Euphresco

Final Report

Project title (Acronym)
Validation of molecular diagnostic methods for the detection and identification of tomato mottle mosaic virus (ToMMV-detect)

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Contents

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2. Short project report

1. *Short executive summary*

In recent years, the emergence of new plant viruses has posed increasing challenges to agricultural production and international trade. Among these, tomato mottle mosaic virus (ToMMV) has gained attention due to its rapid spread, potential for severe crop damage, and its likely seed-borne nature. The lack of harmonised and validated diagnostic tools for ToMMV has created uncertainty for regulatory authorities, seed producers, and diagnostic laboratories, making it difficult to ensure early detection and consistent phytosanitary measures.

This project was initiated in response to a clear policy need: to support plant health authorities and stakeholders with reliable, comparable, and internationally accepted diagnostic methods for ToMMV. The practical problem was the existence of multiple molecular tests for ToMMV, but without coordinated validation or performance comparison across laboratories. This gap hindered effective surveillance, seed testing, and outbreak response.

Through international collaboration, the project brought together a wide network of laboratories to jointly evaluate the performance of available molecular tests. The approach enabled the generation of robust data on test reliability, reproducibility, and diagnostic value under real-world conditions. The project also fostered knowledge exchange and alignment of diagnostic practices across countries.

As a result, the project delivered a clearer understanding of which tests are most suitable for routine use, and laid the groundwork for harmonised diagnostic protocols. These outcomes contribute directly to improved plant health preparedness, support evidence-based regulation, and strengthen the capacity of diagnostic networks to respond to emerging threats like ToMMV.

2. *Project aims*

The aim of the project was to support the development of a harmonised and evidence-based approach to the detection of ToMMV, an emerging plant virus of increasing concern. Although several molecular tests for ToMMV had been developed in recent years, their performance had not been systematically compared, and no common diagnostic protocol had been established. This created uncertainty for both regulatory authorities and diagnostic laboratories, limiting the effectiveness of surveillance and response measures.

From a policy perspective, the lack of validated and comparable diagnostic tools made it difficult to assess the presence and spread of ToMMV, to implement consistent phytosanitary measures, and to ensure fair and science-based regulation of seed trade. Without reliable diagnostics, risk assessments remained inconclusive, and regulatory decisions were harder to justify or harmonise across countries.

For diagnostic laboratories and other end users, the practical challenge was the absence of clear guidance on which tests to use, how they perform under different conditions, and whether results could be trusted across laboratories. This led to inefficiencies, inconsistent results, and reduced confidence in diagnostic outcomes.

The project addressed these challenges by coordinating an international test performance study (TPS), in which a wide range of molecular tests for ToMMV were evaluated under harmonised conditions by participating laboratories. The objective was to generate robust comparative data on test performance, including sensitivity, specificity, and reproducibility, and to identify the most suitable tests for routine use.

By doing so, the project aimed to provide a foundation for the development of a common diagnostic protocol, support regulatory alignment, and strengthen diagnostic capacity across countries. The outcomes are intended to benefit both policy makers, by enabling more consistent and evidence-based regulation, and end users, by improving the reliability and comparability of diagnostic testing for ToMMV.

3. Description of the main activities

The central activity of the project was the design and implementation of an international TPS to evaluate the diagnostic performance of molecular methods for the detection and identification of ToMMV. The study was conducted in accordance with EPPO guidelines (EPPO PM7/122) and involved a coordinated effort among 15 laboratories from 13 countries.

The first phase of the project focused on the identification and selection of molecular tests suitable for inclusion in the TPS. This was achieved through a structured process that included a survey among project partners, a review of scientific literature, and a search of commercial test providers. The selection criteria required that each test had a clearly defined protocol and at least some preliminary validation data. In cases where multiple versions of a test existed, the version with the most extensive validation data was selected.

The final panel of tests included:

- Three conventional RT-PCR assays (Levitzky et al. 2019, Loewe Biochemica GmbH, and Sui et al. 2017),
- Five real-time RT-PCR (RT-qPCR) assays (DAFF DEECA, Fowkes et al. 2022, ISF, and Tiberini et al. 2022 in both singleplex and duplex formats),
- One recombinase polymerase amplification (RPA) assay (Agdia XCS 22800),
- One loop-mediated isothermal amplification (LAMP) assay (Kimura et al. 2023).

All selected protocols were formatted according to the EPPO PM7 standard and compiled into a technical annex for use by all participants. Before distribution, each test was pre-tested at the coordinating laboratory (NIB, Slovenia) using a limited panel of samples, including two ToMMV-positive, two ToBRFV-positive, and two non-template controls. This step ensured that

the protocols were functional and that any ambiguities were resolved in consultation with the original developers.

Given the logistical and budgetary constraints, it was decided that the TPS would be based on extracted RNA rather than plant material. This approach reduced variability and simplified sample handling across laboratories. A panel of 22 RNA samples was prepared, including:

- serial dilutions of two ToMMV isolates (from leaf and seed material),
- RNA from 14 other tobamoviruses,
- three healthy controls.

Each panel also included one positive and one negative control. In total, 40 aliquots of each sample were prepared. Homogeneity and stability of the RNA samples were verified through internal testing, including simulation of transport and storage conditions.

The sample panels were distributed to participating laboratories in May 2024. Each laboratory received one panel per method they had registered to test (e.g., one for RT-PCR, one for RT-qPCR, etc.). Participants were instructed to follow the provided protocols and to document any deviations. They were also asked to test each sample in duplicate and to report results using a standardised Excel template, including raw data such as Cq values, amplification curves, and any observed anomalies.

The data collection phase was followed by a centralised analysis of the results. The performance of each test was evaluated based on:

- diagnostic sensitivity and specificity,
- concordance (true positive and true negative rates),
- reproducibility across laboratories,
- probability of detection at different virus concentrations.

In total, 87 valid datasets were submitted and analysed. The results showed that RT-qPCR assays and the RPA test generally performed best in terms of sensitivity and reproducibility. RT-PCR methods showed high specificity but lower sensitivity. The LAMP assay had the lowest overall concordance and reproducibility.

The study also revealed that some tests produced inconclusive results at high Cq values, particularly in the presence of non-target tobamoviruses such as PaMMV. To address this, a standardised approach to interpreting borderline Cq values was applied during data analysis, ensuring consistency across datasets.

In addition to the technical evaluation, the TPS served as a platform for knowledge exchange and capacity building. Laboratories had the opportunity to test new methods/ tests, compare results, and align their diagnostic practices with international standards. The protocols and validation data generated through the TPS have been made accessible to the broader diagnostic community and will support further dissemination and harmonisation efforts.

In summary, the main activities of the project were:

- identification and selection of molecular tests for ToMMV,
- pre-validation and harmonisation of protocols,
- preparation and distribution of standardised RNA panels,
- execution of the TPS across multiple laboratories,
- centralised analysis of diagnostic performance,
- dissemination of results and recommendations for routine diagnostics.

4. Main results

The TPS comprehensively evaluated 10 molecular diagnostic tests for the detection of ToMMV, including three RT-PCRs, five RT-qPCRs, one RPA, and one LAMP. The study involved 15 laboratories from 13 countries, using harmonized protocols and standardized RNA panels derived from both leaf and seed material.

Key findings:

- Concordance rates:
  The highest concordance rates were observed for the RPA-Agdia test and the RT-qPCR by Tiberini et al. (2022, Singleplex), both achieving nearly 89%. Other RT-qPCR tests showed concordance rates between 85% and 88%. The RT-PCR by Levitzky et al. (2019) reached 84%, while RT-PCR by Sui et al. (2017) had 81%. The lowest concordance rates were recorded for the LAMP test by Kimura et al. (2023) and the RT-PCR by Loewe, at 78% and 77%, respectively.
- Distribution of diagnostic outcomes:
  RT-PCR tests yielded the highest proportion of true negatives, whereas true positives were most frequent in RT-qPCR and RPA-Agdia tests. Inconclusive results were least common in RT-PCR tests, indicating greater clarity in outcomes.
- Diagnostic specificity and sensitivity:
  All RT-PCR tests demonstrated 100% specificity. The RPA-Agdia and RT-qPCR by Tiberini et al. (2022, Singleplex and Duplex) followed with 94–95% specificity. The LAMP test by Kimura et al. (2023) showed 93% specificity, while other RT-qPCRs ranged slightly lower, with the ISF RT-qPCR at 90.5%.
  In terms of sensitivity, RT-qPCRs and RPA-Agdia were the most effective, ranging between 78–80%. RT-PCR and LAMP tests were less sensitive, with RT-PCR by Levitzky et al. (2019) at 62.5% and RT-PCR by Loewe at only 45%.
- Probability of detection – Leaf RNA dilutions:
  Across increasing dilutions of ToMMV isolate (NIB V 373), RT-qPCR and RPA-Agdia tests maintained the highest detection probabilities. RT-PCR tests (especially Loewe and Sui et al. (2017)) and the LAMP test showed a marked decline in detection capability at higher dilutions, indicating reduced effectiveness at low ToMMV RNA concentrations.
- Probability of detection – Seed RNA dilutions:
  At 2x and 20x dilutions of ToMMV isolate from seeds (NIB V 414), RT-qPCRs by Fowkes et al. (2022) and Tiberini et al. (2022, Singleplex) consistently achieved 100% detection, even at higher dilutions. RT-qPCRs from DAFF DEECA, Tiberini et al.

(Duplex), and RPA-Agdia also performed well, with detection rates of 100% at 2x and 83–92% at 20x. The ISF RT-qPCR showed slightly lower performance (100% at 2x, 75% at 20x). RT-PCR and LAMP tests were significantly less reliable, with RT-PCR Loewe detecting only 25% at 2x and failing entirely at 20x dilution.

- Reproducibility:
  Reproducibility across tests ranged from 87% to 97%. The highest reproducibility (97%) was observed for RT-PCR Loewe, RT-qPCRs DAFF DEECA, Fowkes et al. (2022), and ISF. The LAMP test by Kimura et al. (2023) had the lowest reproducibility at 87%. At the sample level, reproducibility was lowest for highly diluted ToMMV samples and for non-target tobamovirus PaMMV.

Detailed performance data are provided in the TPS report available at Euphresco data portal (https://drop.euphresco.net/data/af730655-4022-4e87-a952-b94cfda3a971/). Validation data for all tests included in the TPS are available also in the EPPO Database on Diagnostic Expertise (https://dc.eppo.int/validation_data/validationlist).

Practical implications:

- The availability of validation data for all tested methods, now published in the EPPO Database on Diagnostic Expertise, provides a transparent and evidence-based foundation for laboratories and regulatory authorities when selecting appropriate diagnostic methods for ToMMV.
- The full TPS report, including protocols, sample panel composition, and detailed results, is publicly accessible via the Euphresco data portal and serves as a reference for laboratories, researchers, and policy makers.
- The outcomes of the TPS directly support harmonization of diagnostic protocols, improved plant health preparedness, and evidence-based regulation of seed trade

5. Conclusions and recommendations to policy makers

The project has provided a clear and evidence-based foundation for improving the detection and identification of ToMMV. RT-PCR tests showed the highest diagnostic specificity, while RT-qPCR tests and the RPA Agdia test demonstrated the highest diagnostic sensitivity. Reproducibility was high for all tests, with some showing slightly better consistency, particularly the RT-PCRs and RT-qPCRs.

The highest concordance rates (relative accuracy) were obtained with the RPA-Agdia and the RT-qPCR singleplex Tiberini et al. (2022). These were closely followed by other RT-qPCR tests. The RT-PCR tests by Levitzky et al. (2019) and Sui et al. (2017) showed moderate concordance, while the LAMP assay by Kimura et al. (2023) and, finally, the RT-PCR Loewe test exhibited the lowest concordance and sensitivity, particularly in samples with low virus concentrations or in seed matrices.

These results demonstrate that, as a direct outcome of this project, reliable and harmonised molecular diagnostics for ToMMV are now achievable. Validated protocols and comprehensive performance data are now available to support informed decision-making and to ensure comparability of diagnostic results across countries.

Nevertheless, the study also highlighted some limitations. Not all tests performed equally well under all conditions, and the interpretation of borderline results (such as high Cq values in RT-qPCRs) remains a challenge, particularly in the presence of non-target tobamoviruses. Furthermore, since the TPS focused exclusively on molecular methods, other diagnostic approaches (such as serology or high-throughput sequencing) were not evaluated and may require further study.

The project opened several avenues for future work. These include:

- The development and provision of well-characterised reference materials that could be made available to diagnostic laboratories. Such materials would serve as external quality controls and support the verification of test performance over time, ensuring consistency and reliability across different laboratories and testing conditions.
- Further validation of selected molecular tests on naturally infected field samples and commercial seed lots. While the TPS provided robust data under controlled conditions, additional testing on real-world samples would strengthen confidence in the applicability of these methods in routine diagnostics.
- Investigation of the impact of virus genetic variability on test performance. As new ToMMV isolates continue to emerge, it is important to monitor whether existing tests remain inclusive and specific, and to update protocols if necessary.
- Exploration of diagnostic strategies for environmental sources of infection, such as contaminated water or soil. Preliminary findings suggest that these may play a role in virus transmission, and future work could focus on developing or adapting tests for such matrices.

Recommendation to policy makers:

- Support continued international collaboration in diagnostics: the success of this project highlights the value of coordinated TPS initiatives. Future efforts should build on this model to address other emerging plant health threats.

In conclusion, the project has delivered practical tools and knowledge that are ready to be used. Policy makers now have access to validated methods and harmonised protocols - all of which can directly support better regulation, improved surveillance, and more effective response to ToMMV.

6. *Benefits from trans-national cooperation*

The success of this project was fundamentally rooted in the strong international and multi-stakeholder collaboration that brought together diagnostic laboratories, research institutions, and commercial partners from across Europe and beyond. The international nature of the

consortium enabled the project to address the diagnostic challenges of ToMMV in a way that no single country or institution could have achieved alone.

One of the most significant benefits of this cooperation was the ability to conduct a harmonised TPS across 15 laboratories in 13 countries. This broad participation ensured that the evaluation of diagnostic methods reflected a wide range of laboratory conditions, technical capacities, and regional contexts. As a result, the findings are not only scientifically robust but also broadly applicable and relevant for real-world implementation.

The collaboration also facilitated the sharing of expertise, protocols, and materials. Laboratories with experience in specific molecular methods supported others in adopting and optimising those protocols. This exchange of knowledge helped to build capacity across the network and contributed to the alignment of diagnostic practices. The joint development of harmonised protocols and the collective interpretation of results fostered mutual learning and strengthened trust among partners.

Another key benefit was the pooling of resources and infrastructure. The preparation and distribution of standardised RNA panels, the centralised data analysis, and the coordination of reporting would not have been feasible without the combined logistical and technical support of multiple institutions. This shared effort ensured consistency and quality across all stages of the project.

Beyond the technical achievements, the project also strengthened professional networks and fostered a spirit of collaboration that will benefit future initiatives. The experience gained through this project has laid the groundwork for continued cooperation on emerging plant health threats, and has demonstrated the value of coordinated, cross-border approaches to diagnostics and plant protection.

In summary, the international cooperation enabled:

- A broader and more representative evaluation of diagnostic methods/ tests;
- Capacity building and knowledge exchange among laboratories;
- Efficient use of resources through shared infrastructure and expertise;
- Stronger networks for future collaboration in plant health diagnostics.

These benefits underscore the importance of continued investment in international diagnostic cooperation, particularly in the face of emerging plant pathogens that do not respect national borders.

3. Outreach

3.1. Article(s) for publication in journals

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3.2. Grey literature

The full TPS report has been published and is available online via the Euphresco data portal: https://drop.euphresco.net/data/af730655-4022-4e87-a952-b94cfda3a971/

3.3. Events

The results of the TPS were briefly presented at a national event in Slovenia in November 2024, which focused on the dissemination of outcomes from various plant health research projects. Although the event was not dedicated solely to this project, the TPS and its key findings were highlighted as part of a broader overview of ongoing diagnostic and surveillance initiatives.

4. Open Euphresco data

The following digital objects have been made openly available:

- Validation data for all molecular tests included in the TPS (RT-PCR, RT-qPCR, RPA, LAMP), including diagnostic sensitivity, specificity, reproducibility, and analytical sensitivity. Available at: https://dc.eppo.int/validation_data/validationlist
- Full TPS report, including protocols, sample panel composition, and detailed results. Available at: https://drop.euphresco.net/data/af730655-4022-4e87-a952-b94cfda3a971/