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Determination of The Potential of Some Weeds to be Used as An Indicator Plant in Studies On Plumpox virus-T

Cemile TEMUR ÇINAR[1✉], Elif DERTLİ[2], Yasin KAYA[3], Doğan IŞIK[4]
[1,2,3,4]Department of Plant Protection, Faculty of Agriculture, Erciyes University, 38030, Kayseri, Turkiye
[1]https://orcid.org/0000-0002-0248-1835, [2]https://orcid.org/0000-0001-6612-3546, [3]https://orcid.org/0009-0001-3025-8120
[4]https://orcid.org/0000-0002-0554-2912
✉: cemilecinar27@gmail.com

ABSTRACT
Sharka is the most destructive viral disease of *Prunus* species caused by the plum pox virus (PPV). A unique strain of PPV has been identified from Turkey and named as PPV-Turkey (PPV-T). Being obligate parasitic organisms, viruses cannot be cultured in artificial nutrient media. Weeds play a significant role in virus ecology and epidemiology as they serve as alternative hosts for plant viruses and food for virus vectors. This study investigated the indicator potential of some weeds (such as *Chenopodium album* and *Amaranthus retroflexus*) for PPV-T. *C. album* and *A. retroflexus* plants were inoculated by dusting three leaves on each 3-week-old plant. A homogeneous group of 10 plants grown singly in pots was inoculated with PPV-T. Mock- and non-inoculated plants were used as negative controls. Inoculated plants were monitored daily for symptom development. The PPV-T inoculated leaves were tested by reverse transcription-polymerase chain reaction (RT-PCR) following the appearance of symptoms. The results of the indicator test revealed that *C. album* (6 out of 10 plants) and *A. retroflexus* (10 plants) reacted with the infection. Initially, this indicator reacted with local symptoms, and later symptoms of systemic infection occurred.

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Bazı Yabancı Otların Plum Pox Virüs T ile Ilgili Çalışmalarda Indikatör Bitki Olarak Kullanım Potansiyelinin Belirlenmesi

ÖZET
Plum pox virus (PPV)'ün neden olduğu şarka hastalığı, sert çekirdekli meyvelerin en önemli ve en yıkıcı viral hastalığıdır. PPV, Türkiye'de sert çekirdekli meyve yetiştiriciliği yapılan bazı bölgelerdeki bahçelerde oldukça yaygındır ve ciddi bir sorun teşkil etmektedir. Ülkemize özgü bir ırk olan PPV-T (Turkey) ilk olarak kayısı, şeftali ve erikte ağaçlarında tespit edilmiştir. Virüsler obligat parazit organizmalar oldukları için yapay besin ortamlarda kültüre alınamazlar. Bu nedenle deneysel ortamda çalışma yapabilmek için canlı bir konukçuya ihtiyaç vardır. Yabancı otlar bitki virüs hastalıkları açısından değerlendirildiğinde virüslerin ve vektörlerinin doğal yada alternatif konukçusu olabilir ki buda yabancı otların virüs epidemiyolojisinin doğal bir unsuru olduğunu göstermektedir. Bu projede Türkiye'nin yerli ırkı kabul edilen PPV-T ile ilgili araştırmalarda kullanılabilecek en uygun indikatör yabancı ot konukçunun belirlenmesi amaçlanmıştır. İndikatör bitki olarak kullanılacak *Chenopodium album ve Amaranthus retroflexus* yabancı otlarının üç yaprağına PPV-T mekanik inokülasyonla uygulanmıştır. Her bir uygulama için 10 bitki kullanılmıştır. PPV-T inokulasyonu yapılmamış bitkiler kontrol bitkisi olarak kullanılmıştır. Simtom gelişimini takiben inokülasyon yapılmış ve kontrol bitkileri RT-PCR testi ile analiz yapılmıştır. Kullanılan üç bitkinin ikisinde simptomolojik gözlemler ve RT-PCR sonucunda PPV-T inokulasyonunun başarılı olduğu belirlenmiştir (*C. album* (10 bitkiden 6'sı) ve *A. retroflexus* (10 bitkinin tamamı).

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INTRODUCTION

Plum pox virus (PPV) is the etiological agent of sharka disease and causes the most destructive viral disease in stone fruit trees. The PPV is a member of the genus *Potyvirus* in the family *Potyviridae*. As a significantly regulated pathogen, the detection of PPV is thus of crucial significance to quarantine and elimination of the spreading disease (García et al., 2014; Sheveleva et al., 2021).

The PPV was first reported from Bulgaria in 1917–18 and recognized as a virus infection by Atanasoff (1932). It spread relentlessly across Europe and the Mediterranean basin during the 20th century before appearing in other parts of the world in the last two decades. The PPV isolates are grouped into different strains based on their traits (Kerlan & Dunez, 1979; Candresse et al., 1998). Initially, two main PPV serotypes, serotype D (Dideron) and serotype M (Marcus) were established using polyclonal antisera (Kerlan and Dunez, 1979; Candresse et al., 1998). Strains differ in antigenic and epidemiological properties, host preference, and pathogenicity for different species and cultivars of stone fruit crops (Sheveleva et al., 2021). Currently, 10 strains (D, M, Rec, T, An, EA, W, C, CR, and CV) have been identified based on variances in the full genomic sequences (García et al., 2014; Chirkov et al., 2018). Three PPV strains (PPV-M, PPV-Rec, and PPV-T) have been reported from Turkey each with a distinct distribution (Çağlayan et al., 2012; Morca et al., 2021; Morca et al., 2022).

The PPV was discovered for the first time in Türkiye in 1968 (Sahtiyancı, 1969). Although PPV-D, PPV-M, and Rec strains of PPV were previously reported from Turkey, Ulubas Serce et al. (2009) reported a novel strain of PPV named as PPV-Turkey (PPV-T) and described as the most common strain in the country (Gürcan & Ceylan, 2016; Teber et al., 2019; Temur Cinar et al., 2022).

Although many plant viruses have restricted host ranges, others can infect a large number of plant species. The PPV can infect both woody and herbaceous hosts (van Oosten 1970, 1975). While PPV is mostly found in *Prunus* trees and causes the devastating viral disease known as sharka in stone fruit trees, it has the potential to infect a wide range of experimental herbaceous hosts, such as *Nicotiana* spp. (Hervás, 2020). European and Mediterranean Plant Protection Organization (OEPP/EPPO, 1974) has referred 78 species from 9 families as hosts of PPV. Of these 78 species, 46 were *Solanaceae* (30 *Nicotiana* spp.) and 16 were *Papilionaceae* (Llácer, 2006). Weeds are widely distributed throughout the world and have high environmental adaptability (Prajapati et al., 2014). Weeds may act as an alternative host of phytopathogens, serving as sources of inoculum and playing a significant role in disease epidemiology.

Weeds have an important place in the ecology of cultivated plants and adversely affect their yield and quality. Weeds often become the main hosts for plant viruses and serve as insect vectors at crop harvests (Chen et al., 2013). Viruses rely on other hosts to sustain their survival as they are obligate intracellular parasites (Duffus et al., 1971). Weeds can affect the spread of viruses by serving as breeding substrates for aphids and enabling the vector to pick up the virus from infected plants (Sedhain et al., 2021). The prevalence of weed species in the fields during crop-free times or cultivation seasons makes managing virus diseases even more difficult (Aguiar et al., 2018).

Weeds are also used for inducing plants' resistance to viruses. Many efforts have been made worldwide to obtain stone fruit trees resistant to PPV. Transgenic technology is effective in producing stone fruit plants resistant to PPV. Most research to produce PPV-resistant transgenic plants used herbaceous hosts first. Compared to herbaceous hosts, the cultivation and inoculation of woody hosts is a laborious process. Therefore, in studies related to resistance to PPV, herbaceous model plants are frequently used. (Ilardi, 2011). Therefore, studies related to PPV resistance are commonly conducted using *Nicotiana* spp. (Ilardi, 2011).

Even though plant virologists often concentrate their research on commercially significant crops, there are several instances where non-crop plants are studied. Such plants (mostly weeds) are important for viral reservoirs that cause economic losses in crop plants, and experimental hosts for such discovery, characterization, upkeep, or simpler manipulation of viruses (Adkins et al., 2002). Additionally, it has been determined that fresh leaf tissue is a better source of viral RNA and viral protein compared to frozen leaf tissue. A perennial plant species that is easily manipulated under experimental conditions and susceptible to widely studied plant viruses could find applications in virus culture collections and be used in research involving viruses that lose infectivity during storage (Adkins et al., 2002).

In a study conducted by Saenz et al. (2000), the impact of the differences in the genomic structures of the PPV-M and PPV-D strains on symptom development in experimental herbaceous hosts was investigated. It was found that PPV-D caused local necrotic lesions and systemic chlorotic ring spots in *Pisum sativum*, whereas PPV-M only resulted in systemic chlorotic ring spots. In contrast to the symptoms observed in *P. sativum*, both the PPV-D and PPV-M strains have been found to cause systemic chlorotic ring spots in *Nicotiana clevelandii*. In a study where *C. foetidum* was used as an indicator plant, it was reported that the PPV-D strain caused chlorotic or necrotic lesions (Martínez-Turiño et al., 2021). In studies related to PPV, herbaceous hosts such as *C. foetidum, N. clevelandii*, and *P. sativum* are commonly used as experimental hosts. It has been reported that different strains of PPV cause chlorotic or necrotic lesions in these plants. The research focused on determining the natural hosts of PPV, although *C. album* and *A. retroflexus* were obtained as suspected samples, and weren't determined to be PPV infections (Viröček Marn et al., 2004; Stobbs et al., 2005).

Chenopodium album and *Amaranthus retroflexus* are the most common weeds in orchards (Eşitmez & Işık 2016). *Chenopodium* genus comprises about 250 herbaceous, suffrutescent, and arborescent perennial species (Giusti, 1970) and belongs to the family *Chenopodiaceae* (APG III, 2009). The most prevalent weed species are *C. album. C. album*, which possess unique biological features that help them to tolerate sub-optimal and/or harsh environmental conditions. High seed production, rapid and vigorous development, taller height, short life cycle, and the capacity to germinate under various environmental conditions are some of these characteristics. Numerous plant viruses also use *C. album* as a host plant (Bajwa et al., 2019).

A. retroflexus grows in a wide range of soil types and textures. It thrives in fertile soils and has a high N demand. It tolerates soil pH from 4.2 to 9.1 (Feltner, 1970). It is an aggressive and competitive weed in a variety of row crops. It is an alternative host for several crop pests and diseases (Weaver & MacWilliams, 1980).

The major aim of this study was to discover new weed-virus interactions. Although some information is available about the determinants of pathogenicity and host range of PPV in herbaceous plants such as *C. album* and *A. retroflexus*, there is no information about the experimental and natural weed hosts of the PPV-T strain. In this study, it was evaluated whether *C. album* and *A. retroflexus* are indicators of PPV-T.

MATERIALS and METHODS

Plant Material

The seeds of *Chenopodium album* and *Amaranthus retroflexus* were collected from the experimental fields of Erciyes University Kayseri, Turkey in September 2019. Seeds from different plants were pooled and stored at 5°C. The PPV-T isolates used in this study were initially recovered from an infected apricot tree in Kayseri, Turkey (GenBank accession number: MW413816.1) (Temur Çınar et al., 2022).

Planting and Management

All experiments were performed in the greenhouse of the Department of Plant Pathology at the Faculty of Agriculture at Erciyes University, Kayseri, Turkey. Greenhouse temperatures ranged from 23 to 43 °C (day) and from 12 to 24 °C (night). Weeds seeds were directly sown in plastic pots (100X100 mm size, 600-mL volume) filled with a soilless mix (Potgrond H, perlite). Ten weed seeds were planted per pot and thinned to one to ensure a uniform experiment. For each experiment, a homogeneous group of 10 plants grown singly in pots was inoculated with PPV-T. Mock- and non-inoculated plants were used as negative controls.

Mechanical Inoculation

For this study, *Chenopodium album* and *Amaranthus. retroflex* was used for mechanical transmissions. *C. album, A. retroflexus* plants were inoculated by dusting three leaves on each 3-week-old plant with 600-mesh carborundum and then inoculum was applied to the leaf with a pestle, which was rotated in a circular motion eight to 10 times as if painting the leaf with inoculum. The plants were kept in aphid-proof cages and carborundum was rinsed off the leaves after inoculation to optimize light absorption.

RNA Extraction.

Inoculated plants were monitored weekly for symptom development. Following the appearance of symptoms on PPV-T inoculated leaves 15 days post-inoculation, uninoculated leaves were collected from all plants and tested for the presence of the input virus by reverse transcription-polymerase chain reaction (RT-PCR). Leaf samples from plants were collected 15 days post-inoculation and used for RNA extraction. Total DNA was extracted from leaf samples using a previously reported, modified method (Spiegel et al., 1996). Approximately 50–100 mg of fresh or frozen leaves were ground to a fine powder in liquid nitrogen and mixed with 1 ml of LiCl extraction buffer (0.1M LiCl, 0.1 M Tris–HCl, pH 8.0, 0.01 M EDTA, pH 8.0, and 1% SDS). The 1 µL of mercaptoethanol was added to the extraction buffer before use, which was then incubated for 15 minutes at 65 °C. Following incubation an equal volume of 6 M potassium acetate, pH 6.5, was added and maintained

on ice for 15 min. After centrifugation at 14,000 rpm for 10 min, nucleic acids were precipitated from the supernatant with isopropanol and centrifuged as above. The pellet was washed with cold 70% ethanol by centrifugation at 4000 rpm for 5 min at 4 °C, dissolved in 50 µl of sterile water, and stored at −20 °C (or −80 °C for long-term storage).

Molecular Characterization of PPV

A two-step RT-PCR protocol was used for cDNA synthesis: 10 µl of RNA, 1 µl of Random hexamer, and 1 µl of dNTP and incubated at 65 °C for 5 min then − 20 °C for 5 min. For second step, 1 µl M-MLV (Moloney Murine Leukemia virus reverse transcriptase, 200 unite/µl, Invitrogen), 4 µl 5x Transcriptase buffer, 2 µl 0.1M DTT, ve 1 µl RNAse free of dH_2O incubated as follows 25 °C 10 min, 37 °C 50 min, 70 °C 15 min (Invitrogen). The PCR was carried out in 20 µl total mixtures containing 4 µl of cDNA, 2 µl of 5 mM dNTPs, 2 µl of 25 mM $MgCl_2$, 2 µl of 10X Taq Buffer, and 0.5 µl of 10 µM of each virus-specific primer with 0.5 µl of Taq DNA polymerase (Invitrogen). PCR was performed using PPV universal primer pairs that amplify 243 bp fragments: P1, (5'–3' ACC GAG ACC ACT ACA CTC CC) ve P2, (5'–3' CAG ACT ACA GCC TCG CCA GA) (Olmos et al., 2005). Cycling parameters were 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 53 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 10 min. All RT-PCR products were analyzed by electrophoresis in 2% (p/vol) agarose gels.

RESULTS

Weeds serve as alternative hosts for phytopathogenic viruses. The herbaceous host range of PPV-T is still not fully known. However, *N. benthamiana* has been the sole herbaceous indicator used for PPV greenhouse indexing since serology-based and molecular viral detection methodologies were established.

Occurrence of PPV-T Symptoms in Weeds

Virus-like symptoms, including yellowing and interveinal chlorosis, were observed in only a small number of weed plants found to be positive for PPV-T. The first symptom appeared 13 days post-inoculation. Leaf yellowing and interveinal chlorosis were observed in 4 *C. albumin* and 3 *A. retroflexus* plants infected with PPV-T (Figs. 1-2), whereas no symptoms were observed in PPV-T negative samples. The *C. album and A. retroflexus* were identified as the weeds in terms of PPV-T symptoms with observation rates of 40% and 30%, respectively. However, asymptomatic *C. album* (six) and *A. retroflexus* (seven) plants were PPV-T positive based on the results of RT-PCR-based assays.

C. album and A. retroflexus were very useful for use as an indicator of PPV-T. The most obvious symptoms on the PPV-T infected *C. album* were vein clearing and interveinal chlorosis and *A. retroflexus* were chlorotic local lesions (Figs. 1-2).

Both weed species have been hosts for PPV-T for one month without losing their viability. *C. album* and *A. retroflexus*, which continue to grow actively after infection, can be evaluated as alternative indicator plants in studies on PPV-T. The results with the PPV-T assessed here demonstrate the utility of these two plants as a new experimental host (Figs. 1-2).

RT-PCR Analysis

A 243-bp amplicon using the P1 and P2 primers was amplified for all PPV-T inoculated plants, (Figs. 3-4). A fragment of similar size was observed for the positive PPV-T controls. No amplification was observed with any of the healthy controls included in this study. (Fig. 4).

This study reveals that *A. retroflexus* and *C. album* satisfy their needs as PPV-T hosts.

DISCUSSION

Numerous weed species (both native and introduced) have been discovered to act as PPV hosts and have a considerable impact on the epidemiology and spread of PPV around the world (Llácer, 2006). High incidences of plant viral diseases are influenced by weed hosts of their causative agents (Asala et al., 2014). This study demonstrates that *C. album and A. retroflexus* meet needs as PPV-T hosts and in agreement with results from similar studies (Desvignes, 1999; Llácer, 2006), also suggests that *C. album* and *A. retroflexus* may find more general application as a host for viruses of herbaceous plants which is making another experimental tool available to plant virologists. It was determined that the symptoms observed in herbaceous experimental hosts such as *C. foetidum, N. clevelandii* and *P. sativum* used in studies on PPV-D and PPV-M strains (Saenz et al., 2000; Viröček Marn et al., 2004; Stobbs et al., 2005; Martínez-Turiño et al., 2021) were similar to the symptoms observed in the herbaceous plants *C. album*, and *A. retroflexus* used in this study. Vein clearing and chlorotic rings were the most noticeable signs on the PPV-T-infected *C. album*, while chlorotic lesions were seen on *A. retroflexus*. Similar vein banding and round dots were seen in PPV-M-infected *Senecio sylvaticus* in a study by Morvan & Chastellière (1980). While the PPV-C strain does not induce systemic infection in any *Arabidopsis* spp., it has been reported to cause systemic infection in certain species of *Arabidopsis* spp. This observation suggests that PPV strains may exhibit host specificity towards herbaceous hosts (Decroocq et al., 2009)

Furthermore, *C. album and A. retroflexus* are more adaptable to various environmental conditions than tobacco. The current analysis illustrates this species' value as a long-term host for PPV-T, but more investigation is needed to produce a more comprehensive "virus range" for this species. One widely used *Nicotiana* species is *N. benthamiana*, which has been known for nearly 30 years to be susceptible to many plant viruses (Quacquarelli et al., 1997; Christie et al., 1978; Adkins et al., 2002). Most of these *Solanaceous* species are annual plants with short life cycles, frequently made shorter by virus infection. Although this has no bearing on using these species for virus detection, characterization, and/or replication, it can pose a significant obstacle to the long-term maintenance of viruses like PPV-T in these plants.

Figure. 2. Virus infection symptoms on inoculated *Amaranthus retroflexus* plants. C, D, Necrotic spots on the leaf after inoculation with PPV-T are indicated with an arrow sign

Şekil. 1. PPV-T inokule edilmiş Amaranthus retroflexus bitkilerinde virüs enfeksiyonunun belirtileri. C, D, PPV-T ile inokulasyonun ardından yaprakta klorotik lekeler ok işareti ile belirtilmiştir

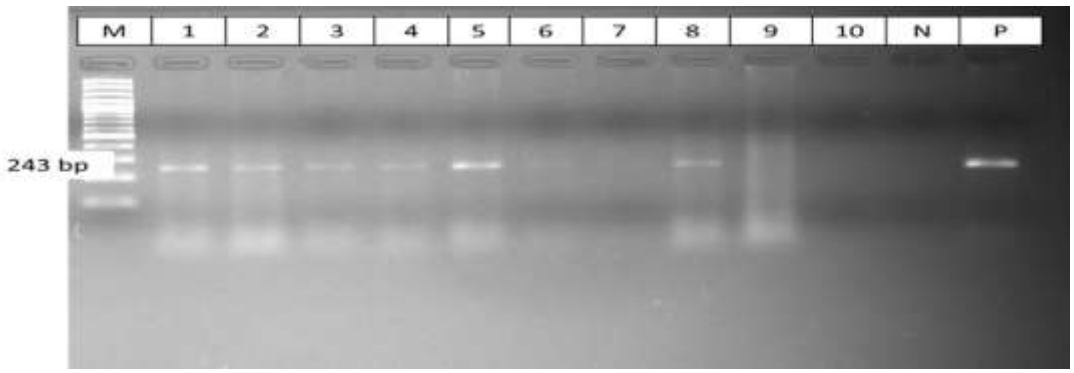

Figure 3. Detection of systemic infection in inoculated *Chenopodium album* plants by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from uninoculated leaves of mock (N), PPV-T positive control sample

(P), and virus-inoculated (lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10). M:100 bp DNA ladder. For each sample, a 243-bp fragment was amplified.

Şekil 3. PPV-T inokule edilmiş Chenepodium album bitkilerinde sistemik enfeksiyonun ters transkripsiyon-polimeraz zincir reaksiyonu (RT-PCR) ile tespiti. Total RNA, virüs inokule edilmemiş (N), pozitif control örneği (P) ve virüs inokule edilmiş (1, 2, 3, 4, 5, 6, 7, 8, 9 ve 10) bitkilerin yapraklardan ekstre edilmiştir. M:100 bp DNA ladder. Her numune için 243 bp'lik bir fragman amplifiye edildi.

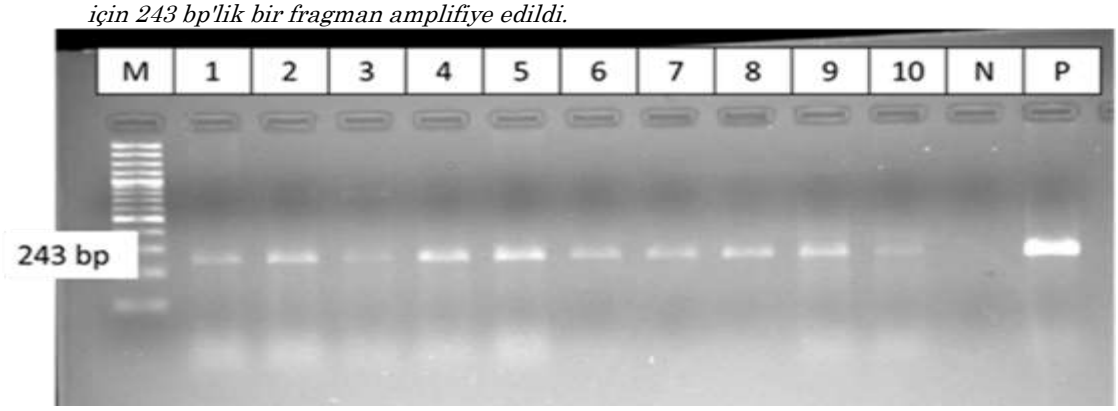

Figure 4. Detection of systemic infection in inoculated *Amaranthus retroflexus* plants by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from uninoculated leaves of mock- (N), PPV-T positive control sample (P), and virus-inoculated (lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10). M:100 bp DNA ladder. For each sample, a 243-bp fragment was amplified.

Şekil 3. PPV-T inokule edilmiş Amaranthus retroflexus bitkilerinde sistemik enfeksiyonun ters transkripsiyon-polimeraz zincir reaksiyonu (RT-PCR) ile tespiti. Total RNA, virüs inokule edilmemiş (N) ve virüs inokule edilmiş, pozitif control örneği (P), (1, 2, 3, 4, 5, 6, 7, 8, 9 ve 10) bitkilerin yapraklardan ekstre edilmiştir. M:100 bp DNA ladder. Her numune için 243 bp'lik bir fragman amplifiye edildi.

C. album possesses unique biological features that help it tolerate sub-optimal and/or harsh conditions. High seed production, rapid and vigorous development, a habit of growing tall, a short life cycle, and the capacity to germinate under various environmental conditions are some of these characteristics (Bajwa et al., 2019). *A. retroflexus* is an aggressive and competitive weed in a variety of row crops. And can be used as an alternative host for several crop pests and diseases (Weaver & MacWilliams, 1980). These herbaceous hosts, such as *Chenopodium* spp., N*. clevelandii*, *A. retroflexus,* and *Pisum sativum* are very useful for concentrating and purifying the virus (Desvignes, 1999) in the current study confirming previous studies reported in this research (Desvignes, 1999; Llácer, 2006). The high adaptability of weeds allows them to be considered as long-term host plants for the like PPV-T.

A pathogen's host range may have unique effects on how it evolves and how its virulence changes over time. For generalists, adaptation to different hosts may be conditioned by different trade-offs in the pathogen's life history and be affected by evolutionary processes that shape pathogen populations (Sacristán et al., 2005; Read, 1994). The creation of effective and long-lasting control measures depends on an understanding of the selection mechanisms that influence the evolution of virulence. The host range of the pathogen (i.e., whether the pathogen is a generalist or a specialist) is predicted to be a major factor in the evolution of virulence (Sacristán et al., 2005). Determining the behavior of PPV-T in different hosts enabled us to find new indicator plants with high adaptability to alternative nature for experimental use. It will also be a preliminary study in studies of the evolutionary development of PPV-T in weed hosts. The current paper reveals that viable experimental hosts for PPV-T have yet to be discovered, even though plant virologists have access to many great experimental hosts.

In conclusion, the indicator test findings revealed that *C. album* (6 out of 10) and *A. retroflexus* (10 plants) both experienced infection after being manually inoculated. This indication initially responded with local symptoms, and then systemic infection signs appeared. Weeds as alternative hosts of plant viruses and nutrient plants of virus vectors play an important role in virus ecology and epidemiology. This study showed that *C. album* and *A. retroflexus* could be experimental hosts for PPV-T, it also showed that PPV-T has the potential to be a natural source of inoculum. The identification of different experimental hosts is a preliminary study in determining the change that PPV has shown for its adaptation in different hosts in the evolutionary process. Plant virologists have access to a wide range of excellent experimental hosts, but the current study shows that there are still plenty of useful experimental hosts to be found.

Credit authorship contribution statement

Cemile Temur Cinar: Data curation, Formal analysis, Writing – review & editing., Elif Dertli: Data curation, Formal analysis, Writing – review & editing., Yasin Kaya: Data curation, Formal analysis,

Writing – review & editing., Dogan Isık: Conceptualization, Methodology, Funding acquisition, Resources, Project administration, Writing – review & editing, Supervision.

Conflict of interest

The authors declare they have no conflict of interest.

Human and animal rights statement

This article does not contain any studies with human participants or animals.

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