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Ph.D. in Biodiversity in Agriculture and Forestry (BAF)

ECOLOGY OF VIRUS AND VIRUS-LIKE DISEASES THAT AFFECT OLIVE TREES IN THE MEDITERRANEAN BASIN

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DOTTORATO

“Resta, goditi lo spettacolo.
Resta coraggiosa.
Resta dolce.
Testa alta,
cuore in mano.”

(Susanna Casciani)

CONTENTS

CASE STUDY: OLEA EUROPAEA GEMINIVIRUS (OEGV)

CASE STUDY: OLIVE LEAF YELLOWING-ASSOCIATED VIRUS (OLYAV)

EXTENDED ABSTRACT

The cultivation of olive tree (*Olea europaea* L.) has significant historical and socio-economic importance, especially in Mediterranean regions (Brunori et al., 2020). Due to the increasing global demand for olives and olive oil, modern agricultural practices, such as establishing high-density orchards and implementing irrigation systems have been employed (Mili and Bouhaddane, 2021; Sobreiro et al. 2023). Conversely, these changes pose challenges related to the increasing incidence of olive tree pests and diseases (Caselli and Petacchi, 2021), resulting in significant yield losses and economic consequences. Olive production in the Mediterranean regions has decreased significantly due to the emergence of biotic agents, affecting both the olive tree and the oil industry (Nuti et al., 2021). The improper use of infected olive material for vegetative propagation has facilitated the spread of systemic pathogens in olive orchards, such as viruses (Lambardi et al., 2023). To manage these threats, preventive measures such as phytosanitary selection and certification programmes have been implemented (Montilon et al., 2023). Within this context, the application of molecular diagnostic techniques for viruses detection has assumed, in the recent past, critical importance for the assessment of the sanitary status (López et al., 2009). These reliable methods are required as they are the basis for efficient and valid certification programmes, therefore research efforts focused on particular attention to the identification, diagnosis and monitoring of viral diseases affecting the olive plants in Mediterranean environment.

For this aim, the present thesis, conducted as part of a three-year PhD program, delves into the investigation on viral and virus-like agents affecting olive cultivation, emphasizing the indispensable role of advanced diagnostic techniques in safeguarding olive orchards' health and sustainability worldwide. The research effort is organised into cohesive chapters as follows. Chapter I highlighted the economic and cultural importance of olive cultivation and underlined the potential viral threats and impacts of both climate change and the implemented modern plantation systems for olive cultivation. In Chapter II, the purpose of the research work is outlined, emphasizing the importance of addressing viral diseases as a global agriculture challenge.

Chapter III screened the presence of the main olive tree viruses in Sicily using the

metagenomic approach based on the Oxford Nanopore MinION sequencer. The implemented diagnostic tool gave interesting preliminary results, demonstrating the potential of innovative diagnostic tools in detecting viral pathogens. Indeed, MinION technology successfully detected olea europaea geminivirus (OEGV), olive leaf yellowing-associated virus (OLYaV) and olive latent virus 3 (OLV-3) that were confirmed, subsequently, by the conventional molecular diagnostic tool end-point RT-PCR, excepting for OLV-3. For these reasons, the case study of OEGV was evaluated in Chapters IV and V, whereas OLYaV was assessed in Chapters VI and VII.

In particular, the development and practical feasibility of a real-time loop-mediated isothermal amplification (LAMP) protocol for the detection and investigation of OEGV incidence in Sicily, as well as a rapid procedure for on-site preparation of olive sample were assessed and are described in Chapter IV. Moreover, with the aim of better understanding the virus transmissibility, Chapter V reported the trials that were conducted to investigate OEGV transmission via grafting using infected propagation material. As results, from one hand, the real-time LAMP assay described in this work revealed to be a potential, rapid, simple, specific and sensitive tool for the recently described OEGV detection, allowing to process of a great number of samples at the same time, especially when associated with the rapidly developed on-site sample preparation method. Moreover, the survey revealed a considerable presence of the virus within the Sicilian olive groves, probably due to the inadvertent movement of clonally propagated and asymptomatic infected germplasms. From the other hand, Preliminary results also demonstrated that OEGV was successfully transmitted through grafting, with symptoms observed within 3-7 months post-grafting. These findings emphasize the importance of rigorous screening methods and cultivar selection to mitigate virus transmission risks in olive propagation.

Chapter VI focused on the development and evaluation of a real-time RT-LAMP protocol for OLYaV detection, comparing it with end-point RT-PCR assay. Moreover, the real-time RT-LAMP protocol developed was used to assess OLYaV incidence in the main olive-producing regions of Spain and Italy. Obtained results showed that the developed real-time RT-LAMP protocol for OLYaV detection was higher sensitive and specific compared to the RT-PCR end-point technique. Furthermore, the investigation conducted in the main Spanish and Italian olive-growing regions represents, to date, the first

comprehensive analysis of OLYaV incidence in these territories.

Actually, the virus incidence analysis showed to be highly present in the Spanish territory than in the Italian one. However, in order to provide comprehensive insights into the prevalence and distribution of the virus in Sicily, Chapter VII describes the study conducted on the dispersion of OLYaV in different Sicilian olive production sites, using the previously developed real-time RT-LAMP. The results revealed a significant prevalence of OLYaV in the sampled olive trees, with a significant number of positive samples identified in asymptomatic plants, suggesting a potential silent spread of the virus through infected propagation material or via vector insects.

Ultimately, a summarized conclusive considerations and future perspectives were reported in Chapter VIII, together with all the thesis possible contributions to the advancement of knowledge in plant virology and the development of strategies that could mitigate viral threats in olive cultivation. The interesting findings in the current PhD thesis aim to significantly contribute to scientific understanding and practical application of control and prevention strategies for managing olive viral diseases. Lastly, the results of the study shed light on the current challenges facing the industry and underscore the critical role of advanced diagnostic techniques in safeguarding the health, productivity and sustainability of olive orchards.

SOMMARIO ESTESO

L'olivo (*Olea europaea* L.) riveste un'importanza storica e socio-economica notevole, in particolare nelle regioni del Bacino del Mediterraneo (Brunori et al., 2020). La crescente domanda globale di olive da tavola e di olio d'oliva ha portato all'adozione di pratiche agricole moderne, quali la creazione di uliveti ad elevata densità e l'implementazione di sistemi di irrigazione (Mili e Bouhaddane, 2021; Sobreiro et al., 2023). Tuttavia, tali cambiamenti comportano enormi sfide legate all'incremento dell'incidenza di parassiti e malattie che colpiscono tale coltura (Caselli e Petacchi, 2021), determinando consistenti perdite di raccolto e ingenti ripercussioni economiche. In risposta, la produzione olivicola nelle regioni mediterranee ha subito una significativa diminuzione a causa dell'emergere di agenti biotici che influenzano l'industria olivicola (Nuti et al., 2021). Inoltre, l'errato impiego di materiale infetto per la propagazione vegetativa dell'olivo ha favorito la diffusione di patogeni sistemici, quali i virus (Lambardi et al., 2023). Al fine di gestire tali minacce, sono state implementate misure preventive, come programmi di selezione e certificazione fitosanitaria (Montilon et al., 2023). In questo contesto, l'applicazione delle tecniche diagnostiche molecolari per la rilevazione di tali agenti patogeni ha assunto, nel recente passato, un'importanza critica per la valutazione dello stato sanitario (López et al., 2009), rappresentando la base per efficienti e validi programmi di certificazione.

Pertanto, gli sforzi di ricerca affrontati nella presente tesi, condotta nel contesto di un programma di dottorato di ricerca, si sono concentrati particolarmente sull'identificazione, diagnosi e monitoraggio delle malattie virali che colpiscono l'olivo in ambiente mediterraneo. Con tale obiettivo, la presente tesi si propone di approfondire lo studio degli agenti virali e virus-simili che influenzano la coltivazione dell'olivo, sottolineando il ruolo indispensabile delle moderne tecniche diagnostiche nel preservare la salute e la sostenibilità degli uliveti. In particolare, gli studi di ricerca affrontati sono stati organizzati in otto capitoli. Il Capitolo I evidenzia l'importanza economica e culturale della coltivazione dell'olivo, ne sottolinea le potenziali minacce virali e descrive gli impatti negativi causati sia dai cambiamenti climatici, che dai moderni sistemi di coltivazione dell'olivo. Nel Capitolo II viene descritto lo scopo del lavoro di ricerca, enfatizzando l'importanza di affrontare le malattie virali come una nuova significativa

sfida globale per l'olivicoltura. Nel Capitolo III viene indagata la presenza di virus che colpiscono la coltura dell'olivo in Sicilia mediante analisi metagenomiche, tramite il dispositivo MinION dell' Oxford Nanopore. Quest'ultimo ha fornito interessanti risultati, dimostrando il potenziale delle innovative tecniche diagnostiche nel rilevare patogeni virali. Infatti, il dispositivo MinION ha rilevato con successo tre virus dell'olivo: l'olea europaea geminivirus (OEGV), l' olive leaf yellowing-associated virus (OLYaV) e l'olive latent virus 3 (OLV-3). Tali risultati sono successivamente stati confermati mediante diagnosi molecolare attraverso l'impiego della tecnica RT-PCR end-point, ad eccezione dell'OLV-3. Pertanto, i principali virus oggetto di studio sono stati l'OEGV e l'OLYaV. In particolare, il caso studio dell'OEGV è stato valutato nei Capitoli IV e V, mentre l'OLYaV è stato valutato nei Capitoli VI e VII.

In particolare, lo sviluppo e la validazione di un protocollo di real-time Loop-Mediated Isothermal Amplification (LAMP) per la rilevazione e lo studio dell'incidenza dell'OEGV in Sicilia e una procedura rapida per la preparazione *in loco* del campione sono stati valutati e descritti nel Capitolo IV. Inoltre, con l'obiettivo di comprendere meglio la modalità di trasmissione di OEGV, nel Capitolo V sono riportati gli esperimenti condotti per indagare la trasmissione di tale virus mediante innesto. In particolare, il protocollo real-time LAMP descritto nel Capitolo IV si è rivelato un metodo rapido, semplice, specifico e sensibile per la rilevazione dell'OEGV, consentendo di processare un gran numero di campioni contemporaneamente, specialmente quando associato al metodo rapido di preparazione del campione. Inoltre, lo studio ha rivelato una presenza considerevole del virus all'interno degli uliveti siciliani, probabilmente a causa del movimento involontario di materiale di propagazione infetto, ma asintomatico. I risultati preliminari, inoltre, hanno dimostrato che l'OEGV è stato trasmesso con successo mediante innesto e i sintomi sono stati osservati entro 3-7 mesi post-innesto. Questi risultati sottolineano l'importanza di rigorosi ed efficaci metodi di screening, al fine di mitigare i rischi di trasmissione virale.

Nel Capitolo VI, invece, viene focalizzata l'attenzione sullo sviluppo e la valutazione di un protocollo real-time RT-LAMP per la rilevazione dell'OLYaV; quest'ultimo viene, inoltre, comparato con la tecnica di diagnosi RT-PCR end-point per valutarne sensibilità e specificità. Successivamente, il protocollo sviluppato è stato impiegato per valutare l'incidenza dell'OLYaV nelle principali regioni olivicole in Spagna e Italia. I risultati

hanno evidenziato che il protocollo di real-time RT-LAMP per la rilevazione di OLYaV si è rivelato maggiormente sensibile e specifico rispetto alla tecnica RT-PCR end-point. Inoltre, l'indagine condotta nelle principali regioni olivicole spagnole e italiane rappresenta, ad oggi, la prima analisi completa dell'incidenza dell'OLYaV in tali territori. In particolare, l'analisi dell'incidenza del virus ha evidenziato una maggiore presenza nel territorio spagnolo rispetto a quello italiano. Tuttavia, per fornire un quadro completo sulla prevalenza e distribuzione del virus in Sicilia, nel Capitolo VII viene descritto lo studio condotto sulla diffusione dell'OLYaV in diverse provincie e siti di produzione siciliani, mediante l'utilizzo del protocollo real-time RT-LAMP precedentemente sviluppato. I risultati hanno mostrato una significativa prevalenza dell'OLYaV negli uliveti campionati, con un numero considerevole di campioni positivi identificati in piante asintomatiche, indicando una possibile diffusione silenziosa del virus attraverso il materiale di propagazione infetto o tramite insetti vettori.

Infine, nel Capitolo VIII sono state esposte le conclusioni e le prospettive future, insieme a tutte le possibili implicazioni per lo sviluppo di strategie mirate a mitigare le minacce virali nella coltivazione dell'olivo. I risultati presentati nella presente tesi mirano a contribuire in modo significativo alla comprensione scientifica e all'applicazione pratica delle strategie di controllo e prevenzione per la gestione delle malattie virali dell'olivo. Infine, gli esiti degli studi condotti mettono in luce le attuali sfide che l'industria olivicola affronta e sottolineano il ruolo cruciale delle moderne tecniche diagnostiche nel proteggere la salute, la produttività e la sostenibilità degli uliveti.

CHAPTER I

Introduction

CHAPTER I – INTRODUCTION

1.1 Cultural and economic importance of olive cultivation

Olive trees (*Olea europaea* L.) has been cultivated by the most ancient civilisations and has played a crucial role in the traditions, environment, and economy of the cultivated regions (Costa, 1998; Zohary et al., 2012; Diez et al., 2015; Bizos et al., 2020). According to archaeological (Friedrich 1980; Kuhn et al. 2010) and ancient texts evidence, olive cultivation began thousand years ago in the Mediterranean basin and its origin could be traced to areas along the eastern Mediterranean coast (Vossen, 2007), where it has been cultivated since prehistoric times (Terral, 2000). In fact, olive tree was one of the first fruit plants to be domesticated and based on archaeobotanical studies, olive domestication started about five to six thousand years ago in the Near East (Zohary and Hopf 2000; Kaniewski et al., 2012). The process of domestication probably occurred through vegetative propagation of olive trees with favourable morphological and agronomic characteristics, such as high fruit-bearing, large fruits and high oil content (Zohary and Spiegel-Roy, 1975; Liphschitz et al., 1991; Terral et al., 2004; Zohary et al., 2012). The spread of olive culture throughout the Mediterranean basin was facilitated by human migrations and trade (Diamond, 2002), then a local process of selection and diversification occurred, generating a large number of varieties that still characterize vegetative landscape and historical-cultural identity (Kostelenos and Kiritsakis, 2017; Langgut et al., 2019). As a matter of fact, olive trees are considered as anthropogenic elements of the landscape, and, thanks to their longevity, they have become a source of historical information (Schicchi et al., 2021), representing a tangible example of sustainable land use (Makhzoumi, 1997). Olive cultivation played a major role in the maintenance of biodiversity and still, representing an important agro-industrial sector with considerable economic, agricultural and ornamental concern and benefit in the economic and social dimensions in rural areas (Vossen, 2007; Palese et al., 2013; Fraga et al., 2020a). Over the centuries, olive growing has played a significant role in the economic development of Mediterranean rural areas, providing substantial sources of income and employment opportunities for the population of agricultural territories (de Graaff and Eppink, 1999). Since antiquity, the wide use of treated products from olive

fruit (fuel, oil as food and soap for cleaning purposes) has contributed to the increase of cultivated areas, and, in the frame of a commercial economy, the olive-growing regions bloomed economically, socially and culturally (Loumou and Giourga, 2003). Today, olive tree continues to be an environmentally and socio-economically important species and the growing demand for olive products (Amira et al., 2017), attributed to consumer awareness of products with high nutritional value (Guo et al., 2018; Flori et al., 2019) and health benefits (Visioli et al., 2002; Kiritsakis, 2007; Covas et al., 2009; Kiritsakis and Shahidi, 2017; Kostelenos and Kiritsakis, 2017; Gorzynik-Debicka et al., 2018; Flynn et al., 2023) is continuously increasing.

Although more than 2,600 cultivars (cvs.) that constitute the rich genetic heritage of the species are included in the World Catalogue of Olive Cultivars (International Olive Council – IOC), only 10% are regarded as commercial (Conte et al., 2020). Owing to the fact that, commercial cultivars are selected according to specific features that are highly related to consumer preferences and their selected use (oil, table or both). Since, each olive cultivar has its own unique chemical and taste characteristics, the selection is determined by different morphological and organoleptic parameters. In particular, table olives are obtained from varieties with low oil content, with medium to large size, appropriate shape, correct flesh/stone ratio, green to black skin and appropriate texture (Conte et al., 2020). Table olives are one of the most popular fermented foods of plant origin, with a world consumption of ca. 2,7 million tonnes, in the years 2021/2022 (IOC, accessed on March 2024). However, the IOC estimates that almost all of the harvested olives are used for oil production. In fact, despite the growth of a number of substitutes, olive oil continues to be essential to the Mediterranean diet and remains a cultural component closely linked to the dietary needs of those who live in this region of the world. Internationally, olive oil production is undergoing constant growth and tied not only to a steady worldwide increase of the olive growing area with more intensive plantations (Rallo et al., 2013; Guerrero-Casado et al., 2021), but also to improvements in irrigation systems (Fernández et al., 2018; Trentacoste et al., 2019) and technological advances (Juliano et al 2023; Anastasiou et al., 2023). According to the latest available data on FAOSTAT (2022), the European Union (EU) is the main market for olive suppliers and among the Mediterranean basin countries, Spain ranked as the main olive producer (2,635,280 ha; 3,940,070 tonnes), followed by Greece (846,660 ha; 3,045,100 tonnes),

Italy (1,076,520 ha; 2,160,400 tonnes), Turkey (901,126 ha; 2,976,000 tonnes), Morocco (1,201,308 ha; 1,968,110 tonnes) and Tunisia (1,799,251 ha; 1,200,000 tonnes) (Table 1).

Table 1 – Total olive cultivated areas and productions of the main producing countries in the Mediterranean basin (FAOSTAT, 2022).

COUNTRY	CULTIVATED AREA [HA]	PRODUCTION [T]
Algeria	457,609	822,973
Egypt	112,851	1,137,075
Greece	846,660	3,045,100
Italy	1,076,520	2,160,400
Libya	220,009	143,410
Morocco	1,201,308	1,968,110
Portugal	379,570	791,660
Spain	2,635,280	3,940,070
Syria	676,338	990,948
Tunisia	1,799,251	1,200,000
Türkiye	901,126	2,976,000

Table olive production and oil industry were initially restricted to the producing regions and consumed only within the limited regions across the Mediterranean basin and the Middle East. Afterward and the last few decades, olive oil started to be consumed in the global market. Nowadays, an expansion in olive cultivation from the Mediterranean basin has been occurring in non-traditional several subtropical countries such as Argentina, Australia, Chile, China, Japan, Peru, Ukraine, Uruguay, (Crimea) and USA (Vossen, 2007; Malheiro et al.; 2015; Petruccelli et al., 2022). It is expected that producers in traditional localities will distinguish their products as competition among olive growers intensifies (Menapace et al., 2011; Dekhili et al., 2011).

1.2 Olive cultivation in Sicily

Among the countries of the EU countries, Italy occupies a prominent position in terms of the dedicated area to olive cultivation and the corresponding harvested production (see Table 1). Olive growing has been a cornerstone of Italian culture and economy since antiquity and is still widespread in many regions (Caracuta, 2020). Olive growing is the most important agricultural activity in Italy, with the majority of olive groves concentrated in the southern regions (see Table 2). In fact, the southern regions of Italy are the primary production area, accounting for about 87% of the total olive production.

Table 2 – Cultivated areas and total olive production in Italy (AgrIstat, 2023).

AREA	CULTIVATED AREA [HA]	PRODUCTION [Q]
Northern Italy		
Emilia Romagna	4,449	31,650
Friuli Venezia Giulia	282	5,653
Liguria	17,130	293,425
Lombardy	2,370	56,207
Piedmont	179	1,194
Trentino Alto Adige	392	15,000
Aosta Valley	1	40
Veneto	4,995	104,600
TOTAL	29,798	507,769
Centre Italy		
Latium	82,997	1,014,740
Marche	4,388	101,316
Tuscany	86,041	573,847
Umbria	27,191	254,507
TOTAL	173,426	1,689,903
Southern Italy		
Abruzzo	41,921	1,434,495
Basilicata	27,610	325,331
Calabria	184,673	5,875,211
Campania	72,043	1,379,265
Molise	14,325	596,740
Apulia	347,400	10,926,400
Sardinia	40,283	832,885
Sicily	167,228	3,595,540
TOTAL	895,483	24,965,867
ITALY	**1,272,133**	**28,853,442**

Within the southern Italian regions actively engaged in olive cultivation, Sicily (167,228 ha; 3,595,540 q) holds a noteworthy position in the production, industry, and export of olives and olive oil. It stands as the third-largest producer, following Apulia (347,400 ha; 10,926,400 q) and Calabria (184,673 ha; 5,875,211 q) (Table 2). Indeed, the olive tree has been cultivated in the Sicilian region since ancient times (Zohary and Spiegel-Roy, 1975; Speciale et al., 2023). Historical records revealed that farmers in southwestem Sicily gained recognition in the oil trade across the Mediterranean region, engaging in the cultivation, propagation, and development of grafting techniques for the olive trees (Zohary, 1993; Besnard et al., 2001; Schicchi et al., 2021; Rodríguez-Ariza et al., 2021). Over the centuries, the olive cultivation in Sicily has extensively expanded across all provinces, from sea level to wherever the climatic conditions have allowed it.

Subsequently, olive becomes the principal fruit tree that dominates the Sicilian agricultural landscapes (Schicchi et al., 2021). The island is currently undergoing a two-fold agricultural evolution. On one hand, there is a growing presence of specialized and mechanized olive farms. On the other hand, there is a simultaneous decline of fragmented and outdated farms characterized by low competitiveness, uneven land divisions, steep terrain, and insufficient irrigation. This dual trend highlights the need for a balanced approach that considers the socio-economic and environmental impacts of modernization while preserving cultural heritage and sustainable practices (Maesano et al., 2021).

Olive cultivation covers 7% of total regional territory and contributes significantly to the physiognomy of the agro-ecosystems. According to the latest data available (AgrIstat, 2023), the Sicilian province with the largest area of olive cultivations is Messina (36,500 ha; 609,500 q), followed by Agrigento (30,008 ha; 993,930 q) and Trapani (27,500 ha; 595,000 q). Agrigento represents the province with the highest production level, followed by Messina and Trapani. The provinces of Syracuse, Caltanissetta and Ragusa are located on less consistent levels, although in these territories there are specific conditions of excellence (Table 3).

Table 3 – Olive production and cultivated area in Sicily by province (AgrIstat, 2023).

PROVINCE	CULTIVATED AREA [HA]	PRODUCTION [Q]
Agrigento	30,008	993,930
Caltanissetta	8,250	90,850
Catania	14,090	140,900
Enna	11,820	591,000
Messina	36,500	609,500
Palermo	22,520	193,860
Ragusa	5,800	142,000
Siracusa	10,740	238,500
Trapani	27,500	595,000

Certainly, variations in soil and climate, cultural practices and cultivar selection among provinces are the main causes of yields fluctuation variations. In addition, new selections and cultivars with higher yields, greater tolerance to biotic and abiotic stresses, adaptability to high-density planting systems, and resistance to climate change are present in more specialized areas. The genetic richness of the Sicilian olive germplasm is well documented (Bòttari and Spina, 1952; La Mantia et al., 2005; Marra et al., 2013; Besnard

et al., 2013) and represents a great patrimony heritage of economical and scientific value, particularly for breeding programmes (Caruso et al., 2014). The Sicilian germplasm is distinguished by a wide genetic diversity, probably related to its past domestication and some biological reproductive peculiarities, such as self-incompatibility (Las Casas et al., 2014).

Currently in Sicily, olive oil production is mainly based on native cvs., such as "Biancolilla", "Cerasuola", "Moresca", "Nocellara del Belice", "Nocellara Etnea", "Ogliarola Messinese", "Santagatese" and "Tonda Iblea" (Marra et al., 2013). The current trend in olive growing is moving toward the use of local cultivars for the production of high-quality olive products (extra-virgin oil and table olives), such as Protected Designation of Origin (PDO), typical of certain geographical areas. For this reason, local government currently supports research and activities aimed at characterizing and recovering local and ancient varieties in order to establish germplasm collections that limit genetic erosion (Faggioli et al., 2005). In Sicily, there are presently six PDO-designated extra virgin olive oils, and efforts are underway in order to obtain PDO recognition for additional Sicilian extra virgin olive oils. These PDO-designated extra virgin olive oils from Sicily are distinguished by their production areas and the olive varieties used, including "Monti Iblei PDO", "Valli Trapanesi PDO", "Val di Mazara PDO", "Monte Etna PDO", "Valle del Belice PDO", "Valdemone PDO" "Colli Ennesi PDO" and "Colli Nisseni PDO". The Sicilian table olive sector, which constitute approximately 10% of the region's total production according to AgrIstat (2023), is mainly based on the "Nocellara del Belice" cv. and, to a lesser extent, "Nocellara Etnea", "Ogliarola Messinese" and "Moresca" cvs., which produce large-sized fruits of high commercial value (Marra et al., 2013).

Within the extensive Italian olive germplasm, certain varieties distinctive to Sicilian olive production have been utilized for crossbreeding in the development of table olives (Sorrentino et al., 2016). However, it is imperative to preserve the genetic and phenotypic variability of these local varieties to uphold biodiversity conservation and support pre-breeding activities (Muzzalupo et al., 2014). This preservation is particularly relevant considering the importance of defining genetic and phenotypic characteristics for EU PDO regulations. Notably there already exists a PDO designation for "Nocellara del

Belice" table olives in Sicily, and exploration of new varieties is essential for potential inclusion in registration processes.

1.3 Olive planting and management systems

Fruit trees cultivation requires sophisticated, commercially viable planting systems and management techniques in order to produce an early, abundant and consistent fruit-bearing (Marra et al., 2016; Beyá-Marshall et al., 2018; Lo Bianco et al., 2021). Moreover, from a social and economic point of view, finding specialized workers became highly difficult due to both high costs and the increasing depopulation in rural areas, thus, minimizing the use of manpower becomes more prominent.

Olive grove management, in particular, has a major impact on orchard productivity, oil quality, production costs and the natural environment. Therefore, several studies were conducted in order to better understand the effect of the various implemented planting density systems on olive production (Tous et al., 1997; Díez et al., 2016; Mairech et al., 2020). Indeed, olive-growing systems are moving from traditional plantings with low density to intensive and super-intensive plantings with extremely high density.

a. Traditional plantings

Over the last years, the majority of Mediterranean olive orchards were traditionally cultivated under rainfed management and mainly located in marginal areas characterized by shallow soils and steep terrain (Loumou and Giourga, 2003; Tous et al., 2010). These orchards are mainly characterized by low planting density and three-dimensional training forms, with the numerous variants adopted locally. This low-density cultivation system rarely exceeds a number of 300 planting trees per hectare, mostly arranged in squares (Lo Bianco et al., 2021). The cultivated trees are characterized by a well-developed root system and their high water and nutrients accumulation capacity of the trunk and large branches. These characteristics allows the tree to overcome environmental stresses such as high light intensity, increased temperatures and prolonged summer-autumn periods of drought, typical climatic characteristics of the Mediterranean areas (Duarte et al., 2008). Furthermore, due to the large size of the trees, the fruits are commonly harvested by spreading nets on the ground, rarely with the aid of machines, on which the drupes fall

naturally (Lavee, 2010). Actually, the use of vibrating combs mounted on mechanical arms or shaking limbs will induce the detachment of olive fruits and make the harvesting process much easier. This operation needs the employment of many workers to collect the fruits, thus a higher harvesting cost (Lo Bianco et al., 2021).

Olive pruning usually involves the use of chainsaws with various power and dimensions, and it is considered as a tiring, dangerous, expensive and often unsustainable technique. Pruning is often conducted at extended intervals, typically every 4–5 years (Castillo-Ruiz et al., 2017). Pesticide treatments necessitate the application of abandoned water volumes, delivered to the upper portions of trees through the use of long-range spears. This procedure frequently leads to the dispersion and dripping of pesticides onto the ground, resulting in considerable environmental pollution impacts (Lo Bianco et al., 2021). Due to the outlined challenges, traditional plantings are facing abandonment, and their sustainability is increasingly related to the cultural role of olive cultivation, characteristic of agroforestry systems, rather than exclusive olive production. This form of olive cultivation offers various ecosystem services such as enhancement of rural landscapes, preservation of cultural heritage, biodiversity promotion and soil conservation. In specific contexts, these ecosystem services may even take precedence over the primary productive function. (Loumou and Giourga, 2003; Nieto et al., 2010; Brilli et al., 2016; Brilli et al., 2019; Lorite et al., 2018).

b. Intensive plantings

An observed transition towards intensive planting systems has been driven by the increasing global demand for olive products, workman scarcity and various socioeconomic constraints, including the imperative to enhance profitability (Visioli et al., 2002; Kiritsakis et al., 2007). This paradigm shift necessitated the extensive irrigation and fertilization of large areas to augment yield per unit area. (Villalobos et al., 2006). Therefore, olive trees were adapted to mechanical pruning and harvesting in order to reduce costs per unit yield and increase production efficiency (Connor and Fereres, 2010). The evolution of irrigation, management and harvesting techniques has changed the olive production systems (Rallo et al., 2013). The establishment of plantations in new areas characterized by increased water availability and favourable edaphoclimatic conditions has facilitated the intensification of olive cultivation. Intensive olive plantations are

characterized by planting densities of 300–1000 trees/ha, organized in square or rectangular configurations. Three major categories of low, medium and high intensive planting densities were identified due to the various adopted range of planting densities (Lo Bianco et al., 2021). Low-density plantings, with a maximum of 400 plants per hectare, use square arrangements and three-dimensional training forms. Harvesting is mechanized, employing either self-propelled machines or those coupled to tractors, equipped with trunk shakers (Sarri and Vieri, 2010). Pruning is facilitated by rod tools without ladders and is limited to the trees with a maximum height of 4,5 meters. Medium-density plantings, ranging from 400 to 800 plants per hectare, adopt training forms such as the vase (suitable for up to 500 plants/ha) or monocone (suitable for planting densities of 400–800 plants/ha) (Lo Bianco et al., 2021). The particular configuration and distribution of the vegetation promotes the transmission of vibrations applied to the trunk by the shaker head, with positive consequences for harvesting efficiency. Moreover, the monocone form, particularly for higher densities (up to 800 trees/ha), minimizes radial expansion, reduce tree spacing along rows and enhance harvesting efficiency through vibration transmission (Fontanazza, 2000). Mechanization is further advanced with self-propelled "side-by-side" trunk shakers (Lo Bianco et al., 2021), while pruning is partially mechanised by topping and, less frequently, by hedging with disc saws mounted on mobile bars and operated by a tractor. Nevertheless, these pruning operations are often "non-selective," requiring manual finishing by skilled operators to maintain canopy structure (Lodolini et al., 2016). In general, intensive olive cultivation ensures higher economic returns and better yields compared to traditional olive cultivation. This economic advantage has led to a trend in many olive-growing countries to convert traditional orchards to more intensive systems (Rallo et al., 2013).

c. *Super-intensive plantings*

Super high-density (SHD) olive groves are rapidly expanding in olive oil-producing countries, leading to significant modifications in the olive growing system and consequential impacts on agronomy, economics, socio-culture, and the environment (Guerrero-Casado et al., 2021). This system, renowned for its high productivity, addresses the escalating global demand for olive oil by producing competitively priced products (Rallo et al., 2013). The adoption of high-density olive trees planting is an example of

entrepreneurial decision-making driven by profit-maximising objectives, which effectively overcomes traditional cultural barriers to the replacement of less productive olive groves (Guerrero-Casado et al., 2021). Modern SHD plantations are characterised by a very high-planting density (around 1600 trees/ha). Some selected cultivars such as "Oliana", "Sikitita" and "Lecciana" showed to be adapted to the SHD cultivation system and allowed even higher planting densities, up to 2500 trees/ha, if compared to the historically Spanish "Arbosana", "Arbequina" and Greek "Koroneiki" cultivated cultivars (Connor et al., 2014; Arquero et al., 2014; Anifantis et al., 2019; Camposeo et al., 2022). This system provided an increased profitability due to the reduced production costs, particularly labor costs that were facilitated by nearly complete mechanization in harvesting, pruning, and phytosanitary treatments (Barranco et al., 2008; Guerrero-Casado et al., 2021). In addition, SHD system has significantly increased olive yield per-hectare because of the high radiation intercepted by the tree canopy, which is made up of many smaller trees (Lo Bianco et al., 2021). However, the SHD system for olive groves faces some limitations, such as its difficult establishment in regions with steep slopes and limited water supplies besides to the few available number of cultivars that could be used since the subsequent loss of local genotypes (Lo Bianco et al., 2021). Actually, reaching a great olive oil and table olive productions, using this super intensive planting system, are highly dependent on the use of cultivars with elite traits such as high-yielding, early maturing, tolerant to both abiotic and biotic stresses, resistant to bruising (in the case of table olives), and amenable to mechanical harvesting (Rallo et al., 2013). Therefore, the process of intensifying olive plantations requires specific cultivars that may differ from those traditionally employed in traditional/intensive plantation systems (Farinelli and Tombesi, 2015; Marino et al., 2017).

To date, the ever-increasing demand for olive oil and table olives leads to the urgent need for modern olive planting systems, but the limited available varietal choices that could be adapted to mechanization forms an obstacle to reach a fully successful production system. The establishment of high and even super-high intensive plantations may decrease habitat heterogeneity and complexity, thus reducing the quality of biodiversity (Guerrero-Casado et al., 2021). Notably, the development of super high-density hedgerow orchards and the adoption of drip irrigation systems are modifying the traditional olive landscape, which has evolved over millennia in the Mediterranean countries. While these benefits are

desirable, the increasing reduction in the number of cultivars or practices such as high inputs of fertilizers or fungicides may have detrimental consequences, namely the reduction in olive genetic diversity or harmful effects on the soil microbiota (Connor et al., 2014; Fernández-González et al., 2019). These alterations, coupled with the high vegetation densities present in high-density orchards, may pose challenges related to increasing the incidence and severity of specific olive pests and diseases, yet have been insufficiently evaluated (Rallo et al., 2013; Cazorla and Mercado-Blanco, 2016). To address these concerns, large-scale research initiatives should be undertaken to exploit the enormous richness of olive germplasm, in order to achieve high levels of food production characterised by unique and high-value quality attributes.

1.4 Impact of climate change on olive cultivation

Climate change is an unequivocal fact that challenges society and each economic sector. Recent reports show that there has been significant global warming over the past 40 years, with annual temperatures now approximately 1.5 °C higher than in the pre-industrial period (1880-1899) (Marques, 2020), well above the current global warming trend (+1.1°C) (Fraga et al., 2020a). On the one hand, the rise in temperature has been accompanied by a series of extreme heat events that are historically unprecedented in terms of duration, intensity and frequency (Kuglitsch et al., 2010; King et al., 2017). On the other hand, there has been a significant decrease in frequency of cold extremes (Frich et al., 2002; Alexander, 2016).

The effects of climate change on agricultural systems, especially in areas undergoing dramatic climatic shifts, are posing significant questions about the compatibility and production of crops in the future. Over the Mediterranean, most climate change scenarios predict an increase in temperature and a decrease in rainfall (Gualdi et al., 2013; Stocker et al., 2013) with a higher frequency of extreme events (Hertig et al., 2014). In particular, southern Mediterranean areas, especially European islands (e.g. Corsica, Sardinia and Sicily) (Ponti et al., 2009), are expected to be most affected by climate change, with reduced crop yields and degraded ecosystems due to increased temperature, increased risk of drought and reduced water availability, that could lead to desertification process (Michalopoulos et al., 2020). These predicted changes in the climate suggest possible effects on natural resources and agricultural productivity. Under future climatic

conditions for perennial crops, the aforementioned projections are expected to cause severe adverse effects, particularly on water relations (Bacelar et al., 2007; Fraga et al., 2020b), oxidative pathways and other physiological processes (Biel et al., 2008; Petridis et al., 2012; Brito et al., 2018), phenological timings (Galán et al., 2005;Villalobos et al. 2006; Fraga et al., 2019), final yield (Villalobos et al. 2006; Quiroga and Iglesias, 2009; Greven et al., 2009; Fernandes-Silva et al., 2010; Fraga et al., 2020c) and quality attributes (Servili et al., 2009; Orlandi et al., 2020; Ben-Ari et al., 2021). Indeed, olive tree is considered a sensitive indicator of climate change degree since its phenology is strongly related to temperature trend (Osborne et al., 2000; Besnard et al., 2007; Fraga et al., 2020a). De Melo-Abreu and co-workers (2004) suggested that the expected increase in temperature may affect the development cycle of olive trees, causing an earlier flowering date (Osborne et al., 2000; Galán et al., 2005; Orlandi et al., 2010; Aguilera et al., 2015; Gabaldón-Leal et al., 2017) or even lead to sterile years due to the lack of vernalisation (Morales et al., 2016). High temperatures can lead to a deficiency in chilling requirements (Gabaldón-Leal et al., 2017; Fraga et al., 2019), resulting in low fruit set which can negatively affect final yield. Additionally, according to Torres et al. (2017), these conditions may result in the production of deformed flower buds and olive fruits, causing problems with blooming and fruit growth (Ayerza and Sibbett, 2001; Gutierrez et al., 2008; Avolio et al., 2012). In addition, increased evapotranspiration can lead to an acceleration of the fruit ripening process, requiring earlier harvests at lower ripening stages (Dag et al., 2014). Moreover, although olive tree is classified as drought-tolerant species (Ponti et al., 2014), water stress may result in a wide range of negative impacts, such as a low flower and fruit setting, low leaf area, limited photosynthesis, flower abscission and/or abortion (Arampatzis et al., 2018). In addition, it is strongly hypothesised that any potential yield gains over the next five decades will be offset by climate change-mediated alterations in disease pressure caused by emerging pathogens and the distribution and spread of their insect vectors (Chaloner et al., 2021).

In fact, in addition to the aforementioned issues, climate change also increases the risk of intensification and spread of plant pests and diseases, which in turn poses a significant threat to the world food supply and plant biodiversity (Velásquez et al., 2018; Scortichini, 2020; Burdon and Zahn, 2020; Muluneh, 2021). A change in short- or long-term climatic conditions can cause a pest or pathogen to expand its range into new areas or retract from

marginal areas (Sutherst et al., 2011). In fact, different studies are reporting that climate change is already affecting several areas worldwide and enhancing the susceptibility of local olive tree cultivars to certain pests and diseases (Ozdemir, 2016; Scortichini, 2020; Caselli and Petacchi, 2021). In general, the set of organisms potentially detrimental to olive plant is composed, to date, of more than 255 species, including insect pests, mites, nematodes and pathogenic agents, including fungi, oomycetes, bacteria and viruses (Bueno and Jones, 2002; Caselli and Petacchi, 2021). In particular, dynamics of pest populations are strongly influenced by weather disturbances. In fact, increasing temperature and humidity have a direct and important impact on reproduction, survival, population and spread dynamics, as well as on the relationships between other pests, the environment and natural enemies (Prakash et al., 2014; Skendžić et al., 2021). Similarly, the spread of pathogens linked to climate change is considered one of the main threats to agriculture health globally. The major meteorological factors that are related to climate change and may influence the plant-pathogen interactions include air and soil temperature, carbon dioxide (CO_2) concentrations, relative humidity, rainfall, wind, and solar radiation intensity (Misra et al., 2020). In particular, changes in temperature and precipitation can directly affect disease incidence and severity by influencing pathogen growth, survival and reproduction (Garrett et al., 2006; Kocmánková et al., 2009; Wolinska and King, 2009; Laine 2023). Additionally, they can alter the expression of host plant disease resistance (Laine, 2009). Elevated CO_2 levels may indirectly impact disease risk due to altered biomass, density, and abiotic conditions within the canopy due to changes in the vegetation (Burdon and Zhan, 2020). These changes may translate directly in altered disease transmission rates, thereby changing epidemiological trajectories (Truscott and Gilligan, 2003). In addition, as reported by Sturrock et al. (2011), climate change affects the life cycles of pathogens and hosts by changing the distribution and phenology of events (e.g., budbreak, release of pathogen spores, activities of vectors, etc.). Modifications in plant host phenology and physiology can also impact their susceptibility to pathogens and the ability of pathogens to infect them. Subsequently, these modifications can affect the attractiveness of the host to vectors, the potential geographic range of vectors and their phenology (including overwintering, density, migration, etc.) (Canto et al., 2009). Climate change can have considerable effects on the rising of emergent plant pathogens also by altering their temporal and spatial distributions

(Burdon and Zhan, 2020), leading to substantial physiological alterations in plants (Gullino et al., 2018). On the other hand, an increase in frequency of outbreaks, introduction of pathogens to new areas, and plant disease intensity are also foreseen (Hunjan and Lore, 2020). In the latter case, the main threat can be represented by the impact of vectors, which find ideal conditions and cause new infection outbreaks. In a fast-changing context for the world's olive cultivation, the impacts of global climate change and the evolution of olive cultivation systems have altered the occurrence and severity of olive diseases, as well as the potential introduction of new or atypical diseases. This evolving scenario poses challenges for olive growers to protect production and adopt economically viable solutions and environmentally friendly strategies in sustainable agriculture. Moreover, agrometeorological studies and an effective environmental management will be required to overcome this challenge in order to predict disease outbreaks and protect and/or improve the worldwide food production with human interventions, adaptation and mitigation strategies.

1.5 Major olive tree diseases

The olive tree demonstrates vulnerability towards socio-economic changes that are rapidly reshaping regional and global agroecosystems (Hall, 2010).

Indeed, both intensive and super- intensive implemented planting systems together with the use of drip irrigation systems and mechanization practices, the excessive use of fertilizers and pesticides, the international agricultural trade of potentially infected materials, coupled with climatic variations, have caused the introduction, reemergence, spread, and establishment of olive pests and diseases (López-Escudero and Mercado-Blanco, 2011; Fernández-Escobar et al., 2013). In addition, the expansion of olive cultivation into new areas and the introduction of non-native cultivars may favour the epidemic explosion of diseases of minor importance into areas with traditional planting systems, as well as the emergence of new diseases (Graniti et al., 2011; Luck et al., 2011). In the last decade, in fact, olive production has been severely challenged by the emergence of biotic agents that have significantly undermined the Mediterranean economy related to the olive tree and oil industry (Montes-Osuna and Mercado-Blanco, 2020). A dramatic example was the case of the recent epidemic of Olive Quick Disease Syndrome (OQDS) caused by *X. fastidiosa* (*X.f.*) subsp. *pauca* bacterium in southern Italy, which decimated olive trees in Apulia (Saponari et al., 2019) and created huge losses in the local olive economy, posing huge challenges for its management (Morelli et al., 2021). Although the emergence of *X.f.* has gained much attention during the few recent years, olive trees remain susceptible to a wide range of threats (Iannotta et al., 2012; Fraga et al., 2020a). Generally, the olive tree is vulnerable to a large number of diseases, mostly caused by fungi (Chliyeh et al., 2014; Keykhasaber et al., 2018), but also by systemic pathogens, including bacteria (Martelli et al., 2000), phytoplasmas (Pollini et al.,1996; Martelli, 1999; Pasquini et al., 2000; Çağlayan et al., 2011) and viruses (Martelli, 1999; Çağlayan and Faggioli, 2024). The main olive pathogens that have an impact on olive groves are listed in Table 4. The viral agents are outlined separately in the section 1.6.2.

Table 4 – Main bacterial, fungal and phytoplasma diseases of olive trees.

AETIOLOGICAL AGENT	DISEASE COMMON NAME
Bacteria	
Pseudomonas savastanoi pv. *savastanoi* (Smith)	Olive knot
Xylella fastidiosa	Olive quick decline syndrome (OQDS)
Fungi	
Venturia oleaginea (Castagne) Rossman & Crous	Olive leaf spot (OLS)
Verticillium dahliae Kleb.	Verticillium wilt of olive (VWO)
Pseudocercospora cladosporioides (Sacc.) Braun	Cercospora leaf spot (CLS)
Colletotrichum species (spp.)	Anthracnose olive (AO)
Camarosporium dalmaticum (Thüm)	Olive fruit rot (OFR)
Marthamyces panizzei (De Notaris)	Parasitic brusca
Armillaria spp.	Root rot disease
Rosellinia spp.	
Phytophthora spp.	
Capnodium spp.	Fruit rots and leaf mould
Limacinia spp.	
Cladosporium spp.	
Alternaria spp.	
Fomes spp.	Trunk rot/decay
Stereum spp.	
Polyporus spp.	
Botryosphaeriaceae spp.	Emerging branch cankers
Phytoplasma	
16S-IB (Aster yellows group)	–
16S-IC (Clover phyllody group)	–
16Sr-III (Peach X disease group)	–
16S-VA (Elm yellows group)	–
16S-XIIA (Stolbur group)	–

The severity of olive diseases across various producing countries underscores the imperative to comprehend their underlying causes and identify factors conducive to their development in olive groves. Furthermore, the evaluation of biological and chemical interventions effectiveness is crucial, along with the assessment of different olive cultivars resistance in diverse cultivation regions (López-Escudero et al., 2004). Moreover, a comprehensive understanding of the pathogens life cycles in olive trees and their interactions with environmental factors is essential for proposing economically viable control measures (Luvisi et al., 2017). Similarly, a thorough knowledge of the

biology of olive tree pathogens, especially viral pathogens and those of unknown aetiology, is essential for the promotion of substantial advances and improvements in detection, diagnosis and identification techniques. This proactive strategy is essential to effectively limit their spread in orchards and nurseries (Martelli, 2013). Pursuing this comprehensive approach requires vigorous collaborative research, implementation of sustainable agricultural practices and vigilant monitoring to proactively mitigate risks and strengthen the resilience of olive production towards the dynamic agro-ecological changes.

1.6 Phytoviruses

Plant viruses are economically important and widely distributed (Awasthi et al., 2016) and, generally, they cause numerous harmful plant diseases, resulting in significant losses in crop production and quality worldwide. Globalization and climate change contribute to the global intensification of viral diseases, and, in the future, this can have serious consequences in food chain (Grešíková, 2022).

Viruses are obligate intracellular biological entities that are capable of infecting eukaryotes, bacteria and archaea, as well as other organisms, occupying a unique position within the biological system (Giunchedi et al., 2007; Raoult and Forterre, 2008).

Based on morphological and biological characteristics, viruses are classified into families, genera and species. These classifications are established by the International Committee on Taxonomy of Viruses (ICTV), using criteria that are carefully reviewed on a case-by-case basis. According to the latest available data, the ICTV lists 11,273 virus species, which are grouped into 2,818 genera, 264 families, 182 subfamilies and a total of 72 orders (ICTV Master Species List 2023). New species are added monthly, thanks to new technologies such as Next-Generation Sequencing systems (NGS), and many of these are capable of infecting plants.

1.6.1 Main features of plant viruses

Viral genome, Viral proteins, Morphology and Viral replication

A complete viral particle (virion) plays a crucial role by delivering its nucleic acid into a host cell, allowing for expression by the host cell's biosynthetic system.

Viral genomes are composed of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) enclosed in a protein envelope and, in some cases, coated with a lipoprotein membrane (Burrell et al., 2017). Since viruses do not have their own metabolic processes, biochemical and physiological activities, they cannot reproduce their genetic material themselves, they exclusively replicate within a living host cell. So that they can survive, viruses take advantages from the host cell mechanisms and synthesize proteins and assemble their own viral components.

Actually, the nucleic acid contains the genetic information essential for encoding all the virus's replicative functions, instructing the host cell to perform tasks necessary for viral replication. The genetic information is contained within one (monopartite genome, all viral genes contained in a single molecule of nucleic acid) or more (segmented genome, viral genes distributed in multiple molecules or segments of nucleic acid) nucleic acids, which can be either single-stranded (ss) or double-stranded (ds) (Burrell et al., 2017).

The genome of most plant viruses is composed of RNA and some with single-stranded DNA. The single strands are classified by their polarity as either positive-sense (positive strand; +) or negative-sense (negative strand; -), depending on whether they are complementary to the viral messenger RNA (mRNA) or not. RNAs with positive polarity can serve directly as messengers for protein synthesis (Murphy et al., 2012). The nature and properties of viral proteins are determined by the nucleotide sequence of the genetic material. These proteins can be classified as either structural or non-structural, depending on their biological functions. Structural proteins are broadly defined as proteins found in virus particles, play essential roles in capsid organization and are intimately associated with the viral genome to form the nucleocapsid. Whereas, non-structural proteins are encoded by the virion genome and expressed inside the virus infected host cells, but not found in the virion particles. This type of proteins performs specific functions related to viral replication or transcription processes, proteolytic cleavage of polypeptides, virion diffusion in host tissues and interplant viral particle transfer. Morphologically, phytoviruses display diverse shapes and sizes. Approximately half of them possess an elongated or spherical shape (isometric or polyhedral), while the rest exhibit a bacilliform morphology. Primarily, the virus morphology is determined by the nature of the capsid structure. Coating proteins encoded by the viral genome limit the number of proteins allocated for specific functions due to their short length. Consequently, the envelope

typically consists of repeated units of one or a few proteins, forming either a helical distribution around the nucleic acid (helical symmetry) or an icosahedral shell (cubic symmetry), known as a "capsid." The latest encapsulates the nucleic acid and its associated proteins (nucleoproteins), collectively are referred as "nucleocapsid." Capsid proteins not only shield the viral nucleic acid but also determine the virus's antigenic characteristics, influencing transmission and, in some cases, specificity towards hosts (Burrell et al., 2017). Since viruses cannot multiply outside the host cell, they must infect host cells and use cellular machinery and energy supplies to replicate and produce the progeny viruses. Distinct genome types exhibit different replication pathways, which are linked to controls for translation and movement within the host cell. Once a plant virus has infected a compatible host, it spreads from cell to cell until it reaches the vascular system. The movement of viral particles between cells occurs through plasmodesmata and is facilitated by the viral genome-encoded movement protein (MP) (Sánchez-Navarro et al., 2006).

Transmission

Transmission from one plant to another is a crucial process for the survival of viruses. Plant viruses have developed various strategies to perform this task efficiently; in many cases involving the existence of specific viral gene products known to facilitate the transmission process. Transmission of phytoviruses can occur in several ways, i.e. by seed, pollen, contact, contaminated tools, vectors and infected propagation material. Seed transmission can occur through either embryonic (seed-borne transmission) or non-embryonic (seed-transmitted transmission) means. In the former, virions reach the embryo through a direct invasion of the embryonic tissue, while in the latter, the virus is confined to the seminal tegument or retained on the outer surface of the seed. Viruses in seeds can persist for a long time, allowing a long-distance spread (Sastry, 2013).

Pollen-mediated transmission is referred to the virus infection of the embryo sac through infected pollen. Viral particles are transported by pollen grains to pollinated plant tissue that can lead to direct transmission of the virus from one plant to another (Mink, 1993). However, cases of transmission by infected pollen are limited to a few virus-host combinations. Vegetative propagation of infected plants represents a very efficient method for virus spread. The wide-spread use of vegetative propagation material for the

multiplication of many horticultural crops results in the spread of the diseases through propagules such as tubers, cuttings, scions and rootstock. Since infection by most viruses is completely systemic, then any vegetative portion is likely to be infected. Importation and exportation of infected vegetative portions that often remains symptomless is a critical concern in virus epidemiology as it spreads virus and viroid diseases from one Country to another (Sastry and Zitter, 2014; Mathioudakis et al., 2020). Transmission by contact occurs through micro-lesions of the plant's epigeal or hypogeal apparatus. Indeed, there are two major types of transmission: direct contact between infected plants and healthy ones, and indirect contact through wounds or injuries caused during cultivation operations, such as contaminated tools. At the root level, transmission by contact occurs through lesions on young roots during growth, which can acquire viruses that persist in the soil. However, from an epidemiological and ecological perspective, most described plant-infecting viruses are transmitted by vector that acquire the virus from infected plants and subsequently inoculate other host plants (Nault, 1997). Vectors can include various species of insects or mites, as well as nematodes, fungi, and protozoa. In fact, insects are the most significant virus vector organisms due to the wide range and severity of viral diseases that have been transmitted (Hogenhout et al., 2008). For these reasons, knowledge and understanding of transmission mechanisms of viral pathogens is crucial for the implementation of appropriate defence strategies for adequate crop protection.

Symptomatology

As obligate intracellular parasites, plant viruses catalyse alterations in the cellular physiology, metabolic function and cellular machinery of host cells to support their own replication. This reorganisation of cellular resources often triggers widespread physiological perturbations in the host. In the most described cases, virus-host plant interactions negatively affect host morphology and physiology (Hull, 2014), leading to the expression of disease symptoms. Although viral diseases do not always result in plant death, they can have a significant impact on plant health and productivity. The most common symptoms of virus-infected plants are those that appear on the leaves, stem, fruit and roots. In general, symptoms of virus infection may include developmental defects such as stunting, malformed leaves, shoots or roots, or a range of foliar symptoms such as chlorosis, mosaics, vein clearing or banding, and even localised or systemic necrosis.

Plants may show severe acute symptoms immediately after infection, resulting in death of young shoots or the entire plant. However, if the host survives the initial shock phase, symptoms tend to become milder (chronic symptoms) at later stages of plant development, leading to partial or even complete recovery. In some viral diseases, however, the severity of the symptoms may increase progressively, leading to a gradual or sudden decline of the plant (slow/rapid decline symptoms). Finally, certain viruses can infect host plants without any visible symptoms (latent viruses); in fact, recent studies have shown that asymptomatic infection of plants with viruses may be a much more common event in nature than originally thought (Barba et al., 2014; Stobbe and Roossinck, 2014; Zhang et al., 2018) Asymptomatic infections may result from tolerance, where plants do not suffer from wild-type (high titer) virus replication, or from viral persistence, where virus titers are reduced to avoid cytopathic effects and host damage (Takahashi et al., 2019). However, this controversial topic is still to be discussed and therefore further research is required not only to understand this phenomenon, but also to identify the genetic traits that could keep viruses in a more dormant state (Little et al., 2010; Råberg, 2014).

Control of viral diseases

The management of plant viruses poses challenges in terms of curative interventions due to their obligate intracellular nature. Thus, strategies for addressing diseases caused by plant viruses necessitate comprehensive risk reduction and prevention measures, considering the dynamic and evolvable nature of these pathogens. The formulation of management strategies for plant viruses should account for the characteristics of host plants, their propagation modes, the biology and ecology of vectors, transmission traits, and the infection cycle of phytoviruses. Additionally, the availability of sensitive, high-throughput virus detection methods is a prerequisite in order to establish an effective risk reduction measure for virus management (Tatineni and Hein, 2023). Therefore, sensitive, high-throughput and user-friendly diagnostic methods for the early identification of viral aetiological agents become crucial for the timely application of appropriate disease control measures (Sankaran et al., 2010; Baldi and La Porta, 2020). Given the variability in virus occurrence and disease intensity from year to year, a multitude of factors must be considered for rational and economically viable disease management (Shtienberg, 2000).

For this purpose, and within the framework of integrated pest management (IPM), the implementation of prophylactic measures based on the use of virus-free plant material obtained through certification programmes is essential to prevent the spread of viruses through infected seed, plants and propagating material (Riyaz and Kathiravan, 2019). Additional strategies for managing viral diseases include vector control through chemical treatments or biological control using natural predators of vectors. Finally, the deployment of resistant plants obtained through genetic improvement programmes or genetic engineering is shaping up to be one of the most effective methods of controlling viral diseases (Rimbaud et al., 2021).

1.6.2 Olive virus and virus-like diseases

Olive has always been considered as very resistant species to diseases caused by different pathogens. However, several pathogens, mainly systemic ones, can affect the trees and, in some cases, invalidate the production. Recent advances in plant pathology and molecular biology have significantly contributed to the discovery of new olive viruses, to characterize their genomes, biology, and epidemiology. Olive trees are infected with systemic pathogens such as viruses and virus-like disease, as well as agents of diseases of unknown aetiology. Viral infections, to which little attention was paid until the relatively recent past, are surprisingly widespread in olive cultivation (Martelli, 2013). The first report of olive trees viral diseases goes back to 1938 (Pesante, 1938). Since then, the number of viral agents reported in olive cultivation has increased over time, mainly in the principal Mediterranean producing countries where olive trees are economically important, but also in new areas where olive cultivation has been established. To date, seven-teen different viruses belonging to ten genera and nine families (with two unassigned species) have been identified in olive plants (Table 5) besides to other eight virus-like diseases (Çağlayan and Faggioli, 2024).

Table 5 – List of viruses identified in olive trees and their geographical distribution.

VIRUS SPECIES	ACRONYM	FAMILY/GENUS	FIRST REPORT	GEOGRAPHICAL DISTRIBUTION
Nepovirus arabis	ArMV	*Secoviridae/Nepovirus*	Savino *et al.*, 1979, Italy	Egypt, Italy, Lebanon, Portugal, Syria, Turkey, USA, Greece
Nepovirus avii	CLRV	*Secoviridae/Nepovirus*	Savino and Gallitelli, 1981, Italy	Croatia, Egypt, Italy, Lebanon, Portugal, Spain, Syria, Tunisia, Turkey, USA, Albania, Greece
Nepovirus oleae	OLRSV	*Secoviridae/Nepovirus*	Savino *et al.*, 1983, Italy	Italy, Portugal, Syria, Egypt, Tunisia, Albania
Stralarivirus fragariae	SLRSV	*Secoviridae/Stralarivirus*	Marte et al., 1986, Italy	Croatia, Egypt, Italy, Lebanon, Portugal, Spain, Syria, Tunisia, Turkey, USA, Albania, Greece
Olive vein yellowing associated virus	OVYaV	*Alphaflexiviride/Potexvirus*	Faggioli and Barba, 1995, Italy	Italy
Olive semilatent virus	OSLV	Unclassified	Materazzi *et al.*, 1996, Italy	Italy
Olive yellow mottling and decline associated virus	OYMDaV	Unclassified	Savino *et al.*, 1996, Italy	Italy
Olive leaf yellowing associated virus	OLYaV	*Closteroviridae/Olivavirus*	Sabanadzovic *et al.*, 1999, Italy	Albania, Croatia, Egypt, Italy, Israel, Lebanon, Greece, Morocco, Spain, Syria, Tunisia, USA, Portugal, Cyprus, Chile, Australia, France, Algeria
Alphanecrovirus oleae	OLV-1	*Tombusviridae/ Alphanecrovirus*	Gallitelli and Savino, 1985, Italy	Italy, Jordan, Portugal, Egypt, USA, Lebanon, Syria, Turkey, Tunisia, Albania
Olive latent virus-2	OLV- 2	*Bromoviridae/Oleavirus*	Savino *et al.*, 1984, Italy	Italy, Syria, Croatia, Lebanon, Tunisia, Egypt
Olive latent virus-3	OLV- 3	*Tymoviridae/Marafivirus*	Alabdullah *et al.*, 2009, Italy	Greece, Italy, Lebanon Malta, Portugal, Syria, Tunisia, Turkey
Alphanecrovirus tessellati	OMMV	*Tombusviridae/ Alphanecrovirus*	Cardoso *et al.*, 2005, Portugal	Portugal, Tunisia
Cucumber mosaic virus	CMV	*Bromoviridae /Cucumovirus*	Savino and Gallitelli, 1983, Italy	Croatia, Italy, Portugal, Spain, Syria, Tunisia, Turkey, USA, Albania, Greece
Tobacco mosaic virus	TMV	*Virgaviridae/Tobamovirus*	Triolo *et al.*, 1996, Italy	Italy
Betanecrovirus nicotianae	TNV-D	*Tombusviridae/Betanecrovirus*	Félix and Clara, 2000, Portugal	Portugal, Tunisia
Olive virus T	OlVT	*Betaflexiviridae /Tepovirus*	Xylogianni *et al.*, 2021, Portugal	Portugal, Greece
Olea europaea geminivirus	OEGV	*Geminiviridae*/Unassigned	Chiumenti *et al.*, 2021, Italy	Italy, Spain, USA, Portugal

Some of these viruses are well characterized and showed to infect many crops, whereas others are apparently restricted to olive trees. Basically, these viruses can be divided into two main groups: viruses identified for the first time in olive trees (viruses named after olives) and viruses already known to infect different crops and subsequently identified in olive trees (other viruses). Some of them are rare and have been detected sporadically, while others are present in different countries and have a high incidence of infection in olive plants.

The greatest number of viruses has been detected in Italy, Portugal and Spain, and, more recently, in other areas where olive trees are cultivated (Fontana et al., 2019). In many cases, infections are asymptomatic (such as for OLRSV, OSLV, OLV-1, OLV-2, OLV-3) and there is no evidence about their threat to other crops. However, displayed symptoms consist of leaf and fruit deformations and leaf discolouration ranging from chlorosis to bright yellowing (Martelli, 2013). Some viruses are involved in the "leaf yellowing complex" (OLYaV, OYMaV, OVYaV) and lately unusual woody cylinder deformations like stem pitting and woody gall disease (OLYaV) have been reported. SLRSV and CLRV are both associated to manifest disease either in olive plants or in other crops. Although, ArMV seems to be latent in olives, it is one of the most harmful pathogens for other crops. CMV and TMV are rare in olive plants, while TNV-D and OMMV are in a restricted geographical area of distribution and related to necrosis symptoms. Finally, OlVT and OEGV are the last viruses found in olive trees using a High-throughput screening (HTS) approach. However, their impact on olive trees is still unclear.

Evidently, the epidemiology of most olive tree viruses is still unknown, although some of these viruses are soil-borne (SLRSV, ArMV, TNV), others can be transmitted mechanically (TMV), by seed (CLRV and OLV-1) (Saponari et al., 2002), by aphids (CMV) and nematodes (ArMV, SLRSV) or only by mechanical inoculation and grafting (OLV-2, OLRSV, OlV-T, OLYaV) (Çağlayan and Faggioli, 2024). There is a very limited knowledge about virus transmission mechanism under field conditions, such as the casa of OLYaV that was reported to be detected in mealybugs and psyllids that fed on infected olive trees (Sabanadzovic et al., 1999; Ruiz-García et al., 2021). All these viruses, however, have been found to be easily transmitted through infected propagation material (Martelli, 2013; Campos et al., 2019; Afechtal and Mounir, 2020; Mathioudakis et al., 2020; Materatski et al., 2021). Recently, experimental studies were carried out for OLYaV and SLRSV possible effects on olive plant propagation and preliminary results showed that both viruses did not either influence young shoots rooting rates or interfere in grafting success. At the same time, significant differences in grafting success were observed only during a temperature stress,

probably due to reduced water need of infected plants (Çağlayan et al., 2011). The absence of negative effects of the two viruses on olive plants propagation can contribute to their transmission through propagative material (Roschetti et al., 2009). Although these studies were helpful in understanding the dispersal of some olive viruses, further in-depth epidemiological studies are needed to obtain a complete understanding of the spread and distribution of these viruses under natural conditions. In particular, the vegetative propagation of olive trees by means of semi-woody cuttings, in fact, has considerably contributed over the years to the spread of viral pathogens, with variable economic effects on both yield and production quality (Xylogianni et al., 2021). The real economic impact of virus infections on olive production has not yet been determined, even though in some recent studies, it was reported that some olive viruses has affected the yield, oil quality and has reduced product quality and/or market value due to visual defects (Godena et al., 2012; Erilmez and Erkan, 2014; Fontana et al., 2019). For these reasons, olive phytoviruses management should not be underestimated or overlooked.

Due to the latency of several infections caused by viruses, visual inspections are showed to be not reliable and laboratory tests were performed to detect and identify olive viruses. In fact, in the past, the assessment of olive plants health status has been conducted for several years exclusively by mechanical transmission to herbaceous hosts, a technique with severe limitations, mainly due to the fact that not all viruses are mechanically transmissible to herbaceous plant species. Likewise, olive viruses serological diagnostic tests showed severe limitations in terms of sensitivity, probably due to the presence of tannins and oxidants in olive tissues (Martelli, 1999). Accordingly, in the last decades, several molecular approaches have been developed and improved to detect olive viruses. In order to obtain pathogen-free material from infected trees, sanitation treatments such as heat therapy, meristem tip culture, and micrografting could be applied, although their application for virus elimination in olive trees is still limited (Çağlayan and Faggioli, 2024). Therefore, in addition to the implementation of preventive measures, more sensitive and reliable detection methods are required, especially for viruses that do not generate symptoms in plant.

1.7 Sanitary certification of olive tree

Phytosanitary legislation and phytopathological diagnosis played a crucial role in mitigating the introduction risk of invasive plant pests, particularly in light of the frequent outbreaks of non-native species. In the past, the selection of high-quality olive germplasm was based on the agronomic and pomological characteristics of the plants and on the quality and yield of the olive product. In particular, the study of the sanitary status of the selected ecotypes was mainly conducted by visual inspection. This led to the proliferation and spread of systemic pathogens, either in latent form or in the form of specific symptoms, which were initially confused with the phenotypic expression of the plant. In fact, the introduction of pests can cause severe damage to agricultural production, leading to genetic erosion of plant species (Martelli et al., 2016). Therefore, the propagation and marketing of plants free from harmful pathogens is achieved through phytosanitary selection and certification programmes, including pomological selection for trueness to type and superior quality traits. In the European Community (EU), the current legal framework on plant protection is constituted by the Regulation (EU) 2016/2031 of 26 October 2016, and the related delegated or implementing regulations (Regulation (EU) 2017/2313, Regulation (EU) 2018/2018; Regulation (EU) 2018/2019; Regulation (EU) 2019/827; Regulation (EU) 2019/2072; Regulation (EU) 2019/1702; Regulation (EU) 2019/829; Regulation (EU) 2019/2148; Regulation (EU) 2020/1201). These lists are subjected to continuous updating as a result of an ongoing re-evaluation process of the risk posed by various harmful organisms on the basis of recent technical and scientific developments. The regulation provides the legal basis for the establishment of measures for pests as lists of Union-regulated nonquarantine pests, Union quarantine pests and priority pests by the member states.

For instance, the Regulation (EU) 2016/2031 describes the current rules for olive trees exchange and propagation materials within the EU, as well as its exportation to other countries and defines the phytosanitary requirements that must be met for this plant species to prevent the introduction and spread of harmful organisms. According to the abovementioned regulation, thirteen olive tree pathogen agents are currently listed in Part J of Annex IV of the Commission Implementing Regulation (EU) 2019/2072 concerning fruit plants propagation material for planting. Specifically, the Union-regulated nonquarantine pests of olive include the viruses SLRSV, ArMV, CLRV, OLYaV, OVYaV and OYMDaV; the nematodes *Meloidogyne arenaria* Chitwood, *Meloidogyne incognita* (Kofoid and White) Chitwood, *Meloidogyne javanica* Chitwood and *Pratylenchus vulnus*, *Xiphinema*

diversicaudatum; the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (E.E. Smith); and the fungal pathogen *Verticillium dahliae* Kleb. The pathogen *X. fastidiosa* is regulated as a priority quarantine pest in the EU and it is listed in the Part B of Annex II of the Commission Delegated Regulation (EU) 2019/1702 of August 1st 2019. The Commission Implementing Regulation (EU) 2020/1201 of August 14 2020, regulates the measures for controlling and eradicating this pathogen in areas where outbreaks occur. These regulations obligate the member states to conduct annual surveys in the territory through visual inspections of the disease symptoms and diagnostic tests for the identification of this pathogen, and also by the subspecies identification of the subspecies in the event of its detection. The pathogen detection and subspecies identification are required to take place following the indicated procedures in the annex of the regulation and have to be carried out by competent authorities or in laboratories authorized by the competent authorities. The movement of fruit plants/propagation material, including olive, out of the delimited areas and within the infected areas is regulated with Council Directive 2008/90/EC of 29 September 2008. According to this law, assessment of sanitary status of olive propagating materials are compulsory and plants are certified as CAC (*Conformitas Agraria Communitatis*). In addition, in order to be imported into Europe, plants from countries where a harmful organism is not present must be accompanied by a phytosanitary certificate containing a further declaration that the country of origin is free from the specific pests in question. The sanitary quality of the olive tree propagation material should be verified for the purpose of certification according to the provisions of EPPO Standard PM 4/17 (3), which provides guidelines for the production of pathogen-tested olive varieties or rootstocks intended for planting. According to those standards, the certification scheme started from the source plants (nuclear stocks), which are propagated through various stages in order to control the phytosanitary status and the trueness to type of the plants produced, also to guarantee the traceability of the entire supply chain (EPPO Standards - PM 4). Nuclear stocks are selected according to the agronomic characteristics of interest and the absence of regulated pests that will serve as candidate mother plants. Subsequently, the selected plants are propagated and used as sources of propagation material, later on and according to an adopted hierarchical scheme, certified mother plants are obtained. The obtained certified mother plants will serve as a stock, from which certified olive trees, with high quality (genetic and sanitary) material, are produced and provided to growers. Propagation of both nuclear and propagation stocks are performed either by seeding or self-rooting for rootstocks, while varieties are multiplied by self-rooting or grafting on pathogen-tested rootstocks with the same or a higher degree of

certification. During all certification steps, plants are subjected to visual inspections and to diagnostic tests to verify that they are free from the Union-regulated olive tree non-quarantine pests and the priority quarantine pathogen *X. fastidiosa*. However, plants intended for export are subjected to mandatory controls for the pathogens covered by the phytosanitary legislation of the importing country. Preventing the introduction and spread of high-risk pests requires compliance with legislative measures and cooperation between countries, which are the objectives pursued by the various authorities responsible for plant protection. In fact, each EU Member State adopts its own enforcement and implementation policies, using the EU Directives as a guide.

Italy has been among the first countries that adopted an effective certification system for the production of plant propagation material with high quality standards (Albanese et al., 2012). In Italy, it is compulsory that the production and marketing of olive propagating material fulfils the requirements established by the Italian Legislative Decree No. 18 of 2 February 2021 in compliance with the aforementioned. Certainly, olive plants of the "pre-basic", "basic" and "certified" categories have to be free from diseases and harmful organisms and their absence has to be ascertained using specific diagnostic tools Table 6.

Table 6 – Diseases and harmful organisms and related information according to the current Italian legislation.

Diseases and/or harmful organisms	Controls			
	Visual inspection		Laboratory test	
	Season	Periodicity	Sample type and era	Diagnostic test
Virus				
ArMV	Spring, autumn	Annual	Cortical tissue from lignified branches and new vegetation	PCR
CLRV				
SLRV				
OLV-1				
OLV-2				
OLYaV				
OLRV				
CMV				
TNV				
Phytoplasmas				
Phytoplasma	Spring	Annual	–	PCR
Bacteria				
Xylella fastidiosa	At any time of the year	Annual	Leaves and twigs	PCR
Pseudomonas savastanoi pv. *savastanoi*	Spring, autumn	Annual	Vascular tissue of 1–2-year-old branch portions	Isolation
Fungi				
Verticillium dahliae	April to September	Annual	–	Isolation

Although EU directives and Italian regulations have been critically reviewed and implemented, it is necessary to continuously update the list of pathogens and diagnostic protocols, including the latest tools for genetic and phytosanitary evaluation. The participation and training of all operators in the sector also play a key role in ensuring the effectiveness of these measures. These measures are one of the most convenient tools for controlling plant pathogens, allowing operators not only to protect the agriculture and economy of a region, but also to safeguard natural ecosystems and biodiversity, which are important for the sustainability of agricultural production.

1.8 Main diagnostic methods for olive tree pathogens and future prospects

Sanitary certification programmes require reliable and sensitive diagnostic tests to identify pathogen-free trees and assess the entire plant production process. In this context, early disease detection is essential to prevent the spread of diseases and minimise damage to crop production. Due to the latency of several infections in olive plants, visual inspections are not reliable, and laboratory tests have to be performed in order to detect and identify olive viruses (Çağlayan and Faggioli, 2024). Biological and serological tests (such as the double-antibody sandwich enzyme-linked immunosorbent assay–DAS-ELISA and direct tissue blot immunoassay–DTBIA), are commonly employed for pathogen detection in various crops, but they faced some limitations when applied to the diagnosis of olive pathogens. These challenges stem from the absence of distinctive woody indicators suitable for bioassays and the application complexities for serological tests resulting from the interference of olive contaminants (Çağlayan and Faggioli, 2024). In fact, attempts to utilize DAS-ELISA for extensive diagnosis in olive trees, in particular for olive viruses detection, have generally proven unsuccessful. This may be attributed to the presence of tannins and oxidants in olive tissue, which render sap preparation for the DAS-ELISA protocol a challenging task (Martelli, 1999; Bertolini et al., 2001). Despite these challenges, serological diagnostic protocols are available, particularly for the detection of bacterium *X. fastidiosa,* SLRSV, CLRV, CMV, and ArMV, as well as *Verticillium dahliae* Kleb. (Henriques et al., 1992 ; Çağlayan et al., 2004 ; Yucel et al., 2005; Loconsole et al., 2014; Gorris et al., 2020),

Over the last 30 years, molecular techniques based on hybridisation or amplification of nucleic acids, such as reverse transcriptase-polymerase chain reaction (RT-PCR), polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) and their variants, have been developed for the major plant pathogens. In particular, these molecular techniques have greatly improved the sensitivity and specificity of olive pathogen diagnosis. Molecular technology has demonstrated success in both routine and extensive detection, presenting potential for adoption in countries seeking to advance olive growing by producing and propagating disease-free planting material.

Since timely and accurate detection methods are indispensable in attaining optimal growing conditions of olive crops, many current diagnostic methods showed to be effective for olive plant pathogen detection and management. Specifically, olive phytoplasma detection are based on PCR and nested-PCR assays on total DNA isolated from olive trees. Additionally, analysis using restriction fragment length polymorphism (RFLP) are employed to identify

phytoplasma pathogens, although it is not obligatory for sanitary certification (Marzachì et al., 1999; Pasquini et al., 2000; Ferreira et al., 2021). Real-time qPCR protocols for phytoplasma identification and group characterization have improved diagnostic techniques in recent years, demonstrating potential for future use (Satta et al., 2017).

Visual observation of foliar and vascular browning symptoms is a preliminary method for *V. dahliae* diagnosis, followed by fungal isolation using conventional cultivation methods in general or special media. However, these methods are time-consuming and usually involve equipment and skilled expertise (Marzachì et al., 1999; Pasquini et al., 2000; McCartney et al. 2003; Ferreira et al., 2021). Hence, to overcome shortcomings related to culturing-based diagnostic methods, nucleic acid-based techniques were employed and considered as one of the most powerful methods for rapid and sensitive detection of *V. dahliae*.

Among molecular techniques, several PCR-based and real-time PCR protocols have been successfully developed and applied in both olive tissues and infested soils even before symptom development caused by pathogens (Mercado-Blanco et al., 2001; López-Escudero et al., 2011; Ceccherini et al.,2013; Gramaje et al., 2013). Moreover, to overcome some of the limitations of these methods, mainly aiming at reducing the costs for insufficiently equipped labs, several LAMP-based procedures were developed with satisfactory results (Moradi et al., 2014; Aslani et al 2017).

Diagnosis of *X. fastidiosa* is crucial for understanding disease distribution and have affordable, reliable, and prompt diagnostic methods to avoid further introductions in free areas. In addition to the serological and enzyme-linked immunosorbent assay protocols mentioned above, several PCR-based detection techniques are available to characterize and differentiate between subspecies and strains, widely used in the laboratory. In general, the diagnostic workflow officially adopted integrates protocols based on both serological and molecular approaches (EPPO – PM 7/24 (3)). Briefly, collected field samples undergo an initial ELISA-based screening test using different commercially available serological kits. Subsequently, samples showing positive or undetermined results to serological test are then sent for confirmation to another laboratory which performs two qPCR assays: a TaqMan-based assay using the primers designed by Harper and co-workers (2010) and a SYBR green-based assay using the primers designed by Francis et al. (2006). The real-time qPCR assay represents the golden standard for bacterium detection and is recognized as an official method in the EU Regulation 2020/1201. In addition, to meet the current demand for a rapid and simple field-based assay to facilitate faster detection of *X. fastidiosa*, several LAMP-based techniques have been developed and used with promising results, which are easily

deployable in-field with limited testing facilities and using crude samples without DNA extraction and purification procedures (Harper et al., 2010; Yaseen et al., 2017; Luchi et al., 2023; Amoia et al., 2023).

The identification of *P. savastanoi* pv. *savastanoi* is traditionally based on visual inspection of typical symptoms (i.e. knots and galls), followed by isolation assays. However, molecular techniques are essential for more sensitive and reliable diagnosis. Notably, the dual role of this bacterium, as both an epiphyte and endophyte, underscores the imperative for stringent control measures in certified plant material. To achieve a heightened level of diagnostic accuracy, various molecular techniques can be employed, as evidenced by previous studies (Bertolini et al., 2003a; Bertolini et al., 2003b; Tegli et al., 2010). These advanced molecular methods enable the detection of *P. savastanoi* even at a latent and symptom-free state. Therefore, the integration of molecular approaches into diagnostic protocols is crucial for ensuring the effective control of *P. savastanoi* in certified plant materials.

Finally, diagnosis of olive tree viruses is based mainly on molecular techniques. While the first cases of olive tree infection were identified by mechanical transmission to herbaceous hosts, followed by serological methods (Savino et al., 1979). Indeed, these methods are generally considered to be insufficiently sensitive to be considered reliable for virus detection in olive plants. Consequently, the application of molecular diagnostic techniques for virus detection has emerged as a more promising and effective alternative compared to other detection methods. In light of this, various molecular protocols have been formulated, encompassing one-step RT-PCR, nested RT-PCR, and multiplex RT-PCR. These techniques have demonstrated sensitivity and reliability in detecting viruses in olive plants, as substantiated by several studies (Grieco et al., 2000; Pantaleo et al., 2001; Bertolini et al., 2001; Bertolini et al., 2003a; Faggioli et al., 2005; Mathioudakis et al., 2020). The implementation of such robust and accurate methods is essential in certification programs for virus detection and characterization in plant materials. Different molecular-based protocols were validated in a ring test conducted by eight Italian laboratories and were subsequently recommended as the official method for assessing the virus status of certified olive propagative material (Loconsole et al., 2010). Recent advancements in understanding the biology and genetics of olive viruses, coupled with the application of cutting-edge molecular techniques, have led to the development of novel diagnostic tools, such as real-time RT-PCR and high-throughput sequencing tests (Campos et al., 2019; Chiumenti et al., 2021; Xylogianni et al., 2021).

Despite these strides, a notable challenge arises from the presence of substantial quantities of oil, polysaccharides, and phenolic compounds in olive extracts, which are known to exert inhibitory effects on PCR-based methods (Amiot et al., 1989; Wilson, 1997; De Nino et al., 1997). This limitation underscores the pressing necessity for innovative methodologies that can streamline sample preparation and effectively counteract the inhibitory effects of these compounds. The development of such methodologies is increasingly imperative to address these challenges and enhance the reliability of subsequent molecular analyses.

Certainly, the domain of plant disease diagnostics is rapidly evolving, characterized by the introduction of innovative diagnostic methods and a growing need for real-time analysis conducted directly in the field. To meet these demands, it is necessary to establish more refined sampling methodologies that can efficiently detect latent infections and pathogen reservoirs. The development of new technologies and the improvement of other existing ones have the potential to provide both qualitative and quantitative data for the detection of plant pathogens, contributing to a more complete comprehension of their biology, epidemiology and ecology. The latest generation of portable thermal cyclers (such as bCUBE from Hyris or Genie®II by OptiGene), which support LAMP- and PCR-based detection systems, offer the advantage of rapid and reliable on-site results, thus transforming the in-field diagnostics landscape (Trippa et al., 2023). This multifaceted approach to plant disease detection represents a promising trajectory for the future. Moreover, the ongoing advancement of sequences methodologies has the potential to revolutionise diagnostic methods, providing opportunities for improved rapidity, cost-effectiveness and precision. Notably, advanced Next-Generation Sequencing (NGS) techniques provide swift detection and identification through high-throughput analyses, presenting a cost-effective and time for pathogen identification by metagenomic approaches (Wu et al., 2015). The diagnostic landscape is currently seeing an increase in modern platforms and portable devices designed for sequences analysis of a wide range of pathogens. Among these, the Oxford Nanopore MinION sequencing technology is a rapidly maturing system that will represent a general trend in the future, allowing on-site detection by sequencing long reads in real-time. Although the current technology still requires improvement in terms of error rates, portable nanopore sequencing devices show promise as a future alternative for point-of-care diagnosis, including plant pathogen detection.

CHAPTER II

Aim of the work

CHAPTER II – AIM OF THE WORK

Viral diseases have emerged as a serious and multifaceted challenge facing global agriculture in the 21st century. The complex interplay of enduring factors, such as climate change and rising human population, has led to profound changes in agricultural landscapes and farming practices. The consequences of these dynamics are particularly stark in subsistence farming contexts, where limited resources for crop management exacerbate the vulnerability to viral threats (Jones and Naidu, 2019). The ramifications of global challenge extend beyond regional boundaries due to the interconnectedness of modern agriculture through globalization and international trade. Viruses and their vectors are now being disseminated to new geographical regions, ushering in unforeseen complexities that reverberate across the spectrum of food production and natural ecosystems. The diverse epidemiological characteristics of viral pathosystems make it challenging to implement a singular, universal approach to mitigate the manifold negative impacts on various agricultural production systems.

Advancements in scientific understanding of viral pathosystems offer hopes in navigating these challenges. The rapid evolution of technological innovation, complemented by innovative communication strategies and the establishment of large-scale global scientific networks, is enabling researchers to fully understand the epidemiological complexity of viral threats. This knowledge is crucial for developing appropriate and context-specific strategies to safeguard global agricultural production and ensure food security. The evolution of plant viruses, driven by mutation events, genetic recombination, reassortment, and migration across diverse geographical regions, engenders profound shifts in viral populations. The multitude of genetic variations introduces a complex interplay of factors influencing evolutionary change, as viruses adeptly adapt to the escalating frequency of environmental shifts. The prompt identification of new pathogens and the analysis of their genetic dynamics are crucial for creating effective risk assessments and intervention strategies. The development of advanced diagnostics is therefore increasingly required, not only for early disease detection, but also for strategic deployment of optimal control measures within the overall sustainable agriculture framework.

Despite the historical perception of olive trees as highly resilient to various pathogens, the emergence of systemic diseases, poses a significant threat. The observed surge in viral diversity within olive cultivation represents a worrisome trend, potentially carrying

economic implications for both tree health and olive production. As of now, the precise impacts on these fundamental facets, especially the economic aspects of olive growing, remain largely unknown. Nonetheless, there is a growing acknowledgment that potential yield losses should not be underestimated. The uncertainty surrounding the economic ramifications of increased viral diversity within olive cultivation underscores the urgency of conducting comprehensive research to shed light on the potential economic implications. Understanding the genetic diversity, transmission mechanisms, and symptomatology of these viruses is crucial for effective disease management and prevention. The dynamics of virus spread within the olive orchards are intricately linked to several factors, including insect vectors, infected but asymptomatic propagation materials and the specific viral strains present. Sanitary selection and sanitation practices emerge as imperative strategies to curtail the spread of viruses, emphasizing the need for rigorous diagnostic protocols and the production of virus-tested or virus-free propagative material. In essence, addressing the virological challenges in olive cultivation necessitates a comprehensive approach that integrates scientific understanding, diagnostic innovation, and strategic agricultural management practices.

The study of a new reported olive plant virus, the investigation into the dispersion and transmission dynamics of viral pathogen and the development of rapid LAMP-based diagnostic protocols constituted the main focus of the present PhD thesis. Specifically, the research delved into two model systems, Olea europaea geminivirus (OEGV) and Olive leaf yellowing associated virus (OLYaV), belonging respectively to *Geminivirus* and *Closterovirus* genera. The research efforts involved a comprehensive examination and comparative analysis of two olive viral pathogens, utilizing advanced methodologies deeply entrenched in contemporary virological research. The overarching objectives of this study were to develop user-friendly, highly specific, and rapid real-time Loop-Mediated Isothermal Amplification (LAMP) diagnostic protocols for both viruses. Simultaneously, the research aimed to investigate the spread of these viruses within the Mediterranean environment. Anticipated outcomes include insights that not only contribute to the scientific understanding of plant health but also offer valuable information for formulating effective and targeted measures to mitigate the risks associated with the spread of these olive pathogens. This study aims to advance our knowledge of plant virology and provide a resource for sustainable strategies to protect olive production in the face of emerging viral threats.

CHAPTER III

Preliminary study index: metagenomic analysis using MinION technology

CHAPTER III – PRELIMINARY STUDY INDEX: METAGENOMIC ANALYSIS USING MINION TECHNOLOGY

3.1 Introduction

The rise of new viral epidemics with the capacity to affect essential crops represents a substantial threat to worldwide food security. The prompt identification of the responsible viral agent is imperative for a swift and efficient response to disease outbreaks. Currently, metagenomic sequencing, represented by next-generation sequencing (NGS), is widely employed for the detection and identification of different pathogens in plant (Hadidi et al., 2016; Roossinck, 2017). In recent years, Oxford Nanopore Technologies (ONT) sequencers have been proposed as an effective diagnostic tool, providing many advantages compared with other sequencing technologies such as single-molecule sequencing, long sequencing read lengths, rapid sequencing speeds, and real-time monitoring of sequencing data (Laver et al., 2015; Deamer et al., 2016). Among ONT sequencing devices, MinION is one of the most widely used. MinION is a pocket-sized sequencer (10 cm × 2 cm × 3.3 cm, 100 g) that is powered by a computer *via* USB port, allowing sequencing and real-time data analysis to be performed on a personal computer (Figure 1).

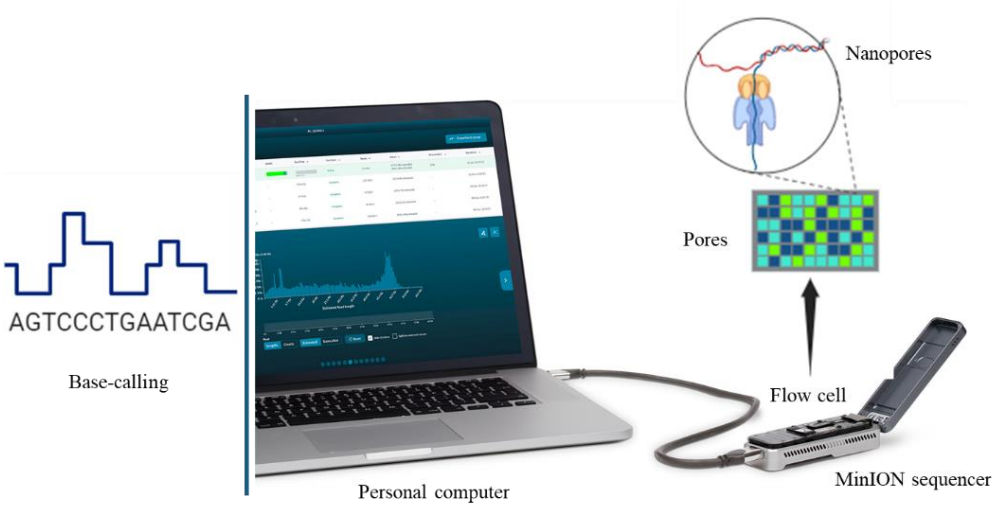


Figure 1 – Schematic diagram illustrating the components of the MinION device, including the flow cell, nanopore and real-time computer process management. The green colour indicates active pores, while the blue colour indicates inactive components. The blue light colour represents recovery pores on the nanopore membrane.

MinION nanopore sequencing identifies nucleotides based on electrical signals rather than optical signals (Deamer et al., 2016); in fact, the Oxford Nanopore sequencing devices use flow cells containing an array of nanopores (1 nm in diameter) embedded in a polymer electro-resistant membrane: each nanopore corresponds to an electrode connected to a channel and a sensor chip, which measures the electric current that flows through the nanopore (Jain et al., 2016). When an electrophoretic force is applied, negatively charged biomolecules (such as DNA or RNA), attached to specific adapters that guide nucleic acids through the nanopores, induce temporary fluctuations in the current flowing, known as a "squiggle". Specifically, the squiggle is then decoded using base calling algorithms to determine the DNA or RNA sequence (Theuns et al., 2018). Moreover, this real-time functionality extends to sequence alignments, facilitated by the EPI2ME™ workflow, allowing for the swift identification of pathogen-related sequences within samples, reducing costs and provides valuable information for farmers, supporting sustainable agriculture (Boykin et al., 2019). Recently, nanopore sequencing has been used for identification of *Xylella fastidiosa* subspecies from naturally infected olive plant material (Faino et al., 2021) and for detection of different viruses in other plant species (Chalupowicz et al., 2019; Fellers et al., 2019; Della Bartola et al., 2020), either following standard MinION protocols or elaborated cost-effective adaptations (Liefting et al., 2021; Sun et al., 2022).

This preliminary study aimed to investigate the presence of olive tree viruses in Sicily using metagenomic analysis as a diagnostic method in olive tree plants, employing the Oxford Nanopore MinION sequencer.

3.2 Materials and methods

3.2.1 Sample collection

During March 2021, a survey was conducted in the experimental collection olive grove of the Department of Agricultural, Food and Forestry Sciences – SAAF (University of Palermo, UniPA), located in the province of Agrigento (Sicily, Italy). A total of 12 samples (referred to as OLV/1 to OLV/12) were collected from both symptomatic and asymptomatic olive plants of different cultivars. The olive trees were sampled according to the hierarchical sampling scheme (Gottwald and Hughes, 2000), with minor adaptations to olive plants. All samples were promptly transported to the "Bruno Rosciglione" plant virology laboratory of the SAAF Department. Samples were stored at -20 °C and processed within the next 24-hour.

3.2.2 Nucleic acid extraction and sample preparation

The extraction of total nucleic acids was carried out separately from all the olive samples collected. In detail, total DNA (tDNA) and RNA (tRNA) extraction was performed on ~3 g of fresh leaf tissue from each sample. Commercial extraction kits, namely GenUP^{TM} Plant DNA and GenUP^{TM} Plant RNA (Biotechrabbit GmbH, Berlin, Germany), were used , respectively, for DNA and RNA extraction, according to the manufacturer's protocol. The eluted RNA and DNA were subsequently resuspended in 50 μL and 100 μL of RNase-free water, respectively. The quality and quantification of both extracted total nuclear acids were assessed with a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted tDNAs and tRNAs were used for subsequent molecular analyses.

3.2.3 Library preparation and nanopore sequencing

The library was prepared using a direct cDNA sequencing kit (SQK-DCS109), following the manufacturer's guidelines (version: DCS_9090_v109_revL_14AUG2019). In detail, cDNA was first generated using RT and the strand-switching method. Specifically, 100 ng of RNA was resuspended in 7.5 μL of RNase-free water and mixed with 2.5 μL of the VNP primer (ONT) that targets the poly-A tail and 1 μL of dNTPs [10 mM]. This mixture was incubated for 5 min at 65°C and immediately snap cooled on a freezer block. In a separate tube a reaction mix comprising 4 μL of 5X RT buffer (ONT), 1 μL of RNaseOUT (40 U/μL, Life technologies), 2 μL of strand-switching Primer (10 μM, ONT) and 1 μL of RNase-free water was prepared. This mixture was added to the snap-cooled RNA prepared above and incubated for 2 min at 42 °C. Subsequently, 1 μL of Maxima H Minus Reverse transcriptase (Life Technologies, Thermo Fisher Scientific) was added and the mixture was incubated at 42 °C for 90 min followed by heat inactivation at 85 °C for 5 min. RNA was then degraded by adding 1 μL of RNase Cocktail Enzyme Mix (Thermo Fisher Scientific) followed by incubation at 37 °C for 10 min. The cDNA was purified using AMPure XP beads as described in the ONT protocol and eluted in 20 μL of nuclease-free water. The second strand was synthetized in a 50 μL reaction composed of 25 μL of 2x LongAmp Taq Master mix (New England Biolabs, Beverly, MA, USA), 2 μL of PR2 primer (ONT), 3 μL of nuclease free-water and 20 μL of reverse-transcribed sample from above. The mixture was incubated at 94 °C for 1 min, 50 °C for 1 min, 65 °C for 15 min and hold at 4°C. The cDNA was then purified using AMPure XP beads and eluted in 21 μL of nuclease-free water. In the final step of the library preparation, the ends of the cDNA fragment were repaired to create blunt

ends and dA-tails were added, by mixing the following reagents in a 0.2 ml PCR tube: 20 µL of the cDNA prepared above, 30 µL of nuclease-free water, 7 µL of Ultra II End-prep reaction buffer (New England Biolabs) and 3 µL of Ultra II End-prep mix (New England Biolabs). After gentle mixing by pipetting, the reaction mix was incubated in a thermal cycler for 5 min at 20°C then 5 min at 65°C. The cDNA was then purified using AMPure XP beads as described into ONT protocol and resuspended in 22.5 µL of nuclease-free water. Sample barcoding was carried out using the Native Barcoding Expansion kit 1-12 (EXP-NBD104), according to manufacturer's instruction. Barcoding was performed in a 50 µL reaction with 22.5 µL of end-prep cDNA, 2.5 µL of native barcode and 25 µL of Blunt/TA ligase Master Mix (New England Biolabs) and the reaction mix was incubated for 10 min at room temperature. The barcoded cDNA was then purified using AMPure XP beads and resuspended in 26 µL of nuclease-free water. The barcoded samples were pooled, and the volume adjusted to 65 µL to which 5 µL of Adapter Mix II (AMII, ONT), 20 µL of 5X NEBNext Quick Ligation Reaction Buffer (New England Biolabs) and 10 µL of Quick T4 DNA ligase (New England Biolabs) were added. The reaction mix was incubated at room temperature for 10 min and purified using AMPure XP beads and resuspended in 13µL of Elution Buffer (EB). After quantification of 1 µL of eluted sample using a Qubit 3.0 Fluorometer with a Qubit 1× dsDNA HS Assay Kit, 12 µL of the library solution were mixed with 37.5 µL of sequencing buffer (SQB, ONT) and 25.5 µL of Loading Beads (LB, ONT) and loaded into a flow cell (FLO-MIN106, ONT) equipped with R9.4.1 chemistry on a MinION (Mk1B, ONT) device, according to ONT protocol. The MinION was operated using MinKNOW and the flow cell was primed following manufacturer's instructions, with a total run time of 24 h.

3.2.4 Bioinformatic analysis

The raw data collected from the sequencing in the MinION device with the MinKNOW v2.0 software, was converted from FAST5 files to FASTQ files for further analysis, with the in-built data processing toolkit Guppy software (ONT). After sequencing the flow cell was washed and storage buffer was added for storage purpose. Downstream analysis of FASTQ files generated with Guppy software were analyzed on EPI2ME software using the application "What's In My Pot?" (WIMP) from ONT. A report of the genome taxonomic classification was created automatically by WIMP application. Moreover, BLASTn algorithm of the National Center for Biotechnology Information (NCBI) were used to

confirm the sequences of plant viruses. Subsequently, as described in the following paragraph, RT-PCR assays were carried out to confirm the presence of viruses detected by bioinformatic analysis, i.e. OLYaV, OEGV and OLV-3.

3.2.5 Detection of olive viruses by RT-PCR assay

To verify the findings of identified plant viruses through ONT sequencing, molecular analyses were conducted on previously extracted tDNA and tRNA samples. Specifically, end-point RT-PCR tests were performed, employing specific primers (Table 7).

Table 7 – Specific primer pairs and reaction conditions for OLYaV, OEGV and OLV-3 detection by end-point PCR.

ID PRIMER	SEQUENCE (5' – 3')	AMPLICON SIZE	PCR PROTOCOL	REFERENCE
OLYaV-H-Fw	ACTACTTTCGCGCAGAGACG	346 bp	95 °C for 5'; 40 cycles: 95 °C for 30'', 30'' at 50 °C, 72 °C for 30''; 72 °C for 10'	Faggioli et al., 2005
OLYaV-C-Rv	CCCAAAGACCATTGACTGTGAC			
OEGV-A2-Fw	GGGGACACCTCCGTACGCTTAC	831 bp	95 °C for 5'; 40 cycles: 95 °C for 30'', 30'' at 64 °C, 72 °C for 1'; 72 °C for 10'	Chiumenti et al., 2021
OEGV-A4-Rev	CTACACTGCCACCAGTGGTGTCC			
OLV3-Fw	CCCGTTGAGCAAGTTGTCTTCC	197 bp	95 °C for 5'; 40 cycles: 95 °C for 30 sec, 30 sec 58 °C,72 °C for 30 sec; 72 °C for 10'	Alabdullah et al., 2009
OLV3-Rv	GCAGTGGCTGGAGAGCATGGAG			

Total RNAs of all samples collected were subjected to a reverse transcription (RT) carried out in a 20 µL final volume with 3 µL of total RNA [50 ng], 0.4 mM dNTPs, 4 µL of 5X First Strand Buffer [50 mM Tris-HCl pH 8.3, 40 mM KCl, 6 mM $MgCl_2$] (Thermo Fisher Scientific, Waltham, MA, USA), 1 µM of respective reverse primer, 20 U of M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume. After an initial denaturation at 65 °C for 10 min, RT was performed at 42 °C for 45 min and 95 °C for 10 min.

All PCR reactions were performed in a final reaction volume of 25 µL, containing 2 µL of the cDNA or tDNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 µM of each primer, 2 U of *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume. Positive controls were included in each analysis and a healthy olive. End-point RT-PCR analyses were performed in a MultiGene OptiMax thermal cycler (Labnet International Inc., Edison, NJ, USA). PCR products were electrophoresed on 1.2 % agarose gel, stained with SYBR™ Safe (Thermo Fisher Scientific, Waltham, MA, USA) and visualized under UV light. All PCR amplicons were Sanger-sequenced in both directions after purification using the UltraClean PCR Clean-

Up kit (Mo-Bio, USA), following the manufacturer's instructions. Finally, the sequences were validated using a BLASTn search.

3.3 Results

3.3.1 Identification of olive viruses by nanopore sequencing

The WIMP workflow was used to rapidly identify and quantify species in metagenomic olive samples. Out of the 4,360 reads, 57% were categorised as bacteria, 22% as viruses, 20% as eukaryotes, and less than 1% as archaea (data not shown). To confirm the identify viruses infecting olive trees, trimmed reads obtained from ONT sequencing were analysed using BLASTn tool. Specifically, three olive viruses were identified by nanopore sequencing in the olive samples collected, i.e. OLYaV, OEGV, and OLV-3.

3.3.2 Detection of olive viruses by RT-PCR assay

To confirm the presence of viruses identified in olive samples, end-point (RT-)PCR tests were performed using virus-specific primers. The RT-PCR results confirmed the presence of OLYaV and OEGV in olive samples, as detailed in Table 8. Interestingly, however, OLV-3 was not detected by end-point RT-PCR. Subsequently, the analysis of obtained sequences using a BLASTn algorithm confirmed the presence of OLYaV and OEGV in olive samples (data not shown).

Table 8 – End-point RT-PCR assay results for OLYaV, OEGV and OLV-3 detection of symptomatic and asymptomatic field samples.

ID SAMPLE	CULTIVAR	SYMPTOMS	RT – PCR ANALYSIS		
			OLYAV	OEGV	OLV-3
OLV/1	Vaddara	0	+	-	-
OLV /2	Pizzutella	0	-	+	-
OLV /3	Cavalieri	1	-	+	-
OLV /4	Cerasuola	0	-	+	-
OLV /5	Zaituna Florida	3	+	-	-
OLV /6	Nocellara del Belice Giafalione	0	+	+	-
OLV /7	Vaddara Frazzano	2	+	+	-
OLV /8	Bottone di Gallo	1	+	-	-
OLV /9	Biancolilla Di Marco	0	-	-	-
OLV /10	Nocellara Licata	3	+	+	-
OLV /11	Ogliarola Messinese	1	+	-	-
OLV /12	Moresca di Noto	0	+	-	-

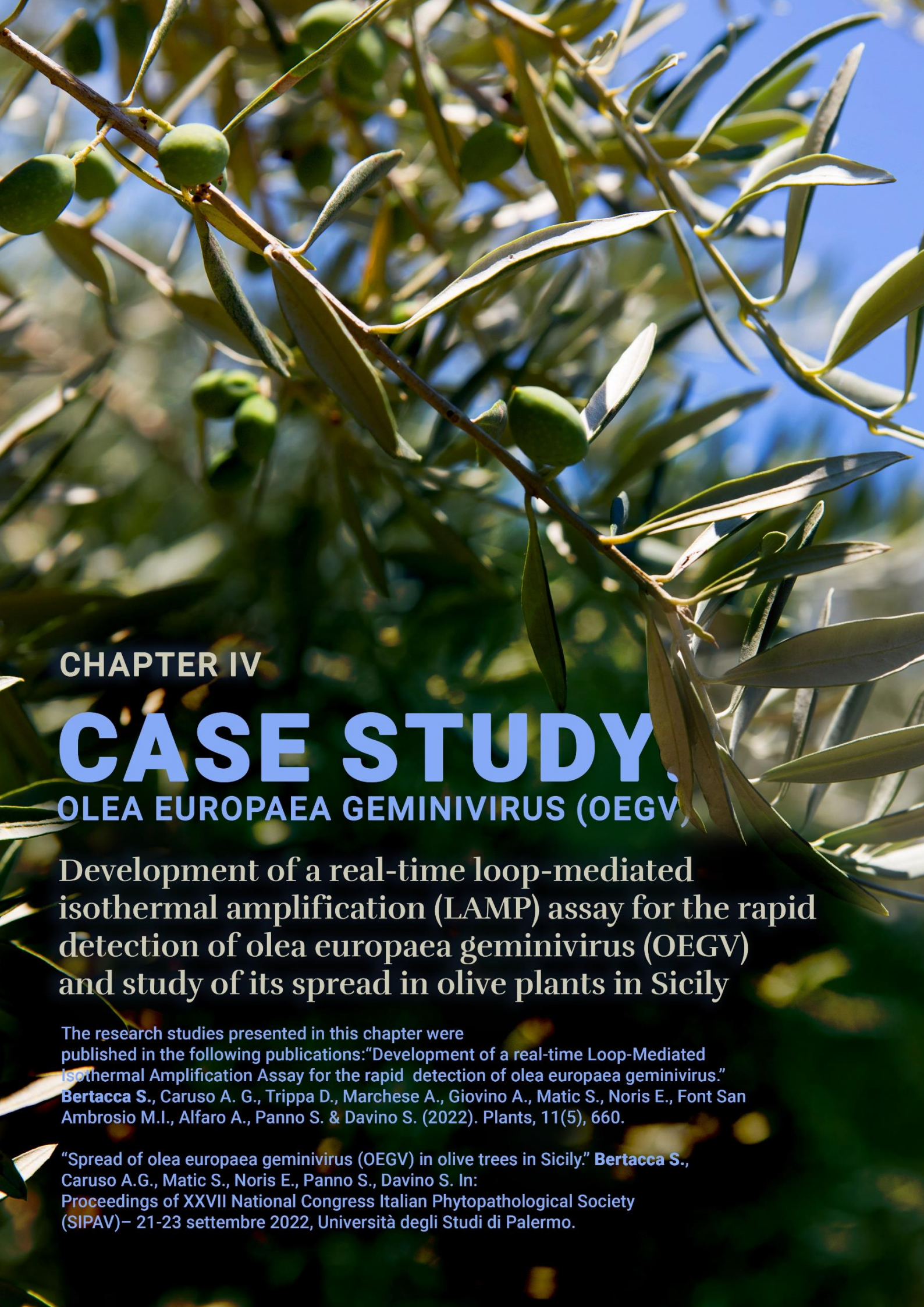
Note: **0**: Symptomless; **1**: Mild leaf discoloration; **2**: Yellow leaf discoloration; **3**: Bright yellow discoloration. **+**: Positive result; **-**: Negative result.

3.4 Conclusion

The development of rapid detection and accurate diagnostic techniques is essential for preventing the spread of plant diseases caused by plant viruses. The ability to simultaneously detect multiple plant viruses in a single sample is an important feature of diagnostic tools, and the ONT platform has been successfully applied to identify viral pathogens (Mehetre et al., 2021). The ONT platform has recently proven to be a reliable, real-time, and portable technology that enhances the study and monitoring of various viral pathogens including human and other animal viruses (Fang and Ramasamy, 2015). However, to date, this technique has rarely been applied in plant virology (Filloux et al., 2018).

Therefore, in order to assess the presence of viruses in symptomatic and asymptomatic olive trees, the potential of the ONT platform to detect viruses infecting olive plants were evaluated. The ONT platform, using massive parallel sequencing technology, offers the potential for genomic studies of known or unknown viruses. In fact, MinION ONT platform successfully detected three olive plant viruses, including OLYaV, OEGV, and OLV-3. Furthermore, the results generated by nanopore sequencing were confirmed by end-point RT-PCR, except for OLV-3. This virus species was detected only by nanopore sequencing, and different attempts to detect OLV-3 by end-point RT-PCR were unsuccessful. Therefore, ONT analysis seems to indicate the presence of OLV-3 in the olive samples. However, due to the lack of confirmation by RT-PCR, the real significance of this finding needs to be further evaluated. In this sense, the establishment of MinION technology parameters that provide a clear threshold leading to a reliable detection of plant viruses by this technique needs to be addressed.

In conclusion, ONT sequencing has been demonstrated to be a powerful tool for detection, diagnosis, and virus discovery; however, as with other methods, it has its advantages and limitations. Plant virologists need to address how to manage the identification of new species of viruses that could affect commercial trade between countries and highlight the urgent necessity to obtain biological data as soon as possible after their identification in order to better assess their relevance.



CHAPTER IV

CASE STUDY:
OLEA EUROPAEA GEMINIVIRUS (OEGV)

Development of a real-time loop-mediated isothermal amplification (LAMP) assay for the rapid detection of olea europaea geminivirus (OEGV) and study of its spread in olive plants in Sicily

The research studies presented in this chapter were published in the following publications:"Development of a real-time Loop-Mediated Isothermal Amplification Assay for the rapid detection of olea europaea geminivirus." **Bertacca S.**, Caruso A. G., Trippa D., Marchese A., Giovino A., Matic S., Noris E., Font San Ambrosio M.I., Alfaro A., Panno S. & Davino S. (2022). Plants, 11(5), 660.

"Spread of olea europaea geminivirus (OEGV) in olive trees in Sicily." **Bertacca S.**, Caruso A.G., Matic S., Noris E., Panno S., Davino S. In: Proceedings of XXVII National Congress Italian Phytopathological Society (SIPAV)– 21-23 settembre 2022, Università degli Studi di Palermo.

CHAPTER IV – DEVELOPMENT OF A REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR THE RAPID DETECTION OF OLEA EUROPAEA GEMINIVIRUS (OEGV) AND STUDY OF ITS SPREAD IN OLIVE PLANTS IN SICILY

4.1 Introduction

4.1.1 Olea europaea geminivirus (OEGV)

In the last year, thanks also to the application of new technologies such as high-throughput sequencing (HTS), a new geminivirus called Olea europaea geminivirus (OEGV) has been identified in olive tree, but its spread and pathogenicity remain unclear. Since its first identification in Apulia in the "Ogliarola" and "Leccino" cvs. (Chiumenti et al., 2021), OEGV was reported in California and Texas (Alabi et al., 2021), Portugal (Materatski et al., 2021) and Spain (Ruiz-García et al., 2021). OEGV is classified as a putative member within the *Geminiviridae* family (Chiumenti et al., 2021), currently including 14 genera and few other still unassigned geminiviruses (). The evolutionary relationship of OEGV with other geminiviruses indicated that OEGV has distinctive genome features, possibly representing a new genus (Chiumenti et al., 2021; Materatski et al., 2021; Ruiz-García et al., 2021).
OEGV is characterized by a bipartite genome containing DNA-A and DNA-B (Figure 2).

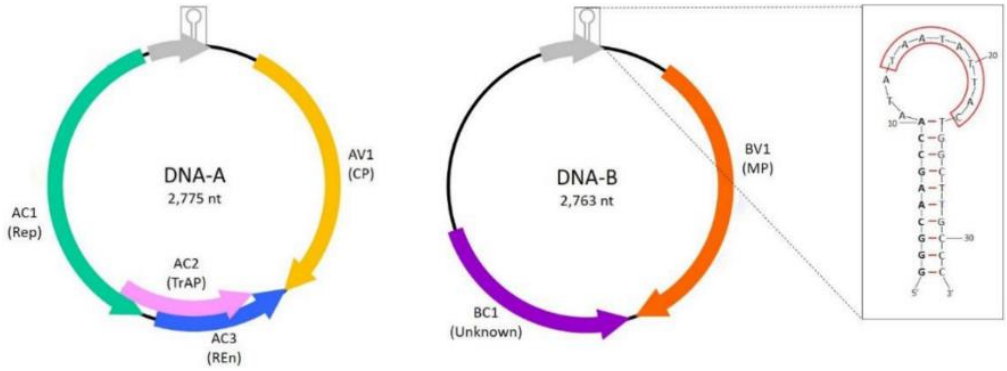


Figure 2 – Genomic organization of OEGV (Chiumenti et al., 2021). Open reading frames (ORFs) are represented by arrows; the orientation indicates if they are encoded on the virion or complementary-sense strand. Grey bold arrows indicate the putative ORFs identified in the intergenic region. A small hairpin structure in grey, indicates the position of the stem-loop. A detail of such hairpin is represented in box on the right of the DNA-B genomic component.

DNA-A (2,775 nucleotides, nts) includes four ORFs, three in the complementary-sense encoding the replication-associated protein Rep (AC1), the transcriptional activator protein TrAP (AC2), the replication enhancer protein Ren (AC3) and one in the virion-sense, (AV1), encoding the coat protein (CP). DNA-B (2,763 nts) includes two ORFs, BC1 in the

complementary sense, with an unknown function and lacking known conserved domains typical of geminiviral proteins, and BV1 on the virion sense, possibly encoding the movement protein (MP). In bipartite geminiviruses, AC4/C4 protein is a symptom determinant involved in cell-cycle control and interacts with CP and/or MP in the replicated genome transport from nucleus to cytoplasm and from cell-to-cell (Materatski et al., 2021). Curiously, no genes encoding AC4/C4 were found on the OEGV genome. In addition, the two DNA molecules present a common region (CR) of 403 nt that contains the TATA box and four replication-associated iterons with a unique arrangement compared to other geminiviruses (Chiumenti et al., 2021; Materatski et al., 2021; Alabi et al., 2021). OEGV does not appear to be clearly associated to any symptom in olive; moreover, a high degree of sequence conservation has been identified (Ruiz-García et al., 2021); in fact, in a recent survey, Alabi and co-workers (2021) detected OEGV-positive olive trees originating from different locations, advancing the concept of a possible worldwide spread of this virus, likely due to the inadvertent movement of germplasms from clonally propagated infected but asymptomatic olive trees.

4.1.2 Real-time Loop-Mediated Isothermal Amplification (LAMP) assay

Loop-Mediated Isothermal Amplification (LAMP) assay represents a rapid, sensitive, and cost-effective molecular diagnostic approach for qualitative and quantitative analysis of nucleic acids (Panno et al., 2020). Distinguished by its capacity for operation outside specialized laboratory settings, LAMP stands as a significant advancement over traditional molecular methods, drastically reducing processing times while ensuring reliable and timely pathogen detection within host plants (Francois et al., 2011).

The real-time LAMP method allows the amplification of target nucleic acid (RNA or DNA) sequence at fixed temperature in a single step, using the thermostable enzyme *Bst* (*Geobacillus stearothermophilus*) polymerase. The reaction consists of an initial step and a combination of a cycling amplification step with an elongation/recycling step (Mori & Notomi, 2009). In general, the isothermal amplification is carried out at 60-65 °C, the optimum temperature for *Bst* polymerase activity (Chander et al., 2014). This enzyme not only synthesizes new DNA strands but also helps to separate the hydrogen bonds between template DNA strands through its strand displacement activity. This feature allows denaturation to occur independently of high temperatures, enabling DNA synthesis to proceed seamlessly at a constant temperature without being hindered by the secondary structure of the DNA template (Notomi et al., 2000). The success of LAMP method relies

heavily on the primer pairs that target six-eight different regions of the DNA template. These regions include F3c, F2c, and F1c in the 3' direction, and B1, B2, and B3 in the 5' direction. The primer set consists of FIP (Forward Inner Primer – F1c+F2) and BIP (Backward Inner Primer – B1c+B2), each with two distinct portions that complement specific target DNA regions. Additionally, the design incorporates F3 (Forward Outer Primer) and B3 (Backward Outer Primer) based on the F3 and B3 relative target regions. Furthermore, to expedite amplification and enhance sensitivity, the reaction mixture may include an additional loop primer pair (LF – Loop Forward Primer, LB – Loop Backward Primer) (Figure 3). These primers facilitate the creation of new binding sites for the *Bst* polymerase, thereby augmenting the efficiency of the amplification process (Notomi et al., 2000).

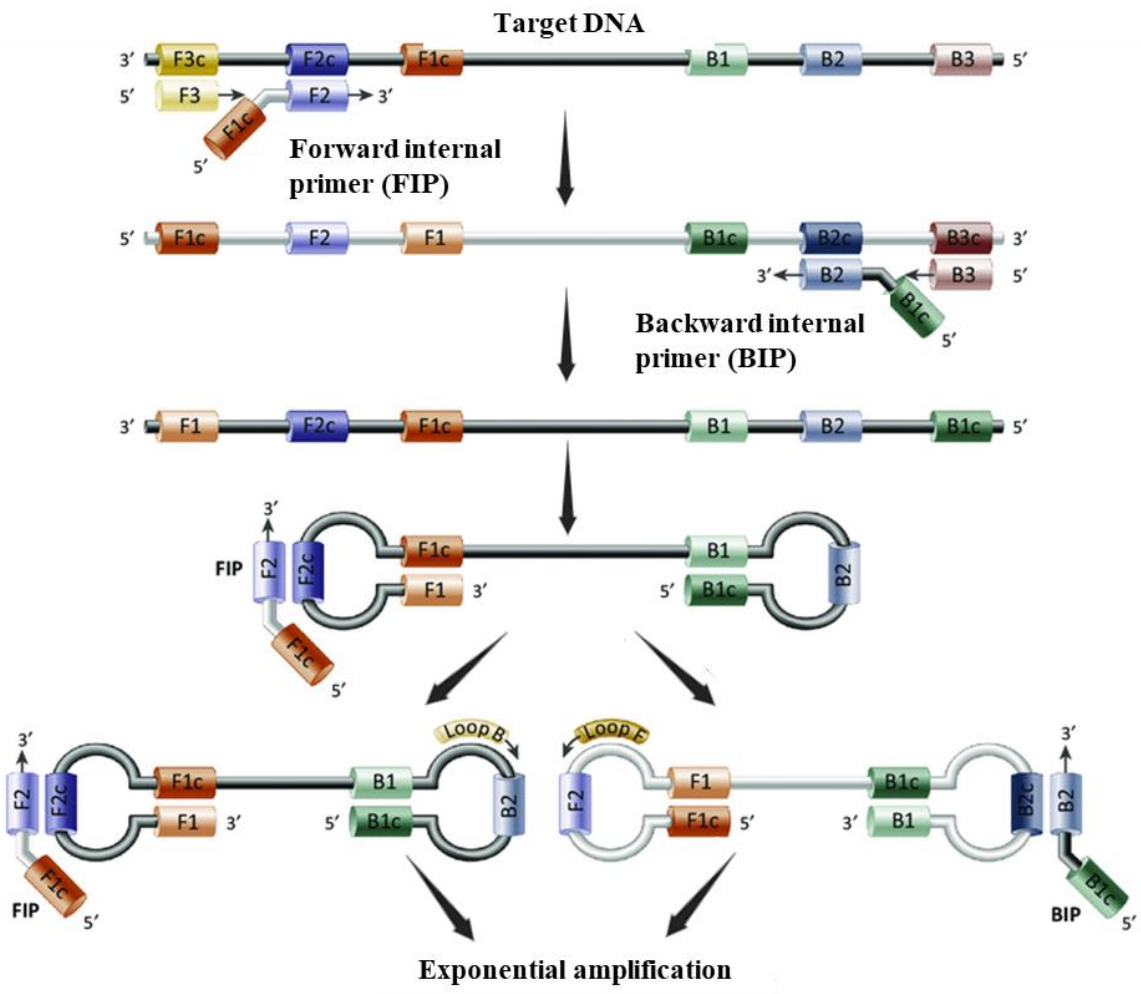


Figure 3 – Schematic diagram of LAMP amplification. F3 and B3: forward and backward outer primers, respectively. FIP (F1c + F2) and BIP (B1c + B2): forward and backward inner primers, respectively. LF and LB: forward and backward loop primers, respectively.

The objectives of this study were to develop a detection protocol based on the LAMP methodology and to assess the presence of OEGV in Sicily. Furthermore, an on-site olive sample homogenization procedure was developed as an alternative to conventional DNA extraction methods, which is useful in evaluating the suitability of the LAMP assay *in situ* OEGV testing.

4.2 Material and methods

4.2.1 Plant material collection

Different surveys were carried out during spring 2021, focusing in particular on two olive producing sites in the Agrigento province (Sicily, Italy). The olive tree samples were randomly collected according to the hierarchical sampling scheme (Gottwald and Hughes, 2000), with minor adaptations to olive plants. All samples were geo-referenced with the Planthology mobile application (Davino et al., 2017), collected from a total of 80 olive trees of 10 different cvs (40 trees randomly sampled for each site). Each sample consisted of 8 branches per plant (two for each plant cardinal point); samples were stored at 4 °C and processed within the next 24 h for subsequent molecular analyses.

4.2.2 DNA extraction and sample preparation

Total DNA was extracted using the DNA extraction GenUP^{TM} Plant DNA kit (Biotechrabbit GmbH, Berlin, Germany), following manufacturer's instructions with slight modifications. In brief, 3 g of tissue were homogenized in an extraction bag (BIOREBA, Reinach, Switzerland) using the HOMEX 6 homogenizer (BIOREBA, Reinach, Switzerland), with 3 mL extraction buffer (1.3 g sodium sulphite anhydrous, 20 g polyvinylpyrrolidone MW 24–40,000, 2 g chicken egg chicken albumin Grade II, 20 g Tween-20 in one L of distilled water, pH 7.4). Aliquots of 400 µL of the extract were added to the same volume of lysis buffer. The eluted DNA was resuspended in 100 µL RNase-free water; following two measurements with a UV–Vis NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), samples were adjusted to approximately 50 ng/µL and stored at -20 °C.

4.2.3 Preliminary screening of OEGV by end-point PCR

The end-point PCR was conducted using the primer pair A2for/A4rev (Chiumenti et al., 2021), amplifying an 831 bp fragment within the AV1 gene. PCR was performed in a final volume of 25 µL, containing 1 µL of total DNA extract, 20 mM Tris-HCl (pH 8.4), 50 mM

KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 µM each primer, and 2 U *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and RNase-free water to reach the final volume. Healthy olive plant DNA and water were used as control samples. The PCR was performed in a MultiGene OptiMax thermal cycler (Labnet International Inc., Edison, NJ, USA) with the following conditions: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 64 °C for 45 s, and 72 °C for 1 min; a final elongation at 72 °C for 10 min. PCR products were electrophoresed on 1.5% agarose gel, stained with SYBR™ Safe (Thermo Fisher Scientific, Waltham, MA, USA) and visualized by UV light.

4.2.4 LAMP primer design

The OEGV DNA-A complete sequence (GenBank Acc. No. MW316657) was used to design LAMP primers by the PrimerExplorer version 5 software (accessed on 5 July 2021), choosing a 540-bp nucleotide sequence elapsing region within the AV1 gene. A set of six primers were selected, including two outer primers (forward and backward outer primer, F3 and B3, respectively), two inner primers (forward and backward inner primer, FIP and BIP, respectively), and two loop primers (forward and backward loop primer, LF and LB, respectively). The specificity of the primer set was tested *in silico* using the Nucleotide-BLAST algorithm (accessed on 5 July 2021) available at the National Centre for Biotechnology Information (NCBI), in order to evaluate possible cross reactions with other viruses. This set of primers was also tested against the full genomic sequences of other geminiviruses reported in Italy using the Vector NTI Advance 11.5 software (Invitrogen, Carlsbad, CA, USA), in order to verify their affinity. The list included Tomato leaf curl New Delhi virus (ToLCNDV) (DNA-A: GenBank Acc. No. MK732932 and DNA-B: MK732933), Tomato yellow leaf curl Sardinia virus (TYLCSV) (GenBank Acc. No. GU951759), Tomato yellow leaf curl virus (TYLCV) (GenBank Acc. No. X15656), TYLCV-IL23 (GenBank Acc. No. MF405078), and TYLCV isolate 8-4/2004 (GenBank Acc. No. DQ144621).

4.2.5 OEGV real-time LAMP assay optimization

The real-time LAMP assay was performed in a 12 µL reaction mixture containing 1.6 µM each of FIP-OEGV and BIP-OEGV, 0.2µM each of F3-OEGV and B3-OEGV, 0.4 µM each of forward loop primer (LF-OEGV) and backward loop primer (LB-OEGV), 6.25 µLWarmStart LAMP 2X Mastermix (New England Biolabs, Beverly, MA, USA), and 0.25

μL of LAMP Fluorescent dye (New England Biolabs, Beverly, MA, USA), 1 μL of total DNA as template and nuclease-free H_2O was added to reach the final volume. DNA extracted from ten samples previously analyzed by end-point PCR was used in the real-time LAMP assay, including a positive control (PC) and a healthy olive plant DNA as negative control (NC). Each sample was analyzed twice. The LAMP assay was conducted at 65 °C (according to manufacturer's instructions) for 60 min and fluorescence was acquired every 60 s, using a Rotor-Gene Q2plex HRM Platform Thermal Cycler (Qiagen, Hilden, Germany). A melting curve was calculated to record the fluorescence using the following protocol:95 °C for 1 min, 40 °C for 1 min, 70 °C for 1 min and an increase of temperature at 0.5 °C /s up to 95 °C. During the amplification, the fluorescence data were obtained in the 6-carboxyfluorescein (FAM) channel (excitation at 450–495 nm and detection at 510–527 nm). The relative fluorescence units (RFU) threshold value was used, and the threshold time (Tt) was calculated as the time at which fluorescence was equal to the threshold value.

4.2.6 Features of real-time LAMP assay: sensitivity and comparison to conventional PCR, reaction time and specificity

To set up the conditions of the LAMP assay, an amplicon obtained by subjecting an OEGV-positive sample to end-point PCR (see above) was purified from agarose gel using an UltraClean™15 DNA purification kit (MO-BIO Laboratories, Carlsbad, CA, USA), following manufacturer's instructions. The purified DNA (named pcr-DNA) was quantified using a UV–Vis NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The number of copies was determined as follows: [Number of copies = (amount of DNA in nanograms × 6.022×10^{23})/(length of DNA template in bp × $1 \times 10^9 \times 650$)].To determine the OEGV real-time LAMP optimal reaction time and sensitivity, ten-fold serial dilutions of the sample (named pcr-DNA) were used as a template for both real-time LAMP assay and end-point PCR. Moreover, to evaluate the specificity of the LAMP assay and to assess potential non-specific cross reactions with other geminiviruses, a LAMP assay was conducted with two OEGV-positive samples together with DNA extracts from other geminiviruses unrelated to OEGV; specifically, ToLCNDV (Acc. No. MK732932), TYLCSV (Acc. No. GU951759), TYLCV (Acc. No. DQ144621), TYLCV-IS76 (Acc. No. MH931766). Each sample was analyzed in duplicate in two independent real-time LAMP assays. In each run, total DNA from a healthy olive plant (NC) was included. The assay was conducted as above described, including the melting curve steps.

4.2.7 Set up of a rapid sample preparation method suitable for the real-time LAMP assay

To set up a simple and inexpensive sample preparation procedure, a method that avoided DNA extraction named "membrane spot crude extract" was used. For this, 1.5 g of vegetable tissue was placed in an extraction bag and homogenized with 3 mL of extraction buffer (see above). Five µL of this extract was spotted on a 1 cm^2 Hybond®-N^+ hybridization membrane (GE Healthcare, Chicago, IL, USA), dried at room temperature for 5 min, and placed in a 2 mL tube containing 0.5 mL of glycine buffer (0.1 M Glycine, 0.05 M NaCl,1 mM EDTA). After 20-s vortexing, samples were heated at 95 °C for 10 min and 3 µL of the sample solution were used for the LAMP assay (Figure 4).

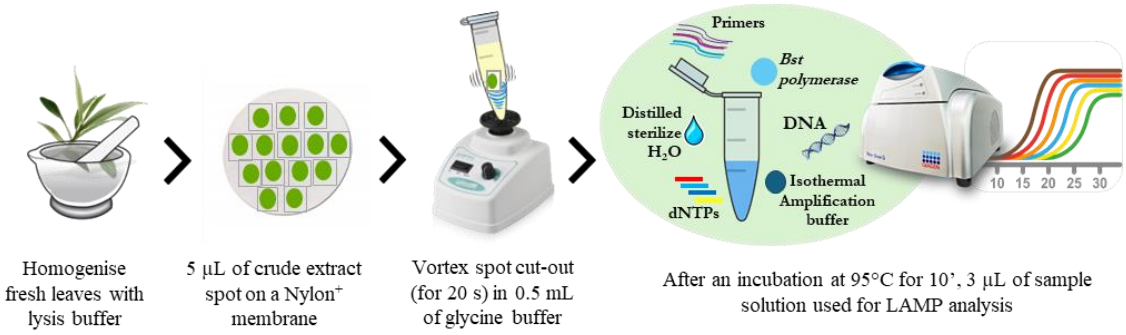


Figure 4 – Workflow of rea-time LAMP test starting from "membrane spot crude extract" rapid sample preparation.

Ten samples previously analyzed by end-point PCR were used in the real-time LAMP assay, including a positive control (PC) and a healthy olive plant DNA as negative control (NC).

4.2.8 Spread of OEGV in different cultivars

During autumn 2021, in different Sicilian areas, a second sampling was carried out to evaluate the OEGV spread in Sicily, this time sampling 10–15-year-old olive trees, belonging to 70 different cvs. A total of 560 samples were collected. For each cv, eight different trees were sampled and grouped, obtaining a total of 70 different batches. Sampling and geo-referencing were as described above. In this case, samples were prepared with the "membrane spot crude extract" method and subjected to real-time LAMP assays in a 12 µL final volume as described above. In the case of positive sample batches, they were resampled and analyzed individually to determine the effective number of positive plants for each cultivar.

4.3 Results

4.3.1 OEGV Detection by end-point PCR

A total of 80 samples representing 10 different cvs. collected from two olive production sites in the Agrigento province were analyzed by end-point PCR. Overall, 44 of them were found to be positive to OEGV, demonstrating a high presence of OEGV in Sicily (Table 9). However, OEGV was not equally distributed among the cvs. tested, and some cvs. tested negative for this virus, at least using the primer set mentioned.

Table 9 – Prevalence and cultivar distribution of OEGV analyzed by end-point PCR.

CULTIVAR	POSITIVE/TESTED SAMPLES	POSITIVE SAMPLES (%)
Cavalieri Standard	8/8	100
Cerasuola Nilo Paceco	8/8	100
Cerasuola Standard	8/8	100
Giarraffa	0/8	0
Nocellara del Belice Giafalione	8/8	100
Pizzutella	8/8	100
Salicina Vassallo	3/8	37.5
Uovo di piccione	1/8	12.5
Vaddara	0/8	0
Zaituna Florida	0/8	0
TOTAL	**44/80**	**55**

Note: Cultivar tested negative to OEGV by end-point PCR are underlined in grey.

4.3.2 OEGV real-time LAMP primer design

A real-time LAMP assay for rapid detection of OEGV was developed using a set of six primers designed on the OEGV-AV1 coding region. The sequences and binding sites of the primers are reported in Table 10 and Figure 5, respectively.

Table 10 – Primers used for OEGV detection by real-time LAMP.

PRIMER NAME	SEQUENCE (5'-3')	LENGTH (NT)	AMPLICON SIZE (BP)
F3-OEGV	CGATACGAGACATACCCAG	19	209
B3-OEGV	TCCATGTTGATCATCCAAGT	20	
FIP-OEGV	CAGCCACTGCTTCATATTATGAACACGA ATTGTGCTTAACGGTT	44	-
BIP-OEGV	GATGTGGCTCGTGTATGATAGACGTCTG GATCCCGACTTTCC	42	-
LF-OEGV	GGCTTCGCTAGTCAACTTAACTG	23	-
LB-OEGV	TCCCGGTAATTCTAATCCCAGAG	23	-

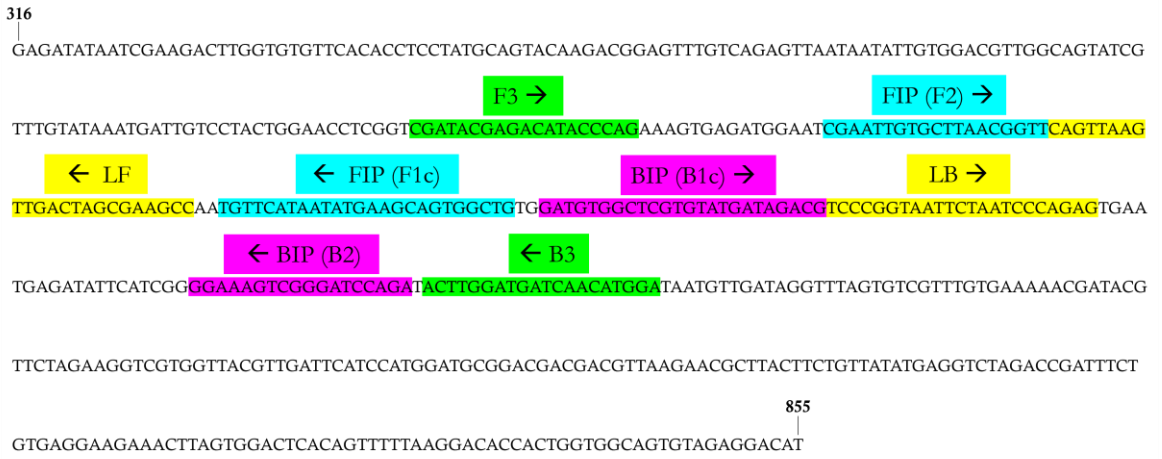


Figure 5 – Location of loop-mediated isothermal amplification (LAMP) primer sets designed on the AV1 coding region of OEGV. F3 and B3 are shown in green, FIP (F1c-F2) in blue, BIP (B1c-B2) in pink, and the two loop primers LF and LB in brown. FIP is a hybrid primer consisting of the F1c and the F2 sequences, while BIP is a hybrid primer consisting of the B1c and B2 sequences. The arrows indicate the extension direction. The numbers at the beginning and end of the sequence represent the genomic position of the first and last nucleotide in the selected sequence (GenBank Acc. No. MW316657).

Both the *in silico* analysis of LAMP primers using Nucleotide-BLAST algorithm and the hybridisation analysis against other geminiviruses performed with the Vector NTI 11.5 program allowed for the exclusion of relevant matches with other organisms and, more specifically, with geminiviruses known to be present in Sicily.

4.3.3 OEGV real-time LAMP assay optimization

To evaluate the performances of the primer set designed for the real-time LAMP assay in the identification of the presence of OEGV in olive DNA extracts, the LAMP assay was conducted using a subset of the samples listed in Table 9, selecting them among those that resulted positive in end point PCR. In the assay, a sample that tested negative was also included (i.e., cv. Giarraffa), together with an appropriate negative control (NC). The assay was conducted at 65 °C. As reported in Table 11 and Figure 6A, the positive samples showed exponential trends between 3 to 13 min. The melting curves of the positive LAMP reactions all had the same peak temperature of approximately 85 °C (Figure 6B). As expected, no signal was obtained with the negative control and, according to the end-point PCR results, the samples from cv. Giarraffa could not be amplified by LAMP, even at late reaction times.

Table 11 – Performance of the real-time LAMP assay for the detection of OEGV in olive samples collected in Sicily.

CULTIVAR	NO. OF DIFFERENT SAMPLES ANALYZED	ID SAMPLE	REACTION TIME (MIN)
Cavalieri Standard	2	1	10
		2	7
Cerasuola Standard	2	3	10
		4	7
Giarraffa	2	5	-
		6	-
Nocellara del Belice Giafalione	2	7	10
		8	13
Pizzutella	2	9	10
		10	9
Positive control	1	PC	3
Negative control	1	NC	-

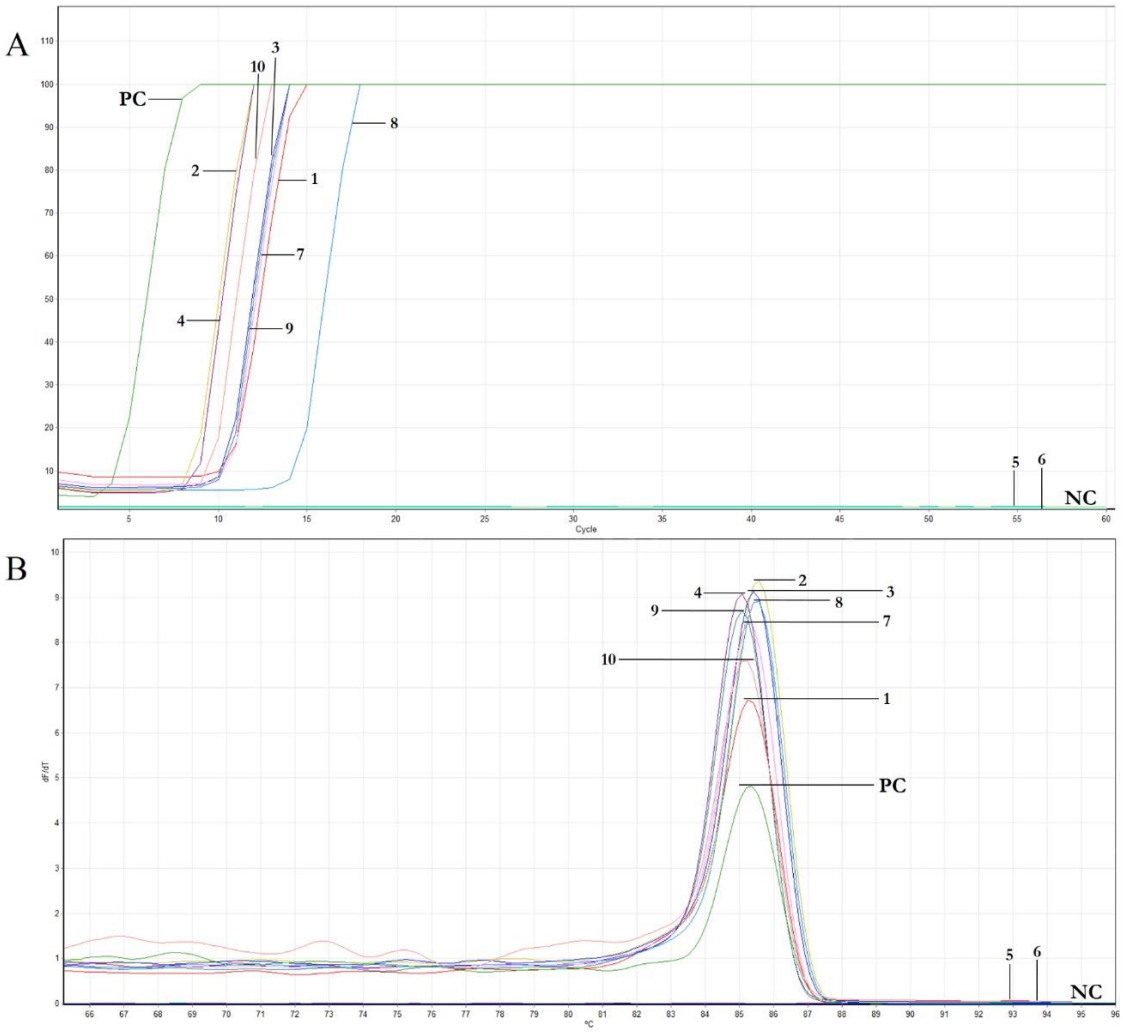


Figure 6 – Results of the real-time LAMP assay for the detection of OEGV. **A**: Amplification curves of real-time LAMP assay; **B**: Melting curves of the amplification curves previously obtained, including positive (PC) and negative control (NC).

4.3.4 Features of real-time LAMP assay: sensitivity and comparison to conventional PCR, reaction time and specificity

To determine the sensitivity of the real-time LAMP assay compared to the end-point PCR and to evaluate the LAMP efficacy, a comparative experiment was conducted using as a template ten-fold serial dilutions of an amplicon obtained by end point PCR from an OEGV-positive sample (pcr-DNA), starting from a concentration of 80.9 ng/µL. As can be observed in Table 12 and Figure 7, DNA concentration up to ~80.9 × 10^{-8} ng/µL was detected the LAMP assay, while the end point PCR positive signals were obtained with DNA concentration up to ~80.9 × 10^{-7} ng/µL, indicating that real-time LAMP was about ten times more sensitive than conventional PCR.

Table 12 – Comparison of the sensitivity of the real-time LAMP and end-point PCR.

STARTING DNA CONCENTRATION (80.9 NG/µL)											
ASSAY	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
END-POINT PCR	+	+	+	+	+	+	+	+	-	-	-
REAL-TIME LAMP	+	+	+	+	+	+	+	+	+	-	-
REACTION TIME (MIN)	3	4	5	6	7	8	9	10	10	-	-

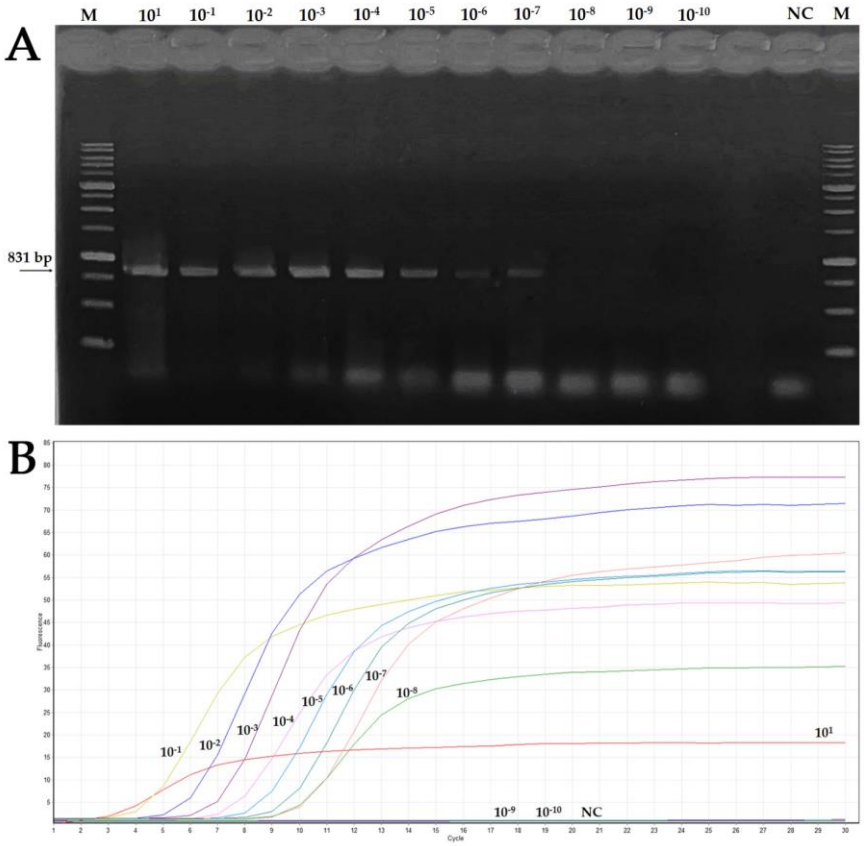


Figure 7 – Sensitivity of the end point PCR (**A**) and real-time LAMP (**B**) for OEGV detection. The assay was conducted using 10-fold serial dilutions of pcr-DNA. Panel A: Agarose gel electrophoresis of PCR products; M: 1Kb ladder marker, NC: negative control. Panel B. Fluorescence of the 10-fold serial dilutions analysed. Fluorescence increased in positive sample curves (from 10-1 to 10-8) after 3 to10 min.

Moreover, even considering the lowest detectable concentration of the pcr-DNA sample in real-time LAMP (~80.9×10^{-8} ng/ μL), the results clearly showed that the time required to carry out the experiment was less than 30 min.

In addition, to evaluate the specificity of the LAMP assay and to assess potential nonspecific cross reactions with other geminiviruses present in the agricultural areas where olive crop samples were collected, a LAMP assay was conducted using the geminiviruses reported in paragraph 4.2.6 as a template. Results showed that no signals were obtained with any of the geminiviruses used as the outgroup, while the two OEGV-positive olive DNA samples used as controls reacted in real-time LAMP with a time value of 10 min and a single peak at 85 °C in the melting curve. This allowed us to confirm the specificity of the assay and to exclude cross-reactivity with unrelated geminiviruses previously isolated in Sicily.

4.3.5 Set up of a rapid sample preparation method suitable for the real-time LAMP assay

With the purpose of identify a method that allows a simple and inexpensive sample preparation useful for real-time LAMP, samples prepared with the two different procedures were tested. For this, the ten samples previously analyzed by end-point PCR and by real-time LAMP assay (Table 11) were considered. As reported in Table 13, all samples tested positive in the LAMP assay when extracted with either procedure. Specifically, samples extracted with the commercial kit showed a fluorescence increase ranging between 3–14 min, while the same samples prepared with the "membrane spot crude extract" method could be detected in 10–24 min. This is worthy of note, as it indicates that the rapid method allows for the detection of the presence of OEGV with a delay of only a few minutes compared to the corresponding extract obtained with the commercial kit. As expected, even with this rapid procedure, no reaction was obtained with the samples from cv. Giarraffa.

Table 13 – Comparison of two different sample preparation methods for the identification of the presence of OEGV in olive samples.

CULTIVAR	ID SAMPLE	TIME VALUE	
		TOTAL DNA EXTRACTION BY COMMERCIAL KIT (MIN)	MEMBRANE SPOT CRUDE EXTRACT (MIN)
Cavalieri Standard	1	10	14
	2	7	12
Cerasuola Standard	3	10	16
	4	7	10
Giarraffa	5	-	-
	6	-	-
Nocellara del Belice Giafalione	7	10	15
	8	13	24
Pizzutella	9	10	16
	10	9	14
Positive control	PC	3	12
Negative control	NC	-	-

Note: (**–**) negative sample.

4.3.6 Spread of OEGV in Sicily

To investigate the spread of OEGV in different olive cultivars grown in Sicily, a new survey was conducted testing 70 samples, each consisting of eight different trees of the same cv. These samples were extracted with the rapid extraction protocol and tested in real-time LAMP, thus representing a total of 560 olive trees analyzed overall. This analysis showed that 30 out of the 70 cultivars (~43%) were positive for OEGV, indicating a relatively high incidence and prevalence of OEGV in the sampling locations and across cultivars. When each of the eight plant samples present in the 30 positive batches were tested individually, the majority (235 out of 240 plants) resulted as being positive for OEGV, except the batch of cv. ‘Calatina’, where only three plants out of eight were positive (Table 14).

Table 14 – Incidence of OEGV evaluated using real-time LAMP assay on sample prepared with the membrane spot crude extraction method.

CULTIVAR ANALYSED	REAL-TIME LAMP	
	CULTIVAR BATCH	POSITIVE PLANTS/ TESTED PLANTS
Abunara	**+**	**8/8**
Aitana	-	NT
Arbequina	**+**	**8/8**
Bariddara	**+**	**8/8**
Biancolilla Caltabellotta	-	NT
Biancolilla Caltabellotta TA PC	**+**	**8/8**
Biancolilla Iacapa	-	NT
Biancolilla Napoletana	-	NT
Biancolilla Pantelleria	-	NT
Biancolilla Schimmenti	-	NT
Biancolilla Siracusana	-	NT
Biancuzza	-	NT
Bottone di Gallo Vassallo	-	NT
Brandofino	-	NT
Calamignara	-	NT
Calatina	**+**	**3/8**
Carasuola Cappuccia	**+**	**8/8**
Castricianella Rapparina	**+**	**8/8**
Cavalieri Standard	**+**	**8/8**
Cerasuola 1 Clone 2	**+**	**8/8**
Cerasuola Nilo Paceco	**+**	**8/8**
Cerasuola Standard	**+**	**8/8**
Conservolia	-	NT
Crastu Collesano	-	NT
Galatina	-	NT
Giarraffa	-	NT
Gordales	-	NT
Iacona	**+**	**8/8**
Indemoniata	-	NT
Koroneiki	**+**	**8/8**
Leucocarpa	-	NT
Lunga di Vassallo	**+**	**8/8**
Manzanilla	-	NT
Minna di Vacca	-	NT
Minuta	**+**	**8/8**
Monaca	**+**	**8/8**
Moresca	-	NT
Murtiddara Vassallo	**+**	**8/8**
Nasitana	**+**	**8/8**
Nocellara del Belice Giafalione	**+**	**8/8**
Nocellara del Belice Clone 1	-	NT
Nocellara del Belice Clone 7	-	NT
Nocellara del Belice Mazara del Vallo	-	NT
Nocellara del Belice Standard	-	NT
Nocellara Etnea	-	NT
Nocellara Messinese Ricciardi	-	NT
Nocellara Messinese Romana	-	NT
Ogliara Maltese	-	NT

Oliva Longa	-	NT
Olivo di Mandanici	**+**	**8/8**
Olivo di Monaci	**+**	**8/8**
Opera Pia	**+**	**8/8**
Passalunara di Lascari	-	NT
Picholine	-	NT
Piricuddara	**+**	**8/8**
Pizzo di Corvo	-	NT
Pizzuta d'Olio	**+**	**8/8**
Pizzutella	**+**	**8/8**
Salicina Vassallo	-	NT
Tonda Iblea	-	NT
Tortella Motticiana	-	NT
Tunnilidda	-	NT
Uovo di Piccione	-	NT
Vaddara	-	NT
Vaddarica	**+**	**8/8**
Verdella	**+**	**8/8**
Verdella Frutto Grosso	**+**	**8/8**
Verdello	**+**	**8/8**
Vetrana	**+**	**8/8**
Zaituna Floridia	-	NT

Note: **(+)** positive sample; **(–)** negative sample; NT: Not Tested.

4.4 Conclusion

A real-time loop-mediated isothermal amplification (LAMP) assay was developed for simple, rapid and efficient detection of olea europaea geminivirus (OEGV). The AV1 (CP) gene of OEGV was targeted for primer design, and the set of six LAMP primers demonstrated good specificity and stability for OEGV detection. Optimization of the LAMP assay, using DNA extracted from OEGV-infected olive samples, revealed that the experiment could be completed in just 30 minutes. The LAMP assay showed no cross-reactivity with other geminiviruses and was allowed to detect OEGV with a 10-fold higher sensitivity than conventional end-point PCR. Moreover, in this study, the conventional extraction method using a commercial kit was compared with a "membrane spot crude extract" method; the data obtained from this comparison suggests that the LAMP-based detection method could be suitable for direct use in the field, confirming that ease of sample preparation is a crucial requirement for future application for on-site detection. The rapid extraction method definitely simplified the surveys of the OEGV spread in different cultivars in Sicily. This survey revealed a considerable presence of the virus in the olive crops in Sicily, probably due to the inadvertent movement of clonally propagated infected but asymptomatic germplasms. In conclusion, the real-time LAMP assay described in this work is a rapid, simple, specific and sensitive technique for detecting the presence of the recently

described OEGV, allowing for the processing of a great number of samples at the same time, especially if associated with the rapid sample preparation method. In particular, this method represents a potential tool for rapidly screening olive plant material useful for large surveys of the spread and pathogenicity of this virus, which to date remain uncertain.

CHAPTER V

CASE STUDY:
OLEA EUROPAEA GEMINIVIRUS (OEGV)

Preliminary study on the transmission of OEGV via grafting

CHAPTER V – PRELIMINARY STUDY ON THE TRANSMISSION OF OEGV VIA GRAFTING

5.1 Introduction

5.1.1 Olive vegetative propagation

Plant vegetative propagation involves different methods of asexual reproduction, whereby a new clonal plant develops from a fragment of the parent plant. These techniques include stem cuttings, air layering, grafting, budding and micro propagation (Awotedu et al., 2021). In particular, olive trees are primarily reproduced today by cutting propagation and micro-propagation (i.e. *in vitro* propagation), although grafting propagation is still widely used, especially in some Mediterranean countries, such as Italy and Greece. In Italy, grafting propagation still accounts for around 65% of the 5 million olive trees produced by nurseries every year (Petruccelli et al., 2012; Lambardi et al., 2023). The main purpose of grafting is to clone genotypes (such as cultivars, selections and elite trees) that cannot be propagated by cuttings or would be prohibitively expensive to propagate. In fact, rooting ability differs markedly among olive cultivars (e.g. several table cultivars are very hard to root or do not root at all) and also micro-propagation is often ineffective (Di Vaio et al., 2012; Lambardi et al., 2023). Furthermore, studies are underway to identify and select clonal rootstocks that can give desired characteristics to olive cultivars, such as the resistance to important diseases (e.g. *Xylella fastidiosa*) or the reduction of the vegetative growth for intensive and super-intensive olive groves, with the expectation that the advent of clonal rootstocks will lead to a return to wider use of grafting approach (Díez et al., 2015; Camposeo et al., 2022).

In light of these considerations, while vegetative propagation offers numerous advantages, it also presents risks, particularly concerning the spread of plant pathogens. Indeed, the widespread adoption of vegetative propagation methods has raised concerns regarding the transmission of viruses through infected propagules (Sastry, 2013). As many viruses have the capacity to infect plants systemically, any propagule used in vegetative propagation is susceptible to contamination. Consequently, vegetative propagation serves as an efficient means of vertical virus transmission, as the virus can spread without the obstacles of establishing infection in a new healthy host plant. The global spread of many plant viruses has been facilitated by the movement of infected propagating material, even over long distances, due to human activities and trade, resulting in viral epidemics and significant negative economic impacts on crop production (Albanese et al., 2012).

In light of this, the transmission dynamics of OEGV remain unclear. Notably, Alabi and co-workers (2021) suggest that the virus may have spread due to the inadvertent movement of clonally propagated infected, but asymptomatic, olive germplasm. Given the lack of biological data for OEGV, its transmissibility was provided by grafting. Thus, this chapter introduces a preliminary study aimed to investigate the transmissibility of OEGV via grafting, utilizing infected propagation material.

5.2 Material and methods

5.2.1 Plant materials

To assess virus transmission via grafting, scions were sourced in late March 2023 from symptomless olive trees previously identified as OEGV-negative in the study outlined within Chapter IV (Section 4.4.6). Specifically, olive scions were carefully obtained from one-year-old side branches of ten distinct olive cultivars (30 scions per cultivar): Bottone di Gallo, Brandofino, Giarraffa, Gordales, Leucocarpa, Nocella del Belice Standard, Nocellara Etnea, Salicina Vassallo, Vaddara, Zaituna Floridia. Plant material was collected ensuring that scions was derived from a single mother plant for each cultivar. Specifically, olive scions were harvested from terminal shoots measuring 25 to 30 cm in length, encompassing the apical and bud regions. Subsequently, all collected scions were wrapped in damp paper and promptly transported to the "Bruno Rosciglione" plant virology laboratory of the SAAF Department and stored at 4 °C until the following grafting trials.

Simultaneously, a total of thirty olive plants, consisting of 20 OEGV-positive and 10 virus-free certified olive plants (Canino cultivar) and tested for the absence of OEGV, were selected as rootstocks for this study. In particular, these specimens were obtained from rooted cuttings and cultivated for three years under greenhouse conditions. The OEGV-positive plants were selected to study the OEGV transmission by grafting, while the virus-free plants served as a control group.

5.2.2 Preliminary screening of plant materials by molecular analysis

In order to confirm the absence of OEGV in the collected scions, total DNA extraction was performed using 3 g of leaf tissue from pooled scions (one pool for each cultivar). For this purpose, the GenUP^{TM} Plant DNA kit (Biotechrabbit GmbH, Berlin, Germany) was utilized following the manufacturer's instructions. Finally, the eluted DNA was resuspended in 100 µL nuclease-free water and, following two measurements with a UV–Vis NanoDrop 1000

spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), samples were adjusted to approximately 50 ng/µL of total DNA. Subsequently, a total of 10 pooled samples (one per cultivar) were subjected to virus detection by real-time LAMP as described in Chapter IV (Section 4.2.5). Finally, the real-time LAMP results were then confirmed by end-point PCR assay, using the OEGV-A2Fw/A4Rev specific primer pair (Chiumenti et al., 2021). Both real-time LAMP and end-point PCR reaction protocols are outlined in Table 15. Each molecular analysis included a positive control (PC), and a healthy olive plant DNA as negative control (NC) were included in both molecular analyses.

Table 15 – Reaction mixtures, volumes and protocols used for real-time LAMP and PCR assays.

REAL-TIME LAMP REACTION MIXTURE	VOLUME [µL]	LAMP PROTOCOL
DNA	2	65 °C for 60 min (fluorescence acquisition every 60 sec) Melting curve: 95 °C for 1 min 40 °C for 1 min 70 °C for 1 min and an increase of temperature at 0.5 °C/s up to 95 °C.
LAMP Primer mix F3-OEGV [0.2 µM] B3-OEGV [0.2 µM] FIP-OEGV [1.6 µM] BIP-OEGV [1.6 µM] LF-OEGV [0.4 µM] LB-OEGV [0.4 µM]	1.25	
LAMP Fluorescent dye	0.25	
WarmStart LAMP 2X Mastermix	6.25	
Nuclease-free water	3.25	
FINAL REACTION VOLUME	**12**	

END-POINT PCR REACTION MIXTURE	VOLUME [µL]	PCR PROTOCOL
DNA	2	95 °C for 5 min 40 cycles: 95 °C for 30 sec, 64 °C for 30 sec, 72 °C for 1 min 72 °C for 10 min Amplicon size: 831 bp
20 mM Tris–HCl [20 mM], KCl [50 mM], $MgCl_2$ [3 mM]	5	
dNTPs [0.4 mM]	1	
OEGV-A2Fw [1 µM]	1	
OEGV-A4Rev [1 µM]	1	
Taq DNA polymerase [2 Units]	0.4	
Nuclease-free water	15.6	
FINAL REACTION VOLUME	**25**	

Finally, olive scions that tested negative for OEGV were selected for the subsequent graft-transmission experiments.

5.2.3 Bark-grafting protocol and transmission assay

In April 2023, olive scions previously confirmed as OEGV-negative through molecular analyses were bark-grafted onto selected rootstocks. Specifically, two scions of the same cultivar were grafted onto OEGV-positive rootstocks, with five plants grafted for each cultivar (5 grafted plants with 2 scions *x* each cultivar).

The selected scions, approximately 10 cm in length and bearing three to four buds each, were carefully sliced at a slant along the base to a length of approximately 2.5 cm. Thin vertical incisions were then made around the cut stem of the rootstock of sufficient length to accommodate the insertion of the scion. Careful attention was dedicated ensuring the smooth peeling of bark during scion insertion, while also guaranteeing precise alignment of the cambium layers of both the scion and rootstock. Thorough cleaning and disinfection of tools was carried out between each grafting operation. Moreover, to promote optimal graft union development, the grafted plants were covered with a transparent polyethylene bag and a waterproof paper bag until 20 days post-grafting.

Subsequently, the newly grafted plants (five grafted plants per cultivar) were kept in insect-proof cages at temperature ambient ranging from 10 to 30 °C and the racks holding the containers were placed in shallow water-filled pans to maintain a higher level of humidity. Plants were grown under these specific conditions and the viability of the scions was checked weekly. Finally, any grafted scions that failed to establish were promptly removed from the trial.

Simultaneously, the same procedure was applied to graft scions onto virus-free rootstocks, using these plants as the control group. In particular, two plants were grafted for each selected cultivar, with two scions used for each graft. The grafted plants were subsequently maintained under optimal growth conditions (25°C and 50% relative humidity) until the conclusion of the transmission experiments.

5.2.4 Evaluation of OEGV graft transmission

To assess the OEGV transmission, monthly molecular analyses were performed on all grafted plants, including those used as negative controls, for a period of up to nine months after grafting. In particular, the grafted plants were subjected to regular visual inspections for the presence of viral symptoms. Thereafter, monthly total DNA extractions were conducted, as described above, from each rootstock and the emerged leaf of each grafted scion. Subsequently, real-time LAMP assays were conducted on extracted DNAs, following the aforementioned protocol. Furthermore, the real-time LAMP results were subsequently confirmed by end-point PCR analysis, using the OEGV-A2Fw/A4Rev primer pair (Chiumenti et al., 2021). Both molecular analyses included a positive control (PC) and a healthy olive plant DNA as a negative control (NC) to ensure accuracy and reliability.

5.3 Results

5.3.1 Preliminary screening of plant materials by molecular analysis

A preliminary investigation utilizing real-time LAMP assay was undertaken to ascertain the absence of OEGV in the collected scions. Out of the ten pooled samples analysed, four tested negatives for OEGV according to the real-time LAMP results. Conversely, the remaining samples displayed amplification curves, indicating their positivity to the virus, and were consequently excluded from the trial (Table 16).

Table 16 – Results of preliminary molecular tests conducted on pooled scion samples.

CULTIVAR	ID SAMPLE	MOLECULAR ASSAY	
		REAL-TIME LAMP	END-POINT PCR
Bottone di Gallo	1P	+	+
Brandofino	2P	+	+
Giarraffa	3P	-	-
Gordales	4P	+	+
Leucocarpa	5P	-	-
Nocellara del Belice Standard	6P	+	+
Nocellara etnea Standard	7P	-	-
Salicina Vassallo	8P	+	+
Vaddara	9P	-	-
Zaituna Floridia	10P	+	+
Positive control	PC	+	+
Negative control	NC	-	-

Note: **(+)** positive sample; **(-)** negative sample. The negative pooled samples are underlined in grey.

Notably, the amplification curves of the positive samples showed a fluorescence increase ranging between 5–38 min, ultimately reaching the reaction plateau within 14 to 50 (Figure 8A), while the melting curve displayed a peak temperature of approximately 85 °C (Figure 8B). The real-time LAMP results were confirmed with end-point PCR assay (Figure 8C). In both molecular analysis no signal was obtained with the negative control (NC).

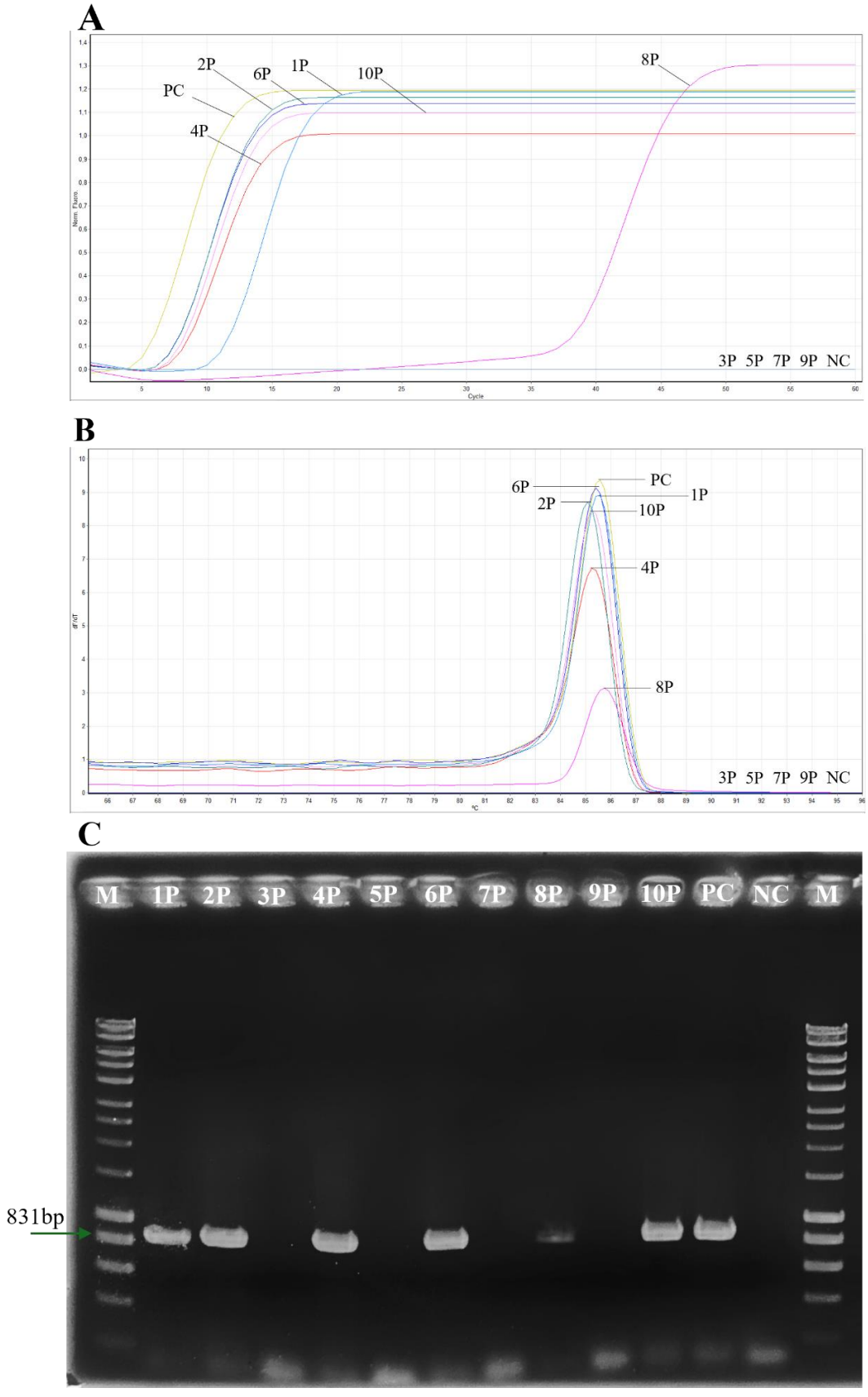


Figure 8 – Preliminary screening by real-time LAMP and end-point PCR assays for OEGV detection. **A**: Real-time LAMP amplification curves; **B**: Melting curves; **C**: Agarose gel electrophoresis of PCR products. M: Nippon Genetics 1 Kb ladder RTU.

5.3.2 Bark grafting protocol and transmission assay

To investigate OEGV transmission via grafting, two scions from each cultivar – Giarraffa, Leucocarpa, Nocellara Etnea, and Vaddara – previously confirmed as OEGV-negative, were bark-grafted onto a total of twenty OEGV-positive rootstocks. The viability of all grafted plants was assessed using different grafting combinations. For instance, in the Giarraffa cutlivar, scion viability ranges within a timeframe spanning from 3 to 4 weeks post-grafting (wpg). Similarly, in the Leucocarpa and Nocellara Etnea cultivars, scion viability varies between 3 and 6 wpg, with occasional graft failures (refer to Table 17 for details). Notably, graft failures were observed in those plants grafted with Vaddara cultivar. In fact, the latter all revealed graft failure and were consequently excluded from the transmission trials.
Similarly, an identical procedure was applied to graft scions onto virus-free plants, which served as a negative control group. These scions showed viability between 3- and 4-weeks post-grafting (wpg) (data not shown).

Table 17 – Grafted plants for OEGV transmission trials and the viability of scions measured in weeks post-grafting.

NO. GRAFTED PLANTS	GRAFTED CULTIVAR	ID SAMPLE	SCION VIABILITY (WPG)
1	Giarraffa	1GIA/1	3
		1GIA/2	3
2		2GIA/1	4
		2GIA/2	4
3		3GIA/1	3
		3GIA/2	3
4		4GIA/1	3
		4GIA/2	4
5		5GIA/1	4
		5GIA/2	4
6	Leucocarpa	1LEU/1	6
		1LEU/2	6
7		2LEU/1	5
		2LEU/2	5
8		3LEU/1	4
		3LEU/2	5
9		4LEU/1	4
		4LEU/2	4
10		5LEU/1	3
		5LEU/2	4
11	Nocellara Etnea	1NOC/1	6
		1NOC/2	6
12		2NOC/1	–
		2NOC/2	–
13		3NOC/1	5
		3NOC/2	6
14		4NOC/1	–
		4NOC/2	–
15		5NOC/1	–
		5NOC/2	5
16	Vaddara	1VAD/1	–
		1VAD/2	
17		2VAD/1	
		2VAD/2	
18		3VAD/1	
		3VAD/2	
19		4VAD/1	
		4VAD/2	
20		5VAD/1	
		5VAD/2	

Note: a dash (–) indicates graft failure.

5.3.3 Evaluation of OEGV graft transmission

To investigate the graft transmissibility of OEGV, all grafted plants were monitored for symptoms and subjected to monthly analysis, using real-time LAMP and end-point PCR techniques.

The results revealed successful transmission of OEGV via bark-grafting to the Giarraffa and Leucocarpa cultivars. A discernible trend of OEGV transmission was observed in the Giarraffa cultivar, with eight out of ten positive detections recorded in multiple samples at different post-grafting intervals. Notably, 2GIA/1 and 2GIA/2 samples exhibit early OEGV detection at three months after grafting (mpg). Regarding plants grafted with Leucocarpa cultivar, OEGV presence was detected in six out of ten samples within a variable range of 5-8 mpg (Table 18). Surprisingly, it is noteworthy that symptomatic manifestations, such as leaf curling, interveinal yellowing, leaf wilting, necrosis on apical leaf and, in some cases, plant death, were observed in plants that tested positive for the virus (Figure 9). Conversely, the Nocellara Etnea cultivar did not exhibit any evidence of OEGV transmission (Table 18). Finally, no OEGV-presence was detected in the grafted plants utilized as negative controls.

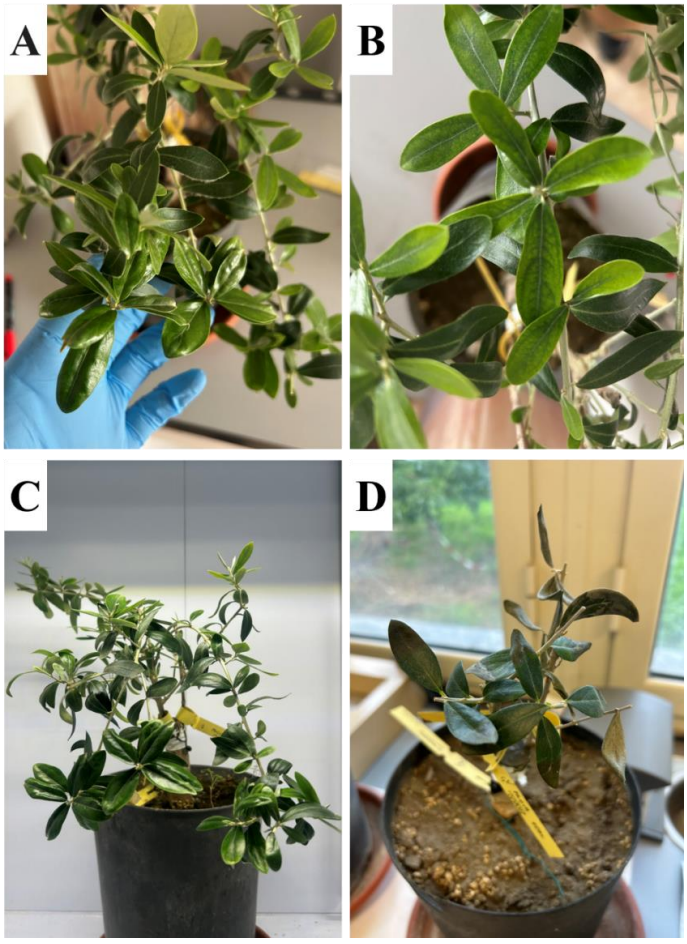


Figure 9 – Symptoms observed on grafted plants testing positive for OEGV.
A-C: leaf curling; **B**: interveinal yellowing; **D**: apical necrosis and general leaf wilt.

Table 18 – Detection by molecular analyses and transmission evaluation of OEGV in plants after grafting.

GRAFTED CULTIVAR	ID SAMPLE	1 MPG		2 MPG		3 MPG		4 MPG		5 MPG		6 MPG		7 MPG		8 MPG		9 MPG	
		R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Giarraffa	1GIA/1	+	NT	+	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+
	1GIA/2	+	NT	+	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+
	2GIA/1	+	NT	+	-	+	+	+	+	+	+	†	†	NT	NT	NT	NT	NT	NT
	2GIA/2	+	NT	+	-	+	+	+	+	+	+	+	+	+	+	†	†	NT	NT
	3GIA/1	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	3GIA/2	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	4GIA/1	+	NT	+	-	+	-	+	-	+	+	+	+	+	+	†	†	NT	NT
	4GIA/2	+	NT	+	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+
	5GIA/1	+	NT	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
	5GIA/2	+	NT	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Leucocarpa	1LEU/1	+	NT	+	NT	+	-	+	-	+	-	+	+	+	+	+	+	+	+
	1LEU/2	+	NT	+	NT	+	-	+	-	+	-	+	-	+	+	†	†	NT	NT
	2LEU/1	+	NT	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	2LEU/2	+	NT	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	3LEU/1	+	NT	+	-	+	-	+	-	+	+	+	+	†	†	NT	NT	NT	NT
	3LEU/2	+	NT	+	-	+	-	+	-	+	+	+	+	+	+	†	†	NT	NT
	4LEU/1	+	NT	+	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+
	4LEU/2	+	NT	+	-	+	-	+		+	-	+	-	+	-	+	+	+	+
	5LEU/1	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	5LEU/2	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Nocellara Etnea	1NOC/1	+	NT	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	1NOC/2	+	NT	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	3NOC/1	+	NT	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	3NOC/2	+	NT	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	5NOC/2	+	NT	+	NT	+	-	+	-	+	-	+	-	+	-	+	-	+	-

Note: OEGV detection is denoted in months post-grafting (mpg). **R**: rootstock; **S**: scion; **NT**: not tested; **(+)** positive sample; **(-)** negative sample. Grafted samples (consisting of rootstock and scion) demonstrating the presence of OEGV in both parts are highlighted in light red. The symbol "†" indicates plant death.

5.4 Conclusion

Olive cultivation relies on vegetative propagation methods to maintain desirable cultivars. Among these techniques, grafting stands out as a traditional and widely employed method, particularly in country such as Italy and Greece, where it accounts for a significant portion of olive tree production. Although vegetative propagation offers many advantages, such as clonal propagation of desired genotypes and the possibility of propagating cultivars with poor rooting ability, it also poses inherent risks, particularly with regard to the spread of plant pathogens.

The study aimed to assess OEGV graft transmissibility, revealing successful transmission via bark-grafting in Giarraffa and Leucocarpa cultivars. Virus detection occurred within 3-7 months post-grafting in Giarraffa and 5-8 months post-grafting in Leucocarpa cultivar, associated with symptomatic leaf curling, yellowing, and plant mortality. Furthermore, the sporadic occurrence of plant death in grafting combinations suggests that factors beyond OEGV infection may contribute to plant death. These factors could involve interactions between the virus and specific cultivars, or variations in the response of individual plants to virus infection. Conversely, Nocellara Etnea showed no virus transmission. These findings suggest potential varietal differences in susceptibility to OEGV graft transmission among olive cultivars, highlighting the need for further exploration into the underlying mechanisms dictating viral transmission dynamics. Moreover, the results underscore the importance of employing stringent screening methods and selecting appropriate cultivars to mitigate the risk of virus transmission in olive vegetative propagation. Ongoing research is essential to understand the transmission mechanisms and to develop effective disease management strategies in olive cultivation.

CHAPTER VI

CASE STUDY:
OLIVE LEAF YELLOWING-ASSOCIATED VIRUS (OLYaV)

Detection by sensitive real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) of olive leaf yellowing-associated virus (OLYaV) and its incidence in Italy and Spain

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"Detection by sensitive real-time Reverse Transcription Loop-Mediated Isothermal Amplification of olive leaf yellowing-associated virus and its incidence in Italy and Spain." Caruso A. G., **Bertacca S.**, Ragona A., Agrò G., Font-San-Ambrosio M. I., Alfaro-Fernández A., Sánchez R.E., Panno S. & Davino S. (2023). Horticulturae, 9(6), 702.

CHAPTER VI – DETECTION BY SENSITIVE REAL-TIME REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) OF OLIVE LEAF YELLOWING ASSOCIATED VIRUS (OLYAV) AND ITS INCIDENCE IN ITALY AND SPAIN

6.1 Introduction

6.1.1 Olive leaf yellowing-associated virus (OLYaV)

Olive leaf yellowing-associated virus (OLYaV) was an unassigned member of the family *Closteroviridae* (Sabanadzovic et al., 1999), but, recently, thanks to the analyses of its full-length genome and its five closteroviral broadly conserved proteins (Ruiz-García et al., 2020), it was demonstrated that it represents a new genus, named *Olivavirus* within the family *Closteroviridae* (ICTV, 2022).

OLYaV has a monopartite positive-sense single-stranded RNA (ssRNA+) of 16,700 nucleotides (nts) that includes 11 open reading frames (ORFs) encoding proteins, some of which have homologies with those of other *Closteroviridae* members (ORF 1a, ORF 1b, ORF2-thaumatin-like protein, ORF4-HSP70h, ORF5-HSP90h, and ORF6-CP), while others have no homologous counterpart in the GenBank database, such as ORF3-p7, ORF7-p17, ORF8-p10, ORF9-p7, ORF10-p23, and ORF11-p10 (Ruiz-García et al., 2020) (Figure 10).

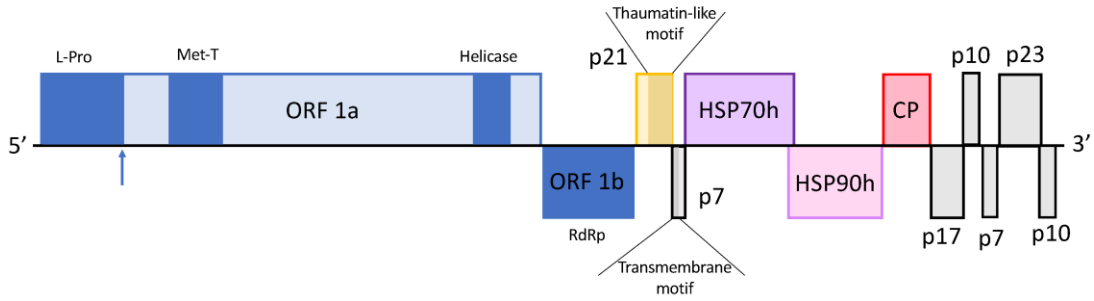


Figure 10 – Genome organization of olive leaf yellowing-associated virus (Ruiz-García et al., 2020). L-Pro: papain-like leader protease; Met-T: viral methyltransferase domain; Helicase: viral helicase domain; RdRp: RNA dependent RNA polymerase. Blue arrow indicates tentative L-Pro cleavage site position.

Among olive viruses, OLYaV seems to be one of the most widespread (Çağlayan et al., 2011; Martelli, 2013); it has been detected in southern Italy (including Sicily) in a large number of cultivars (Faggioli et al., 2005; Savino et al., 1996; Albanese et al., 2003) and in high percentages also in Israel (Martelli, 2011), the USA (Al-Rwahnih et al., 2011), Morocco (Afechtal & Mounir, 2020), Tunisia (El Air et al., 2011), and Croatia (Bjeliš et al., 2007). Moreover, OLYaV has also been officially reported in Lebanon (Fadel et al., 2005), Cyprus (Martelli, 2013), France (Martelli, 2013), Syria (Al Abdullah et al., 2005), Egypt (Youssef

et al., 2010), Chile (Martelli, 2013), Greece (Mathioudakis et al., 2020), Australia (Martelli, 2013), Albania (Çakalli, 1999), Palestine (Samara et al., 2018), Portugal (Campos et al., 2019), Algeria (Martelli, 2013), Brazil (Ruiz-García et al., 2020), Slovenia (Viršcek Marn and Mavric Pleško, 2018), and Spain (Ruiz-García et al., 2021).

To date, no symptomatology has been clearly associated with OLYaV infection (Ruiz-García et al., 2021), as this virus has been detected often in symptomless trees (Martelli, 2013). The symptoms consist of foliar discolorations ranging from chlorosis to bright yellowing (Martelli, 2013). In addition, uncommon woody cylinder deformations such as stem pitting and woody gall symptoms were observed in olive trees (cv. Zarzaleña) (Ruiz-García et al., 2020). It was also demonstrated that total twig length and number, trunk diameter, and leaf area were significantly reduced on olive-infected trees (Cutuli et al., 2014). The OLYaV transmission occurs through the infected plant propagation material (Sabanadzovic et al., 1999, Martelli and Prota, 1997). The vectors for olivaviruses remain to be identified (Mollov et al., 2023), but it is strongly suspected that the OLYaV vectors are the psyllid *Euphyllura olivina* (Costa) (Homoptera: Psyllidae) (Ruiz-García et al., 2021) and unidentified mealybugs of the *Pseudococcus* genus (Sabanadzovic et al., 1999).

In detail, the psyllid *E. olivina* is normally present in abundance during spring, causing up to 60% yield loss, mainly by sooty mould formation with the waxy/sugary substance they emit during nymphal development. To date, it is present in Spain, France, the Canary Islands, Greece, South Africa, and the USA (California) (GBIF—Global Biodiversity Information Facility). Feeding occurs by penetration and suction of olive shoots, inflorescence, and flowers (Guessab et al., 2022).

As with other plant viruses, OLYaV is reported in many olive cultivars in different areas worldwide (Ruiz-García et al., 2021; Martelli et al., 1994; Zellama et al., 2019), and the implementation of preventive measures and selection of healthy and certified propagation material are appropriate strategies to control olive viruses' dispersion (Martelli et al., 1994; Martelli, 1999). The identification of OLYaV-free plants based on visual symptoms is not reliable because many infected olive trees remain asymptomatic (Martelli, 1999). For this reason, due to the high presence of latent infections, field selection must therefore be followed by molecular analyses (Martelli, 1999; Erilmez, 2016). In general, plant virus detection is based on immune-enzymatic assays (e.g., ELISA) and molecular methods. Currently, the available methods for OLYaV detection are based on conventional reverse transcription-PCR (Sabanadzovic et al., 1999; Faggioli et al., 2005; Ruiz-García et al., 2021) and SYBR® Green real-time quantitative PCR (RT-qPCR) assay (Campos et al., 2019);

furthermore, high-throughput sequencing (HTS) has proven to be a robust tool for OLYaV detection (Ruiz-García et al., 2020; Ruiz-García et al., 2021). These methods are valuable to identify latent infections and early disease stages; however, they require expensive equipment, sophisticated laboratory setup, and highly skilled personnel and are impractical for large-scale use.

An alternative is represented by the loop-mediated isothermal amplification technique (LAMP), which allows the amplification of specific genome traits with high specificity, efficiency, and rapidity under isothermal conditions.

In the present study, the OLYaV incidence was evaluated in different cultivars collected from Spain and Italy, which represent the major olive producers in Europe, through a new real-time reverse transcription LAMP (RT-LAMP) diagnostic protocol. The reasons for the LAMP methodology's development are based on the attempt to overcome different drawbacks of end-point RT-PCR (high-cost equipment, long times, inhibitor sensitivity, etc.). Moreover, LAMP could represent a valid method for epidemiological studies thanks to its rapidity, reliability, and specificity.

6.2 Material and methods

6.2.1 Source of viral material

One characterized lyophilized OLYaV isolate from olive leaves, named V64 (GenBank Acc. No. MW056495) (Ruiz-García et al., 2021), stored in the "Bruno Rosciglione" plant virology laboratory of the University of Palermo (SAAF Department) (Palermo, Italy), was used as source material to develop the real-time RT-LAMP assay.

About 100 mg of olive leaf V64 isolate was previously rehydrated with 1 mL of extraction buffer (1.3 g sodium sulphite anhydrous, 20 g polyvinylpyrrolidone MW 24–40,000, 2 g chicken egg chicken albumin Grade II, and 20 g Tween-20 in one L of distilled water, pH 7.4) and subsequently used for total RNA extraction using the NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH & Co., Dueren, Germany) following the manufacturer's instructions, with minor modifications. Briefly, 350 µL of lysis buffer supplied in the kit was added to 350 µL of the extract; the manufacturer's protocol was followed after this preparation step. The eluted RNA was re-suspended in 60 µL of RNase-free water. The concentration of total RNA was measured in duplicate with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), subsequently diluted to ≈50 ng/µL, and stored at −80 °C until molecular analyses.

6.2.2 Primer design and OLYaV detection using canonical RT-PCR

New primer pairs for OLYaV detection by end-point RT-PCR were specifically designed in this work. In detail, the complete genome sequences available in GenBank (Acc. No. MT809205, MW056495, and OK569886) were aligned using the Clustal X2 program (Larkin et al., 2007) to identify the nucleotide regions that show a high homology percentage at the nucleotide level within the reference isolates. A total of six primer pairs, targeting the nucleotide sequence elapsing region between 5'-UTR and ORF-1a, were designed using the OLYaV MW056495 as a reference sequence. The designed primers were evaluated with the Nucleotide-BLAST algorithm (accessed on 15 January 2023) to evaluate possible hybridization with other organisms, while the hairpins and secondary structures were verified using the OligoAnalyzer Tool (accessed on 15 January 2023). Moreover, the primers were also checked using Vector NTI Advance 11.5 software (Invitrogen, Carlsbad, CA, USA) with the complete genome sequences of other viruses belonging to the *Closteroviridae* family, including viruses belonging to the *Olivavirus* genus (Table 19), to evaluate their affinity percentages.

Table 19 – Viruses belonging to Closteroviridae family, and their sequences used for in silico analysis

SPECIES	GENUS	FAMILY	GENBANK ACCESSION NO.
Citrus tristeza virus (CTV)	*Closterovirus*	*Closteroviridae*	EU937521 AF001623 Y18420
Grapevine leafroll-associated virus 1 (GLRaV-1)			NC016509
Grapevine leafroll-associated virus 2 (GLRaV-2)	*Ampelovirus*		DQ286725
Grapevine leafroll-associated virus 3 (GLRaV-3)			AF037268
Tomato chlorosis virus (ToCV)	*Crinivirus*		RNA1: AY903447 RNA2: AY903448
Tomato infectious chlorosis virus (TICV)			RNA1: FJ815440 RNA2: FJ815441
Cucurbit yellow stunting disorder virus (CYSDV)			RNA1: NC004809 RNA2: NC004810
Actinidia virus 1 (AcV1)	*Olivavirus*		KX857665
Persimmon virus B (PeVB)			AB923924

The obtained primer sets were tested by a canonical RT-PCR assay using the OLYaV RNA positive control (PC) (≈50 ng/µL concentration), including a healthy olive plant RNA as a negative control (NC). Each sample was analyzed in triplicate in three independent, two-step end-point RT-PCR assays. In detail, the reverse transcription (RT) was carried out in a 20 µL final volume with 1 µL of total RNA, 0.4 mM dNTPs, 4 µL of 5X First Strand Buffer [50 mM Tris-HCl pH 8.3, 40 mM KCl, 6 mM $MgCl_2$] (Thermo Fisher Scientific, Waltham, MA, USA), 1 µM of reverse primer, 20 U of M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume. After an initial denaturation at 65 °C for 10 min, RT was performed at 42 °C for 45 min and 95 °C for 10 min. PCR was performed in a final reaction volume of 25 µL, containing 2 µL of the obtained cDNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 µM of each primer, 2 U of *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and RNase-free water to reach the final volume, according to the following cycling conditions: 95 °C for 3 min; 40 cycles of 30 s at 95 °C, 30 s at the specific annealing temperature of each primer pair, 45 s at 72 °C, and a final elongation of 10 min at 72 °C. RT-PCR was carried out in a PCR System 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). RT-PCR products were verified by electrophoresis in a 1.5% agarose gel stained with SybrSafe™ (Thermo Fisher Scientific, Waltham, MA, USA) and visualized by UV light. The primer pair that revealed the highest specificity and the absence of any non-specific products was chosen and used for subsequent tests.

The obtained PCR product was cloned into the pGEM-T vector (Promega, Madison, WI, USA) and cloned into *Escherichia coli* One Shot™ Mach1™ competent cells (Invitrogen, Carlsbad, CA, USA). After ampicillin resistance selection of the transformants, the fragment presence was verified by colony-PCR with the specific primer pair previously used. The plasmid DNA was purified using the NucleoSpin Plasmid DNA Purification Kit (Macherey-Nagel GmbH & Co., Dueren, Germany), quantified twice using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and sequenced in both directions using an ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems, Foster City, CA, USA). Finally, the sequence obtained was verified by the BLAST algorithm at the National Center for Biotechnology Information (NCBI) (accessed on 30 January 2023).

6.2.3 OLYaV real-time RT-LAMP primer design

A 429 bp nucleotide sequence elapsing from the 5'-UTR to the ORF1a genes of the OLYaV V64 isolate (GenBank Acc. No. MW056495) used as a reference sequence was chosen to design a specific LAMP primer set. In detail, a set of six primers was designed using the PrimerExplorer version 5 software (accessed on 5 February 2023), including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). Primer set specificity was evaluated in silico using the nucleotide BLAST algorithm (accessed on 5 February 2023) available at the NCBI website to evaluate possible cross-reactions with other organisms. In addition, each primer was tested against the full genomic sequences of other viruses listed in Table 19 to verify their affinity using the Vector NTI Advance 11.5 software (Invitrogen, Carlsbad, CA, USA).

6.2.4 OLYaV real-time RT-LAMP assay optimization

The OLYaV V64 isolate RNA was used as a template for the real-time RT-LAMP assay optimization, including healthy olive plant RNA (NC) as a negative control. The real-time RT-LAMP assay was performed in a volume of 25 µL, containing 0.2 µM each of OLYaV-F3 and OLYaV-B3, 1.6 µM each of OLYaV-FIP and OLYaV-BIP, and 0.4 µM each of OLYaV-LoopF and OLYaV-LoopB, 15 µL of LAMP Isothermal Master Mix (Optigene® Limited, West Sussex, UK), 1 µL of RNA (≈50 ng/µL), and nuclease-free water to reach the final volume. Each sample was analyzed in triplicate in three independent real-time LAMP assays, performed at 65 °C for 60 min (fluorescence acquisition every 60 s), using a Rotor-Gene Q2plex HRM Platform Thermal Cycler (Qiagen, Hilden, Germany). Additional steps for the melting curve were carried out to acquire the fluorescence using the following protocol: 95 °C for 1 min, 40 °C for 1 min, 70 °C for 1 min, and an increase in temperature at 0.5 °C/s up to 95 °C. The fluorescence data were obtained in the 6-carboxyfluorescein (FAM) channel (450–495 nm excitation and 510–527 nm detection). The relative fluorescence units (RFU) threshold value was used, and the threshold time (Tt) was calculated as the time at which fluorescence was equal to the threshold value.

6.2.5 Sensitivity and comparison of OLYaV real-time RT-LAMP assay to conventional RT-PCR

The plasmids obtained were used to determine the OLYaV real-time RT-LAMP sensitivity and to compare the results with an end-point RT-PCR assay. Ten-fold serial dilutions of the purified recombinant plasmid DNA diluted into healthy olive RNA extract were used as templates for both real-time RT-LAMP and end-point RT-PCR assays. The number of copies was determined as follows:

$$\text{No. of copies} = \frac{\text{DNA amount [ng]} \times 6.022 \times 10^{23}}{\text{DNA template lenght [bp]} \times 1 \times 10^{9} \times 650}$$

Additional steps for the melting curve were carried out, as described above (see Section 6.2.4). Each sample was analyzed in triplicate in three independent assays. The results obtained were compared, and the optimal reaction time of the real-time RT-LAMP was determined.

6.2.6 Specificity of OLYaV real-time RT-LAMP assay

To verify the real-time RT-LAMP assay specificity and evaluate non-specific reactions with other viruses belonging to the *Closteroviridae* family, a real-time RT-LAMP assay was conducted using the OLYaV V64 isolate as a positive control and the RNAs of other viruses stored in the "Bruno Rosciglione" virology laboratory. In particular, the following viruses were tested: Citrus tristeza virus (CTV), grapevine leafroll-associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3), tomato chlorosis virus (ToCV), tomato infectious chlorosis virus (TICV), and cucurbit yellow stunting disorder virus (CYSDV). In each run, total RNA from a healthy olive plant (NC) was included. Each sample was analyzed in triplicate in three independent LAMP assays. The experiment was performed with the conditions described above (see Section 6.2.4), reducing the reaction time to 45 min, including the melting curve steps.

6.2.7 Evaluation of OLYaV incidence in Italy and Spain by real-time RT-LAMP assay on symptomatic and asymptomatic olive trees

To understand the effective OLYaV incidence in Italy and Spain, several samplings were carried out in the main Italian and Spanish olive-growing regions, and each sample was analyzed by the real-time RT-LAMP assay developed in this work.

The analyses were performed on 61 symptomatic and 139 asymptomatic field samples, for a total of 200 samples of different cultivars collected during spring 2022. In detail, 80 samples were collected in Spain, of which 40 were in the Valencia region (20 in the Valencia province and 20 in the Castellón province) and 40 were in the Andalusia region (20 in the Granada province and 20 in the Jaén province). Regarding Italy, a total of 120 samples were collected, of which 40 were collected in Sicily (20 in the Agrigento province and 20 in the Trapani province), 20 in the Calabria region (Crotone province), 20 in the Apulia region (Trapani province), 20 in the Latium region (Frosinone province), and 20 in the Umbria region (Terni province) (Figure 11). Table 20 reports the different olive cultivars collected.

Figure 11 – Sampling regions in Spain (left) and Italy (right).

Table 20 – Olive cultivars collected from different Italian and Spanish regions.

COUNTRY	REGION	PROVINCE	NO. SAMPLES COLLECTED	CULTIVAR
Italy	Sicily	Agrigento	20	Giarraffa
		Trapani	20	Nocellara del Belice
	Calabria	Crotone	20	Carolea
	Apulia	Taranto	20	Ogliarola barese
	Lazio	Frosinone	20	Leccino
	Umbria	Terni	20	Frantoio
Spain	Comunitat Valenciana	Valencia	20	Serrana Espadán, Picual
		Castellón	20	Serrana Espadán, Villalonga
	Andalusia	Jaén	20	Picual, Villalonga
		Granada	20	Arbequina

The Planthology mobile application was used to geo-reference all samples collected (Davino et al., 2017); subsequently, the samples were stored at 4 °C and analyzed. Sampling was carried out, collecting eight branches per plant from symptomatic and asymptomatic olive trees. The hierarchical sampling scheme, with minor modifications to olive plants, was applied to collect the samples (Gottwald and Hughes, 2000). Total RNA was extracted, and the concentration was determined as described above. Subsequently, real-time RT-LAMP for easy detection of OLYaV was carried out. Each test included a positive control (PC) and RNA from a healthy olive plant as a negative control (NC). Each sample was analyzed in triplicate in three independent real-time RT-LAMP assays.

6.3 Results

6.3.1 Primer design and OLYaV detection using canonical RT-PCR

In Table 21, the six end-point RT-PCR primer pairs designed are reported. The *in silico* analysis results showed that no relevant matches with other organisms were identified.

Table 21 – OLYaV RT-PCR primer pairs designed in this work.

PRIMER	BINDING SITES (MW056495)	GENOMIC POSITION	SEQUENCE (5'- 3')	TA (°C)	AMPLICON SIZE (BP)
OLYaV-21F	**21-44**	**5' UTR-ORF1a**	**ATCAATTGAAGAAAACCACTCCC**	**59**	**450**
OLYaV-471R	**450-471**		**AGTACCTCCCACGACGTATTG**		
OLYaV-30F	30-54	5' UTR-ORF1a	GAAAACCACTCCCTTCAATTCAAT	61	497
OLYaV-527R	505-527		GAAGAACTATTGATTGGCTTGGG		
OLYaV-36F	36-59	5' UTR-ORF1a	CCACTCCCTTCAATTCAATAACAT	60	428
OLYaV-444R	444-464		CCCACGACGTATTGACCACTC		
OLYaV-46F	46-67	5' UTR-ORF1a	CAATTCAATAACATAACATACC	60	345
OLYaV-371R	371-391		ATGTGACTTTTGACTGAGGTA		
OLYaV-495F	495-517	ORF1a	GAAGAACACTCCCAAGCCAATC	60	646
OLYaV-1141R	1120-1141		CACACTCTCTCTTGTAAGTCCC		
OLYaV-742F	742-763	ORF1a	GCGGCTTGGGTCATTAAGGGT	60	427
OLYaV-1166R	1147-1166		GGGCAAAAATAGTGAGAGCGAC		

All the obtained primer pairs were verified by end-point RT-PCR. Between the six primer pairs, OLYaV-30F/OLYaV-527R and OLYaV-46F/OLYaV-371R did not give the expected amplicon, while the remaining primer pairs gave the expected amplicon, but with non-specific bands, except for OLYaV-21F/OLYaV-471R, which showed the higher specificity without non-specific bands. Therefore, this primer pair was chosen as the best candidate for OLYaV detection by end-point RT-PCR. A 450 bp amplicon size was obtained, as expected PCR product, and the sequence showed a percentage identity of >99.9% with the previously uploaded OLYaV sequences in GenBank, indicating that the assay was specific for OLYaV

detection. The analysis using the Vector NTI 11.5 program against other viruses (see Section 6.2.2) has not demonstrated relevant matches.

6.3.2 OLYaV real-time RT-LAMP primer design

A set of six primers for OLYaV detection by a real-time RT-LAMP assay was designed between the 5'-UTR and ORF-1a coding regions, within the OLYaV-21F/OLYaV-471R amplified region. Table 22 and Figure 12 report the primer sequences and binding sites, respectively. Both the *in silico* analysis of LAMP primers and the hybridization analysis against other viruses showed no cross-reactions with other organisms and excluded relevant matches with other viruses, respectively.

Table 22 – Primers designed for OLYaV detection by real-time RT-LAMP.

PRIMER NAME	SEQUENCE (5'-3')	AMPLICON SIZE (BP)
OLYaV-F3	CTGGTCTCTACGTACAGGGA	195
OLYaV-B3	GGTACTGCCTCAGTTCCCA	
OLYaV-FIP	CCGGTAACGACCTTCAGCCTTCCCCGCTGTGCAGAACAAC	-
OLYaV-BIP	AAGAAAACGGGCTGTAGCCCATGCCGTCAATGTTACGAGC	
OLYaV-LoopF	TCTGTGCAGATCTAGATTTGGGA	-
OLYaV-LoopB	GAGGAAAGCAGCGACCACC	

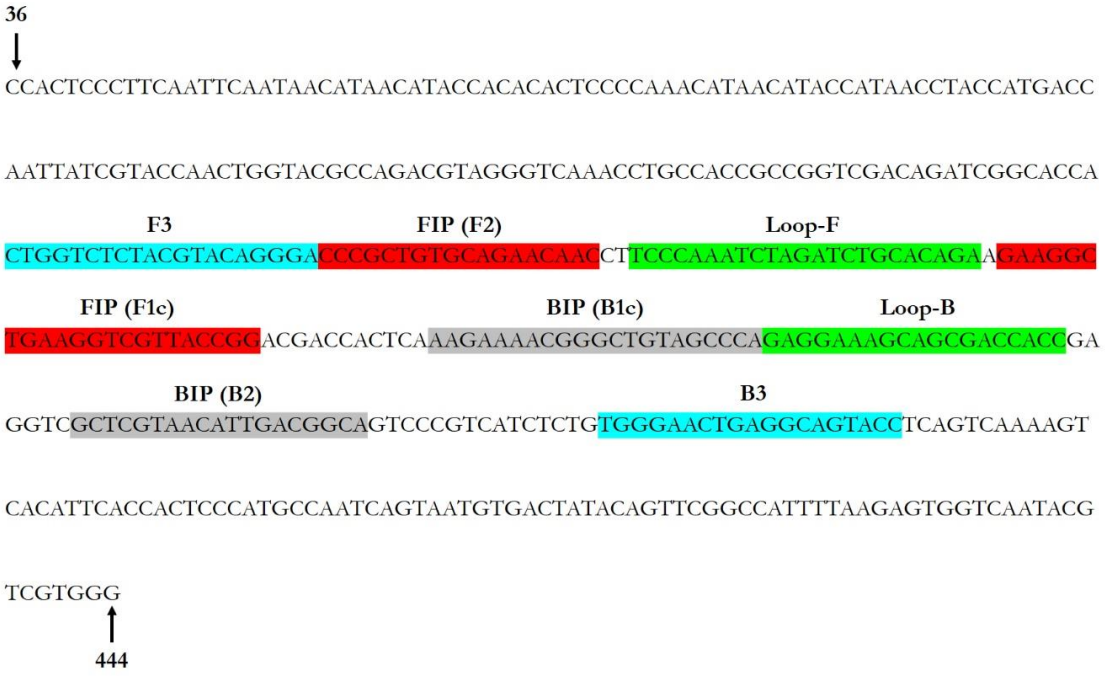


Figure 12 – Genome position of the LAMP primer set designed in this work. F3 and B3 are shown in pale blue, FIP (F1c-F2) in red, BIP (B1c-B2) in grey, and the two loop primers LF and LB in green. Genomic position of the first and last nucleotide in the selected sequence is represented by the number at the beginning and end of the sequence (GenBank Acc. No. MW056495).

6.3.3 OLYaV real-time RT-LAMP assay optimization

As reported in Table 23 and Figure 13A, the amplification curve of the OLYaV V64 isolate showed an exponential trend at 14 min, reaching the reaction plateau in about 24 min, while the melting curve displayed a peak temperature of approximately 90 °C (Figure 13B). No signal was obtained with the negative control (NC).

Table 23 – Real-time RT-LAMP assay performance for OLYaV detection.

SAMPLE	REAL-TIME RT-LAMP REACTION TIME (MIN)		
	ASSAY #1	ASSAY #2	ASSAY #3
OLYaV V64 isolate	13.7	14.1	14.4
Negative control (NC)	-	-	-

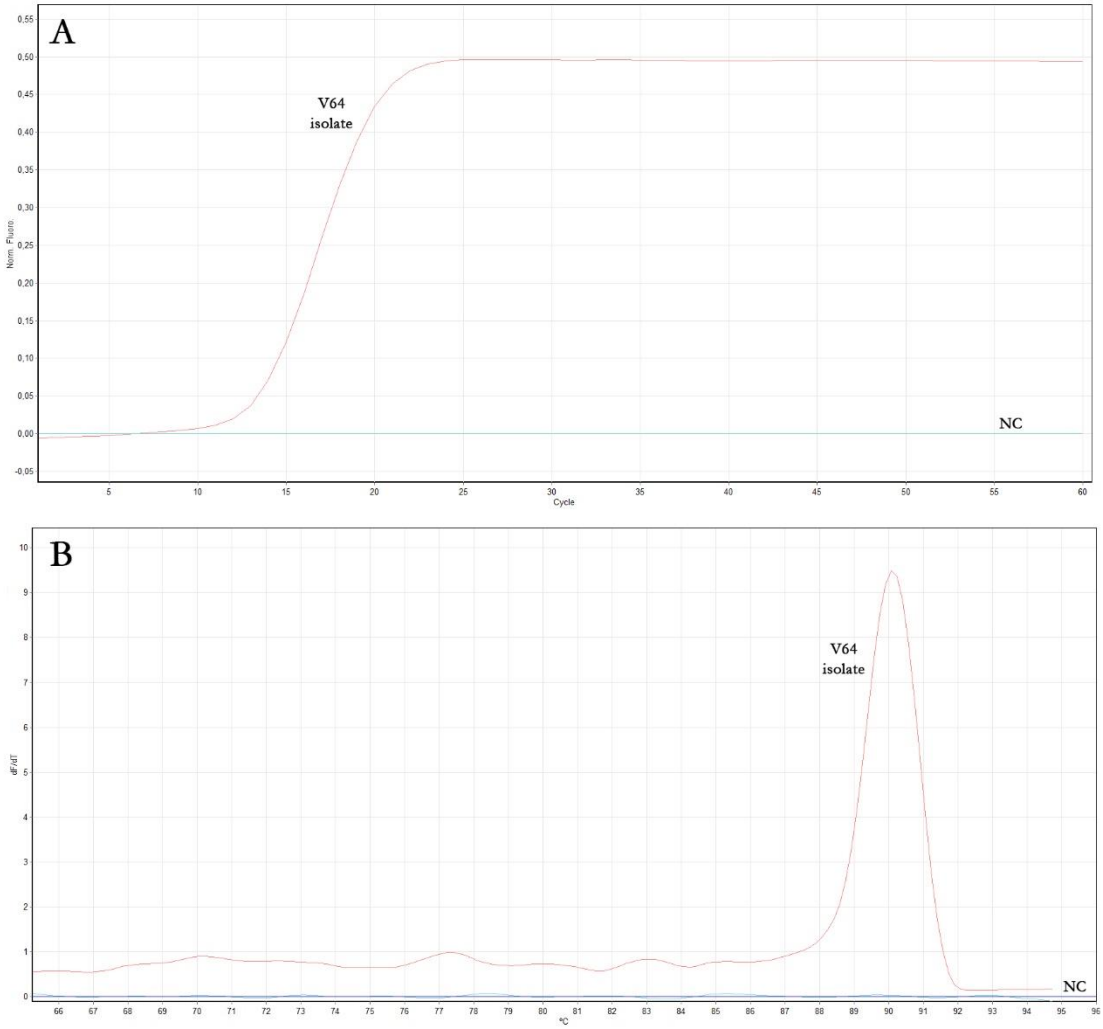


Figure 13 – Real-time RT-LAMP assay results for OLYaV detection. Amplification **(A)** and melting **(B)** curves of OLYaV V64 isolate. **NC**: Negative control.

6.3.4 Sensitivity and comparison of OLYaV real-time RT-LAMP assay to conventional RT-PCR

To ascertain the sensitivity and efficacy of the real-time RT-LAMP assay, a comparative experiment was conducted using as a template ten-fold serial dilutions of a purified recombinant plasmid, starting from a concentration of ≈50 ng/µL (1.34×10^{10} copies). The results obtained in the three replicates for each test returned completely overlapping results. The end-point RT-PCR was able to detect up to $\approx 50 \times 10^{-11}$ ng/µL (Figure 14), while DNA concentrations up to $\approx 50 \times 10^{-12}$ ng/µL were detected with the real-time LAMP assay developed in this work (Figure 15A).

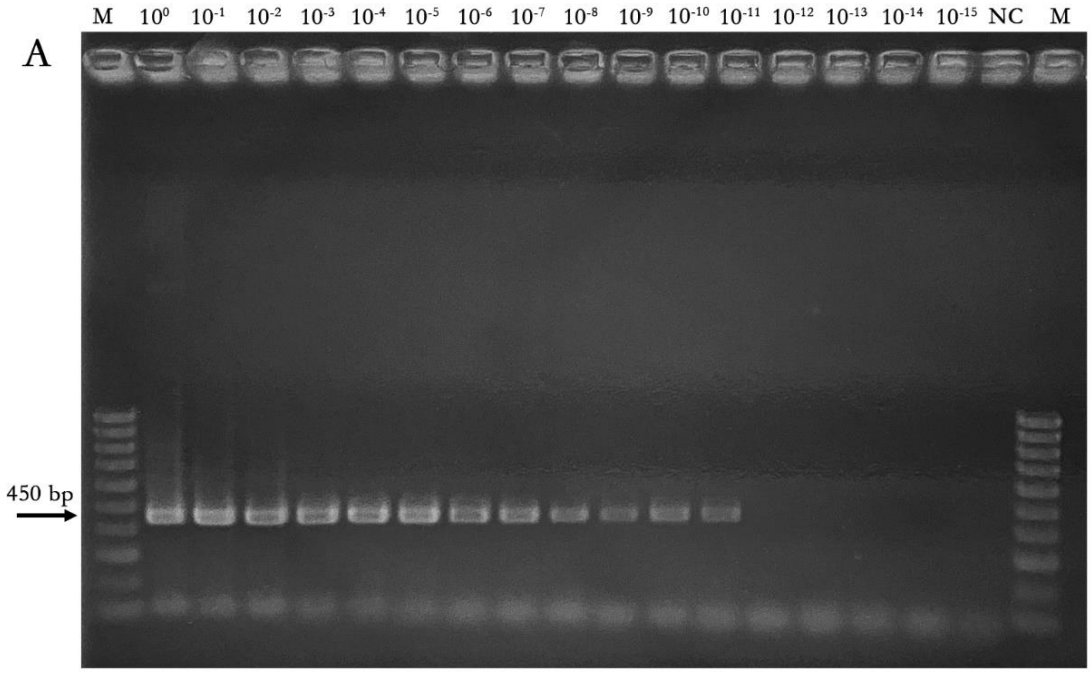


Figure 14 – 1.5% agarose gel electrophoresis of RT-PCR products for OLYaV detection using 10-fold serial dilutions. **M**: Bioline HyperLadder 100 bp. **NC**: negative control.

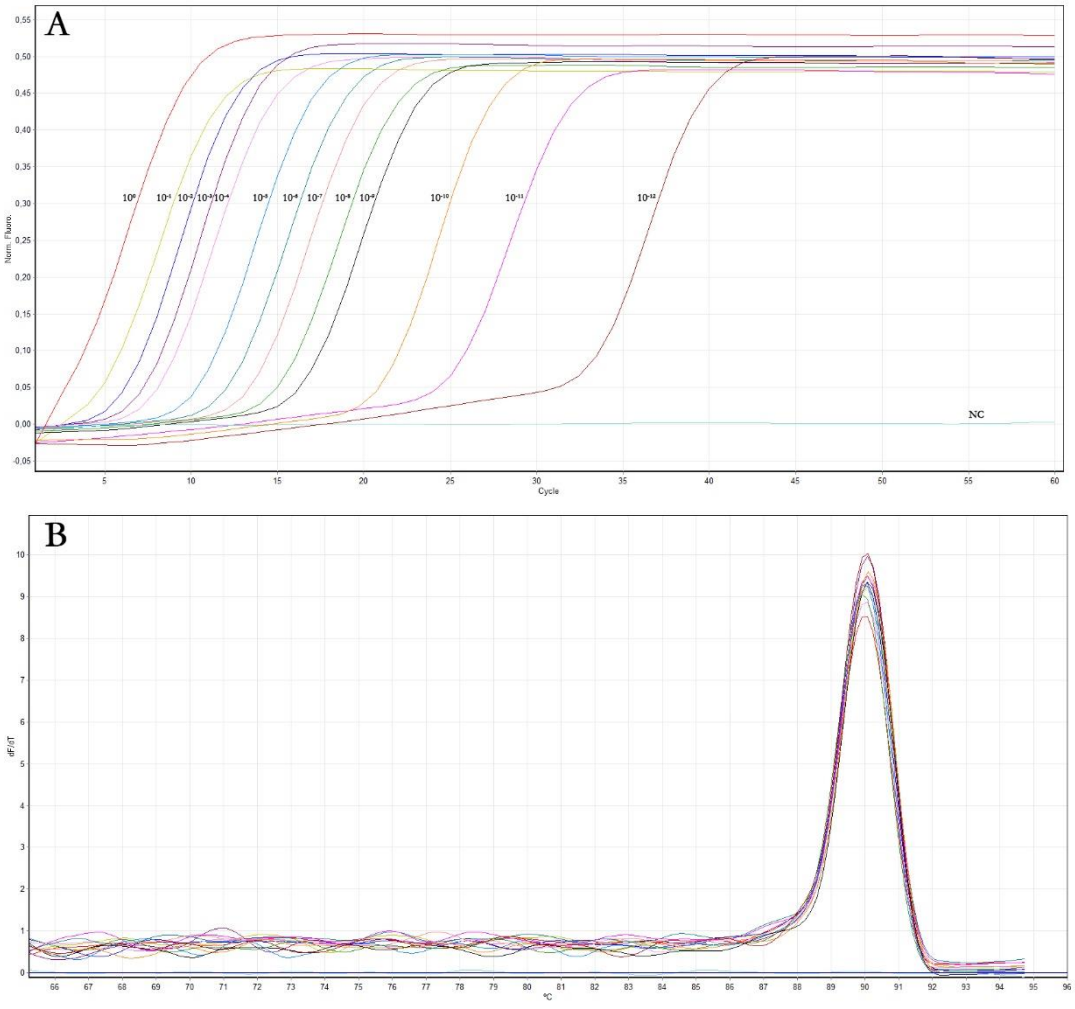


Figure 15 – Sensitivity of real-time RT-LAMP assays for OLYaV detection using 10-fold serial dilutions. **(A)**: Fluorescence of the 10-fold serial dilutions analyzed. Fluorescence increasing in positive sample curves (from ≈50 × 10^{-1} to ≈50 ×10^{-12} ng/µL) after 2 to 33 min **(B)**: Melting curves.

In detail, the LAMP assay was more sensitive and able to detect a total of 1.34 × 10^{-2} genome copies, whereas with the canonical RT-PCR, it was only possible to detect up to 1.34 × 10^{-1} genome copies. Moreover, even considering the lowest detectable concentration in real-time RT-LAMP (≈50 × 10^{-12} ng/µL), the results clearly showed that the time required to obtain reliable results was less than 45 min. The melting curves displayed the same peak temperature at 90 °C (Figure 15B), and the results of the RT-LAMP reaction time plateau were calculated as the mean values obtained from the three replicates (Table 24).

Table 24 – Comparison of real-time RT-LAMP and end-point RT-PCR assays sensitivity using 10-fold serial dilutions of a purified recombinant plasmid (SD: standard deviation).

Assay	**Starting DNA Concentration (50 ng/µL) (1.34×10^{10} copies)**															
	10^{0}	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**	**10^{-11}**	**10^{-12}**	**10^{-13}**	**10^{-14}**	**10^{-15}**
End-point RT-PCR	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
LAMP reaction time plateau (min) (mean values ± SD)	13 ± 0.1	13.9 ± 0.2	15.2 ± 0.2	16.8 ± 0.2	17.4 ± 0.1	20.1 ± 0.4	22 ± 0.2	24.4 ± 0.4	25.1 ± 0.3	26.3 ± 0.4	31.2 ± 0.4	35.1 ± 0.3	43.4 ± 0.4	-	-	-

6.3.5 Specificity of OLYaV real-time RT-LAMP assay

To evaluate the LAMP assay specificity and to assess non-specific cross-reactions, a real-time RT-LAMP assay, using the outgroup reported in Section 6.2.6, was performed. A reaction time value of 15 min and a single peak at ≈90 °C of the melting curve were obtained with the OLYaV V64 isolate RNA, while no amplification was obtained with the other viruses used as outgroups (Figure 16).

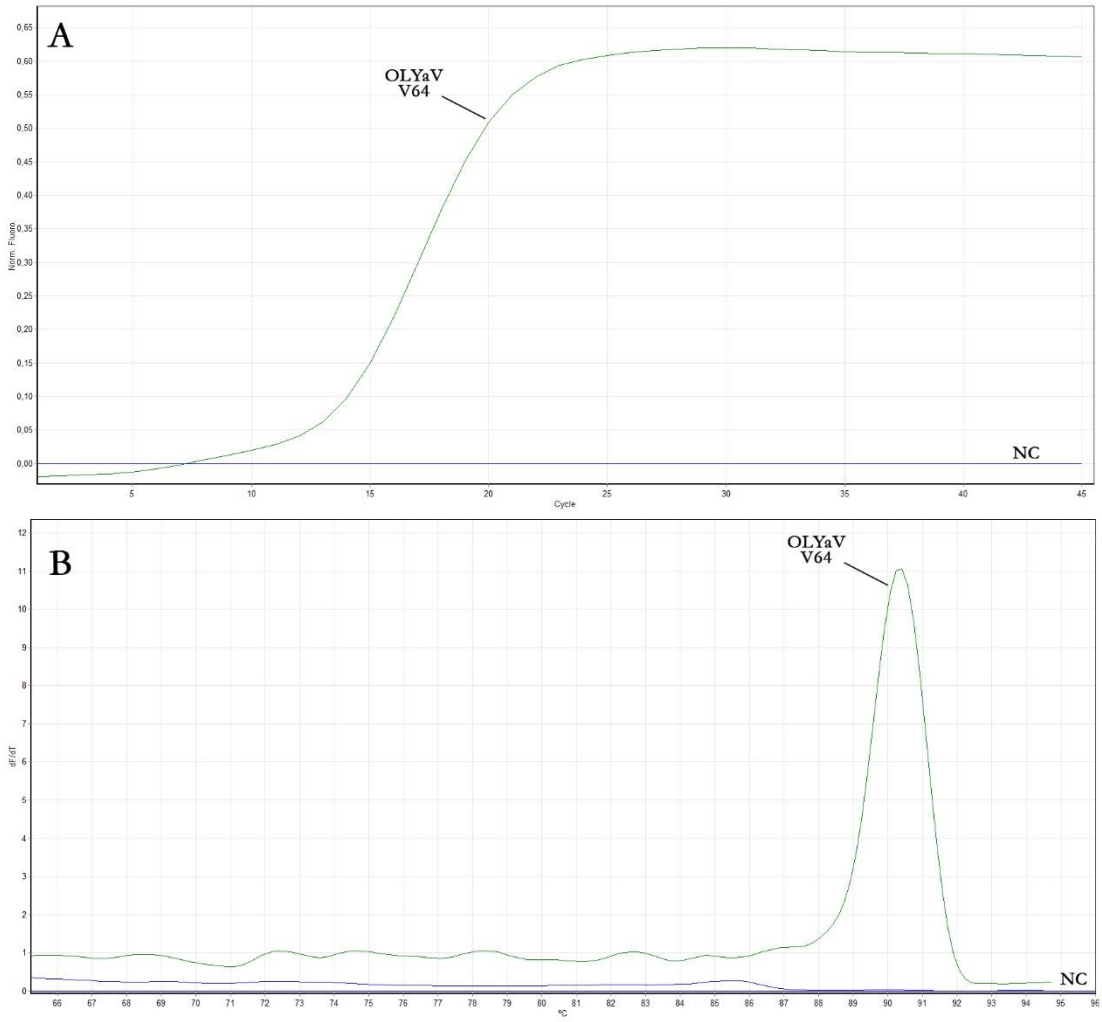


Figure 16 – Specificity of the real-time RT-LAMP assays for OLYaV. Amplification (A) and melting (B) curves of OLYaV V64 isolate, and viruses used as out-group. NC: negative control.

6.3.6 Evaluation of OLYaV incidence in Italy and Spain by real-time RT-LAMP assay on symptomatic and asymptomatic olive trees

To evaluate the OLYaV incidence in Italy and Spain and to confirm the validity of the real-time RT-LAMP assay developed, the analyses were performed on 61 symptomatic (Figure 14 and Figure 15A) and 139 asymptomatic (Figure 15B) field samples, for a total of 200 samples, collected from different olive production sites in Italy and Spain.

Figure 17 – Typical yellowing branches on olive tree infected by OLYaV.

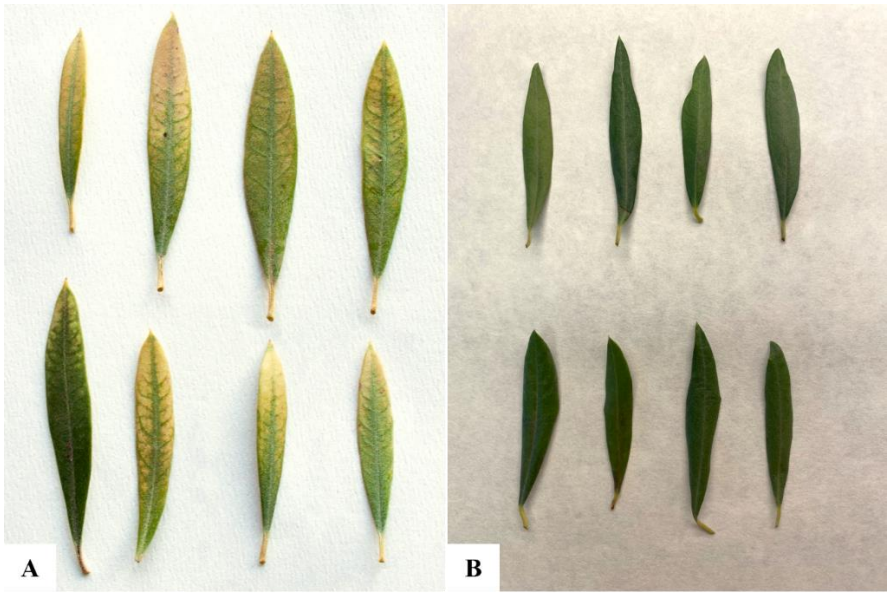


Figure 18 – Typical foliar symptoms caused by olive leaf yellowing associated virus on collected samples.

Seventy-three out of two hundred collected samples resulted positive for OLYaV by real-time RT-LAMP (Tables 25 and Table 26), with an incidence percentage of samples that resulted positive of 36.5%. Moreover, the obtained results showed an improvement in OLYaV-positive sample detection in asymptomatic samples. In Italy, out of 120 samples collected from the 6 different provinces investigated, 36 samples resulted positive for LAMP, with an infection percentage of 30%. In this case, the developed real-time RT-LAMP assay was also able to detect the OLYaV presence in 7 olive asymptomatic plants (Figure 18B). Considering the cultivars analyzed, a different OLYaV incidence was observed. The higher OLYaV incidence was observed in the "Ogliarola barese" cv collected from the Apulia region, with a total of 11 out of 20 samples analyzed, followed by "Giarraffa" (Sicily) and "Carolea" cvs (Calabria), with a total of 8 and 7 samples resulted positive, respectively. A lower incidence was observed in "Nocellara del Belice" (Sicily), "Frantoio" (Umbria), and "Leccino" (Latium) cvs, with a total of 5, 3, and 2 positive samples, respectively. Regarding the OLYaV incidence in Spain, 37 out of 80 samples resulted positive, with an infection percentage of 46.2%; in this case, 5 positive samples collected from asymptomatic plants resulted positive for OLYaV infection. The highest incidence was observed in Valencia (13 out of 20 samples) and Jaén provinces (11 out of 20 samples). Specifically, the following results were obtained for Spanish cultivars: 12 out of 20 samples of the "Serrana Espadán" and "Villalonga" cultivars, respectively, resulted positive for OLYaV, while a lower incidence was revealed in the "Picual" and "Arbequina" cultivars (8 and 5 positive samples out of 20, respectively). Finally, to confirm the RT-LAMP assay results, the melting curve analysis, carried out on asymptomatic samples too, showed the same peak temperature (≈90 °C) in all samples, concordant with the OLYaV-positive control, including those that were asymptomatic and positive by real-time RT-LAMP.

Table 25 – Real-time RT-LAMP assay results for OLYaV detection of symptomatic and asymptomatic field samples collected from different Spanish provinces.

SAMPLING AREA	ID SAMPLE	SYMPTOMATIC	REAL-TIME RT-LAMP	SAMPLING AREA	ID SAMPLE	SYMPTOMATIC	REAL-TIME RT-LAMP
Valencia province (Comunitat Valenciana)	VAL/01	+	+	Jaén province (Andalusia)	JAÉ/01	+	+
	VAL/02	+	+		JAÉ/02	-	-
	VAL/03	-	+		JAÉ/03	+	+
	VAL/04	-	+		JAÉ/04	-	-
	VAL/05	-	-		JAÉ/05	+	+
	VAL/06	-	-		JAÉ/06	+	+
	VAL/07	-	-		JAÉ/07	+	+
	VAL/08	-	-		JAÉ/08	+	+
	VAL/09	+	+		JAÉ/09	-	-
	VAL/10	-	-		JAÉ/10	-	-
	VAL/11	+	+		JAÉ/11	-	-
	VAL/12	-	-		JAÉ/12	-	-
	VAL/13	+	+		JAÉ/13	-	+
	VAL/14	+	+		JAÉ/14	-	-
	VAL/15	-	-		JAÉ/15	+	+
	VAL/16	+	+		JAÉ/16	-	-
	VAL/17	+	+		JAÉ/17	+	+
	VAL/18	+	+		JAÉ/18	+	+
	VAL/19	+	+		JAÉ/19	+	+
	VAL/20	+	+		JAÉ/20	-	-
Castellón province (Comunitat Valenciana)	CAS/01	-	-	Granada province (Andalusia)	GRA/01	-	-
	CAS/02	-	-		GRA/02	-	-
	CAS/03	+	+		GRA/03	-	-
	CAS/04	-	-		GRA/04	-	+
	CAS/05	+	+		GRA/05	-	-
	CAS/06	-	-		GRA/06	-	-
	CAS/07	-	-		GRA/07	-	-
	CAS/08	-	-		GRA/08	-	-
	CAS/09	+	+		GRA/09	-	-
	CAS/10	+	+		GRA/10	+	+
	CAS/11	-	-		GRA/11	-	-
	CAS/12	-	-		GRA/12	-	-
	CAS/13	-	-		GRA/13	+	+
	CAS/14	-	-		GRA/14	-	-
	CAS/15	+	+		GRA/15	-	-
	CAS/16	+	+		GRA/16	-	-
	CAS/17	-	-		GRA/17	-	-
	CAS/18	+	+		GRA/18	-	-
	CAS/19	-	-		GRA/19	-	+
	CAS/20	+	+		GRA/20	+	+
OLYaV positive control	PC	/	+				

Note: asymptomatic samples resulted positive are underlined in grey.

Table 26 – Real-time RT-LAMP assay results for OLYaV detection of symptomatic and asymptomatic field samples collected from different Italian provinces.

SAMPLING AREA	ID SAMPLE	SYMPTOMATIC	REAL-TIME RT-LAMP	SAMPLING AREA	ID SAMPLE	SYMPTOMATIC	REAL-TIME RT-LAMP
Agrigento province (Sicily)	AGR/01	-	-	Crotone province (Calabria)	CRO/01	-	-
	AGR/02	-	-		CRO/02	+	+
	AGR/03	+	+		CRO/03	-	-
	AGR/04	-	+		CRO/04	-	-
	AGR/05	-	-		CRO/05	-	-
	AGR/06	+	+		CRO/06	-	-
	AGR/07	-	-		CRO/07	-	-
	AGR/08	-	-		CRO/08	-	-
	AGR/09	+	+		CRO/09	-	-
	AGR/10	-	-		CRO/10	-	-
	AGR/11	-	-		CRO/11	+	+
	AGR/12	-	-		CRO/12	+	+
	AGR/13	+	+		CRO/13	-	-
	AGR/14	+	+		CRO/14	-	-
	AGR/15	-	-		CRO/15	-	-
	AGR/16	-	-		CRO/16	+	+
	AGR/17	+	+		CRO/17	-	-
	AGR/18	-	-		CRO/18	-	+
	AGR/19	+	+		CRO/19	+	+
	AGR/20	-	-		CRO/20	+	+
Trapani province (Sicily)	TRA/01	-	+	Taranto province (Apulia)	TAR/01	+	+
	TRA/02	-	-		TAR/02	-	+
	TRA/03	-	-		TAR/03	-	-
	TRA/04	-	-		TAR/04	-	-
	TRA/05	+	+		TAR/05	+	+
	TRA/06	-	-		TAR/06	-	-
	TRA/07	-	-		TAR/07	-	+
	TRA/08	-	-		TAR/08	+	+
	TRA/09	-	-		TAR/09	-	+
	TRA/10	-	-		TAR/10	-	-
	TRA/11	-	-		TAR/11	-	-
	TRA/12	-	-		TAR/12	-	-
	TRA/13	+	+		TAR/13	+	+
	TRA/14	-	-		TAR/14	-	-
	TRA/15	+	+		TAR/15	-	-
	TRA/16	+	+		TAR/16	+	+
	TRA/17	-	-		TAR/17	-	-
	TRA/18	-	-		TAR/18	+	+
	TRA/19	-	-		TAR/19	+	+
	TRA/20	-	-		TAR/20	+	+
Frosinone province (Latium)	FRO/01	-	-	Terni province (Umbria)	TER/01	-	-
	FRO/02	-	-		TER/02	-	-
	FRO/03	-	-		TER/03	-	-
	FRO/04	-	-		TER/04	+	+
	FRO/05	-	-		TER/05	-	-
	FRO/06	-	-		TER/06	-	-
	FRO/07	-	-		TER/07	-	-
	FRO/08	-	-		TER/08	-	-
	FRO/09	-	-		TER/09	-	-
	FRO/10	-	-		TER/10	-	-
	FRO/11	+	+		TER/11	-	-
	FRO/12	-	-		TER/12	-	-
	FRO/13	-	-		TER/13	-	-
	FRO/14	-	-		TER/14	-	-
	FRO/15	-	-		TER/15	-	+
	FRO/16	-	-		TER/16	-	-
	FRO/17	-	-		TER/17	+	+
	FRO/18	+	+		TER/18	-	-
	FRO/19	-	-		TER/19	-	-
	FRO/20	-	-		TER/20	-	-

Note: asymptomatic samples resulted positive are underlined in grey.

6.4 Conclusion

A real-time LAMP protocol was developed in order to OLYaV detection. The technique developed has demonstrated a 10-fold higher sensitivity compared to the canonical RT-PCR; in addition, even considering the lowest detectable concentration of 50×10^{-12} ng/µL, the time required to carry out the experiment was less than 45 min. OLYaV was detected in olive orchards independently of the cultivar, crop management, or geographical location (Italy or Spain), probably due to a gene flow that occurred in the past between these two countries, which led to a wide spread of this pathogen over time and a distributed presence in cultivars across the countries. In this sense, OLYaV may represent an issue that should not be underestimated, both in terms of phytosanitary certification of propagation material and for new plantings, particularly new intensive and super-intensive olive orchards. For this reason, it is essential to monitor the commercial cultivars to date most widespread in Italy and Spain, including molecular diagnostic methodologies as reliable and sensitive as possible. The developed real-time RT-LAMP could represent an alternative to the methods currently used for routine OLYaV detection and plant material certification programs, thus representing a potential contribution to improving virus diagnosis. In addition, this work represents the first OLYaV incidence study in the two most important European areas for olive production. The incidence analysis showed that the presence of OLYaV was higher in the Spanish territory than in the Italian territory and that the developed assay was able to detect OLYaV in asymptomatic olive plants.

CHAPTER VII

CASE STUDY:
OLIVE LEAF YELLOWING-ASSOCIATED VIRUS (OLYaV)
OLYaV dispersion assesment in Sicily using a real-time RT-LAMP

The study of this chapter was presented in the following contribution:
"Olive leaf yellowing-associated virus dispersion assessment in Sicily using a real-time RT-LAMP."
A. G. Caruso, **S. Bertacca**, A. Ragona, G. Agrò, S. Davino, S. Panno.
In: Proceedings of XXVIII National Congress Italian Phytopathological Society (SIPaV)–
18-20 settembre 2023, Università degli Studi di Napoli Federico II.

CHAPTER VII – OLYAV DISPERSION ASSESSMENT IN SICILY USING A REAL-TIME RT-LAMP

7.1 Introduction

The Italian olive nursery industry operates within a regulatory framework that is characterised by stringent quality and phytosanitary standards. These standards are delineated in Italian Decrees that govern mandatory and voluntary certification programs for plant propagating material. However, despite regulatory efforts, gaining a comprehensive understanding of the distribution and impact of olive tree viruses requires a deeper exploration of their epidemiology. Olive leaf yellowing associated virus (OLYaV) is a significant concern within the olive industry due to its pervasive prevalence, not only in Italy but also in olive-growing regions worldwide. The severity of the threat to olive cultivation posed by OLYaV is highlighted by the current European legislation mandating the absence of the virus in propagation material. This underscores the urgent need for enhanced epidemiological studies.

In particular, Southern Italy, including Sicily region, faces significant challenges due to the widespread presence of OLYaV, which poses a threat to local olive cultivators. Despite efforts to conduct sanitary selections and procure virus-free cultivars, OLYaV persists and continues to undermine optimal productivity and quality in the olive sector.

Therefore, it is essential to undertake studies to elucidate the epidemiology of OLYaV and other olive tree viruses in order to develop evidence-based interventions to ensure the long-term viability and resilience of the olive industry.

In light of these considerations, the present study aims to address this knowledge gap by conducting a comprehensive survey of different olive production sites in Sicily. This research objective is to provide valuable insights by assessing the distribution of OLYaV in different olive producing sites in Sicily and its potential impact on the olive industry in the region, which can guide proactive measures to mitigate the negative effects of viral infections and promote the sustainable growth of olive production in Sicily.

7.2 Material and methods

7.2.1 Field survey and collected material

During the spring season of 2023, field surveys were conducted in the main olive-growing areas in Sicily. In detail, four Sicilian provinces (Agrigento, Trapani, Palermo and Messina) were sampled (Figure 19).

Figure 19 – Sicilian provinces surveyed for OLYaV spread assessment.

A total of 800 symptomatic/asymptomatic olive trees, belonging to "Giarraffa" and "Nocellara del Belice" cultivars, were sampled. Specifically, 100 plants of each cultivar were sampled for each province. Each sample consisted of 4-8 cuttings, 10 cm long, collected from one-year-old twigs, in accordance with Gottwald and Hughes (2000) with minor adaptation to olive plants. The Planthology mobile application (Davino et al., 2017) was used to geo-reference all collected samples. All samples were labelled and stored in plastic bags at 4°C. Subsequently, the samples were then immediately transported to the "Bruno Rosciglione" plant virology laboratory of the SAAF department, stored at -20 °C and processed within the next 24 h.

7.2.2 Sample preparation

Total RNA was extracted from field-collected olive samples using the NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH & Co., Dueren, Germany), according to the protocol provide. The eluted RNA was resuspended in 60 µL RNase-free water; following two measurements with a UV–Vis NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), samples were adjusted to approximately 50 ng/µL and maintained short term at -20 °C or long term at -80 °C until molecular analysis.

7.2.3 OLYaV dispersion assessment by real-time RT-LAMP

To evaluate OLYaV incidence, real-time RT-LAMP tests were carried out employing the primer set designed and described in Chapter VI (Figure 20). Briefly, the real-time RT-LAMP assay was performed in a volume of 25 µL, containing 0.2 µM each of OLYaV-F3 and OLYaV-B3, 1.6 µM each of OLYaV-FIP and OLYaV-BIP, and 0.4 µM each of OLYaV-LoopF and OLYaV-LoopB, 15 µL of LAMP Isothermal Master Mix (Optigene® Limited, West Sussex, UK), 1 µL of RNA (≈50 ng/µL), and nuclease-free water to reach the final volume. For each analysis, a positive control and a healthy olive plant as negative control were used. The real-time LAMP assays were performed at 65 °C for 60 min (fluorescence acquisition every 60 s), using a Rotor-Gene Q2plex HRM Platform Thermal Cycler (Qiagen, Hilden, Germany) and the fluorescence data were obtained in the 6-carboxyfluorescein (FAM) channel (450–495 nm excitation and 510–527 nm detection). The relative fluorescence units (RFU) threshold value was used, and the threshold time (Tt) was calculated as the time at which fluorescence was equal to the threshold value.

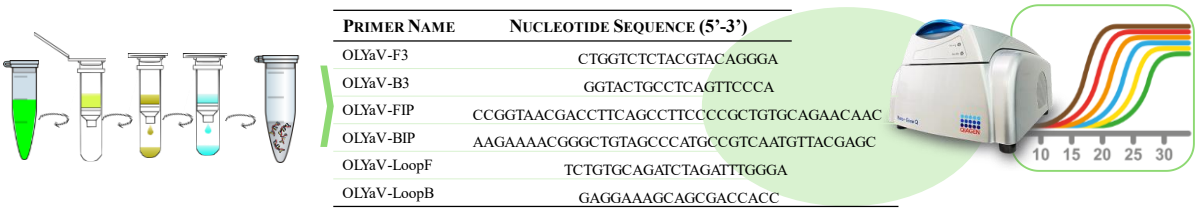


Figure 20 – Diagram for OLYaV dispersion assessment using real-time RT-LAMP.

7.3 Results

7.3.1 OLYaV dispersion assessment by real-time RT-LAMP

Out of the 800 olive samples analyzed, 110 samples of "Giarraffa" cultivar and 89 samples of the "Nocellara del Belice" cvs. resulted positive to OLYaV. This resulted in incidence rates of 27.5% and 22.25% for the "Giarraffa" and "Nocellara del Belice" cultivars, respectively, highlighting the significant impact of OLYaV on olive trees. Moreover, eight and five samples collected from asymptomatic olive plants tested positive to OLYaV. Yellow complex symptoms were observed in all remaining samples (Figure 21).

Figure 21 – Yellow complex symptoms (left) observed on olive samples tested positive to OLYaV.

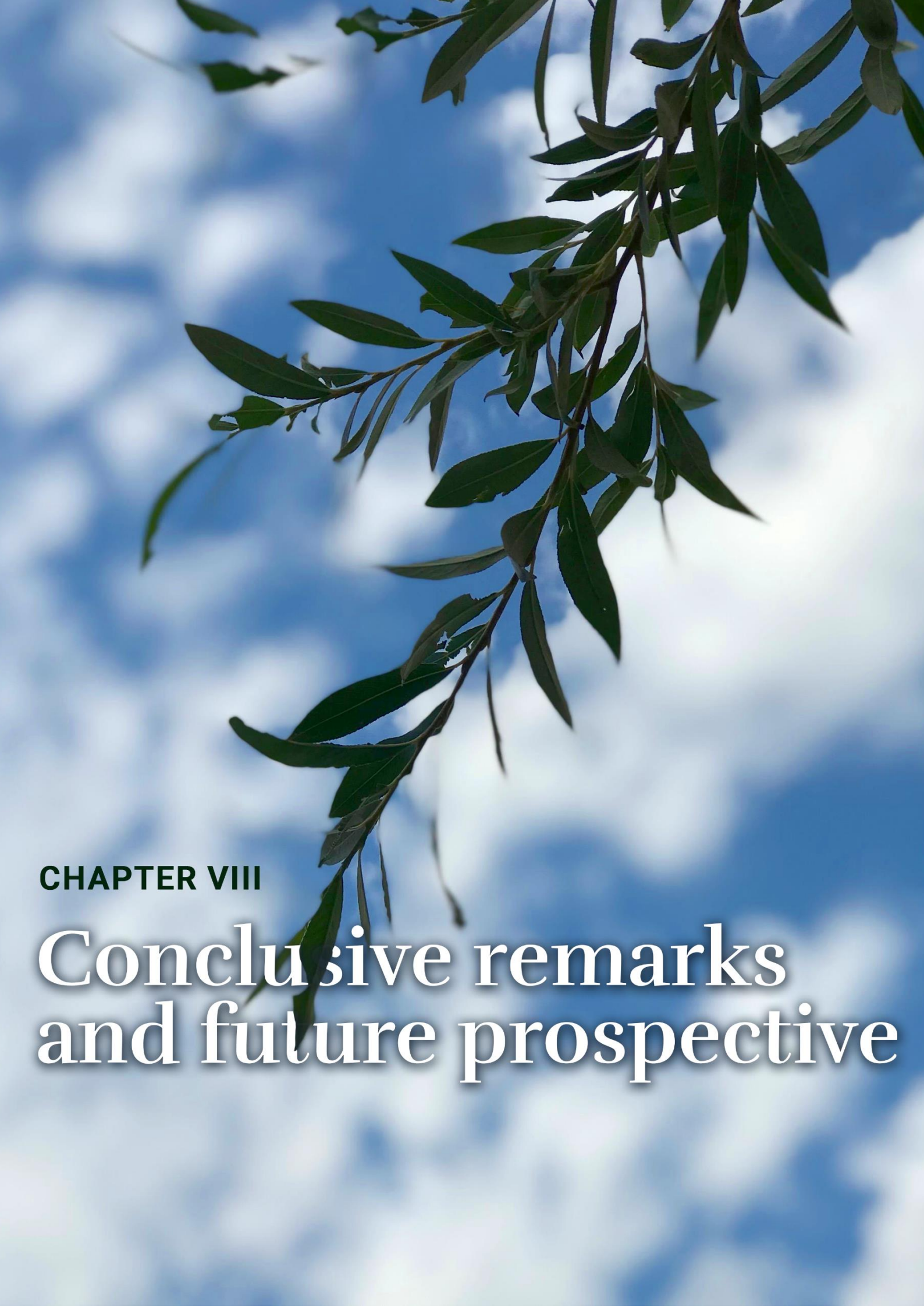
Regarding "Giarraffa" cv, the higher incidence was recorded in Trapani province (34%), followed by Messina, Agrigento and Palermo provinces. Similarly, for the "Nocellara del Belice" cultivar, the highest incidence of 33% was observed in Trapani province, followed by Agrigento, Palermo, and Messina provinces (Table 27).

Table 27 – Real-time RT-LAMP assay results for OLYaV detection of field samples collected from different Sicilian provinces.

CULTIVAR	NO. POSITIVE PLANTS/TESTED PLANTS					
	TRAPANI	AGRIGENTO	MESSINA	PALERMO	TOTAL	PERCENTAGE
Giarraffa	34/100	26/100	29/100	21/100	110/400	27.50 %
Nocellara del Belice	33/100	20/100	17/100	19/100	89/400	22.25 %

7.4 Conclusion

Based on the comprehensive methodology outlined in this study, significant data were collected on the distribution of Olive leaf yellowing-associated virus (OLYaV) in Sicilian olive growing areas. The field surveys conducted across four key provinces in Sicily during the spring season of 2023 provided a robust sampling framework, encompassing 800 symptomatic and asymptomatic olive trees of the "Giarraffa" and "Nocellara del Belice" cultivars. The findings reveal a concerning prevalence of OLYaV within the sampled olive trees, with 27.50 % and 22.25 % incidence rates detected in the "Giarraffa" and "Nocellara del Belice" cultivars, respectively. Notably, the detection of positive samples from asymptomatic olive plants underscores the potential silent spread of the virus, posing significant challenges for disease management and control efforts. Geographically, the distribution of OLYaV incidence rates shows distinct distribution patterns, with the province of Trapani reporting the highest prevalence for both cultivars. This spatial variation highlights the influence of local factors on virus spread and underlines the importance of localised management strategies. In conclusion, this study provides valuable insights into the dispersion of OLYaV in Sicilian olive-growing regions, emphasizing the need for proactive surveillance and targeted intervention strategies to mitigate the impact of this viral pathogen on olive cultivation. The methodology employed in this study provides a strong scientific basis for future investigations to understand and manage OLYaV viral diseases in olive crops.



CHAPTER VIII

Conclusive remarks and future prospective

CHAPTER VIII – CONCLUSIVE REMARKS AND FUTURE PROSPECTIVE

The agricultural sector globally faces persistent threats posed by a series of devastating epidemics instigated by emerging viral pathogens. Although climatic variations and agronomic practices are often linked to the emergence and spread of these pathogens, it is important to note that the underlying dynamics are complex and are the result of evolutionary processes. This process involves important factors such as changes in ecological parameters, the ability of viruses to adapt to new environments and hosts, the complex elements involved in viral replication, and a significant stochastic component. These variables are compounded by anthropogenic factors, notably intense commercial trade, inadequate phytosanitary controls, and unregulated movement of plant material, serving as potential inoculum reservoirs. Viruses, characterized by their rapid evolution through mutation events, genetic recombination, and geographical migration, pose a formidable challenge to agricultural sustainability. Timely identification of new pathogens and a nuanced understanding of their genetic and dispersion dynamics are critical for effective risk assessment and intervention. Therefore, the development of rapid diagnostic tools is imperative to facilitate timely detection of viral infections in agricultural settings.

In the olive cultivation context, viral diseases spreading in orchards endanger both tree health and the economic viability of production. Additionally, these diseases can impact the broader landscape and socio-cultural aspects of olive-growing regions. Beyond economic concerns, such as reduced yields, they can alter the visual appeal of groves and disrupt longstanding cultural practices tied to olive cultivation. Therefore, addressing viral diseases requires a comprehensive approach that considers agricultural, economic, and socio-cultural dimensions. Moreover, the intricate dynamics of virus dispersion within olive orchards, influenced by factors such as insect vectors and infected propagation materials, underscore the need for an integrated approach to disease management. These challenges underline the imperative nature of implementing robust sanitary selection and sanitation practices.

The present PhD thesis has undertaken a comprehensive exploration of the dynamics of a recently identified olive plant virus, alongside an investigation into the dispersal and transmission patterns of two viral agents affecting olive trees. Specifically, this research has focused on olea europaea geminivirus (OEGV) and olive leaf yellowing-associated virus (OLYaV), classified within the *Geminivirus* and *Closterovirus* genera, respectively.

In this work, the overarching objectives were achieved and user-friendly, highly specific, and rapid real-time LAMP diagnostic protocols for both OEGV and OLYaV were developed, while simultaneously investigating their spread within the Mediterranean ecosystem. Our findings could furnish a significant contribution to the study of olive virology and provide valuable information to formulate effective strategies in reducing the risks associated to the spread of these viral agents in olive cultivation.

The application of innovative diagnostic techniques, such as the Oxford Nanopore Technologies (ONT) platform with MinION device, holds significant promise in the detection of olive viral pathogens. The application of this platform has emerged as a reliable tool for identifying viral pathogens, offering real-time and portable capabilities, demonstrating its potential for genomic studies of known and unknown viruses. While its application in plant virology remains relatively underexplored, recent studies evaluating its potential in detecting olive plant pathogens have shown promising results. The ONT platform, with its ability to conduct massive parallel sequencing, offers a valuable avenue for the rapid detection and characterization of viruses that affect olive trees. This platform offers immense potential for genomic investigations of both known and novel viruses. Remarkably, the ONT platform successfully detected olive plant viruses, including OLYaV, OEGV, and OLV-3, thereby contributing to the expansion of understanding of plant virology. The presence of OLYaV and OEGV was confirmed through end-point RT-PCR, validating the results obtained through nanopore sequencing. However, OLV-3 was exclusively detected through nanopore sequencing, as attempts to detect it using end-point RT-PCR were unsuccessful.

Furthermore, the development of rapid real-time LAMP diagnostic protocol for OEGV detection, has provided valuable tools for this viral agent detection. This assay, with its high specificity, stability, and sensitivity, offers a practical solution for efficient detection even in field settings, particularly when coupled with rapid sample extraction methods. Additionally, the successful transmission of OEGV via grafting underscores the need for stringent screening methods and cultivar selection to mitigate the risk of virus transmission, although further and more wide-ranging studies need to be carried out.

Similarly, the development of a real-time RT-LAMP protocol for OLYaV detection highlights its potential as an alternative method for routine virus detection and certification programs. Moreover, the study's findings highlight the widespread presence of OLYaV in olive orchards across Italy and Spain regions, underscoring the need for enhanced monitoring and disease management strategies. Particularly, the incidence and distribution

studies in the main Sicilian olive-growing provinces have provided insights into the prevalence and spread of OLYaV. Geospatial analysis revealed distinct distribution patterns influenced by local factors, emphasizing the importance of localized management strategies. Specifically, the detection of OLYaV in both symptomatic and asymptomatic olive trees highlight the silent spread of the virus, necessitating imperative intervention strategies.

In conclusion, this doctoral thesis contributes to the advancement of knowledge of plant virology and provides valuable insights for the development of effective strategies to mitigate the risks associated with the spread of olive pathogens. The integration of scientific understanding, diagnostic innovation, and strategic agricultural management practices lays the groundwork for sustainable approaches to protect olive production from these viral threats.

Future research efforts are essential to further elucidate transmission dynamics, explore varietal susceptibilities, and develop comprehensive disease management practices to safeguard olive cultivation worldwide. In particular, future research in olive virology should prioritize several key areas to address the ongoing challenges. Firstly, continuous surveillance efforts are essential to monitor the prevalence and spread of known and emerging viral pathogens in olive orchards. Establishing robust monitoring networks will require collaboration among researchers, agricultural authorities and olive growers. Moreover, advancements in diagnostic technologies and the exploration of novel diagnostic platforms are expected to bolster the ability to detect viral infections swiftly and precisely in field conditions. This enhanced capability is deemed essential for the deployment of effective disease management strategies. Finally, research should focus on developing sustainable disease management practices, including the use of resistant olive cultivars, sustainable control methods, and integrated pest management strategies. The future of the olive sector and its resilience in the face of new and emerging viral threats can be secured if these challenges are addressed and innovative approaches are adopted.

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1. Caruso A. G., Tortorici S., Davino S., **Bertacca S.**, Ragona A., Verde G. L., Biondi A., Noris, E., Rizzo R. & Panno S. (2024). The invasive tomato pest *Tuta absoluta* can transmit the emergent tomato brown rugose fruit virus. *Entomologia Generalis.* ….

2. Panno S., Ragona A., **Bertacca S.**, Agrò G., Yahyaoui E., Dimauro B., Caruso A. G. & Davino S. (2024). Outbreak of tomato fruit blotch virus in the most relevant tomato greenhouse production area of Sicily. *Journal Of Plant Pathology*, 1-1. DOI: https://doi.org/10.1007/s42161-024-01623-1

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ABSTRACT O PROCEEDING IN CONGRESS

1. Olive leaf yellowing associated virus dispersion assessment in Sicily using a real-time RT-LAMP. Caruso A. G., **Bertacca S.**, Ragona A., Agrò G., Davino S., Panno S. In: Proceedings of *XXVIII National Congress Italian Phytopathological Society* – Università degli Studi di Napoli Federico II, dal 18/09/2023 al 20/09/2023.

2. Tomato leaf curl New Delhi virus in field dispersion analysis in Sicily (Italy) using ready-to-use LAMP detection kit. Panno S., Ragona A., Agrò G., **Bertacca S.**, Davino S., Caruso A. G. In: Proceedings of *XXVIII National Congress Italian Phytopathological Society* – Università degli Studi di Napoli Federico II, dal 18/09/2023 al 20/09/2023.

3. Efficacy evaluation of different seed disinfection chemical treatments against Tomato brown rugose fruit virus. Caruso A.G., **Bertacca S.**, Ragona A., Agrò G., Davino S., Panno S. In: Proceedings of *XXVIII National Congress Italian Phytopathological Society* – Università degli Studi di Napoli Federico II, dal 18/09/2023 al 20/09/2023.

4. The tomato brown rugose fruit virus can be vectored by *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). A. Ragona, A. G. Caruso, S. Tortorici, **S. Bertacca**, S. Panno, R. Rizzo, G. Lo Verde, A. Biondi, S. Davino. In: Proceedings of *XXVIII National Congress Italian Phytopathological Society* – Università degli Studi di Napoli Federico II, dal 18/09/2023 al 20/09/2023.

5. *Tuta absoluta* as vector of the emerging tomato brown rugose fruit virus". Rizzo R., Tortorici S., Davino S., **Bertacca S.**, Ragona A., Lo Verde G., Caruso A. G., Biondi A., Panno S. In: Proceedings of *XXVVII Congresso Nazionale Italiano di Entomologia* – Università degli Studi di Palermo, dal 12/06/2023 al 16/06/2023.

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7. Genetic structure, molecular variability and spread of Grapevine leafroll-associated virus 1 and 3 in Sicilian autochthonous grapevine cultivars. Caruso A.G., Davino S., Bertacca S., Ragona A., Bova N., Mirabile G., Torta L., Bella P., Matic S., Panno S. In: Proceedings of *XXVII National Congress Italian Phytopathological Society* – Università degli Studi di Palermo, dal 21/09/2022 al 23/09/2022.

8. Insight into Grapevine Virus A spread in Sicily: epidemiological and evolutionary analysis. Caruso A.G., Panno S., **Bertacca S.**, Ragona A., Bova N., Mirabile G., Torta L., Bella P., Matic S., Davino S. In: Proceedings of *XXVII National Congress Italian Phytopathological Society* – Università degli Studi di Palermo, dal 21/09/2022 al 23/09/2022.

9. Survey of main grapevine cultivars for Grapevine fanleaf virus dispersion in Sicily. Caruso A.G., Panno S., **Bertacca S.**, Ragona A., Bova N., Mirabile G., Torta L., Bella P., Matic S., Davino S. In: Proceedings of *XXVII National Congress Italian Phytopathological Society* – Università degli Studi di Palermo, dal 21/09/2022 al 23/09/2022.

10. Exploiting the seed associated endophytes in *Brassica oleracea* genotypes as a potential source for plant growth promoting bacteria and biological control agents. Bova N., Conti S., Miceli A., Moncada A., Vetrano F., **Bertacca S.**, Caruso A.G., Panno S., Davino S., Mirabile G., Torta L., Catara V., Bella P. In: Proceedings of *XXVII National Congress Italian Phytopathological Society* – Università degli Studi di Palermo, dal 21/09/2022 al 23/09/2022.

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12. Fungi associated with a trunk disease in young grapevine plants in Sicily (Italy)". Mirabile G., Agnello A., Bella P., Bova N., **Bertacca S.**, Caruso A.G., Panno S., Davino S., Torta L. In: Proceedings of *XXVII National Congress Italian Phytopathological Society* – Università degli Studi di Palermo, dal 21/09/2022 al 23/09/2022.

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14. *Pleurotus ostreatus* associated with white rot decay in a monumental specimen of *Ficus macrophylla* subsp. *columnaris* in Palermo. Mirabile G., La Mantia G., Bella P., Bova N., **Bertacca S.**, Caruso A.G., Panno S., Davino S., Torta L. In: Proceedings of *XXVII National Congress Italian Phytopathological Society* – Università degli Studi di Palermo, dal 21/09/2022 al 23/09/2022.

ORAL PRESENTATION IN CONGRESS

1. Transmission evidence by Aculops lycopersici (Acari: Eriophyidae) of Tomato fruit blotch virus (ToFBV). **Bertacca S.**, Sanahuja E., Alfaro-Fernández A., Panno S., Davino S., Font-San-Ambrosio M. I. In: Proceedings of *XXVIII National Congress Italian Phytopathological Society* – Università degli Studi di Napoli Federico II, 18-20 settembre 2023.

2. Spread of olea europaea geminivirus (OEGV) in olive trees in Sicily. **Bertacca S.**, Caruso A.G., Matic S., Noris E., Panno S., Davino S. In: Proceedings of *XXVII National Congress Italian Phytopathological Society* – Università degli Studi di Palermo, 21-23 settembre 2022.

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