[Short Communication]**A simple root box acts as an efficient tool to quantify the newly produced cyst nematodes**

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To save time and effort in quantifying the newly produced females of *Heterodera schachtii* (HS), we introduced a simple root box (RB). The RB was composed of two transparent trays, where we examined whether the number of young females on the plant roots in the RB that would be seen from the outside could be used to accurately predict the number of mature females (cysts) in the RB soil. We inoculated 20 to 10,000 individuals of the HS second-stage juveniles into Chinese cabbage seedlings that were planted in the RB. After 20 and 58 days of inoculation at 25°C, we quantified the number of young females that appeared on the roots and the number of mature females (cysts) that appeared in the RB soil, respectively. We detected a high correlation, in which the total number of the HS females that appeared on the roots in the RB was a relatively reliable predictor of the total number of HS cysts in the RB soil. However, we concluded that controlling the moisture and the type of soil in the RB may influence the reliability of the quantification using the RB. *Nematol. Res.* 50(2), 35–38. (2020)

Key words: correlation, *Heterodera schachtii*, inoculation, transparent tray

INTRODUCTION

For the host status evaluation of a crop, it is necessary to quantify the newly produced vermiform individuals, or cysts in the soil after the nematode inoculation of a crop. In the case of cyst nematodes, a laborious procedure is generally used. The egg or second-stage juveniles (J2s) of the nematode are inoculated into a host plant and cultured in a pot until the female adults emerge and mature into cysts. The soil in the pot would be air-dried, poured with water to float the cysts, and then collected. Next, the cysts would be counted under a microscope. Even after the females emerge on the roots, this procedure requires

more than two weeks for the females to mature and for the soil to dry. It is also labor-intensive to perform the cyst collection and counting. To save time and effort, the use of a transplant pot or container was proposed, which allowed the counting of the young females on the roots (Foot, 1977; Phillips *et al.*, 1980; Aiba, 2015). However, during counting, large sets of experimental pots need to be considered. In addition, counting is not reliable if most of the females have emerged deep within the soil and are difficult to be observed from the outside. Hence, a transparent flat bag or pouch should be used, instead, to observe most of the females from the outside and to record the number easily using the digital image (Fassuliotis *et al.*, 1967). However, the bag requires a supporting frame and a folder for daily maintenance. In addition, the bags are typically soft and could easily become deformed, affecting the reliability of the counting data during careless handling.

Thus, the root box (RB), a flat container with a transplant plate on one or both sides, was originally developed for the quantification of the root system structure as well as for the *in situ* evaluation of the biological activities in the system (Lussenhop *et al.*, 1988; Meyer *et al.*, 1988; Alvey *et al.*, 2001). In cyst nematode studies, we can easily record the number of young females emerging on the root systems if the RB has a narrow cross section. In this study, we described our simple RB system and reported its performance for the quantification of newly produced female cyst nematodes in soil. In fact, we examined whether the number of young females of *Heterodera schachtii* (HS), in the digital image taken from the outside of the RB, would accurately reflect the number of newly produced cysts in the soil.

MATERIALS AND METHODS

We used two transparent polystyrene trays that were 220 mm tall with a cross section of 159 × 17 mm (Sanada Seiko Co., Ltd. Osaka, Japan, type L8039) as the main body of the RB (Fig. 1). In one tray, we created four holes that were 4 mm in diameter at the bottom for drainage and we partly cut off the top for the plant shoot growth. After covering the drain holes with a piece of Kimwipe® (Thermo Fisher Scientific, USA), we placed another intact tray on top of the processed one, while fixing them with the painter's tape on both sides. The RB had a narrow inner cross section of 14 × 154 mm, in which we placed approximately 380 mL of the nursery soil to grow plants (Kumiaiippi

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Engeibaido 1 go, Nihon Hiryo Co., Ltd, Tokyo, Japan), containing 240 mg of the total N, 3,000 mg of P_2O_5 , and 240 mg of K_2O per 1,000 g of soil. To prepare the nematode inoculum, we first obtained the HS eggs by extracting the cysts from the soil of the crucifer plant fields in the Hara village, Nagano prefecture, Japan, and crushing them with a homogenizer. To promote hatching and the activation of J2s, we treated the eggs with the stimulant solution that was obtained by soaking the Chinese cabbage roots in tap water.

In a greenhouse at 25°C, we planted 11-day-old Chinese cabbage seedlings (cv. Kigokoro 85, Takii & Co., Ltd, Kyoto, Japan) in the RB, while surrounding the RB with a cardboard sheet to avoid sunlight (Fig. 2). After seven days of planting, we inoculated the active HS J2s representing 20, 40, 100, 200, 500, 1,000, 2,000, 5,000, 7,000, and 10,000 individuals per RB. We

had two replicates for each of the 20, 40, 200, 2,000, and 7,000 individuals, whereas only one replicate for the other treatments, as we could not prepare a sufficient amount of inoculum of J2s. Hence, we chose these inoculation densities for the two replicates, because they were sufficiently low when preparing the replicates (20, 40) or because we observed major differences relative to the other densities in the inoculum series (200, 2,000, 7,000). Next, the plants were watered as required and after 16 days of the nematode inoculation (DNI), the young HS females began to emerge on the root surface of the host plants (Fig. 3). After 20 DNI, we took images of both the front and back sides of the RB to record the number of females using a digital camera (Nikon D5600, Japan). Then, we continued growing the plants for HS female maturation and after 36 DNI, we cut off the above part of the plant and terminated the watering to dry the soil. After 58 DNI, the RB was disassembled to remove the soil. To estimate the number of HS females in the RB, we extracted the HS cysts by pouring tap water into 50 g air-dried soil (a total of 100 g per RB) in a 2-L container to float and collect the HS cysts, where we counted them under a dissection microscope. To examine whether the HS cyst number in the soil

Fig. 1. Specifications and the assembly of the root box. (A) The transparent polystyrene tray was processed to have four drain holes at the bottom as well as an opening at the top. (B) Another intact tray was placed on tray A and fixed with a painter's tape on both sides. All measurements have been shown in millimeters.

Fig. 2. Plant growth in the root box. The boxes were covered with cardboard sheets to avoid sunlight and were supported by bookends.

reflected the inoculated number of J2s, and whether the number of females in an RB image can predict the cyst number in the soil, we performed a correlation analysis. To improve normality, we log-transformed the HS numbers (+0.5) prior to the analysis using the 'BellCurve Excel Toukei' package (Social Survey Research Information Co., Ltd., Japan).

RESULTS AND DISCUSSION

We could easily observe and count the young female HS outside the RB (Fig. 3). Moreover, the correlation between the number of inoculated J2s and that of cysts in the soil as well as between the number of young females in the RB images and that of cysts in soil were both highly correlated ($R^2 = 0.946$ and $P < 0.001$, Fig. 4; $R^2 = 0.940$ and $P < 0.001$, Fig. 5, respectively). This indicated that we successfully established a variety of HS that can load onto the host plants, while reflecting the inoculum series. This also indicated that we can easily predict the number of HS cysts in the soil by counting the young females that appeared on the roots without disassembling RB.

Several scientists have already introduced RB in their soil ecological and nematological studies

(Lussenhop *et al.*, 1988; Meyer *et al.*, 1988; Alvey *et al.*, 2001; Yang *et al.*, 2012), such as examining the effect of elevated CO₂ on the rhizosphere microbial

Fig. 4. Correlations between the number of inoculated J2s of *Heterodera schachtii* in the root box and the number of mature females (cysts) that were detected in the box after 58 days of inoculation. The correlation line was slightly distorted after the back-transformation of the values. Some plots may be obscured due to the overlapping data.

Fig. 3. The root system of the Chinese cabbage. The root system of the plant that was growing in the root box (left) with the females of *Heterodera schachtii* (right), as indicated by the short arrows in the magnified images. In the magnified images 'A' and 'B', the female bodies were obscured by the 'cloud-like' fungal mycelium that was growing on them and the soil powder that was surrounding them, respectively. When clearly seen, the longer diameter length of the females was approximately 700 μm (magnified image 'C').

Fig. 5. Correlations between the total number of young females of *Heterodera schachtii* on both sides of the root box that were detected in the images taken after 20 days of nematode inoculation and the number of mature females (cysts) that were detected in the box after 58 days of inoculation. The correlation line was slightly distorted after the back-transformation of the values. Some plots may be obscured due to the overlapping data.

biomass via plants (Lussenhop *et al.*, 1988) or to quantify the antagonistic fungal populations attacking the HS females on the root surface (Yang *et al.*, 2012). However, most of the RBs that were used were too large for the experimenters (1.6 L or more in volume). Thus, they are difficult to handle and require a larger space to conduct multiple experiments. In addition, they had a greater width of 25 mm or more in terms of cross sections, which may mask some young nematode females on the roots that appear deeper in the soil. Therefore, we propose the use of a more suitable RB for the quantification of cyst nematodes. Our RB is as small as 0.4 L in volume, with a narrow inner width of 14 mm in terms of a cross section. Thus, it can save space, allow easy handling, and more reliable quantification of the newly produced nematode females. In addition, our RB is cost effective, because the estimated cost of the trays that constitute the RB is approximately ¥100 or less.

Furthermore, in this study, we found that the fungal occurrence and disintegration of the soil particles may reduce the reliability of the root data. Fungal mycelia that sometimes develop in the RB soil may obscure the nematode bodies on the roots, thereby underestimating the number of females (Fig. 3, magnified image 'A'). Fungi may show better development if they obtain sufficient nutrients and an optimal level of moisture in the soil. In addition, the female bodies may be obscured if the soil particles easily disintegrate into powder (Fig. 3, magnified image 'B'). Thus, an appropriate selection of the soil type and moisture control is required to prevent the underestimation of nematode numbers.

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