RESEARCH ARTICLE

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Distribution and Molecular Identification of Rice White Tip Nematode *Aphelenchoides besseyi* in Rice Growing Areas in Bulgaria

ABSTRACT

A study was conducted to determine the distribution of *Aphelenchoides besseyi* using molecular identification and the nematode effect on yield components in the major rice growing areas in Bulgaria. One hundred forty one paddy seed samples were collected in 2015 and 2016. Molecular identification was performed based on the previously developed SSU rDNA detection analysis. The specific primer combinations: 5′-GCGGGATTCGTGGTTC*T and 5′-CGACATGCCGAAACATGAG were used. Analysis showed that 23,4 %, from the collected samples, were infested with A. besseyi in the 10 studied locations in Plovdiv, Pazardzhik and Stara Zagora rice growing areas. In the infested samples, nematode densities were in the range 0-396 specimens/100 paddy seed. The effect of *A. besseyi* on cv. Osmancik variety showed that panicles with white tip symptoms were significantly shorter (17.2%) and lighter (39.5%) than those without white tip symptoms.This is the first molecular report for the presence of *A. besseyi* in Bulgaria.

Key words: *Aphelenchoides besseyi*, rice, Bulgaria, morphology, SSU rDNA – based detection analysis, PCR

Introduction

The white tip nematode, *Aphelenchoides besseyi* Christie 1942, is an ectoparasite on rice, Oryza sativa L. It is the causal agent of white tip disease on rice during the growing season (Yoshida et al., 2009). The nematode is widely distributed in rice growing areas in many countries around the world, including Europe (Samaliev & Stoyanov, 2007). The distribution of *A. besseyi* is mainly through the rice paddy, where the nematodes are under a state of anhydrobiosis as adults and fourth-stage larvas within seeds beneath the glumes (Nandakumar et al., 1975). In infested fields, the average yield losses vary from 10 to 30%. Maximum losses of up to 70% for the most susceptible varieties have been reported (Prot, 1992). Along with the risk of yield and quality losses in rice crops, detection of white tip nematodes in rice seeds is of phytosanitary importance for certification of seeds and quarantine programs (Lal & Lal, 2006).

Presence of *A. besseyi* in Bulgaria was reported by Stoyanov for the first time in 1961 - in the rice fields of the village of Trud, Plovdiv region. Later, the nematode was registered in Yambol and Stara Zagora regions (Gateva, 1961; Stoyanov, 1961). The level of A. besseyi in infected grains in the tested localities in Bulgaria was low (99-199 nematodes/250 seeds (in 1961-1963) in comparison to other countries around the world (Stoyanov, 1978). Samaliev reported the presence of *A. besseyi* symptoms (white tip) on rice crops in 2002/2003 in some fields in Plovdiv, Pazardzhik and Stara Zagora provinces (Samaliev, 2007). However, further studies (2005-2009) in the rice growing areas failed to confirm its presence (Samaliev, unpuplished data). Buangsuwon et al. (1971) reported that no symptoms of *A. besseyi* attack were observed in rice plantations in Thailand despite the widespread infection. Recently, Hooper and Ibrahim (1994) have described two new species of *Aphelenchoides*, *A. paranechaleos* and *A. nechaleos* extracted from infected rice samples in Vietnam and Sierra Leone. The identification of *Aphelenchoides* species is very difficult because of their morphological similarity, although, the enzyme profiles of *Aphelenchoides* species in different world regions are useful to their identification (Ibrahim et al., 1994).

Molecular methods can be applied for identification of all life stages, including the immature stages. They can be extremely helpful in the presence of low infestation level, or when adult specimens are atypical or damaged. Polymerase chain reaction (PCR) with species-specific primers can be used for diagnosis of nematodes isolated from plant material. Each development stage can be subjected to molecular tests (IPPC, 2016).

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The analysis of coding and non-coding regions of ribosomal DNA (rDNA) is a popular method for nematode identification (ZijIstra et al., 1995). The internal transcribed spacer area (ITS) is a variable. Thus, it is one of the most frequently used genetic markers for nematode identification. A number of authors (Chizhov et al., 2003; Zhao et al. 2008; Van Megen et al., 2009) have suggested that SSU rDNA contains sufficient phylogenetic signal for the identification of *Aphelenchoides* species (Rybarczyk-Mydłowska et al., 2012).

The present study objective was: to identify the *A. besseyi* recently found in samples from some main irrigated rice areas in Bulgaria, by PCR-based methods; to assess its levels of infestation in the paddy seeds and its effect on yield components on the susceptible rice cv. Osmancik.

Figure 1. *White tip symptoms on rice leaf tops, caused by Aphelenchoides besseyi (location Voyvidinovo).*

Materials and Methods

Nematode samples

The study was conducted in 2015–2016. Paddy panicles of rice plants (one hundred and twenty days of vegetation) without and with white leaf tips (Figure1) were collected during harvest (September and October) from the main rice growing irrigation fields (Plovdiv, Pazardzhik and Stara Zagora), where rice is grown as a monoculture, using systematic sampling methods. At least 30 paddy panicles (30 in 1 ha, 50 in 1 - 5 ha and 100 in each 5 ha larger than 5 ha) were collected from different spots of the same field and were taken to the laboratory, stored at 10°C until processing. Seed samples were also collected from rice processing factories (a sample is taken twice from alongside, the top and the middle, which represents 10% of each 10 tons of rice seed). In total, for both years, 141 seed samples were collected from three rice regions in Bulgaria (Plovdiv, Pazardzhik and Stara Zagora – 77, 47 and 17 samples, respectively) (Table 1).

The nematode extraction

The modified Baermann's funnel method was used for *A. besseyi* extraction (Hooper, 1986). Seeds were previously separated from hulls (lemma and palea) (Moretti et al., 1999). Each sample was kept in water for 48 hours. The nematodes in two aliquant parts of 1 ml water suspension from each extract were counted in counting dishes. Using a stereomicroscope, the average value of both aliquant parts was calculated referring to 100 seeds.

Table 1. *Incidence of Aphelenchoides besseyi in rice seed samples in Bulgaria rice growing irrigation areas (2015 and 2016)*

Number of location	Sampling Location	Number of samples	Number of seed samples infested with *A.besseyi*	Percent of seed samples infested with *A. besseyi*	Average numbers of *A.besseyi* per 100 seeds
	Plovdiv province				
1	Voisil	8	2	25.0	0-30*
2	Trud	13	2	15.38	0-15*
3	Tsalapitsa	14	2	14.29	0-48**
4	Saedinenie	20	2	10.0	0-50*
5	Voivodinovo	15	7	46.67	0-396**
6	Rakovski	7	4	57.14	0-89***
	Pazardjik province				
7	Malo Konare	24	5	20.83	0-142**
8	Saraja	23	6	26.09	0-50**
	Stara Zagora Province				
9	Madjerito	9	3	33.33	0-48*
10	Kalojanovets	8	0	0.0	0

* low infestations(0-50 nematodes per 100 seeds); ** moderate infestation (51-100 nematodes per 100 seeds); *** high infestation (up 100 nematodes per 100 seeds)

Yield losses

To examine the effects of white tip nematodes on yield components, ten plants were separately collected: 1. from crops, cv. Osmancik variety, showing white tip symptoms; 2. from crops without symptoms of *A. besseyi* infection. Panicle length and weight were recorded, fertile and sterile kernels were counted. The weight of 1000 panicle seeds with and without white tip symptoms, as well as the nematode density per 100 seeds, were calculated.

DNA extraction

For the molecular analysis, 5 nematodes were separately hand-moved into 20-µl 10X PCR buffer (Sci Tech S) in a 1.5 ml Eppendorf tube. The nematodes were ground with a pestle and 5 µl of Proteinase K (100 µg/ μL^{-1}) was added into the tube. They were incubated at 600 C for 30 minutes. Samples with DNA content were heated in boiling water for 10 min, in order to deactivate the Proteinase. After that, they were cooled down in an ice bath for 10 min and stored at -20°C until their usage as a PCR template.

PCR amplification

Based on the typical SSU rDNA motives and the previously reported data from the detection analysis (Rybarczyk-Mydłowska et al., 2012), the primer combinations: 5′-GCGGGATTCGTGGTTC*T and 5′-CGACATGCCGAAACATGAG were used in order to detect *A. bessey* species. Expected product length: 325 bp. PCR was carried out in a QB-96 Thermal Cycler (Quanta Biotech, London, UK) with the following profile: PCR reactions were optimized and mixtures (25µl total volume) were composed of 1 µl of template DNA; 1 µl of each primer (10 pmol µl-1) and12.5µl of My Taq Ready Mix (Bioline). The cycling program consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 63°C, and extension at 72°C for 1 min, with a final elongation step for 5 min at 72°C. For the negative control, a sample containing no DNA was used. After the completion of PCR, the products were separated electrophoretically using a 1.5% agarose gel cast in TBE buffer, stained with Ethidium Bromide and photographed under UV light. DNA fragment size was determined by comparing with the 100 bp DNA ladder.

Statistical analyses

The results obtained were analyzed by analysis of variants using SPSS-12 programme.

Results

In 2015 and 2016 among 141 seed samples, 33 samples (23,4 %) were infested with *A. besseyi* in the studied locations in Plovdiv, Pazardzhik and Stara Zagora rice growing provinces (Table 1, Figure 2).

Taking into account the studied locations, the high percent of infected seed samples (46.7%) and the highest level of infection with *A. besseyi* (0-396 nematodes per 100 seeds) was found in samples from Voyvodinovo from fields where the susceptible rice variety cv. Osmancik was grown. In addition, 10 randomly selected plants per m2 were examined and 62 % of them had white tip symptoms on the flag leaves. High levels of infestation (0-142) and the highest percentage of infected seeds (57.14 %) had the samples also from Malo Konare (Table 1).

Of the infested with *A. besseyi* samples, 51.5% had low infestations (under 50 nematodes per 100 seeds), 12.12% had moderate infestations (50-100 nematodes per 100 seeds). High infestation (up 100 nematodes per 100 seeds) had 36.4 % from infested with parasite samples (Table 1).

On rice, populations of *A. besseyi* higher than 30 nematode/100 seeds can cause economical losses (Yamaguchi, 1977). Therefore, about 87.9 % of the infected seed samples, in this experiment, had more than 30 nematodes per 100 seeds (48 to 396 nematodes / 100 seeds), a threshold, creating a need for management programs in order to prevent greater crop losses.

Figure 2. *Map of Bulgaria with pointed out the area with infection of A. besseyi.*

The effect of *A. besseyi* on cv. Osmancik variety included that the panicles with white tip symptoms were significantly shorter (17.2%) and lighter (39.5%) than those without white tip symptoms (Table 2).

The weight of 1000 kernels was reduced by 12.5% and there was significant negative correlation between the number of *A. besseyi* specimens per 100 seeds on one hand, and the weight of 1000 grains in the infected seed samples in 2016, on the other hand (Table 2).

Therefore, if the white tip nematode is not controlled, severe yield loss must be expected.

Table 2. *Population density of Aphelenchoides besseyi in rice grains and decrease (%) in yield components in irrigated rice, cv.Osmancik, in the Plovdiv province (location Voivodinovo), 2016*

Yield component	Plant without white tip symptoms	Plant with white tip symptoms	Decrease in naturally infected plots (%)	CV%	LSD 0.05
Panicle length (cm)	15.36 a*	12.71 b	-17.25	13.3	1.3
Panicle weight (g)	4.80 a	2.9 b	-39.58	34.8	1.0
1000 Kernel weight (g)	34.50 a	30.2 b	-12.46	9.4	2.2
Number fertile grains	112.00 a	69.0 b	+38.39	33.6	21.5
Number sterile grains	25.00 a	46.0 b	-84.00	41.7	10.5
A. besseyi/100 grains	8.00 a	396.0 b	+4950.00	60.2	86.0

*In each lines, means followed by different letters are significantly different according to LSD tests at P = 0.05.

The correct identification of a species should be based on the combination of several methods. Molecular methods enable the rapid and reliable identification of different species. For quarantine species, such as *A. besseyi*, it is recommended to combine methods, plus additional criteria, if available.

For the molecular detection of *A. besseyi* species, a polymerase chain reaction with the species-specific primers was performed. The sample from Voivodinovo region (Number 10) was previously morphologically characterized and identified as *A. besseyi* according to the EPPO standards. Figure 3 presents the PCR amplification and the resulted fragment of 325pb. for the tested samples from all 9 regions, where *A. besseyi* was detected.

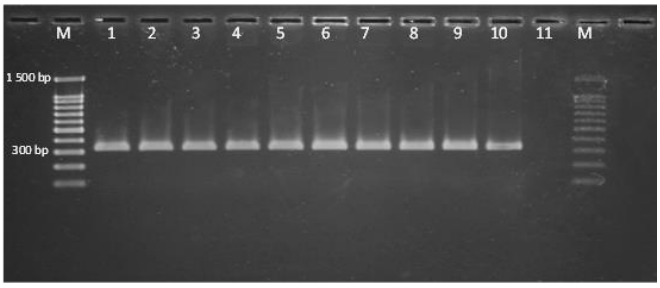

Figure 3. *PCR amplification of A. besseyi – specific primers.M–100bp DNA Ladder, 1-10 PCR amplification of nematode samples and the expected product size of 325 bp.; 1- Voisil, 2- Trud, 3- Tsalapitsa, 4- Saedinenie, 5- Voivodinovo, 6- Rakovski, 7- MaloKonare, 8- Saraja, 9- Madjerito, 10 - Voivodinovo (positive control), 11 negative control.*

Discussion

The distinction between foliar nematodes and fungal feeding *Aphelenchoides* species on the basis of morphological characteristics is often problematic. This is not only due to the absence of a reliable identification key for this genus, but it is also due to the poor description of numerous nominal species (Hunt, 1993). The relatively preserved coding region of SSU rDNA, within the ribosomal DNA, showed to be suitable for detection of foliar nematode species. To assess whether the SSU rDNA genus could be used for the molecular recognition of foliar nematode species, a phylogenetic analysis was conducted. (Rybarczyk-Mydłowska et al., 2012). Ideally, molecular detection of plant-parasitic nematode species would be based on a DNA region showing minimal intraspecific and maximal interspecific variation. The origin of A. besseyi presence in rice growing areas in Bulgaria is currently unknown.

A. besseyi is present in Turkey rice fields, a neighboring country (Öztürk & Enelli, 1997), and in some rice growing countries in Europe (Moretti, 1997; Cotoneo & Moretti, 2001). Rice import from these countries may have been made, probably import of rice paddy for manufacturing. Comparative molecular analyses of *A. besseyi* populations from these regions could be helpful to find the infestation origin.

The presence of *A. besseyi* in Bulgaria represents a threat for the local rice cultivation and production, not only because of its direct impact on crop yield, but also because the export decreases to other countries. Therefore, measures must be taken in order to prevent the spread of *A. besseyi* into new areas, and to keep nematode density below damage levels in infested areas, of the fields in the seven locations with parasite density larger than the threshold, as established in this study. In this context, early detection of infestations, integrated nematode management through quarantine regulations, sanitary principles, crop rotation and physical, chemical and biological control and use of resistant varieties may help reduce the nematode's population density to levels that allow successful and profitable rice production (Yoshii & Yamamoto, 1951; Goto & Fukatsu, 1956; Hoshino & Togashi, 2000; Amin & Al-Shalaby, 2005; Islam et al., 2014).

The PCR-based methods used in the present study will play an important role for further research and for routine identification of *A. besseyi*, in order to restrain the nematode to its current distribution area.

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