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Virology

Biological Characterization of Physostegia Chlorotic Mottle Virus, an Emergent Virus Infecting Vegetables in Diversified Production Systems

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Abstract

In 2014, Physostegia chlorotic mottle virus (PhCMoV) was discovered in Austria in *Physostegia virginiana*. Subsequent collaborative efforts established a link between the virus and severe fruit symptoms on important crops such as tomato, eggplant, and cucumber across nine European countries. Thereafter, specific knowledge gaps, which are crucial to assess the risks PhCMoV can pose for production and how to manage it, needed to be addressed. In this study, the transmission, prevalence, and disease severity of PhCMoV were examined. This investigation led to the identification of PhCMoV presence in a new country, Switzerland. Furthermore, our research indicates that the virus was already present in Europe 30 years ago. Bioassays demonstrated PhCMoV can result in up to 100% tomato yield losses depending on the phenological stage of the plant at the time of infection. PhCMoV was found to naturally infect 12 new host plant species across eight families, extending its host range to 21 plant species across 15 plant families. The study also identified a polyphagous leafhopper (genus *Anaceratagallia*) as a natural vector of PhCMoV. Overall, PhCMoV was widespread in small-scale diversified vegetable farms in Belgium where tomato is grown in soil under tunnels, occurring in approximately one-third of such farms. However, outbreaks were sporadic and were associated at least once with the cultivation in tomato tunnels of perennial plants that can serve as a reservoir host for the virus and its vector. To further explore this phenomenon and manage the virus, studying the ecology of the vector would be beneficial.

Keywords: biological characterization, field experiment, greenhouse assay, host range, leafhoppers, prevalence, rhabdovirus, symptoms, transmission, yield loss

Application of high-throughput sequencing (HTS) technologies enabled the first identification of Physostegia chlorotic mottle virus (PhCMoV, *Alphanucleorhabdovirus physostegiae*) from *Physostegia virginiana* (*Lamiaceae*) in 2018 (Menzel et al. 2018). PhCMoV is a rhabdovirus belonging to the *Alphanucleorhabdovirus* genus and, more precisely, to a cluster that includes eggplant mottle dwarf virus (EMDV), potato yellow dwarf virus, constricta yellow dwarf virus, and joá yellow blotch-associated virus (Dietzgen et al. 2021). PhCMoV is most closely related to EMDV.

With 35 isolates sequenced, PhCMoV is the plant rhabdovirus with the most near-complete genomes available to date. Furthermore, genomic studies showed that although genetic variability ranged between 82 and 100% of nucleotide sequence identity (for the near-complete genome), PhCMoV showed a very low genomic variation in the same environment for an extended period (17 years) on different annual host plants (Temple et al. 2022).

HTS has significantly improved knowledge of plant viral diversity and the evolution of known viruses, as well as enabling the discovery of new plant viral species (Adams et al. 2018; Bejerman et al. 2020, 2021; Lefeuvre et al. 2019). However, genomic information alone does not provide enough indications to assess the phytosanitary risks of novel plant viruses and to develop appropriate management strategies to control epidemics (Massart et al. 2017). In that sense, a hierarchical framework describing the different steps needed to evaluate the biosecurity, commercial, regulatory, and scientific impacts associated with discovery of new plant viruses was published in 2017 and revised in 2023 (Fontdevila Pareta et al. 2023; Massart et al. 2017). The revised framework suggests optimizing the study of symptomology caused by plant viruses by combining experimental data with epidemiological observations, statistical analysis, and testing of asymptomatic and symptomatic plants in the field, as proposed by Fox (2020). Afterward, if the novel virus is still considered a threat to crop production, it is recommended to continue the virus characterization by filling the remaining knowledge gaps related to its genetic diversity, geographic distribution, prevalence, severity, host range, and symptom causality and identifying the vector (Fontdevila Pareta et al. 2023). Studying the transmission mode of a new virus and its vectors is one of the most critical tasks in understanding how to limit the spread of a virus (Jones 2004;

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Jones and Naidu 2019), but it is laborious and consumes considerable time and resources. Indeed, it is one of the least-studied criteria for tomato and fruit tree viruses (Hou et al. 2020; Rivarez et al. 2021). In that context, reviewing the vectors of related viruses can greatly narrow the range of insects to test. In Dietzgen et al. (2020), phylogenetic studies based on the protein L homology of various plant rhabdoviruses showed that these viruses clustered according to their insect vector type. PhCMoV clusters with EMDV, potato yellow dwarf virus, and constricta yellow dwarf virus, which are transmitted by leafhoppers, whereas other plant rhabdoviruses can be transmitted by planthoppers, aphids, mites, or whitefly (Dietzgen et al. 2020). A large study on the vector of EMDV in Iran revealed its transmission by the leafhopper *Agallia vorobjevi* (Dlab.) after testing 27 different arthropods species found in EMDV-infested sites (Babaie and Izadpanah 2003). The transmission of a "cucumber isolate of EMDV" by leafhopper species (*Anaceratagallia laevis* [Ribaut] and *Anaceratagallia ribauti* [Ossiannilsson]) was also demonstrated in France, with better efficiency for *Anaceratagallia laevis* (Della Giustina et al. 2000). These results suggest that leafhopper species within the *Anaceratagallia* or *Agallia* genus are good candidates for PhCMoV transmission studies.

In 2021, prepublication data sharing between scientists resulted in an international collaboration and the first evaluation of the risks associated with PhCMoV. This evaluation, combined with previous reports, highlighted the importance of PhCMoV. Indeed, its sudden detection in multiple European countries was shown to be associated with severe symptoms on economically important crops such as tomato, eggplant, and cucumber (Gaafar et al. 2018; Temple et al. 2022; Vučurović et al. 2021). The original study showed a broad natural host range of PhCMoV across seven families in nine European countries. PhCMoV was associated with severe symptoms on the fruits and with vein clearing on the leaves. Subsequently, in Belgium, where multiple occurrences of the virus were recorded, 2,100 asymptomatic tomato plants from 21 vegetable farms with soil-grown tomatoes were screened for the presence of viruses. No detection of PhCMoV was recorded in the asymptomatic plants, whereas the virus was detected in six of the sites on symptomatic plants, reinforcing the association between virus presence and symptom development in the field (Temple et al. 2023).

The aim of this publication was to pursue the biological characterization of PhCMoV to refine the analysis of the phytosanitary risks it poses and propose management measures to limit its spread. The biological characterization focuses on filling knowledge gaps related to prevalence and epidemiology, disease severity, transmission, host range, and symptomology as suggested in the recent optimized scientific and regulatory framework for plant virus characterization and risk analysis (Fontdevila Pareta et al. 2023).

Materials and Methods

Plant sampling and field observations

Detection: Selection of the best tissue for sampling. For three different tomato cultivars (Black cherry [BC], Saint Jean d'Angely, and Trixi) from site A (Supplementary Table S3), specific sampling of seven different tissues per plant was carried out: (i) an old leaf (sixth from the bottom), (ii) the first regrowth, (iii) a mature fruit and (iv) a regrowth at the middle height, (v) the apex, (vi) the uppermost immature fruit, and the (vii) uppermost mature fruit (Fig. 1). Finally, for the cultivars Saint Jean d'Angely and Trixi, (viii) a leaf from an average-age plant, taken from the middle height of the plant, was also collected. Symptoms on each of the samples were recorded.

For the BC cultivar, 5 asymptomatic plants (AS), 10 plants that only showed symptoms at the bottom of the plant (S), and 10 plants that showed systemic symptoms (S++) were selected. For the cultivar Saint Jean d'Angely, two AS and six S plants were sampled. For the cultivar Trixi, two AS and seven S plants were sampled.

The samples were tested by enzyme-linked immunosorbent assay (ELISA) (see section "Detection of PhCMoV by RT-PCR and double-antibody sandwich ELISA").

Testing of historical samples from Switzerland. Five samples of tomato and one sample of cucumber showing virus symptoms were collected in Switzerland (Tessin, Zurich, and Valais) between 1993 and 2006 and kept in an historical collection (frozen at –20°C). They were tested for the presence of PhCMoV by reverse transcription PCR (RT-PCR). One of the oldest tomato samples (collected in 1993, accession 3216 at Agroscope, Nyon, Switzerland) was sequenced by HTS of total ribodepleted RNA (see section "Detection of PhCMoV by RT-PCR and double-antibody sandwich ELISA").

Ecological survey in the Netherlands. In the Netherlands, in 2020 and 2021, ecological large-scale plant virome surveys were conducted on wild plant species, including *Anthriscus sylvestris*, *Solanum nigrum*, *Viola arvensis, Geranium molle*, and *Hypericum perforatum*, irrespectively of symptoms. Between 3 and 20 plants per species were sampled and pooled before analyzing the presence of viruses by HTS of total ribodepleted RNA (see section on Detection of PhCMoV by HTS).

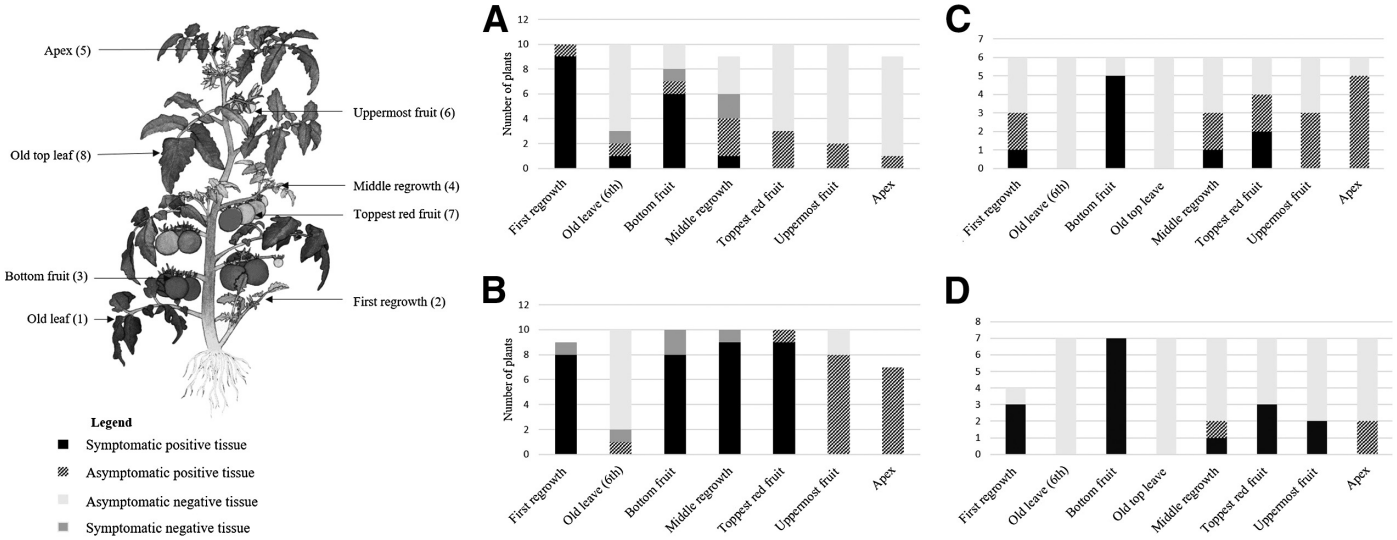

Fig. 1. Detectability of Physostegia chlorotic mottle virus (PhCMoV) in different tissues by enzyme-linked immunosorbent assay (ELISA). **A,** Black cherry with mild symptoms; **B,** Black cherry with severe symptoms; **C,** Saint Jean d'Angely with medium symptoms; and **D,** Trixi with mild symptoms. The status of the plant (positive or negative) was assessed by ELISA.

Farms surveyed in Belgium. During the summer, tunnels of tomato and eggplant crops were visually inspected for PhCMoV-suspicious symptoms (tomato unevenly ripened and deformed fruits and eggplants with vein clearing on newly formed leaves). All the symptomatic plants were counted, collected, and frozen at –20°C. If a PhCMoV-suspicious symptomatic tomato or eggplant was spotted at a site, particular attention was given to the presence of viral-like symptoms (vein clearing, mosaic, deformation, dwarfing) on the other plant species present at the site. The suspected virus-infected plants were photographed, sampled, and tested by RT-PCR (cf. section 2.3.4). In total, 27 farms growing tomatoes in soil for the fresh market were surveyed in the Walloon Region of Belgium, with five of them visited over two consecutive years (between 2020 and 2023). The number of plants per species, year, and site is indicated in Supplementary Table S4.

Prevalence of PhCMoV in tomato in Belgium. The prevalence of plants with PhCMoV-like symptoms was estimated by visual inspection for each site by dividing the number of tomato plants showing PhCMoV symptoms by the total number of tomato plants. The prevalence of symptoms was used as a proxy for virus prevalence.

Laboratory testing

RNA extraction from plants. The protocol used for RNA extraction of historical samples was described in Reynard et al. (2022). For the Belgian samples (survey and transmission experiments), RNA extraction was carried out following the protocol described by Oñate-Sánchez and Vicente-Carbajosa (2008). For *A. sylvestris* and *S. nigrum*, RNA was extracted from about 1 g of frozen leaf tissue, according to Botermans et al. (2013). For *V. arvensis*, *G. molle*, and *H. perforatum*, RNA was extracted using the Maxwell RSC Plant RNA Kit (Promega).

Detection of PhCMoV by HTS. Extracted RNA of the historical accession 3216 and a plant used for mechanical inoculation in control conditions (named "GH24") was processed using the protocol described for Be_GP1 in Temple et al. (2022) prior to Illumina sequencing (total RNA and ribodepletion). RNA of *A. sylvestris* and *S. nigrum* was also analyzed using a protocol based on total RNA and ribodepletion prior to Illumina sequencing, as described for Nd_SL1 in Temple et al. (2022). Finally, for *V. arvensis*, *G. molle*, and *H. perforatum*, RNA extracts were subjected to ribodepletion and cDNA synthesis as described in Liefting et al. (2021). The cDNA was sequenced using the Illumina NovaSeq platform. Reads were trimmed using fastq (default settings) (Chen et al. 2018) and assembled using rnaviralspades (default settings) (Meleshko et al. 2022). PhCMoV genomes were detected using BLASTn with the nucleotide reference database (Altschul et al. 1990).

Detection of PhCMoV by RT-PCR and double-antibody sandwich ELISA. RNA extracts were reverse transcribed in cDNA prior to PCR using the primers and PCR conditions according to Gaafar et al. (2018). Double-antibody sandwich ELISA tests were performed using PhCMoV antibodies JKI-2051 (kindly provided by Heiko Ziebell, JKI) at a dilution of 1:2,000 (vol/vol). The protocol of Clark and Adams (1977) was followed.

Symptomatology study

Greenhouse inoculations: Experimental host range. The PhCMoV isolate GH24 from tomato was reactivated on *Nicotiana benthamiana* before being used for inoculation. Briefly, 1 g of infected frozen leaf material was ground in 10 ml of inoculation buffer (0.02 M phosphate buffer, pH 7.4, containing 2% polyvinylpyrrolidone or 0.2% sodium diethyldithiocarbamate). Plants were inoculated by gently rubbing the inoculum onto carborundum-dusted leaves.

To confirm the PhCMoV host range and to evaluate the associated symptoms, 12 different plants species (*Capsicum annum*, *Tropaeolum majus*, *Lavatera trimestris*, *Stachys affinis*, *Galinsoga parviflora*, *Cucumis sativus*, *Ipomoea purpurea*, *N. glutinosa*, *N. benthamiana*, *Petunia* × *hybrida*, S. *melongena*, and *S. lycopersicum*) including two different cultivars of tomatoes (Suzy and BC) were mechanically inoculated. The number of inoculated plants per species/cultivars varied between 5 and 20 and is indicated in Table 1. Symptoms were monitored 7 to 10 weeks postinoculation (wpi), and the samples were tested by ELISA for the presence of PhCMoV.

Field survey. *Association between PhCMoV presence and symptoms on eggplants.* To better understand the correlation between the PhCMoV-like symptoms (vein clearing and deformations on new leaves) and the presence of the virus in eggplant, 13 symptomatic plants from the cultivar Shakira (Supplementary Fig. S2) and 109 asymptomatic eggplants surrounding the symptomatic plants were sampled. This collection was conducted at site C (Supplementary Table S4) at the end of August 2020 (Temple et al. 2022). The distribution of the symptomatic plants was mapped in the greenhouse (Supplementary Fig. S2). The samples were analyzed by ELISA. The 13 symptomatic and 48 asymptomatic plants immediately surrounding the symptomatic ones, were tested individually, whereas the 61 asymptomatic plants situated away from the symptomatic plants were tested in pools of 2 to 10 plants.

Association between PhCMoV presence and symptoms on several tomato cultivars. At site A (Supplementary Table S4), tomato plants showing symptoms on fruits (deformations, uneven ripening) and leaves (vein clearing on regrowth) were observed in October 2020. In the greenhouse, 14 different tomato cultivars were grown, with approximately 120 plants per cultivar. Half of the plants were planted in April and the other half in June. Whenever possible, at least three symptomatic plants per cultivar were collected and tested by ELISA for the presence of PhCMoV. In total, 61 plants showing symptoms were tested. Ten asymptomatic plants per cultivar were collected, pooled by five, and tested by ELISA.

Severity study: Yield assay

To study the impact of PhCMoV on fruit yield and quality, two cultivars of tomato, BC ($n = 54$ plants) and Cupidissimo F1 (CU, $n = 43$ plants), were mechanically inoculated with PhCMoV isolate GH24 at three different developmental stages: 4 weeks after sowing (BC-1 and CU-1), 8 weeks after sowing (BC-2 and CU-2), or 14 weeks after sowing (BC-3 and CU-3).

At the different time points, between two and five plants were "inoculated" only with the buffer solution as a negative control. The number of plants inoculated with PhCMoV at the three different time points was 20, 18, and 16 for BC and 15, 19, and 9 for Cupidissimo (Table 2).

The plants were randomly distributed in a greenhouse and visually inspected for symptoms each week. When the fruits reached maturity, they were harvested, weighed, and classified as suitable

TABLE 1. Plant species mechanically inoculated with Physostegia chlorotic mottle virus (isolate GH24), symptoms observed, and reverse transcription PCR (RT-PCR) results[a]

Inoculated test plant	Symptoms	ELISA/RT-PCR
Nicotiana glutinosa	vc, d	4/10
Nicotiana benthamiana	vc, d, y	9/9
Petunia hybrida	vc, d	9/9
Cucumis sativus 'Belt alpha'	–	0/10
Capsicum annuum 'Yolo wonder'	–	0/10
Solanum lycopersicum 'Suzy'	vc, d	20/20
Solanum lycopersicum 'Black cherry'	vc, d	20/20
Stachys affinis	vc, m, y	3/5
Tropaeolum majus 'Girerd'	vc, d	2/15
Lavatera trimestris	y, vc, lnl	2/15
Galinsonga pavirflora	–	0/15
Ipomoea purpurea 'Grandpa Ott'	–	0/15
Solanum melongena 'Tsakoniki'	vc, d	3/4

[a] ELISA = enzyme-linked immunosorbent assay, m = mottle, vc = vein clearing, d = deformation, y = yellowing, lnl = local necrotic lesions, and – = asymptomatic.

for the market (asymptomatic) or not (symptomatic, showing deformations, marbling, or anomalies of coloration; Supplementary Fig. S4). At the end of the experiment, regrowth or symptomatic tissues (fruits or leaves) were sampled and tested by ELISA to confirm the presence of PhCMoV. If a negative result was given on an asymptomatic plant inoculated, another organ (bottom fruit) was tested to confirm the absence of the virus. Only ELISA-positive plants were considered for statistical analyses.

The total weight of marketable and nonmarketable fruit was calculated for each plant. Then, the total marketable weight of the plants inoculated at the different time points was compared with the mock-inoculated condition using the Wilcoxon test on RStudio software. A significance threshold of 0.05 was used when testing for differences between control and inoculated plants at each time point.

Vector investigation

Insects trapping. At site A, 16 mature leafhoppers belonging to the *Anaceratagallia* genus were observed in October 2021 around symptomatic common sorrel (*Rumex acetosa*) plants. The specimens were collected from these plants and from the walls of the plastic greenhouse with an insect aspirator.

Transmission assays. Two transmission assays were designed with the collected specimens. For the first assay, 10 *Anaceratagallia* leafhoppers captured as described before at site A were fed on various host plants infected with PhCMoV (eggplant, *Galinsoga* sp., tomato, common sorrel) for 20 days in an insect-proof cage (temperature: 21°C, humidity: 50%, day/night: 16:8). After that, one specimen (LF43-3) was transferred to a healthy eggplant seedling (TR47). Another one (LF43-4) was transferred to a healthy tomato seedling (TR52). After 4 days, the leafhopper on TR47 died and was stored at –20°C. After 13 days, LF43-4 was transferred to another healthy tomato seedling (TR62) for 24 h before being stored at –20°C. The plants were grown in insect-proof cages and tested by RT-PCR for the presence of PhCMoV 7 weeks after the first contact with the leafhopper. The DNA and RNA of the two insects were extracted for species identification by DNA barcoding and PhCMoV testing.

For the second assay, six *Anaceratagallia* leafhoppers were collected at the same site (A) near infected plants and directly transferred in a single cage on three healthy tomato and three healthy eggplant seedlings until they died (between 10 and 23 days later). All the plants were tested for the presence of PhCMoV by RT-PCR. Dead insects were collected and stored at –20°C before DNA/RNA extraction and DNA barcoding/PhCMoV testing. One insect was lost during the process.

DNA and RNA extraction from insect. The entire insect body was ground using a micro pestle in 1.5-ml Eppendorf tubes filled with 0.5 ml of TRIzol (Invitrogen). Half a milliliter of TRIzol was then added to the samples. After overnight incubation at room temperature, 200 µl of chloroform was added. Each tube was then vortexed for 15 s, incubated at room temperature for 3 min, and centrifuged for 15 min at 12,000 × *g* and 4°C. RNA present in the aqueous phase (supernatant) was precipitated in 500 µl of isopropanol before 10 min of incubation at 4°C and centrifugation at 12,000 × *g* and 4°C. Next, the supernatant was removed, and pellets were washed twice in 1 ml of fresh 75% ethanol. At each wash, tubes were spun for 5 min at 7,500 × *g* and 4°C. After the last wash, the remaining ethanol was removed by pipetting and air drying. RNA was resuspended in 30 µl of sterile water. DNA present in the lower phase was precipitated in 300 µl of 100% ethanol. Tubes were mixed by inversions and incubated for 3 min at room temperature before centrifugation for 5 min at 2,000 × *g* and 4°C. The supernatant was removed, and pellets were washed twice in 1 ml of 0.1 M sodium citrate in 10% ethanol for 30 min. At each wash, tubes were centrifuged for 5 min at 2,000 × *g* and 4°C, and the supernatant was discarded. After pipetting away any residual drops, DNA was resuspended in 30 µl of sterile water.

DNA barcoding for insect identification. The amplification step of the PCR was performed using MangoTaq DNA Polymerase (Bioline, Belgium) and the primers LCO1490 and HCO2198 designed by Folmer et al. (1994) for cytochrome c oxidase subunit I (COI) identification. The amplification process involved the following thermal conditions: 94°C for 1 min, 35 cycles of 94°C for 15 s, 48°C for 20 s, 72°C for 20 s, and a final extension step of 3 min at 72°C. The amplified products were purified with the QIAquick PCR Purification Kit (QIAGEN), and amplicons were sent to the Macrogen Europe lab (Amsterdam) for Sanger sequencing. Finally, sequences obtained with forward and reverse primers from each amplicon were assembled on Geneious Prime 2020.0.5 software. Primer sequences were removed, and resulting consensus sequences were analyzed using BLASTn and default settings. The identification of the insect was validated when the percentage of identity with a reference sequence was higher than 95%.

Morphological identification. In summer 2022, one *Anaceratagallia* male specimen was caught at site A using the process as in 2021. First, its genital parts were dissected and pictured to morphologically identify the specimens (Supplementary Fig. S4). For this purpose, the classification key of Tishechkin (2020) was used. Then, DNA was extracted as described above for COI barcoding identification.

Results

Detection: Selection of the best sampling tissue

The seven tissues of the nine asymptomatic plants tested negative for PhCMoV. At least one of the seven tissue samples tested per symptomatic plant was positive. For the BC plants that showed mild symptoms, PhCMoV was best detected in symptomatic lower regrowth and symptomatic lower fruits (Fig. 1). When plants showed severe symptoms, the virus was detected in the upper parts, whether they were symptomatic (bottom fruit, middle regrowth,

TABLE 2. Success of inoculation, symptom appearance, and yield metrics for two tomato cultivars (number of infected plants/inoculated ones, localization of the first symptoms [fruits or leaves], and number of weeks [w] before the apparition of the first symptoms)

Cultivar	Number of inoculated plants	Number of negative	Number of positive	Proportion of infected plants/ inoculated (%)	Mean for all time points	Median number of weeks for apparition of symptoms	Minimum number of weeks for apparition of symptoms	Maximum number of weeks for apparition of symptoms	Number of plants on which the first symptom was observed on fruits	Number of plants on which the first symptom was observed on leaves	Number of plants on which the first symptom was observed on fruits and leaves	No symptoms
Black cherry												
Infected–4 w	20	3	17	85		8	6	9	0	17	0	0
Infected–8 w	18	3	15	83	87	8	6	21	10	1	4	0
Infected–14 w	16	1	15	94		15	5	16	9	1	2	3
Cupidissimo F1												
Infected–4 w	15	8	8	53		9.5	6	11	2	2	4	0
Infected–8 w	19	9	10	53	65	14	4	22	4	2	2	2
Infected–14 w	9	1	8	89		10	9	11	7	0	0	1

topped mature fruit) or not (uppermost fruit, apex). The symptomatic bottom fruit (4) was the most reliable sample in the positive plants of Saint Jean and Trixi (Fig. 1). Overall, most positive tissues exhibited symptoms; still, PhCMoV was also detected on asymptomatic tissues, mainly situated at the top of the plant, especially for the cultivar Saint Jean d'Angely (Fig. 1). All the positive plants' oldest tissues (sixth old leaf, old middle leaf) were asymptomatic and negative. Overall, tissues from symptomatic fruits or regrowth at the bottom of the plants were the best to observe PhCMoV symptoms in various tomato cultivars and to detect the virus.

Country and historical presence extension

Six symptomatic historical samples from Switzerland, dating back to 1993 (Supplementary Table S1) tested positive for PhCMoV. The presence of PhCMoV has been detected in a new country and has been set back by more than a decade in Europe. The genome of the sequenced sample was deposited in GenBank (accession OQ689795).

Host range extension

During the field surveys, 12 new plant species were identified as natural hosts for PhCMoV, extending the number of known PhCMoV host plant species from 9 to 21. These include *A. sylvestris*, *Chenopodium album*, *Capsicum annuum*, *G. molle*, *H. perforatum*, *Malva sylvestris*, *Mesembryanthemum crystallinum*, *Physalis peruviana*, *Rumex acetosa*, *S. nigrum*, *Tropaeolum majus*, and *V. arvensis* (Supplementary Table S2). Four of them belong to two plant families already known to host PhCMoV (*Polygonaceae* and *Solanaceae*), and eight other plant species belong to new families: *Aizoaceae*, *Amaranthaceae*, *Apiaceae*, *Geraniaceae*, *Hypericaceae*, *Malvaceae*, *Tropaeolaceae*, and *Violacea.* When PhCMoV was detected through HTS, the sequences were deposited in GenBank (accession numbers OQ716531, OQ716532, OQ716533, OQ318170, and OQ318171).

Vein clearing and deformations were observed on the leaves of some of the host plants identified in Belgium by RT-PCR (*Chenopodium album*, *Capsicum annuum*, *Mesembryanthemum crystallinum*, *Malva sylvestris*, *Physalis peruviana*, *R. acetosa*, *T. majus*; Supplementary Fig. S1). However, it was not possible to assess whether the symptoms were caused by PhCMoV, other viruses, or abiotic stress because mixed infection cannot be excluded, and no information was collected on putative abiotic stresses for these plants.

Symptomatology

Greenhouse assays: Experimental host range. HTS and bioinformatic analyses confirmed that the original plant used for inoculation was only infected by PhCMoV (isolate GH24, NCBI accession number OQ689794).

Almost 90% of the control plants ($n = 68$, *N. glutinosa*, *N. benthamiana*, *Petunia* × *hybrida*, *S. lycopersicum*) were successfully inoculated and showed symptoms of vein clearing, deformation, and yellowing (Table 1; Fig. 2). For *T. majus* and *L. trimestris*, 2 of 15 plants were successfully inoculated by PhCMoV (Table 1). Infected *L. trimestris* plants showed weak vein clearing on some of the leaves, whereas the symptoms on *T. majus* were more visible (vein clearing, leaf deformation) and resembled the ones observed in the field (Fig. 2). Three out of five plants of *Stachys affinis* were successfully inoculated, and the plants showed vein clearing and discoloration (Fig. 2), in contrast with the symptomless *Stachys affinis* collected in the field and sequenced previously (accession MZ322957; Temple et al. 2022).

Field survey.

Association of PhCMoV detection with symptomatic eggplants. At site C, 13 symptomatic (vein clearing and deformations) and 109 asymptomatic eggplants were collected in a tunnel and tested for PhCMoV (Supplementary Fig. S2). Most symptomatic plants (11/13) were located near the entrance (Supplementary Fig. S2). The 13 symptomatic samples tested positive. In contrast, 108 asymptomatic plants tested negative. The positive asymptomatic plant showed symptoms on the next visit (4 weeks later).

PhCMoV detection on different tomato cultivars and on R. acetosa*.* At site A, 116 tomato plants belonging to 12 different cultivars showed symptoms of PhCMoV.

PhCMoV was detected on all symptomatic plants tested ($n = 61$) and was negative in the 40 asymptomatic plant pools, whatever the cultivar. These results demonstrated a strong association between the presence of the virus and similar symptoms on various cultivars. In the greenhouse, the presence of symptomatic and positive plants of *R. acetosa* was also mapped (Supplementary Fig. S3).

The most impacted cultivar was BC, with a symptom prevalence of 48%, followed by Gipsy noir, Gustafano F1, Saint Jean d'Angely, and Trixi, recording 5 and 10% of symptom prevalence. No symptomatic plants were observed for Charlie's green and Suzy. The prevalence of symptomatic plants was below 4% for the other seven cultivars (Supplementary Table S3).

Incidence and prevalence on farms and in fields

Prevalence of PhCMoV on Belgian farms. During field surveys conducted in two Belgian provinces on vegetable farms serving local markets, PhCMoV symptoms were observed in 9 of 27 farms (33%) (Fig. 3; Supplementary Table S4). Virus presence was confirmed by RT-PCR on all symptomatic host plants tested (*S. lycopersicum*, *S. melongena*, *Galinsoga parviflora*, *Cucumis sativus*, *Stachys affinis*, *Chenopodium album*, *Capsicum annuum*, *Mesembryanthemum crystallinum*, *Malva sylvestris*, *Physalis peruviana*, *R. acetosa*, and *T. majus*). On five farms where PhCMoV was initially detected, subsequent visits in the following years confirmed each time the presence of the virus (Supplementary Table S4). At site C, the virus was detected on symptomatic plants during four consecutive years.

Field prevalence of PhCMoV within the farms. On the nine farms infested by PhCMoV, the ratio of tomatoes with PhCMoV-like symptoms was used as a proxy for evaluating the virus prevalence.

On most farms (7/9), less than 1.5% of the tomato plants were symptomatic (Fig. 3). The symptomatic plants were mainly distributed at the tunnels' entrances or near openings. At two sites (A and P), the prevalence of the virus in tomato reached 7 and 13%, respectively (Fig. 3). Whereas weeds and other annual plants such as tomatoes were commonly present in most of the visited greenhouses, the culture of perennial plants (sorrel, strawberry, aromatics, etc.) was noticed inside tomato tunnels only for sites A and P (Supplementary Table S4).

At site P, 85 and 200 tomato plants, respectively (belonging to 20 cultivars), were grown in two side-by-side small tunnels (4 × 30 m), and the symptomatic plants were mainly observed in one of the two tunnels, where 38 of 85 tomato plants exhibited PhCMoV symptoms. In the other tunnel, only 2 of 200 plants were symptomatic.

After 2021, the producers of site P removed all the perennial plants and weeds that were present in the highly infected tunnel. The following year (2022), the presence of PhCMoV in the tunnel was only sporadic (two to three tomato plants showed the symptoms), despite the cultivation of PhCMoV host plants (tomato, capsicum, and cucumber). A similarly low number of PhCMoV-infected eggplants was observed outdoors in the same two seasons (2021 and 2022).

Severity: Yield assay

Overall, the inoculation success rate was higher for BC than CU (87 versus 63%), but infection was consistently above 50% for each time point and each cultivar. This rate did not decrease with the plant age for the two cultivars (Table 2). For both cultivars, the number of weeks before the observation of the first symptoms varied between plants, whatever the time point (e.g., symptoms could be observed from 4 to 22 wpi for the second time point in CU).

For BC, the first symptoms following the first inoculation time point were spotted on leaves at a median of 8 wpi. They were mostly found on fruits for the second and third inoculation time points at a median of 8 and 15 wpi, respectively.

For CU, the first symptoms following the first inoculation were spotted on leaves and fruit simultaneously, at a median of 9.5 wpi. After the second inoculation, symptoms were observed more often on fruit than on leaves, at a median of 14 wpi, and those of the third inoculation were all spotted first on fruit at a median of 10 wpi (Table 2).

For both cultivars, total asymptomatic fruit weight was significantly reduced when plants were inoculated 4 weeks and 8 weeks after sowing compared with the control (Fig. 4; Supplementary Table S5). The average yield of asymptomatic fruits (marketable fruits) per plant was only 1 and 35% the expected yield of the healthy plants for the BC-infected plants at the first and second inoculation time points, respectively (Fig. 4). The yield reduction was primarily the result of a reduced fruit number, with all fruits exhibiting symptoms (Supplementary Fig. S4; Supplementary Table S5). For the second inoculation time point, the marketable yield was close to 50% of the control (Fig. 4). The same phenomenon was observed for cultivar CU, although yield reduction at the first and second time points compared with the control was less drastic than for BC (Fig. 4). Yield losses were mainly caused by a degradation in fruit appearance, a reduction in the number of fruits per plant, and a decrease in average fruit weight. However, for both cultivars, a late infection no longer significantly impacted the yield (plants inoculated 14 weeks after sowing).

Transmission: Vector candidate

Leafhoppers belonging to the *Anaceratagallia* genus and present on one of the two most affected sites (A) were collected and used in transmission tests to test if they could transmit PhCMoV.

In the first experiment, the two *Anaceratagallia* leafhoppers (LF43-3 and LF43-4) successfully transmitted the virus to two healthy seedlings (TR47 and TR62), which tested positive for PhCMoV 7 weeks after their contact with the viruliferous insects. PhCMoV was also detected in the insect body of the two insect specimens, even though one had been feeding on a healthy plant for the last 14 days before its death.

Comparison of the COI sequence of the two leafhoppers that have transmitted PhCMoV (LF43-3 and LF43-4) with the NCBI database matched with the accession OK275083 "*Anaceratagallia* sp.," which has not been identified at the species level (Supplementary Table S6).

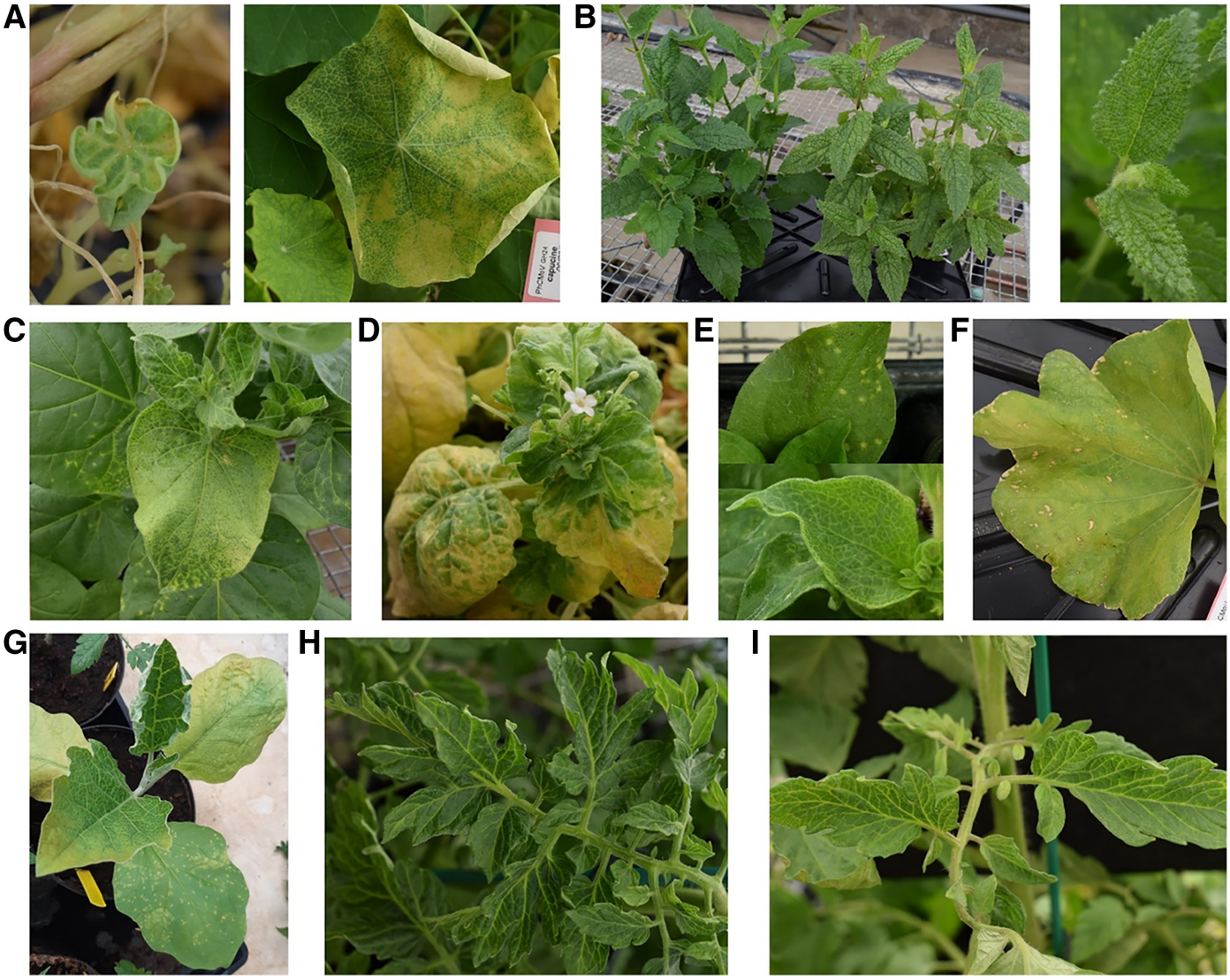

Fig. 2. Symptoms of Physostegia chlorotic mottle virus (PhCMoV) on leaves of different plant species mechanically inoculated by GH24. **A,** *Tropaeolum majus*; **B,** *Stachys affinis*; **C,** *Nicotiana glutinosa*; **D,** *Nicotiana benthamiana*; **E,** *Petunia* × *hybrida*; **F,** *Lavatera trimestris*; **G,** *Solanum melongena*; **H,** *Solanum lycopersicum* 'Black cherry'; and **I,** *Solanum lycopersicum* 'Suzy'.

In the second trial, six additional *Anaceratagallia* leafhoppers were directly put from the field onto six healthy seedlings in a cage (three eggplants and three tomatoes). After 4 weeks, two eggplants were showing vein clearing on new leaves. The symptoms appeared on the third eggplant after 2 more weeks and on two tomato plants 8 weeks after the first contact with the leafhoppers. These five symptomatic plants (out of six) tested positive for PhCMoV. Dead leafhoppers were collected 10 and 23 days after being in contact with the plants, and one of them (LF42b) tested positive for PhCMoV. COI barcoding and sequence homology with the NCBI database were also performed to identify the five remaining insect species. Two specimens (LF42-a and LF42-e) matched accession OK205264 (98% id), namely "*Anaceratagallia* sp.," and one specimen (LF42-b) matched the *Anaceratagallia* sp. accession OK275083 (Supplementary Table S6). The results remained inconclusive for the two other specimens.

Finally, 1 year after the transmission test, a new *Anaceratagallia* specimen was collected, morphologically identified as *A. fragariae* (Supplementary Fig. S5). The COI sequence matched with the accession OK205264 (98% id), which was labeled as *Anaceratagallia* sp. The COI sequence was deposited in GenBank (accession OQ469522).

Discussion

With this study, PhCMoV is now known to be present in 10 European countries. Since its first discovery in 2014, the virus has been detected in diseased plants from economically important species such as tomato, eggplant, and cucumber, highlighting the importance of better understanding its biology (Temple et al. 2022). The framework for the evaluation of biosecurity, commercial, regulatory, and scientific impacts of new viruses revised by Fontdevila Pareta et al. (2023) was followed to fill the knowledge gaps required to understand the phytosanitary risks associated with PhCMoV.

By investigating symptomatic historical samples, PhCMoV was detected in 30-year-old samples from Switzerland, representing its first detection in this country. In parallel, 12 new species were added

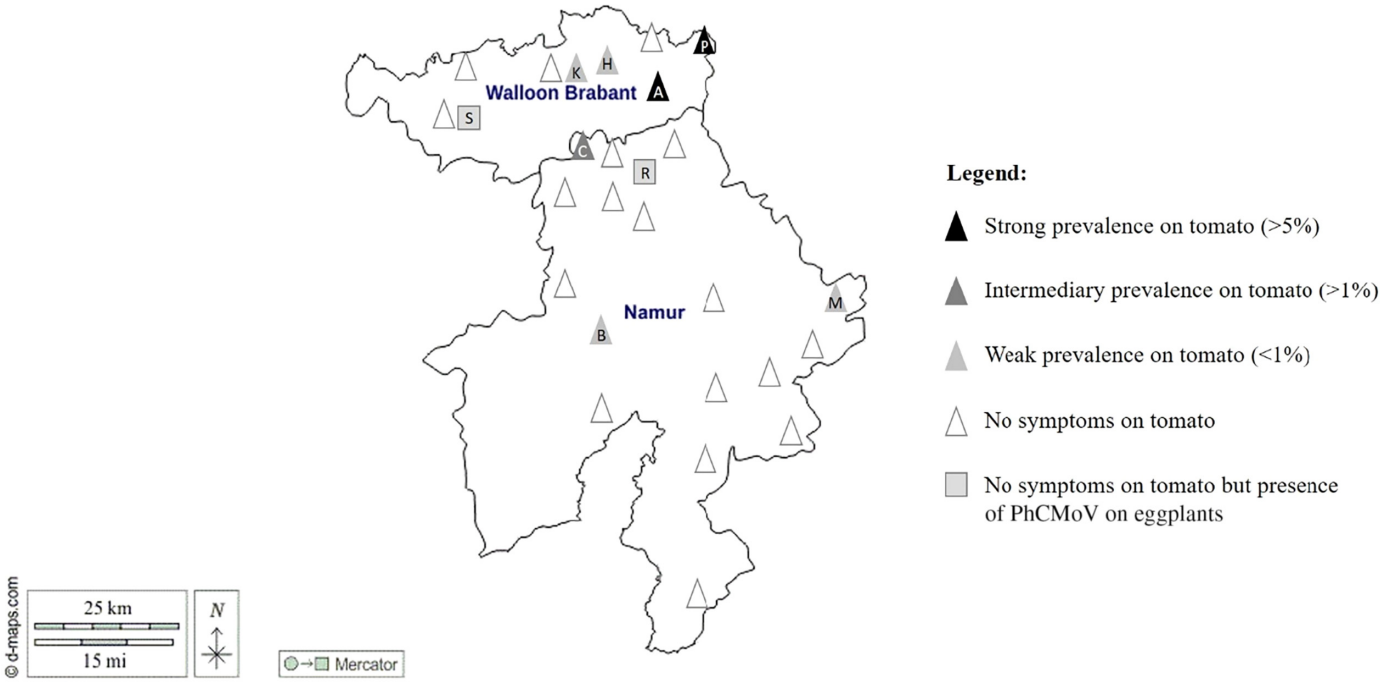

Fig. 3. Distribution and prevalence of Physostegia chlorotic mottle virus (PhCMoV) based on symptom observations in tomato and eggplant (R, S) in the province of Walloon Brabant and Namur (Belgium). The prevalence was calculated based on the number of PhCMoV-symptomatic tomato plants divided by the total number of tomato plants grown at a site (Supplementary Table S7). The different letters correspond to the different sites.

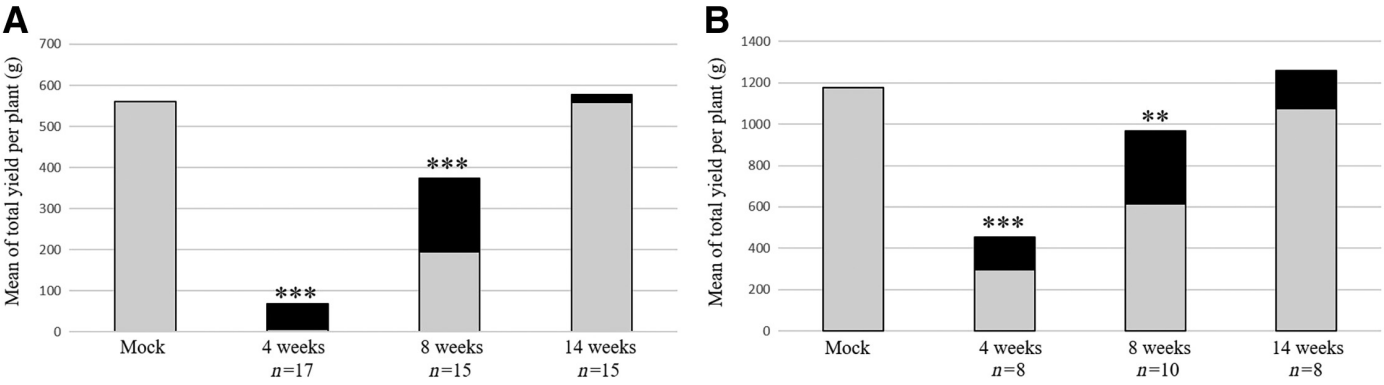

Fig. 4. Mean of total yield (black + gray color), marketable yield (gray color), and unmarketable yield (black) per tomato plant of **A,** Black cherry and **B,** Cupidissimo F1 when the plants were infected at three time points: 4, 8, and 14 weeks after sowing. Mock = control plants inoculated with the buffer only, n = number of plants per conditions. Asterisks indicate statistically significant differences of marketable fruits compared with the mock-treated plants (**, $P < 0.01$; ***, $P < 0.001$). Pictures of marketable (asymptomatic) versus unmarketable fruits are presented in Supplementary Figure S4.

to the virus's natural host range, bringing to 21 the number of plant species susceptible to PhCMoV from 15 plant families. These findings suggest that the actual natural host range is probably wider, given the diversity of the host range identified in 4 years.

To study symptom causality, bioassays were performed in controlled conditions for selected host plants. All the successfully infected plants showed symptoms (72 plants from 12 different plant species). The association of PhCMoV with symptoms in *T. majus* and *L. trimestris*, which belong to two families that host PhCMoV (*Tropaeolaceae* and *Malvaceae*), was assessed, and deformation and vein clearing symptoms were observed. Mechanical inoculations of PhCMoV induced discoloration and yellowing on the leaves of *Stachys affinis.*

Symptoms observed in tomato and eggplant in controlled conditions were identical to those observed in the field (unevenly ripened and deformed fruits, vein clearing and deformed leaves, and dwarfing and shortened nodes for the most impacted plants). For these two host plants, all four criteria to assess symptom causality described by Fox (2020) were fulfilled.

Although the association between PhCMoV and the presence of symptoms is strong on tomato and eggplant, symptoms can be mistaken for those of other plant viruses such as alfalfa mosaic virus for eggplant and tomato brown rugose fruit virus, pepino mosaic virus, or tomato fruit blotch virus for tomato, although none of these viruses was confused with PhCMoV as part of our work (Ciuffo et al. 2020; Temple et al. 2022). Tomato brown rugose fruit virus and pepino mosaic virus exhibit distinct biological properties compared with PhCMoV. These viruses are transmissible through contact and by seeds and can remain stable in the environment (Hanssen and Thomma 2010; Oladokun et al. 2019). Therefore, making a correct diagnosis through laboratory testing in the case of PhCMoV-like symptoms in tomatoes remains crucial.

The symptoms caused by PhCMoV can also be confused with those of EMDV in eggplant, tomato, cucumber, and capsicum. These two viruses have the same transmission mode, and a similar management strategy could be applied (El Maataoui et al. 1985; Roggero et al. 1995). However, except for the South of France, these two viruses have so far been detected in distinct areas: EMDV is endemic and widespread in the Mediterranean Basin, where PhCMoV is so far primarily detected in temperate European countries.

Assessment of the severity of PhCMoV on tomatoes in an experimental setup revealed that it can lead to a 99% reduction in tomato yield. However, the time of inoculation strongly influences its impact on plants. In our experiments, plants infected before the planting date in tunnels (8 weeks after sowing) showed a total loss of marketable fruit yield for one of the two cultivars tested (BC) and a drop of about 75% for the second (CU). A preliminary study on short-lived tomato cultivars (Tom Thumb and Micro-Tom) showed a similar trend in yield loss (Durant 2021). For BC and CU, the impact on yield was reduced when the plants were inoculated at a later developmental stage. This result underlines the importance of safeguarding plants from PhCMoV infection during the early developmental stages. Similar observations were reported in tomatoes infected by tomato yellow leaf curl virus (Levy and Lapidot 2008). Conversely, late infection of tomatoes by pepino mosaic virus had the most pronounced effects on nonmarketability (Spence et al. 2006).

PhCMoV was detected in one-third of the visited diversified farms where vegetables are grown in soil in Belgium. In addition, once the virus was detected on a farm, it was systematically detected the following year(s) (for the five sites that were revisited), suggesting the persistence of the virus in the environment. However, the prevalence of the virus in the field was very limited ($<1\%$) at all but two sites where the prevalence was higher than 7%. The presence of perennial hosts in the direct vicinity of tomatoes in tunnels was noted in the two most affected sites (A and P) and could account for the high virus prevalence on crops. This hypothesis was confirmed by the drastic reduction in the incidence of PhCMoV observed at site P between 2021 and 2022 after elimination of all perennials and weeds in a tomato tunnel.

The spread of plant rhabdoviruses is mainly driven by the specificity and efficiency of their arthropod vector in which they also replicate (Whitfield et al. 2018). The transmission of PhCMoV has been demonstrated for an unidentified species of the *Anaceratagallia* genus. Two distinct species of the *Anaceratagallia* genus were isolated from cultivated sorrel (*R. acetosa*): *A. fragariae* identified morphologically and an unidentified *Anaceratagallia* sp. Based on their COI sequences, these two species were previously described at a same site on a wild strawberry plant (*Fragaria vesca*) in the Czech Republic, suggesting that they cohabit (Fránová et al. 2021).

In the near future, it is crucial to identify the vector of PhCMoV at the species level and to investigate if multiple *Anaceratagallia* species can transmit the virus. Many aspects of the ecology and behavior of *Anaceratagallia* are lacking (Hogenhout et al. 2003; Whitfield et al. 2018). After that, studying the ecology and behavior of PhCMoV vectors will provide a better understanding of the emergence of the disease and could account for the sudden multiple detections of PhCMoV after decades of unnoticed presence. This work will also make it possible to develop more appropriate management strategies specifically targeting plants that are suitable for the vector reproduction or survival during winter. The ability to rear these leafhoppers will also greatly accelerate the research because it is impossible to differentiate species among living individuals and females morphologically. This would also permit testing the transovarial vertical transmission of PhCMoV. In this study, we observed that *A. fragariae* can mate, reproduce, and complete a full life cycle on *R. acetosa* in the laboratory, as shown by Tishechkin (2020). Regarding their behavior, our observations also revealed that *Anaceratagallia* leafhoppers were not very mobile. This was also supported by the distribution of the virus on farms, generally in patches, often close to the entrance of the tunnels. The proximity of the crop to perennial plants in which they can mate and complete an entire life cycle can contribute to the development of the disease.

The rapid detection of PhCMoV in European temperate areas where EMDV has never been reported can be due to agricultural practices. There has been an increase in the number of producers in Belgium who are cultivating a wide range of plant species (20 to 45) over a limited area (<2.5 ha) (Dumont and Baret 2017). The virus was mainly detected in this type of structure where producers often promote sustainable farming, diversity, and natural regulation of pests and where exchanges between natural ecosystems and cultivated plants or between different cultivated plant species are more common than in controlled greenhouses (Boeraeve et al. 2020; Dumont and Baret 2017; Tamburini et al. 2020; Temple et al. 2023).

All the steps of the optimized scientific and regulatory framework for the characterization and risk analysis of a new virus (Fontdevila Pareta et al. 2023) were compiled, and almost all the characterization criteria proposed by Rivarez et al. (2021) have now been met. Overall, this plant rhabdovirus can pose a threat to tomato and other vegetable and ornamental crops. However, with a better understanding of its biology and agricultural practices, management measures can be proposed to mitigate an epidemic. The findings of this work have already resulted in an efficient management solution to solve problematic epidemics of PhCMoV on tomatoes grown under tunnels, as shown at site P.

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