RESEARCH NOTE - NOTA INVESTIGATIVA

EXTRACTION TECHNIQUES OF THE WHITE-TIP NEMATODE *APHELENCHOIDES BESSEYI* FROM PADDY SEEDS IN REGULATORY SAMPLES

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ABSTRACT

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The rice white-tip nematode, *Aphelenchoides besseyi*, is a plant parasitic and seed-transmitted nematode of phytosanitary concern. Its detection methodology is important for seed certification and quarantine. In search of an effective nematode detection and extraction technique, the efficiency of already known and other appropriately modified procedures were compared in laboratory tests. The hydrogen peroxide (H_2O_2) method for the extraction of endoparasitic migratory nematodes from roots was modified and adapted for the extraction of *A. besseyi* from infested paddy seeds. Paddy seeds pre-soaked in 5% H_2O_2 were spread over wet blotter paper (in 2% H_2O_2), incubated in Petri dishes and rinsed with 2% H_2O_2 at regular intervals. The nematodes released from the paddy seeds in the oxygenated aqueous suspension were observed directly. This method resulted in the recovery of the largest number of nematodes which were easily counted after incubation periods of 2, 4, 7 and 10 days.

Key words: Aphelenchoides besseyi, extraction techniques, hydrogen peroxide method, *Oryza sativa*, quarantine, seed certification, seed-transmitted nematode.

RESUMEN

Rajan and A. Lal. 2006. Técnicas de extracción del nematodo de la punta blanca del arroz *Aphelenchoides besseyi* de semillas en muestras regulatorias. Nematropica 36.

El nematodo de la punta blanca del arroz, *Aphelenchoides besseyi*, es un nematodo fitoparásito de importancia fitosanitaria transmitido por semilla. Su detección es importante para la certificación de semilla y los programas de cuarentena. Con el fin de encontrar un método eficiente de extracción e identificación, comparamos procedimientos existentes y modificaciones de éstos en pruebas de laboratorio. Adaptamos el método que utiliza peróxido de hidrógeno (H_2O_2) para la extracción de nematodos migratorios endoparásitos de raíces para extraer *A. besseyi* de semillas de arroz infestadas. Se trataron las semillas en H_2O_2 (5%) y se colocaron en papel filtro mojado con H_2O_2 (2%). Luego se incubaron las semillas en cajas de Petri y se lavaron con H_2O_2 (2%) en intervalos regulares. Los nematodos recuperados en la suspensión acuosa se pudieron observar directamente. Este método permite extraer la mayor cantidad de nematodos después de 2, 4, 7 y 10 días de incubación.

Palabras clave: Aphelenchoides besseyi, técnicas de extracción, método del peróxido de hidrógeno, *Oryza sativa*, cuarentena, certificación de semilla, nematodo transmitido por semilla.

The rice white tip nematode *Aphelenchoides besseyi* Christie, is a seed-transmitted plant parasitic nematode widely distributed in many rice growing areas of the world (CABI/EPPO, 1998). The nematode typically remains coiled up in a dormant condition on the paddy seeds (*Oryza sativa* L), and these infected seeds are the main source of infestation for 'white tip disease' of paddy in nematode-free areas. Paddy

germplasm is exchanged internationally for research and breeding programs. Like any agricultural commodity, the exchanged paddy seed material should meet quarantine standards (International Standards of Phytosanitary Measures of International Plant Protection Convention) and requirements of the importing country. According to the European Plant Protection Organization (EPPO) quarantine requirements, exporting countries are asked to certify that representative samples have been tested by an EPPO recommended method and found free from *A. besseyi*. Several countries have imposed regulations to prevent the entry of nematode-infested seeds. EPPO has listed the species under A2 group, along with a number of other pests, which are regulated on specific hosts. On average, more than sixty thousand seed samples are processed and salvaged each year in a quarantine laboratory in India. Under the experimental, post-entry quarantine, field conditions, at the maturity of the crop many nematodes have been observed under the hulls, lodicules and inside the palea of harvested seeds. Often, plants do not express apparent symptoms of the disease, even when the crop is heavily infested with the nematode.

The availability of moisture is essential for the activation and examination of the nematodes that migrate from the paddy seeds to the water (Taylor, 1969). However, long incubation periods in water often enhance the activity of micro-organisms associated with the seeds, leading to fermentation and cloudiness of the nematode suspension making microscopic quantification extremely difficult. Under warm, microbially contaminated conditions, the mobility of the nematodes is reduced and the resulting nematode population counts for seed lots are often inaccurate.

In regulatory laboratories, the most commonly employed method for extracting nematodes involves manually peeling the husks of soaked seeds under water in a glass Petri dish. As a result the active white tip nematodes float to the surface of the water. This technique is a tedious and time consuming, manual process. Therefore, the objectives of the present study were (i) to compare the efficiency of newly devised techniques with the currently used techniques for the extraction of *A. besseyi* from paddy seeds and (ii) to obtain a nematode suspension free of turbidity and appropriate for examination with a light microscope.

Infected seeds were collected from paddy fields at the Central Rice Research Institute, Cuttack, India. Based on preliminary experiments, a seed lot with low nematode infestation was selected to test the experimental process. The seeds were manually cleaned of debris. Randomly selected seeds in lots of 50 each (Rajan and Lal, 2005) with five replicates were processed using the nematode extraction techniques listed under Table 1. To determine the influence of temperature on nematode recovery, all the techniques were tested and compared in laboratory incubators at two temperatures, 25 and 30°C.

In all cases (except T-13) seeds in five replicates of 50 seeds each were incubated for 10 days and rinsed 4-5 times on the 2^{nd}, 4^{th}, 7^{th} and 10^{th} day to recover the nematodes. Nematode populations in all treatments were observed in a water suspension except those in treatments T-8 and T-9, which were observed in a 2% H_2O_2 water solution. Water/H_2O_2 solutions were replaced on the observation day. The number of nematodes which emerged on each observation day, as well as the cumulative number of active nematodes examined during the entire experimental period were recorded for each treatment at the two experimental temperatures (25-30°C) and are listed in Tables 2 and 3. The analysis of variance of the data on cumulative

Table 1. Treatments/nematode extraction techniques studied.

T-1	Intact seeds soaked in water in a Petri dish (5 cm diameter), suspension observed at regular intervals
T -2	Seeds soaked in water in a Petri dish; after 24 h seeds manually teased open under water, contents transferred to modified Baermann funnel (Huang, 1983; Prot and Gergon, 1994)
T-3	Intact seeds placed on an aluminum wire mesh support, in a Petri dish; mesh touching the level of water (Gergon and Mew, 1991; Prot and Gergon, 1994)
T-4	Seeds pre soaked in water for 48 h and teased open; placed on wet tissue paper
T-5	Dry seeds crushed, contents placed over tissue paper supported on aluminum mesh, in a Petri dish containing water
T-6	Seeds pre-soaked, crushed with mortar and pestle and placed in water for examination; suspension observed at regular intervals and replaced with fresh water each time
T-7	Seeds spread and rolled in a moist 'seed germination paper' and paper roll washed on different days
T-8	Seeds pre-soaked in 2% H_2O_2 (30% w/v); seeds spread and rolled in a moist 'seed germination paper' and paper roll washed at regular intervals
T-9	Seeds pre-soaked in 5% H_2O_2 and spread on soaked (2% H_2O_2) blotter paper in a Petri dish
T-10	Seeds pre-soaked in 0.1% mercuric chloride solution for one day and then kept in a Petri dish containing water
T-11	Seeds soaked, in shallow dishes and supported on nylon sieve (OEPP/EPPO, 1992-EPPO Standard Procedure)
T-12	Individual seeds split longitudinally, soaked in shallow dishes while supported on aluminum wire net (Hoshino and Togashi, 2002)
T-13	Individual seeds (250) split longitudinally, transferred into pipette tips (5 each). Tips placed in glass vials with water (Hoshino and Togashi, 1999)

recovery of nematodes was conducted using ANOVA.

All comparisons of extraction and detection techniques are based on the principle of reactivating the quiescent nematodes with moisture resulting in their migration into water. A large number of nematodes emerged when seeds were exposed to either 5% H_2O_2 (T-9) placement on blotter paper or on blotter moistened with 2% H_2O_2 (T-8). These two techniques provided clean nematode suspensions. The enhanced nematode recovery induced by the oxygenation effect of H_2O_2 was demonstrated by Tarjan (1967) using citrus roots infected by the burrowing nematode *Radopholus similis*. The results of our study show that use of H_2O_2 also enhances the recovery of *A. besseyi*. Soaking of intact seeds in water alone for a period of 48 h, at 25° or 30°C, was not enough to induce nematodes to emerge from the seeds. To enhance early detection, either mechanical teasing/opening of the seed hull, or stimulation using H_2O_2 was required, as demonstrated by several treatments, including T-2, T-8, T-9, T-12 and T-13 (Tables 1-2). Techniques T-2, T-12 and T-13 were effective after the second day of incubation but required dehusting of seeds and, hence, were labor intensive. All other treatments failed to provide nematode recovery after 2 days of incubation.

It is worth mentioning that at both temperatures tested, maximum microbial activity and turbidity of the nematode suspension were observed in the seed lots for T-5 and T-6, where seeds were crushed and

Table 2. Number of *Aphelenchoides besseyi* recovered from paddy seeds as affected by extraction technique at 25°C.

Treatment/ technique	Number of nematodes				Mean number of nematodes extracted during first 10 days[z]
	2nd day	4th day	7th day	10th day	
T-1	0.0	0.0	10.6	13.8	24.4 b
T-2	5.0	18.5	20.0	2.3	45.8 d
T-3	0.0	4.0	25.0	17.4	46.4 d
T-4	0.0	21.0	14.4	0.0	35.4 c
T-5	0.0	4.0	2.6	0.0	6.6 a
T-6	0.0	5.0	2.2	0.0	7.2 a
T-7	0.0	2.3	10.0	12.5	24.8 b
T-8	5.0	12.5	35.0	23.3	75.8 f
T-9	15.0	20.5	32.0	26.3	93.8 g
T-10	0.0	0.0	28.0	7.2	35.2 c
T-11	0.0	5.0	26.0	17.2	48.2 d
T-12	12.0	40.0	5.0	0.0	57.0 e
T-13	10.0	30.0	17.6	0.0	57.6 e

[z]Values are means of five replicates. Means followed by common letters are not different (P = 0.05) according to ANOVA.

opened after soaking. On the contrary, exposure of seeds to mercuric chloride (T-10) resulted in the cleanest suspension, with no microbial activity. However, mercuric chloride had an adverse effect on revival and hence delayed extraction of nematodes for four days.

Though the activity of exchanging seeds or planting material in general is essential for breeding programs, nursery trials, and trade, it also promotes the spread of seed-borne pests. To minimize this risk, quarantine laboratories are required to screen quickly and salvage thousands of samples. The presence of *A. besseyi* on rice seeds can be determined following EPPO Standard PM 3/38 (OEPP/EPPO, 1992), using whole seeds. Moretti *et al.* (1999) recommended the use of rice chaff or hull as an alternative for determining seed infestation. Prot and Gregon (1994) have also described a simple extraction method that is known to be more efficient than the Baermann funnel technique. Many of the suggested mass extraction methods require a very strenuous mode of splitting individual seeds longitudinally (Hoshino and Togashi, 2002; Bueno *et al.*, 2002) which is impractical with large numbers of consignments. Also, these methods rely on the degree of mobility of the juveniles to come out of seed tissue after several days of soaking, and to actively move through the contaminated water and tissue paper.

In these studies, technique T-9 appeared to be the best procedure for detection of the nematodes in a sample size of 50 seeds. The resulting nematode suspensions obtained with this technique were clear, and it was easy to observe and count the nematodes emerging to the surface. The exposure to 5% H_2O_2 stimulated

Table 3. Number of *Aphelenchoides besseyi* recovered from paddy seeds as affected by extraction technique at 30°C.

Treatment/ technique	Number of nematodes				Mean number of nematodes extracted during first 10 days[z]
	2nd day	4th day	7th day	10th day	
T-1	0.0	0.0	15.0	21.4	36.4 c
T-2	0.0	15.0	9.2	0.0	24.2 b
T-3	0.0	7.0	23.0	27.4	57.4 e
T-4	0.0	10.0	27.8	8.0	45.8 d
T-5	0.0	7.4	0.0	0.0	7.4 a
T-6	0.0	9.0	0.0	0.0	9.0 a
T-7	0.0	0.0	20.0	15.8	35.8 c
T-8	10.0	30.0	35.0	23.4	98.4 g
T-9	20.0	45.0	40.0	11.0	116.0 h
T-10	0.0	0.0	37.0	8.6	45.6 d
T-11	0.0	8.0	25.0	24.6	57.6 e
T-12	15.0	35.0	12.6	0.0	62.6 f
T-13	17.0	37.0	15.8	0.0	69.8 f

[z]Values are means of five replicates. Means followed by common letters are not different (P = 0.05) according to ANOVA.

the revival activity of the crypto-biotic nematodes and the technique could be used for determining the "nematode free status" of the consignment with certainty as early as the 2nd day after processing. The hydrogen peroxide technique is therefore a suitable method for adoption by regulatory and seed certification laboratories.

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