 µL of crude enzyme, 20 µL of distilled water, 2.66 mL of 0.1 M KPB, 150 µL of substrate solution (4% (v/v) guaiacol diluted with distilled water at 25 °C) and 150 µL of 1% H_2O_2, was rapidly mixed in a glass cuvette. The reaction mixture was incubated at room temperature (25±3 °C) for 2 min and the activity was determined by measuring the absorbance at 470 nm in a UV spectrophotometer. Enzyme activity (U mL^{-1}) was calculated according to the procedure of Diaz, Bernal, Pomar, and Merino (2001).

2.5 Statistical analysis

Data were statistically analyzed using the SPSS software (version 16.0; SPSS Inc.; Chicago, IL, USA). Different means of gall numbers, reproduction factor, egg masses and number of nematodes inside rice roots were determined by analysis of variance (ANOVA) and the means were compared using Duncan adjustment for multiple comparisons ($p \leq 0.05$). The normal distribution and homogeneities of variance test were done before ANOVA. For defensive enzyme activities, the means were determined by the Student's Paired-Plot Design Test at the 0.05 level.

3. Results and Discussion

3.1 Responses of rice against RKN

The result illustrated that all evaluated rice cultivars exhibited moderate susceptibility to RKN infestation (Table 1). In current study, the most susceptible response was observed in KDML105 rice cultivar, with the greatest number of galls and gall index. However, Rf value was not significant with RD43 and RD57 rice cultivars. On the contrary, RD6 rice cultivar showed the highest resistance to RKN, displaying the lowest of gall numbers and gall index. This result coincided with Arayarungsarit (1985) reported that RD6 rice cultivar was moderately resistant to *M. graminicola* (the number of galls and eggs were less observed and the size of adult females was smaller than that of susceptible rice cultivars). In addition, the susceptibility of KDML105 on root-knot nematodes has been reported by Chongkid and Taengthong (2014) and Thalue, Chinnasri, Sasnarukkit, and Sreewongchai (2019), with yield loss up to 25% both in quantity and quality of rice.

3.2 Nematode development in rice cultivars

At 2 DPI, all rice cultivars, J2s of RKN at the range from 5.1-9.8 nematodes were observed inside one rice root system (Table 2). Lowest nematode numbers successfully penetrating into rice roots were detected in RD6, SPT1 and PT1 cultivars. At 14 DPI, the observation of nematode infected-rice roots revealed that the nematode growth was delayed in RD6 rice cultivar (Figure 1). However, the number of nematodes in the roots were not significantly different between RD6 and PT1 rice cultivars. The minimal percentage of adult females was found in RD6 (29.6%) and SPT1 (41.4%) rice roots, and thus the number of egg masses per root was low as well. Arayarungsarit (1985) reported that the growth of nematodes was delayed and the giant cells were thinner and smaller in infected RD6 rice roots when compared with susceptible rice cultivar. Therefore, RD6 and KDML105 were selected as representatives to determine defensive enzyme activities.

Table 1. Evaluation of rice resistance against *Meloidogyne graminicola* at 15th day after nematode inoculation

Rice cultivar[/1]	Gall number	Gall index	Reaction	Reproduction factor (Rf)
KDML 105	11.9±1.9a[/2]	54.0	S[/3]	33.92±3.2a
PT 1	6.3±0.9c	42.0	MS	11.93±3.1b
SPT 1	5.4±0.6cd	40.0	MS	15.1±2.2b
RD 6	3.2±0.5d	30.0	MS	10.02±3.5b
RD 43	6.3±0.6c	40.0	MS	27.35±3.6a
RD 57	9.2±0.9b	48.0	MS	25.92±4.8a

[1] Khao Dawk Mali 105 (KDML105), Pathum Thani 1 (PT1), San-Pah-Tawng 1 (SPT1), Rice Department No. 6 (RD6), Rice Department No. 43 (RD43), and Rice Department No. 57 (RD57). [/2] means ± standard error ($n=10$) was compared using Duncan adjustment for multiple comparisons ($p<0.05$). Similar lower-case letters in each column indicated that means are not significantly different. [/3] MS; moderately susceptible, S; susceptible

Table 2. Differential development of *Meloidogyne graminicola* in the roots of various rice cultivars

Rice cultivars[/1]	2 DPI[/2]	14 DPI			
	Number of nematodes	Number of nematodes	J3+J4 (%)	Adult females (%)	Egg masses per root
KDML 105	9.8±1.1a[/3]	9.7±0.8ab	21.0±2.8c	79.0±2.8a	4.8±0.4a
PT 1	5.2±0.6c	6.4±0.4d	35.5±5.2b	64.5±5.2b	1.8±0.4cd
SPT 1	6.5±1.0bc	8.5±0.8bc	58.6±2.7a	41.4±2.7c	1.3±0.3d
RD 6	5.1±0.6c	6.9±0.6cd	70.4±2.9a	29.6±2.9c	0.8±0.2d
RD 43	9.0±0.8a	8.7±1.1bc	36.5±5.4b	63.5±5.4b	2.7±0.4bc
RD 57	8.7±0.7ab	11.5±0.7a	30.4±5.6bc	69.6±5.6ab	3.6±0.6b

[1] Khao Dawk Mali 105 (KDML105), Pathum Thani 1 (PT1), San-Pah-Tawng 1 (SPT1), Rice Department No. 6 (RD6), Rice Department No. 43 (RD43), and Rice Department No. 57 (RD57). [/2] DPI = Days post inoculation. [3] means ± standard error (*n=10*) was compared using Duncan adjustment for multiple comparisons ($p<0.05$). Similar lower-case letters in each column indicated that means are not significantly different.

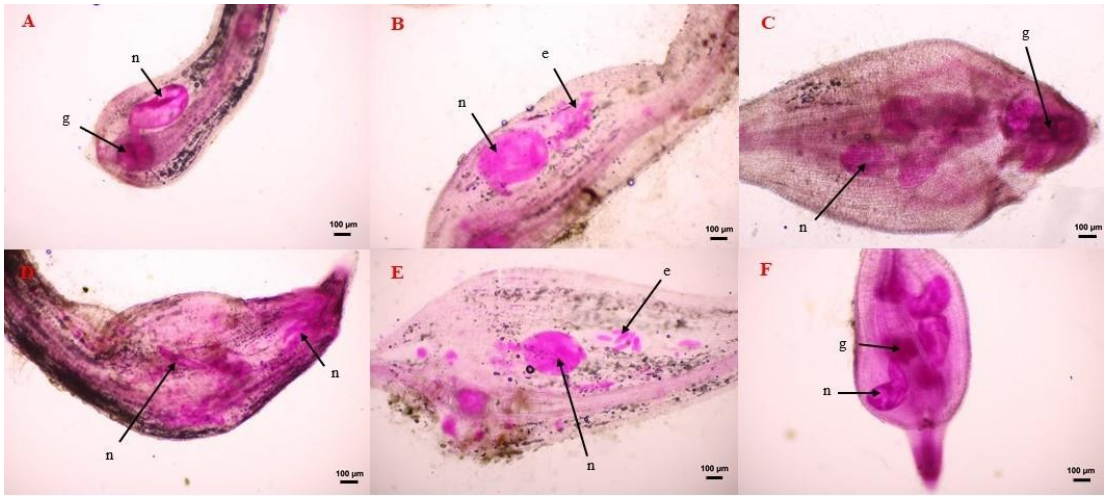

Figure 1. Development of *Meloidogyne graminicola* inside roots of various rice cultivars at 14th post inoculation. A; Khao Dawk Mali 105, B; Pathum Thani 1, C; San-Pah-Tawng 1, D; Rice Department No. 6, E; Rice Department No. 43, F; Rice Department No. 57, n; nematode, e; eggs of nematode, g; giant cell

3.3 Defensive enzyme analyses

PAL, PPO and POD activities in RD6 cultivar (with 100 J2s RKN inoculation) significantly increased in first stage, especially at 2 and 3 DPI, as compared with control (un-inoculation). On the other hand, the enzyme activities in KDML105 (susceptible cultivar) were not significantly different between RKN inoculation and un-inoculation (Figure 2). This result illustrated that nematode resistance shown in RD6 cultivar was correlated with increasing activities of PAL, PPO and POD, and these enzymes may play an important role in the mechanisms of plant resistance against RKN. An increase in all three enzymes is associated with resistance mechanism, which is involved in the biosynthesis of phytoalexins, phenolics and lignins that may contribute to the formation of defense barriers for reinforcing the cell structure (Anita & Samiyappan, 2012; Duan *et al*., 2014). Higher accumulation of phenols and defensive enzymes viz., POD, PPO, PAL and chitinase in rice root tissues resulted in significant reduction of nematode infection (Anita & Samiyappan, 2012). In addition, Arayarungsarit (1985) reported that highest *M. graminicola* population successfully penetrating rice roots was at 2nd day following inoculation. This information supports the previous results regarding nematode development, where lower number of nematodes in roots and more delayed nematode growth were observed in RD6 than in the susceptible rice cultivars (Table 2). These results were similarly observed in *O. glaberrima*, where the number of second-stage juveniles of *M. graminicola* was significantly lowered and giant cell development interrupted (Kyndt, Fernandez, & Gheysen, 2014). Several rice cultivars have been recently reported to be highly resistant to *M. graminicola*. Zhan *et al*. (2018) reported that Zhonghua 11 (*aus*), Shenliangyou 1 (hybrid *aus*), and Cliangyou 4418 (hybrid *indica*) rice cultivars were highly resistant as the nematode populations were significantly reduced and failed to reproduce inside resistant rice roots, when compared with susceptible rice cultivar. Moreover, Lahari *et al*. (2019) examined nematode resistance locus in LD 24 (an *indica* from Sri Lanka) and Khao Pahk Maw (an *aus* from Thailand) crossed with a moderately susceptible cultivar, Vialone Nano (a temperate *japonica* from Italy) by using QTL-seq. The result demonstrated that the same locus located on chromosome 11 was found to be responsible for *M. graminicola* resistance in both cultivars. However, for RD6, there has been no report on root-knot nematode resistant genes in Thailand before. Therefore, further studies are needed, especially to determine the expression of pathogenesis-related (*PR*) genes in RD6 cultivar.

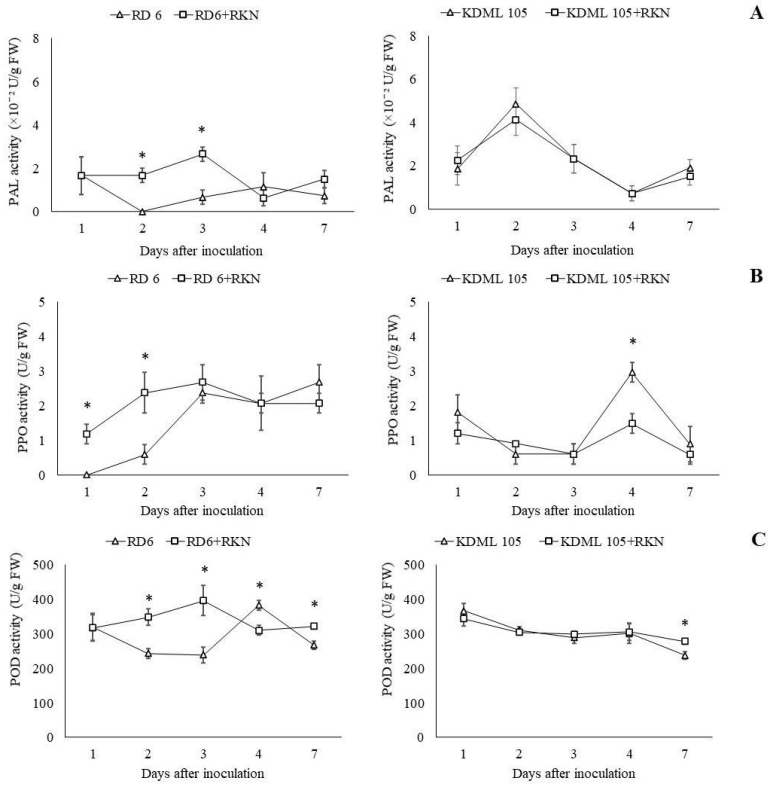

Figure 2. Determination of (A) phenylalanine ammonia lyase (PAL), (B) polyphenol oxidase (PPO) and (C) peroxidase (POD) enzyme activities in Rice Department No. 6 cultivar (RD6) (highest resistance) and Khao Dawk Mali 105 rice cultivars (KDML 105) (high susceptibility) at 1, 2, 3, 4 and 5 days after root-knot nematode (RKN) inoculation. Means (*n=3*) were compared using Student's Paired-Plot Design Test at the 0.05 level. Bar refers to standard error.

4. Conclusions

This study revealed that RD6 cultivar was the most resistant among evaluated six rice cultivars. Moreover, plant defense-related enzymes including PAL, PPO and POD could be induced in RD6 cultivar which may interrupt RKN infection and development in rice roots. RD6 might be useful for further studies on genes responsible for nematode resistance and on possible incorporation into the breeding program to control RKN in rice.

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