Studies on the efficiency of different inoculation methods of rice white-tip nematode, *Aphelenchoides besseyi*

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Summary – To fulfil different research purposes, five methods to inoculate *Aphelenchoides besseyi* onto seedlings and panicles of rice, *Oryza sativa*, were evaluated in this study and the efficiency of the inoculation methods assessed by success rates and recovery rates. Among the three methods leading to the infection of young seedlings without obvious mechanical wounds, i.e., water flotation, seed soaking and leaf spraying, the inoculation of 125 nematodes plant⁻¹ by the water flotation method gave rise to the highest success rate and recovery rate, 95.6 and 8.3%, respectively, in growth chamber experiments. When conducted in the plastic house, seed soaking and leaf spraying of 125 nematodes plant⁻¹ resulted in 75.6 and 66.7% success rate, respectively, and 155.7 and 178.1% recovery rates, respectively. The injection or spraying of 2000 nematodes panicle⁻¹ at the booting or flowering stage gave 100% infection.

Keywords – flotation, injection, nematode recovery, *Oryza sativa*, soaking, spraying, success rate.

Rice (*Oryza sativa*) is one of the major food crops for more than a third of the world's population. Among over 200 plant-parasitic nematodes associated with rice (Prot, 1994), the rice white-tip nematode, *Aphelenchoides besseyi*, is considered to be a major contributor to the seed-borne pathogens of rice (Duncan & Moens, 2013), causing substantial annual losses world-wide of ca US\$ 16 billion (Lilley *et al.*, 2011). It is widely distributed throughout almost all the rice-growing regions of the world but is still a quarantine pest (Fortuner & Orton Williams, 1975). A reduction in rice yield as severe as 30-70% has been reported (Tikhonova, 1966; Muthukrishnan *et al.*, 1974; Lin *et al.*, 2004; Tulek & Cobanoglu, 2010).

As a seed-borne nematode, *A. besseyi* survives anhydrobiotically in stored grain for several years (Tiwari & Khare, 2003). When infected seed is planted, the nematodes become active, emerge from the seed and feed on the above-ground tissues of rice, parasitising the leaves and other young tissues as a foliar nematode. During the early growth stages of rice, *A. besseyi* is found in low numbers in all green tissues, but is more abundant in the stem. A rapid increase in nematode numbers takes place at the flowering stage, when nematodes are mainly found in

the florets of the panicles (Huang & Huang, 1972). This causes typical symptoms of 'white-tip' in leaves, which later become necrotic, then die and disintegrate (Togashi & Hoshino, 2001; Duncan & Moens, 2013). Some *O. sativa* subsp. *japonica* rice varieties do not show the typical white-tip symptoms but manifest smaller grains and erect panicles; other varieties, mainly of *O. sativa* subsp. *indica*, do not have any obvious symptoms (Liu *et al.*, 2008). *Aphelenchoides besseyi* is amphimictic and males are usually abundant (Huang *et al.*, 1979); however, parthenogenetic reproduction has also been found (Nandini *et al.*, 2001). It is a facultative parasite and can feed and multiply on fungi in addition to more than 200 types of plants in 35 genera, of which strawberry (*Fragaria × ananassa*) is another economically important host (Yu & Tsay, 2004).

To develop a control strategy of the nematode, it is necessary to study the parasitism and pathogenicity of *A. besseyi*, the interaction between the nematode and host, the efficiency of nematicides, and the resistance in rice against *A. besseyi*, either in the laboratory or in the field. Therefore, simple and efficient methods to establish successful infection for different purposes are required. At present, the published methods for inoculation include

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injecting the sprout using a syringe (McGawley *et al.*, 1984; Jamali *et al.*, 2006), adding nematode suspensions to the soil (Togashi & Hoshino, 2003; Jamali *et al.*, 2006), leaf spraying, and inoculation of the nematode by inserting a plastic tube at seedling and booting stages (Jamali *et al.*, 2006); however, these methods either cause damage to the host and retain little moisture, or have very low inoculation success rates and are time-consuming. Here, we established four new inoculation methods including water flotation, seed soaking, panicle injection and panicle spraying. Moreover, we re-evaluated the leaf-spraying method (Jamali *et al.*, 2006).

Materials and methods

NEMATODE PREPARATION

Aphelenchoides besseyi was isolated from infected rice seeds of 'AnHui1' (*O. sativa* subsp. *japonica*) by soaking seeds in tap water; 72 h later nematodes were collected and surface-sterilised with 0.1% streptomycin sulphate for 10 min, washed three times with double-distilled water, and subsequently cultured on carrot discs at 25°C for 30 days as described by Tulek *et al.* (2009). The nematodes were then collected in tap water and adjusted to a different concentration before further application. Clean tap water without any nematodes was used as the blank control in all experiments.

PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of a *japonica* rice variety, 'Lijiangxintuan Heigu', were used in all experiments; they were soaked in hot water at 56°C for 15 min to kill the nematodes already in the glumes (Fortuner & Orton Williams, 1975). The seedlings for growth chamber inoculations were grown in autoclaved sand mixed with 0.2% (m/v) superabsorbent polymer (SAP) (Reversat *et al.*, 1999). The growth chamber was set at 27°C under a 16 h light: 8 h dark regime and a relative humidity (RH) of 70-80%.

The seedlings for the plastic house evaluations were grown at approximately 26°C and 60-70% RH in soil plots. Plastic films were used to cover the plants to retain moisture, and the temperature and RH increased to 28-30°C and 70-90%, respectively. Plots were 1.5 m × 2.0 m and independently irrigated, and caged with plastic nets to protect against insects. An earth levee of 25 cm height was made around each plot to maintain the water level and to prevent the spread of nematodes.

INOCULATION METHODS

Water flotation

Hot water-treated seeds were germinated and planted individually into the centre of a 10 ml centrifuge tube containing 4 ml SAP substrate; the tubes were capped and the plants were grown for 3 days in a growth chamber. Subsequently, 2 ml of nematode suspensions in tap water containing either 0, 125, 250 or 500 nematodes were added to each plant to submerge the growth site of the seedling. The tubes were capped for another 5 days to retain moisture. Each inoculum level was conducted on 15 seedlings, and the experiment was independently repeated three times. To evaluate the later effect of inoculation, another 15 seedlings inoculated with 125 nematodes were transplanted into a plastic house plot at 20 days post inoculation (dpi); plants were grown spaced 20 cm apart in rows 15 cm apart. Symptoms were observed and recorded 60 days after transplanting.

Seed soaking

Sixty hot water-treated seeds were soaked in 12 ml nematode suspension in tap water containing either 0, 125, 250 or 500 nematodes seed⁻¹ for 24 h. Subsequently, ten seeds were planted in a 8.5 cm diam. plastic box containing SAP substrate and covered with plastic film for 7 days. Each inoculum level was conducted with six boxes (replicates), and the experiment was independently repeated three times. For plastic house plot inoculation, 15 seeds were soaked in 3 ml suspension containing 1875 nematodes, *i.e.*, 125 nematode seed⁻¹. Each seed was then planted in a plastic house plot. The plants were spaced 20 cm apart in rows 15 cm apart and covered with plastic membrane for 14 days. Symptoms were observed and recorded 60 days after planting. The experiment was independently repeated three times.

Leaf spraying

Eight hot water-treated seeds were germinated and planted in a 8.5 cm diam. plastic box containing SAP substrate, and placed in a growth chamber. When the plants had two leaves, 2 ml nematode suspensions with 0.01% Tween-20 containing either 0, 125, 250 or 500 nematodes plant⁻¹ were sprayed evenly onto the rice leaves of plants in each box using a 4 ml perfume sprayer. Plastic film was placed over the plants to retain moisture for 7 days. Each inoculum level was conducted with six boxes (replicates), and the experiment was independently repeated three times. For plastic house plot inoculation, ten nematode-free seeds were planted in a 15 cm ×

20 cm plot, and 14-day-old seedlings were sprayed with 2 ml nematode suspension with 0.01% Tween-20 containing 125 nematodes plant⁻¹ as in the growth chamber experiment. The plants were covered with plastic film for 7 days and then seedlings were transplanted. The plants were spaced 20 cm apart in rows 15 cm apart and covered with plastic membrane 14 days after spraying. Symptoms were observed and recorded 60 days after transplanting. The experiment was independently repeated three times.

Panicle injection

Hot water-treated seeds were planted in a plastic house plot. At the booting stage, 0.2 ml nematode suspension containing 2000 nematodes was injected into the sheath of each flag leaf enclosing the panicle using a 1 ml sterile plastic syringe. Plastic bags with small holes were used to envelop the top part of the inoculated plants to retain moisture for 5 days. Eight similarly sized panicles from a total of eight rice plants were selected and inoculated; 0.2 ml 0.01% Tween 20 in nematode-free tap water was used as the control. The experiment was independently repeated three times.

Panicle spraying

Hot water-treated seeds were planted in a plastic house plot. At the flowering stage, 0.2 ml nematodes suspension containing 2000 nematodes was sprayed onto each panicle with a 2 ml perfume sprayer. Plastic bags with small holes were used to envelop the panicles to retain moisture for 5 days. Eight similarly sized panicles from a total of eight rice plants were selected and sprayed; 0.01% Tween-20 in nematode-free tap water was used as the control. The experiment was independently repeated three times.

DATA COLLECTION AND ANALYSIS

For growth chamber inoculation, the above-ground part of seedlings at 25 dpi was cut into approximately 1 cm long pieces, immersed in 0.01% Tween-20 and placed on a shaker at 40 rpm for 24 h. Then the suspensions and rinsing water were collected together, viewed under a stereomicroscope (Leica S8AP0; Leica Microsystems) and the number of nematodes was recorded.

For plastic house inoculation, in seed soaking and leaf spraying methods, all the ripe seeds from individual inoculated plants were collected; in panicle injection and panicle spraying methods, ripe seeds were collected from individual inoculated panicles only. The collected seeds were gently ground to separate glumes and grains, then the glumes and grains were soaked in tap water

on a shaker at 40 rpm for 8 h. The suspensions and rinsing water were collected together, and the numbers of nematodes from seeds of each plant or panicle were counted as above.

Plants were considered to be infected if there were nematodes recovered from the seedlings or the harvested seeds. The efficiency of the different methods was evaluated by the success rate and recovery rate. The success rate was defined as the percentage of infected plants out of the inoculated plants, *i.e.*, success rate (%) = 100 × number of infected plants (or panicles)/number of inoculated plants (or panicles). The recovery rate was defined as the rate between average number of nematodes recovered from the infected plants and the number of inoculated nematodes per plants, *i.e.*, recovery rate (%) = 100 × average number of the nematodes recovered from infected plants (or panicles)/number of nematodes inoculated per plant (or panicle).

Statistical analysis was conducted using software R (version 3.3.0) (R Core Team, 2016). Comparisons of differences between inoculations were performed by the Mann-Whitney test, followed by *P* value adjustment with the method of less conservative corrections ($\alpha = 0.05$) of Holm (1979).

Results

INOCULATION CONDUCTED IN A GROWTH CHAMBER

For the water flotation method, mean inoculation success rates with the inocula at 125, 250 and 500 nematodes plant⁻¹ were 95.6, 95.6 and 97.8%, respectively, and there were no significant differences between different inocula (Table 1). The recovery rates of this method with the inocula at 125, 250 and 500 plant⁻¹ were 8.3, 5.3 and 1.6%, respectively, and the inoculum at 125 nematodes plant⁻¹ led to a significantly higher recovery than the other two (Table 1). After transplanting into the plastic house plot, the seedling of 'Lijiangxintuan Heigu' inoculated by water flotation at 125 nematodes plant⁻¹ gave rise to the typical white-tip symptoms. The percentage of plants showing symptoms was 62.2%.

For the seed-soaking method, the mean inoculation success rates with the inocula at 125, 250 and 500 seed⁻¹ were 48.9, 36.7 and 50.0%, respectively (Table 1), and the success rate with the inoculum at 250 nematodes seed⁻¹ was significantly lower than that with the other two inocula. The recovery rates of this method with the inocula at 125, 250 and 500 seed⁻¹ were 6.6, 3.8 and 1.4%, respectively; the inoculum at 125 nematodes seed⁻¹

Table 1. Inoculation efficiency of rice, *Oryza sativa*, by *Aphelenchoides besseyi* with different methods conducted in growth chambers.

Method	Inoculum plant ⁻¹	Success rate (%)	Recovery rate (%)	Number of recovered nematodes from infected plants
Water floatation (n = 45)	500	97.8 ± 3.1a	1.6 ± 0.1a	8.0 ± 0.1a
	250	95.6 ± 3.1a	5.3 ± 1.6b	13.2 ± 2.3b
	125	95.6 ± 3.1a	8.3 ± 3.0c	10.4 ± 2.2c
Seed soaking (n = 18)	500	50.0 ± 8.3b	1.4 ± 0.2a	7.2 ± 3.2a
	250	36.7 ± 7.6c	3.8 ± 0.2d	9.6 ± 2.7c
	125	48.9 ± 6.1b	6.6 ± 0.2e	8.3 ± 1.9a
Leaf spraying (n = 18)	500	44.5 ± 9.7b	1.1 ± 0.2a	5.5 ± 1.4d
	250	54.9 ± 11.8b	1.9 ± 0.3f	4.9 ± 0.2d
	125	45.1 ± 1.0b	4.6 ± 0.8b	5.7 ± 1.9d

Success rate (%) = 100 × (infected plants/inoculated plants); recovery rate (%) = 100 × (average number of nematodes recovered from infected plants/inoculated nematodes per plant). In water-flotation, seed-soaking and leaf-spraying experiments, 15, 60 and 60 seedlings, respectively, were inoculated and investigated at each inoculum amount; data are means ± standard error based on all replicates of three experimental repeats. Different letters in the same column indicate significant difference based on the Mann-Whitney test ($\alpha = 0.05$).

led to a significantly higher recovery in comparison to other inocula (Table 1).

For the leaf-spraying method in the growth chamber, mean inoculation success rates with inocula at 125, 250 and 500 nematodes plant⁻¹ were 45.1, 54.9 and 44.5%, respectively (Table 1), and there were no significant differences between the different inocula. The recovery rates of this method were 4.6, 1.9 and 1.1% at the above inoculum levels, respectively; the recovery resulting from the spraying of 125 nematodes plant⁻¹ was significantly higher than that of other two inocula.

INOCULATION CONDUCTED IN A PLASTICHOUSE

To check if the seed-soaking and leaf-spraying methods could be used on a large scale, the methods with the inoculum at 125 nematodes seed⁻¹ or seedling⁻¹ were used in plastic house experiments. The success rates of the two methods were 75.6 and 66.7%, respectively, and the respective recovery rates were 155.7 and 178.1% (Table 2). The percentages of plants showing white-tip symptoms at later stages of growth were 55.6 and 51.1% from the seed-soaking and leaf-spraying methods, respectively.

The success rates of the inoculation methods by panicle spraying and panicle injection were both 100% (Table 3). The mean number of recovered nematodes from the sprayed panicles was 101.83 panicle⁻¹, while the panicle injection method gave rise to a significantly lower mean number at 53.00 panicle⁻¹ after the recovery test (Table 3).

Discussion

The five inoculation methods, water flotation, seed soaking, leaf spraying, panicle injection and panicle spraying, all resulted in the establishment of infection of rice by *A. besseyi*; the water flotation method was the most efficient indoor method in both terms of rates of infected plants and recovered nematodes from the infected plants. Using this method, nearly all the inoculated seedlings were infected by nematodes and this led to the typical white-tip symptoms at later growth stages of the plants. Such a method could be ideal for laboratory studies of early interactions between *A. besseyi* and rice. In our preliminary test, a lower number of nematodes in the inoculum failed to increase, and even decreased, the success rate and nematode recovery (data not shown); this may be because the establishment of infection needs a minimum number of nematodes. Inoculating nematode suspensions into the soil has also been reported to establish infection (Togashi & Hoshino, 2003; Jamali *et al.*, 2006). However, similar to the method of soil inoculation, the application of nematode suspension to SAP substrate has repeatedly led to an overall low rate of infected plants ranging from 30 to 40%, even when the inoculum was as high as 1000 nematode seed⁻¹ (Table S1). The possible reason for such low infection could be the difficulty for nematodes to access seeds in the soil or the SAP substrate after the dilution of the inoculum in the substrates. To establish the water flotation method, we used SAP in a 10 ml centrifuge tube, which concentrated the nematodes within a small area. This would in part improve inoculation efficiency.

Table 2. Inoculation efficiency of rice, *Oryza sativa*, by seed soaking and leaf spraying of a suspension of *Aphelenchoides besseyi* in glasshouse conditions.

Method	Inoculum plant ⁻¹	Success rate (%)	Recovery rate (%)	Number of recovered nematodes from infected plants	Rate of white-tip symptoms (%)
Seed soaking	125	75.6 ± 3.1a	155.7 ± 38.9a	194.7 ± 54.5a	55.6 ± 3.1a
Leaf spraying	125	66.7 ± 5.4b	178.1 ± 24.4a	222.6 ± 36.9b	51.1 ± 6.3a

For definition of success rate and recovery rate see Table 1. In seed-soaking and leaf-spraying experiments 15 seedlings were inoculated and investigated; data are means ± standard error (n = 45) based on all replicates of three experimental repeats. Different letters in the same column indicated a significant difference based on Mann-Whitney test ($\alpha = 0.05$).

Table 3. Inoculation efficiency of rice, *Oryza sativa*, panicles by panicle injection and panicle spraying of a suspension of *Aphelenchoides besseyi* in plastic-house conditions.

Method	Inoculum panicle ⁻¹	Success rate (%)	Recovery rate (%)	Nematodes recovered from each panicle
Panicle injection	2000	100.0 ± 0.0a	2.7 ± 0.3a	53.0 ± 5.2a
Panicle spraying	2000	100.0 ± 0.0a	5.1 ± 1.2b	101.8 ± 23.8b

For definition of success rate and recovery rate see Table 1. Eight plants were inoculated on one panicle at 2000 nematodes plant⁻¹; data are means ± standard error (n = 24) based on all replicates of three experimental repeats. Different letters in the same column indicated a significant difference based on Mann-Whitney test ($\alpha = 0.05$).

Another benefit of this method is that it could synchronise infection at a certain time after inoculation, which could be used in experiments for observation of infection development, for example.

The success rates of the seed-soaking method and the leaf-spraying method in growth chamber conditions were generally between 40 and 50%, but in the plastic house plot values they were 67 and 76%, respectively. This indicated lower levels of infection rates due to limitations in the recovery test, as well as the possibility of a second infection occurring in the transplanted seedlings. Typical white-tip symptoms were observed in successfully infected plants in the plastic house and the percentage of plants showing symptoms was over 50%, which was consistent with the result of Jamali *et al.* (2006). These two methods were convenient to conduct, and could be used for experiments such as resistance screening, which requires inoculation of a large number of plants.

In addition, it is often necessary to prepare a large quantity of nematode-infected seeds for *in vivo* test of nematicides. Using the panicle-injection and panicle-spraying methods developed in this study, it is possible to obtain a large quantity of infected seeds. The methods also make it possible to keep a bank of *A. besseyi* at low cost by keeping the nematodes in anhydrobiosis in dry seeds.

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Table S1. Inoculation efficiency of rice, *Oryza sativa*, by *Aphelenchoides besseyi* with substrate infestation method.

Inoculum plant ⁻¹	Success rate (%)	Recovery rate (%)	Number of recovered nematodes from infected plants
1000	38.9 ± 3.8a	0.4 ± 0.0a	4.4 ± 0.3a
500	37.8 ± 6.9a	0.6 ± 0.1a	3.3 ± 0.9ab
250	33.3 ± 6.6a	0.9 ± 0.01b	2.2 ± 0.3c
125	41.1 ± 3.8a	2.0 ± 0.2d	2.5 ± 0.3c

Ten seeds were planted in 200 ml superabsorbent polymer substrate in an 8.5 cm diam. plastic box, infested by pouring 5 ml nematode suspensions concentrated at either 0, 125, 250, 500 or 1000 nematodes plant⁻¹. For definition of success rate and recovery rate see Table 1. Data are means ± standard error (n = 9) based on all replicates of three experimental repeats. Different letters in the same column indicated a significant difference based on Mann-Whitney test ($\alpha = 0.05$).