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RESEARCH PAPER

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DETECTION AND MOLECULAR CHARACTERISATION OF THE *Orthotospovirus iridimaculaflavi* N GENE OF THE ONION IN POLAND

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ABSTRACT

Orthotospovirus iridimaculaflavi (iris yellow spot orthotospovirus, IYSV) is one of the most important viral pathogens affecting onions worldwide. The virus is predominantly transmitted by onion thrips and can cause significant losses in cultivated fields. In this study, the identification and molecular characterisation of IYSV isolates originating from onion crops in Poland were carried out. Symptomatic onion plants (*Allium cepa* L.) were observed in Kujawsko-Pomorskie, Lubelskie, and Wielkopolskie regions of Poland. The plants were characterised by various disease symptoms from mild to severe, resulting in deformation and growth reduction. A total of 40 onion samples were collected, and the presence of IYSV was confirmed in three of them. Moreover, the occurrence of *Potyvirus cepae* (onion yellow dwarf virus, OYDV) in single and mixed infections with IYSV was observed. The sequences of IYSV obtained in this study were compared with the sequences retrieved from the GenBank database, and the phylogenetic analysis was subsequently conducted. The maximum-likelihood reconstruction revealed that the Polish isolates mainly grouped with isolates originating from Serbia. To our knowledge, it is the first report of IYSV infecting onions in Poland.

Keywords: iris yellow spot orthotospovirus, IYSV, *Allium cepa* L., RT-PCR, phylogenetic analysis

INTRODUCTION

The onion (*Allium cepa* L.) is one of the valuable vegetables that holds the top vegetable crops cultivated in Poland. Based on the latest statistics released by the Central Statistical Office in 2023, the area of onion cultivation in Poland covered 22,133 hectares, producing approximately 0.6 million tons.

Onions can be infected by numerous pathogens, including bacteria, fungi, phytoplasmas, and viruses, which threaten its cultivation, growth, and production [Schwartz and Mohan 2016]. In previous years, the most common virus infecting onion crops in Poland was *Potyvirus cepae* (onion yellow dwarf potyvirus, OYDV), belonging to the *Potyvirus* genus [Taberska et al. 2021]. This virus causes streaking, yellowing, and bending of leaves. Another common virus in onion cultivation worldwide is *Orthotospovirus iridimaculaflavi* (iris yellow spot orthotospovirus, IYSV). IYSV is a member of the Tospoviridae family, within the order *Elliovirales* and the genus *Orthotospovirus*. The virus was first observed in onion fields in Brazil in 1981 [De Avila et al. 1981] and on iris and leek in the Netherlands [Derks and Lemmers 1996, Cortês et al. 1998]. IYSV is widespread in most regions of the world where *Allium* species are cultivated. Its presence has been documented across several continents, including: North America, South America, Europe, Asia, Africa, and Oceania [Tabassum et al. 2021]. In Poland, it was first detected in 2005 on chrysanthemum crops [Balukiewicz and Kryczyński 2005].

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IYSV infects a variety of hosts, including onions, garlic, chives, leeks, several ornamental plants such as chrysanthemums, and weeds [Bag et al. 2015, Balukiewicz and Kryczyński 2005, Tabassum et al. 2021]. The virus is efficiently transmitted by onion thrips (*Thrips tabaci* Lindeman) and with less efficiency by tobacco thrips (*Frankliniella fusca* Hinds) [Srinivasan et al. 2012]. In recent years, the virus has caused significant economic losses in onion cultivation in the United States and several other countries, which has been associated with the increasing incidence of thrips in these regions [Shin and Rho 2014, Bag et al. 2015, Tabassum et al. 2021].

The virus induces morphological alterations characterised by yellow to bleached-white rhomboid lesions on the leaves, reduces bulb development, and disrupts photosynthetic activity. These effects collectively lead to a significant decrease in crop yield and create entry points for other pathogens and secondary colonizers [Nischwitz et al. 2007]. IYSV particles are helical, measuring 80–120 nm, and consist of RNA, protein, glycoprotein, and lipids [Bag et al. 2015].

The IYSV genome consists of three single-stranded RNAs: small RNA (S RNA), medium RNA (M RNA), and large RNA (L RNA) [Clabbers et al. 2014]. The L RNA is 8880 nucleotides long and contains a single open reading frame (ORF) of 8621 nucleotides in the viral complementary (vc) strand, potentially coding for the viral RNA-dependent RNA polymerase (RdRp). The M RNA is 4821 nucleotides long with two ORFs in ambisense arrangement, potentially coding two glycoproteins (GN and GC) and a non-structural protein (NSm). The S RNA is 3105 nucleotides long and encodes for two non-overlapping ORFs in an ambisense arrangement: the nucleocapsid (N) and the non-structural (NSs) proteins. The S RNA structures of viruses representing the *Orthotospovirus* genus have been found to interact with the viral proteins N and NSs, likely involving the binding of NSs to RNA [Clabbers et al. 2014].

IYSV has not been reported in onion cultivation in Poland so far. However, in recent years, symptoms resembling those caused by orthotospoviruses have been observed in onion crops. Therefore, in this study we analysed the occurrence of IYSV and OYDV in onion crops. Moreover, the phylogenetic relationships of IYSV isolates were established.

MATERIALS AND METHODS

Plant material

During two consecutive vegetative seasons (2023 and 2024), a survey of onion crops in Poland was conducted. A total of 40 leaf samples, including 6 from Lubelskie (50.909722°N; 22.834444°E), 24 from Wielkopolskie (52.1194°N; 16.7886°E), and 10 from Kujawsko-Pomorskie (52.849444°N; 18.476389°E) regions, were collected. The samples were stored at –80 °C until further analyses.

Total RNA extraction

Total RNA from the symptomatic and asymptomatic leaf samples (100 mg of leaf tissue) was extracted using the TRI Reagent procedure (Thermo Fisher Scientific, Wilmington, DE, USA) and dissolved in 30 µl of sterile water. Purity and concentration of obtained RNAs were measured by using the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

RT-PCR (reverse transcription polymerase chain reaction)

First-strand complementary DNA (cDNA) synthesis was done using SuperScript III (One-Step RT-PCR System with Platinum™ Taq DNA Polymerase, Thermo Fisher Scientific, USA). The samples were tested for the presence of OYDV and IYSV using previously published primers (Table 1) [Manglli et al. 2014, Shin and Rho 2014]. The reaction mixture consisted of 22 µl of sterile water, 25 µl of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, USA), 2 µl of forward and reverse primer mixtures, and 1 µl of template cDNA. The PCR reaction was carried out in a thermal profile as previously described [Manglli et al. 2014, Shin and Rho 2014].

To obtain full-length nucleocapsid (N) protein gene sequences of IYSV, the new primer pair (Table 1) was designed using OligoAnalyzer, based on the set of IYSV sequences retrieved from GenBank. The PCR reaction was optimized using designed primers in a T-Professional thermocycler with a temperature gradient block (Biometra, Germany) with a programmed temperature gradient from 45°C to 55°C. The reaction mixture was prepared as described above and followed the manufacturer's protocol.

Obtained PCR products were separated in 1% agarose gel with Midori Green dye (Nippon Genetics Europe, Düren, Germany) with GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, USA). Results were observed with

a UV transilluminator (Vilber, France). The PCR products of appropriate size were purified using NucleoSpin®Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany), according to the manufacturer's protocol. After PCR, the products were sequenced using the Sanger method by an external company (Genomed S.A., Warsaw, Poland) to verify their specificity.

Table 1. Primers used for the detection of IYSV and OYDV in RT-PCR reactions [Manglli et al. 2014, Shin and Rho 2014]

Primers	Sequence 5'– 3'	Product size (bp)	Melting temperature (°C)	Reference
IYSV_N30 IYSV_C40	GCTCGTAAGTTGAGAATCTGC TGGACATTCAGGAGGTTG	307	50	Shin and Rho 2014
IYSV-NgeneR IYSV-NgeneF	CTCTTAAACACATTTAACAAGCA TAAAACAAACATTCAAACAA	1069	53	designed in this study
OYDV-NibCPF1 OYDV-NibCPR1	CATCCAGATCACGAGGGAAT TGTGGCATTTCGGTATTCAA	987	52	Manglli et al. 2014

Selective pressure analysis

IYSV nucleocapsid (N) protein gene sequences of the Polish isolates, consisting of 822 nucleotides, were examined, edited and compiled using BioEdit software [Hall 1999]. Subsequently, the obtained sequences were compared with 46 other IYSV nucleocapsid (N) protein gene sequences available in the GenBank database. The collected sequences were aligned using the MUSCLE algorithm as implemented in MEGA X software [Kumar et al. 2018].

Selective pressure analysis in plant viruses helps to understand how viruses evolve and adapt to their environment [Hasiów-Jaroszewska et al. 2014, LaTourrette and Garcia-Ruiz 2022]. Our analysis of selective pressures was based on ω value estimated for each codon in the nucleotide sequence alignment. The selective pressure affecting individual codons was then analysed using the Datamonkey Adaptive Evolution Server [Weaver et al. 2018] with four algorithms: Mixed Effects Model of Evolution (MEME), Fixed Effects Likelihood (FEL), Fast Unconstrained Bayesian Approximation (FUBAR), and Single-Likelihood Ancestor Counting (SLAC). The selective pressures were quantified by estimating the ratio of non-synonymous (d_N) to synonymous (d_S) substitution rates ($\omega = d_N/d_S$), with $\omega > 1$ indicating diversifying selection, $\omega = 1$ neutral selection and $\omega < 1$ purifying selection [Lemey et al. 2009]. Thresholds for statistical significance were set at $p < 0.05$ for MEME, $p < 0.1$ for FEL and SLAC, and a posterior probability > 0.9 for FUBAR, in line with the Bayesian approach.

Recombination analysis

Prior to phylogenetic analysis, the potential recombination events in the analysed IYSV population were examined by the Recombination Detection Program 4. The analysis was performed by seven different detection algorithms: RDP, Chimaera, BootScan, 3 Seq, GENECONV, MaxChi, SiScan [Martin et al. 2015]. Default parameters and a p-value threshold of 0.05 were used. The recombination events were considered statistically significant if five or more methods had a $p < 0.05$. To confirm the results, an analysis of recombinants was also conducted using the SplitsTree program [Huson and Bryant 2006].

Sequence analysis

Phylogenetic analysis was carried out using the maximum-likelihood algorithm implemented in MEGA X with 49 full-length sequences of the IYSV nucleocapsid (N) protein gene (Table 2). *Orthotospovirus arachinecrosis* (peanut bud necrosis virus, PBNV, accession number: MG913145) was selected as the outgroup. The Tamura-3 nucleotide substitution model with gamma distribution (T92 + G) [Kumar et al. 2018] was applied, along with 1000 random pseudoreplicates. Sequence identity matrices were prepared using BioEdit [Hall 1999] and Sequence Demarcation Tool Version 1.2 (SDTv1.2) [Muhire et al. 2014].

Table 2. Host plant, geographic origin, collection date, and GenBank accession numbers of IYSV isolates used in phylogenetic analysis

Accession no.	Location	Host	Collection date
EU477515	New Zealand	*A. cepa*	2007
OP357939	Germany	*A. ampeloprasum*	–
FJ785835	Greece	*A. cepa*	2008
AY345227	Australia	*A. cepa*	–
MG065699	Iran	*A. porrum*	2015
MF420346	Iran	*A. ampeloprasum*	2015
MF431883	Iran	*A. cepa*	2015
HQ148173	Iran	*A. cepa*	2009
MF431884	Iran	*A. cepa*	2016
HQ148174	Iran	*A. cepa*	2009
MF420345	Iran	*A. cepa*	2015
DQ233479	USA	*A. cepa*	–
AB871456	Japan	*A. cepa*	–
AB505813	Japan	*A. chinense*	2008
AB121025	Japan	*E. russellianum*	–
AB871444	Japan	*A. cepa*	–
AB871438	Japan	*A. cepa*	–
AB180922	Japan	*Alstroemeria*	–
AB180920	Japan	*A. cepa*	–
AB871451	Japan	*A. cepa*	–
AB180918	Japan	*E. russellianum*	–
AB871455	Japan	*A. cepa*	–
GU901211	Sri Lanka	*A. porrum*	2009
FJ514257	USA	*A. sativum*	2008
DQ233476	USA	*A. cepa*	–
AY345226	Australia	*A. cepa*	–
AF271219	Israel	*E. russellianum*	–
DQ270004	India	*A. cepa*	–
KJ868797	India	*A. cepa*	2014
EU310292	India	*A. cepa*	–
EU310280	India	*A. cepa*	–
EU310270	India	*A. cepa*	–
EU310300	India	*A. cepa*	–
KC161369	Egypt	*A. cepa*	2012
AF067070	Brazil	*A. cepa*	–
JX861126	Bosnia and Herzegovina	*A. cepa*	2012
JQ973065	USA	*A. cepa*	2011
MH172159	Pakistan	*A. sativum*	2017
KT272884	Serbia	*A. cepa*	2014
KT272879	Serbia	*A. cepa*	2014
JQ973066	USA	*A. cepa*	2009
EU727180	Serbia	*A. cepa*	2007
EU287943	Canada	*A. cepa*	–
MH172160	Pakistan	*A. cepa*	2017
KX147286	USA	*A. cepa*	2015
MF359019	Zimbabwe	*A. sativum*	2015

Note: – data not available

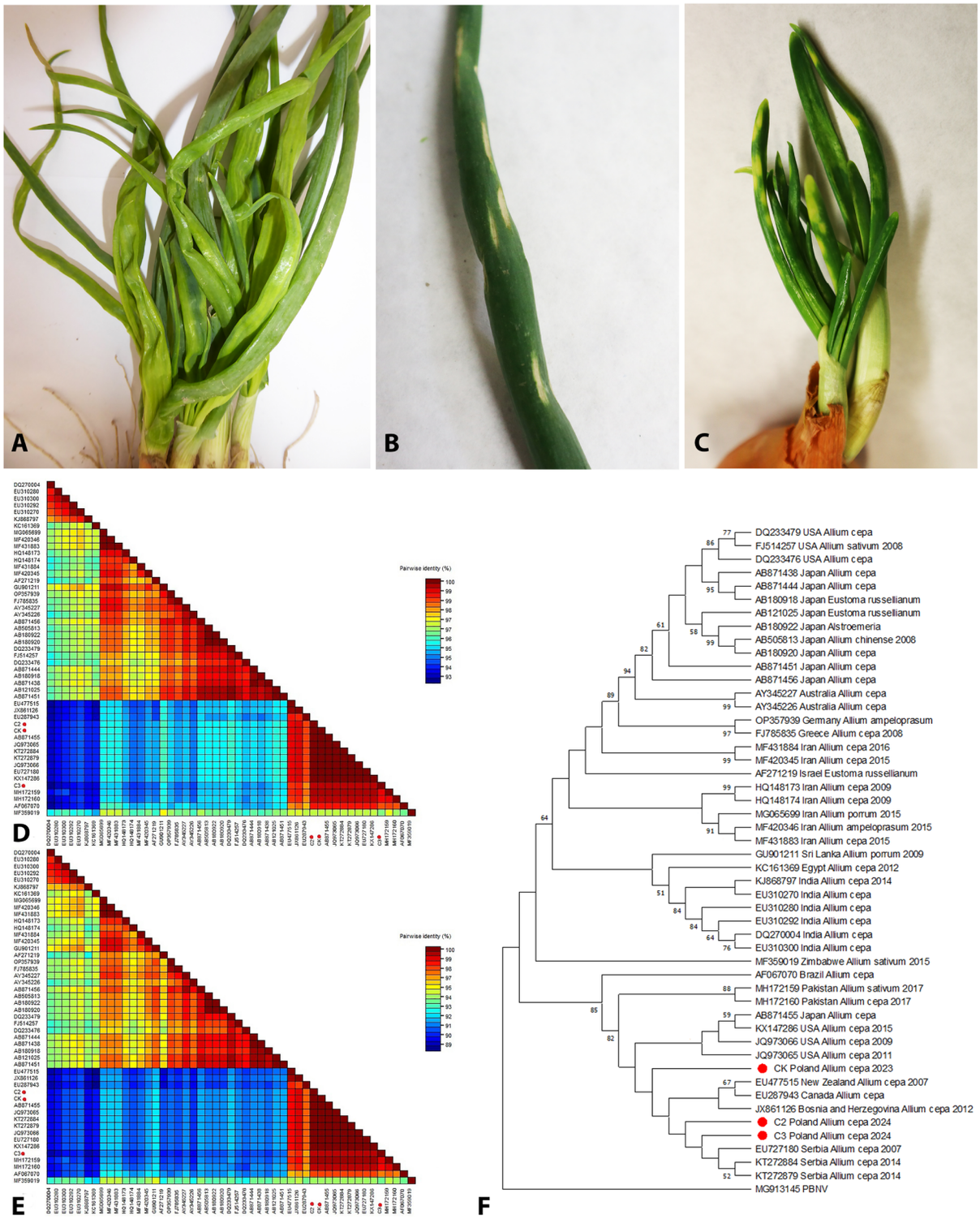
RESULTS

Detection of the viruses in collected samples

During 2023–2024, the monitoring of onion fields in Lubelskie, Wielkopolskie, and Kujawsko-Pomorskie regions of Poland was carried out. Samples were collected from symptomatic and asymptomatic onion plants.

The presence of the viruses was analysed using RT-PCR method with the specific primer pairs. OYDV was detected in five analysed samples collected from Lubelskie and Wielkopolskie regions, with the symptoms in the form of bending of leaves (Figure 1A) and yellow stripes on leaf blades. IYSV was confirmed in three samples from

Figure 1. A) Onion infected with OYDV; B) Onion infected with IYSV; C) Onion infected with IYSV and OYDV; D) Two-dimensional visualization of nucleotide and E) amino acids sequence identity of 49 IYSV isolates examined in this study. The Polish isolates are marked by red dots. The matrices were performed using SDTv1.2.; F) A phylogenetic tree of IYSV isolates created in MEGA X. The Polish isolates, obtained in this study, are marked by red dots



the same regions. These plants displayed characteristic symptoms in the form of irregular yellow-to-bleached, white-coloured changes on leaves (Figure 1B). Moreover, in one of the analysed samples, the presence of mixed infection of OYDV and IYSV was noticed. The infected plant showed symptoms of severe mosaic and leaf deformation (Figure 1C). In the remaining 32 samples, none of the tested viruses was detected.

To obtain the full-length sequence of the nucleocapsid (N) protein gene of the identified IYSV isolate, RT-PCR with newly designed primers was carried out. For the primers designed in this experiment, a temperature of 53°C was selected, as it yielded a product with the highest efficiency and a length of 1069 bp. The specificity of the obtained products was confirmed by Sanger sequencing.

As a result of sequencing, three sequences of the gene encoding the IYSV nucleocapsid (N) protein were obtained: CK 2023 (GenBank accession number PV036881) originated from Lubelskie region, C2 2024 (accession number PV036879) originated from Wielkopolskie region, and C3 2024 (accession number PV036880) originated from Wielkopolskie region.

Recombination and selective pressure analysis

The recombination analyses did not detect any recombination events in the analysed data set, allowing all sequences to be included in further phylogenetic analyses. The strength of selection acting on individual codons was evaluated using four algorithms: MEME, FUBAR, FEL and SLAC. The MEME algorithm detected the presence of positive selection ($\omega > 1$) in 8 codons (at positions 2, 85, 141, 179, 265, 266, 274, and 277), FUBAR detected the presence of positive selection in 3 codons (positions 111, 141, and 227), and FEL identified 4 codons (positions 2, 111, 141, and 264) under positive selective pressure. The SLAC algorithm did not indicate any positive selection. Negative selection ($\omega < 1$) was detected in 94 codons using the FEL algorithm, 80 codons using FUBAR, and 38 codons using SLAC.

Sequence Demarcation Tool analysis results

Distance matrix analyses of the obtained sequences with 46 full-length IYSV nucleocapsid (N) protein gene sequences retrieved from the GenBank database, performed using the Sequence Demarcation Tool, showed that nucleotide sequence identity among all analysed isolates ranged from 92% to 99%, whereas amino acid sequence identity ranged from 88% to 100% (Figure 1D, 1E). The Polish isolates obtained in this study exhibited 99% nucleotide sequence identity with each other.

Phylogenetic analysis

The phylogenetic reconstruction based on the full-length nucleocapsid (N) protein gene sequence of IYSV revealed the presence of two phylogenetic groups in the analysed population, composed of different isolates. In the first clade, isolates primarily from India, Japan, Iran, and Australia are located (Figure 1F). The Polish isolates clustered in the second clade with the isolates from Serbia, Bosnia and Herzegovina, Canada, New Zealand, the United States, Japan, Pakistan, and Brazil (Figure 1F). The only sequences that were present in both clades were those originating from Japan.

DISCUSSION

In this study, a survey of onion crops in Poland was conducted. The presence of OYDV in symptomatic plants collected in different regions of Poland (Wielkopolska and Lubelskie) was identified. OYDV infection reduces seed setting and causes premature plant death, leading to losses in onion and garlic crops ranging from 25 to 60% [Barg et al. 1994, Lot et al. 1998, Dovas et al. 2001]. The presence of OYDV in onion cultivation in Poland has been observed earlier [Taberska et al. 2021]. The virus can be transmitted through propagation materials and in a non-persistent manner by over 50 species of aphids, including *Myzus persicae*, which is the most important vector of the virus [Abd El-Wahab 2009, Jayasinghe et al. 2021]. *M. persicae* is a species present in Poland that can cause significant yield losses [Ruszkowska et al. 2017]. Climate warming promotes an increased number of aphids, which can transfer the virus from neighbouring weeds to agricultural fields, and also can adapt to new environmental conditions rather quickly. Climate change impacts vectors in several ways, including alterations in their phenology, overwintering patterns, population density, migration, and their predators. Additionally, global warming affects the primary infection of the host, the progression of the infection within the host, and/or the potential for horizontal transmission of the virus to new hosts through the vector [Harrington et al. 1995, Ruszkowska 2007].

For the first time in onion crops in Poland, the presence of another important pathogen, IYSV was also identified. IYSV was first reported in Poland in 2005 on chrysanthemum plants [Balukiewicz and Kryczyński 2005]. The

virus is transmitted by onion and tobacco thrips [Srinivasan et al. 2012]. Onion thrips are common in both greenhouses and fields in Poland [Pobożniak et al. 2021]. Adult insects overwinter in the upper layer of soil, on weeds, grasses, and tobacco residues. The source of the virus can be infected seedlings and bulbs. The virus has been previously detected in other European countries (Germany, the Czech Republic), which confirms its widespread distribution in Europe.

In this study, the presence of both viruses, OYDV and IYSV, was detected in single and mixed infections. So far, mixed infections have only been observed in the case of OYDV and the viruses from the *Allexivirus* or *Potyvirus* genus, for example, *Potyvirus ampeloprasi* (leek yellow stripe virus, LYSV) [Pappu et al. 2005, Kumar et al. 2010]. Mixed infections of OYDV and LYSV not only exacerbate the health of plants and reduce yields, but can also improve the transmission efficiency of the viruses by vectors, making it more difficult to control these pathogens in *Allium* crops [Lot et al. 1998, Jayasinghe et al. 2021]. In our study, it was difficult to determine whether the presence of mixed infections may have an impact on disease symptoms. The presence of disease symptoms was observed both on samples in which OYDV or IYSV was confirmed, as well as in those samples in which the presence of viruses was not detected. This may be due to the low concentration of viruses in the tested samples. Further research is required using high-throughput sequencing, which can detect all DNA and RNA viruses present in a sample in a single assay, providing an exhaustive view of a plant's viral phytosanitary status [González-Pérez et al. 2024].

In this study, we performed the phylogenetic analysis based on the full-length sequence of the nucleocapsid (N) protein gene of IYSV. The sequences obtained in this experiment show a 99% identity, indicating low variation between isolates from two different regions of Poland. The analysis of the obtained sequences, generated through sequencing, revealed a low level of genetic diversity among samples originating from different countries. The selection pressure analysis showed that the nucleocapsid (N) protein gene has been mainly evolving under the action of purifying selection operating thus highlighting its functional role during IYSV infection. Interestingly, one codon 141 was identified as positively selected by three different methods used. This suggests that changes at this site might be beneficial to the virus and contribute to its success in infecting and replicating within the host plant.

The variability index between sequences was minimal, suggesting a high degree of genetic similarity. These findings may indicate limited genetic diversification within the studied population, regardless of the geographical origin of the samples. In the garlic-infecting isolate from Zimbabwe, the authors conducted a detailed analysis of three complete genes: nucleocapsid (N), nonstructural protein (NSs), and movement protein (NSm) [Karavina et al. 2019]. The N gene sequence did not form distinct clusters associated with specific geographical locations, suggesting a potential absence of local variants of this gene. In contrast, the NSs and NSm genes clustered closely with homologous sequences of other IYSV isolates available in databases. Their similarity to sequences from various locations may indicate the conserved functions of these genes, which are essential for the virus's infection cycle and survival across different geographical conditions [Karavina et al. 2019]. IYSV sequences were divided into three groups based on their origin. The first group consisted of sequences from North America, labeled as NL, the second group included sequences from Asian countries, labeled as BR, and the third group, named "other" exhibited the greatest genetic diversity. The sequences obtained in this study are classified in the phylogenetic tree within the NL group, which, as established in the publication, is the globally dominant [Tabassum et al. 2021].

CONCLUSIONS

In summary, the presence of IYSV in Poland has been detected for the first time in onion. Phylogenetic analysis revealed that the Polish IYSV isolates from Wielkopolskie and Lubelskie regions primarily clustered with isolates originating from Serbia. The virus is transmitted by onion thrips, a globally prevalent species, positioning IYSV as a potentially significant threat to onion cultivation both in Poland and worldwide. Another important question is regarding the occurrence of mixed infections and their impact on pathogenicity and virus transmission. There is a very limited number of complete genomic sequences of IYSV, therefore, further studies are required to elucidate the distribution, host specificity, and evolutionary dynamics of IYSV.

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