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NEW EXTRACTION METHOD OF ENTOMOPATHOGENIC NEMATODES USING THE BASIC FLOTAC AND THE MINI-FLOTAC TECHNIQUES

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El Khoury Y., Salvemini C., Tarasco E., Cringoli G. - New extraction method of entomopathogenic nematodes using the basic FLOTAC and the Mini-FLOTAC techniques.

The FLOTAC basic technique is a recognised method used mainly in the veterinary field to extract parasites from the faeces of animals found in the stomach and intestine. This study is the first application of the FLOTAC and Mini-FLOTAC techniques to extract infective juveniles of entomopathogenic nematodes from infested *Galleria mellonella*. The EPN used for this purpose was an endemic Italian nematode belonging to the Rhabitidae family *Steinernema carpocapsae*. *Galleria mellonella* larvae were infected and extraction was performed a few days after infection using both techniques. A comparison was then made between the two methods. The results showed that infective juveniles could be extracted from the infected larva as early as three days after infection. The difference between the FLOTAC basic technique and the Mini-FLOTAC technique was not significant in the extraction of entomopathogenic nematodes. Due to its sensitivity and operating easiness the Mini-FLOTAC technique is a promising method for the extraction of entomopathogenic nematodes. It allows faster extraction of nematodes from the inhabited soil and insect host than the conventional White trap method.

Key Words*:* Microbial biocontrol, Entomopathogenic nematodes, Nematode extraction, FLOTAC, Mini-FLOTAC.

INTRODUCTION

Nematodes are animals that live in a wide range of environments including soil and fresh and salt water. There are species of nematodes that feed on fungi, bacteria, protozoans, other nematodes, and plants, and can also parasitize insects, humans, and animals. A 10% of known nematodes are parasites of plants causing severe yield losses and thus economic damage. Another small portion corresponds to parasites of insects. Entomopathogenic nematodes (EPNs) are obligate parasites of insects; they complete their cycle at the expense of the insects they parasitize (Poinar, 1990). Entomopathogenic nematode infective juveniles IJs penetrate the insect body through natural openings or through the release of hystolytic enzymes (Ishibashi and Kondo, 1990; Peters and Ehlers, 1994). They are symbiotically associated with *Xenorhabdus* or *Photorhabdus* bacteria, which are responsible for host death by bacterial septicemia within 48-72 hours (Kaya and Gaugler, 1993; Boemare, 2002; White, 2019). Steinernematidae Filipjev, 1934 and Heterorhabditidae Poinar, 1976 (order Rhabditida) are the major genera of EPNs that are considered great biological control agents against agricultural pests and are widely used as part of an integrated pest management approach (Georgis, 1992; Gaugler *et al*., 2002; Lacey *et al*., 2015). In order to collect, identify, and study native EPNs, numerous surveys and soil investigations have been conducted worldwide (Stock *et al*., 2008; Tarasco *et al*., 2009; Noujeim *et al*., 2011; Tarasco *et al*., 2015; Abdel-Razek *et al*., 2018). Indigenous EPNs populations have received increasing attention mainly because of their beneficial characteristics such as better adaptation to local biotic and abiotic conditions (Ulu and Susurluk, 2014; El Khoury *et al*., 2018). The most popular method for extracting EPNs from soil is the 'galleria bait method' developed by Bedding and Akhurst (1975). This technique uses the last instar stage larvae of the greater wax moth *Galleria mellonella* Linnaeus, 1758, (Lepidoptera, Pyralidae), which attracts the free-living 3rd stage infective juveniles (IJs). The dead larvae are then transferred to a White trap (White, 1927) at 25± 2 °C and IJs emerge after 7 to 15 days.

Expanding the library of EPN species or strains to achieve an adequate match of nematodes with target pests is one of the major challenges in EPN use (Abd-Elgawad, 2021). It is difficult to generalise the results of a specific case study because the EPN samples are very diverse. However, it is clear that only 5-34% of samples test positive for EPN in various general surveys worldwide (Hatting *et al*., 2009; Khatri-Chhetri *et al*., 2010; Valadas *et al*., 2014; Stock, 2015; Tarasco *et al*., 2015; Hussaini, 2017). It needs to be increased immediately to improve the match between EPN and host and perhaps introduce new strains. Such an approach is based on the fusion of a few elements, namely time and a good sampling technique, targeted site selection, and the use of multiple extraction techniques (Abd-Elgawad, 2020). Hence, extraction techniques should be evaluated and developed to use these criteria or other novel ideas to maximise EPN sampling and recovery frequencies. The extraction process must be highly sensitive, accurate, and

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easily reproducible to rapidly diagnose the presence of nematodes (McSorley and Walter, 1991).

FLOTAC technique is an effective extraction technique for human and animal parasites (Cringoli *et al.*, 2017; Troccoli *et al.*, 2022). It was developed by Cringoli *et al.*, (2010) for the rapid detection and counting of parasitic elements such as cysts, oocysts, eggs, and larvae in animal and human faeces. There are two devices that can be used for this technique: the basic FLOTAC and the Mini-FLOTAC. The first consists of a cylindrical device with two 5-ml flotation chambers that is centrifuged. Its efficiency was demonstrated in a recent study by Troccoli *et al.*, (2022) for the extraction of root-knot nematodes *Meloidogyne* spp. from tomato roots and soil samples. The second consists of a cylindrical device with two 1-ml flotation chambers and does not require centrifugation. Therefore, the purpose of our study is to verify the possible use of FLOTAC techniques in the extraction of IJs and adult stages of EPN from previously infested insect larvae.

MATERIALS AND METHODS

Entomopathogenic nematodes population

An indigenous strain of the entomopathogenic nematode EPN *Steinernema carpocapsae*, Weiser, 1955, was collected using the "Galleria baiting technique" (Bedding and Akhurst, 1975) during a soil survey in different habitats in Italy (Tarasco *et al.*, 2015). It was previously identified morphometrically and molecularly and stored in the Entomology Laboratory of the Department of Soil, Plant and Food Sciences of the University of Bari Aldo Moro, Bari, Italy. To obtain fresh infective juveniles (IJs), nematodes were inoculated into *Galleria mellonella* larvae at the last instar stage at a temperature of 22±2 °C on a 100 x 10 mm Petri dish with a 90 mm filter paper treated with 2,000 IJs in 1.5 ml of water, as described by Tarasco *et al.*, (2015). Dead larvae in the last larval stage were put on modified White traps (White, 1927); juveniles hatched after 10-15 days from *G. mellonella* carcasses were collected and stored in tap water at 13 °C to be used in the experiments.

Infestation of *Galleria mellonella* larva

The infective juveniles IJs of *S. carpocapsae* were counted and the concentration of the solution was adjusted to 1000IJs/ml. For each strain, Petri dishes (60 mm) with filter paper were inoculated with 500 IJs in 0.5 ml of water each. Each Petri dish contained one larva of *G. mellonella* in the last instar stage. The presence and infectivity of EPN was assessed daily until day 3 post-treatment. Twelve replicates were considered, one larva per replicate.

NEMATODES EXTRACTION FROM LARVA

Extraction by the FLOTAC basic technique (FBT)

Dead larvae were weighed (approx. 0.5 g each) and washed thoroughly using 6% sodium hypochlorite water solution to remove any possible nematode attached on the external part of the carcass. Clean larvae were placed in the conical collector of the fill-FLOTAC device filled with 15 ml of tap water (dilution ratio 1:30) and smashed for 3 minutes (Fig. I, A). The suspension was then transferred into an Eppendorf tube and was centrifuged for 3 min at 170 g (1500 rpm). The formed supernatant was discarded leaving the pellet, and the tube was refilled with 15 ml of magnesium sulphate solution (1.165 specific gravity). The suspensions were thoroughly homogenised and the two flotation chambers of the FLOTAC apparatus (5 ml each) were filled. The FLOTAC apparatus was centrifuged for 5 min at 120 g (1,000 rpm) at room temperature, and examined under the optical microscope after a clockwise turn (translation) of the upper reading disc (Fig. I, B) (Cringoli *et al.*, 2010).

Extraction by the modified Mini-FLOTAC technique

To prepare the larva and the dilution for the Mini-FLOTAC technique, the procedure followed was the same as for the basic FLOTAC technique described above. Following the first centrifugation, the supernatant was discarded leaving only the pellet, and the Eppendorf tube was refilled with 15 ml of magnesium sulphate solution (1.165 specific gravity). The suspension was thoroughly homogenised and the two flotation chambers of the Mini-FLOTAC apparatus (1 ml each) were filled. After ten minutes, the nematodes floated in the apparatus and were counted under an optical microscope (Cringoli *et al.*, 2010, 2017).

Statistics

Data from the two series of experimental runs of the extraction methods were statistically analysed and means were compared by the student's t test (P=0.05). Statistical analysis was performed using SPSS Version 11.0 statistical software package.

RESULTS

The accuracy and sensitivity of the Basic FLOTAC and the Mini-FLOTAC techniques were evaluated. The overall results showed that entomopathogenic nematodes were detected in 100% (12/12) of the replicates analysed in both techniques employed. The results of the nematodes extracted by both techniques were different. In terms of mean numbers of nematodes extracted, *S. carpocap-*

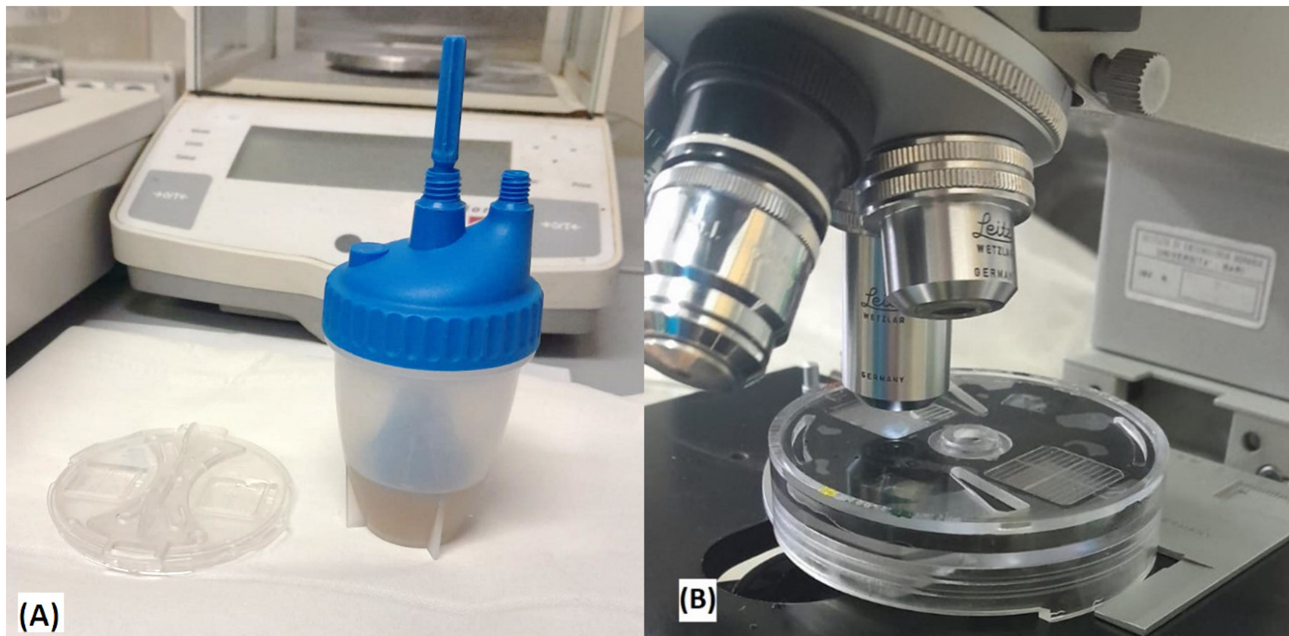

Fig. I - Mini-FLOTAC and Fill-FLOTAC apparatus (A), FLOTAC apparatus under the microscope (B)

sae IJs extracted from *G. mellonella* larvae were higher in Mini-FLOTAC than in FLOTAC. However, statistically there were not significant differences between the two extraction techniques (Sig>0.05) (F=1.32 df=22). FLOTAC basic technique extracted a mean of 339 nematodes, ranging from 198 to 948, versus a mean of 447 nematodes extracted by Mini-FLOTAC ranging from 75 to 1095 (Table 1). Values of Standard Deviation (SD) indicated a high variability of data among the replications from both methods.

DISCUSSION AND CONCLUSION

Sensitive and accurate extraction technologies such as the FLOTAC techniques play an important role in ecological studies to detect the presence of EPN populations, and the changes that may occur in the soil nematofauna due to biotic or abiotic disturbances (Troccoli *et al.*, 2022). In addition, the reproducibility of extraction techniques helps to increase the population of EPN species, thus ensuring a greater diversity of potential biocontrol agents. The FLOTAC technique have shown to be an effective method for rapid and reliable diagnosis of infectious and parasitic diseases in humans and animals due to its high sensitivity, precision, and accuracy (Knopp *et al.*, 2011; Levecke *et al.*, 2012; Steinmann *et al.*, 2012; Cringoli *et al.*, 2017; Ramos *et al.*, 2016).

In our experiments, Mini-FLOTAC proved to be more effective than FBT in extracting *S. carpocapsae* from *G. mellonella* larvae, but the difference was not statistically significant. In addition, nematode suspensions extracted with Mini-FLOTAC were cleaner and free of residues compared to those prepared with FBT, allowing for easier microscopic examination. Another advantage of the modified Mini-FLOTAC is that it eliminates the need for a second centrifugation, resulting in a faster operation. Previous parasitology studies using the Mini-FLOTAC have shown that a large number of animal samples can be analysed quickly and reliably, even in the absence of a centrifuge and other basic equipment (Cringoli *et al.*, 2017; Lozano *et al.*, 2021). Our results are not aligned with the results of Lima *et al.*, (2015), where eggs and/or oocysts of gastrointestinal parasites were detected in 63 % and 90 % of samples using Mini-FLOTAC and FLOTAC, respectively. The lower number of nematodes counted in the FLOTAC apparatus may be due to the second centrifugation, during which the present nematodes may have exploded and died. This affected the results in two ways: the number of IJs was reduced and the FLOTAC chambers were less readable because of the debris.

The speed of FLOTAC techniques provides an important advantage over the water trap methods. In the latter, the larvae are in humid conditions at 25± 2 °C for 7-15 days, which expose them to the risk of contamination with fungi or other parasites. Any contamination can prevent the nematodes from completing their biological cycle and escape the *G. mellonella* larva into the water, where they should be collected. The extraction of EPN in a one-day procedure could avoid these complications. Nevertheless, the extraction efficiency of FBT is significantly affected by the type and density of the flotation solution, which varies greatly for sucrose, magnesium sulphate, and zinc sulphate, the most commonly used compounds (Coolen and D'Herde, 1977; Cringoli *et al.*, 2010). FBT is strongly recommended for processing large numbers of soil samples from nematode surveys conducted across extensive geographical areas, where all potentially present EPN must be reliably recognized. However, when large numbers of EPN Infective juveniles are needed, standard extraction procedures are more favourable because they allow large numbers of EPN to multiply and grow after 10-15 days of infection. Both approaches have proven effective and are characterised

Table 1 - Numbers of S. carpocapsae Ijs and adults extracted from 12 larvae replicates samples using FBT and Mini-Flotac

Replicates	IJs g^{-1} larva	
	FLOTAC basic technique	**Mini-FLOTAC technique**
1	189	360
2	309	405
3	276	210
4	216	330
5	948	1095
6	279	345
7	237	660
8	378	285
9	276	480
10	300	585
11	363	555
12	303	75
Mean	339	447
SD	199	262

by high sensitivity, precision, and accuracy in the diagnosis of viral and parasitic diseases in humans and animals (Levecke *et al.*, 2012; Ramos *et al.*, 2016) and more recently in the diagnosis of plant parasitic nematodes (Troccoli *et al*., 2022).

The results of this study suggest that FBT and Mini-FLOTAC, in combination with the Galleria bait method, are two promising extraction techniques to perform large EPN surveys in different environments. They should be investigated to increase the extraction efficiency of beneficial organisms so that they can be researched and employed in integrated pest management. Also, further experiments should be conducted with different flotation solutions and EPN species and strains.

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