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A review: Cultivation of plant-parasitic nematodes: General principles and methods

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Abstract
The ability to culture organisms is always of great benefit to their scientific study, and sometimes simply essential. Cultures ensure that a steady supply of live material is available for teaching and research, eliminating the need for repeated sampling of inaccessible or temporally fluctuating habitats in search of living specimens. Cultures also provide all stages of development for observation, demonstration and experiments and allow for more standardized methods and for easier corroboration.

Keywords: Nematode, Culture, Xenic, Monoxenic, *In vitro* culture

Introduction
A wide range of nematode species can be maintained in plant pots in the greenhouse of lath house and some plant-parasitic nematodes (PPNs) can also be cultured using more artificial in *vitro* systems [1]. Depending on the host-parasite relationships, several approaches should be considered for establishing plant-parasitic nematode cultures. As obligate parasites, they must feed on host cells for development and reproduction. So far, no successful continuous axenic cultures of PPNs have been developed, and the cultivation of these nematodes therefore depends directly on the nature of the host cells and their possible modes of maintenance in a culture system.

Collecting live material
From field populations
To establish seed cultures, soil samples containing the desired nematode species must be collected from rhizospheres in natural sites or agricultural fields. It is obviously of crucial importance to determine as accurately as possible to which plant the sampled rhizospheres belong and it may be necessary to collect these plants, or their fruits or seeds, for maintenance in the greenhouse. In the laboratory, a portion of the collected soil should than be processed and extracted to determine the nematode diversity and population density. If present in sufficient numbers, the target species can be picked out from the extract and transferred directly to seed culture systems. If present in low numbers, attempts can be made to inoculate pots with a probable host plant, in the hope that populations of the target species will eventually build up to useful numbers.

From culture collections
Perhaps even more so than for any other group of nematodes, numerous plant-parasitic species are already being cultured in laboratories around the world. Depending on your needs, it may be much more convenient to obtain an established culture from colleagues than to have to start one up yourself, all the more so because those colleagues will be able to advise you extensively on the appropriate culture conditions. Nevertheless, care must be taken to comply with quarantine regulations and to avoid turning your own laboratory into a bridgehead for invading plant pests.

Quarantine and clean handling procedures
If you wish to culture economically important plant parasites that are not yet omnipresent in your area, then you should make a maximum effort to contain those cultures and avoid accidental release of the nematodes into the environment. This requires a number of facilities [2], such as:

a. Hot water available at all sinks in your laboratory. Rinsing all equipment thoroughly with hot water (preferably above 65°C, or even higher when dealing with species from xeric habitats) in a plugged sink will kill nematodes that would otherwise be flushed down the drain alive and well.
b. A collection tank receiving water from the extraction sink. This water must be treated chemically or thermally to kill all organisms released during extraction.
c. A big autoclave for sterilizing the remainders of discarded soil samples, extracted soil and plant material.
d. Restricted access to quarantine cultures, for example; by keeping doors to laboratories, cupboards, and containers locked and clearly labelled as quarantine areas.

Xenic and monoxenic soil-based culture systems
Ectoparasitic plant parasites do not penetrate the host roots but feed externally, moving from feeding site to feeding site. Only the stylet is inserted into one or several layers of the root tissue. Most ectoparasites have the ability to feed on multiple host-plant species, for example; they are polyphagous. Also, they interact directly with other micro-organisms in the soil environment and are strongly influenced by soil factors such as temperature, pH, moisture content, host requirements, etc., which may play an important role in the establishment of certain species [3]. In contrast, nematodes living and feeding inside the root are more specialized in terms of host specificity, feeding mechanisms, and complexity of the host-parasite relationship. In some of the most important groups of such endoparasites, the infective stage penetrates the host tissue, establishes a feeding site, and permanently (in the case of females) or temporarily (in the case of males) becomes sedentary [4].

While inside the root, endoparasites are better protected against fluctuations in soil conditions than ectoparasites, and it has been suggested that in-*vitro* cultures of the latter are harder to maintain because they are more dependent on biotic and physico-chemical conditions [5]. This may explain why for instance Belonolaimus longicaudatus has proven quite difficult to culture under in-*vitro* conditions [6]. Soil texture may affect the development of some ectoparasitic nematodes in different ways [7]. We have observed that mixing an original soil sample with an artificially created soil often results in loss of the target species, apparently due to dilution or alteration of some unknown soil factor. However, in other instances, such a mixture seems not to affect the increase of target nematode populations.

Plots and microplots

Small plots or concrete containers in the open air can provide a convenient intermediate between collecting samples in the field versus maintaining cultures in-*vitro*. Such systems are obviously much more exposed to the local weather and other uncontrollable influences and will evidently work better with local nematode species than with exotic ones. However, they also provide the nematodes with a large quantity of more or less natural soil, a broad range of organisms and a presumably stable network of well-balanced ecological interactions between these. As such, they are generally agnotobiotic.

The partitions of adjacent plots or walls of neighbouring containers should be raised above the soil surface, and must reach sufficient depth underground, in order to minimize cross-contamination between adjacent nematode population. The appropriate depth depends very much on the rhizosphere dimensions of the host plants and the vertical movements of the target nematodes but we would recommend that partitions or walls should reach at least 1.5 m depth. Also, at the bottom edge of the partitions each plot is preferably provided with a grille or strong mesh as well as a layer of gravel, both acting as barriers against passage of roots, rodents, nematodes, etc.

After initial establishment of host plants and target nematodes, much less maintenance and supervision is required than in greenhouse or laboratory cultures. Nevertheless, the plots should be weeded, protected to some degree against carry-over of other nematode species, and replanted with host plants when necessary. They should also be screened at least seasonally to monitor fluctuations in the density of the target species and to verify that it is indeed permanently established. Such screening must consist of some form of soil extraction and is therefore in itself more time-consuming than for most in-*vitro* systems but the extract can often serve other purposes as well, for example, in preparation of teaching and demonstration material.

Cultures in pots and greenhouses

Step 1: Compared to the open field or microplots, greenhouses often provide more controlled conditions that are also more advantageous for optimal growth of both host and parasite. If the host plant can be maintained in the greenhouse, then pot cultures represent a simple and inexpensive way of maintaining live PPNs for considerable periods of the. Irrigation of plants growing under these conditions is very critical, and may require the installation of an automatic irrigation system, consisting of microsprinklers or emitters that dispense water at selected time intervals according to the specific needs of the plants.

As a relatively natural system, pot cultures can be used both as seed and stock cultures and in the latter case they should be inspected routinely to determine the population fluctuations. Water schedules have to be designed according to water quality and quantity requirements, which have to be determined for each combination of plant and nematode species. Some species require high levels of moisture while others are sensitive to salts or to other minerals present in the water. As a rule, avoid drastic fluctuations since these may affect nematodes and plants to a lethal degree.

The pots should be weeded regularly, since weeds may adversely affect both the host plant and its parasites. The greenhouse floor, if not completely covered with concrete or another hardened surface, should also be kept weed-free as the presence of weeds will allow the uncontrolled establishment and build-up of nematodes (both those that have escaped from pots as well as those that may enter the greenhouse environment through soil or accidental introduction) which may subsequently contaminate pots. Great care should also be taken to avoid cross-contamination between pots (unless nursery conditions are to be replicated for experimental purposes) [8]. Especially during automated or manual watering, for example; by putting all planted pots on benches with surfaces from which any spilled water drains away immediately.

A few clean host plants in fresh pots should be kept at hand, ready for inoculation with nematodes from old cultures in the event of the latter's decline. Individual pot cultures may last for many years, but unknown factors or accidents, for example; failure of the temperature regulation of the greenhouse, omissions in the watering schedule, inappropriate use of pesticides inside the greenhouse, can never be excluded and losses of individual cultures of whole strains will inevitably occur. If permanent survival of strain is critical to your work, then it is as always best to use several culture systems and to spread the risk over several culture series.

Step 2: Temperature fluctuations within and between pots in an experimental series should be kept at a minimum. This can be achieved by submerging them partly in a large thermo-regulated water bath or temperature tank. Water is maintained in constant circulation by a water pump to homogenize the temperature across the tank.

Step 3: Leaf parasites can be maintained by inoculation of nematodes in suspension onto the leaves of suitable hosts grown in pots.

Step 4: The pot soil itself can be sterilized with steam or methyl bromide prior to planting of the host and inoculation with selected microorganisms and nematodes, allowing for gnotobiotic micro or mesocosm studies of ecological interactions in simplified soil ecosystems [9]. Depending on the degree of containment required, ordinary plant pots can be replaced by containers that effectively seal off the soil from external influences and contaminants and sterile diluted medium can be used for watering the host plant. If only a moderate degree of containment is required, regular tap water can be used after filtration on filters and sieves appropriate for removal of bacteria, fungi, nematodes and other organisms.

Step 5: Greenhouses are also well-suited for maintenance and containment of cultures of quarantine organisms [2], provided access is strictly controlled and quarantine procedures are rigorously adhered to.

Step 6: Baujard [10] developed a miniaturized system pf pots actually PVC tubes, inserted through a grid of circular openings in an otherwise closed incubator, which was equipped with a thermostat and two light bulbs to control air temperature around the PVC tubes.

Step 7: Another approach to increased control consists of using soilless substrates; combinations of various mineral, synthetic and organic substances can be used in pot cultures of some migratory endoparasitic nematodes.

In *Vitro* culture systems

During the last few decades, in *vitro* culturing of PPNs on plants or plant tissues has become a common technique. Various procedures for obtaining and maintaining gnotobiotic cultures of endoparasitic nematodes are available in the literature [11,12,13]. We will provide the most practical techniques available and focus on some of the more recent information. It should be noted that no axenic culture technique has so far been developed for Tylenchina. Part of the problem appears to be that the economically important species do not initiate feeding behaviour until they detect contact with plant roots or plant cell walls.

In *vitro* aseptic dual cultures with entire plants

Step 1: Certain plants can be fitted inside an in *vitro* system in their entirety. Monoxenic cultures of several PPNs have been successfully established on the cruciferous plant, Arabidopsis thaliana. Because of its small genome size and other agronomic features, this plant is an effective model host for studies of plant/nematode interactions. Cultures of various endo- and ectoparasites have been established on A. thaliana in order to study life cycle and plant resistance [14,12].

Step 2: Soilless plastic pouches for seed germination have been used widely to obtain pure cultures of PPNs [15]. The bags offer several advantages; they minimize space usage, are handy for direct observations of host-parasite interactions, and the obtained nematode specimens or plant tissues are ideal for ultrastructural observations (TEM/SEM) because materials are free of soil particles and other solid contaminants, thus avoiding optical obstruction of important structures under SEM and preventing knife damage when sectioning specimens for TEM observations.

Surface-sterilized seeds are germinated in the pouches. Once the roots have developed, surface-sterilized second-stage juveniles are inoculated in the pouches, which are maintained in a growth chamber at light and temperature settings as appropriate for the plant and nematode species. The plants in the pouches need to be systematically watered with a nutrient solution and care must be taken to avoid contamination by bacteria or fungi. Several sedentary endoparasites, including Meloidogyne spp., Globodera spp., Nacobbus aberrans, have been successfully cultured in seed-pack growth pouches [16,17]. So far, attempts at culturing the ectoparasite Belonolaimus longicaudatus on corn roots with this technique have not provided positive results.

In *vitro* aseptic dual cultures with excised plant tissues

Step 1: Excised roots of various plant species can be established under axenic conditions in Petri plates or flasks with media containing nutrients and other chemical compounds necessary to sustain plant development. Agar is the most commonly used substrate, in combination with different media. Other gelling agents have been tested with Gellan gum [18]. The choice of a medium is not only important with respect to essential elements provided for plant development, but also affects the nematodes infection rate [6]. This type of system allows manipulation of many components of the culture environment that are more difficult to control under field conditions or in pots. According to host specificity, nematodes are surface-sterilized with different types of disinfectants and including antibiotics and other compounds.

Step 2: Several migratory endoparasitic nematodes can be cultured and maintained in sealed Petri plates with carrot discs. This is particularly common practice for cultivation of burrowing nematodes, Radopholus spp., and lesion nematodes, Pratylenchus spp. [19].

Step 3: Huettel [20] provided an updated review of nematode cultivation on plant callus tissue. Carrot callus tissue allows efficient culture of migratory endoparasites such as Pratylenchus brachyurus, P. agilis, P. scribneri and Radopholus similis. The carrot callus procedure yields high numbers of nematodes and reduces the need for additional sterilization during continuous maintenance of the cultures [21]. Alfalfa callus tissue can also be used for at least some nematodes.

Conclusion

There is ample scope for combining steps of different approaches and the possibilities for developing new protocols are still wide open. New technology and novel approaches will undoubtedly play an important role in simplifying and optimizing culture methods and we expect that many nematode species will be brought into culture in the years to come. However, it remains an open question whether or not these species will mostly belong to nematode families that are already well represented in culture collections.

I therefore hope that my review will both stimulate the development of novel rearing techniques for previously non-culturable species, as well as contributing to the increasingly widespread cultivation of the great diversity of more amenable nematodes.

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