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A Facile and Modified Scheme for Synchronization and Isolation of Nematode Eggs

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Abstract: Nematodes are common pests that damage agricultural crop plants. Some of them are beneficial while others are parasitic and harmful to plants, animals and humans. Several in vitro studies have aimed to develop chemicals to kill parasitic nematodes, while others have been conducted to use beneficial nematodes as biocontrol agents. However, the preparation of large quantities of nematode eggs in a laboratory setting is challenging. Traditional egg isolation protocols involve the use of sieves to filter eggs or the use of chemicals that can be harmful to nematodes while isolating the eggs. Our method utilizes 1.5 × 1.5 cm sized chunks of bacterial or fungal feed to lure nematodes. A subsequent gentle washing of the consumed chunk with distilled water provides a rapid and straightforward method of collecting eggs in 6-well polystyrene plates and removing unwanted nematodes. Approximately 4000 *Bursaphelenchus xylophilus* eggs from a fungal chunk and 2400 *Caenorhabditis elegans* eggs from a bacterial chunk were obtained when tested. This study shows a protocol for the isolation of eggs and synchronization of nematode stages that is relatively straightforward, rapid, eco-friendly, and efficient. The protocol also provides a chemical-free and a reliable, simple means of separating eggs from adults and induces the synchronization of nematodes based on the simple concept that gravid nematodes can be provoked to lay eggs by providing additional feed.

Keywords: eggs; *Bursaphelenchus xylophilus*; *Caenorhabditis elegans*; juveniles; nematode

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1. Introduction

Bursaphelenchus xylophilus is a notorious plant parasitic nematode and has been used for decades for drug screening [1]. In vitro experiments with the nematode are often conducted with mixed developmental stages [2–6] but although widely practiced, this technique causes variable results [7]. Such variations resulting from unsynchronized populations of worms compromise test reproducibility and may over- or understate the efficacies and potencies of tested compounds [7]. Thus, synchronized worms are preferred especially when the ages and sizes of worms are important. For example, juveniles have less developed biological systems than adults, intermediate stages may undergo molting [7,8], and certain nematicides may specifically target juveniles or adults [9].

Most of the techniques used to isolate eggs include a basic synchronization step involving egg preparation by bleaching [10,11]. Bleaching involves treating gravid with sodium hypochlorite and then repeatedly washing them with M9 buffer. The egg laying method and subsequent separation by centrifugation provides an alternative that allows eggs to be isolated without exposing nematodes or eggs to stress [12]. This method provides a straightforward means of isolating nematode eggs, especially those of parasitic nematodes. For example, the pinewood nematode, *B. xylophilus*, is a parasitic nematode that feeds on the fungus *Botrytis cinerea* [8]. Under laboratory conditions, the nematode is reared by subculture on fully grown *B. cinerea* plates. It requires seven to eight days for the

worms to consume all the fungi in a 90 mm diameter Petri plate, which delays experimental procedures. Subsequently, nematodes are retrieved from plates by repeated aspiration with sterile distilled water [13]. However, it is nigh impossible to retrieve nematodes free of fungal contamination from half-consumed plates, and when fungi are completely consumed, the probability is dependent on the mating frequency [14]. Eggs usually hatch within 1 or 2 days and after seven days of incubation at 22–25 °C, most of the eggs laid by gravid adults have already hatched, which results in a mixture of instar stages and adults with few or no eggs. In fact, the difficulties associated with securing supplies of nematode eggs are major problems for nematologists working with pinewood nematodes. As a result, the majority of studies on *B. xylophilus* have focused on nematicides that kill mixed stages and few studies have been conducted on *B. xylophilus* juveniles. The use of mixed stages, although realistic, fails to provide sufficient information about the modes of action of chemicals. For example, certain nematicides like ivermectin kill juveniles and not adults [9], while others neutralize or paralyze adults without affecting juveniles [7]. Furthermore, as a result of these difficulties, researchers working on *B. xylophilus* do not indulge in detailed studies about modes of action and barely report chemical killing efficacies.

We believe that *B. xylophilus* juveniles and adults respond in different ways to chemicals, and thus we decided to develop a simple protocol for isolating *B. xylophilus* eggs or juveniles routinely for regular assessment. The decanting and sieving methods used to isolate *B. xylophilus* eggs are time consuming. The Baermann funnel technique is widely used to isolate nematodes from environmental sources [15] and relies on larval movement and subsequent settling of eggs to the bottom of the microtiter plate. Several other automated egg isolation techniques have recently been employed to isolate eggs from different parasitic nematodes based on the use of a low-density salt solution to cause egg flotation and subsequent centrifugation [16]. Conversely, our protocol implements the use of *B. cinerea* lawns on potato dextrose agar (PDA) to attract gravid females which subsequently lay eggs on the fungus.

2. Materials and Methods

2.1. Isolation of Eggs from B. xylophilus

In laboratory conditions, *B. cinerea* is the feed for *B. xylophilus*. *B. cinerea* is prepared by growing on PDA plates for 7 days. *B. cinerea* plates were inoculated with *B. xylophilus* and incubated at 22 °C and humidity was set to 75 ± 5% for 8 days. One square piece (~1.5 × 1.5 cm) cut from the *B. cinerea* on PDA and was placed on top of 8 d old *B. xylophilus* plates with fungal lawns facing upwards. The plate was then incubated at 22 °C ± 2 °C and humidity was set to 75 ± 5% for 12 h or for 24 h. Highly active nematodes tend to climb onto fungal lawns, feed, and reproduce. After 12 h of incubation the fungus was consumed by *B. xylophilus*, which can be verified by the disappearance of fungal mycelia. Consumed square lawns (1.5 × 1.5 cm) of *B. cinerea* on PDA agar was transferred from a 24 h-old plate into a 6-well microtiter plate and repeatedly aspirated with sterile distilled water for 15–20 min. Sterile distilled water (1 mL) was then pipetted into each well and the lawns were repeatedly washed or aspirated mildly. After aspiration, water is removed and discarded, and the plate was allowed to dry. Eggs that stick to the bottom of plates are visible to the naked eye as small dots.

Mild aspiration is a critical step as it dislodges eggs from consumed pieces but not nematodes which require strong aspiration to separate them from PDA agar. This allows the eggs to settle on the bottoms of 6-well polystyrene plates. After mild aspiration, consumed lawns (containing nematodes) are carefully removed and discarded. Any nematodes (all stages) accidently dislodged would float or swim in the sterile distilled water, whereas all eggs sink to plate bottoms. The adherent coating on polystyrene plates also helps retain eggs on plate bottoms. Finally, the remaining floating nematodes were removed by pipette. Eggs can be observed by the naked eye and via the microscope for checking the various embryogenic stages. The settled eggs were suspended again in sterile distilled water and incubated for 6 or 24 h at 22 ± 2 °C and humidity was set to 75 ± 5%. The eggs in the

bottom of the 6-well plate were manually counted using a simple fluorescent microscope equipped with a camera, a monitor, and integrated image analysis software (The iRiS™ Digital Cell Imaging System, Anyang-si, Korea).

2.2. Isolation of Eggs from C. elegans

The *C. elegans* [*fer-15*(*b26*); *fem-1*(*hc17*)] strain was maintained on nematode growth medium (NGM) with *Escherichia coli* OP50 as feed at 16 ± 2 °C, as previously described [17]. Isolation of eggs from *C. elegans* was similar to the protocol we used in *B. xylophilus*, but food source for the nematode was the *E. coli* OP50. A square chunk of *C. elegans* on NGM agar was transferred to a 6-well microtiter plate and repeatedly aspirated with sterile M9 buffer for 15–20 min. After aspiration, the buffer was removed and discarded, and the plate allowed to dry. Eggs that stick to the bottoms of plates were visible to the naked eye as small dots. The settled eggs were suspended again in M9 buffer and incubated for 6 or 24 h at 25 ± 2 °C and humidity was set to 75 ± 5%. Isolated eggs of *C. elegans* and *C. elegans* were counted as described above. All experiments were performed with at least three replicates. The results have been averaged and the results from at least six repetitions and data were used to compile all figures.

3. Results and Discussion

The previous protocol used to obtain *B. xylophilus* and its eggs is relatively complicated and time-consuming [18]. As per this protocol, *B. xylophilus* is sub-cultured in fully grown *B. cinerea* plates. A drop of sterile distilled water containing about 50 nematodes is placed on fully grown *B. cinerea* PDA plates. The nematodes then reproduce and proliferate on the plates for 7–8 days, consuming the fungi and eventually clearing the plates (Figure 1). After 9 days of growth, nematodes and eggs are collected by repeated mild aspiration using distilled water. This is a critical step, because eggs require vigorous aspiration whereas nematodes can be easily collected by mild aspiration. After 7 or 8 days of growth, 90 mm diameter Petri plates contain approximately 3.2 ± 0.4 × 10^5 nematodes [8], but the numbers of eggs vary. Furthermore, it is not recommended to use plates before seven days have elapsed because unconsumed fungi and fungal spores/mycelium interfere with the subsequent isolation/collection steps, which as mentioned above requires aspirating the plates with sterile distilled water. A diagrammatic scheme for the isolation of nematode eggs using the devised technique is provided in Figure 2. Approximately 4240 ± 210 eggs (54%) are retrieved from a single piece after 24 h. We also observed that 36% of nematodes (2430 ± 350) in these pieces were females, while other stages constituted 10% (870 ± 70) (Figure 3c).

When we experimented with *C. elegans*, the method was equally successful and approximately 2410 ± 296 eggs (62%) were retrieved from a single NGM agar chunk after 72 h. We also estimated that 28% of the nematodes (1110 ± 183) in these pieces were adults, while other stages constituted 10% (370 ± 14) (Figure 3f). Moreover, form the total isolated eggs of *C. elegans* this technique managed to isolate 75% of the worms in L1 stage (1800 ± 45) from J2 stage eggs in 6 h of incubation at room temperature in M9 buffer (Figure 2). Ultimately, this simple scheme can be used to improve eggs and juvenile isolation efficacy in a short period of time. Furthermore, the method helps to remove any eggs that hatch instantaneously or after a period of 6 h (which we standardized). After 6 h of incubation, most of the eggs in late embryogenesis hatched, while the eggs in early or mid-embryogenesis were further incubated for 24 h to obtain juveniles that were in the same developmental stage.

We found that isolated eggs carry over bacterial culture. For example, isolated eggs of *C. elegans* carried 6.8 ± 1.4 × 10^2 cells/mL of *E. coli* OP50 as a food source of *C. elegans*. To remove the bacterial cells from the eggs, isolated eggs were treated with 1% bleach for 90 s and then washed with sterile M9 buffer. Bleach treated and untreated samples were diluted as required and plated onto Luria-Bertani agar plates. After overnight incubation at 37 °C, colony-forming units were enumerated. We found that 1% bleach treatment for a

short time killed all attached *E. coli* OP50 cells (data not shown). Also, there was no effect of bleach on egg hatching as four times more bleach is used in conventional synchronization procedures for *C. elegans* [11]. However, bacterial contamination of *C. elegans* plates is a critical problem. In general, it is quite impossible to control contamination. However, the use of chuck can reduce the potential source of contamination.

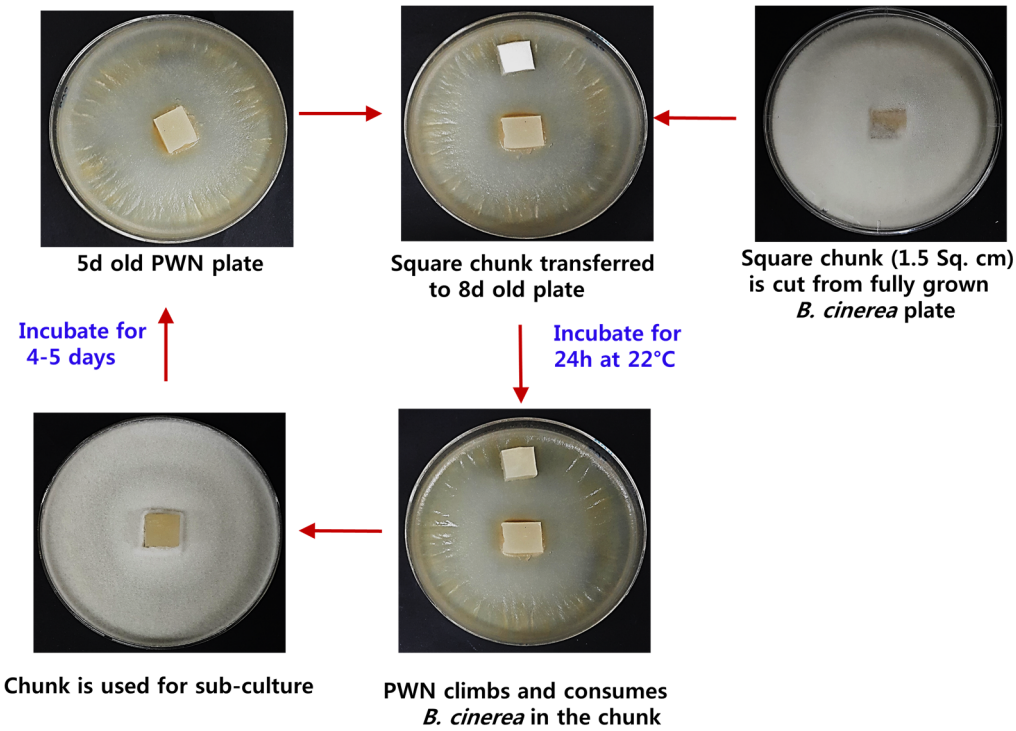

Figure 1. A simple procedure for subculturing the pinewood nematode, *Bursaphelenchus xylophilus* by *Botrytis cinerea* lawn on potato dextrose agar (PDA).

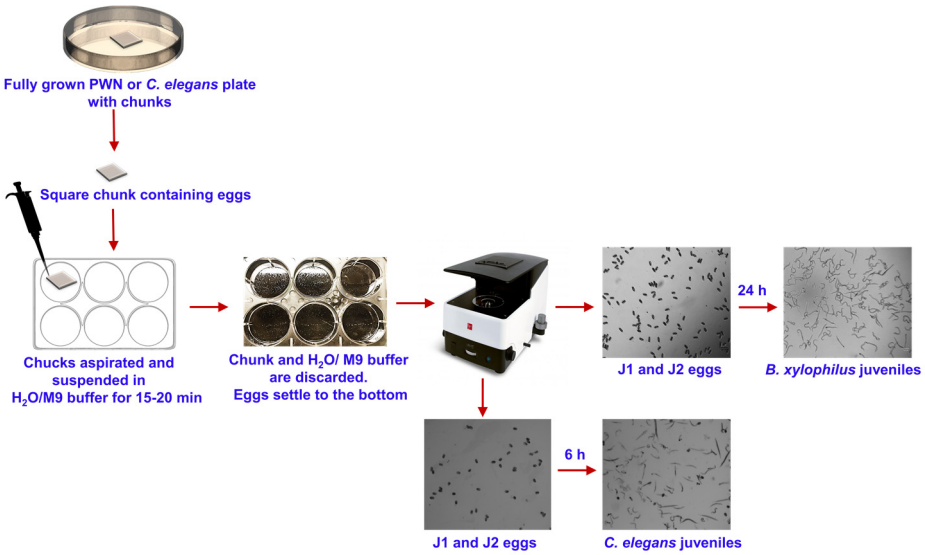

Figure 2. Pictorial representation of technique used to isolate nematode eggs and juveniles from small chunks of *B. cinerea* lawns on PDA agar or *Escherichia coli* OP50 lawns on nematode growth medium (NGM) agar.

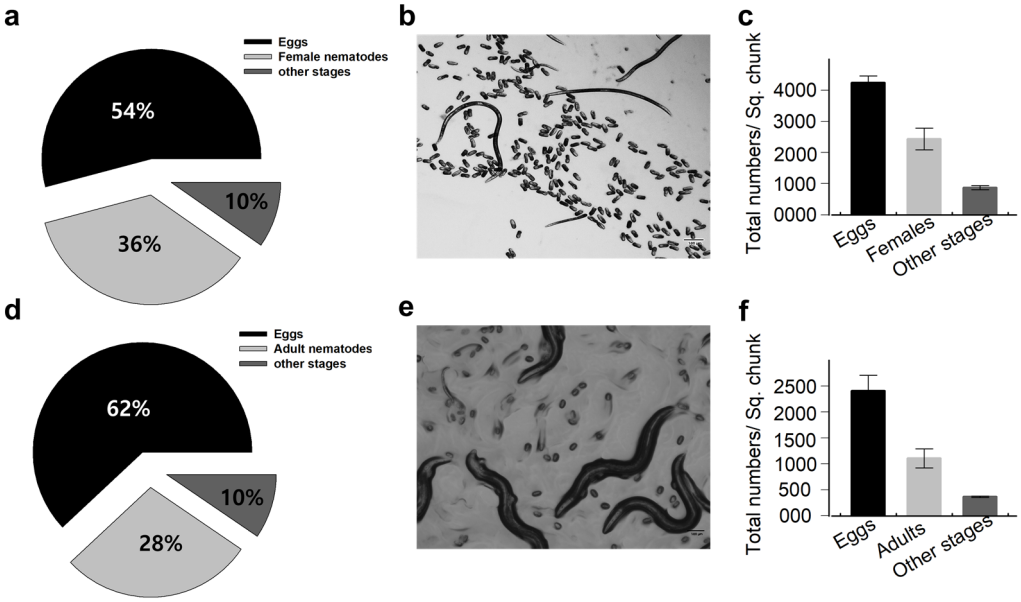

Figure 3. *B. xylophilus* egg counts in fungal lawns and of *Caenorhabditis elegans* [*fer-15*(*b26*); *fem-1*(*hc17*)] egg counts in bacterial lawn. (**a**) Pie chart showing the percentage of (**a**) *B. xylophilus*; (**d**) *C. elegans* adults, eggs, and other stages. (**b**) Representative microscopic image of *B. xylophilus*; (**e**) *C. elegans* eggs with few nematodes that settled at the bottom of the microtiter plate. (**c**) Estimate of the *B. xylophilus*; (**f**) *C. elegans* eggs, and nematode stages isolated by the current method.

Most researchers labor to separate nematodes and eggs and even then, egg counts vary as it is difficult to determine the numbers of eggs and embryonic stage nematodes during isolation steps. For instance, eggs in the J2 developmental stage might hatch before J1 stage eggs, which reduces egg counts during isolation. Several methods such as the Baermann technique have been adopted to achieve this separation process [19–21], but they did not address the aforementioned criterion.

Bioassays with nematodes is the primary method for screening the toxicity of drugs and worm-based survival assays [22–24]. *C. elegans* is considered an alternative to animal models, is portrayed a model organism in studies on molecular biology, genetics and the toxicities of drugs and chemicals [25–27]. For these assays with *C. elegans*, there is an increasing demand for procedures with rapid synchronization and isolation of eggs. Synchronized worms that were obtained by conventional procedures are generally not fast enough and also include use of higher amounts of harmful chemicals such liquid bleach (sodium hypochlorite) and caustic soda (sodium hydroxide). In contrast, our method is relatively straightforward, rapid, eco-friendly, and efficient with less use of chemicals. It is also based on the simple concept that gravid female nematodes (*B. xylophilus*) can be provoked to lay eggs by providing additional feed. We believe the devised method can be employed to isolate the eggs of other nematodes. We hope that the egg isolation technique described will aid researchers working with pinewood nematodes by reducing the amount of time required for experimental assays.

4. Conclusions

Agriculturalist and nematologists use nematodes as in vivo models. Although it is feasible to maintain nematodes in any nematology laboratory, it is quite tedious to achieve synchronization of nematode stages. We propose a chemical-free method for isolating eggs by supplying fresh feeds (as square chunks) to nematodes that linger on a depleted food source, thus provoking the active nematodes to crawl up and lay eggs. The method is simple and curtails the time and labor required by conventional procedures.

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