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A method to obtain disinfected *Globodera* infective juveniles directly from cysts

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Summary – *In vitro* inoculation methods are an efficient tool for the accurate study of the interaction between plants and parasitic nematodes. Obtaining disinfected nematodes is a crucial step in most of these methods. Most disinfection protocols involve surface sterilization of hatched juveniles, a procedure that usually leads to high mortality. This paper describes a new protocol for fast, inexpensive and easy disinfection of nematodes of the genus *Globodera,* directly from cysts. Mortality is low and varies between 10 and 40 % at the most. The surface-sterilized juveniles successfully infected *in vitro*-grown potato roots and reproduced on them.

Résumé – ***Une méthode pour l'obtention de juvéniles de* Globodera *désinfectés à partir de kystes*** – Les systèmes d'inoculation *in vitro* sont des outils performants et précis pour l'étude des interactions plantes-nématodes. L'obtention de juvéniles stériles est une étape cruciale pour la plupart de ces systèmes. La majorité des protocoles publiés comprennent une désinfection des juvéniles, ce qui conduit à une mortalité élevée. Nous décrivons ici une nouvelle méthode pour désinfecter, rapidement, facilement, et à faible coût des nématodes du genre *Globodera,* en partant de kystes. La mortalité des juvéniles désinfectés est faible (entre 10 et 40 % au maximum). Les juvéniles stérilisés infestent les racines de pomme de terre cultivées *in vitro* et s'y développent normalement.

Key-words : axenic, cysts, *Globodera,* hatching, juveniles, nematodes.

The study of plant-nematode interactions is greatly facilitated by the use of controlled inoculation and culture methods. They allow synchronized infections, accurate observations of early stages and greater experimental precision than greenhouse experiments. For several reasons *in vitro* inoculation must be made with disinfected nematodes. First, bacterial and fungal contamination prohibit the preservation of the cultures for an extended period. Second, contaminating microorganisms prevent the accurate study of strictly plant-nematodes interactions. Third, when studying the expression of plant genes upon nematode infection, using for example the bacterial reporter gene ß-glucuronidase *(gus),* contamination with microorganisms that possess endogenous GUS activity may give false results.

Successful disinfection of cyst nematode species such as *Heterodera* and *Globodera* has been obtained using various chemical disinfecting solutions and different types of sterilizing equipment, as reviewed by Zuckerman (1971). Some of these methods have been improved (Johnson & Viglierchio, 1969; Müller, 1978; Brodie & Spivey, 1988; Sanft & Wyss, 1990; Kumar & Forrest, 1990).

In all of these procedures, the surface sterilization is performed on hatched second-stage juveniles, preceded or not by surface sterilization of the cysts. These methods are usually long and rather complex and the mortality associated with direct juvenile disinfection is generally high. This article describes an efficient, inexpensive, and easy protocol for obtaining surface-sterile infective juveniles by direct disinfection of cysts.

Methods and results

Cysts isolated from infected soil samples were surface sterilized in a modified syringe, similar to the one described by Forrest (1986), but larger (20 ml) and with a 30-µm nylon mesh. This mesh retains *Globodera pallida* cysts and eggs, but allows the juveniles to pass through after hatching. The modified syringe is constructed by cutting off about 1 cm of the top of a standard 20 ml plastic syringe and heat-welding a 30 µm nylon mesh onto the cut surface. After checking the connection and removing the excess of mesh, the modified syringe and its plunger are autoclaved at 120 °C for 20 min.

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Approximately 30 cysts of *Globodera pallida,* preserved dry at 4 °C, are collected and put into the syringe. The plunger is inserted and the surface sterilization process starts (Fig. 1). All manipulations take place in a sterile laminar flow bench and using sterile materials. Ethanol (90 %) is drawn into the syringe and the suspension is gently mixed by inverting the syringe for 15 s. Immediately after expelling the ethanol by depressing the plunger, a 1.3 % NaOCl aqueous solution (Janssens Chimica, Geel, Belgium) is drawn up and again gently mixed until the cysts have bleached completely and start to open. Depending on the origin and age of the cysts, the bleaching time required to obtain this stage is between 7 and 9 min. When using clean cyst batches, lower disinfecting times (3 to 7 min) are often sufficient to obtain disinfected juveniles. For cysts containing many fungal spores it is necessary to treat with NaOCl until they start to break open, allowing the sterilizing fluid to pass more thoroughly between the eggs of the cysts to reach the fungal spores. Longer disinfecting times result in higher mortality of the juveniles. After treatment with NaOCl, the cysts are rinsed three times with sterile water for 2 min. After expelling the last rinse solution, the lower 1.5 cm of the modified syringe is cut off using a hot scalpel and placed in a sterile 8 cm diameter hourglass (Duran® glass, Schott Glaswerke, Mainz, Germany), which is in turn placed in a Petri dish (100 × 20 mm; Falcon®, Becton Dickinson, Plymouth, England) (Fig. 1). The cut part of the syringe acts as a sieve in which the nematodes can rehydrate and hatch. For this purpose, the hourglass is filled with 5 ml of sterile water, the Petri dish is closed, sealed with Parafilm® " M " (American National Can™, Greenwich, CT, USA) and incubated at 20 °C in darkness for 3 days. The sieve is then transferred to another sterile hourglass, containing 5 ml of filter-sterilized potato root diffusate (PRD), using a 0.2 µm minisart filter (Sartorius, Göttingen, Germany), and incubated at 20 °C in darkness. The hatched juveniles move through the mesh and concentrate at the bottom of the hourglass. By transferring the sieve to new hourglasses with PRD every 3 days, separate hatches can be harvested.

Mortality was estimated by counting the number of dead juveniles in samples of 100 juveniles. Depending on the origin of the nematodes and the sterilizing conditions used, mortality varied from approximately 10 to 40 %. The majority of the dead juveniles were collected in the hourglass containing the rehydration fluid. Later hatches in PRD contained more than 90 % viable juveniles. Contamination of the purified juveniles was checked by transferring four drops with approximately 100 nematodes/drop using a sterile pipette to a Petri dish containing Yeast Extract Agar. After a 4-day incubation period at 23 °C, no bacterial or fungal development could be observed. By adjusting the bleaching time between 3 and 9 min, the disinfection protocol was successfully used with cysts from different origins and ages. The harvested nematodes were kept at 4 °C for several days and, after checking for residual contamination, drops of 5-10 µl containing 20 active juveniles were transferred using a sterile pipette to young roots of

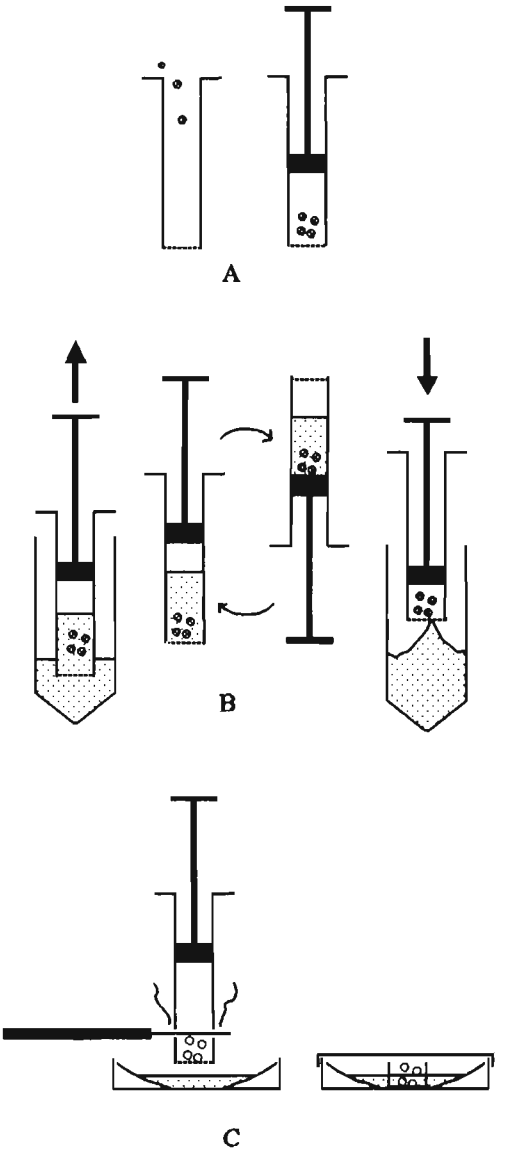

Fig. 1. *Cyst-nematode disinfection protocol. A : The cysts are put in the autoclaved syringe and the plunger is reinserted; B : A 90 % ethanol solution is applied to the cysts and the modified syringe is gently inverted for 15 s. The ethanol is expelled and the procedure is repeated using a 1.3 % NaOCl solution for 3 to 9 min. The NaOCl solution is expelled and the cysts are rinsed three times with sterile water; C : The lower part of the syringe is cut off using a hot scalpel, then placed in sterile water, and later in sterile potato root exudate.*

an *in vitro* potato culture to check the infecting capacity of the juveniles. The development of the nematodes within this monoxenic system was assayed microscopically. The infection of *in vitro* roots was successful in all cases. Ten to 50 % of the inoculated juveniles developed into brown cysts after 8 to 10 weeks.

Discussion

The advantages of this protocol are its speed, its technical simplicity and its efficiency. No hatching of the juveniles is required before the disinfection procedure. The hatching of the nematodes after disinfection is rapid. The NaOCl treatment may accelerate rehydration and hatching of the juveniles; they already hatch substantially during the rehydration of the eggs in water. Although this first hatch within the rehydration solution contains a large amount of dead juveniles, viable juveniles are also obtained. The subsequent hatches all contain mostly viable and infective juveniles. After 2 to 3 days in the potato root diffusate, i.e., 5 to 6 days after the disinfection, massive hatching occurs, giving rise to many viable infective juveniles. Without this disinfection procedure, the first hatched juveniles can only be harvested approximately 10 days after the start of the rehydration. The protocol is also simple and inexpensive : little material is needed and common laboratory chemicals are used. No complicated filtering or hatching apparatus is needed. The nematodes can be collected in a simple way with no losses due to manipulations or transfer of the nematodes; a single recipient is used for disinfection and hatching.

The success of the disinfection is mainly due to the opening of the cysts during the surface sterilization. The cyst wall is chemically ruptured by NaOCl after 5 to 9 min. This increases the contact between the NaOCl and the eggs and also facilitates the later hatching of the juveniles. No mechanical opening is needed. To prevent losses when the cysts are opening, a mesh aperture size of 30 µm, through which the juveniles, but not the eggs can pass, was chosen. This protocol was most successful when recent cyst batches were used. This most likely resulted from the higher viability of the juveniles. The flexibility of the system which can be used with a range of different disinfection times can, however, make the protocol useful for older cyst batches as well. The higher level of mortality in that case (40 %) is acceptable as it can be interpreted as the result of a selection against less viable or less infectious juveniles (Sanft & Wyss, 1990).

Nematode juveniles within cysts are dehydrated survival structures and in that respect they can be compared to plant seeds. Our protocol was designed in analogy to standard seed surface sterilization protocols. Like plant seeds, juveniles inside cysts show a high degree of resistance to bleach, probably because of their dehydrated state. Hatched juveniles are much more sensitive to bleach, a treatment of 1 min with a 1.3 % NaOCl solution results in 90 to 95 % mortality (Niebel & Mugniéry, unpubl.). Additionally the damage to external sensory organs, such as the amphids of the nematodes, is probably higher when disinfecting juveniles instead of eggs. Damage to these organs probably results in a decreased detection and recognition of root infection sites. Because this protocol presents a disinfection procedure for dehydrated structures it can probably be used not only for *Globodera pallida* but also for many other cyst nematodes, which can survive desiccation as cysts.

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