Final Report

Project title (Acronym)
Detecting virus-carrying *Xiphinema* spp. as an alternative to *Xiphinema* identification up to species level in trade (XiphiVIR)

Project duration:

Start date:	2020-07-01
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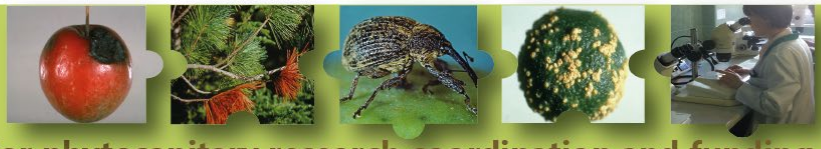
Contents



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2. Short project report

2.1. Executive Summary

Nematodes of the genus *Xiphinema* (dagger nematodes) are migratory root ectoparasites with a broad host range. There are more than 260 species, of which approximately 60 belong to the *Xiphinema americanum* group (*X. americanum* sensu lato). Some of the species can specifically transmit certain nepoviruses of phytosanitary concern. Several subgroups of nepoviruses exist, each with their own specific vector species. Based on morphological and morphometrical data, the identification up to *Xiphinema* species level is quite difficult. Most of the time, it is impossible due to lack of specimens or appropriate nematode stadia. Currently, there is also a lack of molecular information of all *Xiphinema* species to develop reliable diagnostic tools such as PCR or barcoding. Direct damage caused by *Xiphinema* species is usually limited, the main issue is that some specimens are potential carriers of viruses. Whereas the whole *X. americanum* group had a quarantine status before the new Plant Health Legislation (December 2019), only 7 species retained this status and two species became regulated non-quarantine organisms. Still, their identification up to species level is very difficult. Focusing on a direct detection of these viruses in the nematodes could be an alternative to the difficult task of correctly identifying the *Xiphinema* species. This way, only specimens of the *X. americanum* group that are actually carrying viruses can be considered as a Plant Health risk, and consignments carrying *Xiphinema* spp. free of viruses could be allowed to enter trade. The aim of the project was to optimise a generic classic nepovirus detection test for nematodes of the genus *Xiphinema* and ultimately to validate it through the organisation of an interlaboratory test.

Based on literature, methods were selected to develop a diagnostic procedure for nepovirus detection in *Xiphinema* nematodes. Nematodes (*X. diversicaudatum*, *X. index*, and *X. americanum s.s.*) and nepoviruses (*Arabis* mosaic virus (ArMV), grapevine fanleaf virus (GFLV) and tomato ringspot virus (ToRSV)) were procured, virus transmission assays were carried out and the selected methods were compared and validated in the laboratory. This resulted in a recommended diagnostic protocol consisting of the Automated Zonal Centrifuge (AZC) for nematode extraction from soil, a method of choice for physical disruption of the isolated nematodes (slicing, bead beating and bead beating with collagenase pre-treatment) and the KingFisher MagMax Kit for RNA extraction. Due to inadequate sensitivity of generic nepovirus subgroup detection tests, nepovirus detection still relies on specific single nepovirus real-time PCR tests. The MinION nanopore technology was evaluated as an alternative for the future. MinION nanopore sequencing failed to detect GFLV and ToRSV in single nematode specimens. However, its potential was demonstrated by successful ArMV detection in six *X. diversicaudatum* individuals.

2.2. Project aims

The project aimed to address the following questions:

- What are the tests currently available to detect (nepo) viruses in plants and nematodes, and which of these provide the best options as a performant generic test to detect the different nepovirus subgroups?
- Can an optimised and validated test(s) for detection of viruses inside the nematodes be developed? Emphasis will be on specificity, sensitivity and robustness of the method.
- How does this test for the detection of viruses inside nematodes perform in the framework of an interlaboratory test? Through the interlaboratory test, other parameters such as repeatability and reproducibility will also be assessed.
- Does nanopore sequencing (MinION technology) allow to identify (nepo)viruses in nematode samples?

2.3. Description of the main activities

2.3.1. WP1. State of the art on virus detection methods and establishing *Xiphinema* cultures

Predefined tasks

- Task 1.1. Screening of the available tests to detect (nepo)viruses in plants and nematodes (ILVO, KIS, IORPIB, Fera, TVRI)
- Task 1.2. Selection of the most performant generic tests for detection of nepovirus subgroups (ILVO, KIS, IORPIB)
- Task 1.3. Collection of *Xiphinema* spp. (ILVO, KIS, Fera, TVRI, USDA-ARS)
- Task 1.4. Establishment and maintenance of *Xiphinema* spp. cultures (ILVO, IORPIB, Fera, TVRI, USDA-ARS)

Description of activities by partners

ILVO:

- Selection of tests to develop a diagnostic protocol for nepovirus detection in *Xiphinema* nematodes.
- Collection and maintenance of nematodes (*X. diversicaudatum, X. index and X. americanum s.l.*) and nepoviruses (*Arabis* mosaic virus (ArMV), grapevine fanleaf virus (GFLV) and tomato ringspot virus (ToRSV)).
- Perform virus transmission tests to obtain viruliferous nematodes.

Fera:

- Collection of *X. index* and *X. pachtaicum* from soil in Sicily (Italy), start cultures on grapevine in glasshouse and provide other partners with these specimens.
- Molecular and morphological *Xiphinema* species identification.

IORPIB:

- Provide other partners with *X. diversicaudatum* populations.
- Provide breeding protocols of *X. diversicaudatum*.

KIS:

- Provide other partners with *X. index* and *X. rivesi* populations.

TVRI:
- Investigation of vertical *Xiphinema* nematode distribution in soil with tractor mounted soil auger.
- Rear *X. index* and *X. pachtaicum* on fig tree.
- Identification of *Xiphinema* nematode species in Turkey.

USDA-ARS:
- Improvement of knowledge on the epidemiology of *Xiphinema* nematodes and nepoviruses in small fruit production fields in the Pacific Northwest (PNW) of the United States.
- Provide other partners with populations of the *X. americanum s.l.* group.

2.3.2. WP2. Detection of viruses in *Xiphinema*: test optimisation and validation

Predefined tasks

Optimization and validation of selected test(s) for nepovirus detection in *Xiphinema* nematodes. Establish a standardized protocol that covers nematode extraction from soil, physical disruption, RNA extraction, virus detection and virus identification. Emphasis will be on specificity, sensitivity and robustness of the method.

- Task 2.1. Test optimization and laboratory validation (ILVO, KIS, IORPIB, USDA-ARS)

Description of activities by partners

ILVO:
- Method comparison and validation: the classical sieving and decanting method (Flegg Modified Cobb method) was compared with automated zonal centrifugation (AZC) for nematode extractions from soil. The AZC uses magnesium sulphate as the separation fluid. For physical disruption, slicing, bead beating and bead beating with collagenase pre-treatment were compared. To optimize the RNA extraction, the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was compared with a CTAB extraction (protocol according to Anses) and a KingFisher MagMax RNA isolation (ThermoFisher Scientific, Waltham, USA). Finally, different existing generic nepovirus subgroup real-time PCR tests were compared with specific single species real-time PCR tests for nepovirus detection.

Fera:
- Evaluation of the potential of various tests for nepovirus detection in *Xiphinema* nematodes.

KIS:
- Comparison of direct RT-PCR and regular RNA extraction with RT-PCR using *X. index* specimens collected on GFLV infected vineyard. Plan additional tests on nematodes from a blackberry orchard spreading strawberry latent ringspot virus (SLRSV).

TVRI:
- Comparison of Cobb's decanting and sieving method with the centrifugal flotation method using sugar as separation fluid (475 g sugar/ l water) for nematode extractions from soil.
- Evaluation of the potential of ELISA for GFLV detection in *X. index*.

2.3.3. WP3. Test performance study (TPS) on the validated methods

Predefined tasks

Organize a test performance study for the validated tests from WP2 for virus detection in nematodes. Through the test performance study, other parameters such as repeatability and reproducibility were assessed.

- Task 3.1. Organisation and implementation of the TPS (ILVO; KIS, Fera, USDA-ARS)

- Task 3.2. Assessment of the interlaboratory test results and adjustment of the validated test (ILVO)

Description activities partners

ILVO:

- The tasks in this work package could not be performed due to time restraints. However, during the final meeting of the Euphresco project (October 2022), several partners provided their availability to participate in a TPS after the project end. The TPS will be organized by ILVO.

2.3.4. WP4. Assess the feasibility to use HTS to detect viruses in nematodes

Predefined tasks

Assessment of the feasibility to use Illumina and nanopore sequencing (MinIon technology) for fast and reliable identification of nepoviruses in nematode samples.

- Task 4.1. Preparation of nematode samples with known virus status (ILVO, KIS, USDA-ARS)
- Task 4.2. Inventory of the available MinION strategies and selection of the best option for a first MinION run (ILVO)
- Task 4.3. Organization of an Illumina and MinION sequencing run on the samples prepared in T4.1. (ILVO, KIS, Fera)
- Task 4.4. Interpretation of results and recommendations (ILVO)

Description activities partners

ILVO: Performed one MinION and Illumina sequencing run on three samples:

- Sample 1: 4 pooled samples of *X. diversicaudatum* carrying ArMV (total of 12 individuals)
- Sample 2: 2 pooled samples of *X. index* carrying GFLV (total of 2 individuals)
- Sample 3: 1 sample of *X. americanum s.s.* carrying ToRSV (1 individual)

2.4. Main results

2.4.1. WP1. State of the art on virus detection tests and establishment of *Xiphinema* cultures

ILVO:

- Successful virus transmission of *Arabis* mosaic virus (ArMV), grapevine fanleaf virus (GFLV) and tomato ringspot virus (ToRSV) from *Chenopodium quinoa* bait plants to *X. diversicaudatum*, *X. index* and *X. americanum s.s.* nematodes, respectively.

Fera:

- Successful reproduction of nematodes over the months, but no grapevine fanleaf virus transmission to grapevines in greenhouse.
- Successful molecular identification of *X. americanum s.s.* population originating from Michigan by COI barcoding. Morphological identification was not possible due to lack of fresh and live material.

KIS:

- Provided soil with predetermined populations of *X. index* and *X. rivesi* to ILVO for nematode culture establishment.

TVRI:

- Vertical *Xiphinema* nematode distribution in soil: peak population of *X. pachtaicum, X. turcicum, X. ingens, X. pyrenaicum* was found at 20-40 cm and of *X. index* at 60-80 cm soil depth.
- Molecular identification of 12 *Xiphinema* species in Turkey.

USDA:

- *Xiphinema* nematodes are widespread in the Pacific North West of the USA, with nematodes found in 75% of the fields. Diagnostic symptoms of the nematode transmitted viruses tomato ringspot (ToRSV) and tobacco ringspot (TRSV) viruses were only observed in 4% of fields surveyed. Plant material collected for virus determination with PCR is yet to be conducted and will occur once methodologies are established to be reliable. This will be partially achieved by using next generation sequencing to obtain virus genomes.

2.4.2. WP2. Detection of viruses in *Xiphinema*: test optimisation and validation

ILVO:

As a first step in the development of a diagnostic protocol, the classical sieving and decanting method was compared with the automated zonal centrifugation (AZC) in an experiment with *X. diversicaudatum* for nematode extractions from soil; the AZC resulted in the highest nematode yields (p-value = 0.0016). Comparison of the tests for physical disruption, RNA extraction and nepovirus detection were carried out over three experiments with three different nematodes and nepoviruses: *X. diversicaudatum*, *X. index* and *X. americanum s.s* carrying ArMV, GFLV and ToRSV respectively. The experiment with *X. index* and GFLV included the largest number of nematodes: 528 tubes with different nematode numbers (1, 3 and 10) and life stages (J = juveniles and A = adults), enabling statistical analysis. In this experiment the GFLV specific real-time PCR of Čepin *et al*., 2010 was used to evaluate the different physical disruption and RNA extraction methods. After data inspection and model building the model 'Ct_Mean ~ Population + RNA + Tube' was selected, based on both Akaike information criterion and ANOVA criteria, with Ct_Mean, population, RNA and tube referring to the estimated mean Ct, the repetition (SL.1.1, SL.1.2 and SL.2), the RNA extraction method (RN = RNeasy Plant Mini Kit, CT = CTAB, KF = KingFisher MagMax RNA extraction kit) and the nematodes present in the micro centrifuge tubes (A = adult, J = Juvenile and number of individuals), respectively. In Figure 1 the cumulative effects of the model parameters Tube and RNA are shown in function of the estimated Mean Ct or 'emmeans' (left figure) and the proportion of positive samples (right figure). Both the proportion analysis and the emmeans Ct evaluation identify CTAB as the least performing RNA extraction method. The KingFisher MagMax RNA extraction kit and the RNeasy Plant Mini Kit are both well performing methods, with KingFisher scoring slightly better than RNeasy by a non-significant difference. Furthermore, the results show that virus detection in adult *Xiphinema* nematodes was better than in juveniles. For samples with 3 adults, detection was possible with 95% certainty, independent of the RNA extraction method. For samples with only 1 adult, this was only true for RNA extractions with the KingFisher MagMax Kit. For juveniles, on the other hand, very poor results were obtained for tubes with 1 and 3 juveniles. Nonetheless, when increasing the number of individuals to 10, the detection improved and the results even approximated those obtained by samples with 3 adults. Overall, similar results were obtained between different experiments.

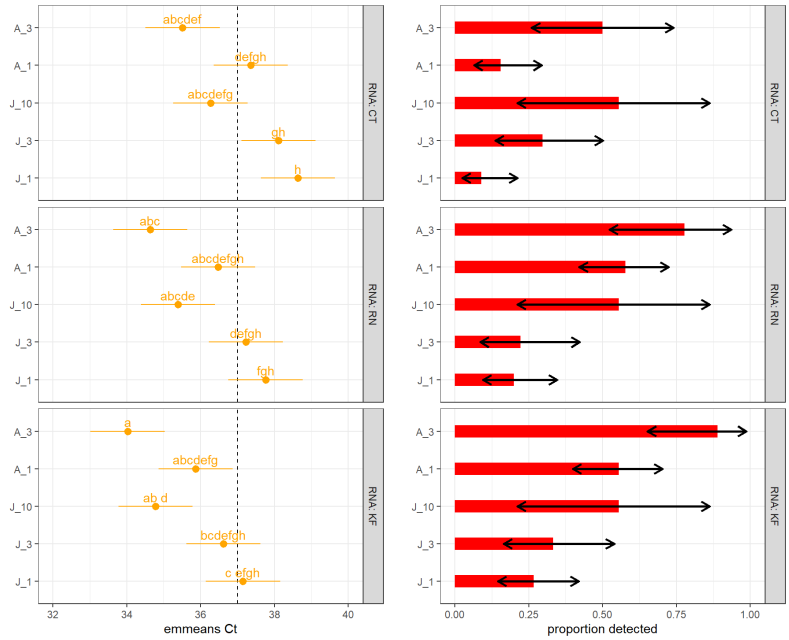

Figure 1 Model analysis of 'Ct_Mean ~ Population + RNA + Tube' with combined effect of RNA and Tube factors in function of the 'emmeans Ct' or estimated means Ct (left) and the proportion of samples where virus was detected (B). RNA represents the RNA extraction method (CT = CTAB, RN = RNeasy Plant Mini Kit, KF = KingFisher MagMax RNA extraction kit) and Tube stands for the nematodes present in the micro centrifuge tube (A = adult, J = Juvenile and number of individuals (1, 3 and 10)). The detection limit was determined at 37 and is shown as the dashed line in the emmeans CT graph (left). Confidence intervals are presented as orange lines (left) or black arrows (right) and statistical significance is visualized by a letter code (left). Negative samples are flagged as 38.5 (left). Meloidogyne arenaria and GFLV infected leaf samples were used as negative (NC) and positive (PC) extraction controls, respectively.

Figure 2 shows the occurrence of a 'population/plant and operator' effect. Repetitions SL.1.1 and SL.1.2 contained nematodes which fed on *C. quinoa* plant 1, while SL.2 contained nematodes which fed on *C. quinoa* plant 2. The experiments in repetition SL.1.1 and SL.2 were performed by operator 2 and those in SL.1.2 by operator 3. Depending on the operator and the plant on which the nematodes fed, different results were obtained, with significant differences related to both factors "plant" and "operator". The operator effect is related to differences in handlings and/or protocol interpretations during the physical disruption, RNA extraction and virus detection between lab technicians. Variations caused by the plant effect are a direct consequence of differences in virus titers between plants on which the nematodes fed.

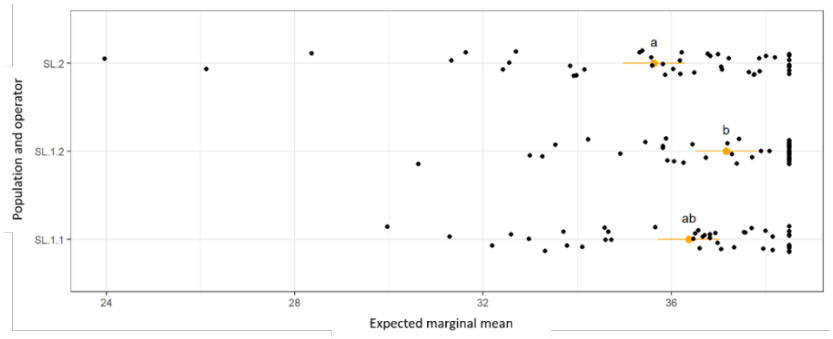

Figure 2 Population (plant) and operator effect visualized in function of the estimated marginal mean Ct, with SL.1.1 and SL.1.2 containing nematodes which fed on C. quinoa plant 1 and SL.2 containing nematodes fed which on C. quinoa plant 2. The experiments in repetition SL.1.1 and SL.2 were performed by operator 2 and those in SL.1.2 by operator 3.

Different existing specific and generic real-time PCR tests were tested over the three different experiments, yet only the specific tests were sensitive enough for nepovirus detection in *Xiphinema* nematodes. The nested PCR of Pantaleo *et al*., (2001), the real-time PCR of Čepin *et al*., (2010) and the nested PCR of Martin *et al*., (2009) were able to detect ArMV, GFLV and TorSV in *X. diversicaudatum*, *X. index* and *X. americanum s.s.* respectively.

Fera:
Tests were performed on 6 individuals of *X. americanum* group from California (CDFA) but virus detection failed (samples were from a DNA collection). KIS:

- Two RNA extraction methods and direct GFLV detection in nematodes were compared. A natural population of *X. index* from GFLV infected vineyard was used for this purpose. For method 1, RNA was extracted from 5 or 10 nematodes using RNeasy Plant Mini Kit (Qiagen). For method 2, nematodes were crushed on cellulose membrane and crude extracts were prepared as described in Caglayan *et al*., (2012). These were further extracted with MagMax Total RNA extraction kit (Thermo). Five or 10 µl of cDNA were used in PCR for GFLV detection with EV00N1 and CPS primers. Two samples were analysed for each extraction method and for direct RT-PCR. The results show that direct RT-PCR was the most reliable in this experiment, followed by membrane extraction. The use of 10 µl of cDNA seems to have inhibitory effect on PCR detection of GFLV. We are continuing this experiment on higher number of nematode samples.
- The SLRSV infection was confirmed in blackberry orchard and the soil infestation with *X. divesicaudatum* was confirmed. Similar experiment as above will be conducted on these nematodes.

TVRI:

- The best method to extract *Xiphinema* nematodes from soil samples was the Cobb's decanting and sieving method. Many nematodes were disrupted after extractions with the sugar centrifuge method (475 g sugar/ L water).
- ELISA GFLV detection was successful in 30 *X. index* specimens

2.4.3. WP4. Assess the feasibility to use HTS (focus nanopore sequencing technology) to detect viruses in nematodes

ILVO:

- Unfortunately both the quality and quantity of the RNA were insufficient for Illumina sequencing that was outsourced to an external provider (Admera Health). In spite of the poor quality control results, MinION sequencing was performed. Although MinION sequencing failed for GFLV and ToRSV it was successful for ArMV. In total 871 reads per million (rpm) were generated, exceeding the minimum threshold and indicating a reliable detection (100-500 rpm considered “plausible to be present”, >500 rpm considered “very plausible to be present”).

2.5. Conclusions and recommendations to policy makers

2.5.1. Conclusions

Can we offer an optimised and validated method for virus detection inside nematodes?

For **nematode extractions from soil**, automated zonal centrifugation resulted in the highest nematode recovery and is therefore the recommended method. In case laboratories are not equipped with such a device, manual extractions with the Flegg Modified Cobb method are a good alternative.

Secondly, any of the three **physical disruption** methods can be chosen, as the same results were obtained for slicing, bead beating and bead beating with collagenase pre-treatment.

For **RNA extraction**, CTAB is the least performing method. Good results were obtained for both the KingFisher MagMax RNA Extraction Kit and the RNeasy Plant Mini Kit. Nonetheless, the KingFisher MagMax Kit is preferred over the RNeasy Plant Mini Kit, by scoring slightly better and by being more cost-effective (KingFisher MagMax Kit: 2 euro / sample; RNeasy Plant Mini Kit: 9 euro / sample).

Currently only specific real-time PCR tests were sensitive enough for **nepovirus detection** in *Xiphinema* nematodes. The nested PCR of Pantaleo *et al*., (2001), the real-time PCR of Čepin *et al*., (2010) and the nested PCR of Martin *et al*., (2009) were able to detect ArMV, GFLV and TorSV in *X. diversicaudatum*, *X. index* and X. *americanum s.s.* respectively. The recommended diagnostic procedure is presented in Figure 3.

When using this procedure for diagnostics it is important to be aware of an **operator and plant effect**, as significant differences in results were obtained between operators and plants on which nematodes acquired the virus. To minimize the operator effect it is recommended to provide a training on good laboratory practices, tailored to the needs of this diagnostic procedure.

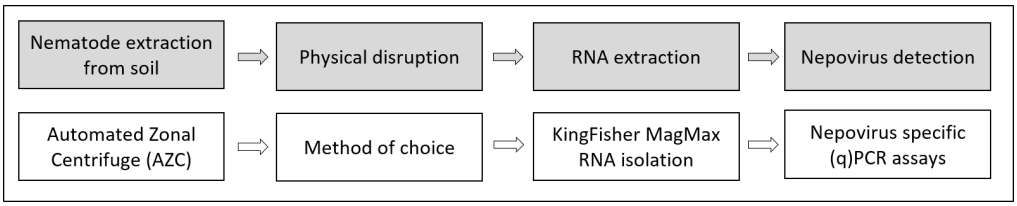

Figure 3 Recommended diagnostic procedure for nepovirus detection in Xiphinema nematodes.

Is nepovirus detection possible in single nematode specimens?

In samples, especially those of imported materials, the number of *Xiphinema* specimen is usually low (<10) and often only juveniles are found. Whether nepovirus detection is possible and reliable in single nematode specimens is dependent on the nematode life stage, the RNA extraction method and the detection test. In the experiment with *X. index* and GFLV, GFLV detection was possible with 95% confidence in single adult *Xiphinema* specimens, extracted with the KingFisher MagMax Kit and tested with the real-time PCR of Čepin et al., (2010). Similar results were obtained in the other experiment (no statistics are provided because of the small sample size).A higher sensitivity was observed in all experiments when increasing the number of individuals in the sample. When comparing adults with juveniles, juveniles are inferior test subjects, possibly because less virus particles are retained in the stylet/oesophagus compared to adults. Additionally, nematodes lose virus particles during moulting, which might have also influenced the virus titers. Nonetheless, when increasing the number of individuals, reliable results could still be obtained. For instance, in the experiment with *X. index* and GFLV the results obtained with 10 juveniles approximated those with 3 adults.

Is nanopore sequencing an alternative option for the future?

MinION nanopore sequencing failed to detect GFLV and ToRSV in single nematode specimens. However, its potential to detect viruses in nematodes was demonstrated by successful ArMV detection in six *X. diversicaudatum* individuals. Better results might be obtained for nepovirus detection in single nematodes without successive freezing and thawing. Furthermore, the analytical sensitivity of the test could be increased by working with a different library preparation kit (PCR cDNA instead of direct cDNA library preparation). However, to obtain this, extensive optimization and fine-tuning is required. Unfortunately, the comparative analysis with Illumina sequencing could not be performed due to poor quality and quantity RNA extracts. Presumably transportation decreased this quality and quantity even more, whereby sequencing could not be performed.

2.5.2. Recommendations

Recommendation 1

Recommendation on the diagnostic procedure for nepovirus detection in *Xiphinema* nematodes (Figure 3), with a side note on awareness for an operator and plant effect and with the recommendation to minimize the operator effect by providing training sessions.

Recommendation 2

When possible, adult *Xiphinema* nematodes should be used instead of juveniles for nepovirus detection. In case only juveniles are present, at least 10 individuals should be used. Nepovirus detection is possible in single adult nematode species with the right RNA extraction method and detection test. However it is recommended to use 3 or even more individuals when these are present in the sample, as this won't imply higher costs but will improve the sensitivity significantly.

2.6. Benefits from trans-national cooperation

Various benefits were gained in the XiphiVIR projects by a trans-national cooperation. The cooperation included the transnational partners in the Euphresco project, but by extension also the participation from a large number of institutes from the network that has been created through cooperation in other projects. In this project there was a high risk of (viruliferous) *Xiphinema* nematode shortage. *Xiphinema* nematodes have a slow reproduction cycle and are very sensitive to environmental fluctuations such as soil temperature and soil humidity. This makes culturing and virus transmission studies very hard. However, the network was an opportunity to exchange nematodes frequently allowing to avoid this risk. Furthermore, in the consortium the partners were able to share their expertise and exchange knowledge. Additional benefits will be gained once the test performance study, to which at least four partners already committed, will be organized. Cooperation of project partners will be continued after the end of the project.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Everaert *et al*. Nepovirus detection in *Xiphinema* nematodes. In preparation.

4. Open Euphresco data

None.

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