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Short Notes

First report of strawberry mild yellow edge virus in Bosnia and Herzegovina

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Summary. In 2023, a survey assessed 120 strawberry leaf samples collected from regions of Bosnia and Herzegovina (BiH) for presence of arabis mosaic virus (ArMV), strawberry mottle virus (SMoV), strawberry latent ringspot virus (SLRSV), and strawberry mild yellow edge virus (SMYEV), which are among the most common and internationally widespread viruses affecting strawberry plants. All samples were tested using Double Antibody Sandwich ELISA (DAS-ELISA) and RT-PCR. SMYEV was detected in 19 samples, 17 of which were from the variety Clery and two from Alba. ArMV, SLRSV, and SMoV were not detected in any of the samples. Sequence analyses of 405 nucleotides within the replicase and 25k triple gene block protein genes from 19 SMYEV isolates showed nine sequence variants. The least nucleotide similarity (83%) was observed with a Chinese SMYEV isolate, FJ1 (GenBank accession number OK562580), while greatest similarity (99.3%) was found with a German isolate, MY-18 (GenBank accession number NC_003794). Phylogenetic analysis showed that the BiH SMYEV isolates formed a distinct cluster closely related to the Argentinian isolate Berra-2 (GenBank accession number KX150372) and the German isolate, MY-18 (GenBank accession number NC_003794). This is the first report of SMYEV in BiH. Further research is required to determine whether additional viruses or other pathogens may be contributing to the symptoms observed in the field.

Keywords. Strawberry, DAS-ELISA, RT-PCR, sequence analysis.

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INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is an important crop in Bosnia and Herzegovina (BiH). In 2020, the 1,337 hectares, with an average yield of 8.5 tonnes ha^{-1}. More than 30 viruses and phytoplasmas are known to infect strawberries, often causing poorly defined or latent symptoms in single infections. However, mixed infections are also frequently associated with stunted

plant growth and substantial yield losses (Maas, 1998; Babini *et al*., 2004; Kwon *et al*., 2019). Among the most economically significant strawberry viruses are arabis mosaic virus (ArMV; *Secoviridae*, *Nepovirus*, *Nepovirus arabis*), strawberry mottle virus (SMoV; *Secoviridae*, *Sadwavirus*, *Sadwavirus fragariae*), strawberry latent ringspot virus (SLRSV; *Secoviridae*, *Stralarivirus*, *Stralarivirus fragariae*), and strawberry mild yellow edge virus (SMYEV; *Alphaflexiviridae*, *Potexvirus, Potexvirus fragariae*). These viruses can cause severe damage, particularly when occurring in mixed infections with other viruses.

Strawberry cultivation is widespread in BiH, with the regions of Ilidža, Čelić, Gornji Vakuf, Čapljina, and Živinice recognized as key production areas. In 2023, virus-like symptoms and significant decline in strawberry yields were observed in several locations of these regions. In response, a preliminary laboratory investigation was conducted to assess the presence of major viruses known to infect strawberries. The results from this diagnostic survey are presented in this paper.

MATERIALS AND METHODS

Source of plant material

A total of 120 strawberry leaf samples were collected from 6-month-old plants from Ilidža, Čelić, Gornji Vakuf, Čapljina and Živinice. The samples were from several strawberry varieties: 62 from Clery, 28 from Mjesecarke, 12 from Alba, and ten from Asia. Additionally, two samples were collected from each of the varieties Senga Sengana, Elsanta, Joly, and Arosa. Most of the sampled plants were asymptomatic, while 32 plants had virus-like symptoms, including vein clearing, and leaf mottling, and yellowing. During field inspections, all plant samples were carefully examined, using a hand lens in the field and a stereomicroscope in the laboratory, for the presence of insects, particularly strawberry mites which commonly infest this crop, but no mites were detected. The collected samples were brought to the laboratory for serological and molecular analyses to detect virus presence.

DAS-ELISA, RT-PCR and bioinformatic analysis

All the strawberry leaf samples were initially screened serologically using the Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA), as described by Clark and Adams (1977). Polyclonal antibodies specific to ArMV, SLRSV, and SMYEV were used according to the manufacturer's protocol (BIOREBA AG). For molecular detections, total RNA was extracted from 100 mg of leaf vein tissue, which was homogenized in 1 mL of grinding buffer containing 4.0 M guanidine thiocyanate, 0.2 M sodium acetate (pH 5.2), 25 mM EDTA, 1.0 M potassium acetate, and 2.5% (w/v) polyvinylpyrrolidone-40 (PVP-40). RNA purification was carried out using silica particle-based extraction, following the protocol described by Foissac *et al.* (2001). Total RNAs were extracted from grapevines infected with SLRSV (isolate T-29, France) and ArMV (isolate Fr2-, Germany), both previously characterized by Digiaro *et al*. (2007) and maintained in the grapevine collection at IAM Bari, Italy, as well as from the SMoV DSMZ isolate PV-1268. These samples were used as positive controls in the RT-PCR assays. In parallel, healthy plant material derived from certified grapevine and strawberry plants was used as negative controls in the RT-PCR assays. RNA samples with A260/280 ratios between 1.8 and 2.1, A260/230 ratios >1.8, and concentrations above 50 ng μL^{-1} were employed for downstream complementary DNA (cDNA) synthesis.

Reverse transcription was carried out performed using 0.5 µg of total RNA and 0.5 µg of random hexamer primers (Sigma-Aldrich) and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Thermo Fisher Scientific), incubated at 39°C for 1 h, followed by enzyme inactivation at 70°C for 10 min. The resulting cDNA was subjected to RT-PCR using virus-specific primers targeting all four viruses (Thermo Fisher Scientific) (Table 1), using the PCR conditions detailed in Table 1. Amplification products were analyzed by electrophoresis on 1.2% agarose gel with 1× Tris-Borate-EDTA buffer. After purification, the PCR amplicons were bidirectionally sequenced at Eurofins Genomics (Konstanz, Germany), by Sanger sequencing using both forward and reverse primers. The obtained sequences were compared using the BLASTn algorithm on the NCBI platform (Altschul *et al*., 1990). Sequence similarity matrices and a phylogenetic tree were constructed using the Maximum likelihood method with 1000 bootstrap replicates, implemented in Geneious Prime v2020.2.5 program (Dotmatics, Boston, United States of America, Auckland, New Zealand).

RESULTS AND DISCUSSION

Virus detection and sequence analysis

ELISA and RT-PCR assays exclusively detected SMYEV. ArMV, SLRSV, and SMoV were not detected by DAS-ELISA, and were also not detected when RT-PCR was used as a supplementary method to detect potentially

Table 1. List of primers used in RT-PCR assays for amplifying partial genome sequences of SMYEV, ArMV, SLRSV and SMoV.

Virus	Primers	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
SMYEV	SMYEV-C	TGCACTCTGTGTTGACCTTC	405	Kaden-Kreuziger *et al.* (1995)
	SMYEV-B	ATACTCGTCTACGAAGGCT		
ArMV	ArMV-5A	TACTATAAGAAACCGCTCCC	302	Faggioli *et al.* (2005)
	ArMV-3A	CATCAAAACTCATAACCCAC		
SLRSV	SLRSV-5D	CCCTTGGTTACTTTTACCTCCTCATTGTCC	291	Faggioli *et al.* (2002)
	SLRSV-3D	AGGCTCAAGAAAACACAC		
SMoV	Smdetncr4a	TAAGCGACCACGACTGTGACAAAG	460	Thompson & Jelkmann (2003)
	Sm2ncr1b	ATTCGGTTCACGTCCTAGTCTCAC		

low virus concentrations. Both methods yielded consistent results, identifying 19 of the 120 samples as SMYEV-positive, corresponding to an infection rate of 16 %, of which six (32%) were symptomatic. The variety Clery accounted for most of the SMYEV infections (17 samples), while only two infected samples were detected in the Alba variety.

All sequences obtained in this study have been deposited in GenBank under accession numbers PP489404 and PP530291 to PP530298. Comparative nucleotide analyses gave the lowest sequence similarity (83%) with the Chinese SMYEV isolate, FJ1 (GenBank accession number OK562580), and greatest similarity with the German SMYEV isolate, MY-18 (GenBank accession number NC_003794). Among the 19 SMYEV-positive samples, nine sequence types were distinguished, partly spanning the replicase and the 25k triple gene block protein genes. The intra comparison of the nucleotide sequences resulted in similarities from 89.4% to 99.3%, indicating the coexistence of genetically divergent and closely related viral strains in BiH.

SMYEV prevalence in BiH strawberries

SMYEV was detected in Ilidža, Čelić, and Živinice. The predominance of infections in the Clery variety may indicate greater susceptibility of this variety to virus infections, or reflect effects of regional propagation practices, such as the exchange of infected monovarietal plant material, on virus spread. The absence of ArMV, SLRSV, and SMoV in all tested samples indicates either limited presence of these viruses in the surveyed areas or the effectiveness of current disease management strategies, as these viruses are typically associated with severe symptoms in strawberry crops and growers often remove affected plants at the first signs of infection.

The two SMYEV-infected strawberry plants of the Alba variety, as well as 11 infected Clery plants, all had no visible symptoms. Only six infected plants exhibited mild leaf yellowing, which resembled symptoms typically associated with SMYEV (Figure 1). The causal agent of the virus-like symptoms observed in the other 13 symptomatic plants were unclear, as none of the four assessed viruses tested were detected in these samples. Involvement of other potential pathogens, such as strawberry-infecting viruses not assessed in this study, as well as phytoplasmas or viroids, cannot be excluded. Hence, further investigations are required to clarify the underlying etiology of the symptomatic plants that gave negative virus detections.

Phylogenetic analyses

The phylogenetic analyses based on the nucleotide sequences of SMYEV isolates showed presence of two distinct clusters (Figure 2). All the BiH SMYEV isolates grouped within Cluster II, alongside the German isolate MY-18 (GenBank accession number NC_003794). This clustering pattern, derived from partial RNA replicase and 25K triple gene block sequences, aligned with previously reported phylogenetic groupings. Notably, similar genetic stratification into two major groups has also been observed when analyses are based on coat protein (CP) sequences of SMYEV (Cho *et al*., 2011). This consistent clustering, supported by RNA replicase and 25K triple gene block sequences, reinforces previous findings, and highlights the genetic stability of SMYEV differentiation into two major phylogenetic groups. These groups probably reflect geographic origins rather than differences in host ranges or pathogenicity. Further research is required to determine whether these genetic clusters correlate with biological virus traits, such as virulence or transmission dynamics.

The present study is the first to record occurrence of SMYEV in BiH. Although SMYEV infection alone is generally not considered highly detrimental to most

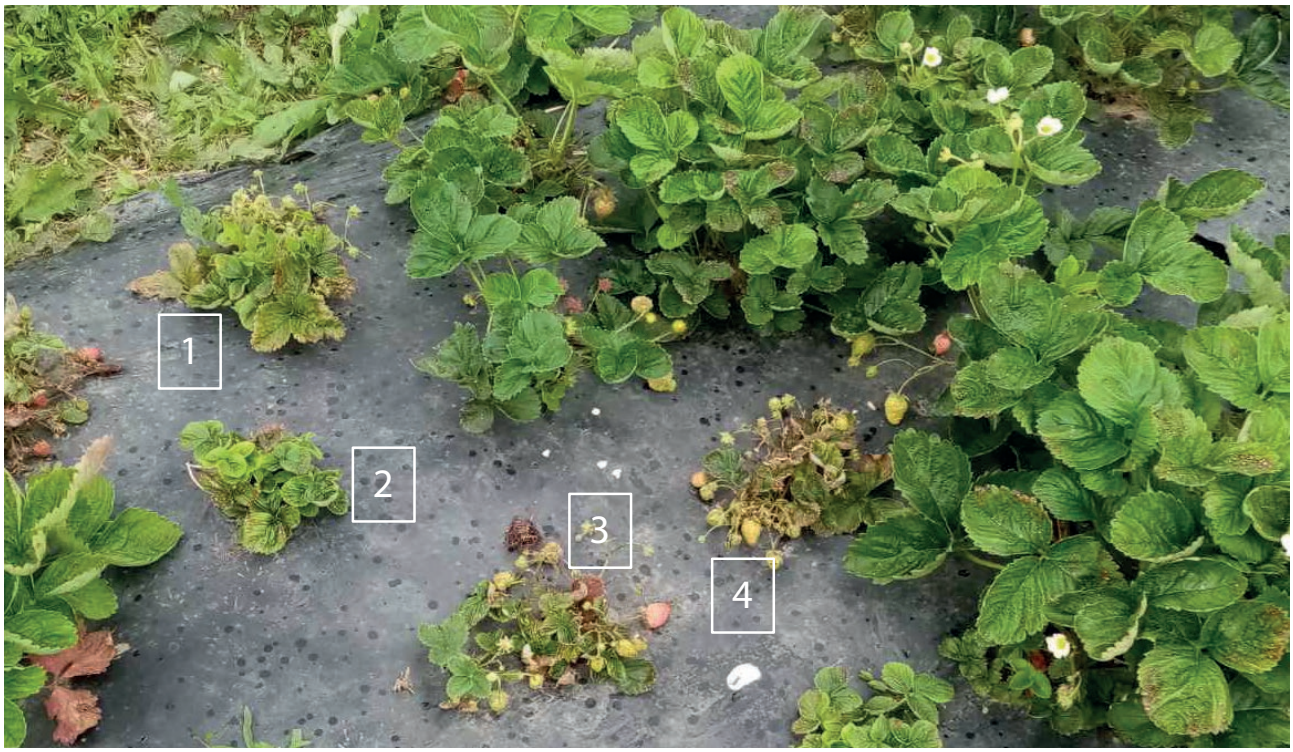

Figure 1. Strawberry plants (variety Cleary) infected with SMYEV, confirmed by DAS-ELISA and RT-PCR, showing leaf yellowing and stunting.

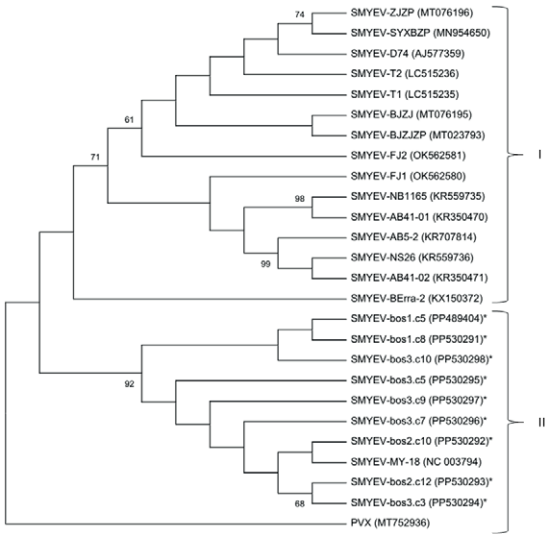

Figure 2. Maximum-likelihood phylogenetic tree constructed based on the nucleotide sequence alignment of partial RNA replicase and 25K triple gene block regions from SMYEV isolates originating from Bosnia and Herzegovina (indicated by asterisks), and for homologous sequences retrieved from the GenBank. Sequence accession numbers are shown in parentheses. Potato virus X (PVX) was included as an outgroup. Bootstrap support values greater than 60% (based on 1,000 replicates) are indicated at the corresponding branch nodes; values below <60% are not shown.

strawberry varieties, it rarely occurs as a single infection, thereby complicating the assessment of its actual economic impact. This is reflected in the findings of 19 SMYEV-infections, of which only 6 (32%) exhibited SMYEV-like symptoms, while the remaining 13 plants were asymptomatic. Consequently, further research is needed to investigate potential mixed infections involving other common strawberry-infecting pathogens (viruses, phytoplasmas and viroids), to better understand the overall pathological landscape and its implications for the strawberry production in the country.

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