Rose viruses: Understanding the current status and protecting the future of the UK rose sector

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Thesis submitted for the degree of Doctor of Philosophy

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May 2021

Abstract

The rose (*Rosa* spp.) is the national flower of England and one of the most valued ornamental flowering shrubs grown around the globe. Despite the spread of rose viruses and their importance in rose cultivation, they have not been studied in detail in the United Kingdom (UK) since the 1980s. Molecular methods have evolved since then, and as a result they have rarely been deployed to study these viruses. In the UK many viruses have been reported previously infecting roses such as arabis mosaic virus (genus *Nepovirus*) and strawberry latent ringspot virus (family *Secoviridae*). However, numerous viruses have been identified infecting roses in recent years, especially with the application of high-throughput sequencing (HTS). Diagnosis is fundamental to facilitate the management of plant diseases, and early detection is essential for successful biosecurity campaigns, for example against rose rosette virus (RRV; genus *Emaravirus*), which is devastating roses in the USA and Canada, and was recently discovered in India (2017). In this project, different molecular (PCR, qPCR and HTS) and serological methods (ELISA) have been used to understand the baseline of viruses present in roses in the UK. Detailed experiments were performed to compare various targeted and non-targeted methods, including two different pipelines for HTS data analysis, Angua and EDNA. RT-qPCR showed the highest sensitivity for the detection of known viruses, whereas ELISA was identified as the technique with the lowest sensitivity. The Angua and the EDNA pipelines showed non-significant differences in sensitivity from RT-qPCR except in the detection of viral agents with lower titre. The advantages of HTS as a potential future front-line diagnostic tool are described. The performance of this study has allowed the estimation of the prevalence of some previously reported viruses in the UK but also the identification of *Rosa* spp. as a new host for viruses that are widespread in the country. This work resulted in three first virus records in the UK (rose cryptic virus-1, rose spring dwarf virus, and sweetbriar rose curly top virus), and the discovery of a new virus species (rosa ilarvirus-1; genus *Ilarvirus*). Furthermore, this research has proven a connection between the movement of plant pathogens and the trade in roses as cut flowers.

To my aunts Rosy (Rosa) and Macu

A mis tías Rosy (Rosa) y Macu

Acknowledgements

I would like to express my sincere thanks to DEFRA and the Royal Horticultural Society, funding bodies of this project, and to Fera Science Ltd., all of which have made this work possible.

I would like to deeply thank my supervisors Professor Neil Boonham, Dr Adrian Fox, Dr Gerard Clover, and Dr Rebekah Robinson for all their help, support and hard work. Thank you for being such an amazing team, for guiding me, for the discussions, and for being consummate professionals. I admire you all and I feel extremely lucky to have had the opportunity to work with all of you. Thanks for teaching me about more than just plant health. Thanks for showing me the best motto: 'If you want to go fast, go alone. If you want to go far, go together'.

I would like to thank Dr Ian Adams and Sam McGreig for being involved in the project and for being constantly available to help me during all these years. Thank you to all my PhD colleagues and all the great people I have met during these years for sharing this experience with me. Especially thank you to Professor Francisco Ochoa-Corona, Dr Liz Peña and Dr Andrés Espindola for making me feel part of Virus Chasers, opening your home to me and sharing your knowledge, muchas gracias. I would like to thank my 'new' work buddies for their support and help in my old and new adventure.

I would like to thank my family and friends for being always there to support me. Thank you to my second family: Hollie, Ben, Mathieu, Aimee, Alex, Clara, Garbi, Bea, Diego… Living in another country can be difficult at times, but you have made my days incredible and I have felt part of a family all this time. Thanks a lot for the good moments and laughs, of which there are many. Thank you to my friends from Uni, mis Calostrochos, for all their support ¡os echo de menos! Thank you to my life-long friends Elena, Sabela, Inés. Thanks for being always there ¡os quiero!

I would like to thank my aunt Macu for introducing me to Science since I was a toddler. I admire you and love you. I would also like to thank my aunt Fany for always being there, ¡te quiero! Mamá, Papá, Nico, thank you so much for all your encouragement and support during these years, and for helping me become the person I am. ¡Os echo de menos cada día, os quiero!

Finally, thank you Matías for being part of this adventure from the beginning. We came into the unknown and here we are. Thank you for your help, support and for being an amazing partner and best friend during all these years ¡te quiero!

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Main Thesis Chapters

Abbreviations

Apple mosaic virus	ApMV	**Rosa ilarvirus-1**	RIV-1
Arabis mosaic virus	ArMV	**Rose cryptic virus 1**	RoCV1
Asparagus virus 2	AV2	**Rose mosaic disease**	RMD
Blueberry shock virus	BlShV	**Rose rosette virus**	RRV
Citrus leaf rugose virus	CLRV	**Spinach latent virus**	SLV
Citrus variegation virus	CVV	**Strawberry latent ringspot virus**	SLRSV
Cucumber mosaic virus	CMV	**Sweetbriar rose curly top virus**	SRCTV
Cytochrome c oxidase	COX	**Tobacco rattle virus**	TRV
Elm mottle virus	EMoV	**Tobacco streak virus**	TSV
High throughput sequencing	HTS	**Tobacco ringspot virus**	ToRSV
Impatiens necrotic spot virus	INSV	**Tomato necrotic streak virus**	TomNSV
Lilac ring mottle virus.	LRMV	**Tomato ringspot virus**	TRSV
Prunus necrotic ringspot virus	PNRSV	**Tomato spotted wilt virus**	TSWV
Raspberry ringspot virus	RpRSV	**Tulare apple mosaic virus**	TAMV

Chapter 1 Introduction to the Thesis

Roses (*Rosa* spp.) are one of the most important ornamental flowering shrubs grown worldwide, noted for their beauty and scent, their desirable aesthetics, landscaping and industrial products (Dobhal *et al.*, 2016). Repeat flowering varieties were introduced into Europe from China in the 18th century (Joyaux, 2003) transforming the concept of roses, showing a broader range of colours, growth types, flower sizes, and fragrances. From that time, extensive rose breeding has taken place across the world, creating a massive industry (Debener and Byrne, 2014). Cultivation of roses is economically important around the globe. World rose production was estimated to be valued at 24 billion euros in 2008 (Heinrichs, 2008). The estimated annual production of cut flowers is around 18 billion, 60-80 million potted plants (miniature roses and bare-root grafting plants), and 220 million plants for landscaping (Blom and Tsujita, 2003; Pemberton *et al*., 2003; Roberts *et al*., 2003).

Roses are susceptible to numerous diseases (including exotics that could enter via trade) which require management, increasing the cost of production. They are vulnerable to infections by bacteria, fungi, viruses, nematodes, and phytoplasmas, causing leaf and flower mosaics, distortion, spotting, discolouration, necrosis, reduced growth or death of the plant. In the United Kingdom (UK), the garden industry contributes £9 billion to the economy every year. Defra (Department for Environment, Food & Rural Affairs) valued general ornamental plant production at £1.1 billion in 2015, pointing out that diseases caused losses of £630 million annually in the UK to ornamental plant production, of which £40 million was due specifically to viral diseases (Department for Environment Food and Rural Affairs, 2016).

The discovery of tobacco mosaic virus as an infectious agent initiated the study of virology as a subject at the end of the 19th century (Creager *et al.*, 1999). Since then, a wide variety of viruses have been identified infecting plants, animals, fungi and bacteria. Most crops and ornamentals are under the threat of different plant viruses and viroids, causing mild or devastating symptoms with consequences for the economy and food security (Yadav and Khurana, 2015). The globalisation of trade has increased the movement of viruses and their respective vectors around the world, whilst climate change has facilitated their establishment in new locations (Trebicki, 2020). Thus, the need for diagnostic methods to rapidly identify viruses and other plant pathogens to support plant health measures is increasingly evident. However, this is more challenging due to the variability in populations (Jenkins *et al.*, 2002) common in RNA viruses because of the lack of proof reading activity in the RNA polymerase, the short generation times, virus–host interactions (Roossinck, 2003), and the generation of

recombinants (Fuentes *et al.*, 2021). Robust diagnostic tools are essential to underpin the production of healthy plant material and for the precise identification of viral diseases due to the similarities with symptoms caused by environmental stresses and other plant pathogens.

Serological and molecular methods are both commonly deployed as targeted methods for viral detection and diagnosis. ELISA (enzyme-linked immunosorbent assay) is a widely used technique for routine virus testing in phytosanitary, quarantine and virus-certification programmes (Boonham *et al.*, 2014). Based on the specificity of antibodies to interact with proteins, mostly the capsid protein of the target virus, ELISA is chosen for its simplicity, accuracy, and its economic cost (Hull, 2014). ELISA requires the production of high-quality antisera with lack of cross reactivity to diverse pathogens and plant proteins. The cost of production can be seen as a disadvantage (Boonham *et al.*, 2014) compared to nucleic acid-based methods, which are less costly to develop, more sensitive and easier to manipulate to achieve the desired specificity, albeit with a higher on-going testing cost (Schaad and Frederick, 2002; Arif and Ochoa-Corona, 2013; Arif *et al.*, 2014). Since its introduction (Mullis *et al.*, 1986), PCR has proven to be a very powerful tool for virus detection and diagnostics. RT-PCR is a sensitive and relatively rapid method for detection of RNA viruses, and multiple variations of the basic technique have been designed to improve sensitivity and specificity for the detection of plant viruses (Waterhouse and Chu, 1995), such as quantitative PCR (qPCR). qPCR adds further benefits in a routine testing laboratory as it is quicker than a conventional PCR, with no need to use agarose gel electrophoresis, with an improved sensitivity, quantification (for some applications), and adaptability to automation. PCR assays require the design of highly specific primers (and probes in the case of qPCR based on TaqMan® chemistry (Livak *et al.*, 1995) targeted to the pathogen of interest to overcome mismatches by mutations, genetic drift or selection pressure of sequence variants, frequently occurring in RNA viruses (Jenkins *et al.*, 2002; Metzgar, 2011). One of the most recent methods used in plant virology diagnostics is high-throughput sequencing (HTS; (Adams *et al.*, 2009; Al Rwahnih *et al.*, 2009; Kreuze *et al.*, 2009). Unlike PCR or ELISA methods, HTS offers the possibility of generic detection of viruses and other pathogens (Boonham *et al.*, 2014) at the species/strain level , and allows a generic approach to virus identification that does not require any prior knowledge of the pathogens present, being able to detect novel unidentified viruses (Adams and Fox, 2016; Fox *et al.*, 2019). Initially, its widespread use as a front line diagnostic tool was limited by the high cost of analysis, though the methods continue to evolve, with different platforms and library preparation methods being developed (Pecman *et al.*, 2017).

Despite the spread of rose viruses and the importance of rose cultivation, rose viruses have not been studied in detail in the UK since the 1980s with the work of B.J. Thomas (Thomas, 1984a). The molecular methods for detection of viruses in the 1980s were less advanced than the resources now available. The advance of molecular techniques could help us to clarify virus diseases and to study their aetiology more effectively. If we can understand the current situation in the UK, we can identify the gaps in our knowledge and identify future priorities. This will help to develop more sustainable rose cultivation in the UK. Improving our current understanding will also improve our response to new and emerging diseases such as rose rosette virus (RRV; genus *Emaravirus*), which has spread rapidly in the USA but is not yet present in the UK.

This thesis project fills some of the gaps regarding viruses infecting roses in the UK, using some of the currently available state-of-art diagnostic techniques. The hypothesis proposed at the beginning of this project were that (i) there was under-reporting of known viruses in roses in the UK; that (ii) RRV was present in the UK and was confused with other diseases or abiotic stress; and that (iii) there were undescribed viruses affecting roses that may be limiting production in the UK. Throughout the chapters of this thesis, answers have been obtained to the proposed hypotheses.

Chapter 2 Facing *Rose rosette virus*: A Risk to European Rose Cultivation

Abstract

Roses (*Rosa* spp.) are one of the most valuable ornamental flowering shrubs around the globe. They are susceptible to numerous pathogens which require management, increasing the cost of cultivation. Rose rosette virus (RRV; genus *Emaravirus*) is a devastating virus that has been spreading since the 1940s in the United States and Canada. It is an emerging risk to European and worldwide rose cultivation, causing symptoms such as witches' broom, malformations, excessive thorn production, and eventually plant death. RRV is transmitted by the eriophyid mite *Phyllocoptes fructiphilus* and by grafting. Research is being undertaken to understand RRV and to find control measures and resistant cultivars, as they are not currently available. Early detection of the disease is the key to prevent the establishment and spread of RRV and its vector. Different molecular and serological diagnostic methods have been designed and implemented, including ELISA, RT-PCR, RT-qPCR, LAMP and high-throughput sequencing. RRV infected plants can remain asymptomatic for long periods, so these diagnostic assays are necessary in conjunction with visual assessment to facilitate early detection. Significant social, economic and environmental impacts are expected if RRV and its vector establish and spread in Europe. Rose trade between countries is the most likely pathway of introduction of RRV into Europe. This chapter describes the current knowledge about RRV, the molecular and serological methods available for the detection of this virus, pathways to entry, and the possible impact if it establishes and spreads in Europe.

Published in: **Vazquez-Iglesias, I.,** Ochoa-Corona, F.M., Tang, J., Robinson, R., Clover, G.R.G., Fox, A. and Boonham, N. (2020) 'Facing *Rose rosette virus*: A risk to European rose cultivation', *Plant Pathology*, 69, pp. 1603-1617.

2.1 Introduction

Roses (*Rosa* spp.) are one of the most important ornamental species worldwide (Boskabady *et al.*, 2011), not only for their industrial properties (Dobhal *et al.*, 2016), but for their fragrance, beauty and aesthetics. They are considered the national flower of several countries in Europe, including England. Repeat flowering varieties were introduced into Europe from China in the 18th century (Joyaux, 2003) transforming the concept of roses, showing a broader range of colours, growth types, flower sizes, and scents. From that time, extensive rose breeding has taken place across the world, creating a massive industry (Debener and Byrne, 2014).

Cultivation of roses is economically important around the globe. The estimated annual production of cut flowers is around 18 billion stems, 60-80 million potted plants (miniature roses and bare-root grafting plants), and 220 million plants for landscaping (Blom and Tsujita, 2003; Pemberton *et al.*, 2003; Roberts *et al.*, 2003). The world rose production was estimated to be valued at €24 billion (around £21 billion) in 2008 (Heinrichs, 2008). In the United States (US), total wholesale production of shrub roses was estimated to be worth $204 million in 2014 (around £166 million), with 1808 growers producing 36.6 million plants (United States Department of Agriculture, 2015). In terms of plants for planting, including bare-rooted plants, pot plants, cuttings/budwood, rootstock and tissue culture, Serbia (36.34%) and China (30.81%) are the main countries from which the European Union (EU) imports roses (Table 2.1). Rosehips are also traded, used for different products such as rosehip jelly, water or perfume (Leghari *et al.*, 2016).

2.2 Pest and Pathogens Affecting Roses in Europe

Roses are susceptible to numerous diseases which require management, increasing the cost of production. In the United Kingdom (UK), the garden industry contributes £9 billion to the economy every year. Defra (Department for Environment, Food & Rural Affairs) valued general ornamental plant production at £1.1 billion in 2015, pointing out that diseases caused losses of £630 million annually in the UK to ornamental plant production of which £40 million was due specifically to viral diseases (Department for Environment Food and Rural Affairs, 2016).

Roses are vulnerable to infections by bacteria, fungi, viruses, nematodes, and phytoplasmas, causing leaf and flower mosaics, distortion, spotting, discolouration, necrosis, reducing growth or death of the plant. Several fungal pathogens affect roses with a worldwide distribution. Black spot is the causal agent of the most serious fungal disease of roses grown outdoors in Europe and worldwide (Yasin *et al.*, 2016). Rust is caused mainly by the fungi *Phragmidium tuberculatum* and *Phragmidium mucronatum* (Helfer, 2005), among other *Phragmidium*

species, and is another common disease. Powdery mildew caused by *Peronospora pannosa*, is the major fungal pathogen of roses grown in greenhouses, but can also be detected in the field (Schulz and Debener, 2010).

Table 2.1- Average number of rose plants imported from non-EU countries to the EU during the years 2014 to 2018. The table shows the percentage of the total imports originating from each country per year (Eurostat, 2019).

EU imports of rose plants	Average kg per year	Percentage total imports (%)
2014-2018 (Jan - Dec)		
SERBIA	281740	36.34
CHINA	238880	30.81
SOUTH AFRICA	120920	15.60
UZBEKISTAN	33280	4.29
KENYA	20920	2.70
SWITZERLAND	20560	2.65
NORWAY	13040	1.68
MOLDOVA	9120	1.18
ETHIOPIA	6120	0.79
SOUTH KOREA	5900	0.76
NORTH MACEDONIA	5880	0.76
UKRAINE	5540	0.71
TURKEY	4220	0.54
MOROCCO	3860	0.50
UNITED STATES	1140	0.15
SRI LANKA	920	0.12
INDIA	760	0.10
BELARUS	620	0.08
JAPAN	620	0.08
RUSSIAN FEDERATION (RUSSIA)	620	0.08
ECUADOR	200	0.03
LEBANON	120	0.02
ISRAEL	100	0.01
COLOMBIA	60	0.01
SURINAME	40	0.01
THAILAND	40	0.01

The management of pests and diseases in rose production is primarily achieved using agro-chemicals. Restrictions imposed by plant protection legislation and the increasing ecological awareness of consumers, have pushed breeders in line with plant pathologists to identify and characterise resistant cultivars (Schulz and Debener, 2010). Increasing disease resistance is especially necessary for garden roses, to inspire confidence amongst amateur rosarians, gardeners and landscapers for their use in public areas (Leus *et al.*, 2008).

The control of diseases in greenhouses is also important, because controlled environments enable a year-round supply of rose plants and cut flowers, even in seasons when outdoor temperatures or light conditions are not suitable for growth (Raviv *et al.*, 2010). Rose varieties are commonly grown in greenhouses using rootstocks, that favour a rapid economic multiplication of scions from desirable rose cultivars, which cannot be raised on their own roots (Tubbs, 1973). Rootstocks play an important role for economic aspects of propagation, flower production, flower quality, adaptation to different kinds of soil and disease resistance (Fuchs, 1994). One of the most used rootstocks is *Rosa multiflora*.

2.3 Rose Viruses Reported in Europe

Several viruses have been reported affecting roses in Europe including arabis mosaic virus (ArMV; genus *Nepovirus*), strawberry latent ringspot virus (SLRSV; family *Secoviridae*), apple mosaic virus (ApMV, genus *Ilarvirus*), and prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*(King *et al.*, 2012). Rose mosaic disease (RMD), is one of the most common diseases of roses worldwide, and is caused by single or mixed infections of these viruses (Vazquez-Iglesias *et al.*, 2019). Differences have been established between viruses involved in RMD occurring in North America and Europe, although PNRSV has been identified as the most frequent virus associated with this disease in both continents (Horst *et al.*, 1983; Manners, 1997; Sertkaya, 2010). RMD is thought to have propagated in roses by grafting from infected rootstocks or scions subsequently spreading among rose cultivars (Sertkaya, 2010). Viruses associated to RMD are considered transmitted by seeds, pollen, aphids, thrips, contaminated soil or pruning tools, but no conclusive scientific evidence is available regarding transmission pathways (Horst and Cloyd, 2007). Golino *et al.* (2007) showed evidence of ApMV and PNRSV transmission via roots between roses growing close together in experimental fields.

Symptoms of RMD (Fig. 2.1) vary depending on the variety, and include chlorotic line patterns, ring spots, mottles in leaves, yellow net and mosaic. Infected plants are less vigorous and more likely to die over winter (Horst *et al.*, 1983). PNRSV-infected plants have reduced quality with weaker shoots and fewer, smaller blooms, and are more likely to die after transplanting, generating losses in production. However, virus-infected plants can remain symptomless for much of the growing season depending on the variety (Thomas, 1982).

Rose cryptic virus-1 (RoCV1), also known as rosa multiflora cryptic virus (Martin and Tzanetakis, 2008), is a *Partitivirus* first reported in the USA (Sabanadzovic and Ghanem-Sabanadzovic, 2008) and subsequently in Canada (James *et al.*, 2015), New Zealand (Milleza *et al.*, 2013) and recently in the UK (Vazquez-Iglesias *et al.*, 2019). Cryptic viruses escaped detection for many years because most cause no visible symptoms or, in a few cases, very mild

symptoms (Milleza *et al.*, 2013). Cryptic virus occur in very low concentrations in infected plants (Hull, 2014). There are no known natural vectors, and no graft transmission or cell-to-cell movement. The reported mode of transmission is by cell division, by pollen or seed (Boccardo *et al.*, 1987).

Rose yellow vein virus (RYVV) is a circular dsDNA virus which has recently been reported in Turkey (Karanfil *et al.*, 2018), but was first described in the USA and New Zealand (Perez-Egusquiza *et al.*, 2013). RYVV belongs to family *Caulimoviridae*, genus *Rosadnavirus* (King *et al.*, 2012) causing vein banding or central vein chlorosis in infected leaves (Milleza *et al.*, 2013; Mollov *et al.*, 2013).

Figure 2.1- Classic symptoms of Rose mosaic disease include yellow netting and mosaic on leaves. This image is courtesy of A. Fox.

2.4 Rose Rosette Virus

Rose rosette virus (RRV) is a virus in the order *Bunyavirales*, genus *Emaravirus*, and is the causal agent of rose rosette disease (RRD; Laney *et al.*, 2011), a damaging disease of roses in North America. RRV is a multipartite RNA virus consisting of 7 single-stranded negative sense RNA particles (RNA1-RNA7), encoding for an RNA-dependent RNA polymerase (RdRp), a

glycoprotein, a nucleocapsid, a movement protein, and p5, p6 and p7 proteins respectively (Laney *et al.*, 2011; Di Bello *et al.*, 2015).

RRV was first described in the 1940s in Manitoba, Canada (Conners, 1941). At the same time, similar reports were made in Wyoming and California (Thomas and Scott, 1953). RRV is considered the most important viral disease of roses in the USA (Dobhal *et al.*, 2016). Early studies suggested the cause of RRD could be related to phytoplasma (Gergerich and Kim, 1983), but the association of double-membrane bound bodies and dsRNA (Doudrick and Millikan, 1983) with rosette-affected material indicated the involvement of a virus (Laney *et al.*, 2011).

Numerous plant species have been assessed for the presence of RRV, but *Rosa* remains the only host genus identified (Laney *et al.*, 2011). This occurrence may explain the small variation between RRV isolates (Laney *et al.*, 2011), as host-driven diversity has not developed in RRV. Similarly, studies in European mountain ash ringspot-associated virus (EMARaV, the type species of *Emaravirus*) have also shown small sequence diversity (Kallinen *et al.*, 2009). It may be hypothesized that the reported RRV low variability may be due to the virus replication in the vector, creating an evolutionary bottleneck where only variants replicating in both plant and mite are transmissible, such as the case of EMARaV and the mite *Eriophyes pyri* Pagenstecher reported by Mielke-Ehret *et al.* (2010). However, further research is being undertaken to look for isolate variation in RRV (Byrne *et al.*, 2019; Katsiani *et al.*, 2020).

2.5 The Beginning of RRD Dissemination in North America

Rosa multiflora was introduced to North America from Japan during the early 1800s, as an ornamental for breeding proposes and as a rootstock (Rheder, 1936). Due to its hardiness and resistance to pest and disease, it was used widely in amenity planting. For example 14 million multiflora roses were planted in West Virginia alone between 1940-1960 (Dugan, 1960). *R. multiflora* was subsequently considered a weed (Hindal *et al.*, 1988) and in the early 2000s, the number of hectares covered by *R. multiflora* in the eastern US reached 18 million (Loux *et al.*, 2005).

RRV was considered an agent for the biological control of *R. multiflora*, on the assumption that rose plants would die in a period of 5 years (Epstein and Hill, 1999). Even though the USA government was aware that the mite was a vector of the virus, they assessed the risk of spreading of RRD to other ornamental roses to be low (Amrine, 1996). However, as different types of roses grew in popularity, hundreds of thousands of RRV-susceptible plants were planted in private gardens and commercial beds, making it more likely that the virus would spread (Amrine, 1996).

2.6 RRV Geographical Distribution

RRV is currently present from the eastern coast of the USA to the Rocky Mountains and California (Center for Invasive Species and Ecosystem Health, 2019). It was thought to be restricted to North America until 2017, when it was reported for the first time in India (Chakraborty *et al.*, 2017). *Rosa multiflora* is a widespread susceptible host, serving as a reservoir for both virus and vector. Beyond *R. multiflora,* RRD has been reported in different rose species such as *R. arkansana, R. bracteata, R. canina, R. corymbifera, R. gallica, R. glauca, R. rubiginosa, R. spinossisima, R. villosa, R. woodsia,* and in multitude of types: climbers, hybrid teas, floribundas, miniatures, shrub and antique roses (Martin, 2014).

2.7 Symptoms

Symptoms of RRV (Fig. 2.2) are highly variable between rose cultivars, stage of the disease and environmental factors (Epstein and Hill, 1995; Epstein and Hill, 1999). Moreover, roses may harbour other viruses such as PNRSV and/or ApMV and their synergistic effect on symptom expression has not been determined. Symptoms of RRD include reddening on newly emerging shoots, excessive lateral shoot growth, excess thorn production, leaf mosaic and mottling. Flowers tend to bunch together, forming witches' broom or rosetting, with malformed flowers (Laney *et al.*, 2011; Dobhal *et al.*, 2016). The virus moves throughout the plant affecting the roots, and plants show reduced growth and vigour compared to uninfected plants (Epstein and Hill, 1999). Other symptoms that may be expressed are darkening of canes, short internodal distances, blind shoots, rough leaf texture and an increased susceptibility to infection, especially by fungal diseases (Hong *et al.*, 2012). Infected plants die within 3-5 years of becoming infected (Di Bello *et al.*, 2018).

Roses infected with RRV can show few or no symptoms during early stages of infection (Dobhal *et al.*, 2016), and can remain asymptomatic for 30 to 146 days after transmission (Allington *et al.*, 1968). Hence, by the time the first recognisable symptoms appear, the disease could have spread to nearby plants (Hong *et al.*, 2012).

Figure 2.2- Symptoms of rose rosette virus (RRV) in different rose cultivars in Oklahoma, US: (A) reddening in the leaves and stems, (B) witches' broom or rosetting, and (C) excess of thorn production and thicker stems. Picture D shows a healthy-looking stem (left) compared with an RRV infected (right). Pictures A-C were taken by the author in the USA, and picture D is courtesy of F. Ochoa-Corona.

2.8 RRV Transmission

Members of the genus *Emaravirus* are transmitted by eriophyid mites (Mielke-Ehret and Mühlbach, 2012). In early epidemiological studies, researchers theorised symptoms of RRV might be caused by eriophyid mite feeding toxicity (Slykhuis, 1980). Later experiments showed RRD was mite transmissible (Allington *et al.*, 1968) and the pathogenicity of RRV was demonstrated by Di Bello *et al.* (2015). The eriophyid mite *Phyllocoptes fructiphilus* Keifer (Fig. 2.3) is currently the only competent vector species identified (Keifer, 1966; Allington *et al.*, 1968), although research has been undertaken with other *Phyllocoptes* species. The mite *P. adalius* is difficult to discriminate from *P. fructiphilus* morphologically since the prodorsal

shield, which is used to distinguish them, is not visible with the naked eye (Druciarek *et al.*, 2016). The identification of eriophyids is based on morphological observations, and sometimes ecological characteristics give important clues. Light and electron microscopy techniques are used to identify *P. fructiphilus* and differentiate it from other mite species. It is commonly found in the flowers, under stipules or vegetative bud scales (Otero-Colina *et al.*, 2018). Whilst *P. adalius* is similar to *P. fructiphilus,* and a significant pest in its own right, causing serious damage due to feeding, it has been shown to not be an RRD vector (Amrine, 2002). Another mite species also considered for RRV transmission is *Eriophyes eremus* (Fig. 2.3). This eriophyid mite is also found in roses, and was first described in Israel (Druciarek and Lewandowski, 2016). *E. eremus* was found in several states of the USA in 2018, colonising native, naturalised, and ornamental rose cultivars (Otero-Colina *et al.*, 2018), and like *P. fructiphilus*, it is also a micro-environment shelter-seeking mite. Interest in *E. eremus* arose after being found in large numbers and as the only mite species feeding on a symptomatic RT-qPCR positive plant (Solo, 2018). However, finding an *E. eremus* colony upon a rose specimen that tested positive to RRV may be circumstantial. Otero-Colina *et al.* (2018) have shown that no damage has been observed in association with this mite.

Eriophyids are small, typically between 140-170 µm, and unlike most mite species possess four, rather than eight legs. These mites are typically found in the angles formed between leaf petioles and axillary buds, feeding on plant tissues and overwintering on plants. Eriophyids are thought to survive for only 8 hours without a host. Eriophyids have a short life cycle of eight days, and during that time can lay an egg a day (Kassar and Amrine, 1990). They do not have wings, but they can be transported by insects during pollination, dispersed by the wind, or by contact with clothing (Hong *et al.*, 2012; Byrne *et al.*, 2015). Jesse *et al.* (2006) showed roses with a higher density of leaves had a greater number of mites, because of a larger microhabitat availability, and they described a preference for sunny environments.

Currently, *P. fructiphilus* has only been described in North America, and is thought to be widely distributed in the USA on wild and commercial roses (Amrine, 2002). Although RRV has been reported in India, *P. fructiphilus* has not been detected and it is unknown if there is a vector present (Chakraborty *et al.*, 2017; EPPO, 2019a). Although mite transmission is the primary mechanisms for spread in the field, RRV can also be transmitted by grafting (Amrine *et al.*, 1988) and potentially by pollen (Babu *et al.*, 2017a).

2.9 Early Detection and Biocontrol

The diagnosis of RRD in the early stages of infection is difficult. Symptoms are often confused with other pest problems, herbicide damage, nutrient deficiencies or fungal infections.

When glyphosate, a broad-spectrum systemic herbicide, contacts green tissue during autumn treatments, it is translocated to the buds and witches' broom symptoms with yellow leaves may appear during the following spring; this is easily confused with the rosetting caused by RRV (Hong *et al.*, 2012). Also, manure contaminated with picloram + 2,4-dichlorophenoxyacetic acid, a systemic herbicide, can also cause the same symptoms when applied around roses (Davis *et al.*, 2015).

Nevertheless, early detection is crucial, and identification and eradication of infected plants are necessary for effective control of RRD (Hong *et al.*, 2012). Pruning out symptomatic parts of plants does not eliminate the virus and should be avoided to minimise the persistence of the virus after overwintering in the root system (Di Bello *et al.*, 2018). Ideally, all multiflora roses in a 100 m radius should be removed, because they serve as a source of inoculum for RRV (Department for Environment Food and Rural Affairs, 2016). The use of acaricides could decrease mite populations, reducing the risk of RRV dissemination. Acaricides may be useful to treat rose plants surrounding areas where RRV-infected plants have been removed (Hong *et al.*, 2012). However, it is difficult to completely eliminate mites, because eriophyids hide in inaccessible areas of the plant (Otero-Colina *et al.*, 2018).

There is no complete resistance or immunity reported in rose cultivars for RRD. Resistance to any pathogen depends on host genotype, the RRV virus isolate, the environment, the vector biology, and seasonality. The development of new resistant varieties is a long process that takes years. The stability of the prospective resistance is not known until later phases of testing, in which varieties are assessed in different locations within a range of environmental conditions and diversity of pathogens (Debener and Byrne, 2014). Amrine (2002) observed that rose species or varieties differ in RRV symptom expression and that there are likely to be differences in susceptibility or resistance to the virus. When a rose genotype shows resistance and robustness in a field with high RRV infestation, the molecular mechanism that makes this phenotype resistant can be studied to enable the use of resistant genetic material in breeding programmes (Byrne *et al.*, 2015; Byrne *et al.*, 2019). Other rose species including *Rosa acicularis, R. arkansana, R. blanda, R. californica, R. carolina, R. palustris, R. pisocarpa, R. setigera* and *R. spinossisima* have shown elevated levels of resistance to RRV infection. *R. bracteata* and *R.* 'Meizeli' [The McCartney Rose] are resistant to feeding by the mite vector, although both are susceptible to the virus (Hong *et al.*, 2012). Since the RRV genome is known, there are possibilities to apply gene editing technology in the future. Research groups in the USA are making efforts to develop RRD-resistant roses: identifying genes linked to resistance,

discriminating susceptible and resistant plants to the virus and to the mite, aiming to incorporate traits into elite rose germplasm (Byrne *et al.*, 2015; Dobhal *et al.*, 2016; Roundey *et al.*, 2016).

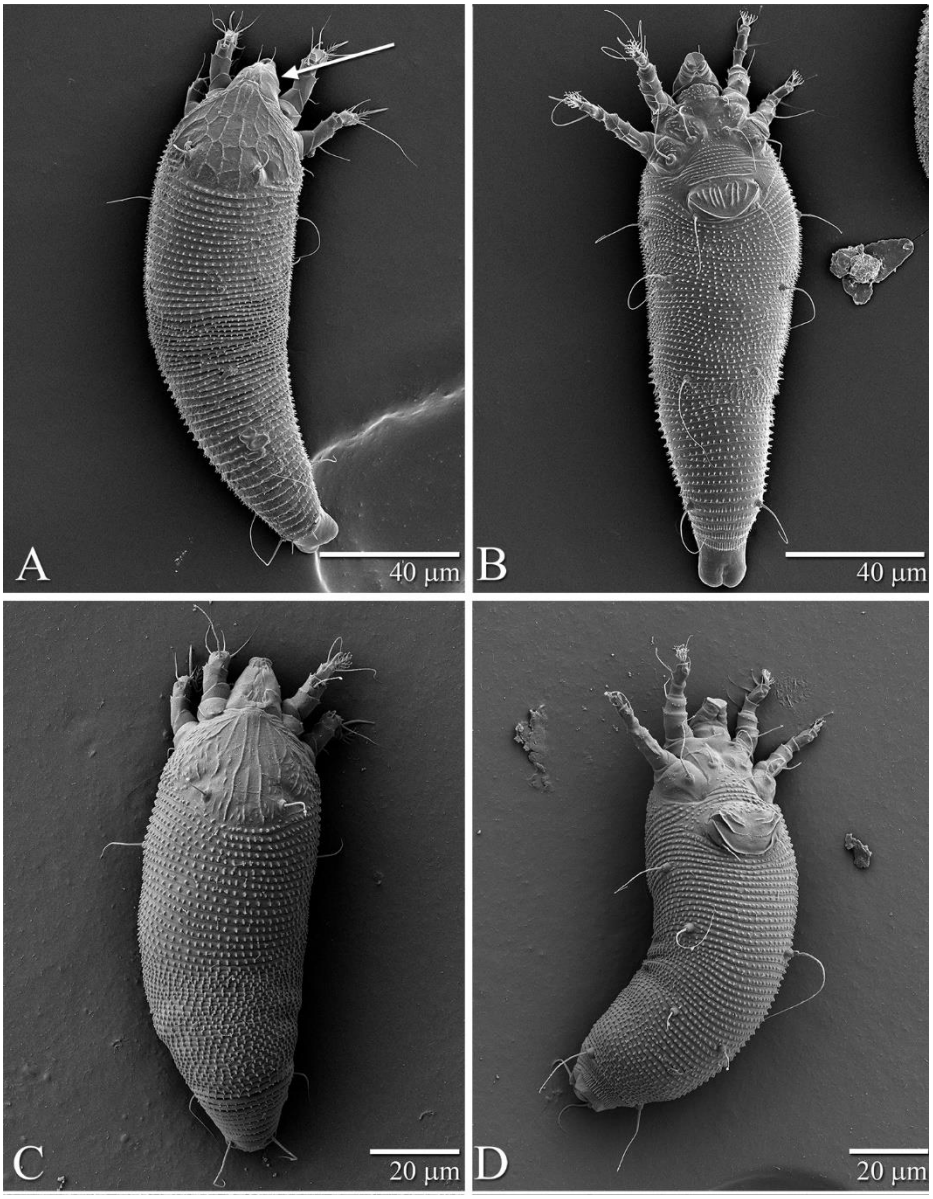

Figure 2.3- Low-temperature scanning electron microscopy images of female adults of (A-B) *Phyllocoptes fructiphilus*, A) dorsum, B) venter; (C-D) *Eriophyes eremus*, C) dorsum, D) venter. Image reproduced with the permission of USDA-ARS, Electron & Confocal Microscopy Unit, Beltsville, MD (Otero-Colina *et al.*, 2018).

2.10 Diagnostic Techniques

Several techniques have been developed in the last few years for detection and diagnosis of RRV. Jordan *et al.* (2018) are developing polyclonal, monoclonal and/or single-chain antibodies and associated serology-based protocols, that is ELISA (enzyme-linked immunosorbent assay), immuno dip-stick (lateral flow) and immune-capture RT-PCR, for specific, reliable and sensitive detection of RRV. ELISA is a versatile technique, widely used for routine virus testing in phytosanitary, quarantine or virus certification applications

(Boonham *et al.*, 2014). However, ELISA requires the costly production of high-quality antisera with lack of cross reactivity to diverse pathogens and plant proteins, which may be seen as a disadvantage (Boonham *et al.*, 2014) compared to nucleic acid-based methods, which are less costly to develop, more sensitive and easier to manipulate to achieve the desired specificity (Schaad and Frederick, 2002; Arif and Ochoa-Corona, 2013; Arif *et al.*, 2014). However, in the long run, once antisera are developed, it allows an increased throughput of samples and lower costs than nucleic acid-based methods.

Reverse transcription-polymerase chain reaction (RT-PCR) is considered a sensitive and relatively rapid method for detection of RNA viruses. The first reported RRV detection method consists of an end-point reverse transcription polymerase chain reaction (RT-PCR) with primers designed to amplify a fragment of RNA1 of the RRV genome (Table C.1; (Laney *et al.*, 2011). Subsequent work showed the initial method to be inconsistent compared to other assays (Babu *et al.*, 2016), which led to the development of additional methods.

Di Bello *et al.* (2018) developed an RT-PCR assay designed in a highly conserved region of RNA3 of the RRV genome (Table C.1). They proposed that RNA3 would be a better target because it codes for a nucleoprotein, so this gene would be transcribed at higher levels than the virus polymerase (RNA1). They used previously published sequences of 23 isolates available in GenBank for primer design and additional sequences from 107 isolates collected in different US states, thereby incorporating intra-virus variation into the primer design. This assay was used in conjunction with primers designed to amplify the NADH dehydrogenase ND-2 subunit gene as an internal positive control in a multiplex PCR. Evaluation of the sensitivity was performed in comparison to the RT-PCR developed by Laney *et al.* (2011), and it was found to have higher sensitivity.

A different end-point RT-PCR was developed by Dobhal *et al.* (2016). The primers were designed to be compatible with two RT-qPCR chemistries: TaqMan RT-PCR and SYBR Green combined with High Resolution Melting (HRM) analysis aimed at providing flexibility to diagnosticians with different resources or diagnostic preferences, since these techniques can be used with a single set of primers (Table C.1). These proposed primers were designed using the nucleocapsid protein gene fragment (RNA3) of RRV as a template. The sequences of all RRV isolates available in NCBI GenBank at that time were considered. To verify the specificity of the primers, an *in silico* analysis was performed. Moreover, a panel of eleven reference control viruses was used for exclusivity assessment of the three techniques. The limit of detection was determined to be 1 fg using serial dilutions of a constructed artificial RRV positive control. Positive amplification was obtained with RRV infected samples, and sequencing of the

amplicon confirmed RRV was specifically amplified. The presence of phenolic compounds, carbohydrates, pigments, and other putative compounds in rose tissue were found to interfere during nucleic acid extraction (Dobhal *et al.*, 2016). The use of PCR amplification facilitators BSA (bovine serum albumin) and PVP (polyvinylpyrrolidone) in the PCR reaction mix improved amplification and helped avoid false negatives. BSA and PVP did not cross react or influence the specificity of the primer or the negative control.

Quantitative PCR (qPCR) based on TaqMan chemistry provides a greater specificity and speed compared to conventional end-point PCR for the detection of a pathogen, or a group of pathogens (Jenkins *et al.*, 2002; Metzgar, 2011). In the case of RRV, both techniques have a comparable limit of detection. Babu *et al.* (2016) developed multiple primer/probe sets (Table C.1) targeting three different regions of the RRV genome. Four primer/probe sets (RRV_2-1; RRV_2-2; RRV_3-2; RRV_3-5) and their corresponding product were tested *in silico*. Then the sensitivity (1 fg) was determined for the different assays. The specificity of the primer/probe sets in the presence/absence of other common rose-infecting viruses, and their reproducibility, was tested three times within a 30-day interval. By comparison with end-point RT-PCR the RT-qPCR was more sensitive, detecting positive infected samples that gave negative results when using RT-PCR (Laney *et al.*, 2011). In addition, positive detection of samples from different states of the USA indicated that the primer/probe sets had broad specificity.

Another TaqMan RT-qPCR assay for RRV detection was developed in 2017 at the Plant Health and Environment Laboratory (PHEL), New Zealand (Joe Tang personal communication; Table C.1). The primers and probe were designed based on the alignment of 27 RRV sequences of the nucleocapsid gene of RNA3 sourced from GenBank; the product size of the assay is 103 bp. An *in silico* assessment of this assay indicates that it is likely to detect all reported RRV isolates, and this is supported by results obtained showing two RRV isolates were successfully detected while samples of non-target emaraviruses (fig mosaic virus and raspberry leaf blotch virus) and healthy rose plant tested negative. The described TaqMan RT-qPCR assay is currently the assay implemented by PHEL for RRV routine testing. Since 2018, a total of 214 rose samples have been tested for presence of RRV, including post-entry quarantine and domestic growers. RRV is not reported in New Zealand to the present day.

RPA (recombinase-polymerase amplification) and LAMP (Loop-mediated Isothermal Amplification) are simplified isothermal amplification techniques (Notomi *et al.*, 2000; Piepenburg *et al.*, 2006). Their advantages compared to PCR are (i) the reduction of reaction time to circa 20 min; (ii) the reaction runs at a constant low temperature (37-42°C for RPA, LAMP 65°C), so there is no need for thermal cycler investment, enabling the use of simpler

equipment; and (iii) the potential to be transferable to the field for use as a simplified screening assay (Sen and Ashbolt, 2011).

Babu *et al.* (2017b) developed a basic gel-based RT-RPA assay. The method uses three different primer sets (RPA-131; RPA-267; RPA 321) designed to detect different regions of the RRV genomic RNA (Table C.1). The specificity of the three sets of primers was assessed beforehand *in silico* against other common rose infecting viruses, and sensitivity was found to be 1 fg/µl. The method worked well with different tissue sources (leaves, petals and stems), and with samples from different states of the USA.

A probe-based reverse transcription-recombinase-polymerase amplification (RT-exoRPA) assay for RRV was also described by Babu *et al.* (2017a). Primers were designed in the conserved regions of RRV genomic RNA3 (Table C.1; RPA-267). Analysis *in silico*, assessment of the specificity, and limit of detection (1 fg/µl) were undertaken to assess this primer set. The developers of this technique envisioned commercial growers and nursery personnel performing the method on-site. Thus, a quick viral RNA extraction method named direct antigen-capture was developed. which can be completed in circa 5 min and allows the use of different types of plant tissues (Babu *et al.*, 2017a).

RRV was detected in pollen (anthers) of RRV infected roses with the RT-exoRPA analysis (Babu *et al.*, 2017a). This finding suggested a new potential transmission pathway of the virus; however, further research is needed to confirm the finding and its significance. Sample collection still poses questions regarding which plant parts are best for sampling. The detection of RRV from the primary and secondary roots suggests they can be a good matrix for RRV detection, where the virus could overwinter, and allow testing plants even in the absence of leaves, green stems and petals (Babu *et al.*, 2017a). Roots can be tested in wintertime, and petals and symptomatic leaves during the rest of the year. This type of sampling proved to work well as sample source at the Oklahoma State University, Microbial Forensic Laboratory (Ochoa-Corona, personal communication). However, a statistically tested sampling technique for asymptomatic plants is yet to be demonstrated.

LAMP primers for RRV were designed after analysis of RRV P3 and P4 gene sequences using 'Primer Explorer' software (https://primerexplorer.jp/e/; (Salazar-Aguirre *et al.*, 2016); Table C.1). Alignment of the P3 and P4 RRV genes allowed precise LAMP primer design for broad detection of most reported isolates to 2016 (Salazar-Aguirre *et al.*, 2016). RRV-LAMP primers do not cross-react with cDNA reverse transcribed from ten reference isolates of frequently rose co-infecting viruses or RRV related viruses: high plains wheat mosaic virus

(genus *Emaravirus*), maize stripe virus (genus *Tenuivirus*), impatiens necrotic spot virus (genus *Orthotospovirus*), tomato spotted wilt virus (genus *Orthotospovirus*), groundnut ringspot virus (Genus *Orthotospovirus*), ApMV, ArMV, PNRSV, tobacco ringspot virus (genus *Nepovirus*) and tobacco mosaic virus (genus *Tobamovirus*). LAMP for RRV was tested successfully using tissue samples of symptomatic and asymptomatic RRD-infected roses from Oklahoma. Healthy tissue and non-template controls were included in all reactions.

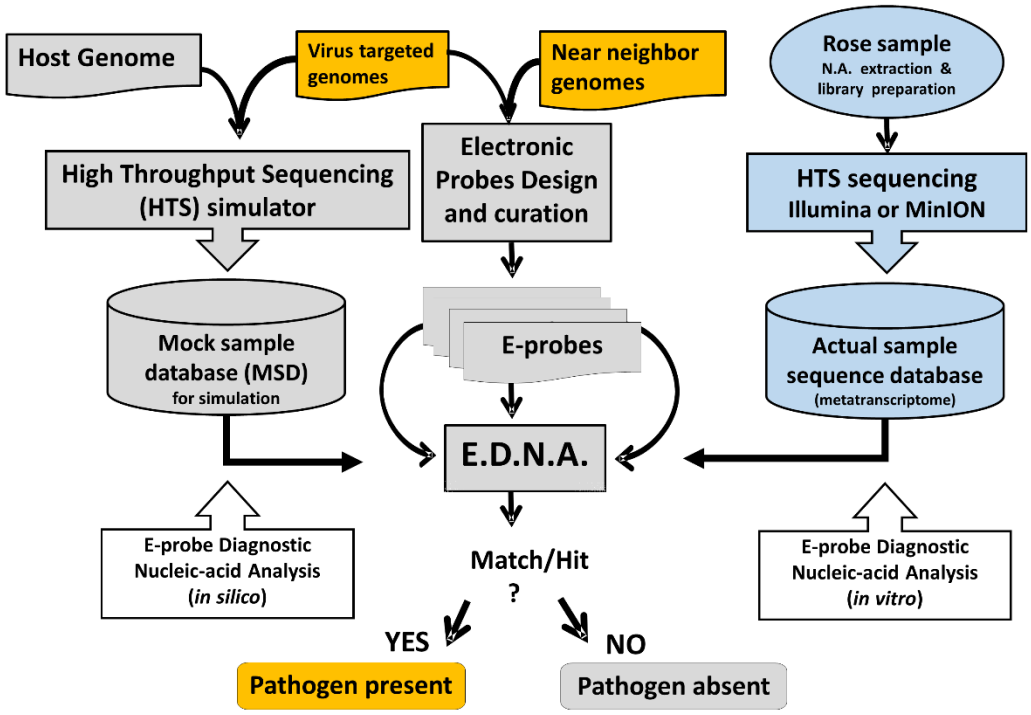

Figure 2.4- Flow chart representation of the EDNA pipeline, showing the *in silico* development stage (left -top) where databases of rose genomic sequences (host), and sequences of rose-infecting viruses and related viruses are built. Subsequently e-probes are designed and curated for specificity and high-throughput sequencing (HTS). Mock or simulated samples are also generated for simulation of pre-tests *in silico* (left-centre). The *in vitro* and routine diagnostic stage (right) corresponds to the actual *in vitro* HTS assay to include sample processing, nucleic acid extraction, and library preparation followed by actual sample HTS using either Illumina or MinION platforms (right-centre). The obtained output database is screened with EDNA (centre-bottom). Electronic probe hits determine virus detection. This diagram was elaborated by F. Ochoa-Corona and modified by the author.

High-throughput sequencing (HTS) has revolutionized diagnostics since 2009 (Adams *et al.*, 2009; Al Rwahnih *et al.*, 2009; Kreuze *et al.*, 2009). HTS offers the possibility of generic detection of viruses and other pathogens (Boonham *et al.*, 2014), and allows a generic approach to virus identification that does not require previous knowledge of the targeted pathogens. HTS can deliver a species/strain specific result (Adams and Fox, 2016). HTS continues to evolve, and different platforms and sample preparation methods have been developed (Pecman *et al.*,

2017). A novel bioinformatic pipeline called electronic diagnostic nucleic acid analysis (EDNA) is being developed for the detection and diagnosis of 24 reported viruses infecting rose worldwide (Peña-Zuñiga *et al.*, 2017). This computational tool combines HTS and bioinformatics, minimises and ignores non-relevant sequence data and focuses on predetermined specific pathogen-associated sequences. It enables the detection of multiple viruses in a single sample or run (Fig. 2.4) of either Illumina or Oxford Nanopore MinION raw metagenomic outputs.

2.11 Potential Entry Pathways to Europe

There are several potential entry pathways into the EU for RRV and its vector. Roses for planting are imported from different countries (as dormant plants free from leaves), including India and the USA. Although the percentage of imported plants from these countries is not high, the risk is elevated since 2000 kg of roses are imported to Europe yearly from countries where this virus is present. Details about the rose species and varieties imported are unknown. *R. multiflora* is a regulated plant species in 13 US states, where its importation, distribution, trade, and sale have been banned (New York Invasive Species, 2019). Only dormant *Rosa* plants free from leaves, flowers and fruit can be imported into the EU from non-European countries. However, the risk of RRV introduction and its vector persist since both can survive on dormant plants. (EPPO, 2018). Thus, RRV has been regulated in the EU since November 2019, and roses imported from US, Canada and India need to follow specific measures to avoid the introduction of RRV and *P. fructiphilus* (Andriukaitis, 2019). Moreover, roses may be imported illegally through internet trading or smuggling (Tuffen, 2016).

If RRV-infected plants were imported without the vector, the virus would be limited to that plant, except if used for propagation. Nevertheless, as reported all plants showing symptoms of RRD are generally infested by *P. fructiphilus* (Otero-Colina *et al.*, 2018) if imported from North America. The presence of just one female will be enough to initiate a population. In the case of introduction of nymph and adult stages of the vector, adults could be dispersed by wind or by other media, spreading the infection (Tuffen, 2016).

Other possible but less likely pathways are by natural spread or by the rosehip trade. The countries with the presence of RRV and *P. fructiphilus* are far from Europe, so vector transmission by wind is unlikely. Rosehips are generally used for domestic consumption therefore are unlikely to act as a pathway to the wider environment. The spreading of RRV by pollen needs to be further assessed by research (Babu *et al.*, 2017a).

2.12 Cut Flowers: A Risk?

RRV has not been reported infecting cut rose varieties yet, though it is highly probable they are susceptible. There are non-commercially available resistant or tolerant species. The possibility of finding flowers with symptoms in the market is low, because they would likely be graded out due to quality issues. Nevertheless, flowers could be taken from asymptomatic parts of an infected plant. The quality standards are high for cut roses, and under controlled conditions the use of agrochemicals could reduce the mite population.

The EU is a significant importer of fresh cut roses. This fact increases the risk of entrance of any exotic pathogen if phytosanitary measures are not effective. During the first 10 months of 2017, rose imports into the EU were valued at €624 million (£507 million), ten times more than the value of exported roses to non-EU countries. According to Eurostat (2019), the Netherlands was the top EU exporter of cut flowers (70% of the total extra-EU exports of roses), as it is a major producer in Europe and receives cut flowers from other producer countries to redistribute them to the European market. After the Netherlands, other key exporters in Europe are Lithuania (11%), Germany (8%) and Latvia (7%). The Netherlands was also the top importer of fresh cut roses from outside the EU (77% of the total EU imports of roses). Other major importers were the UK (10%), Germany (6%) and Spain (5%). There is a minor trade of cut flowers with the USA (200 kg in 2016; Eurostat, 2019), and there is no trade with Canada. The trade with India has increased from 300,000 to 900,000 kg in 2012-2016, mainly exported to the Netherlands and the UK (EPPO, 2018).

The possibility of RRV being introduced by cut flowers is unlikely, though not impossible if infected plants and vectors were found in an exporter's production site. Cut flower shelf-life is around 2 weeks. Cut flowers are mostly used indoors which reduces the risk of mites moving outdoors to transmit the virus in gardens. However, when the cut flowers are disposed of outdoors, e.g. in compost, mites may still be able to reach garden roses and transmit the virus.

2.13 RRV Impact on the USA Industry and Environment

RRV has led to a significant decline of garden roses and urban landscapes of cities in the USA (Laney *et al.*, 2011). The outbreak of RRV has been particularly evident in Tulsa and Oklahoma City (Oklahoma) and has affected the rose industry in other states (Ochoa-Corona and Vazquez-Iglesias personal observation). RRV infects randomly in the field with other viruses creating new combinations of mixed infections in a large number of rose varieties and hybrids (Peña-Zuñiga *et al.*, 2017), and threatens to decimate the USA rose industry (Byrne *et al.*, 2015).

In the USA about 35% of rose sales are specifically used by the landscape industry. Recently, this market has reduced the use of roses by about 10% per year due to RRV and associated virus complexes. There are approximately 2,000 businesses that produce garden roses to sell in the USA. These growers produced 36.6 million garden-rose bushes in 2014 generating sales worth $203.5 million (£165.5 million), creating approximately $777 million (£632 million) for the USA economy (Pemberton *et al.*, 2018). The overall losses caused by RRV to present are being estimated and the official magnitude of the economic loss caused by the RRD is yet to be determined (communication with rose stakeholders at technical meetings).

2.14 Potential Impact in Europe

For RRV to become established in Europe, its vector *P. fructiphilus* would also need to be introduced. The economic impact of RRV is expected to be high. Breeders, nurseries, retailers of garden and pot roses, and landscapes would be affected. Symptomatic rose plants would be unmarketable and eradication measures which include destruction of plants in a range of 100 m, even if they remain asymptomatic, will damage the economy of this sector (EPPO, 2018). The cost associated with replacement of rose plants in private and public landscaping will be high and the rose industry will be seriously affected by the introduction of alternative ornamentals into both the garden and landscape industry.

Bulgaria and Turkey are the largest producers of rose oil worldwide, which relies primarily on species like *R. damascena*, which is reported to be a RRV host (EPPO, 2018). In Bulgaria, the rose oil industry provides labour for ca. 65000 people, mostly seasonal workers (Kovacheva *et al.*, 2010). In Turkey, 8200 families grew oil roses in 2005 (Gunes, 2005).

The environment is also expected to be affected by RRV. In Europe there are several wild species known to be susceptible to the virus, for example *R. canina* and *R. rubiginosa* (EPPO, 2018). Roses are used for hedges, game cover, slope stabilisation and erosion control. Invertebrates that rely on *Rosa* spp. would also be affected, for example the gall-forming wasp *Diplolepis spinosissimae.* This insect causes the so-called Robin's pincushion. A negative impact on pollinators is expected, as there are species which feed on roses. Pollinators have alternative sources available, but some have a specific relationship with these plants (Tuffen, 2016).

The introduction of RRV to Europe would also cause serious social impact, from affecting the mental and physical health benefits associated with gardening (Soga *et al.*, 2017) to the loss of employment and income in the nursery industry and other associated sectors such as tourism which rely heavily on public gardens and attractive urban landscapes. The availability of rose

products with cultural importance like jam, rosehips, rose water, rose petals or flower buds is likely to be reduced.

Rose germplasm repositories and unique European rose germplasm collections will be threatened, such as the "Europa-Rosarium Sangerhausen" (Germany), which is the largest rose collection in the world and plays an important role as a source of budwood and support for research.

2.15 Conclusions

Roses have a significant cultural value for a number of European countries (EPPO, 2018), and are a valuable flower crop worldwide affected by a range of pathogens. RRV is a devastating mite-transmitted virus which could potentially be introduced into Europe. The first finding of RRV outside North America has triggered interest and raised concern. The introduction pathway for RRV to India remains unknown. In view of the intercontinental distance between RRV-infected countries and Europe, the virus or the vector is unlikely to be introduced by natural spread, but other pathways of entry are possible.

Creating awareness plays a critical role in preventing RRV establishment. Thus, European governments should inform stakeholders and interested parties about this virus, the disease that it causes, and the economic consequences, including members of the public. Simple tips to follow include spacing of plants to prevent mites crawling from plant to plant or implementing good hygiene measures to avoid spread (clean equipment before pruning, cleaning clothes, etc.). Breeders, nurseries and botanic gardens should be informed and aware of RRV and routine checks should be performed during the year. In the event of an outbreak, notification to the authorities to allow regulatory response must be prompt.

Controls within rose-trading countries are key to prevent the introduction of RRV. Early detection and surveillance programmes are necessary, because plants can have long latent periods, during which the mite vector can spread the virus. Regular inspection throughout the growing season with destruction of symptomatic plants appears to be the most effective control measure for RRV at present. Visual diagnosis of RRV requires serological or molecular confirmation during the early stages of infection (Fig. 2.5). Several diagnostic methods have been developed and incorporating these into early detection strategies is essential to intercept the virus and vector before it is able to establish. The different diagnostic methods available enable techniques to be chosen depending on the resources available for each laboratory (Babu *et al.*, 2018). All the techniques available are useful for detecting an outbreak, within the limit of detection and capacities of the assay. RT-qPCR is a good option, due to its high sensitivity compared with RT-PCR or ELISA. HTS has potential as a front-line diagnostic tool, in

particular for screening multiple virus infections in propagation material, but further research work is in process. RRV testing must be rapid to target non-symptomatic infections since these are common. In the case of an outbreak an eradication and tracing programme should be followed. First, suspect plants are to be tested to confirm the presence of RRV, and if positive, infested and adjacent plants (including the roots) are to be destroyed. Inspect for presence of the vector and forbid movement of rose plants from the site of the RRV outbreak. Precautions should be taken to avoid spreading the vector during the response (e.g. bagging plants before any manipulation to avoid dispersing the vector). In addition, nearby host plants must be treated with acaricides. If occurring in a glasshouse, the whole glasshouse should be disinfested (EPPO, 2018).

Following an outbreak, delimiting surveys of the area surrounding the infected plants are needed including visual surveys. Trace-forward and back analysis should be conducted to identify possible areas where infected plants might be present. Surveys of *Rosa* plants in late spring and summer should be performed each year and for at least 2 years after the outbreak of the first infection due to the long incubation period of RRV. Similar surveys are required for vector infestations; all surveys must be negative before declaring the outbreak eradicated. No *Rosa* spp. should be moved out of demarcated areas until the eradication is declared successful (EPPO, 2018).

Another measure to limit the spread of RRV is to import roses from a RRV and *P. fructiphilus* pest-free area (PFA), as England and Wales (UK) have now declared (Department for Environment Food and Rural Affairs, 2019). Other considerations should be taken, for example the packing conditions to prevent infestation by *P. fructiphilus* during transport, and pre- or post-entry quarantine period for at least one growing season. This should include visual inspection for RRV and *P. fructiphilus* and molecular testing for RRV.

2.16 Future Directions

Further research is needed to identify other possible RRV vectors or transmission pathways, as well improve understanding of RRV variability and diversity. The susceptibility of cut rose varieties needs to be assessed, as they could play an important role in the spreading of RRV and its vector if infection can occur. Resistant varieties adapted to European hardiness zones need to be developed and released to reduce the impact and spreading of RRV in advance. An effective educational programme is required to inform the general public and create awareness regarding RRV, all of which will help to develop a quick response in case of an RRV outbreak.

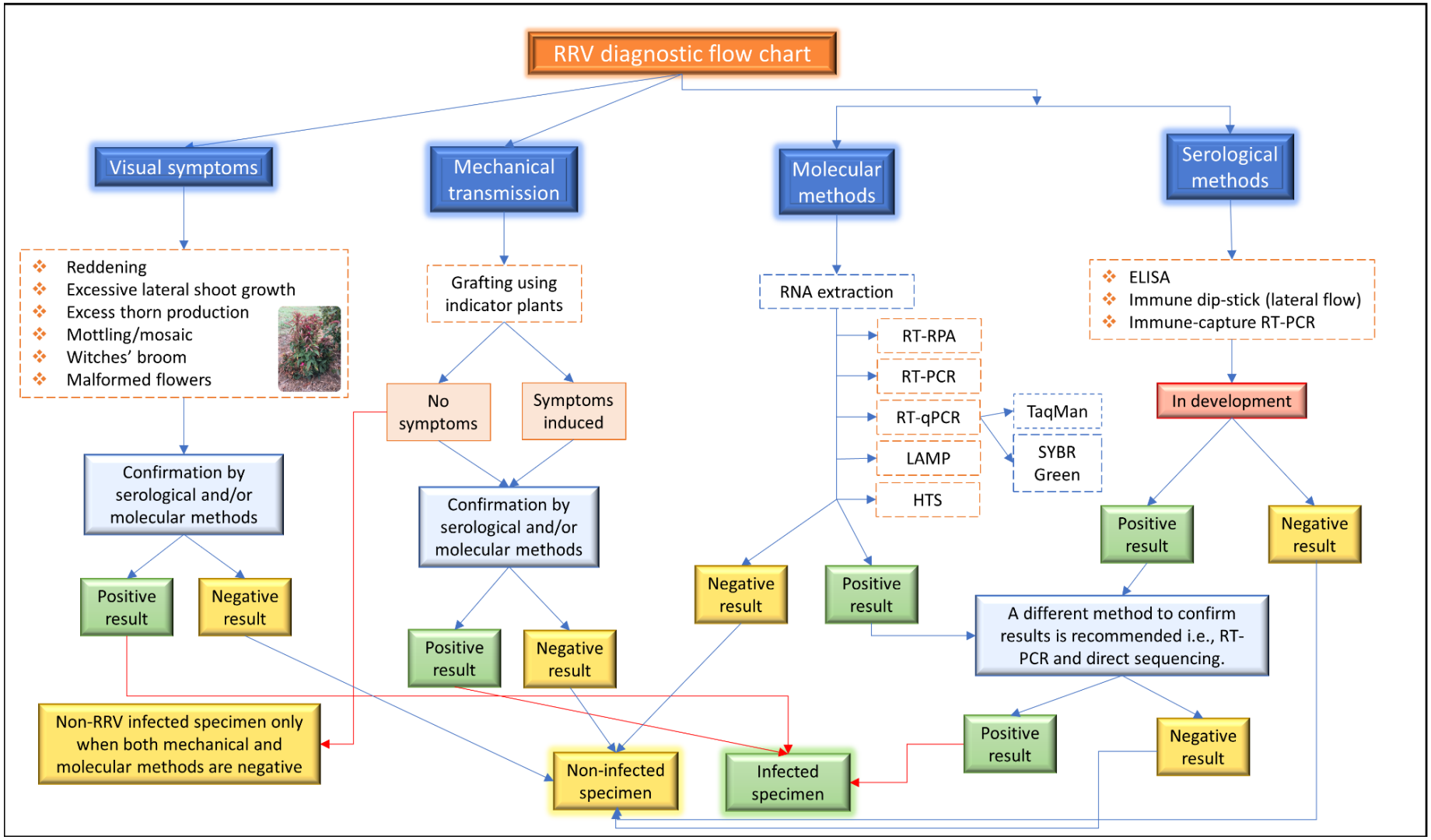

Figure 2.5- Flow chart representation of a purposed decision scheme for detection and the identification of rose rosette virus. This diagram was elaborated by the author.

Chapter 3 A Survey of Rose Viruses in the United Kingdom

Abstract

Roses (*Rosa* spp.) are valuable ornamental shrubs grown worldwide and are also the national flower of England. Despite the importance of their cultivation, the large contribution they make to the United Kingdom (UK) garden industry, and the spread of rose viruses, they have not been studied in detail in the UK since the 1980s. As a result, the most recent molecular methods for virus detection have not been employed to assess their disease status. In this study, a survey of rose viruses was performed using molecular and serological methods in the UK. The aims of the study include an update on the presence and incidence of rose viruses, analysis of symptoms and mixed infections, and identification of the viruses involved in rose mosaic disease, one of the most common diseases in roses worldwide. Previously reported viruses were found during this study, including strawberry latent ringspot virus (family *Secoviridae*), arabis mosaic virus (genus *Nepovirus*), and prunus necrotic ringspot virus (genus *Ilarvirus*), but also two novel viruses (rose cryptic virus-1 (genus *Partitivirus*) and rose spring dwarf-associated virus (genus *Luteovirus*)) and two viruses infecting roses for the first time in the UK (cucumber mosaic virus (genus *Cucumovirus*) and tomato spotted wilt virus (genus *Orthotospovirus*)).

3.1 Introduction

Roses (*Rosa* spp.) are ornamental shrubs grown around the globe and are susceptible to numerous pathogens, which require management and increase the cost of cultivation. The United Kingdom (UK) Department for Environment, Food & Rural Affairs (Defra) estimated disease caused losses of £630 million within UK ornamental plant production, of which £40 million could be attributed specifically to viral diseases (Department for Environment Food and Rural Affairs, 2016). Several viruses have been reported infecting roses in the UK. Those are arabis mosaic virus (ArMV; genus *Nepovirus*), strawberry latent ringspot virus (SLRSV; family *Secoviridae*), prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*), and apple mosaic virus (ApMV; genus *Ilarvirus*), either in single of mixed infections (Ikin and Frost, 1974; Thomas, 1980). Previously described symptoms include vein mottling, with or without chlorotic ring spots on the foliage and a reduction in vigour for ArMV (Thomas, 1980). For SLRSV, infected plants had shown strap-like leaves with small yellow angular flecks (Ikin and Frost, 1976), although they could also be asymptomatic or show symptoms similar to those caused by ArMV (Thomas, 1980). Ring spots, irregular chlorotic line patterns, mosaics, distortion and puckering were identified as being induced by PNRSV and ApMV (Fulton, 1952; Golino *et al.*, 2007). Variations in symptom expression are attributable to different factors including environment, virulence of virus strains, host genotype and the possible presence of undetected viruses (Thomas, 1984a).

Rose mosaic disease (RMD) is one of the most common diseases in roses worldwide. It has a complex pathology, being caused by single or mixed infections of SLRSV, ArMV, ApMV, and PNRSV. In the 1960s, ApMV was recognized as a synonym for RMV (Fulton, 1968). However, since then differences have been established between viruses involved in RMD occurring in North America and Europe, although PNRSV has been identified as the most frequent virus associated with this disease in both continents (Ikin and Frost, 1974; Horst and Cloyd, 2007). ApMV and PNRSV are commonly found in the USA causing RMD (Thomas, 1984a). SLRSV was the virus with most economic significance in the UK rose industry in the 1970s (Ikin and Frost, 1974). However, it was not reported in the USA until three decades later (Martin *et al.*, 2004). Other nepoviruses have been considered as part of the RMD complex, such as tobacco ringspot virus (TRSV; genus *Nepovirus*) and tomato ringspot virus (ToRSV; genus *Nepovirus;* (Horst and Cloyd, 2007). RMD is believed to have originated by grafting from infected rootstocks or scions, and subsequent spread between rose cultivars (Sertkaya, 2010). Viruses implicated in RMD have been considered to be transmitted by seed, pollen, aphids, thrips, contaminated soil or by pruning tools, but no conclusive scientific evidence is

available regarding transmission routes (Horst and Cloyd, 2007). Golino *et al.* (2007) showed evidence of ApMV and PNRSV transmission via roots between roses grown close together in experimental fields.

All these viruses cause a range of symptoms recognized as RMD (chlorotic line patterns, ring spots, mottles in leaves, yellow net, mosaic, etc.). Infected plants are less vigorous, with a reduction in the number of flowers and length of their stems (Manners, 1998), and the plants are more likely to die over winter (Horst *et al.*, 1983). Asymptomatic plants have been identified for all the viruses involved in RMD (Thomas, 1980; Sertkaya, 2010). In the 1970s it was known that ArMV and SLRSV cause similar symptoms to PNRSV in mixed infections (Thomas, 1980). Since that time, visual diagnostics were understood to be unreliable, not only for the identification of asymptomatic species, but due to the similarity of symptoms between viruses in single and mixed infections. Diagnosis is fundamental to facilitate the management of plant diseases (van der Want and Dijkstra, 2006; Aboul-Ata *et al.*, 2011) and early detection is essential for successful eradication campaigns. Despite the widespread nature of rose viruses and their importance in cultivation, they have not been studied in detail in the UK since the 1980s (Thomas, 1984a), and as a result molecular methods for virus detection have rarely been deployed to study them.

In this study, molecular and serological methods such as ELISA, RT-qPCR (TaqMan) and high-throughput sequencing (HTS) have been used to perform a survey of rose viruses in the UK. The aim was to study the presence and incidence of viruses infecting roses, as single and mixed infections, and their related symptoms, as well as clarify the viruses involved in RMD in the UK.

3.2 Materials and Methods

A total of 237 samples were collected during 3 consecutive years (2017-2020) as part of a survey of viruses in the UK (Table A.2). Samples consisted of leaves from different parts of the plant, from symptomatic and asymptomatic roses from the UK. Symptoms were consistent with virus infections such as mottling, yellow veining, distortion, and ring spots. Samples were conserved at -20 °C before nucleic acid extraction.

ELISA analysis was performed for a range of viruses using commercially available kits following the manufacturer's instructions, as follows: alfalfa mosaic virus (AMV; BIOREBA AG, Switzerland), ArMV (Leibniz Institute DSMZ GmbH, Germany), ApMV (Loewe Biochemica GmbH, Germany), cucumber mosaic virus (CMV; Agdia Inc., US), impatiens necrotic spot virus (INSV; BIOREBA AG, Switzerland), PNRSV (Loewe Biochemica GmbH, Germany), raspberry ringspot virus (RpRSV; Leibniz Institute DSMZ GmbH, Germany),

SLRSV (BIOREBA AG, Switzerland), TRSV (Agdia Inc., US) and tomato spotted wilt virus (TSWV; Leibniz Institute DSMZ GmbH, Germany). Samples were ground and 5 ml of 2% PVP in PBST buffer (phosphate-buffered saline with Tween detergent) was added immediately after. In each case a negative control corresponding to the same host plant species as the test sample was used. These were previously tested for the same viruses to determine that they were negative. The result was considered positive when the absorbance at 405 nm after 1 h for a given sample was greater than 3× the mean absorbance of the corresponding negative control. Each sample was tested in duplicate.

RNA was extracted from the leaf samples either by using a magnetic bead-based extraction method and the KingFisher® mL platform (Thermo Scientific) or using a CTAB (cetyl trimethylammonium bromide) method as described by Adams *et al.* (2009), except that the incubation with 4 M LiCl was performed overnight at 4 °C

Cycling for RT-qPCR was performed on extracted RNA in 96 well plates using either a Viia7, ABI7500 or QuantStudio (Applied Biosystems). Negative controls where water replaced template were included in all runs and all samples were tested in duplicate. Results were scored positive if a C_T value <40 was recorded in both duplicates. Nucleic acid samples were tested using an RT-qPCR simplex assay designed to amplify the plant cytochrome c oxidase (COX) subunit I gene (Weller *et al.*, 2000; Hughes *et al.*, 2006) to verify the nucleic acid extractions. For the COX assay and ApMV, CMV, INSV, TRSV, ToRSV, TSWV, rose cryptic virus-1 (RoCV1), rose rosette virus (RRV), and tobacco rattle virus (TRV) reactions consisted of iTaq One-step (2x), iScript™ reverse transcriptase (Bio-Rad), 375 nM of each primer (Table A.3), 125 nM of probe and 1 µl of extracted RNA (concentration as extracted) in a final reaction volume of 20 µl. The cycling conditions used were: 10 min at 50 °C, 2 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions for ArMV, PNRSV, RpRSV, and SLRSV contained qScriptXLT 1-Step ToughMix 2X (QUANTAbio) and 2 µl of extracted RNA (concentration as extracted) in a final reaction volume of 20 µl. For SLRSV and ArMV reactions contained 375 nM of each primer (Table A.1) and 125 nM of probe, and for PNRSV and RpRSV 112.5 nM primers and 37.5 nM probe respectively (Table A.3). The cycling conditions used were 10 min at 50 °C, 3 min at 95 °C, then 40 cycles of 10 s at 95 °C and 1 min at 60 °C.

Cycling for rose-spring dwarf associated virus (RSDaV) RT-PCR was performed using a 2720 Thermal Cycler (Applied Biosystems). RT-PCR was performed using Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific), 0.5 µl of RevertAid RT (Thermo Fisher Scientific), 333 nM of previously published primers (Salem *et al.*, 2008a) and 1 µl extracted

RNA in a final volume of 30 µl. Cycling conditions were 30 min at 40 °C, 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 1 min followed by a final extension of 10 min at 72 °C and a hold step of 4 °C. The expected product was 418 bp estimated using agarose gel electrophoresis and Quick-Load® Purple 1 kb Plus DNA Ladder (NEB).

3.3 Results

3.3.1 Identified viruses: single and mixed infections

A survey of viruses infecting roses was carried out in the UK. This involved testing a total of 237 samples for 15 different viruses during 3 consecutive years (2017-2020). In the first year (2017-2018), ELISA was used to test 85 samples for alfalfa mosaic virus (AMV; genus *Alfamovirus*), ApMV, ArMV, cucumber mosaic virus (CMV; genus *Cucumovirus*), impatiens necrotic spot virus (INSV; genus *Orthotospovirus*), PNRSV, raspberry ringspot virus (RpRSV; genus *Nepovirus*), SLRSV, TRSV, and tomato spotted wilt virus (TSWV; genus *Orthotospovirus*). In subsequent years (2018-2020) RT-qPCR (TaqMan) was incorporated and testing for rose cryptic virus-1 (RoCV1; family *Partitiviridae*), rose rosette virus (RRV; genus *Emaravirus*), ToRSV, and tobacco rattle virus (TRV; genus *Trobravirus*) was also done. RT-PCR was also used to test for rose spring-dwarf associated virus (RSDaV; genus *Luteovirus*). A further 34 samples were analysed using HTS, with the aim of comparing the different diagnostic methods (Chapter 5). In total, 170 samples (71.73%) were positive for at least one of the seven viruses tested for during the surveys. Three of those viruses were previously reported in the UK. These were ArMV (36.70% incidence), SLRSV (27.00%) and PNRSV (9.28%), all involved in RMD. Two novel viruses were found in the UK: RoCV1 (54.85%) and RSDaV (1.17%). In addition, two viruses were identified for the first time infecting roses in the UK: CMV (3.80%) and TSWV (1.27%). Mixed infections were commonly seen (Fig. 3.1) involving more than one of the viruses identified during this survey.

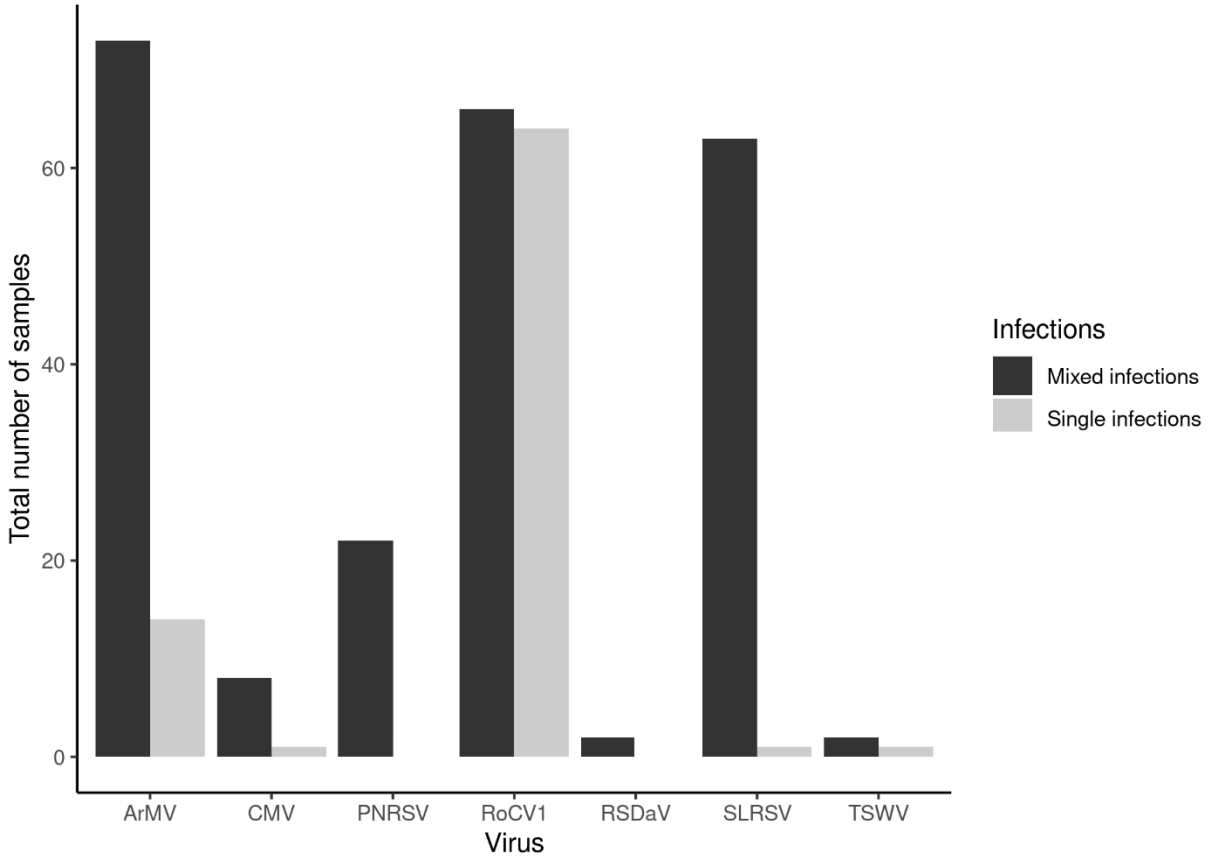

Figure 3.1- Total number of single and mixed infections for each of the viruses found during the survey.

In this study, SLRSV was found in 64 of the 237 samples tested (27.00%). SLRSV was identified in a single infection in only one sample. However, it was found coinfecting a sample with other viruses on numerous occasions (63 samples; Table 3.1). It was commonly detected in mixed infections with ArMV (12 samples); ArMV+RoCV1 (16 samples); or ArMV+RoCV1+PNRSV (14 samples). ArMV was found in 87 of 237 tested samples (36.70%), and was the second most prevalent virus after RoCV1. It was a single infection in 14 samples and coinfected with other viruses in 73. It was commonly found with RoCV1 (15 samples); SLRSV (12 samples); SLRSV+RoCV1 (16 samples); or SLRSV+RoCV1+PNRSV (14 samples).

RoCV1 was found for the first time in the UK (Vazquez-Iglesias *et al.*, 2019) in 130 of 237 tested samples (54.85%). Of the infected samples, 64 were infected with RoCV1 alone (49.23%), whilst 66 were found in mixed infections, mainly with SLRSV (10 samples); ArMV (17 samples); with both viruses (16 samples); or with SLRSV+ArMV+PNRSV (14 samples). PNRSV was found in 22 of 237 tested samples (9.28%), always in mixed infections (Table 3.1).

The most common coinfections involving PNRSV were with with SLRSV+ArMV (4 samples) or including RoCV1 (14 samples).

TSWV was found infecting 3 of the 237 tested samples (1.27%). One sample was a single infection, whilst 2 were coinfected with SLRSV+ArMV+RoCV1+CMV. In the case of CMV, it was detected in 9 of the 237 samples (3.80%). A singly infected sample was identified, and mixed infections with RoCV1 (2 samples), ArMV+RoCV1 (1 sample) and also including SLRSV+TSWV were detected for the remainder (Table 3.1). RSDaV was found for the first time in the UK (Vazquez-Iglesias *et al.*, 2020b) infecting 2 samples (1.17%) of 171 tested for this virus. It was found in conjunction with ArMV (1 sample) and RoCV1 (1 sample).

Table 3.1- Single and mixed infections found during the rose virus survey and related symptoms identified in the infected samples. Arabis mosaic virus (ArMV); cucumber mosaic virus (CMV); prunus necrotic ringspot virus (PNRSV); rose cryptic virus-1 (RoCV1); rose spring dwarf-associated virus (RSDaV); strawberry latent ringspot virus (SLRSV); and tomato spotted wilt virus (TSWV).

Virus present	Number of samples	Asymptomatic	Symptomatic									
			Yellow netting and vein banding	Ring spots and/or oak leaf pattern	Mosaic	Mottling	Chlorosis	Rings	Central vein chlorosis	Distortion	RRV-like symptoms	Clear yellow spots, pink leaves, think texture
RoCV1	64	**48**	0	0	0	**4**	0	0	0	0	**10**	**2**
ArMV+RoCV1	17	**7**	**6**	0	0	**1**	0	0	0	**2**	0	**1**
SLRSV+ArMV+RoCV1	16	0	**12**	**1**	**1**	**2**	0	0	0	**1 (and mottling)**	0	0
ArMV	14	**7**	**3**	0	**1**	**2**	0	0	0	**1**	0	0
SLRSV+ArMV+RoCV1+PNRSV	14	**1**	**6**	0	**1**	**3**	**2**	**1**	0	**1 (and yellow netting)**	0	0
SLRSV+ArMV	13	**2**	**7**	0	**1**	**1**	0	**1**	**1 (and yellow netting)**	0	0	0
SLRSV+RoCV1	10	**2**	**2**	0	**2**	**3**	**1**	0	0	0	0	0
SLRSV+ArMV+PNRSV	4	0	0	**1**	**1**	**1**	**1**	0	0	0	0	0
CMV+RoCV1	2	**1**	0	0	0	0	0	0	0	0	**1**	0
SLRSV+ArMV+RoCV1+TSWV+CMV	2	0	**1**	0	0	0	0	0	1	0	0	0
SLRSV	1	0	**1**	0	0	0	0	0	0	0	0	0
CMV	1	**1**	0	0	0	0	0	0	0	0	0	0
TSWV	1	**1**	0	0	0	0	0	0	0	0	0	0
ArMV+PNRSV	1	0	**1**	0	0	0	0	0	0	0	0	0
ArMV+RSDaV	1	**1**	0	0	0	0	0	0	0	0	0	0
RSDaV+RoCV1	1	0	**1**	0	0	0	0	0	0	0	0	**1 (and yellow netting)**
ArMV+RoCV1+CMV	1	**1**	0	0	0	0	0	0	0	0	0	0
ArMV+RoCV1+PNRSV	1	0	**1**	0	0	0	0	0	0	0	0	0
SLRSV+ ArMV+CMV	1	0	**1**	0	0	0	0	0	0	0	0	0
SLRSV+ RoCV1+PNRSV	1	0	0	0	0	**1**	0	0	0	0	0	0
SLRSV+ArMV+ PNRSV+CMV	1	0	**1**	0	0	0	0	0	0	0	0	0
SLRSV+ArMV+RoCV1+CMV	1	0	**1**	0	0	0	0	0	0	0	0	0

3.4 Description of Symptoms

Plants coinfected with multiple viruses showed variable symptoms. However, they were similar between the different virus combinations. Yellow netting and vein banding (Fig. 3.2) were the most common symptoms found (47 samples). These symptoms are recognised as RMD symptoms, but it is still a challenge to identify which viruses are involved, as single but also multiple mixed infections have been identified causing them (Table 3.1). These symptoms were identified in samples singly infected with SLRSV (1 sample; Fig. 3.3A), ArMV (3 samples; Fig. 3.4B), and in most of the plants coinfected with multiple viruses (Table 3.1; Fig. 3.5): ArMV+PNRSV (1 sample); SLRSV+RoCV1 (2 samples); SLRSV+ArMV (7 samples); ArMV+RoCV1 (6 samples); SLRSV+ArMV+RoCV1 (12 samples); ArMV+RoCV1+PNRSV (1 sample); SLRSV+ArMV+CMV (1 sample); SLRSV+ArMV+RoCV1+PNRSV (6 samples); SLRSV+ArMV+PNRSV+CMV (1 sample); SLRSV+ArMV+RoCV1+CMV (1 sample); SLRSV+ArMV+RoCV1+TSWV+CMV (1 sample). The only exceptions that did not show these symptoms were single infections with RoCV1, TSWV or CMV, and plants infected with SLRSV+ArMV+PNRSV (4 samples); ArMV+RSDaV (1 sample); SLRSV+ RoCV1+PNRSV (1 sample, Fig. 3.7); CMV+RoCV1 (2 samples); or ArMV+RoCV1+CMV (1 sample).

Figure 3.2- Vein banding (A) and yellow netting (B) commonly found in infected roses in the United Kingdom. Pictures were taken by the author.

Figure 3.3 -Samples (A) infected with strawberry latent ringspot virus (SLRSV) showing yellow netting and (B, C, D) infected with SLRSV and rose cryptic virus-1 showing Rose mosaic disease symptoms. Pictures were taken by the author.

Ring spots and oak leaf pattern symptoms (Fig. 3.6A) were found in plants with mixed infections of SLRSV+ArMV+PNRSV/RoCV1 (1 sample respectively). Mosaic symptoms were seen on the leaves of samples singly infected with ArMV (1 sample) or in mixed infections with SLRSV+ArMV (1 sample); SLRSV+RoCV1 (2 samples); and SLRSV+ArMV+PNRSV/RoCV1 (1 sample respectively, Fig. 3.8A,C) or mixed infections with the four viruses (1 sample; Fig. 3.9). Mottling was seen on leaves of plants with single infections of ArMV (2 samples, with distortion; Fig. 3.4A); RoCV1 (4 samples); SLRSV+ArMV (1 sample); SLRSV+ArMV+RoCV1 (2 samples, one with distorted leaves; Fig. 3.8B); SLRSV+RoCV1 (3 samples); SLRSV+ArMV+RoCV1+PNRSV (3 samples, one with distorted leaves); SLRSV+ArMV+PNRSV (1 sample); SLRSV+ RoCV1+ PNRSV (1 sample; Fig. 3.7); and ArMV+RoCV1 (1 sample).

Figure 3.4-Samples infected with arabis mosaic virus showing (A) yellowing and distortion, and (B) yellow netting. Pictures were taken by the author.

Figure 3.5-. Samples showing yellow netting and vein banding, Rose mosaic disease symptoms, when infected with (A, B) SLRSV+ArMV+RoCV1+TSWV+CMV, (C) ArMV+RoCV1+PNRSV, (D) PNRSV+ArMV, and (E) SLRSV+ArMV+CMV. Arabis mosaic virus (ArMV); cucumber mosaic virus (CMV); prunus necrotic ringspot virus (PNRSV); rose cryptic virus-1 (RoCV1); strawberry latent ringspot virus (SLRSV); and tomato spotted wilt virus (TSWV). Pictures were taken by the author.

Figure 3.6-Samples infected with strawberry latent ringspot virus+arabis mosaic virus+prunus necrotic ringspot virus showing (A) oak leaf pattern (indicated by red circles) and (B) chlorosis. Pictures were taken by the author.

Figure 3.7- Mottling on an infection with strawberry latent ringspot virus+rose cryptic virus-1+prunus necrotic ringspot virus. Picture was taken by the author.

Chlorosis was identified in mixed infections of SLRSV+RoCV1 (1 sample); SLRSV+ArMV+PNRSV (1 sample); and also including RoCV1 (2 samples; Fig. 3.6B). Yellow rings at the end of the leaves were seen in one sample infected with SLRSV+ArMV+RoCV1+PNRSV and also in a sample with yellow netting and vein banding infected with ArMV+SLRSV. One of the samples infected with SLRSV+ArMV showed yellow netting and vein banding, but also a yellow central vein was distinguished in some of the leaves (1 sample, Fig. 3.8D). This central vein was also recognised in a SLRSV+ArMV+RoCV1+TSWV+CMV infection (1 sample; Fig. 3.5A). Some samples showed distortion when infected with ArMV alone (1 sample), and with ArMV+RoCV1 infection (2 samples; one of them with mottling). In addition, one sample infected with ArMV+RoCV1 showed yellow netting and vein banding and also had some unidentified symptoms including clear yellow spots, thin texture and a pink colour in the leaves, but they were not believed to be caused by a viral infection. The same plant appeared asymptomatic the following year, therefore symptoms were probably caused by environmental conditions or phytotoxicity. Those unusual symptoms were also identified in a single sample infected with RoCV1+RSDaV and 2 samples single infected with RoCV1.

Figure 3.8- Samples infected with SLRSV+ArMV+RoCV1 showing (A) mosaic, (B) mottling and (C) patching. Sample (D) was infected with SLRSV+ArMV, displaying a yellow middle vein. Arabis mosaic virus (ArMV); rose cryptic virus-1 (RoCV1); and strawberry latent ringspot virus (SLRSV). Pictures were taken by the author.

Samples (10) singly infected with RoCV1 and RoCV1+CMV (1 sample) were tested by RT-qPCR because they had similar symptoms to those caused by RRV. However, none of them gave positive results with any tests performed, and the symptoms were probably caused by herbicide damage.

Figure 3.9-Sample (A, B) showing mosaic, infected with strawberry latent ringspot virus+arabis mosaic virus+rose cryptic virus-1+prunus necrotic ringspot virus. Pictures were taken by the author.

Asymptomatic samples were found infected with ArMV (7 samples), RoCV1 (48 samples), TSWV (1 sample) and CMV (1 sample) and in addition, in mixed infections with SLRSV+RoCV1 (2 samples), SLRSV+ArMV (2 samples); SLRSV+ArMV+RoCV1+PNRSV (1 sample); ArMV+RoCV1 (7 samples); ArMV+RSDaV (1 sample); ArMV+RoCV1+CMV (1 samples); and CMV+RoCV1 (1 sample).

3.5 Discussion

A survey was performed to determine the occurrence and prevalence of some of the main viruses known to infect roses in the UK. Previously reported viruses were found during this study, including SLRSV, ArMV, and PNRSV, but also two novel viruses (RoCV1 and RSDaV) and two viruses infecting roses for the first time in the UK (TSWV and CMV). These results confirm that there has been under-reporting of known viruses in roses in the UK. Both SLRSV and ArMV are the viruses with the highest prevalence in the UK in conjunction with RoCV1, and probably the ones causing the biggest impact in the UK rose industry as was believed to happen with SLRSV in the 1970s (Ikin and Frost, 1974).

RoCV1 was found for the first time in the UK (Vazquez-Iglesias *et al.*, 2019)**.** This virus has been reported in the USA (Martin and Tzanetakis, 2008; Sabanadzovic and Ghanem-Sabanadzovic, 2008; Lockhart *et al.*, 2011), Canada (James *et al.*, 2015), and New Zealand (Milleza *et al.*, 2013). There is no known natural vector for RoCV1; it is assumed to be pollen- and seed-transmitted like other cryptoviruses. RoCV1 is generally considered not to cause

symptoms, although very mild symptoms have been described (Milleza *et al.*, 2013). Therefore, it is probable that RoCV1 has spread during commercial trade of planting material. RSDaV has previously been found in the USA (Salem *et al.*, 2008a), Chile (Rivera and Engel, 2010), and New Zealand (Milleza *et al.*, 2013). This is the first report of RSDaV in the UK and in Europe. As described by Rivera and Engel (2010), RSDaV was found in plants with yellow vein chlorosis (RMD symptoms), as seen in other mixed infections in this study. However, in this study one sample was asymptomatic and the other one showed symptoms believed not to be caused by viral infection. RSDaV is transmitted by aphids, and previous studies identified the rose-grass aphid (*Metapolophium dirhodum* Walker) and yellow rose aphid (*Rhodobium porosum* Sanderson) as vectors (Salem *et al.*, 2008a). *M. dirhodum* is present in the UK (CABI, 2020b) and *R. porosum* is widely distributed across Europe (Müller and Steiner, 1988; Barjadze *et al.*, 2011). Thus, commercial trade could explain again the arrival of RSDaV to the UK**.** TSWV was previously found infecting roses in Iran (Moini and Izadpanah, 2000). It is transmitted by *Frankliniella occidentalis* (Pergande), a widespread thrips species that has caused damage on both agricultural and horticultural crops over the last five decades (Baker *et al.*, 1993; Kirk and Terry, 2003). CMV it is a widespread virus as it is its most efficient vector, *Myzus persicae* Sulzer (Babu *et al.*, 2017b; CABI, 2020a). CMV is transmitted by 80 aphid species and has a wide range of hosts (1200 species), including *Rosa* spp. (Shi *et al.*, 2016). It is not surprising to find roses infected with TSWV and CMV in the UK, as both viruses and respective vectors are present in the country (CABI, 2020a).

RMD is one of the most common diseases in roses worldwide. Previous reports identified PNRSV as the most frequent virus associated with RMD in North America and Europe (Ikin and Frost, 1974; Horst and Cloyd, 2007), but PNRSV was not the virus with the highest incidence in this study (9.28%). Results indicate that both SLRSV and ArMV are the main viruses causing RMD in the UK, and that yellow netting and vein banding are the most common symptoms found. Different mixed infections have shown these symptoms in at least one infected sample (Table 3.1), excluding SLRSV+ArMV+PNRSV, SLRSV+RoCV1+PNRSV, ArMV+RoCV1+CMV, CMV+RoCV1, and ArMV+RSDaV. It is possible that PNRSV might have an influence in mixed infections, as in infections with SLRSV+ArMV+PNRSV (4 samples) and SLRSV+RoCV1+PNRSV (1 sample) these symptoms were not observed. However, in other mixed infections with ArMV and PNRSV, or including RoCV1, RMD symptoms were seen. The absence could be related to an interaction with SLRSV, however other mixed infections including SLRSV and PNRSV have shown these symptoms (e.g. SLRSV+ArMV+RoCV1+PNRSV). As RoCV1 is believed not to cause symptoms and its

influence in mixed infections is also unknown, it is probable that this fact it is a coincidence. In addition, the number of samples found with these infections is limited and may not be representative. It remains difficult to identify which symptoms are caused by the different viruses, as mixed infections have caused similar symptoms in the plants. Furthermore, symptoms are often confused with other pest problems, herbicide damage, or nutrient deficiencies. Asymptomatic infected plants have been identified for both single and mixed infections. This could reflect an adaptation of the host. Different samples (12) were identified as long-lived roses and despite being infected with different viruses, they did not show any symptoms, which may support this theory.

AMV, INSV, RpRSV, RRV, ToRSV, TRSV, and TRV were not found during this survey. ApMV, also involved in RMD, was not found in the UK, but as described in Chapter 6, it was found in samples imported into the UK. One of the reasons why this survey took place was to investigate the presence of RRV in the UK. RRV is a devastating virus that has spread in the USA and Canada since the 1940s (Conners, 1941) and was recently reported in India (Chakraborty *et al.*, 2017). It is an emerging risk to European and worldwide rose cultivation, which will have significant social, economic, and environmental impacts if it establishes and spreads in Europe (Vazquez-Iglesias *et al.*, 2020a). It was not found during the survey; plants with suspected symptoms were found not to be infected with RRV and it was hypothesised that the symptoms may have been as a result of exposure to herbicide (Hong *et al.*, 2012).

Mixed infections were commonly found during the survey (Fig. 3.1). The most common mixed infections found involved ArMV and SLRSV in conjunction with RoCV1, but also the three viruses with PNRSV. Mixed infections allow diverse and complex interactions among viruses (Díaz-Muñoz, 2019). Previously reports have shown that naturally occurring double or triple infections can cause devastating synergistic diseases, such as maize lethal necrosis disease (Adams *et al.*, 2017), although not all the relationships between viruses are synergistic, e.g. helper dependence and antagonistic virus interactions may occur (Syller, 2012). No evidence was found during the survey to suggest that a synergistic relationship was occurring between the different viruses in mixed infections in roses. Reports of mixed infections have increased in the last two decades (1998–2019; Alcaide *et al.*, 2020); for reports since 2009 this could be due in part to the widespread use of HTS methods which uncover previously unknown mixtures of viruses.

Chapter 4 New Viruses

Abstract

In this chapter are described two novel viruses identified in the United Kingdom for the first time: rose cryptic virus-1 (genus *Partitiviridae*) and rose spring-dwarf associated virus (genus *Luteovirus*). In addition, the identification of a new virus species from imported roses, tentatively named 'rosa ilarvirus-1' (genus *Ilarvirus*) is described.

Published in:

Vazquez-Iglesias, I., Adams, I.P., Hodgetts, J., Fowkes, A., Forde, S., Ward, R., Buxton-Kirk, A., Kelly, M., Santin-Azcona, J., Skelton, A., Harju, V., Boonham, N., Robinson, R., Clover, G. and Fox, A. (2019) 'High throughput sequencing and RT-qPCR assay reveal the presence of rose cryptic virus-1 in the United Kingdom', *Journal of Plant Pathology*, 101(4), pp. 1171-1175.

Vazquez-Iglesias, I., Scrace, J., McGreig, S., Pufal, H., Robinson, R., Clover, G.R.G., Adams, I., Boonham, N. and Fox, A. (2020) 'First report of Rose spring dwarf-associated virus in *Rosa* spp. in United Kingdom', *New Disease Reports*, 42(13).

Submitted to *Journal of Virological Methods*:

Vazquez-Iglesias, I., McGreig, S., Pufal, H., Robinson, R., Clover, G.R.G., Fox, A, Boonham, N. and Adams, I.P. (2021) A novel high-throughput sequencing approach reveals the presence of a new virus infecting *Rosa*: rosa ilarvirus-1 (RIV-1).

4.1 High Throughput Sequencing and RT-qPCR Assay Reveal the Presence of Rose Cryptic Virus-1 in the United Kingdom

Roses (*Rosa* spp.) are one of the most important ornamental crops worldwide. They are grown for their desirable aesthetics and scent for gardens and landscaping, and their use in industrial products (Dobhal *et al.*, 2016). The garden industry contributes £9 billion to the UK economy every year. The UK's Department for Environment, Food & Rural Affairs (Defra) valued ornamental plant production at £1.1 billion in 2015, with an estimated disease loss of £630 million within UK ornamental plant production of which £40 million could be attributed specifically to viral diseases (Department for Environment Food and Rural Affairs, 2016).

A number of viruses have been reported affecting roses in the UK including arabis mosaic virus (ArMV; genus *Nepovirus*), prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*) and strawberry latent ringspot virus (SLRSV; family *Secoviridae;* (Thomas, 1984b). Rose mosaic disease, one of the most common diseases in roses, is caused by single or mixed infections of these viruses, and others such as apple mosaic virus (ApMV; genus *Ilarvirus*). Symptoms include chlorotic line patterns, ring spots, mottles in leaves, yellow net and yellow mosaic. Infected plants are less vigorous and more likely to die during winter (Horst *et al.*, 1983).

Rose cryptic virus-1 (RoCV1), also known as rosa multiflora cryptic virus (Martin and Tzanetakis, 2008), is a partitivirus, related to other species in the genus *Alphacryptovirus*¸ but not yet classified within this genus (King *et al.*, 2012). Partitivirus genomes are generally composed of two monocistronic dsRNA segments of 1.4-3.0 kbp, which are encapsidated in isometric particles about 30-40 nm in diameter. The larger RNA encodes a viral RNA-dependent RNA polymerase (RdRp), and the smaller one encodes a coat protein (CP; (King *et al.*, 2012). However, RoCV1 has been shown to contain a third dsRNA segment (Sabanadzovic and Ghanem-Sabanadzovic, 2008), the origin of which is not clear, though several hypotheses have been published: (i) it may represent a co-infection by two different partitiviruses, (ii) the virus may possess two different versions of dsRNA2, or (iii) the third dsRNA element may represent a satellite virus (Nibert *et al.*, 2014). Cryptoviruses are reported to be widespread in nature and have escaped detection for many years because most of them cause no visible symptoms or, in a few situations, very mild symptoms (Hull, 2014). RoCV1 has been found in asymptomatic plants as well as plants with mottling, leaf spots and necrosis (Milleza *et al.*, 2013), as well as in mixed infections with rose spring dwarf-associated virus (Salem *et al.*, 2008b) or rose rosette virus (Martin and Tzanetakis, 2008). In common with other partitiviruses there are no known natural vectors for RoCV1; it cannot move from cell to cell but can be transmitted by pollen and seed (Boccardo *et al.*, 1987). It was first reported in the USA and it

is thought to be widespread there, being reported from several locations (Martin and Tzanetakis, 2008; Sabanadzovic and Ghanem-Sabanadzovic, 2008; Lockhart *et al.*, 2011). Subsequently it has been reported in New Zealand, where it is the most prevalent virus infecting roses (Milleza *et al.*, 2013), and in Canada (James *et al.*, 2015). This is the first report describing the detection of RoCV1 in rose in the United Kingdom.

Rose samples were submitted to the plant clinic at Fera Science Ltd (York, UK) in 2007 (LS11S16) and 2012 (LS13S9), suspected of having a viral infection based on symptoms: distortion of new growth, and wrinkled and streaked leaves. ELISA analysis was performed for ArMV, PNRSV, ApMV, SLRSV and impatiens necrotic spot virus (INSV), as described in Material and Methods section of Chapter 3. Sample LS11S16 gave a positive result for ApMV and sample LS13S9 gave a positive result for SLRSV.

Subsequently, high-throughput sequencing (HTS) using nucleic acid extracted from samples LS11S16 and LS13S9 was performed. Ribosome-depleted indexed sequencing libraries were prepared from extracted RNA using the ScriptSeq complete plant leaf kit (Illumina, US) which were then sequenced on an Illumina MiSeq instrument using a V3, 2x300 cycle run kit (Illumina, US). Sequence reads were trimmed for quality using Sickle (Joshi and Fass, 2011), assembled using Trinity (Grabherr *et al.*, 2011) and then compared to the GenBank nr and nt databases using BLAST+ (Camacho *et al.*, 2009). Reads of viral origin were inspected using MEGAN community edition (Huson *et al.*, 2016). Sequences of RoCV1 were detected in both samples: for LS11S16, the total number of reads was 654746 and 1893 mapped to RoCV1 (GenBank Accession numbers MK075821, MK075822, MK075823, MK075824, MK075825). For LS13S9, the total number of reads was 317946 and 62 mapped to RoCV1 (GenBank Accession numbers MK075826, MK075827, MK075828).

As part of a survey of rose viruses in the UK, 251 leaf samples were collected in autumn 2017 and spring/early summer 2018 from roses in the Royal Horticultural Society gardens at Harlow Carr (Harrogate, North Yorkshire, UK) and Wisley (Woking, Surrey, UK). In addition, samples of rose leaves were taken from a nursery in the Midlands, a public garden near London, the Royal National Rose Society Garden (St Albans, UK) and from samples submitted to the plant clinic at Fera Science Ltd. (York, UK).

ELISA analysis was performed for the same viruses as described above, and also tested for the following: alfalfa mosaic virus (AMV), raspberry ringspot virus (RpRSV), cucumber mosaic virus (CMV), tomato spotted wilt virus (TSWV), and tobacco ringspot virus (TRSV). ELISA results showed that six samples were infected with SLRSV (2.4%); ten samples were infected with ArMV (4%); and three samples with both viruses (1.2%).

To further investigate the incidence of RoCV1 in the UK, primers and a probe for the specific detection of RoCV1 by RT-qPCR (TaqMan) were designed (RoCV1-2-Fw-5′-TGATCGACCAAAGTTGCAACC-3′/RoCV1-2-Rv-5′-GAAGATAAGACAATGCAGTCA CTTTCTT-3′/RoCV1-2-Pe-5′-FAM-ATTCGGACTGAATTTGCTA-MGBNFQ-3′; 110 bp) using Primer Express 3.0.1 (Applied Biosystems) in regions conserved within the RoCV1 genome (dsRNA1 segment) and divergent from other species. Sequences used for assay design included those generated using HTS, and sequences obtained from GenBank® of target (NC_010346, KM598758.1, JX492318.1, EU413666.1, EU350962.1, EU024675.1) and non-target viruses (fragaria chiloensis cryptic virus, DQ093961.2), to avoid non-specific amplification of sequences from closely related viruses.

Samples collected for the survey were analysed using the new assay as described in the Material and Methods section of Chapter 3. Nucleic acid was extracted from the leaf samples using a magnetic bead-based extraction method and the KingFisher® mL platform (Thermo Scientific). Nucleic acid samples were also tested using an RT-qPCR simplex assay designed to the plant COX1 gene (see Material and Methods Chapter 3).

Previously published primers for the dsRNA2 segment (Sabanadzovic and Ghanem-Sabanadzovic, 2008) were used to confirm the results obtained by RT-qPCR. A two-step RT-PCR was performed on a single sample, 7B3, which tested positive for RoCV1 dsRNA1 using RT-qPCR. Generation of cDNA was performed using SuperScript™ II Reverse Transcriptase (Thermo Scientific) following the manufacturer's instructions. cDNA (10% by volume) was added in a total volume of 30 µl, containing dNTP mix, 5x Phusion ® HF Buffer, Thermo Scientific® Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) and primers (300 nM**).** Cycling was done using a C1000™ Thermal Cycler (Bio-Rad) as follows: 30 min at 40℃, 5 min at 94℃, followed by 40 cycles of 30 s at 94℃, 30s at 50℃ and 45s at 72℃, and a final extension step of 72℃ for 10 min.

Using agarose gel electrophoresis, a PCR product of the expected size (610 bp) was obtained and PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen, Germany), following the manufacturer's instructions before being sequenced (Eurofins Genomics, Germany) using both PCR primers (sequence deposited in GenBank, Accession numbers MK075829).

The resulting sequence was compared to published nucleic acid data (>99.8% identity; GenBank Accession numbers KM598759.1, EU024677.1, EU350963.1, EU413667.1) and amino acid data (99-100% identity; Accession numbers ABY60413.1, ABV89764.1,

YP_001686787.1) for RoCV1 dsRNA2, confirming that the sample 7B3 was infected with RoCV1.

The remaining samples were then tested for RoCV1 using the RT-qPCR method for dsRNA1. The results showed 43% (75 samples of 251, including 7B3) were positive (C_T between 17.4 and 38.4). Of these, two samples (0.8%) were found to be co-infected with RoCV1 and SLRSV, eight (3.2%) with RoCV1 and ArMV and two (0.8%) with all three viruses. All the samples tested positive with the plant COX assay previously, confirming the success of the nucleic acid extraction.

Following conventional PCR with the dsRNA2 primers, products from three RoCV1 positive samples (4B3, 13A3, 16B1) were sent for Sanger sequencing (sequences deposited in GenBank, Accession numbers MK075830, MK075831, MK075832). Comparison of the nucleic acid sequences with published data confirmed the presence of RoCV1 with more than 99% identity.

Furthermore, eight samples (11C, 16A1, 19A3, 21A1, 69A2, 74A2, 89 and 99) with high C_T values (27.8-37.6) for RT-qPCR were analysed by conventional PCR. For three of these samples PCR gave a band of the expected size (610 bp) while the remaining samples gave negative results. To investigate if these samples were false-positive results following RT-qPCR or false negatives following testing using conventional PCR, the DNA amplified using the RT-qPCR from the eight samples was cloned using a pGEM®-T Easy Vector System (Promega), following the manufacturer's recommended protocols. Ligation of the insert was performed, bacterial cultures grown overnight after transformation, clones containing inserts were identified, and plasmid DNA was purified. Following sequencing of the plasmid inserts, comparisons were made with published sequence for RoCV1 dsRNA1. The results showed that the sequences of all inserts have a high sequence identity (>97%) to GenBank nucleic acid accessions KM598759.1, EU024677.1, and amino acid accessions ABY60413.1, ABV89764.1. The results showed that the samples were infected, giving positive results with the RT-qPCR and negative results with the conventional RT-PCR most likely due to lower sensitivity of conventional RT-PCR.

ELISA analysis of the samples in the UK rose survey demonstrated the presence of SLRSV and ArMV, both previously described in the UK (Thomas, 1980). Whilst the symptoms were frequently as described in the literature, SLRSV-infected plants (Fig. 4.1 A) and plants with mixed infections (SLRSV + ArMV) displayed a chlorotic mosaic whilst ArMV-infected plants had yellow vein clearing symptoms (Fig. 4.1 B). Nevertheless, not all the samples followed this

pattern: some samples infected with ArMV had chlorotic mosaic symptoms typical of SLRSV infections. Further analysis is required to confirm these results.

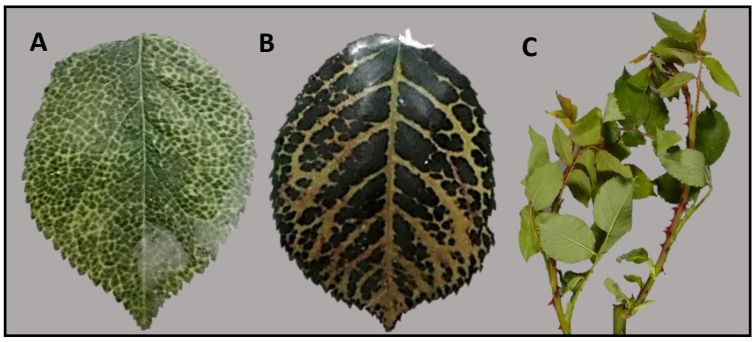

Figure 4.1- Rose leaves showing rose mosaic disease symptoms, infected with (A) strawberry latent ringspot virus and rose cryptic virus-1 (RoCV1) (B) arabis mosaic virus and RoCV1 and (C) an asymptomatic rose stem infected with RoCV1. Photos were taken by the author.

Based on the provenance of samples, these results suggest that RoCV1 has been in the UK at least since 2007. Following the genus demarcation criteria from the International Committee on Taxonomy of Viruses (Vainio *et al.*, 2018), < 24% amino acid sequence identity within the RdRP gene indicates a new species. Analysis of the sequences of samples LS11S16 and LS13S9 showed that the amino acid sequences for RNA1 (RdRP protein) have between 98.85-100% identity with published sequences.

RoCV1 coat protein nucleic acid sequences (dsRNA2) from this work (GenBank Accession numbers MK075822, MK075824, MK075827, MK075829, MK075830, MK075831, MK075832) showed little difference (<0.5%) between isolates. For samples LS11S16 and LS13S9 dsRNA 3 was detected (deposited as MK075823, MK075825, MK075828). Comparison of the nucleic acid sequence showed 99% identity with published sequences.

RoCV1 has been reported in the USA (Martin and Tzanetakis, 2008; Sabanadzovic and Ghanem-Sabanadzovic, 2008; Lockhart *et al.*, 2011), Canada (James *et al.*, 2015), and New Zealand where it was the most prevalent virus in roses with a 48% incidence (Milleza *et al.*, 2013). By using HTS we have shown the presence of RoCV1 in the UK for the first time. Follow-on testing using RT-qPCR (TaqMan) has shown that a large percentage (43%) of the samples tested were infected with RoCV1. There is no known natural vector for RoCV1; it is assumed to be pollen and seed transmitted like other cryptoviruses. Therefore, it is probable that RoCV1 has spread between the USA, New Zealand, Canada and the UK during commercial trade of planting material.

In New Zealand, infected roses were associated with mottling, leaf spots and necrosis and also one with flower break but it was also found in asymptomatic samples (Milleza *et al.*, 2013). In this study, several samples were asymptomatic, while others had vein banding, mottling or

leaf distortion symptoms. It is possible that these symptoms could be caused by another, as yet unidentified virus (or pathogen), by herbicide damage or adverse cultural conditions.

A sample with symptoms similar to those caused by rose rosette virus was positive for RoCV1; further studies will be undertaken to investigate if those symptoms were due to mixed infections with other, as yet undescribed viruses. We also found roses with symptoms of rose mosaic disease which were infected with RoCV1, where ELISA analysis indicated that they were co-infected with ArMV, SLRSV or all three viruses. It is unknown whether the symptoms caused by ArMV or SLRSV were impacted by co-infection with RoCV1, although we found samples with mosaic symptoms which were not infected with RoCV1. Cryptoviruses are not thought to cause direct economic losses in their plant host, although it is not clear what impact they have in mixed infections.

4.2 A Novel High-Throughput Sequencing Approach Reveals the Presence of a New Virus Infecting *Rosa*: Rosa Ilarvirus-1 (RIV-1)

4.2.1 Introduction

Roses are susceptible to numerous diseases which require management, increasing the cost of cultivation. Exotic pathogens can be introduced via trade in plants, plant products or even imported through illegal internet trading or smuggling (Tuffen, 2016). Despite the spread of rose viruses and their impact on cultivation, they have not been studied in detail in the United Kingdom (UK) since the 1980s (Thomas, 1984a), and as a result molecular methods for virus detection have rarely been deployed to study them. The advance of molecular techniques could help clarify the taxonomy of rose viruses and allow the aetiology of viral diseases to be determined more effectively. Diagnosis is fundamental to facilitate the management of plant diseases (van der Want and Dijkstra, 2006; Aboul-Ata *et al.*, 2011) and early detection is essential to successful biosecurity responses. Better understanding of rose viruses moving in trade will enable the identification of future risks to the UK and facilitate the development of effective plans for interception, eradication and management. Improving current understanding will also improve the response to new and emerging diseases such as those caused by rose rosette virus, which has spread rapidly in the USA (Laney *et al.*, 2011) but is not yet present in the UK.

Serological and molecular methods are both commonly deployed for viral detection and diagnosis as targeted methods. ELISA (enzyme-linked immunosorbent assay) is a widely used technique for routine virus testing in phytosanitary, quarantine and virus-certification programmes. RT-PCR is a sensitive and relatively rapid method for detection of RNA viruses, and quantitative PCR (qPCR) adds further benefits in a routine testing laboratory primarily due

to its higher sensitivity and speed, with no need to use agarose gel electrophoresis (Boonham *et al.*, 2014). PCR and qPCR assays require the design of highly specific primers (and in the case of qPCR based on TaqMan chemistry, probes) targeted to the pathogen of interest, yet designed to avoid mismatches caused by mutations, genetic drift or selection pressure of sequence variants, something that happens frequently in RNA viruses (Jenkins *et al.*, 2002; Metzgar, 2011).

However, unlike PCR or ELISA methods, high-throughput sequencing (HTS) offers the possibility of generic detection of viruses and other pathogens (Boonham *et al.*, 2014) at species/strain level (Adams and Fox, 2016) and allows a generic approach to virus identification that does not require any prior knowledge of the pathogen presence. Deployment of HTS as a diagnostic tool is becoming more widespread since the first reports in 2009 (Adams *et al.*, 2009; Al Rwahnih *et al.*, 2009; Kreuze *et al.*, 2009). Initially its widespread use was limited by the high cost of analysis, but the methods are continuously evolving, with different platforms and library preparation methods being developed (Pecman *et al.*, 2017). For most HTS methods, the high run cost is mitigated by pooling or tagging samples, resulting in a reduction in the sample cost. Whilst this approach is effective in screening or surveillance work where large numbers of samples are processed, sequencing single or small numbers of samples in a diagnostic laboratory remains problematic due to cost and is used as a diagnostic method of last resort, when other methods have failed.

From a diagnostic perspective the MinION sequencer (Oxford Nanopore Technologies, UK) has the advantage of enabling lower throughput of samples, at a cost appropriate to a diagnostic laboratory and with data generated in a short space of time (24-48 h run). This becomes useful when a quick turnaround of results is required, for example when testing urgent samples in the case of an outbreak. It has been used for the detection and characterization of a number of plant viruses (Filloux *et al.*, 2018; Chalupowicz *et al.*, 2019; Gaafar *et al.*, 2019). A further adaption, the Flongle (flow cell dongle), further reduces cost and throughput, potentially making it ideal for single or small numbers of samples in a diagnostic setting. One of the disadvantages is the requirement of expert operators for both laboratory and bioinformatic analysis work. However, with the appropriate training and practice, application would get simpler, as has previously happened with other diagnostic techniques such as RT-qPCR.

In this study, both MinION and Illumina platforms were used to investigate the cause of discrepant results following prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*) testing in imported roses. RT-qPCR testing gave positive results for PNRSV in 10 samples, but subsequent testing using a PNRSV specific end-point RT-PCR only confirmed the presence of

PNRSV in one sample (ID168). In further confirmatory testing using an ilarvirus-specific end-point RT-PCR two samples were positive (ID168 and ID188). Following sequencing of the amplification products, one sample was confirmed to be infected with PNRSV (ID168) whilst the other sample was infected with an ilarvirus that was not PNRSV (ID188). Analysis using the Flongle flow cell led to the identification of a novel virus infecting roses, for which we propose the name "rosa ilarvirus-1" (RIV-1; genus *Ilarvirus*). This was confirmed using MiSeq sequencing. This report further supports the deployment of HTS as a front-line diagnostic tool as highlighted previously (Fox *et al.*, 2019), and that innovations such as the Flongle are potentially well suited to this application.

4.2.2 Materials and Methods

Samples

A total of 35 plant samples were collected by inspectors from the Animal and Plant Health Agency (APHA) at Heathrow Airport (London, UK) between February and May 2019. Samples consisted of leaves from 22 symptomatic and 13 asymptomatic cut roses being imported into the UK. Symptoms were consistent with virus infections such as mottling, yellow veining, distortion, and ring spots.

Nucleic acid extraction and RT-qPCR (TaqMan)

RNA was extracted using a CTAB (cetyl trimethylammonium bromide) method (see Material and Methods Chapter 3). RT-qPCR was performed to test for the plant COX1 gene and for the presence of prunus necrotic ringspot virus (PNRSV; see Material and Methods Chapter 3).

Conventional PCR

Cycling for RT-PCR was performed using a 2720 Thermal Cycler (Applied Biosystems). For generic detection of ilarvirus, RT-PCR was performed using Reddymix (Thermo Fisher Scientific), 1 µl of Verso RT (Thermo Fisher Scientific), BSA (50 mg/ml), 400 nM of primers Ilar2F5 and Ilar2R9 (Untiveros *et al.*, 2010) and 1 µl extracted RNA in a final volume of 25 µl. Cycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 44 °C for 1 min, and 72 °C for 1 min followed by a final extension of 10 min at 72 °C and a hold step of 4 °C. The expected product was 380 bp estimated using agarose gel electrophoresis and Quick-Load® Purple 1 kb Plus DNA Ladder (NEB). For PNRSV detection, Hot-Start Verso mastermix (Thermo Fisher Scientific), 1 µl of Verso RT (Thermo Fisher Scientific), 400 nM of primers PNRSV-C and PNRSV-D (Sanchez-Navarro *et al.*, 1997) and 1 µl extracted RNA in a final volume of 25 µl were used. Cycling conditions were 15 min at 50

°C, 15 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min followed by a final extension of 5 min at 72 °C and a hold step of 4 °C. The expected product was 700 bp estimated using agarose gel electrophoresis and Quick-Load® Purple 1 kb Plus DNA Ladder (NEB).

ELISA

ELISA was performed for a range of ilarviruses to check for any cross-reaction between the virus infecting sample ID188 and viruses belonging to this group. Samples were tested for: PNRSV, apple mosaic virus (ApMV), tobacco streak virus (TSV; BIOREBA AG, Switzerland); prune dwarf virus (PDV; Loewe BiochemicaGmbH, Germany); asparagus virus 2 (AV2; Leibniz Institute DSMZ GmbH, Germany); and blueberry shock virus (BlShV; Agdia Inc., US), as described in materials and methods in Chapter 3.

High-throughput sequencing – Flongle (MinION-Oxford Nanopore Technologies)

For the sequencing using Flongle (Oxford Nanopore Technologies, UK), DNA was removed from the nucleic acid extract (RNA from sample ID188) using DNase TURBO DNA-free™ Kit (Invitrogen) and the RNA was 3´ tailed using poly(A) polymerase (NEB) following the manufacturers' protocols. Library preparation was completed to enable direct RNA sequencing using (SQK-RNA002) version DRS_9080_V2_REVL_14Aug2019 protocol by Oxford Nanopore Technologies. Genomic DNA by Ligation (SQK-LSK109) version GDE_9063_revQ_14Aug2019 (Oxford Nanopore Technologies, UK) was followed for sample loading into the Flongle flow cell.

Fastq files were generated from the outputs from the Flongle using the basecaller Guppy (Oxford Nanopore, v3.1.5), which separated reads into pass and fail groups based on a quality score of 7. The quality-passed reads were then clustered using isONclust (v0.0.5; (Sahlin and Medvedev, 2019). Subsequently, BLAST was used to analyse a representative from each cluster, to reduce the time and computer resource required for searches. Clusters which had a high BLAST score (top hits) to a plant virus taxon as the representative sequence were combined, and Minimap2 (v2.14; (Li, 2018), Miniasm (v0.3; (Li, 2016), Racon (v1.4.3; (Vaser *et al.*, 2017) and Medaka (v0.11.0; Oxford Nanopore Technologies, UK) were used to generate consensus sequences.

High-throughput sequencing – MiSeq (Illumina)

For the sequencing on the MiSeq (Illumina, US), DNA was removed from the sample (RNA from sample ID188) using RNase-Free DNase Set (Qiagen, Germany). Ribosome depleted, indexed sequencing libraries were generated using TruSeq® Stranded Total RNA Library Prep

Plant kit (Illumina, US) and Illumina TruSeq RNA UD Indexes. The library was sequenced using a MiSeq instrument (Illumina, US) and a MiSeq Reagent Kit v3 (600-cycle; Illumina, US).

Sequence reads were trimmed for quality using Sickle (v1.33; Joshi and Fass, 2011), assembled using Trinity (v2.8.4; Grabherr *et al.*, 2011), and mapped using BWA (v0.7.17.4; Li and Durbin, 2009) and SAMtools (v1.9; Li *et al.*, 2009) on the public accessible Galaxy server (Afgan *et al.*, 2018). They were compared to the GenBank nr and nt databases using BLAST+ (Camacho *et al.*, 2009). Reads of viral origin were inspected using MEGAN community edition (v6.12.3; Huson *et al.*, 2016). Pairwise analysis and Neighbour joining trees were generated using MEGA7 (v7.0.21; Kumar *et al.*, 2016).

4.2.3 Results

Of 35 samples tested, 10 gave positive results using the PNRSV RT-qPCR with mean C_T values in the range 11.59-38.66. To confirm these results, conventional RT-PCR (using PNRSV-specific and generic ilarvirus primers) was performed on the 10 positive samples (ID152, ID164, ID168, ID170, ID171, ID180, ID188, ID190, ID193 and ID198; see Table 4.1).

Following PNRSV RT-PCR, a band of the expected size (700 bp) was obtained for only one sample (ID168), of the 10 tested. This sample had the lowest C_T value (11.59) by RT-qPCR. Following testing using the generic ilarvirus primers, two samples (ID168 and ID188) produced a band of the expected size (380 bp; Table 4.1).

The sequence of the 380 bp product from the ilarvirus generic PCR from sample ID168 had 98.03 - 99.16 % identity with the nucleotide (GQ865664.1; MH727232.1; MH727231.1) and 97.46 - 99.15 % identity with the amino acid sequence of PNRSV (ADE58478.1; ADE58477.1; ADE58479.1). The product from sample ID188 had 84.43 - 85.63% nucleotide identity with tulare apple mosaic virus (TAMV; genus *Ilarvirus*, group 2; GQ865660.1; AF226161.1) and 83.58% with tomato necrotic streak virus (TomNSV; genus *Ilarvirus*, group 2; NC039074.1). Amino acid comparisons showed a match with the replicase from TAMV and TomNSV (97.35% identity; ADE58475.1; YP00950887), as well as with other members of the genus *Ilarvirus*, group 2: citrus leaf rugose virus (CLRV; 96.46% identity; NP613281.1;AFR46583.1), elm mottle virus (EMoV; 85.44% identity; ADE58476.1), asparagus virus 2 (AV2; 84.96% identity; ADE58463.1), spinach latent virus (SLV; 84.07% identity; ADE58474.) and citrus variegation virus (CVV; 84.96% identity; ADE58472.1). Further testing was performed to identify the virus present in sample ID188.

Table 4.1- Results following RT-PCR using prunus necrotic ringspot virus (PNRSV)-specific and ilarvirus-generic primers for the 10 samples used for the RT-qPCR validation.

Sample	RT-qPCR-PNRSV assay (average C_T value)	RT-PCR (PNRSV assay)	RT-PCR (ilarvirus assay)
ID152	37.16	Negative	Negative
ID164	38.66	Negative	Negative
ID168	**11.59**	**Positive**	**Positive**
ID170	29.92	Negative	Negative
ID171	34.68	Negative	Negative
ID180	34.54	Negative	Negative
ID188	**23.93**	Negative	**Positive**
ID190	33.06	Negative	Negative
ID193	35.59	Negative	Negative
ID198	32.26	Negative	Negative

Sample ID188 was symptomatic (leaf distortion and yellow spot). Unfortunately, no photographs were taken, and it is not clear if these were viral symptoms or if they had other causes such as arthropod damage. The original leaf sample was tested by ELISA for different viruses within the *Ilarvirus* genus and tests for PNRSV, ApMV, TSV, PDV, AV2 and BlShV, were all negative.

Sample ID188 was run on a Flongle flow cell for MinION (Oxford Nanopore Technologies, UK). The number of pores active at the beginning of the run was low (48 pores). However, 9229 reads were obtained (average quality of 7.4). A "What's in my pot" (3.4.0; (Juul *et al.*, 2015) run on EPI2ME (Oxford Nanopore Technologies, UK) analysed 5605 reads and classified 1418, 7 of which were identified as viral (4 genus *Ilarvirus*, 2 genus *Orthotospovirus* and 1 from *Myoviridae*). Guppy was then used as a basecaller and sequences were subjected to a database search using BLAST-n and 7 further reads were identified as being viral in origin.

The results of the BLAST-n analysis (Table A.4) showed that the sequences of longer fragments (600, 604, 910, 335, 82, 1033, and 499 bp) have higher identity with TomNSV (NC_039076.1; KP861235.1; NC_039074.1), whereas smaller reads (166, 203, 154, 190, 239 and 145 bp) have sequence identity with viruses within ilarvirus group 2 (TomNSV, TAMV, SLV, EMoV, AV2, CLRV). The closest overall match for each fragment was to TomNSV, although the most similar fragment only had a sequence identity of 87.57%, indicating that the virus from the rose sample was probably a new species.

To confirm the conclusions from the MinION sequencing, RNA for sample ID188 was sequenced using the MiSeq on a run including 23 other libraries. The whole run generated approximately 9.2 million reads, resulting in a total of 208,708 reads for sample ID188. 3079 reads of the total generated for sample ID188 were virus in origin (1.48%). Trinity (Grabherr *et al.*, 2011) was used for assembly of genome fragments and ORF Finder (National Center for Biotechnology; (Wheeler *et al.*, 2003) to identify open reading frames. RNA1 (3346 bp; GenBank accession number MT017861) was reconstructed from 401 reads, RNA2 (3063 bp; GenBank accession number MT017862) from 485 reads and RNA3 (2329 bp; GenBank accession number MT017863) from 2193 reads. RNA1 was found to contain one ORF coding for a replicase, whilst RNA2 and RNA3 had two ORFs. RNA2 codes for the RNA-dependent RNA polymerase and protein 2b, and RNA3 for the movement and coat proteins. The genome structure conforms to that described for the genus *Ilarvirus* by the International Committee on Taxonomy of Viruses (ICTV; (Bujarski *et al.*, 2019)**.**

Clustering analysis (Fig. 4.2) of the proteins coded by RNA1 (replicase), RNA2 (RNA-dependent RNA polymerase and protein 2b) and RNA3 (coat protein) grouped the new virus with TomNSV, CLRV and TAMV, forming a distinct cluster, separated from the other members of the ilarvirus group with high bootstrap support (100%). In the case of the movement protein (RNA3), CLRV is not part of this group, instead clustering with CVV. Previous studies in ilarvirus group 2 highlighted the same difference (Scott *et al.*, 2003).

Pairwise analysis based on the nucleotide sequences (Table 4.2) shows that the new virus has most similarity with TomNSV. The second most similar virus varies depending on the RNA in the pairwise analysis: RNA1 and RNA3 are similar to TAMV (79.13% and 75.08% respectively), whereas RNA2 is most similar to CLRV (72.62%).

Sequence data generated from the MinION was aligned to that generated by the MiSeq for RNA3 using IGV (Robinson *et al.*, 2011). The coverage depth shows a maximum of 9 reads covering the fragment between positions 16-150 bp. However, MinION reads reach position 1045 bp, and some positions are only covered by one MinION read. The number of reads obtained from the Flongle flow cell was significantly fewer than from the MiSeq platform. However, a consensus sequence was constructed for RNA3 with sequences obtained with the Flongle (positions 1 to 1045 bp). Results show a 95.98% identity with the RNA3 sequences obtained with MiSeq.

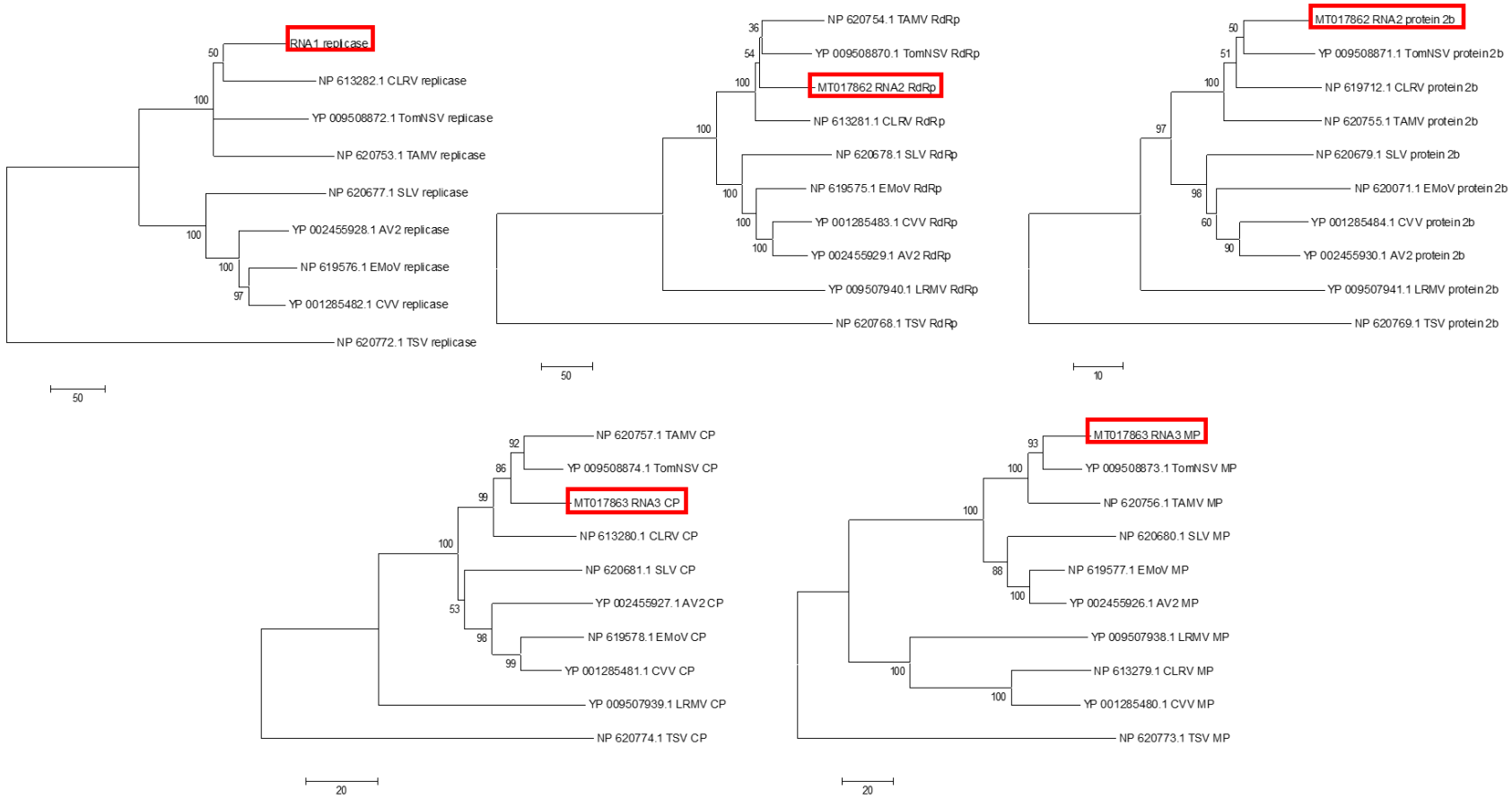

Figure 4.2.- Neighbour joining trees (1000 bootstrap) comparing the protein sequences translated from RNA 1, 2, and 3 of sample ID188 with ilarviruses within group 2. The outgroup, tobacco streak virus (TSV; NP_620772.1; genus *Ilarvirus*) belonging to group 1 was included to root the tree. The evolutionary distances are in the units of the number of base substitutions per branch.

Table 4.2- Pairwise analysis tables (A, B, C) showing nucleotide sequence comparison (percentage of identity) of the three respective RNAs (1, 2, 3) reconstructed from the MiSeq data for the virus in sample ID188.

A	RNA1	CLRV	EMoV	SLV	TAMV	CVV	AV2	TomNSV
RNA1		78.33	68.46	68.16	79.13	68.34	68.86	82.16
CLRV	78.33		68.79	68.8	76.35	68.36	68.57	78.4
EMoV	68.46	68.79		79.43	68.83	85.98	84.53	69.19
SLV	68.16	68.8	79.43		68.25	79.64	78.15	68.51
TAMV	79.13	76.35	68.83	68.25		68.7	68.18	80.16
CVV	68.34	68.36	85.98	79.64	68.7		84.46	68.26
AV2	68.86	68.57	84.53	78.15	68.18	84.46		69.44
TomNSV	82.16	78.4	69.19	68.51	80.16	68.26	69.44	

B	RNA2	CLRV	EMoV	SLV	TAMV	CVV	AV2	LRMV	TomNSV
RNA2		72.62	65.05	63.67	72.17	65.73	65.55	54.86	76.04
CLRV	72.62		66.56	65.62	71.24	65.59	66.54	56.12	73.22
EMoV	65.05	66.56		74.66	66.77	75.75	78.22	57.52	65.4
SLV	63.67	65.62	74.66		65.48	74.69	76.17	56.75	63.53
TAMV	72.17	71.24	66.77	65.48		66.47	67.7	56.13	75.72
CVV	65.73	65.59	75.75	74.69	66.47		84.22	56.8	64.78
AV2	65.55	66.54	78.22	76.17	67.7	84.22		57.1	65.7
LRMV	54.86	56.12	57.52	56.75	56.13	56.8	57.1		54.25
TomNSV	76.04	73.22	65.4	63.53	75.72	64.78	65.7	54.25	

C	RNA3	CLRV	EMoV	SLV	TAMV	CVV	AV2	LRMV	TomNSV
RNA3		61.04	64.86	63.82	75.08	56.47	64.27	45.75	80.47
CLRV	61.04		55.8	56.34	60.57	64.49	55.35	49.8	60.76
EMoV	64.86	55.8		72.79	64.74	68.48	80.06	47.04	65.97
SLV	63.82	56.34	72.79		64.59	62.19	72.96	46.41	66.04
TAMV	75.08	60.57	64.74	64.59		57.22	64.42	47.29	78.74
CVV	56.47	64.49	68.48	62.19	57.22		67.18	50.39	57.64
AV2	64.27	55.35	80.06	72.96	64.42	67.18		47.72	65.61
LRMV	45.75	49.8	47.04	46.41	47.29	50.39	47.72		47.54
TomNSV	80.47	60.76	65.97	66.04	78.74	57.64	65.61	47.54	

4.2.4 Discussion

In this study, different commonly used diagnostic techniques have been employed to troubleshoot other methods and resolve the identification of a virus infecting a rose sample (sample ID188). Discrepant results were obtained when tests were performed for PNRSV. Whilst the RT-qPCR gave positive results for PNRSV in 10 samples (ID152, ID164, ID168, ID170, ID171, ID180, ID188, ID190, ID193 and ID198), subsequent testing using a PNRSV specific RT-PCR (Sanchez-Navarro *et al.*, 1997) only confirmed the presence of PNRSV in one

sample (ID168). In further confirmatory testing using an ilarvirus-specific RT-PCR (Untiveros *et al.*, 2010) two samples were positive. Following sequencing of the amplification products, one sample was confirmed to be infected with PNRSV (ID168) whilst the other sample was infected with an ilarvirus that was not PNRSV (ID188). Though only a limited number of samples were used, the RT-PCR tests (Sanchez-Navarro *et al.*, 1997; Untiveros *et al.*, 2010) appear specific, though none of the tests could be used independently to identify the PNRSV-positive samples without further work. RT-qPCR products of the 10 PNRSV-positive samples were sent for Sanger sequencing and confirmed to be infected with PNRSV (99.12-100% identity, GenBank accession numbers MN656197.1 and AY948440.1). These results showed that sample ID188 was infected with PNRSV in coinfection with a potential new ilarvirus. This clarifies that the RT-qPCR primers (Marbot *et al.*, 2003) did not cross react with the new ilarvirus found by HTS. The higher level of sensitivity of RT-qPCR can explain the detection of smaller amounts of PNRSV than the RT-PCR methods used. Further analysis of the HTS data (MiSeq) revealed the presence of 2 paired-end reads identified as PNRSV. It is possible that the first analysis did not identify PNRSV because a contig could not be assembled using Trinity (Grabherr *et al.*, 2011) due to the low number of PNRSV reads. This could be explained by a low level of infection or to possible cross-contamination.

The amplification product of the sample ID188 which was positive for an ilarvirus but negative for PNRSV was sequenced and found to be most similar to TAMV, TomNSV, and other members within ilarvirus group 2 (CLRV, EMoV, AV2, SLV and CVV). Using the Flongle flow cell, 14 reads were identified as viral. Sequence similarity was found between some of the reads and multiple RNAs belonging to TomNSV (Table A.4), which is not surprising as the 3′-termini of the different RNAs are generally highly conserved in members of the *Bromoviridae* family. The similarity of the reads to other members of the *Ilarvirus* genus suggested that the sample was infected with a new virus within the ilarvirus group 2. The highest sequence identity for each viral read was to TomNSV. However, the species demarcation percentage criterion for *Ilarvirus* is not defined. Since this virus shows variation in sequence compared with other ilarviruses in group 2 and was identified in *Rosa,* not known as a host for other members of ilarvirus group 2, we consider it a new virus and propose the name “rosa ilarvirus-1” (RIV-1). Unfortunately, because the plant sample was from an imported cut flower sampled as part of a surveillance programme, it was not possible to undertake any biological characterisation (symptoms, transmission, host range and impact either to the industry or the environment). The MiSeq data confirmed the results, and the additional sequence generated enabled the reconstruction of the three RNAs comprising the genome of a

new virus (GenBank accession numbers MT017861, MT017862, MT017863) with the same number of ORFs described as for other members of ilarvirus group 2 (Bujarski *et al.*, 2019).

Workflows in a diagnostic laboratory vary depending on the study. Surveillance samples are usually tested in large numbers for the presence of the target pest using a single method. This could be for phytosanitary purposes such as demonstrating freedom from the pest or to estimate the extent of spread following an outbreak. The same approach is often used for certification purposes, for example testing seeds or screening propagation material, though frequently a range of different tests are performed, depending on the material and the requirements of trading partners. Samples that are sent to a laboratory to evaluate the cause of specific symptoms are often subjected to investigational testing, where a range of tests, often of different types (molecular, serological or biological) are deployed either in series or in tandem to elucidate the cause.

The reagent cost of RT-qPCR or RT-PCR is approximately 10 € per sample and assay, assuming the sample needs to be tested in duplicate with a positive and negative control, whilst ELISA is approximately 2 € per sample and assay. Staff time however has a much more significant impact on the cost of testing, with an average cost estimated to be 200-400 € per day in Europe. The third aspect that needs to be considered is the turnaround time for a sample, that is the cumulative time between receipt of sample and reporting of the results. In the case of suspected PNRSV in roses, deployed in an investigational workflow (Fig. 4.3) and considering time, staff and reagent costs, the MinION (Flongle) shows significant promise. The HTS methods deployed here generated more definitive results more rapidly than first testing using a specific RT-qPCR followed by confirmation using specific and genus level RT-PCR assays and sequencing of the amplified products. RT-qPCR and RT-PCR require the design of specific primers and probes, the sequences of which need to be reviewed over time, as new virus isolates or species appear (Zheng *et al.*, 2008). In this example if no further studies had been conducted, virus identification based solely on PCR, qPCR or ELISA would have been incorrect and incomplete.

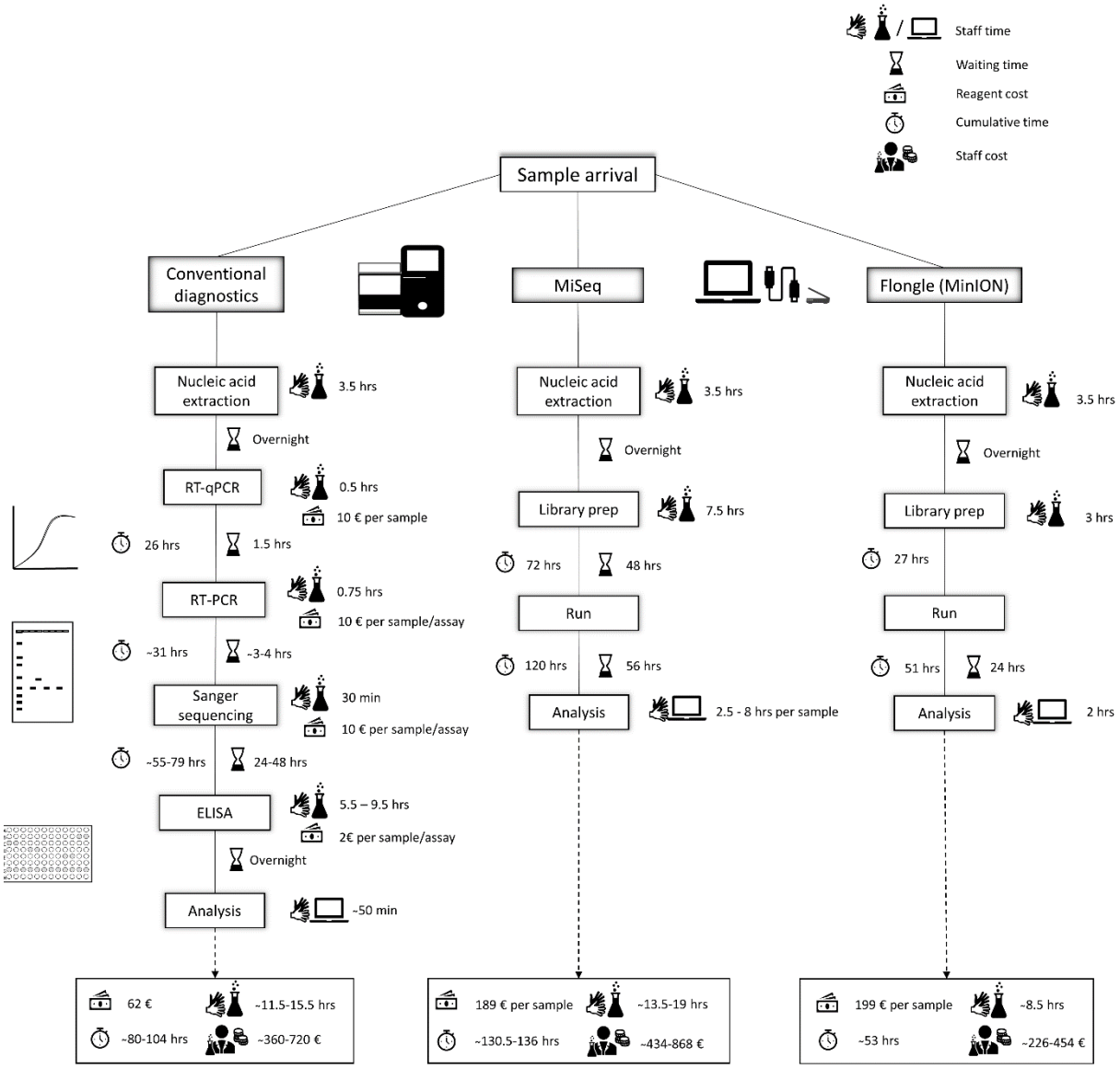

Figure 4.3- Comparison among different diagnostic techniques of reagent costs, staff time and cost, and total time invested from the sample arrival until results. This diagram was created by the author.

The main difference between the Flongle and the MiSeq approach is the impact of scaling on costs. The MiSeq platform becomes cost effective when multiple samples are run together. For example, if 24 samples are individually tagged the reagent price per sample is 189 € (TruSeq® Stranded Total RNA Library Prep Plant 2715 €, indexes for TruSeq RNA 160 €, Superscript II 110 €, and the MiSeq Reagent Kit v3 600-cycle 1550 €). This would be expected to generate approx. 560 million nt/sample (around 22.5 million read pairs total run, 300 bp length). The MinION Flongle can be used to sequence a single sample with a reagent cost of 199 € (direct RNA kit 94.5 €, a poly(A) kit 13 €, Superscript III kit 10 € and a flow cell 82 €).

This is expected to provide an average of 5400 million nt/sample (200k reads, 4-50 kbp length from a typical QC pass flow cell). Library preparation for the Flongle took 3 h compared with two days for the MiSeq and the run was completed in 24 h (although viral reads were identified in the first six hours of the run) compared with 56 h for the MiSeq. Calculation of the time taken to complete the data analysis varies depending on the pipeline used, the number and diversity of reads, removal of host sequences (for MinION) or the depth of the analysis (i.e. if only a BLAST-n or both BLAST-n/-x analysis are performed). The data of a MinION run (x1 sample) can be analysed in 2 h, and between 2.5-8 h per sample are needed for the MiSeq.

Most molecular diagnostic techniques, including some HTS platforms such as the MiSeq, have the limitation of not being deployable close to the point of sampling, which can improve the speed of decision making. The MinION platform has a number of potential advantages in this regard. The equipment is small with low capital costs and can be run from a laptop, the workflow has fewer steps and little hands-on time and real-time base-calling and cloud-based analysis means that detection is possible without access to a high-performance computing cluster. This technology could be deployed in simple laboratories at key locations (e.g. border controls), where decisions are made by biosecurity staff.

As a front-line diagnostic tool, HTS has a number of significant advantages. The methods can be used to achieve a definitive result with a single test, without recourse to a number of different assays or testing methods. The results can be definitive regardless of whether the target is a well characterised virus or a virus new to science. The genome data generated by HTS can allow a rapid design of high-throughput targeted diagnostics, which may be useful for surveillance in the case of an outbreak of a new virus. This study demonstrated that the Flongle adapter further enhances the potential of HTS for front-line diagnostic testing by reducing the cost and time taken, and with a throughput better suited to small and potentially large numbers of diagnostic samples compared with other platforms.

4.3 First Report of Rose Spring Dwarf-Associated Virus in *Rosa* spp. in United Kingdom

In July 2019, a sample of *Rosa* sp. was submitted to Fera Science Ltd. via the Royal Horticultural Society (RHS) gardening advice service. The sample (ID220) was sent in following the appearance of symptoms including mottling, yellow/white patching, thin texture and a pink colour in the leaves.

RNA was extracted using a CTAB method (see Material and Methods section Chapter 3). The sample was tested for common rose viruses using RT-qPCR (Table A.3). A positive result was achieved for rose cryptic virus-1 (family *Partitivirus*). Subsequently, the sample was

analysed by high-throughput sequencing (HTS) using a TruSeq Stranded Total RNA Library Prep Plant kit (Illumina, US) for library preparation. A MiSeq instrument and a MiSeq Reagent Kit v3 (600-cycle; Illumina, US) were used to run the library. The run generated 569,452 reads for the sample, and data was analysed as described by Fox *et al.* (2019). Three fragments of rose spring dwarf-associated virus (RSDaV; genus *Luteovirus*) were identified (234, 251 and 229 bp; GenBank Accession Nos. MT993839-MT993841). A BLAST+ search of GenBank found sequences with high sequence identity in both nucleotide (92.11-93.59% identity, EU024678.1) and amino acid comparisons (94.34-100%, YP_001949737.1, YP_001949736.1, YP_001949738.1). RT-PCR amplification using specific primers (Salem *et al.*, 2008a) was performed to confirm the result, and a product of the expected size (418 bp) was obtained.

To assess the spread of RSDaV in the UK, 171 roses were analysed using the RT-PCR assay. Samples were collected as part of a survey of rose viruses in the UK and both asymptomatic and symptomatic leaf samples, consistent with virus infection symptoms (mottling, yellow veining, distortion, and ring spots) were included. Only one sample (ID140) tested positive for RSDaV, and no symptoms were identified. Previous analysis showed this sample was positive for arabis mosaic virus (genus *Nepovirus*) by ELISA and RT-qPCR.

The RT-PCR products (418 bp) from both RSDaV-positive samples (ID220, ID140) were sequenced, and nucleotide comparisons showed a 98.51-99.02% identity with sequences in GenBank (HM236366.1, HM2363641, HM236362.1, HM236364.1). Amino acid comparison showed a 98.51-100% identity with previously published sequences (ADK78852.1, ADK78851.1).

RSDaV has previously been found in the USA (Salem *et al.*, 2008a), Chile (Rivera and Engel, 2010), and New Zealand (Milleza *et al.*, 2013). This is the first report of RSDaV in Europe. Further samples (4) were submitted to the RHS and Fera Science Ltd. Plant Clinic, showing the previously described symptoms. They were tested by RT-PCR and also sequenced by HTS and tested negative for RSDaV. The cause of these symptoms is not believed to be of viral origin.

Chapter 5 Comparison of Methods for Virus Detection

Abstract

In this chapter, two different pipelines were used to analyse the data obtained from a high-throughput sequencing (HTS) run in the MiSeq platform (Illumina, US). The two pipelines used were: Angua, routinely used at Fera Science Ltd., and EDNA (Electronic Diagnostic Nucleic acid analysis), a novel pipeline under development which aims to simplify the bioinformatic analysis of data, created at Oklahoma State University. Samples (35) were also tested using ELISA and RT-qPCR to confirm the results obtained after the analysis of the HTS data. In this chapter, results obtained by the two pipelines and the diagnostic techniques are compared and explored. Overall, RT-qPCR was identified as the method with the highest sensitivity, whereas ELISA was identified as the technique with the lowest sensitivity. Both pipelines, the Angua and the EDNA, showed non-significantly different results for the analysis of SLRSV, ArMV, PNRSV and RoCV1-infected samples compared with RT-qPCR. In addition, correlation was observed among the results obtained for the different diagnostic techniques.

5.1 Introduction

Plant virus diagnostic methods evolve with the development of new technologies. A large variety of diagnostic techniques with different characteristics are available: targeted vs non-targeted methods, molecular or serological, etc. The decision about which method should be used depends on the question being addressed (quarantine and virus-certification programmes, identification of a virus or a strain, surveys, etc.) and the different scenarios (available resources, time limitation, etc.; (Hull, 2014). It is important to be aware of the performance characteristics of different methods, such as sensitivity or specificity, the number of samples that can be processed by one operator in a given period of time, the resources available (cost of the machinery and consumables), operator training and/or the adaptability to field conditions.

One of the most recent methods used in plant virology diagnostics is high-throughput sequencing (HTS; (Adams *et al.*, 2009; Al Rwahnih *et al.*, 2009; Kreuze *et al.*, 2009). HTS offers the possibility of generic detection of viruses (Adams and Fox, 2016) that does not require previous knowledge of the targeted pathogens (Boonham *et al.*, 2014), being able to identify novel unidentified viruses (Fox *et al.*, 2019) and their variants (Adams *et al.*, 2013). HTS continues to evolve, and different platforms and sample preparation methods have been developed (Pecman *et al.*, 2017). For instance, the MinION platform (Oxford Nanopore Technologies) has a small size and can be run from a laptop, which allows it to be deployed in simple laboratories close to the point of sampling, something not easily possible with other HTS platforms.

HTS has numerous advantages such as the identification of unknown viruses or as support for the development of other diagnostic assays. For example, the genome data generated by HTS can allow a rapid design of primers and probes for an RT-qPCR assay. However, results of the analysis of a HTS run depend on multiple factors such as the pipeline performance, the database accuracy and extensiveness, and the scientific expertise of the user (Massart *et al.*, 2019). Sensitivity of the technique not only relies on the intrinsic sensitivity of the sequencing, but also on the bioinformatic pipeline used. The analysis of HTS data can be a long and complex process and requires significant expertise to understand the many steps linked together in an analysis pipeline. Different bioinformatic pipelines have been developed for the identification of plant virus sequences within HTS data (Villamor *et al.*, 2019). However, the number of user-friendly bioinformatic pipelines available that could facilitate the analysis of the HTS data is limited. In this study, two different pipelines were used to analysis HTS data: Electronic Diagnostic Nucleic acid analysis (EDNA) and Angua. Angua is a pipeline routinely used for the analysis of HTS data at Fera Science Ltd. This pipeline allows an exhaustive analysis of the

data which requires server capacity and takes several days. This pipeline is one of the many available for the analysis of HTS data from potential virus-infected samples (Villamor *et al.*, 2019), which requires command line knowledge.

On the other hand, EDNA is a novel bioinformatic pipeline for the detection and identification of viruses infecting roses under development (Peña-Zuñiga *et al.*, 2017) built at Oklahoma State University. EDNA enables the detection of multiple viruses in raw outputs of a sample run using either Illumina or Oxford Nanopore platforms (Vazquez-Iglesias *et al.*, 2020a). This computational tool focuses on the identification of predetermined specific virus sequences. The software identifies the genetic signatures of target viruses using previously designed electronic probes (e-probes) in metagenomic datasets. EDNA aims to simplify the bioinformatic analysis of complex HTS metagenome data by eliminating the need for data assembly, accelerating the diagnostic process. Multiple pathogens can be identified in a sample by performing just one test, obtaining the results in a couple of minutes. The software can be accessed through a standard personal computer, with no need for a specific dedicated server capacity.

In this study, these two pipelines, Angua and EDNA, were used for the analysis of HTS data obtained after running 34 samples using the Illumina MiSeq platform, following confirmation with ELISA and RT-qPCR. Results obtained by the different pipelines and diagnostic methods were compared.

5.2 Materials and Methods

Samples (34) were collected during 3 consecutive years (2017-2020) as part of a survey of viruses in the UK or were collected by inspectors from the Animal and Plant Health Agency (APHA) at Heathrow Airport (London, UK) between February and May 2019 (Table A.2). Samples consisted of leaves from symptomatic and asymptomatic roses. Where symptoms were observed they were consistent with virus infections such as mottling, yellow veining, distortion, and ring spots. For this experiment, samples were chosen on the basis of the results obtained in preliminary tests (ELISA, RT-qPCR), to be included in a HTS run. In addition, asymptomatic material was also selected to look for non-targeted viruses, as well as imported samples coming from overseas.

5.2.1 ELISA

ELISA analysis was performed for a range of viruses using commercially available kits following the manufacturer's instructions, as follows: arabis mosaic virus (ArMV), apple mosaic virus (ApMV), prunus necrotic ringspot virus (PNRSV), strawberry latent ringspot virus

(SLRSV), and tomato spotted wilt virus (TSWV), as described in the Material and Methods section in Chapter 3.

5.2.2 Nucleic acid extraction

RNA was extracted from the leaf samples either by using a magnetic bead-based extraction method and the KingFisher® mL platform (Thermo Scientific) or using a CTAB (cetyl trimethylammonium bromide, see Material and Methods Chapter 3).

5.2.3 RT-qPCR (TaqMan)

Nucleic acid samples were tested using an RT-qPCR for the COX1 gene, ApMV, ArMV, PNRSV, rose cryptic virus-1 (RoCV1), SLRSV, and TSWV, as described in Material and Methods in Chapter 3.

DNA amplified using the RT-qPCR assays for TSWV and ApMV was cloned using a pGEM®-T Easy Vector System (Promega), following the manufacturer's recommended protocols. Clones containing inserts, white colonies, were identified, and a colony PCR was performed. Plasmid DNA was purified using a QIAquick® PCR Purification Kit (Qiagen, Germany) following the manufacturer's instructions before being sequenced (Eurofins Genomics, Germany) using universal M13 primers.

5.2.4 High-throughput sequencing library preparation

Preparation of the libraries for sequencing is described in the Material and Methods section in Chapter 4.2.

5.2.5 Angua pipeline analysis

HTS data was analysed using both the Angua (https://fred.fera.co.uk/smcgreig/angua) and the EDNA pipeline (Stobbe *et al.*, 2013). For Angua pipeline analysis, sequence reads were trimmed for quality using Sickle (v1.33; Joshi and Fass, 2011), and assembled using Trinity (v2.8.4; Grabherr *et al.*, 2011).They were compared to the GenBank nr and nt databases using BLAST+ (Camacho *et al.*, 2009). Reads of viral origin were inspected using MEGAN community edition (v6.12.3; Huson *et al.*, 2016). Pairwise analysis and Neighbour joining trees were generated using MEGA7 v7.0.21; Kumar *et al.*, 2016).

5.2.6 EDNA pipeline analysis

The EDNA pipeline analysis (Stobbe *et al.*, 2013) of the HTS data was performed using the MiFi software (http://bic.okstate.edu/). MiDetect was employed with an e-probe library containing all the available e-probe sets for rose viruses: alfalfa mosaic virus (AMV; 28 e-probes); apple chlorotic leafspot virus (ACLSV; 21 e-probes); ApMV (7 e-probes); apple stem grooving virus (ASGV; 11 e-probes); ArMV (30 e-probes); blackberry chlorotic ringspot virus

(BCRV; 12 e-probes); impatient necrotic spot virus (INSV; 20 e-probes); iris yellow spot virus (IYSV; 21 e-probes); PNRSV (23 e-probes); prune dwarf virus (PDV; 15 e-probes); rose leaf rosette-associated virus (RLRaV; 59 e-probes); rose leaf curl virus (RoLCuV; 7 e-probes); rose yellow leaf virus (RoYLV; 10 e-probes); rose yellow mosaic virus (RoYMV; 24 e-probes); rose yellow vein virus (RYVV; 46 e-probes); raspberry ringspot virus (RpRSV; 29 e-probes); rose rosette virus (RRV; 22 e-probes); rose spring dwarf-associated virus (RSDaV; 19 e-probes); RoCV1 (5 e-probes); rose necrotic mosaic virus (RoNMV; 1 e-probe); SLRSV (34 e-probes); tobacco ringspot virus (TRSV; 42 e-probes); tobacco streak virus (TSV; 19 e-probes); tomato ringspot virus (ToRSV; 48 e-probes); TSWV (22 e-probes); and tomato yellow ring virus (TYRV; 4 e-probes). All the e-probes were 30 nucleotides long.

When a sample is analysed using the EDNA pipeline, the number of matches and hits are returned for each of the e-probes within an e-probe set for the detection of a virus. In this context, a match is defined as an instance where an individual e-probe was found in the raw data, such that the total number of matches must be equal to or less than the total number of e-probes available in a set for the detection of a virus, whereas a hit is defined as any instance where a read from the raw data has homology with an e-probe. A single match could be made up of multiple hits. It is critical to distinguish between a true and a false signal. Thus, decoy e-probe sets are used to determine the chances that a relatively random sequence from the raw data (e.g. from the host) would find a match with any of the e-probes by chance (Stobbe *et al.*, 2013). The results of the e-probes are compared to the decoy set. No significant difference between the two sets suggests no evidence for the presence of the virus sequences, whilst a significant difference indicates a positive result for the virus test. However, there is a grey area when the EDNA p-value is between 0.05 and 0.1. In this case, EDNA 'suspects' the sample could be infected with the virus, and further testing will be needed.

5.2.7 Further analysis

Mapping of raw reads against virus genomes was performed using BWA-ME (Li and Durbin, 2009), visualising the results with SAMtools (Li *et al.*, 2009) through the Galaxy server (Afgan *et al.*, 2018). Identification of a single or low number of matching raw reads does not determine the sample is positive.

Alignment of the different virus isolates with publicly available sequences, and alignment of the e-probe sequences with virus sequences were performed using Geneious® software v11.0.2. Results obtained among the different diagnostic methods and pipeline analysis were compared using tables adapted from Hughes *et al.* (2006); Fig. 5.1). Comparison was performed individually for each of the viruses (SLRSV, ArMV, RoCV1 and PNRSV) found by all the

techniques. Sensitivity was calculated by dividing the positive agreements (X) for both techniques by the sum of the positive agreements (X) and the number of discrepancies (Y or Z). The number of discrepancies was chosen as the higher of both values (Y or Z) due to the lack of knowledge of which samples were truly positive or negative. Thus, if Z>Y, sensitivity of technique 1 vs technique 2 will be represented in the percentage of sensitivity, and vice versa. Statistical differences between the different diagnostic techniques were calculated performing a McNemar chi-square test (McNemar, 1947). The differences were considered statistically significant at p-value ≤ 0.05.

Virus	Technique 2			
Technique 1		+	-	Total
	+	X	Y	X+Y
	-	Z	W	Z+W
	Total	X+Z	Y+W	

Figure 5.1- Example of table adapted from Hughes *et al.* (2006) for the comparison of the different diagnostic techniques. Numbers in the tables represent a positive agreement (X), a negative agreement (W), and discrepancies (Z or Y) between two different diagnostic techniques.

Correlation among the two pipelines, Angua and EDNA, and the confirmatory testing with ELISA and RT-qPCR was calculated using Spearman's correlation rank test using "corrplot" package in RStudio (Wei and Viliam, 2017).

5.3 Results

Samples (34) were tested by high-throughput sequencing (HTS) and subsequently analysed using two different pipelines: Angua and EDNA. These samples (34) were also tested by ELISA and RT-qPCR (TaqMan) to confirm the results obtained by HTS (Table 5.1).

5.3.1 Concordant results

A total of 6 samples gave a positive result after analysis with both pipelines and confirmatory testing for the detection of strawberry latent ringspot virus (SLRSV; family *Secoviridae*); 8 for arabis mosaic virus (ArMV; genus *Nepovirus*); 5 for rose cryptic virus-1 (RoCV1; family *Partitivirus*); and 1 for prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*). In the case of RoCV1, an ELISA test was not performed, as antibodies kits were not available. For 8 SLRSV-positive samples, 5 ArMV-positives and 1 PNRSV-infected sample, results were positive by both pipelines but only confirmed by RT-qPCR as the samples gave negative results following ELISA testing (Table 5.1).

Table 5.1- List of samples and results obtained with the different HTS pipelines and virus testing by ELISA and RT-qPCR. In the table are indicated the positive (+) and the negative (-) results for Angua and EDNA pipelines and ELISA, the C_T values obtained after RT-qPCR

testing, the total number of reads identified for the viruses and satellite viruses (if possible), the total number of matches and hits of the e-probes after EDNA analysis.

Sample	Virus	Angua pipeline	EDNA pipeline	ELISA	RT-qPCR (C_T value)	Identified virus reads	Identified satellite virus reads	EDNA Matches	EDNA Hits
ID152	ApMV	-	-	-	36.94	0	NA	0	0
ID196	ApMV	-	-	-	36.57	0	NA	0	0
ID66	ArMV	+	+	+	27.15	331	123	9	45
ID68	ArMV	+	+	+	24.32	5781	234	19	211
ID74	ArMV	+	+	+	29.21	1584	1248	16	180
ID91	ArMV	+	+	+	29.32	41080	45174	19	214
ID210	ArMV	+	+	+	34.55	23320	0	17	228
ID237	ArMV	+	+	+	30.24	50	141	5	9
ID245	ArMV	+	+	+	22.02	2057	4	13	167
ID256	ArMV	+	+	+	29.21	5029	1271	19	257
ID258	ArMV	+	+	+	15.95	42137	980	21	234
ID65	ArMV	+	+	-	32.15	136	11	10	24
ID70	ArMV	+	+	-	29.81	614	16	12	117
ID90	ArMV	+	+	-	30.31	2544	615	13	166
ID128	ArMV	+	+	-	30.42	364	102	12	60
ID252	ArMV	+	+*	-	32.55	8	6	2	2
ID145	ArMV	+	+	+	Negative	9035	1100	18	179
ID95	ArMV	+	+	-	Negative	286	1225	13	165
IDPool1	ArMV	+	+	-	Negative	425	0	11	75
ID266	ArMV	+	-	+	30.18	12	35	0	0
ID231	ArMV	-	+*	-	29.55	8	0	1	1
ID148	ArMV	+	-	-	34.47	2	0	0	0
ID244	ArMV	+	-	-	38.6	2	0	0	0
ID99	ArMV	-	-	-	37.24	0	2	0	0
ID100	ArMV	-	+	-	Negative	22	31	4	5
ID237	PNRSV	+	+	+	12.17	12	NA	3	6
ID148	PNRSV	+	+	-	22.16	36	NA	7	20
ID65	PNRSV	-	+	-	Negative	4	NA	3	3
ID152	PNRSV	-	-	-	36.21	0	NA	0	0
ID153	PNRSV	-	-	-	38.63	2	NA	0	0
ID164	PNRSV	-	-	-	38.86	0	NA	0	0
ID188	PNRSV	-	-	-	23.93	2	NA	0	0
ID196	PNRSV	-	-	-	33.92	0	NA	0	0
ID244	PNRSV	-	-	-	35.88	0	NA	0	0
ID256	PNRSV	-	-	-	33.1	0	NA	0	0
ID258	PNRSV	-	-	-	36.4	0	NA	0	0
ID266	PNRSV	-	-	-	33.01	0	NA	0	0
ID11B	RoCV1	+	+	NA	20.05	119	NA	4	32
ID68	RoCV1	+	+	NA	22.72	86	NA	5	23
ID91	RoCV1	+	+	NA	20.63	235	NA	5	45
ID188	RoCV1	-	+*	NA	37.18	8	NA	1	1
ID210	RoCV1	+	+	NA	24.7	198	NA	4	14
ID231	RoCV1	+	+	NA	31.17	14	NA	2	4
ID252	RoCV1	+	+	NA	20.85	910	NA	5	75
ID66	RoCV1	-	-	NA	35.45	0	NA	0	0
ID74	RoCV1	-	-	NA	36.65	0	NA	0	0
ID99	RoCV1	-	-	NA	35.4	0	NA	0	0
ID102	RoCV1	-	-	NA	38.12	2	NA	0	0
ID152	RoCV1	-	-	NA	34.94	0	NA	0	0
ID153	RoCV1	-	-	NA	35.71	0	NA	0	0
ID237	RoCV1	-	-	NA	34.18	0	NA	0	0
ID244	RoCV1	-	-	NA	36.11	0	NA	0	0
ID245	RoCV1	-	-	NA	29.54	0	NA	0	0
ID256	RoCV1	-	-	NA	28.99	2	NA	0	0
ID258	RoCV1	-	-	NA	32.8	0	NA	0	0
ID266	RoCV1	-	-	NA	36.37	0	NA	0	0
ID70	SLRSV	+	+	+	20.05	635	218	23	251
ID91	SLRSV	+	+	+	10.95	34790	10377	27	358
ID128	SLRSV	+	+	+	19.94	3200	0	23	331
ID237	SLRSV	+	+	+	13.69	208	22	19	109
ID244	SLRSV	+	+	+	23.06	48	0	13	41
ID258	SLRSV	+	+	+	12.43	14952	0	18	193
ID66	SLRSV	+	+	-	16.62	1343	0	13	181
ID68	SLRSV	+	+	-	23.37	21	0	7	20
ID90	SLRSV	+	+*	-	37.05	6	2	2	2
ID231	SLRSV	+	+	-	22.33	14	0	4	12
ID245	SLRSV	+	+*	-	25.66	4	0	2	2
ID256	SLRSV	+	+	-	23.58	111	14	8	19

ID145	SLRSV	+	+	+	Negative	2676	1200	18	204
ID11B	SLRSV	-	+	-	32.34	12	4	4	4
ID252	SLRSV	-	+*	-	37.47	2	2	1	1
ID65	SLRSV	-	+	-	Negative	10	2	3	3
ID100	SLRSV	-	+	-	Negative	20	12	4	8
ID74	SLRSV	-	-	-	26.89	0	0	-	-
ID74	TSWV	-	-	-	36.15	0	NA	0	0
ID152	TSWV	-	-	-	36.45	0	NA	0	0
ID153	TSWV	-	-	-	35.96	0	NA	0	0
ID112	-	-	-	-	-	-	-	0	0
ID114	-	-	-	-	-	-	-	0	0
ID223	-	-	-	-	-	-	-	0	0

***** P-value appeared as not computed (negative for the virus) in the results table. Further analysis was performed by looking at the match/hit numbers, and some were identified. These samples were considered positive for EDNA as the e-probes matched with the raw data.

A sample (ID145) gave a positive result for SLRSV and ArMV after analysis with both pipelines. These results were confirmed using ELISA testing, but this sample was negative for both viruses when tested using RT-qPCR. An alignment of the ArMV (Fig. 5.2) and SLRSV (Fig. 5.3) fragments identified using the Angua pipeline was performed for samples that gave positive results and negative results when tested using RT-qPCR. In the case of samples ID256 and ID128, two different isoforms were included in the alignment. Isoforms are variants obtained after *de novo* assembly using Trinity software as part of the Angua pipeline. These variants represent the same gene of the virus, but the software generates different options after assembly when the reconstruction path of the virus genome is not clear. The alignment showed the presence of polymorphisms with reference to the primers and probe. These variations were present in samples that gave positive results for RT-qPCR as well as the sample (ID145) that gave negative results, even showing more polymorphisms.

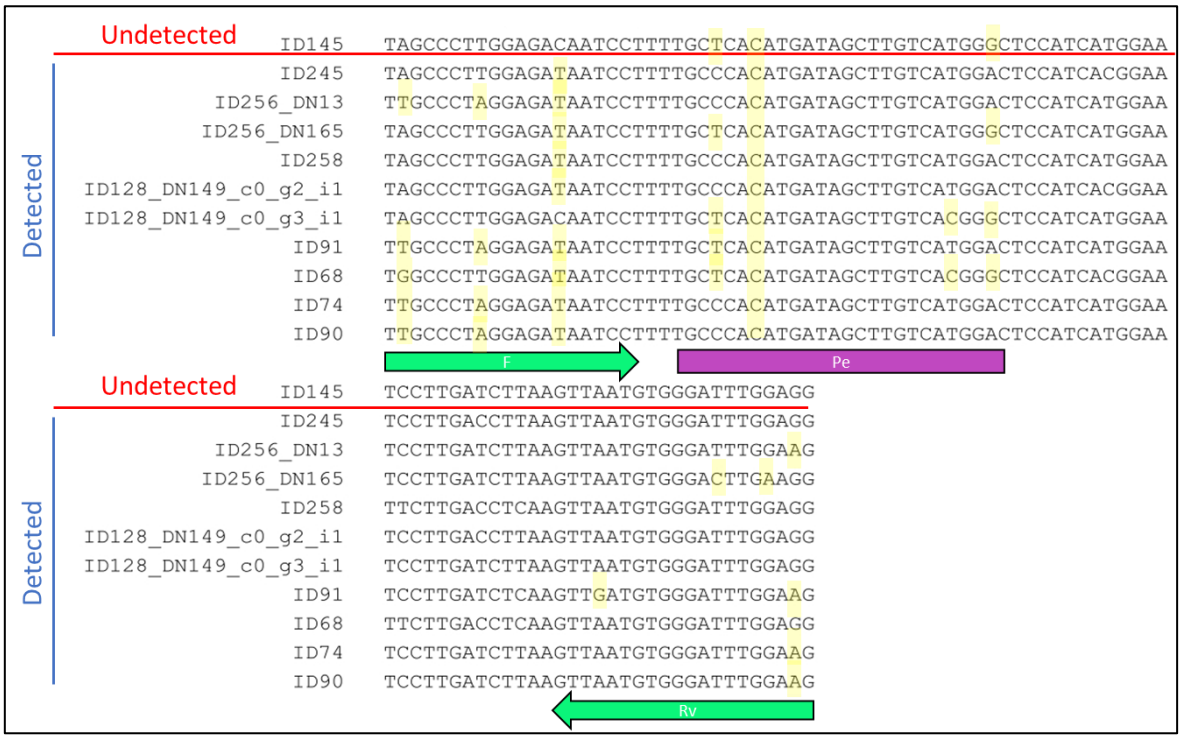

Figure 5.2- Alignment of the arabis mosaic virus region amplified by RT-qPCR obtained after the Angua pipeline analysis of the undetected sample ID145 with detected samples (ID245, ID256, ID258, ID128, ID91, ID68, ID74, and ID90). Highlighted in yellow are the nucleotide variations in reference to the primers (green) and probe (purple) sequences. Both sequences of ID256 and ID128 represent different isoforms of the same assembled read.

Figure 5.3- Alignment of the amplified region of strawberry latent ringspot virus by RT-qPCR of the undetected sample ID145 obtained after the Angua pipeline analysis with detected samples (ID128, ID258, ID70). Highlighted in yellow are the nucleotide variations in reference to the primers (green) and probe (purple) sequences. Different ID128 and ID70 sequences represent different isoforms of the same assembled read.

5.3.2 Correlations among the different testing methods

Results obtained among the different pipelines and confirmatory diagnostic methods were compared using tables adapted from Hughes *et al.* (2006; Tables 5.2 A-D). Comparison was performed individually for each of the viruses (SLRSV, ArMV, RoCV1 and PNRSV) and sensitivity was also calculated. The statistical difference between the techniques was analysed with a McNemar chi-square test. For SLRSV and ArMV testing, ELISA sensitivity was significantly different from the other diagnostic techniques. Overall, RT-qPCR was the most sensitive technique, followed by HTS (EDNA and Angua pipeline analysis). For the detection of SLRSV and ArMV, differences between analysis with both pipelines and RT-qPCR were not significant. The Angua pipeline was less sensitive than the EDNA pipeline analysis for the detection of SLRSV, not detecting the virus in samples ID11B and ID252, (confirmed by RT-qPCR), and ID65 and ID100 (positives only after analysis with the EDNA pipeline). However, in the case of ArMV, both pipelines showed a similar sensitivity. Angua detected ArMV in samples ID148, ID244, and ID266 (confirmed by RT-qPCR; ID266 also using ELISA), which were negative after analysis with the EDNA pipeline. However, the EDNA pipeline identified ArMV in samples ID231 (confirmed by RT-qPCR) and ID100 (unconfirmed), when the Angua pipeline was negative. For the detection of PNRSV, RT-qPCR showed the highest sensitivity. The Angua and the EDNA pipeline showed a similar sensitivity. Only one sample (ID65) was identified as positive after analysis with EDNA when negative after Angua pipeline, and unconfirmed using RT-qPCR or ELISA. For RoCV1 a comparison with ELISA was not possible. RT-qPCR showed a significantly higher sensitivity than HTS after the Angua and the EDNA analysis. In this case, sample ID188 was identified as positive for RoCV1 after EDNA analysis, being negative for the Angua pipeline.

Table 5.2- Comparison in pairs of Angua and EDNA high-throuput sequencing pipelines, ELISA, and RT-qPCR results for the detection of strawberry latent ringspot virus (SLRSV; A), arabis mosaic virus (ArMV; B), prunus necrotic ringspot virus (PNRSV; C) and rose cryptic virus-1 (RoCV1; D). Percentage of sensitivity among the techniques was also included.

A.

SLRSV	**RT-qPCR**			
ELISA		+	-	Total
	+	6	1	7
	-	9	3	12
	Total	15	4	19

Sensitivity 40%

SLRSV	**EDNA**			
RT-qPCR		+	-	Total
	+	14	1	15
	-	3	1	4
	Total	17	2	19

Sensitivity 82%

SLRSV	**Angua**			
RT-qPCR		+	-	Total
	+	12	3	15

SLRSV	**EDNA**			
Angua		+	-	Total
	+	13	0	13

	-	1	3	4
	Total	13	6	19

Sensitivity 80%

	-	4	2	6
	Total	17	2	19

Sensitivity 76%

SLRSV	**Angua**			
ELISA		+	-	Total
	+	7	0	7
	-	6	6	12
	Total	13	6	19

Sensitivity 54%

SLRSV	**EDNA**			
ELISA		+	-	Total
	+	7	0	7
	-	10	2	12
	Total	17	2	19

Sensitivity 41%

B.

ArMV	**RT-qPCR**			
ELISA		+	-	Total
	+	10	1	11
	-	9	5	14
	Total	19	6	25

Sensitivity 53%

ArMV	**EDNA**			
RT-qPCR		+	-	Total
	+	15	4	19
	-	4	2	6
	Total	19	6	25

Sensitivity 79%

ArMV	**Angua**			
RT-qPCR		+	-	Total
	+	17	2	19
	-	3	3	6
	Total	20	5	25

Sensitivity 85%

ArMV	**EDNA**			
Angua		+	-	Total
	+	17	3	20
	-	2	3	5
	Total	19	6	25

Sensitivity 85%

ArMV	**Angua**			
ELISA		+	-	Total
	+	11	0	11
	-	9	5	14
	Total	20	5	25

Sensitivity 55%

ArMV	**EDNA**			
ELISA		+	-	Total
	+	10	1	11
	-	9	5	14
	Total	19	6	25

Sensitivity 53%

C.

PNRSV	**RT-qPCR**			
ELISA		+	-	Total
	+	1	0	1
	-	10	1	11
	Total	11	1	12

Sensitivity 9%

PNRSV	**EDNA**			
RT-qPCR		+	-	Total
	+	2	9	11
	-	1	0	1
	Total	3	9	12

Sensitivity 18%

PNRSV	**Angua**			
RT-qPCR		+	-	Total
	+	2	9	11
	-	0	1	1
	Total	2	10	12

Sensitivity 18%

PNRSV	**EDNA**			
Angua		+	-	Total
	+	2	0	2
	-	1	9	10
	Total	3	9	12

Sensitivity 67%

PNRSV	**Angua**			
ELISA		+	-	Total
	+	1	0	1
	-	1	10	11

PNRSV	**EDNA**			
ELISA		+	-	Total
	+	1	0	1
	-	2	9	11

	Total	2	10	12			Total	3	9	12

Sensitivity 5% **Sensitivity** 33%

D.

RoCV1	**EDNA**			
RT-qPCR		+	-	Total
	+	7	12	19
	-	0	0	0
	Total	7	12	19

Sensitivity 37%

RoCV1	**Angua**			
RT-qPCR		+	-	Total
	+	6	13	19
	-	0	0	0
	Total	6	13	19

Sensitivity 32%

RoCV1	**EDNA**			
Angua		+	-	Total
	+	6	0	6
	-	1	12	13
	Total	7	12	19

Sensitivity 86%

Spearman's rank correlation test was used to assess the statistical dependence among the results obtained for both pipelines and the confirmatory diagnostic techniques (Fig. 5.4). Results obtained using ELISA (positive/negative), the -C_T value from the RT-qPCR, the identification of a virus after analysis with the Angua pipeline (positive/negative), the number of reads identified after mapping, and the matches and hits detected after the use of the EDNA pipeline were included as variables. Spearman's test identified a significative correlation in all cases (p-value<0.05). RT-qPCR results (C_T value) and ELISA showed a moderate correlation with the remaining variables. As expected, the correlation between the number of reads identified and the results obtained after analysis with the Angua and the EDNA pipeline (matches and hits) is strong.

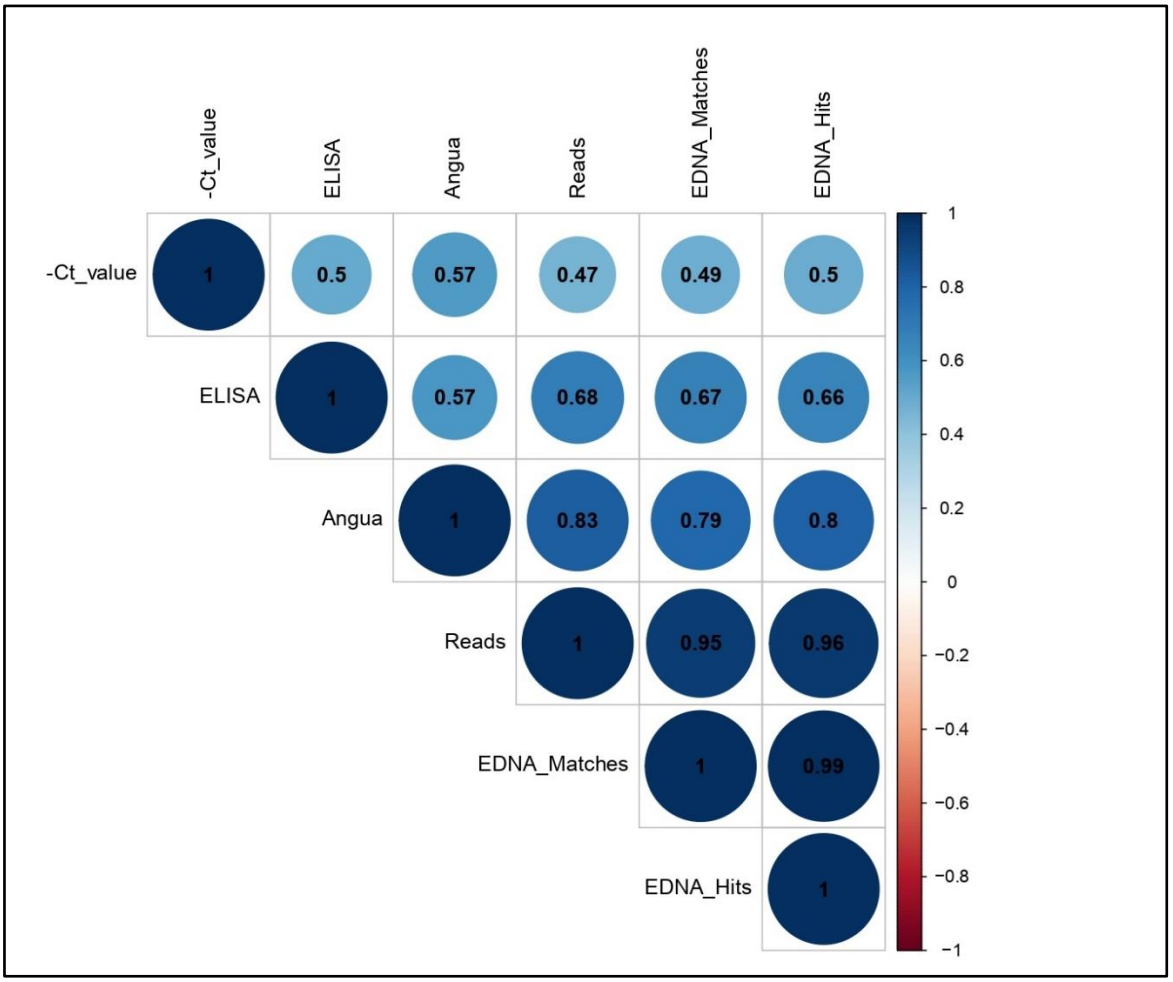

Figure 5.4- Correlation matrix showing Spearman's correlation coeficients indicating dependance among the -C_T value obtained after RT-qPCR testing, results from ELISA, and high-throughput sequencing after analysis with the Angua and the EDNA pipeline (matches and hits), as well as the number of reads identified after mapping the raw reads against the virus genome.

The C_T values obtained using RT-qPCR were used as indicators to graphically represent the correlations with ELISA and both pipelines. Overall, there is a trend which shows that with low C_T values, ELISA tends to be positive (Fig. 5.5). In the case of the Angua pipeline (Fig. 5.6), results show a similar tendency, as well as with the EDNA pipeline (Fig. 5.7). In all three cases, the inflection point is at a C_T values of 30-31, when results tend to be negative for the three methods (ELISA and both pipelines).

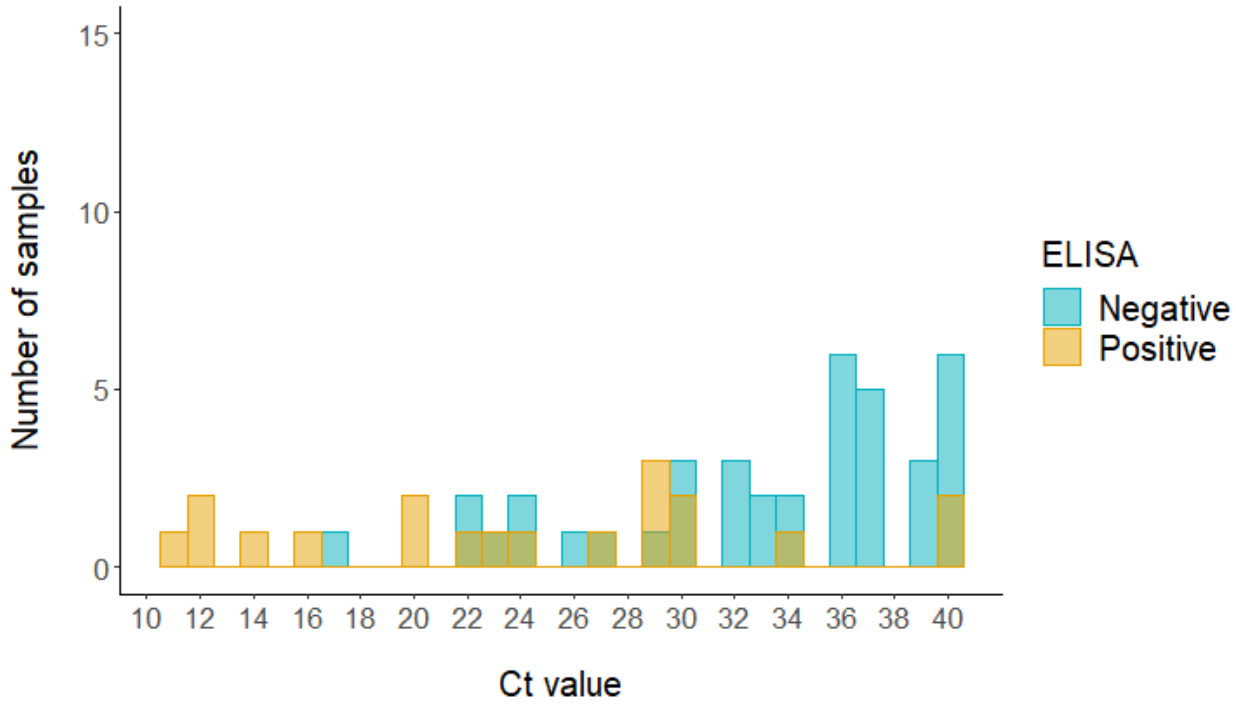

Figure 5.5- Number of cases (frequency) of samples positive (yellow) or negative (blue) for ELISA testing in comparison with the C_T values obtained after RT-qPCR testing. Negative samples for RT-qPCR are represented with a C_T value of 40.

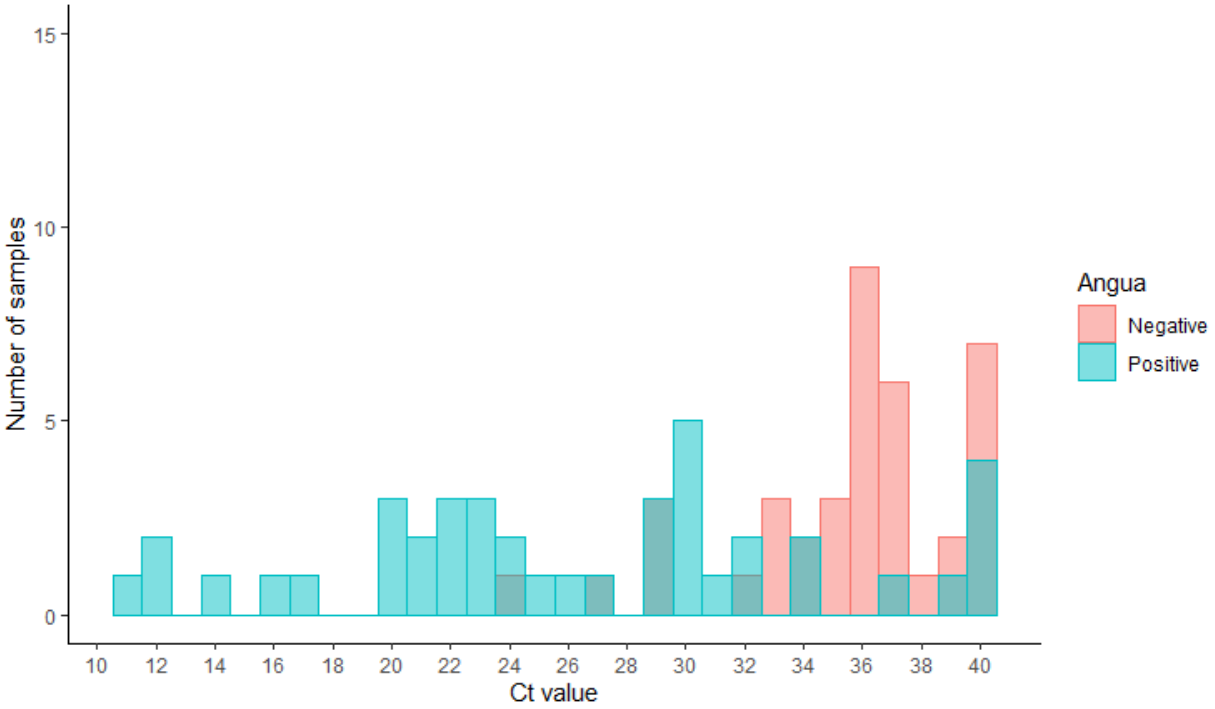

Figure 5.6- Number of cases (frequency) of samples positive (blue) or negative (red) after analysis with the Angua pipeline in comparison with the C_T values obtained after RT-qPCR testing. Negative samples for RT-qPCR are represented with a C_T value of 40.

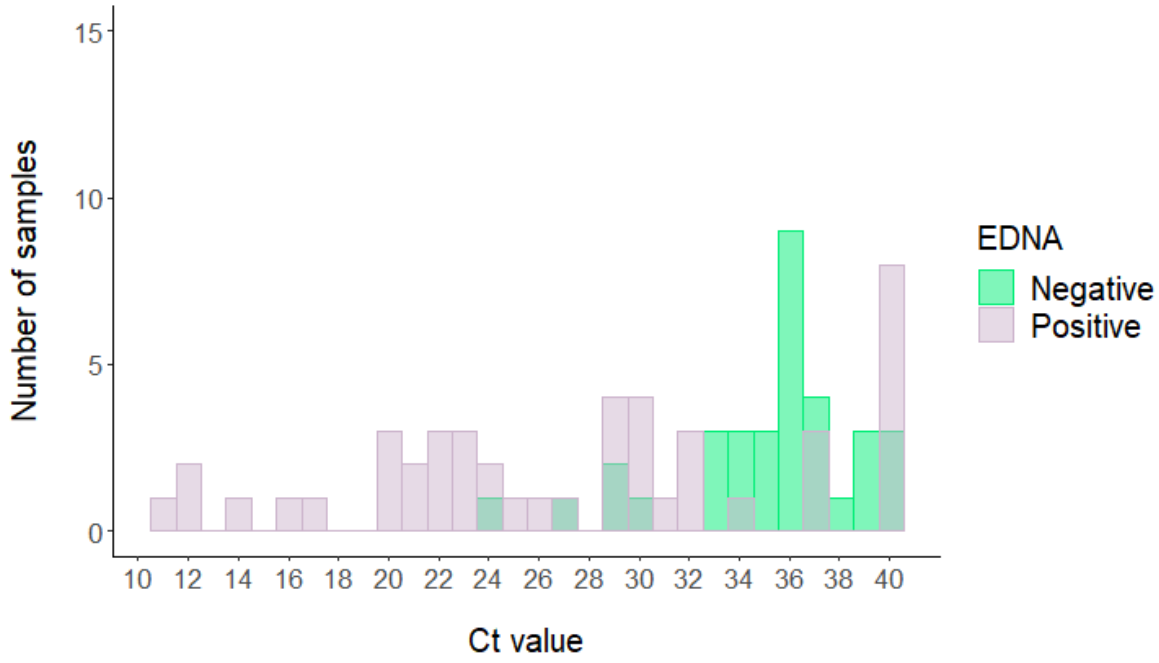

Figure 5.7-. Number of cases (frequency) of samples positive (purple) or negative (green) after analysis with the EDNA pipeline in comparison with the C_T values obtained after RT-qPCR testing. Negative samples for RT-qPCR are represented with a C_T value of 40.

5.3.3 Discordant results: exploring the different cases

Some of the results obtained were negative for both the Angua and the EDNA pipeline analysis, whilst positive for ELISA and/or RT-qPCR diagnostic techniques, and vice versa. Different scenarios were identified:

Case 1- Negative results obtained after the EDNA pipeline analysis.

ArMV: sample ID266 gave a negative result after the EDNA analysis but positive when analysed using the Angua pipeline, ELISA and RT-qPCR. After the Angua pipeline analysis, one fragment of ArMV genome and a fragment of an ArMV satellite were identified. Further analysis was performed, which revealed that the ArMV fragment identified using the Angua pipeline was not represented by any e-probes used in the EDNA pipeline (Fig. 5.8).

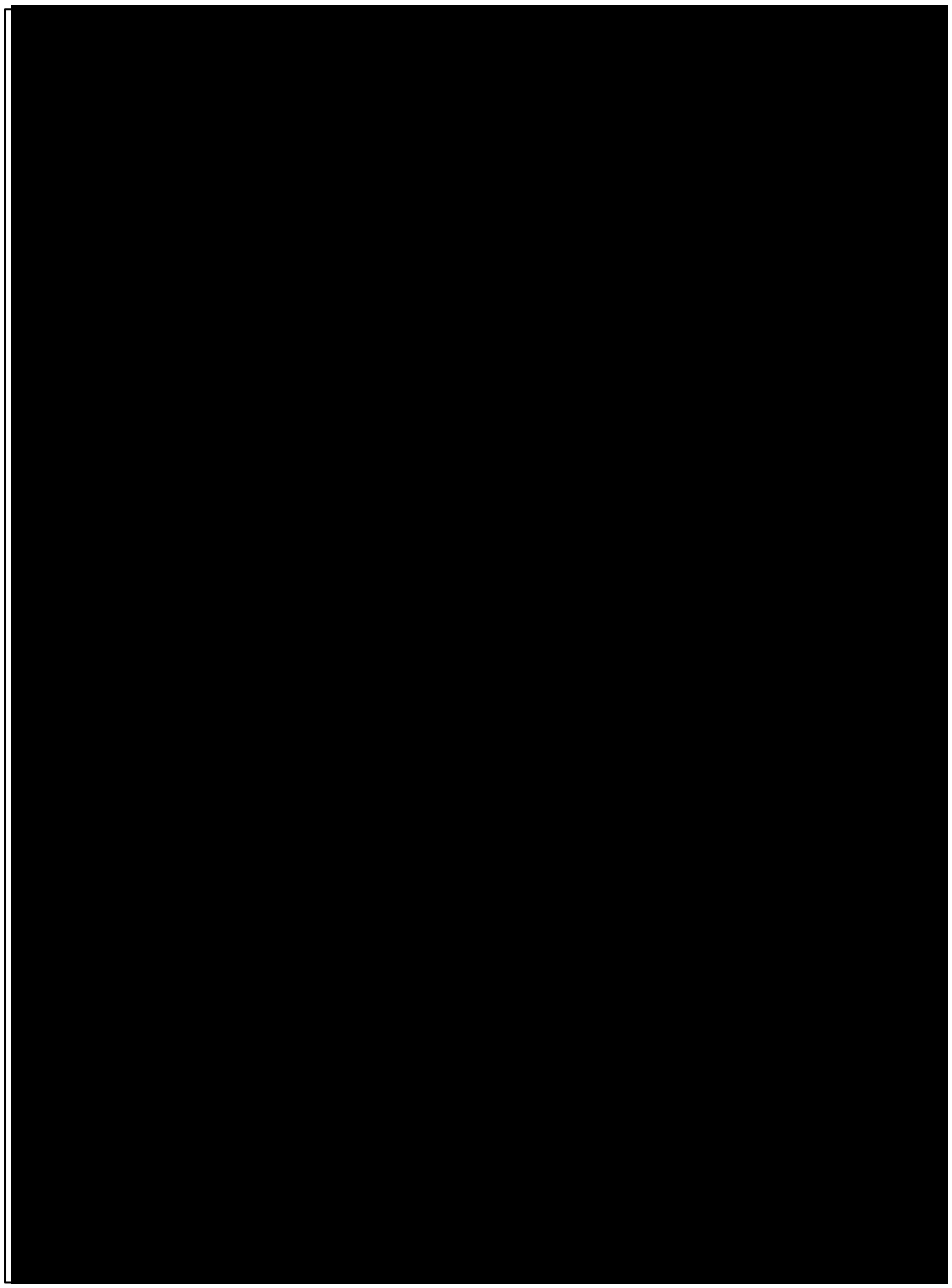

Figure 5.8- Alignment of a section of the arabis mosaic virus (ArMV) fragment identified in sample ID266 after the Angua pipeline analysis with other publicly available ArMV sequences (EF426853.1; MH802021.1; NC_006056.1). EDNA e-probes were aligned with these sequences (yellow boxes), revealing that the e-probes do not cover the region identified with the other pipeline.

Case 2- Negative results obtained after the Angua pipeline analysis.

RoCV1: a single sample (ID188) was negative for RoCV1 after testing using the Angua pipeline but positive for the remaining tests and the EDNA pipeline analysis. This sample gave a positive result (1 match) after analysis using the EDNA pipeline and was confirmed by RT-qPCR. The alignment of the e-probe with the matching read showed a 100% identity over the whole length of the fragment. In this case, ELISA was not performed, as antibodies were not available for RoCV1 testing. A Blast+ search was performed with the read matching the EDNA

e-probe, confirming RoCV1 (100% identity with MK075826.1, KM598758.1, EU024675.1, EU413666.1).

Case 3- Negative results after the EDNA pipeline analysis and ELISA testing.

ArMV: samples ID148 and ID244 were positive for ArMV following analysis with the Angua pipeline, and results were confirmed by RT-qPCR. In both cases ELISA gave negative results and the samples were negative following analysis with the EDNA pipeline. Only one fragment of sequence was identified following analysis with the Angua pipeline for both samples, and no e-probes were found to match this region (Figs. 5.9, 5.10).

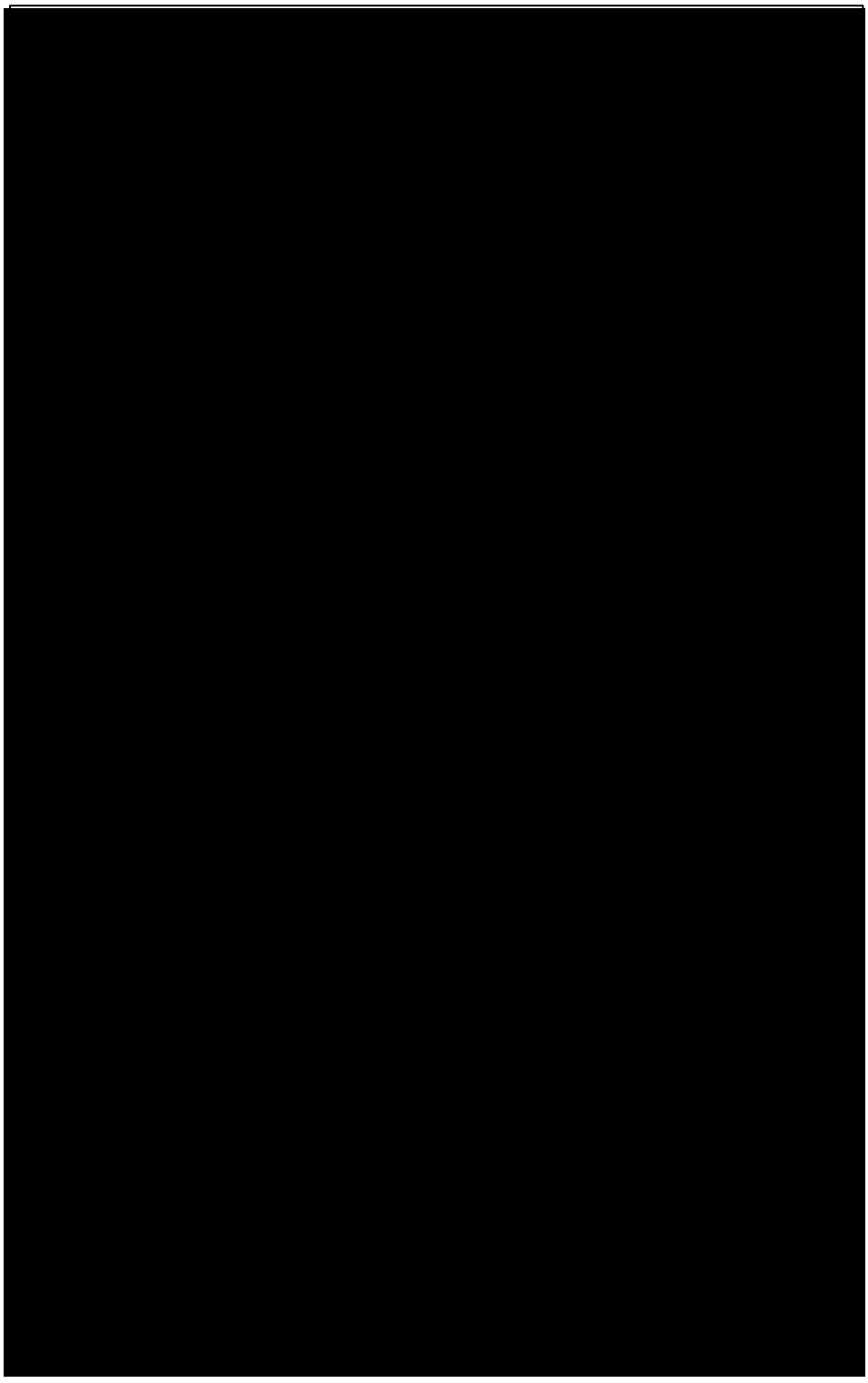

Figure 5.9- Alignment of a section of the arabis mosaic virus (ArMV) fragment identified in sample ID244 after the Angua pipeline analysis with other publicly available ArMV sequences (EF426853.1, MH802021.1, and NC_006056.1). EDNA e-probes were aligned with these sequences (yellow boxes), revealing that the e-probes do not cover the region identified with the other pipeline.

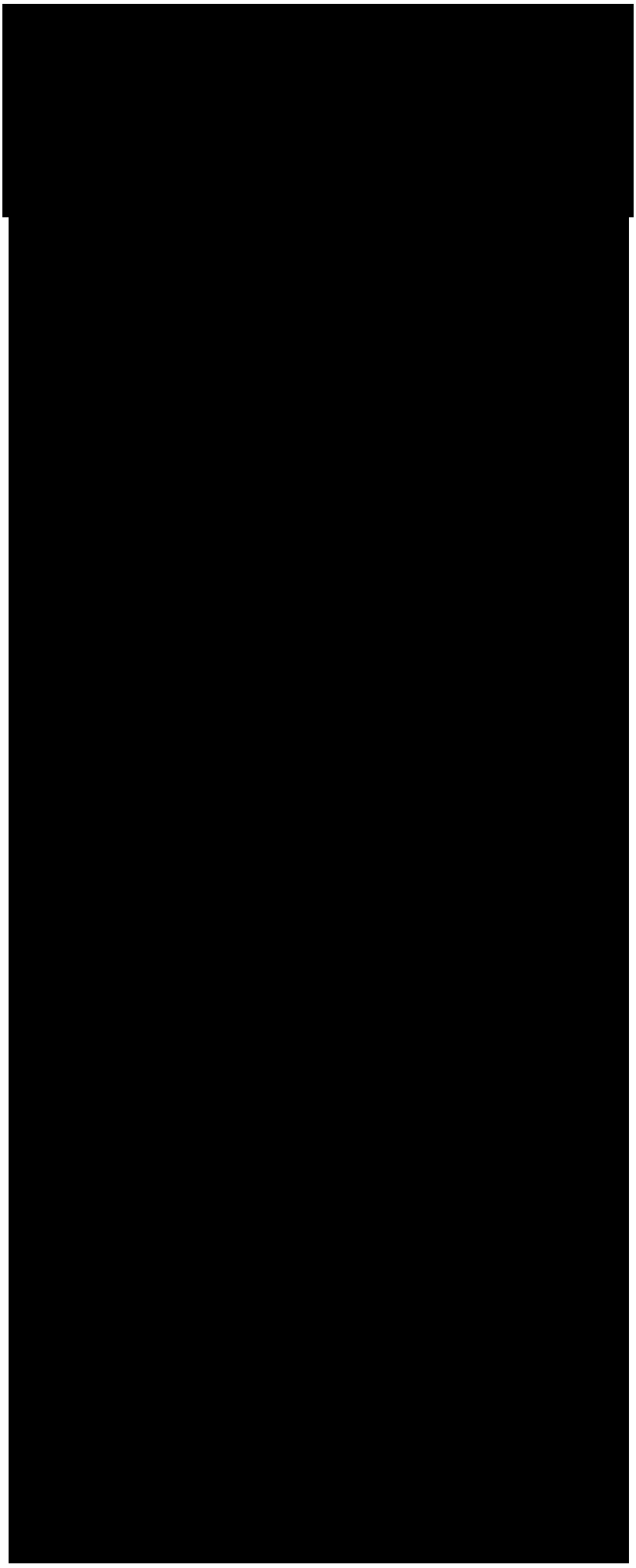

Figure 5.10- Alignment of a section of the arabis mosaic virus (ArMV) fragment identified in sample ID148 after the Angua pipeline analysis with other publicly available ArMV sequences (EF426853.1, MH802021.1, and NC_006056.1). EDNA e-probes were aligned with these sequences (yellow boxes), revealing that the e-probes do not cover the region identified with the other pipeline.

Case 4- Negative results after analysis using the Angua pipeline analysis and ELISA testing.

SLRSV: samples ID11B and ID252 gave a positive result for SLRSV following the EDNA analysis and confirmed by RT-qPCR. In both cases ELISA was negative, and analysis using the Angua pipeline did not identify any SLRSV sequences. Sample ID11B gave a positive result after the EDNA pipeline analysis identified 4 matches and hits, showing a 90-97% identity over the whole length of the fragment, and a 97% identity with 27 of the total 30 nucleotides of the e-probe for one of the reads. The sequence fragments matching EDNA e-probes were used in a Blast+ search and one of them was confirmed to be SLRSV with a 79.46-86.55% nucleotide identity (AY860979.1, MF196996.1). For sample ID252, a match was identified after analysis with the EDNA pipeline, showing a 93% identity with 27 of the total 30 nucleotides of the e-probe. This fragment matching the EDNA e-probe was included in a Blast+ search and confirmed as SLRSV on the matching read sequence with 86.67- 87.04% nucleotide identity (MF797000.1, X77466.1).

ArMV: sample ID231 gave a positive result for ArMV after the EDNA pipeline analysis (1 match) and was confirmed after RT-qPCR testing. The alignment of the fragment and the e-probe showed a 93% identify over the whole length of the fragment. A Blast+ search was performed with the matching read, and 91.28% identity with ArMV (EU617326.1) was identified.

Case 5- Positives after analysis with the Angua and the EDNA pipelines but negative by ELISA and RT-qPCR

ArMV: samples IDPool1 and ID95 gave a positive result for ArMV following analysis using both pipelines whilst negative by confirmatory testing. An alignment of the fragments identified using the Angua pipeline and the RT-qPCR primers and probe assay was performed (Fig. 5.11), with the aim of evaluating any variation which could affect their binding. Sequence variation was observed between the primers and probe and the fragments identified, although similar variation was observed for samples which gave positive results in RT-qPCR for the forward primer and the probe. However, the reverse primer region showed higher variation in the samples which gave positive results in RT-qPCR.

Figure 5.11- Alignment of the region of arabis mosaic virus amplified by RT-qPCR with the undetected samples IDPool1 and ID95 obtained after the Angua pipeline analysis and detected samples (ID245, ID256, ID258, ID128, ID91, ID68, ID74, and ID90). Highlighted in yellow are the nucleotide variations in reference to the primer (green) and probe (purple) sequences. Different ID256 and ID128 sequences represent different isoforms of the same assembled read.

SLRSV: different samples were identified as positive after analysis with the EDNA pipeline. Samples ID65 and ID100 were suspected (0.05<EDNA p-value<0.1) of being infected with SLRSV following the EDNA pipeline analysis (3 matches and 3 hits, and 4 matches and 8 hits, respectively). The alignment of the matching fragments and the e-probes showed a 90-100% identity over the whole fragment length. A Blast+ search was performed with the reads matching EDNA e-probes, and 2 of the 3 reads were confirmed to be SLRSV for ID65, with 83.57-95.18% identity (AY860979, MF796996.1). Mapping of the HTS raw data was performed and 4 paired-end reads matching the SLRSV genome were identified for sample ID65. A Blast+ search was performed and all reads were confirmed to match with SLRSV (75.17-95.18% identity in nucleotide comparison with MF796977.1, MF797005.1, MF796996.1, AY860979.1, and X75165.1 and 52-90% identity in amino acid comparison with AWO67808.1, YP_227374.1, AWO67809.1). Low percentage of identity could be due to the use of raw data for the BLAST search, including reads with low quality, rather than assembled reads. For sample ID100, the alignment of the matching fragments and the e-probes showed a 90-93% identity over the whole length of the fragment for three reads, 97-100% identity with 29 of the 30 nucleotides of the e-probes in three reads, and 96% identity with 27 nucleotides of the e-probes in two reads. A total of 4 reads were identified as SLRSV, with an 85.71-96.39%

identity in nucleotide comparison (MF796999.1, AY860979.1, MF796993.1). Mapping raw data against the virus genome showed 10 paired-end reads that were subsequently confirmed by a Blast+ search to be SLRSV (80.14-94.39% identity in nucleic acid comparison with MF797005.1, MF796993.1, MF796977.1, MF796999.1, MF796984.1, MF796978.1, HM591197.1, MF796996.1, X75165.1 and 70-94.67% in amino acid with AWO677804.1, AWO67822.1, AWO67802.1, YP_227370.1, YP_227367.1, AHB30925.1, AWO67823.1, YP_227376.1, AWO67808.1, YP_227368.1, YP_227374.1, AAY56484.1).

PNRSV: sample ID65 was also suspected (0.05<EDNA p-value<0.1) of being infected with PNRSV after the EDNA pipeline analysis (3 matches and 3 hits). Alignment of the e-probes and the matching reads showed a 93-97% identity over the whole fragment length, and a 93% identity with 28 of the 30 nucleotides of the e-probe for one of the reads. A Blast+ search confirmed PNRSV in 2 of the 3 reads identified matching the e-probes, with an 87.33% identity to MF069038.1. Paired-end reads (2) were identified mapping against the PNRSV genome. A Blast+ search confirmed PNRSV with an 87.33% identity in nucleotide comparison (GenBank accession numbers MF069038.1, MN656195.1) and amino acid comparison (71.43% identity with AZJ51141.1, AXY66745.1).

ArMV: sample ID100 gave a positive result for ArMV only after analysis with the EDNA pipeline (4 matches). Alignment of the matching fragments with the e-probes showed a 90-93% identity over the whole fragment length, and for one of the reads a 93% identity with 28 of the 30 nucleotides of the e-probe. Mapping was performed and 22 paired-end reads were found matching the ArMV genome. A Blast+ search was used, and results confirmed a match with ArMV in 21 paired-end reads. Nucleic acid comparison revealed a 73.60-95.65% identity with sequences belonging to ArMV (GenBank accession numbers X81815.1, EU617326.1, MH802018.1, EU617327.1, EF426853.1, AY090016.1, KC138733.1, GQ3695229.1, AB279740.2, MH802015.1, MG731671.1, MH614321.1, GQ3695230.1, EU433920.1, KJ481191.1, KJ481198.1, KJ481195.1, MH802021.1, D10086.1). Low percentage of identity could be due to the use of raw data for the BLAST search, including reads with low quality, rather than assembled reads. Alignment with the mapped reads and the RT-qPCR primers was performed. Of the 22 paired-end reads found, only 4 paired-end reads potentially matched with the primers and probe fragment. As shown in Fig. 5.12, the reverse primer and the probe have some variation.

Case 6- Positives only after RT-qPCR testing

SLRSV: sample ID74 gave a positive result for SLRSV when tested using RT-qPCR with a C_T value of 26.89.

ArMV: sample ID99 gave a positive result for ArMV when tested using RT-qPCR with a C_T value of 37.24. The samples were negative by all the other methods used. Mapping of the HTS reads was performed, but none were found matching the virus genomes.

RoCV1: samples ID66, ID74, ID99, ID153, ID152, ID237, ID244, ID245, ID258, and ID266 gave a positive result when tested using RT-qPCR for RoCV1, with a C_T value ranging between 29.54 and 37.48. Raw data from the HTS was mapped against the RoCV1 genome, but no reads were identified giving a significant match to the virus. Sample ID102 gave a positive result for RoCV1 when tested using RT-qPCR with a C_T value of 38.12. However, one paired-end read was identified when further analysis was performed mapping the raw data against the RoCV1 genome. A Blast+ search was performed with the identified paired-end read and showed a match with RoCV1 exclusively in one of the ends of the paired-end read, with 90.50% identity in nucleotide acid comparison (MK075828.1) and amino acid comparison with a 75% identity (QCQ67916.1). The low quality and short length of the read could explain why the percentage of identity of the amino acid sequence is lower than the nucleic acid. In addition, sample ID256 gave a positive result for RoCV1 when tested using RT-qPCR with a C_T value of 28.99. Following analysis with the EDNA pipeline, no matches were found, and no reads were identified matching RoCV1 after analysis with the Angua pipeline. After mapping against the RoCV1 genome, one matching paired-end read was found. A Blast+ search using the identified read confirmed RoCV1 with 100 % identity in nucleic acid (MK075827.1) and amino acid comparison (QCQ67915.1).

Figure 5.12- Alignment of the amplified region of arabis mosaic virus by RT-qPCR of the undetected sample ID100 with detected samples (ID245, ID256, ID258, ID128, ID91, ID68, ID74, and ID90). Highlighted in yellow are the nucleotide variations in reference to the primer (green) and probe (purple) sequences. Different ID256 and ID128 sequences represent different isoforms of the same assembled read. The different ID100 sequences represent the different reads identified after mapping raw data against the virus genome.

PNRSV: samples ID152, ID164, ID196, ID258, ID256, ID244, and ID266 gave positive results for PNRSV only by RT-qPCR with C_T values between 33.10 and 38.86. Raw reads were mapped but no matches were identified. Samples ID153 and ID188 gave positive results for PNRSV by RT-qPCR with C_T values 38.63 and 23.93 respectively. After mapping raw reads, one paired-end read was identified matching PNRSV in both cases. A Blast+ search confirmed PNRSV in nucleotide comparison with a 96.25-100% identity (for ID153: MH727232.1, MF069039.1, and for ID188: KU144863.1, KU144861.1, DQ992417.1, DQ992416.1). Amino acid comparison showed a 92.78-100% identity (for ID153: ALU66005.1, ATB18112.1, and for ID188: AFP87358.1, AFP87362.1, AFP87355.1).

TSWV: samples ID74, ID152 and ID153 gave positive results following testing for TSWV by RT-qPCR (C_T values 36.15, 36.45, 35.96), but negative results with the other three methods. Sequences from the HTS were mapped against the TSWV genome, but no reads were identified matching the virus. Further work was performed to confirm the presence of this virus in the samples. DNA amplified using RT-qPCR from both samples was cloned using a pGEM®-T Easy Vector System (Promega) following the manufacturer's recommended protocols. Clones containing inserts were identified, and a colony PCR was performed. Plasmid DNA was

purified using a QIAquick® PCR Purification Kit (Qiagen, Germany), following the manufacturer's instructions before being sequenced (Eurofins Genomics, Germany) using universal M13 primers. TSWV was confirmed, as the sequences of all inserts had high sequence identity (98.70–100%) to GenBank accessions KU884648.1, KC494483.1, DQ479968.1, AY879111.1, and AY879110.1).

ApMV: samples ID152, and ID196 showed a positive result when tested using RT-qPCR for ApMV, with C_T values of 36.94 and 36.57 respectively, but negative results with the other three methods. Sequences from the HTS data were mapped against the ApMV genome, but no reads were identified matching the virus genome. Further work was performed to confirm the presence of this virus in the samples. As described for TSWV, DNA amplified using the RT-qPCR from both samples was cloned and subsequently sequenced. A Blast+ search was performed with the obtained sequences, after removing the primer sequences, and ApMV was confirmed with a 98.44-100% identity (GenBank accession numbers HG328282.1, KX646548.1).

5.4 Discussion

During this study, two different pipelines were compared for the analysis of HTS data: Angua and EDNA. Both pipelines showed non-significantly different results for the analysis of SLRSV, ArMV, PNRSV and RoCV1-infected samples. Analysis with the EDNA pipeline showed slightly higher sensitivity for the detection of SLRSV - detecting two samples (ID11B and ID252) which were confirmed by RT-qPCR, as well as with one sample (ID188) for RoCV1, confirmed by RT-qPCR, although the p-value appeared as not computed (no detection). However, the Angua pipeline analysis showed slightly better results for ArMV (ID266, ID148. ID244), and PNRSV (ID65). Although sensitivity was calculated, and results were supported in tables adapted from Hughes *et al.* (2006; Tables 5.2 A-D), these results only show a general estimation of the sensitivity of the techniques. Further experiments would be required to compare the analytical sensitivity, using a panel of known positive and negative samples for different viruses. Performing further studies will make it possible to calculate the specificity of the different pipelines and methods. Correlation was observed among the results obtained for the different diagnostic techniques, after performing a Spearman's rank correlation test (Fig. 5.4).

ELISA and RT-qPCR were used as confirmatory diagnostic methods. Consistency among both pipelines and both diagnostic techniques was obtained for 6 samples in the case of SLRSV, 8 samples for ArMV, 1 sample for PNRSV and 5 for RoCV1. However, ELISA was identified as the technique with the lowest sensitivity when compared to all the other tests for each of the

viruses. In general, nucleic acid-based methods are known to be more sensitive than serological techniques such as ELISA (Vigne *et al.*, 2018; Rubio *et al.*, 2020). Thus, when a negative result was obtained for a sample using ELISA that was positive by other methods, the lower sensitivity of ELISA was attributed as the cause. The only sample where the results contradicted this assumption was sample ID66, which gave a negative result for SLRSV following ELISA testing, yet the C_T value was 16.62 when tested using RT-qPCR. Correlation was identified between both techniques; thus, a positive result would be expected for ELISA (Fig. 5.5). The SLRSV sequence obtained after Angua pipeline analysis was compared with other detected SLRSV isolates (Chapter 6; Fig. 6.7), with a positive result for ELISA. No variation was observed in the sequence. Positive and negative controls were included in the assay. Thus, it is possible that the false negative result is due to an uneven distribution of the virus in the sample, resulting in the testing of virus-free tissue, or due to a mishandling of the sample during the testing.

RT-qPCR was identified as the method with the highest sensitivity for the detection of ApMV, PNRSV, RoCV1, and TSWV. For ApMV, 2 samples were exclusively detected using RT-qPCR; 9 for PNRSV; 12 for RoCV1; and 2 for TSWV. Differences were found between RT-qPCR and both HTS pipelines when testing for ArMV and SLRSV, but these were not significant. During this study, only one sample (ID145) gave a negative result following RT-qPCR but was positive for all the remaining diagnostic techniques, including ELISA. Nucleotide sequences of ArMV and SLRSV were compared with those available in databases (Figs. 5.2, 5.3), and variations in primer and probe matching sites were identified. However, those sequence differences were identified in other samples that gave positive results. As ID145 was negative for both viruses by RT-qPCR, it is possible that an inhibitor interfered during the RT-qPCR reactions.

When the Angua pipeline analysis reported a negative result, mapping of the raw reads and subsequent a Blast+ search with the identified reads (if any) was performed. In some occasions when the Angua pipeline was negative and mapping detected reads matching a virus genome, a Blast+ search revealed an overall low percentage of coverage (SLRSV 34-75%; ArMV 27-100%; RoCV1 73%; and PNRSV 60-75%) and a percentage of identity ~71-95%. This can be explained as raw data was used in the BLAST search. Thus, reads with low quality, rather than curated and assembled reads, were included in the search. The lack of detection by the Angua pipeline is due to a low sequencing depth, discarding reads that cannot form a contig.

In samples ID153, ID252, ID231, ID256, when mapping was performed, a 99-100% cover and an identity of 98-100% was obtained. These samples were infected with multiple viruses,

and further investigation was done. In all cases except sample ID153 three viruses were identified. RT-qPCR detected the viruses, two of them with high C_T value (C_T value >29) and one with low (C_T value <23). The Angua pipeline only gave a positive result with two of the three viruses, not detecting one of the viruses with a high C_T value. This happened with infections with SLRSV+ArMV+RoCV1. For samples ID252 and ID256, 2 reads were identified after mapping for SLRSV and RoCV1 respectively. Furthermore, 2 reads were identified mapping the SLRSV satellite for ID252. In this case, the Angua pipeline did not identify the viruses due to the lack of sufficient depth on reads to form a contig. In samples such as ID148 or ID244, ArMV was detected after analysis with the Angua pipeline despite the identification of a low number of reads (2 paired-end) after mapping. However, sample ID231, which was positive for RoCV1+PNRSV+TSWV after RT-qPCR (C_T value >31), was negative after Angua pipeline analysis, although 8 reads were identified after mapping. In this case, the Angua pipeline did not detect ArMV because reads were assembled in contigs smaller than the set minimum contig length (200 bp). This also could explain why viruses in samples such as ID65 and ID100 (both for SLRSV analysis and in ID65 also for PNRSV) were detected exclusively by the EDNA pipeline. However, the mapping of raw reads against the virus genome identified some reads which were used subsequently in a Blast+ search, which confirmed the virus (SLRSV and PNRSV) with a low coverage percentage (49-74% cover). The Angua pipeline did not detect the virus because the identified reads were not assembled into contigs, which is probably due to a low sequencing depth.

The EDNA pipeline gave a negative result when the remaining techniques gave positive results on a single occasion (ID266, ArMV detection). The Angua pipeline detected only one fragment, and further analysis revealed that that the region was flanked by the e-probes but was not covered by them. A similar situation occurred with 2 negative samples (ID148 and ID244) for ArMV by both ELISA and the EDNA pipeline. Satellite reads were identified by using either the Angua pipeline (ArMV satellites in 13 samples, and SLRSV satellites in 5 samples) or by mapping the raw data against publicly available genomes (ArMV satellites in 18 samples, and SLRSV satellites in 10 samples). Currently there are no e-probes designed for satellite viruses. In the future the design of e-probes targeting satellites should be considered, as an added benefit for virus detection.

Although the EDNA pipeline gave positive results for some samples, further investigation of the results was needed to prove them. This is the case with samples such as ID188 for the detection of RoCV1, ID245 and ID90 for SLRSV, and ID252 for ArMV. On these occasions, a negative result was generated following EDNA analysis, showing no computed p-value. This

result is possible when the diagnostic and the decoy e-probes scores are identical or equal to 0. However, in all these cases, matches (1-2) were detected with the diagnostic e-probes and none of them with the decoy ones. The EDNA p-value should therefore have been calculated. Thus, those results were considered positive by the EDNA pipeline, as the virus was detected despite having no computed p-value. EDNA is still under development, and the reason why p-values are wrongly calculated is unknown. Readjustments need to be performed in the pipeline, to make sure p-values are correctly calculated. The EDNA pipeline was designed to be a user-friendly software, so no further analysis should be required.

The *Rosa* genome was recently published (Raymond *et al.*, 2018). Additional updates will become available in databases, such as different varieties. Designed e-probes are submitted to a curation process before being available for virus detection. During this curation process, e-probes are used to detect different titres of viruses present in a simulated host genome (Stobbe *et al.*, 2013), and are subjected to a Blast+ search to avoid mismatches. In this study, samples such as ID65 and ID100 for the analysis of SLRSV had some e-probes matching reads that could not be confirmed as part of the SLRSV genome. It is unknown if these e-probes could be matching sequence of the host genome or other non-viral unidentified sequences. Furthermore, the EDNA pipeline had available another e-probe set for each virus with a length of 20 nucleotides. Some of the decoy e-probes were randomly allocated, matching the raw HTS data, and giving positive results when no matches or not a sufficiently significant number of matches were identified with the diagnostic e-probes. Matches with reads belonging to the host genome were also identified using some of these e-probe sets. The negative control (non-templated control) included in the HTS run showed also some match results after the EDNA analysis with the 20-nucleotide e-probes. Specificity is controlled by the length of the e-probes as it is in the design of primers for a PCR. For each additional nucleotide, a primer becomes four times more specific (Dieffenbach *et al.*, 1993). However, for PCR primers the annealing temperature of the reaction can be used to modulate specificity and reduce low quality/non-specific matches, as well as the proximity of the primers and the extension time. Thus, oligonucleotides between 18 and 24 bases tend to work well in PCR reactions. In the case of the EDNA pipeline, longer e-probes have been shown to be more specific. Thus, only the 30-nucleotide e-probe results were used for this study.

E-probes should be designed specifically to target the pathogen of interest, and to avoid mismatches by mutations, genetic drift or selection pressure of sequence variants, frequently occurring in RNA viruses. A regular review of the e-probes should be performed, as is needed for the primers and probes of RT-qPCR. During this study, variations in the sequences where

primers/probe should bind have been recognised for ArMV (Figs. 5.2; 5.11; 5.12) and SLRSV (Fig. 5.3). Although a positive result was obtained for some of the samples, a redesign of these should be made to avoid those variations, focusing on other regions where the sequences are more conserved between isolates. Further reviews of the isolates available should be made regularly to avoid missing them with any assay.

A failure in the detection of ArMV and/or SLRSV using RT-qPCR for samples ID145, IDPool1, ID95 could be due to the presence of secondary metabolites (e.g. tannins or polysaccharides) which could have interfered during the qPCR. Two different extractions methods were used for isolation of the nucleic acid in the samples: a bead-based extraction method (KingFisher®) and CTAB. All three samples were extracted using a bead-based extraction method (KingFisher®). However, CTAB extraction has been shown to be an optimal method for extraction of leaf material containing high concentrations of polyphenols, tannins and polysaccharides, such as members of the *Rosaceae* family (Porebski *et al.*, 1997). In the case of sample ID100 (Fig. 5.12), the reverse primer and the probe have some variation. However, there are differences in the sequence where the forward primer should have bound. Hence, it is possible this is the reason why the RT-qPCR did not detect ArMV.

In this study there were samples that gave a positive result after analysis with the pipelines, but were negative with both RT-qPCR and ELISA. These are ID95 and IDPool1 for ArMV, ID65 and ID100 for SLRSV, and ID65 for PNRSV. HTS-based approaches are likely to meet contamination problems, thus the detection of a single or a few viral reads might not be considered sufficient to consider a sample positive. This could be the case of PNRSV detected in sample ID65, as only 4 reads were identified. It could be possible that those reads are cross-contamination from other infected samples during the library preparation. However, for samples ID95 and IDPool1 the number of reads identified was higher (1511 and 425 respectively). A second diagnostic technique should have confirmed these results. As described before, is possible that secondary metabolites interfered with the testing. The EDNA pipeline detected SLRSV in samples ID65 and ID100, but the data obtained in this study is not enough to reach a clear conclusion for the samples detected exclusively by HTS. One of the challenges of HTS is still to identify how many reads are needed to consider a sample positive, as well as identify false positives and false negatives. Currently, the best approach is still to confirm the positive results by using a second test.

In this study, RT-qPCR has shown an overall higher sensitivity for the detection of known viruses (SLRSV, ApMV, ArMV, PNRSV, RoCV1, SLRSV, and TSWV), whereas ELISA was identified as the technique with the lowest sensitivity. The pipelines used for the analysis of

HTS data performed well for the detection of viruses such as SLRSV and ArMV, but they showed difficulty in the detection of viral agents with lower titre (e.g. RoCV1 and TSWV). Sensitivity of HTS is affected by different factors such as low titre, small genome size, and contribution of host or environmental nucleic acid (Massart *et al.*, 2019). Thus, sensitivity of HTS depends on the bioinformatic pipelines used for the analysis of data as well. As previously reported, higher sensitivity was achieved by the pipelines that used a dual approach, combining de novo assembly and direct mapping against reference viral databases (Massart *et al.*, 2019). Failures in the detection of viruses were identified for both pipelines. Thus, enhancement is needed and should be routinely performed for both the Angua and the EDNA pipeline. Including a step which can identify single reads or reads that do not form a contig and then scan these for viruses would increase the sensitivity of the Angua pipeline, as well as the modification of the minimum contig length parameters. The EDNA pipeline uses software still under development and it could be improved to develop all its potential for a quick diagnostic. This pipeline is capable of analysing the HTS raw data for different viruses in a couple of minutes, whereas the Angua pipeline analysis could take 2.5-8 hours per sample. In addition, the EDNA pipeline uses user-friendly software, which facilities the analysis for operators who are not familiar with command-line interfaces. The EDNA pipeline could be used in conjunction with portable HTS platforms such as MinION (Oxford Nanopore Technologies). This will allow a fast diagnostic test next to the point of sampling, for example at borders, which could accelerate the testing process, or for situations when a rapid result is required (e.g. an outbreak).

HTS has potential to become a front-line diagnostic tool, as it has the advantage of being a non-targeted method, allowing the detection of new potential threats. Further work is still needed to reach that stage. One of the main limitations is still the cost and investment required (Chapter 4), not only regarding the platforms and reagents, but also in molecular and bioinformatic expertise. Nevertheless, HTS has proven to have a comparable cost to other combinations of methods such as ELISA and RT-qPCR (Chapter 4) when samples are subjected to investigational testing to evaluate the cause of specific symptoms. However, ELISA is still an important and cost-effective technique used for routine virus testing in phytosanitary, quarantine and virus-certification programmes, and RT-qPCR enables an inexpensive, rapid and sensitive testing for targeted viruses. The use of HTS could support the design of RT-qPCR assays, using the identified sequences as templates or assist the design of the primers/probe by providing broader and more up-to-date information on the diversity of viral genomic sequences.

In addition, HTS could be routinely used to review the available assays and modify them accordingly when new virus strains are discovered.

Chapter 6 Trade Study: Pathways of Entrance and Spread of Viruses

Abstract

The growth of international travel and long-distance trade, in conjunction with climate change, is a threat to biodiversity and ecosystems due to the potential movement and establishment of invasive organisms. In this chapter, cut roses imported from Colombia, Ecuador, India, Kenya, Rwanda, and Uganda were tested for rose rosette virus (RRV; genus *Emaravirus*) and other common rose viruses by RT-qPCR. Some of these samples (8) were analysed by high-throughput sequencing, and two novel viruses were discovered: a tentative new virus species, 'rosa ilarvirus-1' (RIV-1; genus *Ilarvirus*), and a novel rose waikavirus previously reported in New Zealand, 'sweetbriar rose curly top virus' (SRCTV; genus *Waikavirus*). For positive samples, a phylogenetic analysis was performed, comparing the virus sequences with other isolates from public databases, as well as with isolates identified in the United Kingdom in this study when possible, to evaluate the potential connection between the spread of rose viruses and ornamental trade.

6.1 Introduction

The growth of international travel and long-distance trade, in conjunction with climate change, is a threat to biodiversity and ecosystems due to the potential movement and establishment of invasive organisms (Aukema *et al.*, 2011). The use of new technologies for the transport of ornamentals has enhanced the survival of the transported material, but also the survival of pests (Dehnen-Schmutz *et al.*, 2007). Europe is a centre for international trade and has experienced the establishment of a large number of species which have had a negative impact on plant health (Keller *et al.*, 2011), such as *Hymenoscyphus pseudoalbidus*, the cause of ash dieback disease (Pautasso *et al.*, 2013). Recent outbreaks of *Xylella fastidiosa* (European Commission, 2018), tomato brown rugose fruit virus (Oladokun *et al.*, 2019) in Europe and rose rosette virus (RRV; genus *Emaravirus*) in India (Chakraborty *et al.*, 2017) have also demonstrated the potential for the movement of pests into new countries, probably due to trade.

Risks associated with plant importation are well known (Kahn, 1980; Wilson and Graham, 1983; Ebbels, 2003). Thus, updated lists of plant pests and associated hosts from different countries are published and available (Jones and Baker, 2007). Checking these lists and performing surveillance work is fundamental for plant health and quarantine legislation (Shivas *et al.*, 2006). However, organisms not yet described cannot be regulated (Brasier and Webber, 2010). In the past, unknown pathogens were discovered when causing damage in other parts of the globe, outside their native countries (Brasier, 2005). In the last decade, high-throughput sequencing (HTS) has proven to be a powerful tool for the discovery of unknown pathogens (Adams *et al.*, 2009; Kreuze *et al.*, 2009), including new viruses affecting roses (Diaz-Lara *et al.*, 2020b; Diaz-Lara *et al.*, 2020a). Previous to the use of HTS as a diagnostic tool, Jones and Baker (2007) published a list of the plant pathogens discovered in Great Britain between 1970 and 2004. Of the pathogens described, approximately 11% of the 234 pathogens recorded for the first time were viruses, and 53% of pathogens were found in ornamental crops. Furthermore, Fox and Mumford (2017) reported that detections of pest in ornamental plants dominated the 2000s, as a result of the increase in the trade of ornamental species, the change of diagnostics towards more rapid and sensitive targeted methods (e.g. ELISA or PCR), and also increased awareness of the risk posed by some viruses infecting ornamentals that can cause devastating symptoms in commodity crops.

The estimated annual production of one of the most important ornamental crops worldwide, roses, is around 18 billion cut stems, 60-80 million potted plants (miniature roses and bare-root grafting plants), and 220 million plants for landscaping (Blom and Tsujita, 2003; Pemberton *et al.*, 2003; Roberts *et al.*, 2003). Global rose production was estimated to be valued at €24 billion

(around £21 billion) in 2008 (Heinrichs, 2008). The high profitability of the export of cut roses has led to increased production in different countries but especially in Kenya, Ecuador, and Colombia (In *et al.*, 2016). The roses grown in these countries are high quality but also competitively priced due to the optimal growing conditions and low labour costs (Japan External Trade Organization, 2011; Park *et al.*, 2011).

With the aim of surveying for rose viruses, samples were obtained from cut roses imported from Colombia, Ecuador, India, Kenya, Rwanda, and Uganda. They were tested for RRV and other common rose viruses using RT-qPCR. Some of the samples were analysed by HTS as part of a comparison of methods (Chapter 5). In this study we describe the discovery of a tentative new virus species, rosa ilarvirus-1 (RIV-1; genus *Ilarvirus*), and the finding of a novel rose waikavirus, 'sweetbriar rose curly top virus' (SRCTV; genus *Waikavirus*), in samples imported from India and Colombia respectively. Furthermore, a phylogenetic analysis was performed using the virus sequences obtained from positive-identified samples, comparing them with other isolates from public databases, as well as with isolates identified in the UK when possible (Chapters 3 and 5).

6.2 Materials and Methods

Cut rose samples (41) imported from India (32), Ecuador (4), Colombia (1), Kenya (2), Uganda (1), and Rwanda (1) were collected at Heathrow airport (UK; Table A.2). RNA was extracted using a CTAB (cetyl trimethylammonium bromide) method (see Material and Methods section in Chapter 3). RT-qPCR was performed to test for the COX1 gene, apple mosaic virus (ApMV), prunus necrotic ringspot virus (PNRSV), rose cryptic virus-1 (RoCV1), rose rosette virus (RRV), and tomato spotted wilt virus (TSWV), as described in the Material and Methods section in Chapter 3.

Cycling for SRCTV virus RT-PCR was performed using a 2720 Thermal Cycler (Applied Biosystems). RT-PCR was performed using Reddymix (Thermo Fisher Scientific), 0.5 µl of Verso RT (Thermo Fisher Scientific), 400 nM of primers (Joe Tang, personal communication) and 1 µl extracted RNA in a final volume of 25 µl. Cycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 35 s followed by a final extension of 10 min at 72 °C and a hold step of 4 °C. The expected product was 190 bp estimated using agarose gel electrophoresis and Quick-Load® Purple 1 kb Plus DNA Ladder (NEB). PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen, Germany), following the manufacturer's instructions before being sequenced (Eurofins Genomics, Germany) using both PCR primers.

DNA amplified using the RT-qPCR assays for TSWV and ApMV was cloned and sequenced as described in the Material and Methods section in Chapter 5.

Libraries for sequencing in the MiSeq (Illumina, US) were prepared as described in the Material and Methods section in Chapter 4.2. Analysis of the HTS data using both pipelines, Angua and EDNA, was performed as described in Material and Methods section in Chapter 5.

The alignment of the different virus isolates sequences with publicly available sequences was performed using Geneious® software v11.0.2. Phylogenetic trees were generated using the FASTTREE application v2.1.10 (Price *et al.*, 2009) through Galaxy server (Afgan *et al.*, 2018) and visualised using MEGA7 v7.0.21 (Kumar *et al.*, 2016).

6.3 Results

6.3.1 Virus identified in imported samples

Cut rose samples (41) imported from India, Ecuador, Colombia, Kenya, Uganda, and Rwanda were collected at Heathrow airport (UK). Samples consisted of leaves from both asymptomatic (6) and symptomatic samples (35). Symptoms were consistent with virus infections, such as distortion (3), mottling (14), both mottling and deformation (12), ringspots (2), RRV-like symptoms (3; red growth, thicker stems), or with a yellow central vein (1). Samples were tested by RT-qPCR for apple mosaic virus (ApMV; genus *Ilarvirus*), arabis mosaic virus (ArMV; genus *Nepovirus*), prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*), rose cryptic virus-1 (RoCV1; family *Partitiviridae*), rose rosette virus (RRV; genus *Emaravirus*), strawberry latent ringspot virus (SLRSV; family *Secoviridae*), and tomato spotted wilt virus (TSWV; genus *Orthotospovirus;* Table 6.1). No positive samples were identified for SLRSV, ArMV or RRV. A total of 9 samples were analysed by HTS as part of the comparison of the methods (Table 6.1; Chapter 5).

Table 6.1- Total number of positive samples found for apple mosaic virus (ApMV), prunus necrotic ringspot virus (PNRSV), rose cryptic virus-1 (RoCV1), rosa-ilarvirus-1 (RIV-1), sweetbriar rose curly top virus **(**SRCTV), and tomato spotted wilt virus (TSWV) after testing roses imported into the United Kingdom.

Country of origin	Virus	Total positive samples
Colombia	PNRSV	1
	Waikavirus (SRCTV)	1
Ecuador	ApMV	2
	PNRSV	4
	RoCV1	2
	TSWV	1
India	PNRSV	21
	RoCV1	17
	Ilarvirus (RIV-1)	1

Kenya	PNRSV	2
Rwanda	PNRSV	1
	RoCV1	1
Uganda	PNRSV	1
	RoCV1	1
	TSWV	1

A total of 30 samples, imported from Colombia (1), Ecuador (4), India (21), Kenya (2), Rwanda (1), Uganda (1), were positive for PNRSV. RT-qPCR products of the 10 PNRSV-positive samples (ID152, ID153, ID170, ID171, ID180, ID188, ID190, ID193, ID196, ID198) were sent for Sanger sequencing and were confirmed to be infected with PNRSV (99.12-100% identity, GenBank accession numbers MN656197.1 and AY948440.1). Samples (21) from India (17), Ecuador (2), Rwanda (1) and Uganda (1) were positives for RoCV1. Samples from Ecuador (1 sample) and Uganda (1 sample) were positive for TSWV by RT-qPCR. These results were confirmed by cloning the DNA amplified using the RT-qPCR from both samples using a pGEM®-T Easy Vector System (Promega). Plasmid DNA was purified and subsequently sequenced. Comparisons were made with published sequences, and TSWV was confirmed, as results showed that all inserts have identical nucleic acid (100% to GenBank accessions LC549181.1 and LC495143.1 and amino acid (100% identity; QNT22142.1 and ASM93563.1) sequences. Similar work was performed for samples (2) imported from Ecuador which were positive for ApMV after RT-qPCR testing. RT-qPCR amplified fragments were cloned, and subsequent Sanger sequencing confirmed the ApMV positive results, with 98.44-100% nucleotide identity (GenBank accession numbers HG328282.1, KX646548.1) and 100% amino acid identity (ADD66806.1, ADD66807.1, ADD66807.1, AMH87252.1, AGV30978.1).

6.3.1 Novel viruses identified in imported samples

A new ilarvirus, RIV-1, was identified in a sample originating from India (ID188; Chapter 4). ID188 was analysed using HTS with both Flongle (Oxford Nanopore Technologies) and MiSeq (Illumina) platforms. Within the data generated using the Flongle (9229 reads), 14 reads were viral. The similarity of the reads to other members of the *Ilarvirus* genus suggested that the sample was infected with a previously undescribed virus within the ilarvirus group 2. The MiSeq data confirmed the results and the additional sequence generated enabled the reconstruction of the three RNAs comprising the genome of a new virus (GenBank accession numbers MT017861, MT017862, MT017863) with the same number of ORFs described as for other members of ilarvirus group 2.

A sample originating from Colombia (ID181) was analysed using HTS on the MiSeq (Illumina) platform. A 1180 bp fragment was identified, sharing homology with members of

the genus *Waikavirus.* Nucleic acid comparisons showed 68.87% identity with red clover associated virus-1 (MH325329.1), 74.68% with brassica napus RNA virus (NC_040586.1), and 67.30% with bellflower vein chlorosis virus (KT238881.1). Amino acid comparison showed 42% identity with maize chlorotic dwarf virus (AAR14150.1, NP_734453.1), 39% with brassica napus RNA virus 1 (YP_009552078.1), 37% with bellflower vein chlorosis virus (YP_009165498.1, YP_009167335.1), 38% with red clover associated virus 1 (AXY44617.1), and 37% with rice tungro spherical virus (QCT85329.1). Further research was performed, and comparison with a new waikavirus affecting roses in New Zealand was done (Joe Tang, personal communication). An RT-PCR was performed with primers designed by Joe Tang (personal communication). The RT-PCR product (190 bp) was subsequently Sanger sequenced, sharing 100% identity with the waikavirus discovered in New Zealand, tentatively named 'sweetbriar rose curly top virus' (SRCTV).

6.3.2 Phylogenetic analysis of the identified isolates

The sequences from these isolates were compared to other publicly available sequences in databases (GenBank), as well as with isolates identified in the UK (Chapters 3 and 5).

For samples ID159 and ID187 a fragment of RNA1 (coding for a replicase) of PNRSV was identified. Sequences were compared with other publicly available sequences. Three main clusters were identified (Fig. 6.1). The sequences of the samples imported from India and Kenya group with other virus isolates identified in roses (*Rosa* spp.; Fig. 6.1 cluster C) and are closely related to cherry (*Prunus* sp.) isolates from the USA. This cluster differs from other cherry (*Prunus* sp.) isolates from China (Fig. 6.1 cluster B), and isolates from other hosts (cherry plum (*Prunus cerasifera*), almond (*Prunus dulcis*), Armenian plum (*Prunus armeniaca*), and peach (*Prunus persica*)) from different countries.

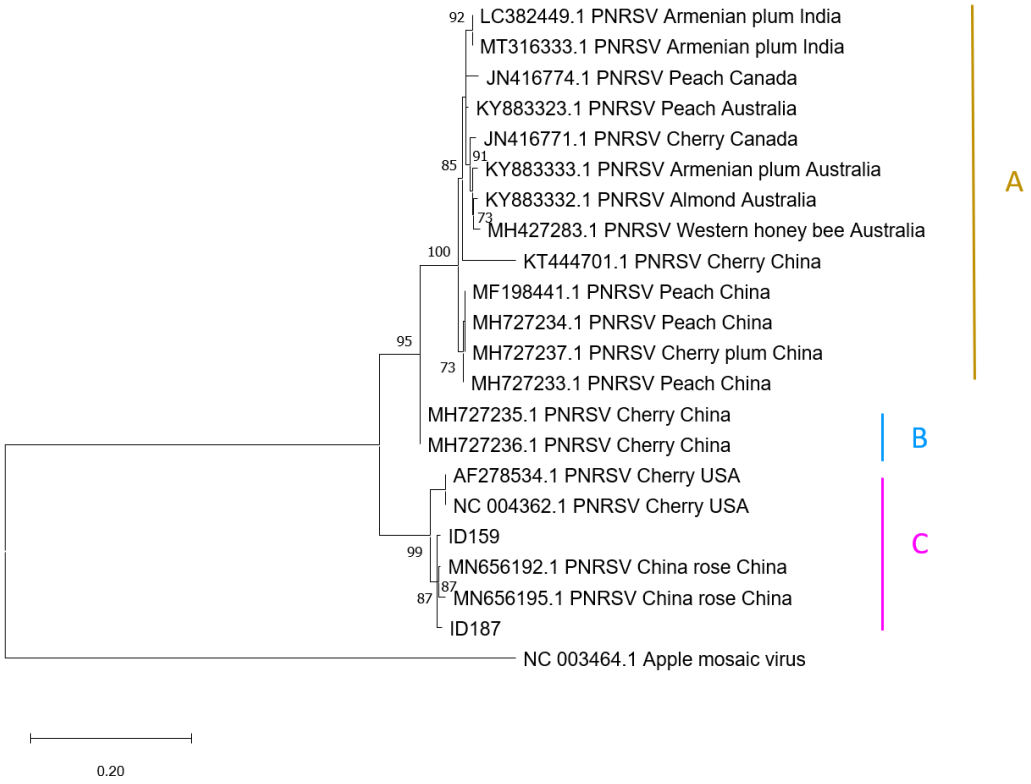

Figure 6.1- Maximum likelihood tree (1000 bootstrap) of different prunus necrotic ringspot virus (PNRSV) isolates based on sequence of the RNA1 fragment (replicase; 2306 bp), including sequences from two samples (ID159 and ID187) identified during the analysis of imported cut roses in the United Kingdom. Apple mosaic virus (genus *Ilarvirus)* was included as outgroup. Only bootstrap values higher than 70% are represented.

For the comparison of PNRSV RNA2 (RdRp), only the sequence from one sample ID159 (imported from India) was included in the phylogenetic analysis. The isolate from this sample belongs to a group formed with other rose isolates (Fig. 6.2 cluster B) from China but also from cherry (*Prunus* sp.) and cherry plum (*Prunus cerasifera*) as hosts from the same country. The other main cluster (Fig. 6.2 cluster A) is composed of isolates from peach (*Prunus persica*) from China and other hosts from different countries.

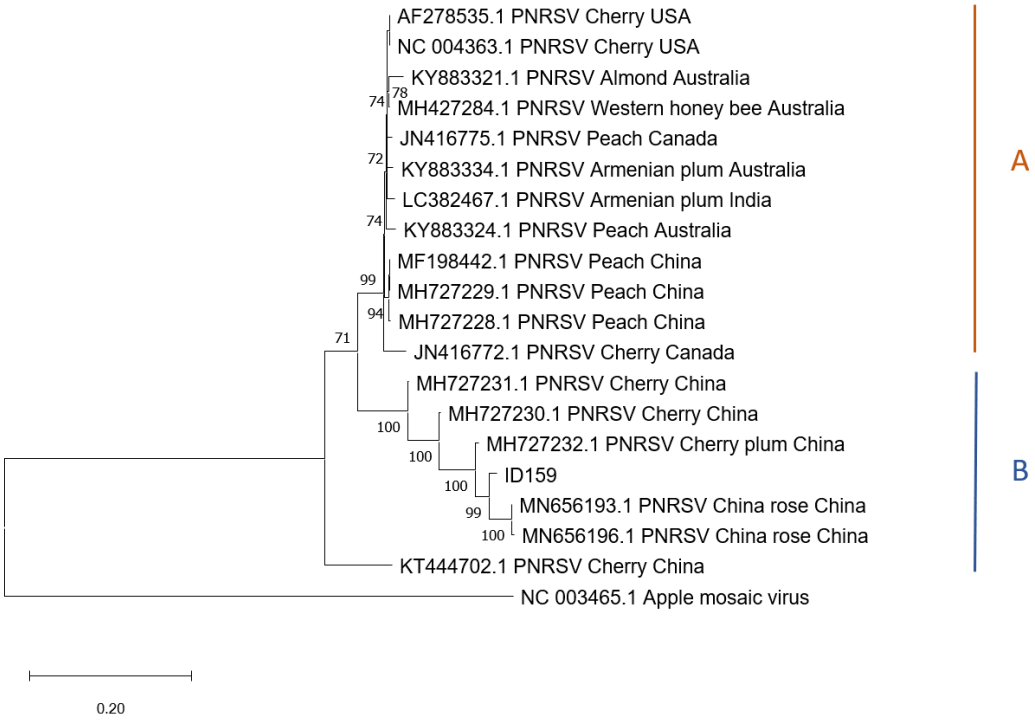

Figure 6.2- Maximum likelihood tree (1000 bootstrap) of different prunus necrotic ringspot virus (PNRSV) isolates based on sequence of the RNA2 fragment (RdRp; 2865 bp), including the sequences from an imported Indian sample (ID159) identified during the analysis of imported cut roses in the United Kingdom. Apple mosaic virus (genus *Ilarvirus)* was included as outgroup. Only bootstrap values higher than 70% are represented.

For the isolate comparison of PNRSV RNA3 (MP and CP), the sequences of three samples (ID181, ID187, ID159) were included in the phylogenetic tree, imported from Colombia, Kenya, and India respectively. In this case, a UK PNRSV isolate was also included. These isolates group together with other *Rosa* spp. isolates from China and from Poland (Fig. 6.3 cluster B). These isolates are closely related to peach isolates (*Prunus persica*) from Canada and Mexico, and isolates from cherry (*Prunus* sp.) and cherry plum (*Prunus cerasifera*) from China, as well as apple (*Malus domestica*) and cherry isolates (*Prunus* sp.) from the USA. One rose isolate from China (FJ610344.1) is outside this cluster, in Fig. 6.3 cluster A, which tends to cluster together by host (peach (*Prunus persica*), plum (*Prunus domestica)*, sweet cherry (*Prunus avium*) and cherry (*Prunus* sp.).

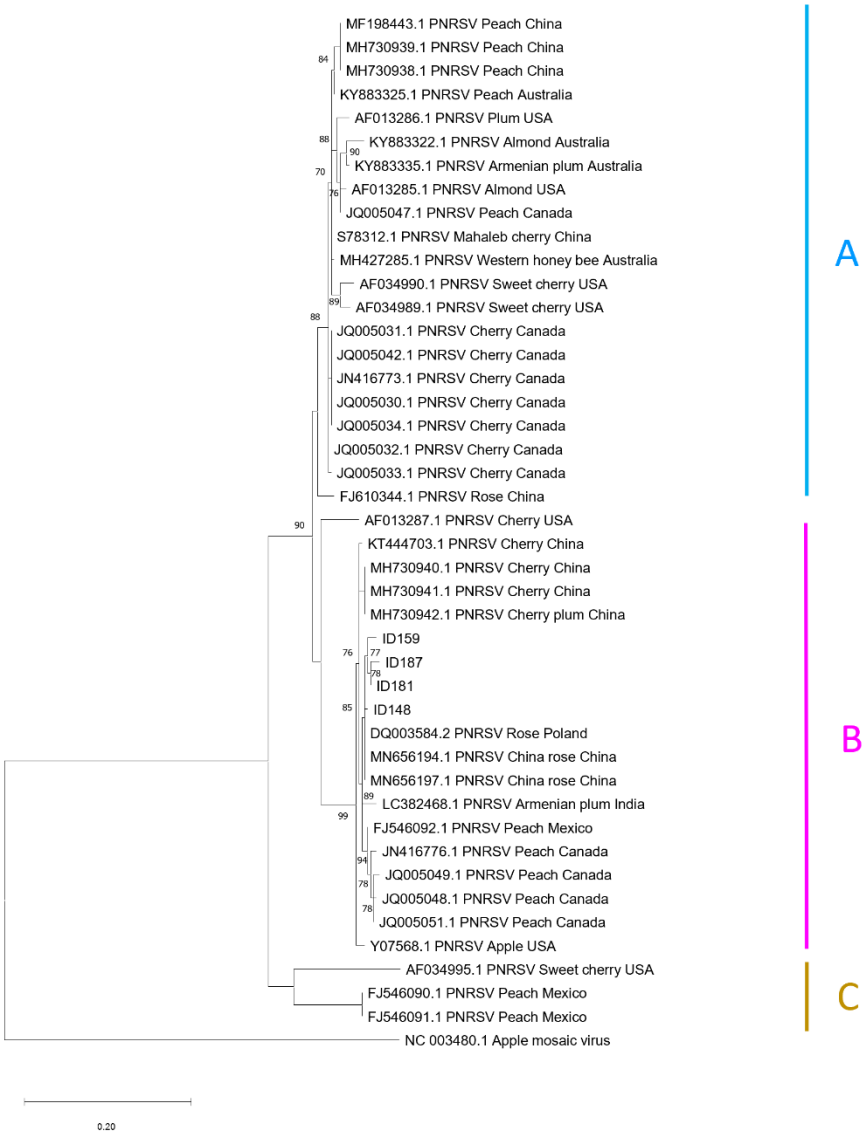

Figure 6.3- Maximum likelihood tree (1000 bootstrap) of different prunus necrotic ringspot virus (PNRSV) isolates based on sequence of the RNA3 fragment (MP and CP; 1103 bp), including sequences from imported cut rose samples (ID159, ID187, ID181) and an isolate from the United Kingdom (ID148). Apple mosaic virus (genus *Ilarvirus)* was included as outgroup. Only bootstrap values higher than 70% are represented.

The RT-qPCR amplified fragments of TSWV (segment S, nucleocapsid protein) of samples ID152 and ID153 (Ecuador and Uganda), which were subsequently sequenced, were compared against isolates from the UK (ID74 and ID75) and other sequences available in GenBank. Sequences from both UK and imported TSWV-positive samples shared homology (Fig. 6.4 clusters F and G). However, the sequences from imported samples group with sequences from other isolates found in ornamental crops, including chrysanthemum (*Chrysanthemum* sp.) from China and valerian (*Valeriana officianales*) from Japan, but also with tomato (*Solanum lycopersicum*) from Argentina and Serbia and pepper (*Capsicum annum*) from Argentina, while the UK isolates are closely related to a pepper (*Capsicum annum*) isolate from Spain and an

alstroemeria (*Alstroemeria* sp.) isolate from Argentina. Regarding other isolates of TSWV, isolates from Asia mainly cluster together (Fig. 6.4 cluster B), suggesting a variation of isolates depending on their geographical distribution. However, this is less clear in clusters C and D, which involve isolates from Europe, America and Asia.

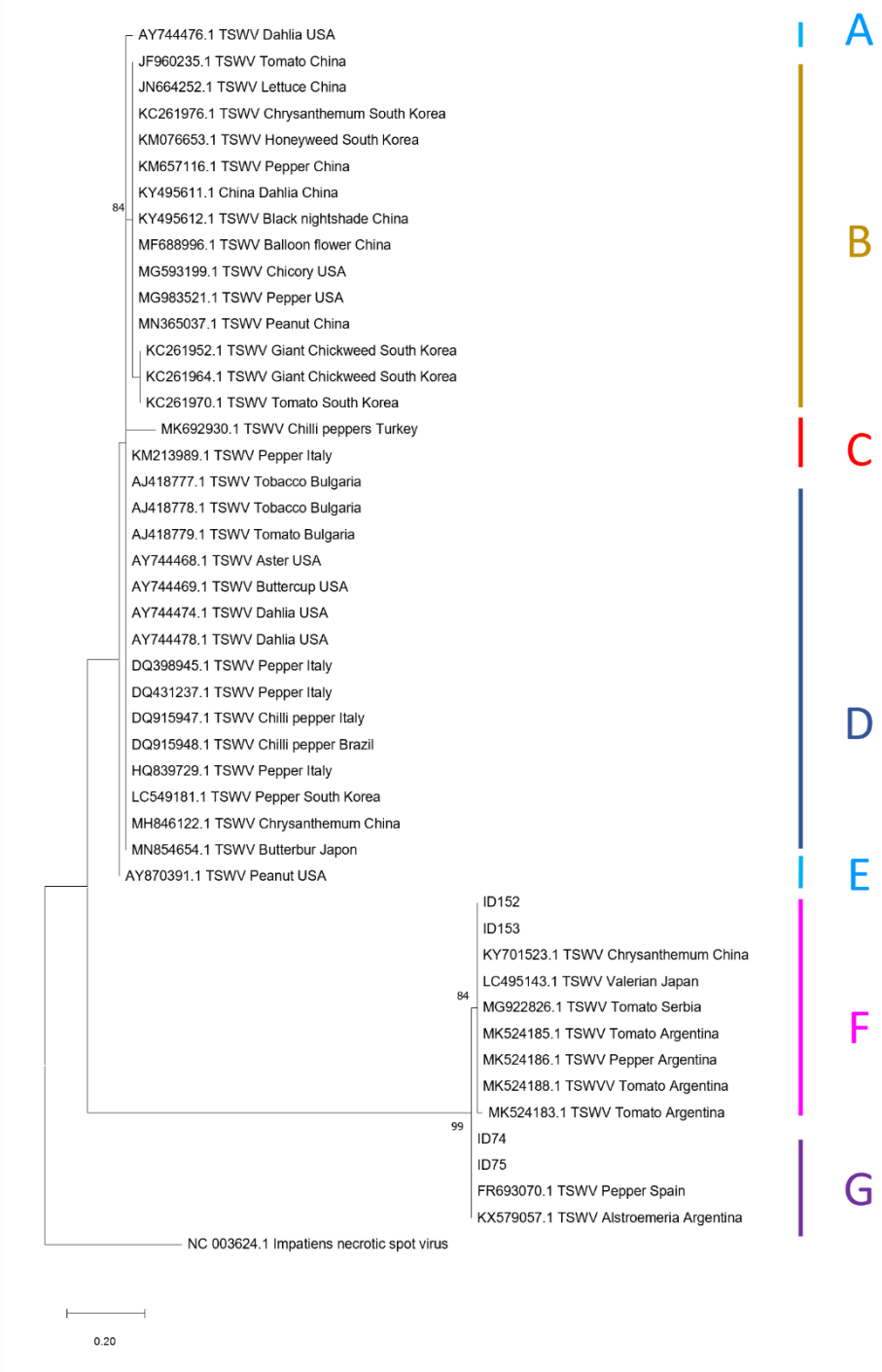

Figure 6.4- Maximum likelihood tree (1000 bootstrap) of different tomato spotted wilt virus (TSWV) isolates based on sequence of the RNA3 fragment (MP and CP; 83 bp), including sequences from imported cut rose samples (ID152 and ID153) and an isolate from the United Kingdom (ID74 and ID75). Impatiens necrotic spot virus (genus *Orthotospovirus)* was included as outgroup. Only bootstrap values higher than 70% are represented.

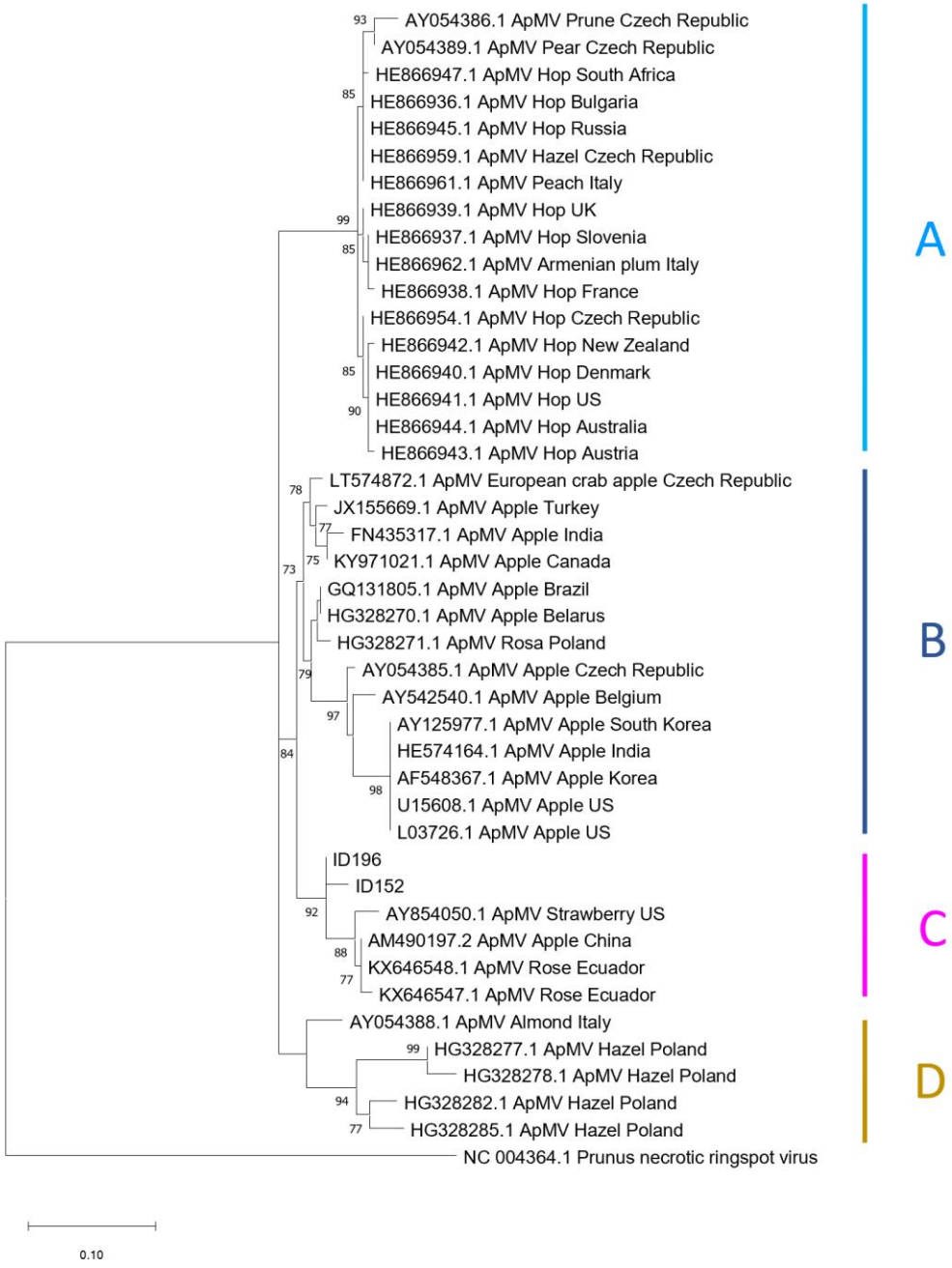

Figure 6.5- Maximum likelihood tree (1000 bootstrap) of different apple mosaic virus (ApMV) isolates based on sequence of the RNA3 fragment (CP; 66 bp), including sequences from imported cut rose samples (ID152, ID196). Prunus necrotic ringspot virus (genus *Ilarvirus)* was included as outgroup. Only bootstrap values higher than 70% are represented.

Samples ID152 and ID196 (Ecuador) tested positive for ApMV by RT-qPCR. Sequences obtained from the amplified fragments (CP) were compared with other isolates. In this case, rose isolates cluster together (Fig. 6.5 cluster C) with other isolates from the same host from Ecuador, but also with apple (*Malus domestica*) isolates from China and a strawberry (*Fragaria* sp.) isolate from the USA. The rose isolate from Poland is not part of this group. Other clusters

identified (A, B, D) are grouped based on the host, primarily hop (*Humulus lupulus*, A), apple (*Malus domestica*, B) and hazel (*Corylus* sp., D).

RoCV1 sequences from imported samples were compared against the isolates from the UK and the small number of sequences available in databases. For RNA1 (RdRp) and RNA2 (CP) fragments, the sequence from imported sample ID159 (India) was compared against 4 UK isolates (for RNA1: ID11B, ID68, ID91, and ID210; for RNA2: ID11B, 210, ID252 and ID256). For RNA3 (capsid proteins), the sequences from two imported samples (ID159 and ID188; India) were compared against 5 UK isolates (ID68, ID91, ID210, ID235, ID252). Overall, sequences have been found conserved between isolates, showing a small variation. Percentage of identity between isolates was found in a range of 97.02-100% for RNA1, 96.41-100% for RNA2, and 98.66-100% for RNA3 (Table A.5).

6.3.3 Phylogenetic analysis of SLRSV and ArMV isolates

Neither SLRSV nor ArMV was detected in the imported tested samples. However, a comparison of the isolates identified during the survey of rose viruses in the UK (Chapter 3) and the sequences publicly available in databases was performed. The aim of the comparison was to detect any pattern or relationship between the different isolates around the globe.

For SLRSV, RNA1 fragment (polyprotein: protease, helicase, VPg, and polymerase) comparison revealed that UK rose isolates group together (Fig. 6.6 cluster A). ID145, ID128, ID66 and ID258 are closely related to a bramble (*Rubus* sp.) isolate from New Zealand, while ID91 clusters with a wild bean (*Phaseolus* sp.) isolate from the Netherlands. Within this clade, *Rosa* isolates from the UK share homology with isolates from mint (*Mentha* sp.) from the USA, lily (*Lilium* sp.) from the Netherlands, and clematis (*Clematis* sp.*)* from Norway (Fig. 6.6 cluster A). There is a well differentiated cluster (Fig. 6.6 cluster B) which groups lily (*Lilium* sp.) isolates from the Netherlands. Isolates from different host (roses, lilies) from the Netherlands appear grouped in cluster A (Fig. 6.6), which suggests a possible geographical variation of this virus.

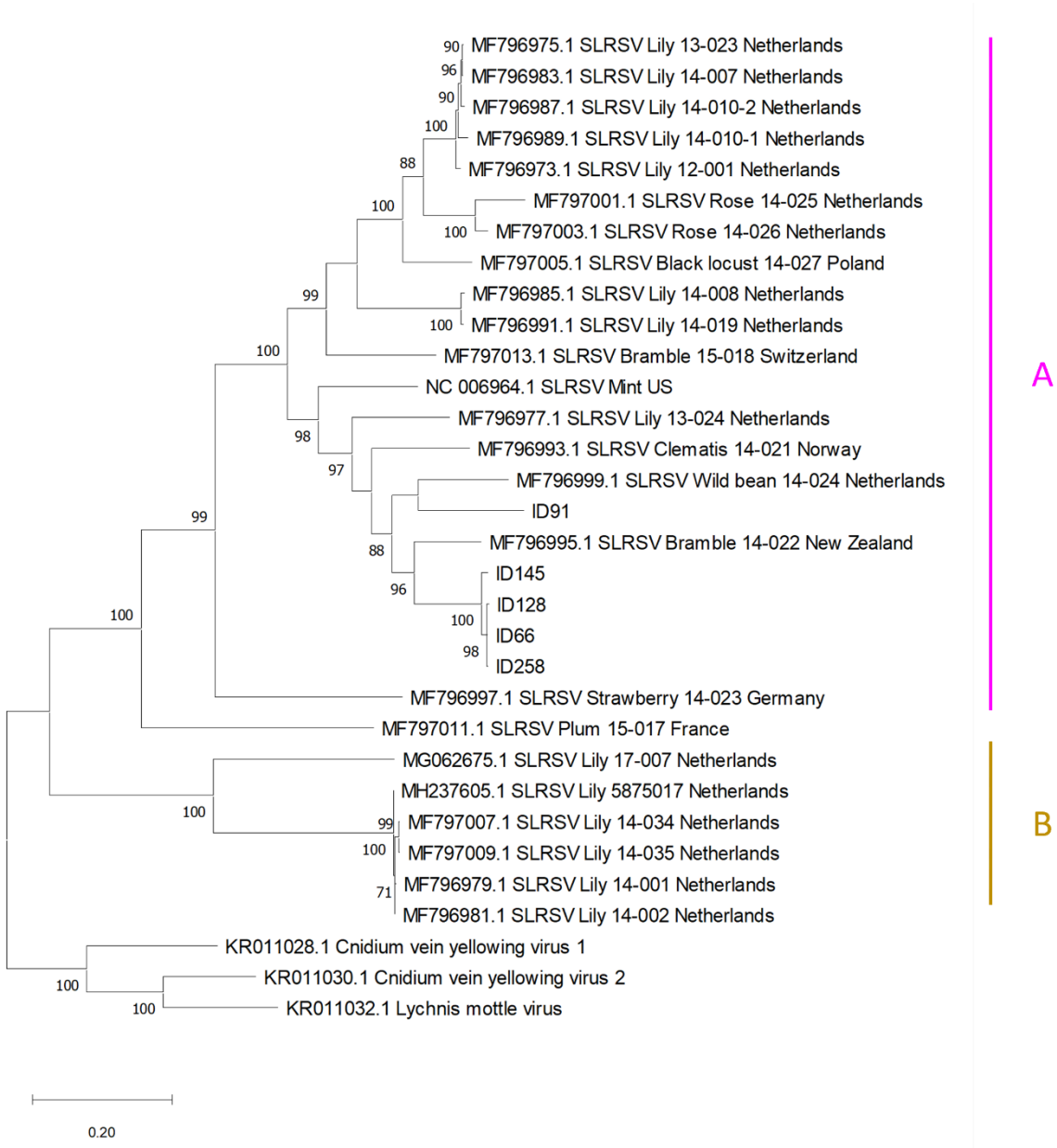

Figure 6.6- Maximum likelihood tree (1000 bootstrap) including different strawberry latent ringspot virus (SLRSV) isolates from the United Kingdom (ID91, ID145, ID128, ID66, and ID258) based on sequence of the RNA1 fragment (polyprotein; 6917 bp). Cnidium vein yellowing virus 1 and 2 and lychnis mottle virus were included as an outgroup. Only bootstrap values higher than 70% are represented.

For SLRSV RNA2 fragment (CP and MP), the comparison of sequences had a similar clustering pattern as for the RNA1 fragment. All the UK rose isolates cluster together (Fig. 6.7 cluster A). In this case, they are closely related to a strawberry isolate from the UK, but also to clematis (*Clematis* sp.) and wild bean (*Phaseolus* sp.) isolates from Norway and the Netherlands respectively, as happened with RNA1. In this case, ID91 is the isolate with homology to the bramble (*Rubus* sp.) isolate from New Zealand and mint (*Mentha* sp.) from the USA. In cluster A, there are also isolates from Netherlands from different hosts (lily, roses), but also isolates from Poland and New Zealand. A different cluster (Fig. 6.7 cluster B) is formed

by a plum (*Prunus* sp.) isolate from France and a strawberry (*Fragaria* sp.) isolate from Germany. Some of the lily (*Lilium* sp.) isolates from the Netherlands are part of a differentiated cluster (Fig. 6.7 cluster C), as happened with RNA1.

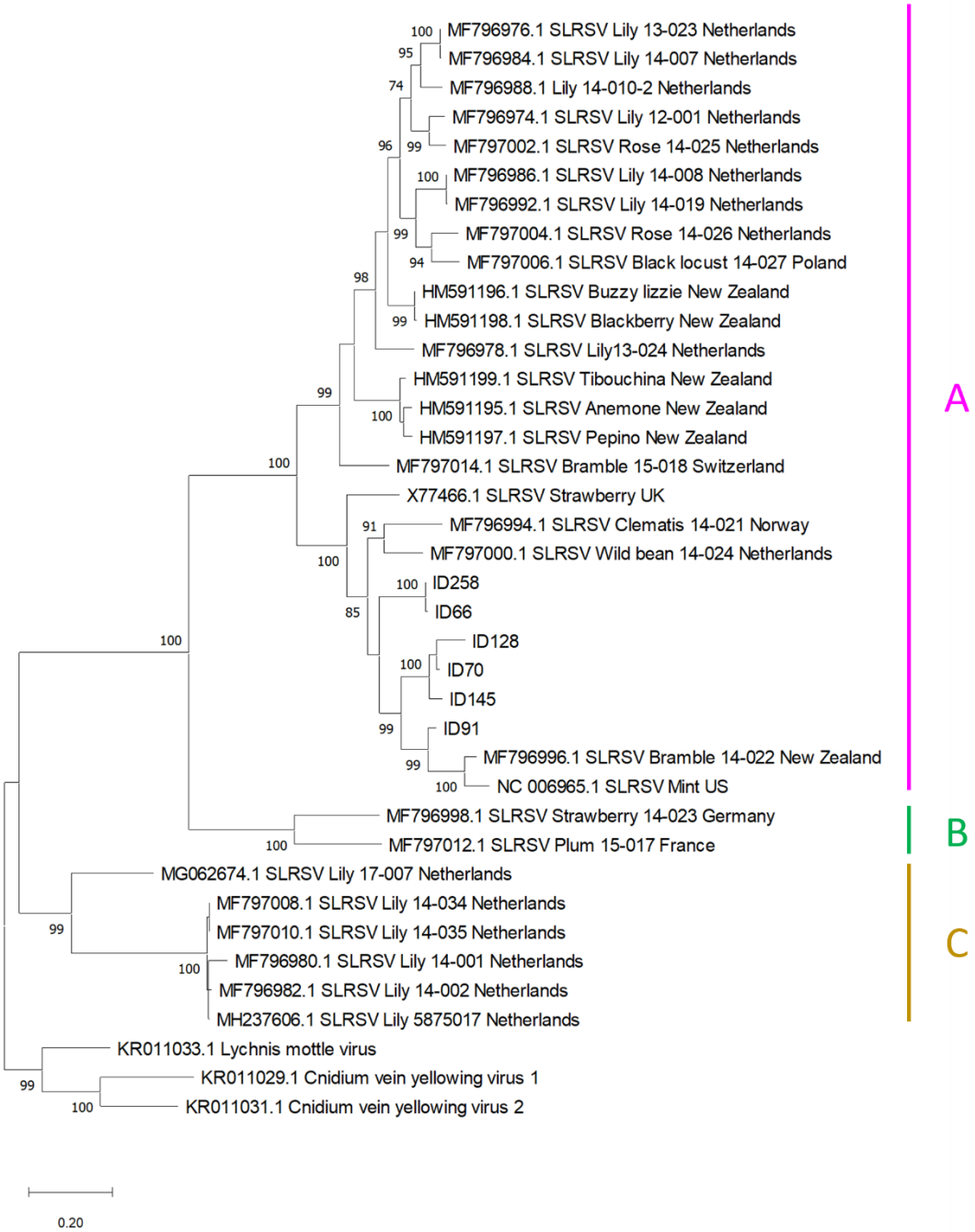

Figure 6.7- Maximum likelihood tree (1000 bootstrap) of different strawberry latent ringpost virus (SLRSV) isolates from the United Kingdom (ID258, ID66, ID128, ID70, ID145, and ID91) based on sequence of the RNA2 fragment (CP and MP; 2268 bp). Cnidium vein yellowing virus 1 and 2 and lychnis mottle virus were included as an outgroup. Only bootstrap values higher than 70% are represented.

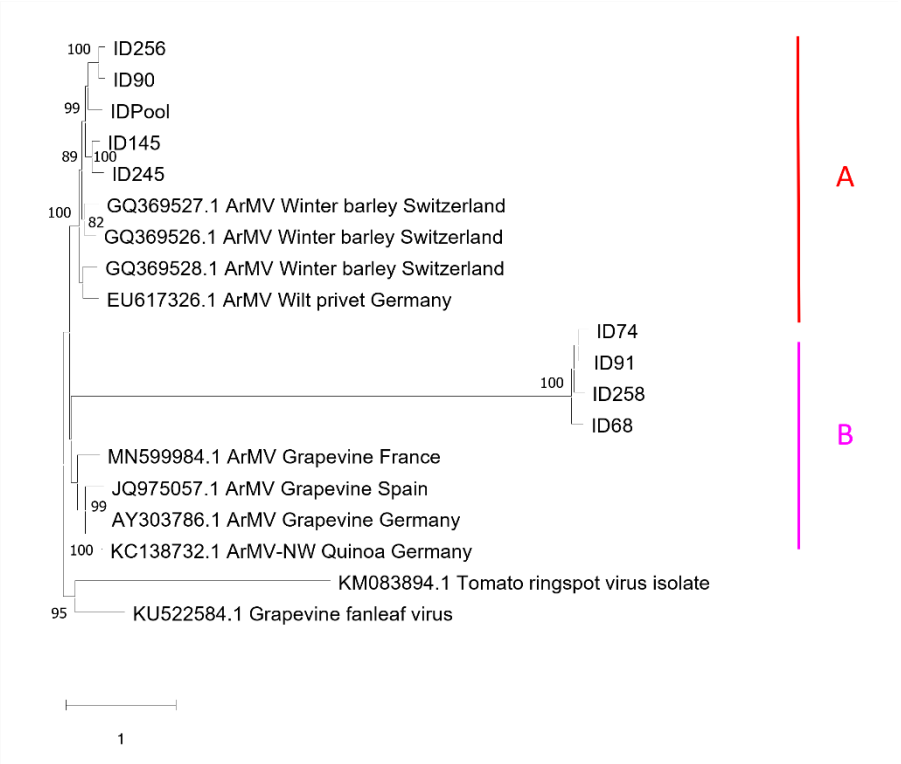

Figure 6.8- Maximum likelihood tree (1000 bootstrap) of different arabis mosaic virus (ArMV) isolates from the United Kingdom (ID256, ID90, IDPool1, ID145, ID245, ID74, ID91, ID258 and ID68) based on sequence of the RNA1 fragment (polyprotein: protease, helicase, VPg, and polymerase; 7146 bp). Tomato ringspot virus and grapevine fanleaf virus were included as an outgroup. Only bootstrap values higher than 70% are represented.

ArMV RNA1 (polyprotein: protease, helicase, VPg, and polymerase) phylogenetic analysis divides UK rose isolates into two clades (Fig. 6.8). The first cluster (A) groups together the UK isolates with isolates from winter barley (*Hordeum vulgare*) from Switzerland and privet (*Ligustrum vulgare*) from Germany, whilst the other UK isolates are closely related to grapevine (*Vitis vinifera)* isolates from France, Spain and Germany (cluster B).

In the case of ArMV RNA2 (MP and CP), UK rose isolates cluster together in one clade (Fig. 6.9 cluster A) but seem to have different origin. They can be classified in 4 main clusters. The first is sequences from samples ID128, ID145, and ID95, which share homology with a UK lilac (*Syringa* sp.) isolate. They are all closely related to isolates from daffodil (*Narcissus* sp.) from the Netherlands and French grapevine (*Vitis vinifera;* Fig. 6.9 A1). Secondly, samples ID66, ID91, ID90, ID256, ID74 are closely related to other daffodil (*Narcissus* sp.) isolates from the Netherlands, all of them sharing a common origin with sequences from samples IDPool1 and ID258 (Fig. 6.9 A2). Thirdly, isolates ID70, ID68, ID245 are related to cluster 1 and 2 but form their own group (Fig. 6.9 A3), although bootstrap values are weak (less than 70%) for groups A2 and A3. Finally, isolate ID210 seems to share homology with an USA isolate (Fig. 6.9. A4).

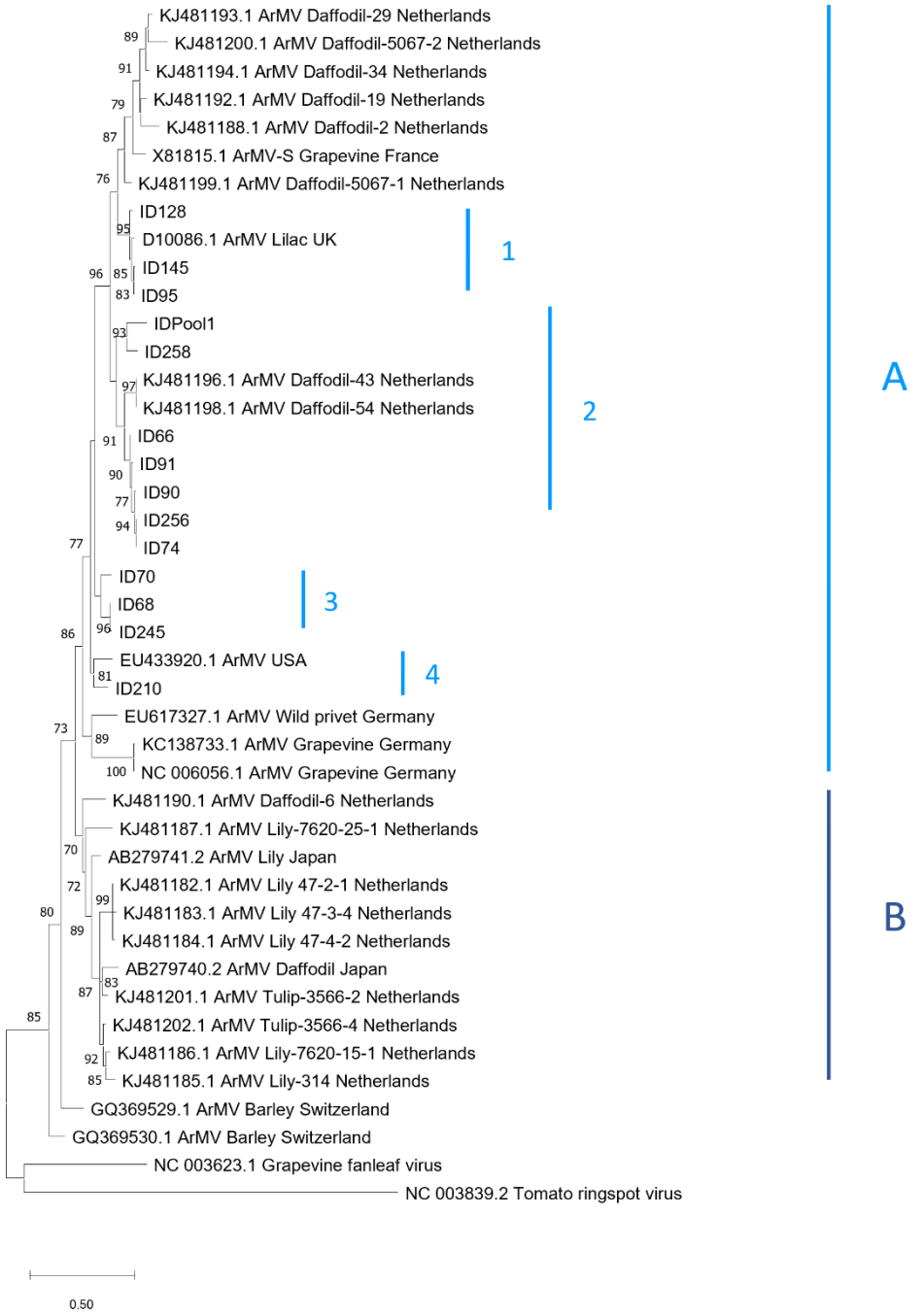

Figure 6.9- Maximum likelihood tree (1000 bootstrap) of different arabis mosaic virus (ArMV) isolates from the United Kingdom (ID128, ID145, ID95, IDPool1, ID258, ID66, ID91, ID90, ID256, ID74, ID70, ID68, ID245 and ID210) based on sequence of the RNA2 fragment (MP and CP; 486 bp). Tomato ringspot virus and grapevine fanleaf virus were included as an outgroup. Only bootstrap values higher than 70% are represented.

6.4 Discussion

Imported cut rose samples were tested as part of surveillance for RRV and further testing was performed for other common rose viruses using RT-qPCR. Positive samples were identified for RoCV1, ApMV, PNRSV, and TSWV. RoCV1 was identified for the first time in roses imported from Ecuador, India, Rwanda and Uganda. RoCV1 isolates showed low sequence variation. Isolates from the UK and from imported samples shared a high percentage of identity (96.41-100%). *Rosa* spp. is the only host known for RoCV1, which could explain the low variation between isolates, as the virus has not suffered the required mutations to adapt and infect other hosts. There are no known natural vectors for RoCV1, and no graft transmission or cell-to-cell transport, except at cell division; pollen and seed transmission are the only known modes for transmission (Boccardo *et al.*, 1987). Cryptoviruses escaped detection for many years because most of them cause no visible symptoms or, in a few situations, very mild symptoms were reported (Hull, 2014). RoCV1 has been found infecting a high percentage of plants in the UK and New Zealand (Milleza *et al.*, 2013; Vazquez-Iglesias *et al.*, 2019). Therefore, with its finding in Ecuador, India, Uganda and Rwanda roses, it is probable that RoCV1 has spread during commercial trade of planting material and it is currently distributed worldwide.

Samples infected with TSWV were imported from Ecuador and Uganda, where the virus was previously reported in other hosts (Tananси Muwanika *et al.*, 2013; Sivaprasad *et al.*, 2017; Sivaprasad *et al.*, 2018). TSWV has been considered widespread since the 1980s, due to the rapid spread of *Frankliniella occidentalis*, its most efficient vector (Kirk and Terry, 2003). This virus is transmitted in a persistent manner and multiplies in its thrips vectors (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993), which facilitates its long-distant spread. TSWV has been reported in numerous hosts and mixed infections of different isolates occur commonly in the same infected plants (Qiu and Moyer, 1999). TSWV fragments from the imported samples and a UK isolate share homology (Fig. 6.4 clusters F and G) and seem to have a common origin. Phylogenetic analysis suggests variation of isolates can depend on the geographical distribution. Previous studies indicated that both reassortment and recombination have contributed to the molecular diversity and evolution of TSWV based on partial sequences or regional whole-genome isolates (Moritz *et al.*, 2004; Tsompana *et al.*, 2005; Tentchev *et al.*, 2011).

Samples imported from Ecuador were found to be infected with ApMV, which was previously reported in that country (Paz *et al.*, 2020). Variation in the CP gene sequences of the ApMV isolates from different plant species and geographical origins resulted in their classification into separate clusters. Previous studies classified ApMV based on the variation

of the CP gene sequence into three (Lakshmi *et al.*, 2011) or five main subgroups (Lee *et al.*, 2002). The isolates NCGR 9026 from American strawberry (AY854050) and XX1-CHN found in Chinese apple (AM490197) were not classified to any of these groups previously (Cieślińska and Valasevich, 2016), but with this study it has been demonstrated that they share homology with rose isolates. In this study, 5 subgroups were clearly distinguished: Czech Republic isolates (prune and pear), and isolates predominantly from hop, apple, rose, and hazel, although in some of these subgroups other hosts were included in a smaller proportion. ApMV is transmitted by vegetative propagation of infected plant material and by mechanical inoculation. This could explain the spread of the virus from apple to rose, as no natural vector has been identified for ApMV. There are previous reports of potential spread via root grafting or weeds (Cieślińska and Valasevich, 2016).

PNRSV was previously identified in roses from India (Kulshrestha *et al.*, 2013), Ecuador (Çelik and Ertunç, 2019), and Colombia (Cutler *et al.*, 2018). However, this represents the first record of PNRSV in Uganda, Kenya and Rwanda. Other African countries have reported PNRSV, such as Algeria, Egypt, Morocco, South Africa, and Tunisia (CABI, 2020a), but it is probably more widespread than reported in this continent, as PNRSV is considered widespread around the globe (Fajardo *et al.*, 2015). PNRSV is transmitted by plant propagation, by root grafting in orchards, and by pollen and seeds. In addition, it is transmitted by different thrips species (Golino *et al.*, 2007). Finding PNRSV infecting roses in these countries is not unexpected, as this virus is involved in Rose mosaic disease (RMD), one of the most common diseases in roses worldwide. PNRSV isolates in roses imported from Kenya, India and Colombia shared homology with other isolates from the same host but from China (RNA1, RNA2, RNA3) and Poland (RNA3). Overall there is a phylogenetic relationship with isolates from cherry, mainly from China (RNA2 and RNA3), but also from the USA (RNA1). A higher number of sequences were publicly available for RNA3 fragment, and a UK isolate was also included in this case (ID148). The UK rose isolate also shared homology with the other rose isolates. Based on RNA3 comparison, rose isolates also have a common origin with peach isolates from North America.

SLRSV and ArMV were not detected in the analysis of imported samples. However, a comparison of the UK rose isolates with the available isolates in the database was performed to identify homologies between the isolates and provide evidence for the movement of infected material around the globe. In the case of SLRSV, comparison of both RNA fragments (1 and 2; Figs. 6.6 and 6.7) showed that the UK rose isolates shared homology with isolates from New Zealand, the Netherlands, the USA, and Norway identified in different hosts. For RNA2, a UK

strawberry (*Fragaria* sp.) isolate shared homology with the UK rose isolates. For ArMV, phylogenetic analysis of RNA1 and RNA2 includes the UK rose isolates in clusters with different European isolates such as winter barley isolates from Switzerland or grapevine from France. The only exception is isolate ID210, which seems to have a higher homology with a US isolate.

Two novel viruses were identified in imported flowers entering the UK. For one of them, RIV-1 (sample ID188; Chapter 4), detected in cut roses from India, it was the first report. This virus is not currently regulated, as it is a newly identified virus species. It is unknown how widespread this virus is in both in India and around the globe. The other novel virus identified was a *Waikavirus*, tentatively named 'Sweetbriar rose curly top virus', previously reported in New Zealand (Joe Tang, personal communication). This virus was identified in a sample imported from Colombia (ID181). The sample did not show any symptoms in the leaves; however, this variety was a tinted tricolour rose. If any symptoms were present in the petals, they could have gone unnoticed.

The results obtained in this study suggest that rose viruses are more widespread than previously reported, and that trade is likely to contribute to their global spread. Phylogenetic analysis performed during this study has shown the relationships between different rose isolates around the globe, which clustered together in the case of PNRSV (Fig. 6.1, 6.2) or ApMV (Fig. 6.5). Furthermore, the group of isolates from different hosts and countries reveals homology between isolates from different countries, proving an exchange of isolates potentially by trade, as could be distinguished for example with TSWV isolates (Fig. 6.4). It is possible that viruses were introduced by infected seed, rootstock, or material for grafting, and then multiplied and spread locally, maybe with virus adaptation to the host, before they started dispersing around different countries through distribution routes of breeding lines, germplasm or seeds. Globalisation has opened the door to long-distance trade, causing a huge impact on the economy. In the case of ornamentals, it allows exchange of new varieties and the development of new ones. Although some of the viruses are asymptomatic in ornamentals, these viruses are a risk for other species when they establish in a new country, posing a risk for biodiversity and ecosystems (Aukema *et al.*, 2011).

Diagnostics are fundamental to facilitate the management of plant diseases (van der Want and Dijkstra, 2006; Aboul-Ata *et al.*, 2011) and early detection is essential to successful biosecurity responses. In this study, HTS has proven its potential as a diagnostic tool, as without this analysis the two novel viruses would probably still be unidentified and not reported in the UK. Furthermore, in this study 8 samples were included in the HTS run and 2 of them were

found to be infected with novel viruses. It is not known if the remaining 33 samples were infected with these viruses or if they were infected with other novel viruses. Nevertheless, we can conclude that there are unreported rose viruses potentially spreading though trade. Non-targeted methods such as HTS could be useful as a diagnostic tool to help to strengthen plant health, identifying and stopping the spread of unknown species between countries, including viruses, new vectors and/or other pests. Further investigation should be performed to identify the best way to take advantage of this testing, as new technologies such as Flongle, a low-cost flow cell (Chapter 4), could be used to perform quick and cost-effective tests *in situ*, for example at an airport border. Nevertheless, one of the disadvantages of the discovery of these new species is that the impact they could cause remains unknown. Further work is needed to perform a biological characterisation of the new virus species, which will help to develop the required plant health regulations.

Chapter 7 General Discussion

The aim of this project was to test the following hypotheses: (i) there has been under-reporting of known viruses in roses in the United Kingdom (UK); (ii) rose rosette virus (RRV; genus *Emaravirus*) was present in the UK and was confused with other diseases or abiotic stress; and (iii) there were undescribed viruses affecting roses that may be limiting production in the UK. Throughout this PhD thesis, answers have been obtained to the proposed hypotheses.

Despite the importance of rose cultivation, rose viruses have not been studied in detail in the UK since the 1980s (Thomas, 1984a), and as a result molecular methods for virus detection have rarely been deployed to study their presence, variation, and diversity. Performing a survey of rose viruses allowed us to confirm that there was under-reporting of known viruses in the UK. Viruses such as apple mosaic virus (ApMV; genus *Ilarvirus*), arabis mosaic virus (ArMV; genus *Nepovirus*), prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*), and strawberry latent ringspot virus (SLRSV; family *Secoviridae*) have been previously reported in the UK (Thomas, 1984b) and are considered widespread in Europe (CABI, 2020a). However, in this study, it was possible to estimate the current prevalence of these viruses in the UK (e.g. ArMV 36.7% prevalence, SLRSV 27.0% prevalence). The epidemiology of Rose mosaic disease (RMD) was studied and revealed that the main viruses involved in the UK are SLRSV and ArMV, establishing a difference from RMD from North America, which is mainly caused by PNRSV and ApMV (Ikin and Frost, 1974; Horst and Cloyd, 2007). Mixed infections were commonly found during the surveillance work on rose viruses (Chapter 3). Different symptomatic and asymptomatic samples were analysed, and it remains difficult to identify which symptoms are caused by the different viruses or virus mixtures, as all have been shown to cause either similar symptoms or none in infected plants. In the last two decades (1998-2019) reports of mixed infections have increased (Alcaide *et al.*, 2020). This could be due to the use of improved diagnostic methods, including HTS since 2009, which have uncovered previously unrecognised mixtures of viruses.

Viruses such as cucumber mosaic virus (CMV; genus *Cucumovirus*) and tomato spotted wilt virus (TSWV; genus *Orthotospovirus*) are widespread worldwide (CABI, 2020a) and so are their vectors. TSWV is transmitted by at least 10 species of thrips (Jacobson and Kennedy, 2013), and more than 1000 plant species have been reported as hosts (Roselló *et al.*, 1996; Parrella *et al.*, 2003), it being one of the most economically important members of the genus *Orthotospovirus* (Riley and Pappu, 2000). CMV is transmitted by at least 75 species of aphids (Roossinck, 2001), and has developed mechanisms to infect more than 1,200 species that belong

to more than 80 plant families (Ouedraogo *et al.*, 2019). Within this study, CMV and TSWV have been found for the first-time infecting roses in the UK. Even though these viruses are known to cause damage, there are few reports of significant damage in ornamentals (Yoon *et al.*, 2020). However, these ornamental hosts could potentially serve as reservoir of viruses which could then have an impact in other crops.

Performing a survey of rose viruses with the application of high-throughput sequencing (HTS) allowed us to confirm the presence of undescribed viruses affecting roses in the UK**.** Viruses such as rose cryptic virus-1 (RoCV1; family *Partitivirus*) and rose spring-dwarf associated virus (RSDaV; genus *Luteovirus*) were detected for the first time in the UK. RoCV1 is not believed to cause significant damage to roses, but the effect of this virus in conjunction with others during mixed infections is still unknown (Vazquez-Iglesias *et al.*, 2019). RoCV1 was identified with a high incidence in New Zealand and Turkey (48.8 and 51% respectively; (Milleza *et al.*, 2013; Karanfil, 2021), similar to the incidence reported in the UK (Vazquez-Iglesias *et al.*, 2019, Chapter 4). Furthermore, 53% of the roses tested (32 samples tested) that were imported from India, 50% of roses imported from Ecuador (4 samples tested), and 100% from Uganda and Rwanda (1 sample tested; Chapter 6) were positive for RoCV1. There is no known natural vector for RoCV1; it is assumed to be pollen- and seed-transmitted like other cryptoviruses. RoCV1 is transmitted from cell to cell during cell division. Thus, the main transmission route could result from infected breeding lines. Through trade, RoCV1 may have been spreading and it is likely to be more prevalent around the world than currently reported. Further study on variety incidence is needed to understand the high prevalence and spread of this virus.

In New Zealand, Turkey, and Chile, RSDaV was identified with a higher incidence (20-24%) than in the UK (1.17%; (Rivera and Engel, 2010; Milleza *et al.*, 2013; Karanfil, 2021). In the USA, 55% of tested samples were found infected with RSDaV, although samples were collected from a unique site in this study (Salem *et al.*, 2008a). This virus may have been spreading through trade during the last decades and is likely to be more prevalent around the world than currently reported. RSDaV, thought to be the cause of Rose spring dwarf disease (Salem *et al.*, 2008a), has an impact in the industry due to the development of symptoms (e.g. rosetting) in the infected plants, although in this this study infected plants with those symptoms were not identified (Vazquez-Iglesias *et al.*, 2020b, Chapter 4). RSDaV is transmitted by the rose-grass aphid (*Metapolophium dirhodum*), present in the UK (CABI, 2020b), and the yellow rose aphid (*Rhodobium porosum*), widely distributed across Europe (Müller and Steiner, 1988; Salem *et al.*, 2008a; Barjadze *et al.*, 2011). The lower incidence of this virus in the UK could

be explained by the limitation of inoculum source in the country, environmental conditions not being optimal for virus transmission or aphid replication, or the absence of efficient aphid biotypes. However, if this virus is being spread via trade, new sources of inoculum could be arriving in the country. Furthermore, trade could also lead to the potential entrance of aphids, or new or more efficient vector biotypes. In addition, climate change could result in the development of more favourable conditions for the aphids to transmit the virus in the UK. Further studies on the epidemiology of the virus are required, to further study the spread of the virus, to identify other possible vectors, and to use modelling to study the possibilities of the aphids establishing and spreading in the UK.

On the basis of survey work carried out during this study it was shown that RRV is not present in the UK and has not been confused with other diseases or abiotic stress. A review was performed on RRV literature, going through host susceptibility, symptomology and vectors, as well as the diagnostic techniques available, to help to develop a quick response in case of an outbreak (Vazquez-Iglesias *et al.*, 2020a, Chapter 2). The work developed in this thesis has brought information about RRV closer to Europe, raising awareness about this virus by publishing the RRV literature review article, by sharing information about the virus in different public events, and by working closely with Plant Health and Seed Inspectors (PHSI). Hence, further investigation and surveillance work could be performed in different countries.

With this work it was possible to establish a network with the PHSI, who have started to look for RRV characteristic symptoms in plant material arriving into the UK at airports. Susceptibility of cut rose varieties has not been assessed, and they could play an important role in the spreading of RRV and its vector. In this project, roses imported from different production countries where RRV has been reported, such as India (Chakraborty *et al.*, 2017), were tested (Chapters 4 and 6) and were negative for RRV. Further work was done to undertake surveillance on cut roses imported into the UK, which allowed us to discover a connection between the rose industry and virus spread. A total of 8 imported samples were included in an HTS run, resulting in the discovery of one novel and one new virus entering the UK (Chapters 4 and 6): rosa ilarvirus-1 (RIV-1; genus *Ilarvirus*) and sweetbriar rose curly top virus (SRCTV; genus *Waikavirus*). These viruses were identified when HTS was employed in samples imported from India and Colombia respectively. In addition, RoCV1 was identified for the first time in roses imported from Ecuador, India, Rwanda and Uganda, and PNRSV also in Uganda, Kenya and Rwanda. PNRSV is transmitted by plant propagation. International commercialisation of infected plant propagation material could lead to the spread of viruses through trade. Further study is required to assess the prevalence of viruses such as PNRSV and SLRSV, ArMV or

RSDaV in countries such as Kenya, which supplies a high volume of roses sold in the EU (Eurostat, 2019).

Early detection is important to enable eradication, containment and control of pests such as RRV. However, other strategies, including the use of remote sensing and mathematical models, could be more suitable for the management of other pests when the impact is unknown (Martinelli *et al.*, 2015; Thompson *et al.*, 2018). Identification of known pathogens can be readily performed using published methodologies. For instance, the European and Mediterranean Plant Protection Organization (EPPO) publishes a list of validated diagnostic methods for the detection of pests that a laboratory could easily follow and perform to help avoid the entrance of unwanted organisms into a country. However, the detection of unknown pests is more difficult and may be critical. Since the start of the modern globalisation in the 19th century there has been increased movement of material between countries, included unwanted organisms. The main means of spread is through trade, especially in ornamentals (Fox and Mumford, 2017). Roses are cultivated around the globe, being one of the most valuable ornamental flowering shrubs (Boskabady *et al.*, 2011). During this study, the application of HTS has been used to identify viruses affecting roses both in the UK or imported via trade into the UK. Results suggest that it is highly possible that there is still a significant number of unknown virus species to be discovered travelling and spreading around the globe.

Information on the presence and diversity of viruses was obtained by testing samples with different targeted and non-targeted diagnostic techniques. Well-established diagnostic methods for the detection and identification of plant viruses were used in this study: ELISA and RT-qPCR. These diagnostic techniques were used to confirm results obtained by HTS (Chapter 5). Overall, RT-qPCR showed a higher sensitivity for the detection of known viruses, whereas ELISA was identified as the technique with the lowest sensitivity (Chapter 5). The Angua and the EDNA pipelines, used for the analysis of HTS data, showed non-significant differences in sensitivity from RT-qPCR for the detection of viruses such as SLRSV and ArMV. However, they showed difficulty in the detection of viral agents with lower titre such as RoCV1, as previously reported (Massart *et al.*, 2019). Failures in the detection of viruses were identified for both pipelines. Thus, improvement is needed and should be routinely performed for both the Angua and the EDNA pipeline. This could include a step to analyse single reads or reads that do not form a contig and scan these for viruses, increasing the sensitivity of the Angua pipeline. In the case of EDNA, an example could be the adjustment of the p-value calculation, to avoid extra steps during the analysis of data.

The potential of HTS has been emphasised in the different chapters of this thesis (Chapters 4, 5, 6), regarding its use as a non-targeted method and as a front-line diagnostic tool, and its cost-effectiveness. In this research, the MiSeq platform (Illumina) was used to test different samples, but for the RIV-1-infected sample, a comparison with the low-cost Flongle flow cell for the MinION platform (Oxford Nanopore) was performed (Chapter 4). The comparison between the two platforms highlighted the characteristics of the latter, with a low capital cost, little hands-on time, simplicity of connection to a laptop, and portable technology which could be used close to the point of sampling, such as at border control points. These characteristics of HTS could be beneficial to help to stop the spread of pathogens and could lead to its use as a front-line diagnostic tool. The use of pipelines such as EDNA (Chapter 5) could enable the transition of HTS to be used as a front-line diagnostic tool, due to its speed and user-friendly software. As EDNA works with pre-designed e-probes, detection of unknown viruses is not possible. By performing random checks on sequencing data, it could be possible to identify future threats before they cause any substantial damage. This analysis could be performed in the corresponding laboratory/institution, using more potent tools such as servers. In addition, a deeper examination of the data could also be performed in a hypothetical situation where a targeted virus is identified, and further research or information is required. The use of EDNA in conjunction with portable HTS platforms such as MinION could help to reinforce plant health.

Despite showing the lowest sensitivity, ELISA is still an important and cost-effective technique used for routine virus testing in phytosanitary, quarantine and virus-certification programmes. RT-qPCR enables an inexpensive, rapid and sensitive testing for targeted viruses. The implementation of HTS does not imply that other molecular techniques have to disappear. For example, HTS could support the design of RT-qPCR assays, using the identified sequences as templates, or assist the design of the primers/probe by providing a broader and up-to-date information on the diversity of viral genomic sequences. In addition, HTS could be routinely used to review the available assays and modify them accordingly when new virus strains are discovered. Despite all the advantages of HTS, one of the main limitations is the cost of this tool. However, HTS methods are continuously evolving and new platforms and library preparation methods are being developed. Technologies such as the Flongle flow-cell further reduce cost and throughput, which could make possible its use it as a front-line diagnostic tool. As described in Chapter 4, Flongle has shown promising results regarding turnaround time and staff cost. Overall, reagent cost is still more expensive than the use of conventional diagnostics (€199 vs €62; Chapter 4); however, when a sample is required to be tested for a panel of viruses,

HTS could be cost-effective, as a single test could be used, rather than recourse to a number of different assays or testing methods.

Future prospects will include surveillance work on RRV to protect UK and European rose cultivation, avoiding its entrance and establishment. Controls withing rose trading countries are key to prevent the introduction of RRV, but checks are performed on consignments partially (e.g. 30% of the total shipment) due to the high volume of imports. Thus, importing roses from an RRV and *P. fructiphilus* pest-free area (PFA), as England and Wales (UK) have now been declared (Department for Environment, Food & Rural Affairs, 2019) is another measure to limit the spread of RRV. Further work will include research on the biological characterisation and impact of the new virus discoveries, not only in this study, but applicable to all the new viruses reported in the last decades (Hou *et al.*, 2020). With the use of HTS more and more viruses are being identified, and mixed infections are commonly found, complicating the causal identification of a disease. Alternative strategies have been proposed for when Koch's postulates are not possible to fulfil, using epidemiological observations and appropriate statistics (Fox, 2020). One of the key future aspects is to validate HTS as a diagnostic method as detailed in EPPO PM7/98 (EPPO, 2019b) for other molecular diagnostic assays, due its advantages as a front-line diagnostic tool. One of the challenges is to know how many reads are necessary to consider a sample positive, as the detection threshold is unknown and reads could be associated with contamination. Sensitivity is key not only for the intrinsic sensitivity of the sequencing, but for the bioinformatic pipeline used, to be able to detect the viral sequences in all the data generated from a sample (Maree *et al.*, 2018). As described by Boonham *et al.* (2014), for the establishment of new techniques it is necessary to provide a platform that is easy to establish and use, despite any other benefits. For that to be possible, an improvement of the pipelines is required, to give an accurate result in the minimum time.

The outcomes of this PhD project have had an impact on plant health. The performance of this study has allowed the estimation of the prevalence of some previously reported viruses in the UK such as SLRSV, ArMV, and PNRSV, but also the identification of *Rosa* spp. as a new host for viruses that are widespread in the country and the globe (TSWV and CMV). Novel viruses were discovered in garden roses (RoCV1 and RSDaV), and a novel and a new virus species (SRCTV, and RIV-1 respectively) were identified after testing imported cut flowers arriving into the UK. The results of this project have provided a realistic insight into the range of viruses affecting roses in the UK. Some of these viruses cause mild symptoms in ornamentals such as roses, but roses could serve as a reservoir of viruses that could affect other crops. Furthermore, this research has proven a connection between the movement of plant pathogens

and the trade in roses as cut flowers. With this work, it was possible to establish a network with the PHSI and hence start looking for RRV symptoms in roses imported into the UK. A review on RRV was published (Vazquez-Iglesias *et al.*, 2020a, Chapter 2), gathering relevant information about RRV, providing information about the virus to Europe to raise awareness and help to stop its entrance and spread, protecting rose cultivation.

Appendix A

Table A.1– List of viruses studied in this research, including their acronyms, virus family and genus, genome size, host range and potential vectors.

Virus name	Acronym	Family	Genus	Genome size	Host range	Potential vectors
Apple mosaic virus	ApMV	*Bromoviridae*	*Ilarvirus*	ssRNA (+) multipartite: RNA1 (3476 bp), RNA2 (2979 bp), and RNA3 (2056 bp)	Diverse, woody and herbaceus plants. More than 65 species including *Malus domestica, Prunus persica* or *Fragaria* sp.	Vegetative propagation, pollen, seed. No known natural vector.
Arabis mosaic virus	ArMV	*Secoviridae*	*Nepovirus*	ssRNA (+) bipartite: RNA1 (7330 bp) and RNA2 (3820 bp)	Diverse, including *Fragaria* sp., *Vitis vinifera*, *Syringa vulgaris* or *Humulus lupulus*	Nematodes (*Xiphinema diversicaudatum*), and seed
Cucumber mosaic virus	CMV	*Bromoviridae*	*Cucumovirus*	ssRNA (+) multipartite: RNA1 (3357 bp), RNA2 (3050 bp), and RNA3 (2216 bp)	Wide host range including (>1200 species) including crops and ornamentals. Some examples are *Cucumis sativus*, *Daucus carota*, *Solanum lycopersicum* or *Narcissus*	Different species of aphids and by seed (for some species)
Prunus necrotic ringspot virus	PNRSV	*Bromoviridae*	*Ilarvirus*	ssRNA (+) multipartite: RNA1 (3330 bp), RNA2 (2590 bp), and RNA3 (1960 bp)	*Prunus* sp., *Rosa* sp., *Humulus lupulus*, *Malus domestica*, and *Morus alba*	Vegetative propagation, pollen, seeds, and thrips
Rosa ilarvirus1	RIV-1	*Bromoviridae*	*Ilarvirus*	ssRNA (+) multipartite: RNA1 (3346 bp), RNA2 (3063 bp) and RNA3 (2329 bp)	*Rosa* sp.	Unknown
Rose cryptic virus 1	RoCV1	*Partitivirus*	*Alphacryptovirus* (pending approval)	dsRNA multipartite: RNA1 (1750 bp), RNA2 (1490 bp) and RNA3 (1450 bp)	*Rosa* sp.	Pollen- and seed-transmitted. No known natural vector
Rose rosette virus	RRV	*Fimoviridae*	*Emaravirus*	ssRNA (-) multipartite: RNA1 (7030 bp), RNA2 (2250 bp), RNA3 (1540 bp), RNA4 (1540 bp), RNA5 (1670 bp), RNA6 (1400 bp), and RNA7 (1650 bp)	*Rosa* sp.	Eriophyid mite *Phyllocoptes fructiphilus*, grafting, and potentially by pollen
Rose spring dwarf-associated virus	RSDaV	*Luteoviridae*	*Luteovirus*	ssRNA (+) monopartite: 5810 bp	*Rosa* sp.	Rose-grass aphid (*Metapolophium dirhodum*) and the yellow rose aphid (*Rhodobium porosum*)
Strawberry latent ringspot virus	SLRSV	*Secoviridae*	Unassigned	ssRNA (+) bipartite: RNA1 (7500 bp) and RNA2 (3800 bp)	Diverse, including species *Rosaceae* family, *Sambucus nigra*, *Asparagus setaceus* or Apium graveolens	Nematodes (*Xiphinema diversicaudatum*), vegetative propagation, and seed
Tomato spotted wilt virus	TSWV	*Tospoviridae*	*Orthotospovirus*	ssRNA (-) multipartite: L (8900 bp), M (4820 bp), and S (2920 bp)	Wide host range including (>1000 species) including crops and ornamentals. Some examples are *Solanum lycopersicum*, Solanum tuberosum, *Impatiens* sp. or *Rosa* sp.	Different thrips species

Table A.2– List of samples used in this study

Sample	Location	Sample	Location	Sample	Location	Sample	Location
ID1A	RHS garden -Wisley	**ID23A**	RHS garden -Wisley	**ID70**	Nursery-Midlands	**ID115**	Nursery-Midlands
ID1B	RHS garden -Wisley	**ID23B**	RHS garden -Wisley	**ID71**	Nursery-Midlands	**ID116**	Nursery-Midlands
ID2A	RHS garden -Wisley	**ID24A**	RHS garden -Wisley	**ID72**	Nursery-Midlands	**ID117**	Nursery-Midlands
ID2B	RHS garden -Wisley	**ID24B**	RHS garden -Wisley	**ID73**	Nursery-Midlands	**ID118**	Nursery-Midlands
ID3A	RHS garden -Wisley	**ID25**	RHS garden -Wisley	**ID74**	Nursery-Midlands	**ID119**	Nursery-Midlands
ID3B	RHS garden -Wisley	**ID30**	RHS garden-Harlow Carr	**ID75**	Nursery-Midlands	**ID120**	Nursery-Midlands
ID3C	RHS garden -Wisley	**ID31**	RHS garden-Harlow Carr	**ID76**	Nursery-Midlands	**ID121**	Nursery-Midlands
ID4A	RHS garden -Wisley	**ID32**	RHS garden-Harlow Carr	**ID77**	Nursery-Midlands	**ID122**	Nursery-Midlands
ID4B	RHS garden -Wisley	**ID33**	RHS garden-Harlow Carr	**ID78**	Nursery-Midlands	**ID123**	Nursery-Midlands
ID5A	RHS garden -Wisley	**ID34**	RHS garden-Harlow Carr	**ID79**	Nursery-Midlands	**ID124**	Nursery-Midlands
ID5B	RHS garden -Wisley	**ID35**	RHS garden-Harlow Carr	**ID80**	Nursery-Midlands	**ID125**	Nursery-Midlands
ID6A	RHS garden -Wisley	**ID36**	RHS garden-Harlow Carr	**ID81**	Nursery-Midlands	**ID126**	Nursery-Midlands
ID6B	RHS garden -Wisley	**ID37**	RHS garden-Harlow Carr	**ID82**	Nursery-Midlands	**ID127**	Nursery-Midlands
ID7A	RHS garden -Wisley	**ID38**	RHS garden-Harlow Carr	**ID83**	Nursery-Midlands	**ID128**	Nursery-Midlands
ID7B	RHS garden -Wisley	**ID39**	RHS garden-Harlow Carr	**ID84**	Nursery-Midlands	**ID129**	Nursery-Midlands
ID8A	RHS garden -Wisley	**ID40**	RHS garden-Harlow Carr	**ID85**	Nursery-Midlands	**ID130**	Nursery-Midlands
ID8B	RHS garden -Wisley	**ID41**	RHS garden-Harlow Carr	**ID86**	Nursery-Midlands	**ID131**	Nursery-Midlands
ID9A	RHS garden -Wisley	**ID42**	RHS garden-Harlow Carr	**ID87**	Nursery-Midlands	**ID132**	Nursery-Midlands
ID9B	RHS garden -Wisley	**ID43**	RHS garden-Harlow Carr	**ID88**	Nursery-Midlands	**ID133**	Nursery-Midlands
ID10	RHS garden -Wisley	**ID44**	RHS garden-Harlow Carr	**ID89**	Nursery-Midlands	**ID134**	Nursery-Midlands
ID11A	RHS garden -Wisley	**ID45**	RHS garden-Harlow Carr	**ID90**	Nursery-Midlands	**ID135**	Nursery-Midlands
ID11B	RHS garden -Wisley	**ID46**	RHS garden-Harlow Carr	**ID91**	Nursery-Midlands	**ID136**	Nursery-Midlands
ID11C	RHS garden -Wisley	**ID47**	RHS garden-Harlow Carr	**ID92**	Nursery-Midlands	**ID137**	Nursery-Midlands
ID12A	RHS garden -Wisley	**ID48**	RHS garden-Harlow Carr	**ID93**	Nursery-Midlands	**ID138**	Nursery-Midlands
ID12B	RHS garden -Wisley	**ID49**	RHS garden-Harlow Carr	**ID94**	Nursery-Midlands	**ID139**	Nursery-Midlands
ID13	RHS garden -Wisley	**ID50**	RHS garden-Harlow Carr	**ID95**	Nursery-Midlands	**ID140**	Nursery-Midlands
ID13B	RHS garden -Wisley	**ID51**	RHS garden-Harlow Carr	**ID96**	Nursery-Midlands	**ID141**	Nursery-Midlands
ID14	RHS garden -Wisley	**ID52**	RHS garden-Harlow Carr	**ID97**	Nursery-Midlands	**ID142**	Nursery-Midlands
ID14B	RHS garden -Wisley	**ID53**	RHS garden-Harlow Carr	**ID98**	Nursery-Midlands	**ID143**	Royal National Rose Society Garden
ID15	RHS garden -Wisley	**ID54**	RHS garden-Harlow Carr	**ID99**	Nursery-Midlands	**ID144**	Royal National Rose Society Garden
ID15B	RHS garden -Wisley	**ID55**	RHS garden-Harlow Carr	**ID100**	Nursery-Midlands	**ID145**	Royal National Rose Society Garden
ID16	RHS garden -Wisley	**ID56**	RHS garden-Harlow Carr	**ID101**	Nursery-Midlands	**ID146**	Royal National Rose Society Garden
ID16B	RHS garden -Wisley	**ID57**	RHS garden-Harlow Carr	**ID102**	Nursery-Midlands	**ID147**	Royal National Rose Society Garden
ID17	RHS garden -Wisley	**ID58**	RHS garden-Harlow Carr	**ID103**	Nursery-Midlands	**ID148**	Private garden
ID17B	RHS garden -Wisley	**ID59**	RHS garden-Harlow Carr	**ID104**	Nursery-Midlands	**ID149**	PHSI
ID18	RHS garden -Wisley	**ID60**	RHS garden-Harlow Carr	**ID105**	Nursery-Midlands	**ID150**	RHS garden -Wisley
ID18B	RHS garden -Wisley	**ID61**	RHS garden-Harlow Carr	**ID106**	Nursery-Midlands	**ID152**	PHSI
ID19	RHS garden -Wisley	**ID62**	RHS garden-Harlow Carr	**ID107**	Nursery-Midlands	**ID153**	PHSI
ID19B	RHS garden -Wisley	**ID63**	RHS garden-Harlow Carr	**ID108**	Nursery-Midlands	**ID155**	PHSI
ID20	RHS garden -Wisley	**ID64**	Member RHS	**ID109**	Nursery-Midlands	**ID156**	Nursery-Midlands
ID20B	RHS garden -Wisley	**ID65**	Nursery-Midlands	**ID110**	Nursery-Midlands	**ID159**	PHSI
ID21	RHS garden -Wisley	**ID66**	Nursery-Midlands	**ID111**	Nursery-Midlands	**ID164**	PHSI
ID21B	RHS garden -Wisley	**ID67**	Nursery-Midlands	**ID112**	Nursery-Midlands	**ID168**	PHSI
ID22	RHS garden -Wisley	**ID68**	Nursery-Midlands	**ID113**	Nursery-Midlands	**ID170**	PHSI
ID22B	RHS garden -Wisley	**ID69**	Nursery-Midlands	**ID114**	Nursery-Midlands	**ID171**	PHSI

Sample	Location	Sample	Location
ID180	PHSI	**ID231**	Nursery-Midlands
ID181	PHSI	**ID23ID2**	Nursery-Midlands
ID187	PHSI	**ID233**	Nursery-Midlands
ID188	PHSI	**ID234**	Nursery-Midlands
ID190	PHSI	**ID235**	Nursery-Midlands
ID193	PHSI	**ID236**	Nursery-Midlands
ID196	PHSI	**ID237**	Nursery-Midlands
ID198	PHSI	**ID238**	Nursery-Midlands
ID199	PHSI	**ID239**	Nursery-Midlands
ID201	PHSI	**ID240**	Nursery-Midlands
ID202	PHSI	**ID241**	Nursery-Midlands
ID203	PHSI	**ID242**	Nursery-Midlands
ID205	PHSI	**ID243**	Nursery-Midlands
ID206	PHSI	**ID244**	Nursery-Midlands
ID207	Private garden	**ID245**	Nursery-Midlands
ID208	PHSI	**ID246**	Nursery-Midlands
ID209	PHSI	**ID247**	Nursery-Midlands
ID210	PHSI	**ID248**	Nursery-Midlands
ID211	PHSI	**ID249**	Nursery-Midlands
ID212	PHSI	**ID250**	Nursery-Midlands
ID215	PHSI	**ID251**	Nursery-Midlands
ID216	RHS member	**ID252**	Nursery-Midlands
ID217	PHSI	**ID253**	Nursery-Midlands
ID218	PHSI	**ID254**	Nursery-Midlands
ID219	PHSI	**ID255**	Nursery-Midlands
ID220	RHS member	**ID256**	Nursery-Midlands
ID221	PHSI	**ID257**	Nursery-Midlands
ID222	PHSI	**ID258**	Nursery-Midlands
ID223	RHS member	**ID259**	Nursery-Midlands
ID224	RHS member	**ID260**	Nursery-Midlands
ID225	PHSI	**ID261**	Nursery-Midlands
ID226A	Private garden	**ID262**	Nursery-Midlands
ID226B	Private garden	**ID263**	Nursery-Midlands
ID226C	Private garden	**ID264**	Nursery-Midlands
ID230	Nursery-Midlands	**ID265**	Nursery-Midlands
		ID266	Nursery-Midlands

Table A.3– List of primers and probes for the detetection of apple mosaic virus, arabis mosaic virus, cucumber mosaic virus, impatiens necrotic spot virus, prunus necrotic ringspot virus, raspberry ringspot virus, rose cryptic virus-1, rose rosette virus, strawberry latent ringspot virus, tobacco rattle virus, tobacco ringspot virus, tomato ringspot virus, and tomato spotted wilt virus using RT-qPCR

Virus	Genus	Primers (5'-3')	Amplicon size (bp)	Reference
Apple mosaic virus	*Ilarvirus*	ApMV-F-TGG TGG AGG ATT ACG ATG AAA GTA	66	Malandraki *et al.* (2017)
		ApMV-R-TTT GAA ACC CTT TCG GTC CAT		
		ApMV-Pe-[FAM]-CGA AAG GTC CGA ATC-[MGB-NFQ]		
Arabis mosaic virus	*Nepovirus*	ArMV-CP-F-TAG CCC TTG GAG ACA ATC CT	93	Wei *et al.* (2011)
		ArMV-CP-R-CCT CCA AAT CCC ACA TTA AC		
		ArMV-CP-Pe-[FAM]-TGC CCA TAT GAT AGC TTG TCA TGG AC-[BHQ1]		
Cucumber mosaic virus	*Cucumovirus*	CMV-F-GCT TGT TTC GCG CAT TCA A	87	Skelton *et al.* (2018)
		CMV-RI-GAG GCA GRA ACT TTA CGR ACT GT		
		CMV-RII-TGA AGG TAC TTT CCG AAC TGT AAC C		
		CMV-Pe-[FAM]-TTA ATC CTT TGC CGA AAT TTG ATT CTA CCG T GTG-[TAMRA]		
Impatiens necrotic spot virus	*Orthotospovirus*	INSV-120F-CTT CTT TAC CAA CAA CCG TGA AAA	79	Department for Environment Food & Rural Affairs (2005)
		INSV-198R-AGA TTG CCT ATT CTT GAG GAA GGA		
		INSV-145Pe-[FAM]-ATT CAG AAC ATG ACT ACT GC-[MGB]		
Prunus necrotic ringspot virus	*Ilarvirus*	PNRSV10F-TTC TTG AAG GAC CAACCG AGA GG	348	Marbot *et al.* (2003)
		PNRSV10R-GCT AAC GCA GGT AAG ATT TCC AAG C		
		PNRSVPe-[FAM] ATG TCT TGC TGG TCG ATG 3[MGB-NFQ]		
Raspberry ringspot virus	*Nepovirus*	RpRSV-1699F-GTT GTG TTG CTT CCC AGG GTA T	81	Monger and Mumford (2010)
		RpRSV-1780R-YAA AAC CAR SGG TGC ATA TTC TTT		
		RpRSV-1723Pe-[FAM]-TGC AGA CCT GGG AAA AGG AGG TTA ATC CT-[BHQ1]		
Rose cryptic virus 1	*Alphacryptovirus* (pending approval)	RoCV1–2-Fw-TGA TCG ACC AAA GTT GCA ACC	110	Vazquez-Iglesias *et al.* (2019)
		RoCV1–2-Rv-GAA GAT AAG ACA ATG CAG TCA CTT TCT T		
		RoCV1–2-Pe-[FAM]-ATT CGG ACT GAA TTT GCT A-[MGB-NFQ]		
Rose rosette virus	*Emaravirus*	RRV-F-GAT TAC CTT GTA GCC AAT TAC TTC TAA CTG	122	This study
		RRV-R-CAT CTT CAA TGA TAT GCT CAA TTT AGT TAA		
		RRV-Pe-[FAM] TGT GTT TGC ACT GTT GAC-[MGB-NFQ]		
Strawberry latent ringspot virus	Unassigned (Family *Secoviridae*)	SLRSV-194F - CAT CTC CAA ART GCT CMT TTC A	~80	Monger and Mumford (2010)
		SLRSV-192F- ACC TCC TTC AAA GTG TTC CTT TCA		
		SLRSV-271R-GYC CRC TAG CTT CTG CCT CRC		
		SLRSV-275R-TGT AGT CCA CTC GAT TCT GTC TCA C		
		SLRSV-224Pe-[FAM]-TTG GGT GYC CRT GCA ARC AGC ATA CT-[BHQ1]		
Tobacco rattle virus	*Tobravirus*	TRV-1466F-CAT GCT AAC AAA TTG CGA AAG C	88	Mumford *et al.* (2000)
		TRV-1553R-TAC AGA CAA ACC ATC CAC AAT TAT TTT		
		TRV-1489Pe-[FAM]-ACG TGT GAC ACC AAC CAT GTC AGC AAC T-[TAMRA]		
Tobacco ringspot virus	*Nepovirus*	TRSV-F-GGG GTG CTT ACT GGC AAG G	91	EPPO (2017)
		TRSV-R-GCA CCA GCG TAA GAA CCC AA		
		TRSV-Pe-[FAM]-TGA TTT GCG GCG TAC TG-[MGB]		
Tomato ringspot virus	*Nepovirus*	ToRSV-F-GAA TGG TTC CCA GCC ACT T	82	Tang *et al.* (2014)
		ToRSV-R-AGT CTC AAC TTA ACA TAC CAC		
		ToRSV-Pe-[FAM]-AGG ATC GCT ACT CCT CCG TCA AC-[BHQ1]		
Tomato spotted wilt virus	*Orthotospovirus*	TSWV-F- CTC TTG ATG ATG CAA AGT CTG TGA	83	EPPO (2004)
		TSWV- R -TCT CAA AGC TAT CAA CTG AAG CAA TAA		
		TSWV Pe-[FAM]-AGG TAA GCT ACC TCC CAG CAT TAT GGC AAG-[TAM]		

Table A.4- Summary results of the 14 viral sequence reads using Flongle (Oxford Nanopore Technologies, UK) following BLAST-n of GenBank.

Reads	Length (bp)	BLASTn	Identity (%)	Accession number
1	*166*	TomNSV RNA 3	87.57	NC_039076.1
		TomNSV RNA 2	87.43	NC_039074.1
		TomNSV RNA 1	86.47	NC_039075.1
		SLV	83.33	KY695012.1
		TAMV	84.05	AF226161.1
		EMoV	82.74	U57048.1
2	*203*	TomNSV RNA 3	84.43	NC_039076.1
		TomNSV RNA 2	83.33	NC_039074.1
		TomNSV RNA 1	83.50	NC_039075.1
		EMoV	82.16	U34050.1
		TAMV	80.88	AF226161.1
3	*600*	TomNSV RNA 3	75.00	NC_039076.1
4	*604*	TomNSV RNA 3	73.83	NC_039076.1
5	*154*	TomNSV RNA 2	85.71	NC_039074.1
		TomNSV RNA 3	84.62	NC_039076.1
		SLV	84.52	U93193.1
		TomNSV RNA 1	84.18	NC_039075.1
		AV2	83.77	EU919667.1
		EMoV	83.77	U57048.1
6	*190*	TomNSV RNA 3	82.67	NC_039076.1
		TomNSV RNA 2	83.50	NC_039074.1
		TomNSV RNA 1	81.19	NC_039075.1
		TAMV	77.95	AF226161.1
		AV2	77.16	EU919666.1
7	*335*	TomNSV RNA 3	75.54	NC_039076.1
8	*910*	TomNSV RNA 2	74.35	NC_039074.1
		CLRV RNA 2	71.89	JX256248.1
9	*239*	TomNSV RNA 3	83.25	NC_039076.1
		TomNSV RNA 2	82.91	NC_039074.1
		TomNSV RNA 1	82.54	NC_039075.1
		TAMV	78.57	AF226161.1
10	*822*	TomNSV RNA 3	70.11	NC_039076.1
				KP861235.1
11	*145*	TomNSV RNA 3	76.16	NC_039076.1
		TomNSV RNA 2	76.16	KP861235.1
		TAMV	74.17	AF226162.1
12	*1033*	TomNSV RNA 3	71.62	NC_039076.1
13	*499*	TomNSV RNA 2	84.31	NC_039074.1
14	140	TomNSV RNA 3	73.50	NC_039076.1

Table A.5- Percentage of identity among the different RNA (1, 2, 3) fragments of rose cryptic virus-1 isolates identified in imported samples and isolates from the United Kingdom.

RNA1	ID11B	ID68	ID91	ID210_i	ID210_ii	ID159_i	ID159_ii	ID159_iii	ID188	ID252	MK075826.1 UK	MK075821.1 UK	EU350962.1 USA
ID11B		100	100	100	0	100	100	0	99.54	100	100	99.76	98.39
ID68	100		100	100	99.58	100	100	99.74	99.67	100	100	99.76	99.33
ID91	100	100		100	99.58	100	100	99.74	99.67	100	100	99.76	99.04
ID210_i	100	100	100		0	100	0	0	92.86	100	100	99.67	97.02
ID210_ii	0	99.58	99.58	0		0	0	99.73	97.22	99.58	99.58	0	99.58
ID159_i	100	100	100	100	0		0	0	98.36	100	100	99.58	97.21
ID159_ii	100	100	100	0	0	0		0	0	100	100	100	0
ID159_iii	0	99.74	99.74	0	99.73	0	0		0	99.74	99.74	0	99.74
ID188	99.54	99.67	99.67	92.86	97.22	98.36	0	0		99.67	99.67	0	99.34
ID252	100	100	100	100	99.58	100	100	99.74	99.67		100	99.76	99.33
MK075826.1 UK	100	100	100	100	99.58	100	100	99.74	99.67	100		99.75	99.33
MK075821.1 UK	99.76	99.76	99.76	99.67	0	99.58	100	0	0	99.76	99.75		93.1
EU350962.1 USA	98.39	99.33	99.04	97.02	99.58	97.21	0	99.74	99.34	99.33	99.33	93.1	

RNA2	ID11B	ID159	ID210_i	ID210_ii	ID252	ID256_i	ID256_ii	EU350963.1 USA	EU024677.1 USA	MK075832.1 UK	MK075831.1 UK	MK075830.1 UK	MK075829.1 UK
ID11B		99.1	100	100	100	100	100	99.53	100	99.81	100	100	100
ID159	99.1		98.44	0	99.1	0	0	95.45	99.07	0	0	0	0
ID210_i	100	98.44		0	100	0	0	99	100	100	100	100	100
ID210_ii	100	0	0		100	100	100	100	100	99.58	100	100	100
ID252	100	99.1	100	100		100	100	99.54	100	99.81	100	100	100
ID256_i	100	0	0	100	100		100	100	100	0	0	0	100
ID256_ii	100	0	0	100	100	100		100	100	100	100	100	100
EU350963.1 USA	99.53	95.45	99	100	99.54	100	100		99.54	99.81	100	100	100
EU024677.1 USA	100	99.07	100	100	100	100	100	99.54		99.81	100	100	100
MK075832.1 UK	99.81	0	100	99.58	99.81	0	100	99.81	99.81		99.81	99.81	99.81
MK075831.1 UK	100	0	100	100	100	0	100	100	100	99.81		100	100

MK075830.1 UK	100	0	100	100	100	0	100	100	100	99.81	100		100
MK075829.1 UK	100	0	100	100	100	100	100	100	100	99.81	100	100	
MK075827.1 UK	100	99.1	100	100	100	100	100	99.54	100	99.81	100	100	100

RNA3	ID68	ID91	ID159_i	ID159_ii	ID188	ID210	ID235	ID252_i	ID252_ii	MK075828.1 UK
ID68		99.91	99.35	99.44	99.66	100	98.7	99.91	99.91	99.91
ID91	99.91		99.73	99.44	98.66	100	100	100	100	100
ID159_i	99.35	99.73		0	0	0	99.57	99.64	99.73	99.72
ID159_ii	99.44	99.44	0		100	99.38	0	99.44	99.44	99.44
ID188	99.66	98.66	0	100		99.59	0	98.67	98.67	98.67
ID210	100	100	0	99.38	99.59		0	100	100	100
ID235	98.7	100	99.57	0	0	0		100	100	100
ID252_i	99.91	100	99.64	99.44	98.67	100	100		100	100
ID252_ii	99.91	100	99.73	99.44	98.67	100	100	100		100
MK075828.1 UK	99.91	100	99.72	99.44	98.67	100	100	100	100	

Appendix B Publications

B.1 Facing *Rose rosette virus:* A Risk to European Rose Cultivation

Received: 24 April 2020 | Accepted: 17 July 2020

DOI: 10.1111/ppa.13255

PLANT PATHOGEN IMPACTS – REVIEW

Plant Pathology WILEY

Facing *Rose rosette virus*: A risk to European rose cultivation

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Abstract

Roses (*Rosa*) are one of the most valuable ornamental flowering shrubs around the globe. They are susceptible to numerous pathogens that require management, increasing the cost of cultivation. Rose rosette virus (RRV; genus *Emaravirus*) is a devastating virus that has been spreading since the 1940s in the United States and Canada. It is an emerging risk to European and worldwide rose cultivation, causing symptoms such as witches' broom, malformations, excessive thorn production, and eventually plant death. RRV is transmitted by the eriophyid mite *Phyllocoptes fructiphilus* and by grafting. Research is being undertaken to understand RRV and to find control measures and resistant cultivars, as they are not currently available. Early detection of the disease is the key to prevent the establishment and spread of RRV and its vector. Different molecular and serological diagnostic methods have been designed and implemented, including ELISA, RT-PCR, RT-qPCR, LAMP, and high-throughput sequencing. RRV infected plants can remain symptomless for long periods, so these diagnostic assays are necessary in conjunction with visual assessment to facilitate early detection. Significant social, economic, and environmental impacts are expected if RRV and its vector establish and spread in Europe. Rose trade between countries is the most likely pathway of introduction of RRV into Europe. In this review we describe current knowledge about RRV, the molecular and serological methods available for the detection of this virus, pathways to entry, and the possible impact if it establishes and spreads in Europe.

KEYWORDS

diagnostics, ornamentals, roses, RRV

1 | INTRODUCTION

Roses (*Rosa* spp.) are one of the most important ornamental species worldwide (Boskabady *et al*., 2011), not only for their industrial properties (Dobhal *et al*., 2016), but for their fragrance, beauty, and aesthetics. They are considered the national flower of several countries in Europe, including England. Repeat flowering varieties were introduced into Europe from China in the 18th century (Joyaux, 2003) transforming the concept of roses, showing a broader range of colours, growth types, flower sizes, and scents. From that time, extensive rose breeding has taken place across the world, creating a massive industry (Debener and Byrne, 2014).

Cultivation of roses is economically important around the globe. The estimated annual production of cut flowers is around 18 billion stems, 60–80 million potted plants (miniature roses and bare-root grafting plants), and 220 million plants for landscaping (Blom and Tsujita, 2003; Pemberton *et al*., 2003; Roberts *et al*., 2003). The world rose production was estimated to be valued at €24 billion (around

£21 billion) in 2008 (Heinrichs, 2008). In the USA, total wholesale production of shrub roses was estimated to be worth $204 million in 2014 (less than £166 million), with 1,808 growers producing 36.6 million plants (United States Department of Agriculture, 2015). In terms of plants for planting, including bare-rooted plants, pot plants, cuttings/budwood, rootstock, and tissue culture, Serbia (36.34%) and China (30.81%) are the main countries from which the European Union (EU) imports roses (Table 1). Rosehips are also traded, used for different products such as rosehip jelly, water, or perfume (Leghari *et al.*, 2016).

2 | PEST AND PATHOGENS AFFECTING ROSES IN EUROPE

Roses are susceptible to numerous diseases that require management, increasing the cost of production. In the UK, the garden industry contributes £9 billion to the economy every year. Defra (Department for Environment, Food and Rural Affairs) valued general ornamental plant production at £1.1 billion in 2015, pointing out that diseases caused losses of £630 million annually in the UK to ornamental plant production, of which £40 million was due specifically to viral diseases (Department for Environment, Food and Rural Affairs, 2016; Little, 2016).

TABLE 1 Rose plants imported from non-EU countries to the EU from Jan 2014 to Dec 2018

	Mean	
Source country	kg	% of total
Serbia	281,740	36.34
China	238,880	30.81
South Africa	120,920	15.60
Uzbekistan	33,280	4.29
Kenya	20,920	2.70
Switzerland	20,560	2.65
Norway	13,040	1.68
Moldova	9,120	1.18
Ethiopia	6,120	0.79
South Korea	5,900	0.76
North Macedonia	5,880	0.76
Ukraine	5,540	0.71
Turkey	4,220	0.54
Morocco	3,860	0.50
United States	1,140	0.15
Sri Lanka	920	0.12
India	760	0.10
Belarus	620	0.08
Japan	620	0.08
Russian Federation	620	0.08
Ecuador	200	0.03
Lebanon	120	0.02
Israel	100	0.01
Colombia	60	0.01
Suriname	40	0.01
Thailand	40	0.01

Note: The table shows the percentage of the total imports originating from each country (Eurostat, 2019).

Roses are vulnerable to infections by bacteria, fungi, viruses, nematodes, and phytoplasmas, causing leaf and flower mosaics, distortion, spotting, discolouration, and necrosis, reducing growth or leading to death of the plant. Several fungal pathogens affect roses with a worldwide distribution. Black spot is the causal agent of the most serious fungal disease of roses grown outdoors in Europe and worldwide (Yasin *et al.*, 2016). Rust is caused mainly by the fungi *Phragmidium tuberculatum* and *Phragmidium mucronatum* (Helfer, 2005), among other *Phragmidium* species, and is another common disease. Powdery mildew caused by *Peronospora pannosa* is the major fungal pathogen of roses grown in greenhouses, but can also be detected in the field (Schulz and Debener, 2010).

The management of pest and diseases in rose production is primarily achieved using agrochemicals. Restrictions imposed by plant protection legislation and the increasing ecological awareness of consumers have pushed breeders in line with plant pathologists to identify and characterize resistant cultivars (Schulz and Debener, 2010). Increasing disease resistance is especially necessary for garden roses, to inspire confidence amongst amateur rosarians, gardeners, and landscapers for their use in public areas (Leus *et al.*, 2008).

The control of diseases in greenhouses is also important, because controlled environments enable a year-round supply of rose plants and cut flowers, even in seasons when outdoor temperatures or light conditions are not suitable for growth (Raviv *et al.*, 2010). Rose varieties are commonly grown in greenhouses using rootstocks that favour a rapid economic multiplication of scions from desirable rose cultivars, which cannot be raised on their own roots (Tubbs, 1973). Rootstocks play an important role for economic aspects of propagation, flower production, flower quality, adaptation to different kinds of soil, and disease resistance (Fuchs, 1994). One of the most used rootstocks is *Rosa multiflora*.

3 | ROSE VIRUSES REPORTED IN EUROPE

Several viruses have been reported affecting roses in Europe, including arabis mosaic virus (ArMV; genus *Nepovirus*), strawberry latent ringspot virus (SLRSV; family *Secoviridae*), apple mosaic virus (ApMV, genus *Ilarvirus*), and prunus necrotic ringspot virus (PNRSV; genus *Ilarvirus*; King *et al.*, 2012). Rose mosaic disease (RMD) is one of the most common diseases of roses worldwide, and is caused by single or mixed infections of these viruses (Vazquez-Iglesias *et al.*, 2019). Differences have been established between viruses involved in RMD occurring in North America and Europe, although PNRSV has been identified as the most frequent virus associated with this disease in both continents (Horst *et al.*, 1983; Manners, 1997; Sertkaya, 2010). RMD is thought to have propagated in roses by grafting from infected rootstocks or scions, subsequently

spreading among rose cultivars (Sertkaya, 2010). Viruses associated with RMD are considered to be transmitted by seeds, pollen, aphids, thrips, contaminated soil, or pruning tools, but no conclusive scientific evidence is available regarding transmission pathways (Horst and Cloyd, 2007). Golino *et al*. (2007) showed evidence of ApMV and PNRSV transmission via roots between roses growing close together in experimental fields.

Symptoms of RMD (Figure 1) vary depending on the variety, and include chlorotic line patterns, ring spots, mottles in leaves, yellow net, and mosaic. Infected plants are less vigorous and more likely to die over winter (Horst *et al*., 1983). PNRSV-infected plants have reduced quality with weaker shoots and fewer, smaller blooms, and are more likely to die after transplanting, generating losses in production. However, virus-infected plants can remain symptomless for much of the growing season, depending on the variety (Thomas, 1982).

Rose cryptic virus-1 (RoCV1), also known as Rosa multiflora cryptic virus (Martin and Tzanetakis, 2008), is a partitivirus first reported in the USA (Sabanadzovic and Ghanem-Sabanadzovic, 2008) and subsequently in Canada (James *et al*., 2015), New Zealand (Milleza *et al*., 2013), and recently in the UK (Vazquez-Iglesias *et al*., 2019). Cryptic viruses escaped detection for many years because most cause no visible symptoms or, in a few cases, very mild symptoms (Milleza *et al*., 2013). Cryptic viruses occur in very low concentrations in infected plants (Hull, 2014). There are no known natural vectors, and no graft transmission or cell-to-cell movement. The reported mode of transmission is by cell division, pollen, or seed (Boccardo *et al*., 1987).

Rose yellow vein virus (RYVV) is a circular double-stranded (ds) DNA virus that has recently been reported in Turkey (Karanfil *et al*., 2018), but was first described in the USA and New Zealand (Perez-Egusquiza *et al*., 2013). RYVV belongs to family *Caulimoviridae*, genus *Rosadnavirus* (King *et al*., 2012), causing vein banding or central vein chlorosis in infected leaves (Milleza *et al*., 2013; Mollov *et al*., 2013).

4 | ROSE ROSETTE VIRUS

Rose rosette virus (RRV) is a virus in the order *Bunyavirales*, genus *Emaravirus*, and is the causal agent of rose rosette disease (RRD;

FIGURE 1 Classic symptoms of rose mosaic disease include yellow netting and mosaic on leaves

Laney *et al*., 2011), a damaging disease of roses in North America. RRV is a multipartite RNA virus consisting of seven single-stranded negative-sense RNA particles (RNA1–RNA7), encoding an RNA-dependent RNA polymerase (RdRp), a glycoprotein, a nucleocapsid, a movement protein, and p5, p6, and p7 proteins, respectively (Laney *et al.*, 2011; Di Bello *et al*., 2015).

RRV was first described in the 1940s in Manitoba, Canada (Conners, 1941). At the same time, similar reports were made in Wyoming and California (Thomas and Scott, 1953). RRV is considered the most important viral disease of roses in the USA (Dobhal *et al.*, 2016). Early studies suggested the cause of RRD could be related to phytoplasma (Gergerich and Kim, 1983), but the association of double-membrane-bound bodies and dsRNA (Doudrick and Millikan, 1983) with rosette-affected material indicated the involvement of a virus (Laney *et al.*, 2011).

Numerous plant species have been assessed for the presence of RRV, but *Rosa* remains the only host genus identified (Laney *et al*., 2011). This occurrence may explain the small variation between RRV isolates (Laney *et al*., 2011), as host-driven diversity has not developed in RRV. Similarly, studies in European mountain ash ringspot-associated virus (EMARaV, the type species of *Emaravirus*) have also shown little sequence diversity (Kallinen *et al*., 2009; von Bargen *et al*., 2013). It may be hypothesized that the reported RRV low variability could be due to the virus replication in the vector, creating an evolutionary bottleneck where only variants replicating in both plant and mite are transmissible, such as the case of EMARaV and the mite *Eriophyes pyri* reported by Mielke-Ehret *et al*. (2010). However, further research is being undertaken to look for isolate variation in RRV (Byrne *et al*., 2019; Katsiani *et al*., 2020).

4.1 | The beginning of RRD dissemination in North America

R. multiflora was introduced to North America from Japan during the early 1800s, as an ornamental for breeding proposes and as a rootstock (Rheder, 1936). Due to its hardiness and resistance to pests and diseases, it was used widely in amenity planting. For example, 14 million multiflora roses were planted in West Virginia alone between 1940 and 1960 (Dugan, 1960). *R. multiflora* was subsequently considered a weed (Dale *et al.*, 1988) and in the early 2000s, the number of hectares covered by *R. multiflora* in the eastern USA reached 18 million (Loux *et al*., 2005).

RRV was considered an agent for the biological control of *R. multiflora*, on the assumption that rose plants would die within a period of 5 years (Epstein and Hill, 1999). Even though the US government was aware that the mite was a vector of the virus, they assessed the risk of spreading of RRD to other ornamental roses to be low (Amrine, 1996). However, as different types of roses grew in popularity, hundreds of thousands of RRV-susceptible plants were planted in private gardens and commercial beds, making it more likely that the virus would spread (Amrine, 1996).

4.2 | RRV geographical distribution

RRV is currently present from the eastern coast of the USA to the Rocky Mountains and California (Center for Invasive Species and Ecosystem Health, 2019). It was thought to be endemic to North America until 2017, when it was reported for the first time in India (Chakraborty *et al*., 2017). *R. multiflora* is a widespread susceptible host, serving as a reservoir for both virus and vector. Beyond *R. multiflora*, RRD has been reported in different rose species such as *R. arkansana*, *R. bracteate*, *R. canina*, *R. corymbifera*, *R. gallica*, *R. glauca*, *R. rubiginosa*, *R. spinossisima*, *R. villosa*, *R. woodsia*, and in a multitude of types: climbers, hybrid teas, floribundas, miniatures, shrub, and antique roses (Martin, 2014).

4.3 | Symptoms

Symptoms of RRV (Figure 2) are highly variable between rose cultivars, stage of the disease, and environmental factors (Epstein and Hill, 1995, 1999). Moreover, roses may harbour other viruses such as PNRSV and/or ApMV and their synergistic effect on symptom expression has not been determined. Symptoms of RRD include reddening on newly emerging shoots, excessive lateral shoot growth, excess thorn production, leaf mosaic, and mottling. Flowers tend to bunch together, forming witches' broom or rosetting, with malformed flowers (Laney *et al*., 2011; Dobhal *et al*., 2016). The virus moves throughout the plant affecting the roots, and plants show reduced growth and vigour compared to uninfected plants (Epstein and Hill, 1999). Other symptoms that may be expressed are darkening of canes, short internodal distances, blind shoots, rough leaf texture, and an increased susceptibility to infection, especially by fungal diseases (Hong *et al*., 2012). Infected plants die within 3–5 years of becoming infected (Di Bello *et al*., 2017).

Roses infected with RRV can show few or no symptoms during early stages of infection (Dobhal *et al.*, 2016), and can remain symptomless for 30–146 days after transmission (Allington *et al*., 1968). Hence, by the time the first recognizable symptoms appear, the disease could have spread to nearby plants (Hong *et al*., 2012).

4.4 | RRV transmission

Members of the genus *Emaravirus* are transmitted by eriophyid mites (Mielke-Ehret and Mühlbach, 2012). In early epidemiological studies, researchers theorized symptoms of RRV might be caused by eriophyid mite feeding toxicity (Slykhuis, 1980). Later experiments showed RRD was mite transmissible (Allington *et al*., 1968) and the pathogenicity of RRV was demonstrated by Di Bello *et al*. (2015). The eriophyid mite *Phyllocoptes fructiphilus* (Figure 3) is currently the only competent vector species identified (Keifer, 1966; Allington *et al*., 1968), although research has been undertaken with other *Phyllocoptes* species. The mite *Phyllocoptes adalius* is difficult to discriminate from *P. fructiphilus* morphologically because the prodorsal

FIGURE 2 Symptoms of rose rosette virus (RRV) in different rose cultivars in Oklahoma, USA: (a) reddening in the leaves and stems; (b) witches' broom or rosetting; and (c) excess thorn production and thicker stems. (d) A healthy-looking stem (left) compared with an RRV-infected stem (right)

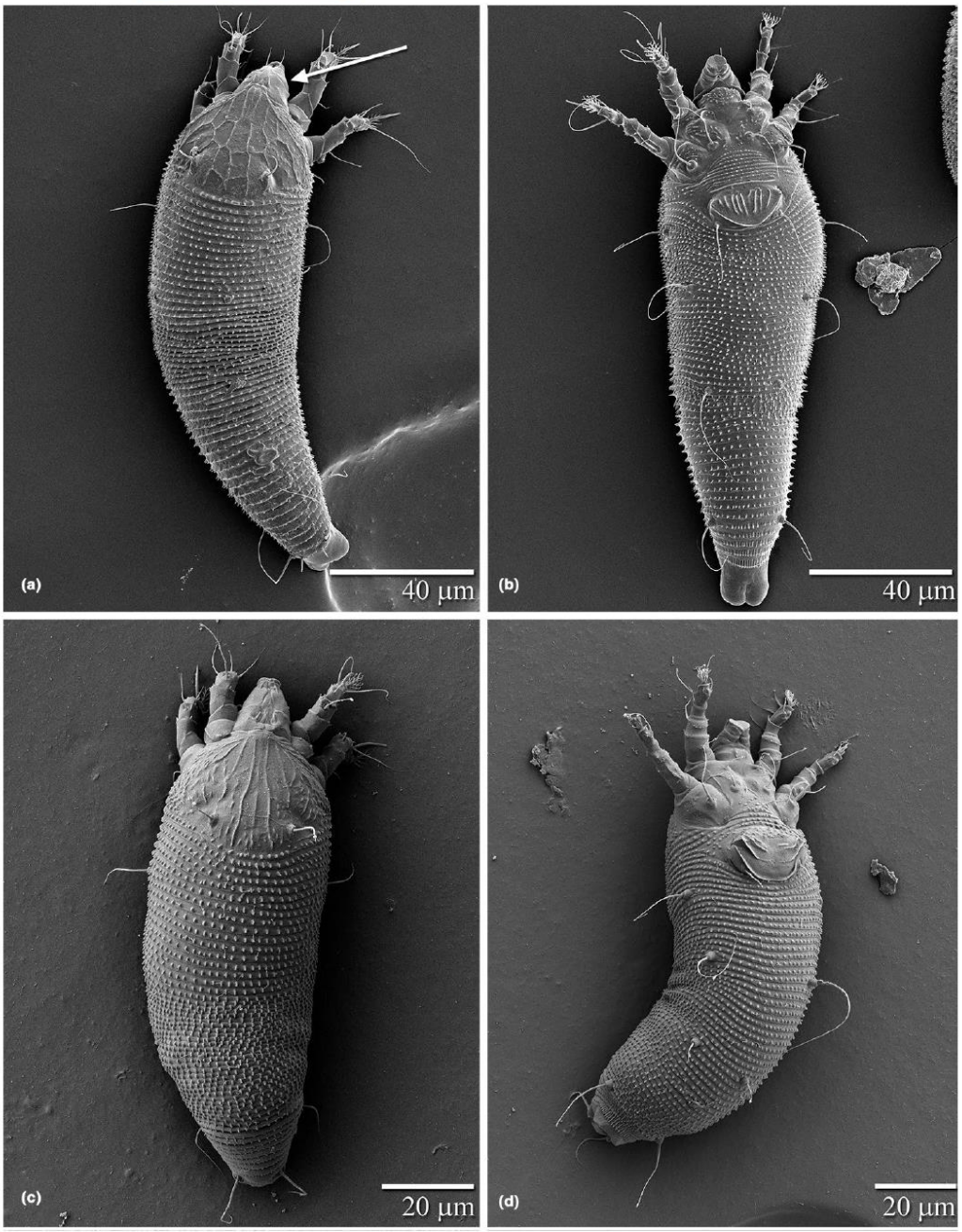

FIGURE 3 Low-temperature scanning electron microscopy images of female adults of (a, b) *Phyllocoptes fructiphilus*, (a) dorsum, (b) venter; (c, d) *Eriophyes eremus*, (c) dorsum, (d) venter. Image reproduced with the permission of USDA-ARS, Electron and Confocal Microscopy Unit, Beltsville, MD (Otero-Colina *et al.*, 2018)

shield, which is used to distinguish them, is not visible with the naked eye (Druciarek *et al.*, 2016). The identification of eriophyids is based on morphological observations, and sometimes ecological characteristics give important clues. Light and electron microscopy techniques are used to identify *P. fructiphilus* and differentiate it from other mite species. It is commonly found in the flowers, under stipules or vegetative bud scales (Otero-Colina *et al.*, 2018). Whilst *P. adalius* is similar to *P. fructiphilus*, and a significant pest in its own right, causing serious damage due to feeding, it has been shown to not be an RRD vector (Amrine, 2002). Another mite species also considered for RRV transmission is *Eriophyes eremus* (Figure 3). This eriophyid mite is also found in roses, and was first described in Israel (Druciarek and Lewandowski, 2016). *E. eremus* was found in several states of the USA in 2018, colonizing native, naturalized, and ornamental rose cultivars (Otero-Colina *et al.*, 2018), and like *P. fructiphilus*, it is also a microenvironment shelter seeking mite. Interest in *E. eremus* arose after being found in large numbers and as the only mite species feeding on a symptomatic, quantitative reverse transcription PCR (RT-qPCR)-positive plant (Solo, 2018). However, finding an *E. eremus* colony upon a rose specimen that tested positive to RRV may be circumstantial. Otero-Colina *et al.* (2018) have shown that no damage has been observed in association with this mite.

Eriophyids are small, typically between 140–170 μm, and unlike most mite species possess four, rather than eight legs. These mites are typically found in the angles formed between leaf petioles and axillary buds, feeding on plant tissues and overwintering on plants. Eriophyids are thought to survive for only 8 hr without a host. Eriophyids have a short life cycle of 8 days, and during that time can lay an egg a day (Kassar and Amrine, 1990). They do not have wings, but they can be transported by insects during pollination, dispersed by the wind, or by contact with clothing (Hong *et al.*, 2012; Byrne *et al.*, 2015). Jesse *et al.* (2006) showed roses with a higher density of leaves had a greater number of mites, because of a larger microhabitat availability, and they described a preference for sunny environments.

Currently, *P. fructiphilus* has only been described in North America, and is thought to be widely distributed in the USA on wild and commercial roses (Amrine, 2002). Although RRV has been reported in India, *P. fructiphilus* has not been detected and it is unknown if there is a vector present (Chakraborty *et al.*, 2017; EPPO, 2019). Although mite transmission is the primary mechanism for spread in the field, RRV can also be transmitted by grafting (Amrine *et al.*, 1988) and potentially by pollen (Babu *et al.*, 2017a).

4.5 | Early detection and biocontrol

The diagnosis of RRD in the early stages of infection is difficult. Symptoms are often confused with other pest problems, herbicide damage, nutrient deficiencies, or fungal infections. When glyphosate, a broad-spectrum systemic herbicide, contacts green tissue during autumn treatments, it is translocated to the buds and witches' broom symptoms with yellow leaves may appear during the following spring; this is easily confused with the rosetting caused by RRV (Hong *et al.*, 2012). Also, manure contaminated with picloram + 2,4-dichlorophenoxyacetic acid, a systemic herbicide, can also cause the same symptoms when applied around roses (Davis *et al.*, 2015).

Nevertheless, early detection is crucial, and identification and eradication of infected plants are necessary for effective control of RRD (Hong *et al.*, 2012). Pruning out parts of plants with symptoms does not eliminate the virus and should be avoided to minimize the persistence of the virus after overwintering in the root system (Di Bello *et al.*, 2017). Ideally, all multiflora roses in a 100 m radius should be removed, because they serve as a source of inoculum for RRV (Department for Environment, Food and Rural Affairs, 2016). The use of acaricides could decrease mite populations, reducing the risk of RRV dissemination. Acaricides may be useful to treat rose plants surrounding areas where RRV-infected plants have been removed (Hong *et al.*, 2012). However, it is difficult to completely eliminate mites, because eriophyids hide in inaccessible areas of the plant (Otero-Colina *et al.*, 2018).

There is no complete resistance or immunity reported in rose cultivars for RRD. Resistance to any pathogen depends on host genotype, the RRV isolate, the environment, the vector biology, and seasonality. The development of new resistant varieties is a long process that takes years. The stability of the prospective resistance is not known until later phases of testing, in which varieties are assessed in different locations within a range of environmental conditions and diversity of pathogens (Debener and Byrne, 2014). Amrine (2002) observed that rose species or varieties differ in RRV symptom expression and that there are likely to be differences in susceptibility or resistance to the virus. When a rose genotype shows resistance and robustness in a field with high RRV infestation, the molecular mechanism that makes this phenotype resistant can be studied to enable the use of resistant genetic material in breeding programmes (Byrne *et al.*, 2015, 2019). Other rose species including *R. acicularis*, *R. arkansana*, *R. blanda*, *R. californica*, *R. carolina*, *R. palustris*, *R. pisocarpa*, *R. setigera*, and *R. spinossisima* have shown elevated levels of resistance to RRV infection. *R. bracteata* and *Rosa* 'Meizeli' (the "McCartney rose") are resistant to feeding by the mite vector, although both are susceptible to the virus (Hong *et al.*, 2012). Because the RRV genome is known, there are possibilities of applying gene-editing technology in the future. Research groups in the USA are making efforts to develop RRD-resistant roses: identifying genes linked to resistance, discriminating susceptible and resistant plants to the virus and to the mite, aiming to incorporate traits into elite rose germplasm (Byrne *et al.*, 2015; Dobhal *et al.*, 2016; Roundey *et al.*, 2016).

4.6 | Diagnostic techniques

Several techniques have been developed in the last few years for detection and diagnosis of RRV. Jordan *et al.* (2018) are developing polyclonal, monoclonal and/or single-chain antibodies and associated serology-based protocols, that is, ELISA (enzyme-linked

immunosorbent assay), immunodip-stick (lateral flow), and immunocapture reverse transcription (RT)-PCR, for specific, reliable, and sensitive detection of RRV. ELISA is a versatile technique, widely used for routine virus testing in phytosanitary, quarantine, or virus certification applications (Boonham *et al*., 2014). However, ELISA requires the costly production of high-quality antisera with lack of cross reactivity to diverse pathogens and plant proteins, which may be seen as a disadvantage (Boonham *et al*., 2014) compared to nucleic acid-based methods, which are less costly to develop, more sensitive, and easier to manipulate to achieve the desired specificity (Schaad and Frederick, 2002; Arif and Ochoa-Corona, 2013; Arif *et al*., 2014). However, in the long run, once antisera are developed, it allows more throughput processing of samples and lower costs than nucleic acid-based methods.

RT-PCR is considered a sensitive and relatively rapid method for detection of RNA viruses. The first reported RRV detection method consists of an end-point RT-PCR with primers designed to amplify a fragment of RNA1 of the RRV genome (Table S1; Laney *et al*., 2011). Subsequent work showed the initial method to be inconsistent compared to other assays (Babu *et al*., 2016), which has led to the development of additional methods.

Di Bello *et al*. (2017) developed an RT-PCR assay designed in a highly conserved region of RNA3 of the RRV genome (Table S1). They proposed that RNA3 would be a better target because it codes for a nucleoprotein, so this gene would be transcribed at higher levels than the virus polymerase (RNA1). They used previously published sequences of 23 isolates available in GenBank for primer design and additional sequences from 107 isolates collected in different US states, thereby incorporating intravirus variation into the primer design. This assay was used in conjunction with primers designed to amplify the NADH dehydrogenase gene as an internal positive control in a multiplex PCR. Evaluation of the sensitivity was performed in comparison to the RT-PCR developed by Laney *et al*. (2011), and it was found to have higher sensitivity.

A different end-point RT-PCR was developed by Dobhal *et al*. (2016). The primers were designed to be compatible with two quantitative RT-PCR chemistries: TaqMan RT-PCR and SYBR Green combined with high-resolution melting (HRM) analysis, aimed at providing flexibility to diagnosticians with different resources or diagnostic preferences, because these techniques can be used with a single set of primers (Table S1). These proposed primers were designed using the nucleocapsid protein gene fragment (RNA3) of RRV as a template. The sequences of all RRV isolates available in NCBI GenBank at that time were considered. To verify the specificity of the primers, an in silico analysis was performed. Moreover, a panel of 11 reference control viruses was used for exclusivity assessment of the three techniques, and the limit of detection was determined to be 1 fg. Positive amplification was obtained with RRV-infected samples, and sequencing of the amplicon confirmed RRV was specifically amplified. The presence of phenolic compounds, carbohydrates, pigments, and other putative compounds in rose tissue were found to interfere during nucleic acid extraction (Dobhal *et al*., 2016). The use of PCR amplification facilitators bovine serum albumin (BSA) and polyvinylpyrrolidone (PVP) in the PCR mix improved amplification and helped avoid false negatives. BSA and PVP did not cross react or influence the specificity of the primer or the negative control.

Quantitative PCR (qPCR) based on TaqMan chemistry provides a greater specificity and speed compared to conventional end-point PCR for the detection of a pathogen, or a group of pathogens (Jenkins *et al*., 2002; Metzgar, 2011). In the case of RRV, both techniques have a comparable limit of detection. Babu *et al*. (2016) developed multiple primer/probe sets (Table S1) targeting three different regions of the RRV genome. Four primer/probe sets (RRV_2-1, RRV_2-2, RRV_3-2, RRV_3-5) and their corresponding product were tested in silico. Then the sensitivity (1 fg) was determined for the different assays. The specificity of the primer/probe sets in the presence/absence of other common rose-infecting viruses, and their reproducibility, was tested three times within a 30-day interval. By comparison with end-point RT-PCR, the RT-qPCR was more sensitive, detecting positive infected samples that gave negative results when using RT-PCR (Laney *et al*., 2011). In addition, positive detection of samples from different states of the USA indicated that the primer/probe sets had broad specificity.

Another TaqMan RT-qPCR assay for RRV detection was developed in 2017 at the Plant Health and Environment Laboratory (PHEL), New Zealand (author's unpublished data; Table S1). The primers and probe were designed based on the alignment of 27 RRV sequences of the nucleocapsid gene of RNA3 sourced from GenBank; the product size of the assay is 103 bp. An in silico assessment of this assay indicates that it is likely to detect all reported RRV isolates, and this is supported by results obtained showing two RRV isolates were successfully detected while samples of nontarget emaraviruses (fig mosaic virus and raspberry leaf blotch virus) and healthy rose plant tested negative. The described TaqMan RT-qPCR assay is currently the assay implemented by PHEL for RRV routine testing. Since 2018, a total of 214 rose samples have been tested for the presence of RRV, including postentry quarantine and domestic growers. RRV is not reported in New Zealand to date.

Recombinase-polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) are simplified isothermal amplification techniques (Notomi *et al*., 2000; Piepenburg *et al*., 2006). Their advantages compared to other PCR methods are: (a) the reduction of reaction time to around 20 min; (b) the reaction runs at a constant low temperature (37–42°C for RPA, 65°C for LAMP), so there is no need for thermal cycler investment, enabling the use of simpler equipment; and (c) the potential to be transferable to the field for use as a simplified screening assay (Sen and Ashbolt, 2011).

Babu *et al*. (2017b) developed a basic gel-based RT-RPA assay. The method uses three different primer sets (RPA-131, RPA-267, RPA-321) designed to detect different regions of the RRV genomic RNA (Table S1). The specificity of the three sets of primers was assessed beforehand in silico against other common rose-infecting viruses, and was found to be 1 fg/µl. The method worked well with different tissue sources (leaves, petals, and stems), and with samples from different states of the USA.

A probe-based RT-recombinase-polymerase amplification (RT-exoRPA) assay for RRV was also described by Babu *et al.* (2017a). Primers were designed in the conserved regions of RRV genomic RNA3 (Table S1; RPA-267). Analysis in silico, assessment of the specificity, and limit of detection (1 fg/µl) were undertaken to assess this primer set. The developers of this technique envisioned commercial growers and nursery personnel performing the method on-site. Thus a quick viral RNA extraction method, named direct antigen-capture, was developed, which can be completed in around 5 min and allows the use of different types of plant tissues (Babu *et al*., 2017a).

RRV was detected in pollen (anthers) of RRV-infected roses with the RT-exoRPA analysis (Babu *et al*., 2017a). This finding suggested a new potential transmission pathway of the virus; however, further research is needed to confirm the finding and its significance. Sample collection still poses questions regarding which plant parts are best for sampling. The detection of RRV from the primary and secondary roots suggests they can be a good matrix for RRV detection, where the virus could overwinter, and allows the testing of plants even in the absence of leaves, green stems, and petals (Babu *et al*., 2017a). Roots can be tested in winter, and petals and leaves with symptoms during the rest of the year. This type of sampling proved to work well at the Oklahoma State University, Microbial Forensic Laboratory (Francisco Ochoa-Corona, personal communication). However, a statistically tested sampling technique for symptomless plants is yet to be demonstrated.

LAMP primers for RRV were designed after analysis of RRV P3 and P4 gene sequences using Primer Explorer software (https://primerexplorer.jp/e/; Salazar-Aguirre *et al*., 2016; Table S1). Alignment of the P3 and P4 RRV genes allowed precise LAMP primer design for broad detection of most reported isolates up to 2016 (Salazar-Aguirre *et al.*, 2016). RRV-LAMP primers do not cross-react with cDNA reverse transcribed from 10 reference isolates of frequently coinfecting viruses in rose or RRV-related viruses: high plains wheat mosaic virus, maize stripe virus, impatiens necrotic spot virus, tomato spotted wilt virus, groundnut ringspot virus, ApMV, ArMV, PNRSV, tobacco ringspot virus, and tobacco mosaic virus. LAMP for RRV was tested successfully using tissue samples of RRD-infected roses with and without symptoms from Oklahoma. Healthy tissue and nontemplate controls were included in all reactions.

High-throughput sequencing (HTS) has revolutionized diagnostics since 2009 (Adams *et al*., 2009; Al Rwahnih *et al*., 2009; Kreuze *et al*., 2009). HTS offers the possibility of generic detection of viruses and other pathogens (Boonham *et al*., 2014), and allows a generic approach to virus identification that does not require previous knowledge of the targeted pathogens. HTS can deliver a species/strain-specific result (Adams and Fox, 2016). HTS continues to evolve, and different platforms and sample preparation methods have been developed (Pecman *et al*., 2017). A novel bioinformatic pipeline called electronic diagnostic nucleic acid analysis (EDNA) is being developed for the detection and diagnosis of 24 reported viruses infecting rose worldwide (Peña-Zuñiga *et al*., 2017). This computational tool combines HTS and bioinformatics, minimizes and ignores nonrelevant sequence data, and focuses on predetermined specific pathogen-associated sequences. It enables the detection of multiple viruses in a single sample or run (Figure 4) of either Illumina or Oxford Nanopore MinION raw metagenomic outputs.

4.7 | Potential entry pathways to Europe

There are several potential entry pathways into the EU for RRV and its vector. Roses for planting are imported from different countries (as dormant plants free from leaves), including India and the USA. Although the percentage of imported plants from these countries is not high, the risk is elevated because 2,000 kg of roses are imported to Europe yearly from countries where this virus is present. Details about the rose species and varieties imported are unknown. *R. multiflora* is a regulated plant species in 13 US states, where its importation, distribution, trade, and sale have been banned (New York Invasive Species, 2019). Only dormant *Rosa* plants free from leaves, flowers, and fruit can be imported into the EU from non-European countries. However, the risk of RRV introduction and its vector persist because both can survive on dormant plants (EPPO, 2018). Thus, RRV has been regulated in the EU since November 2019, and roses imported from the USA, Canada, and India need to follow specific measures to avoid the introduction of RRV and *P. fructiphilus* (Andriukaitis, 2019). Moreover, roses may be imported illegally through internet trading or smuggling (Tuffen, 2016).

If RRV-infected plants were imported without the vector, the virus would be limited to that plant, except if used for propagation. Nevertheless, as reported, all plants showing symptoms of RRD are generally infested by *P. fructiphilus* (Otero-Colina *et al*., 2018) if imported from North America. The presence of just one female will be enough to initiate a population. In the case of introduction of nymph and adult stages of the vector, adults could be dispersed by wind or by other media, spreading the infection (Tuffen, 2016).

Other possible but less likely pathways are by natural spread or by the rosehip trade. The countries with the presence of RRV and *P. fructiphilus* are far from Europe, so vector transmission by wind is unlikely. Rosehips are generally used for domestic consumption therefore are unlikely to act as a pathway to the wider environment. The spreading of RRV by pollen needs to be further assessed by research (Babu *et al.*, 2017a).

4.8 | Cut flowers: a risk?

Rose rosette virus has not been reported infecting cut rose varieties yet, though it is highly probable they are susceptible. There are no-commercially available resistant or tolerant species. The possibility of finding flowers with symptoms in the market is low, because they would probably be graded out due to quality issues. Nevertheless, flowers could be taken from symptomless parts of an infected plant. The quality standards are high for cut roses, and under controlled conditions the use of agrochemicals could reduce the mite population.

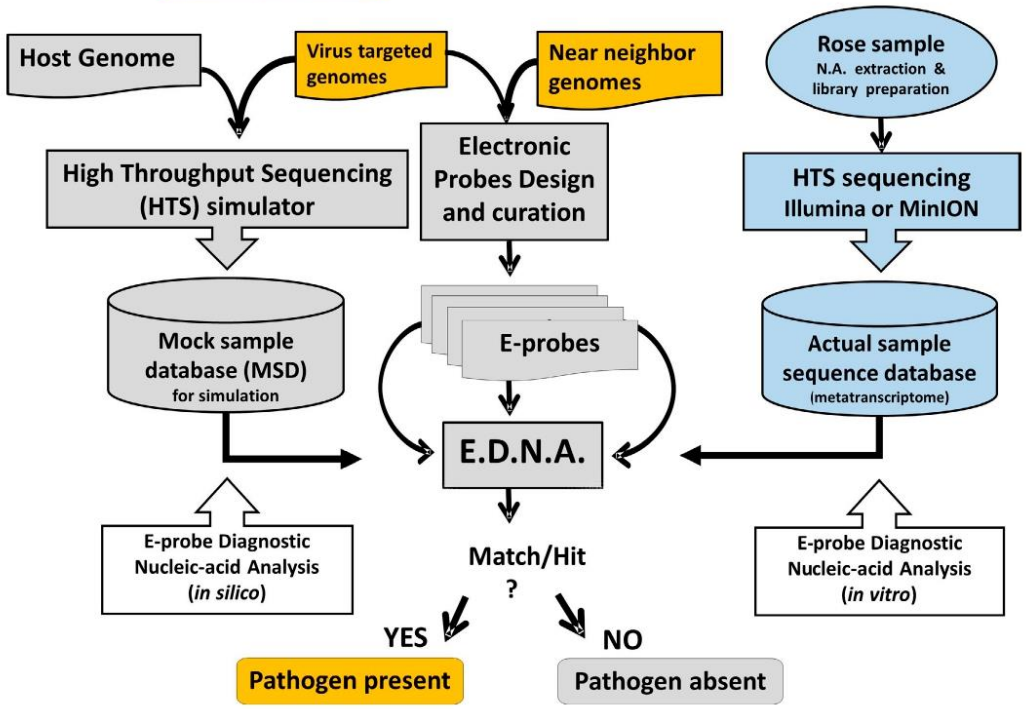

FIGURE 4 Flow chart representation of the electronic diagnostic nucleic acid analysis (EDNA) pipeline, showing the in silico development stage (left-top) where databases of rose genomic sequences (host), and sequences of rose-infecting viruses and related viruses are built. Subsequently, e-probes are designed and curated for specificity and high-throughput sequencing (HTS). Mock or simulated samples are also generated for simulation of pretests in silico (left-centre). The in vitro and routine diagnostic stage (right) corresponds to the actual in vitro HTS assay, to include sample processing, nucleic acid extraction, and library preparation, followed by actual sample HTS using either Illumina or MinION platforms (right-centre). The obtained output database is screened with EDNA (centre-bottom). Electronic probe hits determine virus detection

The EU is a significant importer of fresh cut roses. This fact increases the risk of entrance of any exotic pathogen if phytosanitary measures are not effective. During the first 10 months of 2017, rose imports into the EU were valued at €624 million (£507 million), 10 times more than the value of exported roses to non-EU countries. According to Eurostat (2019), the Netherlands was the top EU exporter of cut flowers (70% of the total extra-EU exports of roses), as it is a major producer in Europe and receives cut flowers from other producer countries to redistribute them to the European market. After the Netherlands, other key exporters in Europe are Lithuania (11%), Germany (8%), and Latvia (7%). The Netherlands was also the top importer of fresh cut roses from outside the EU (77% of the total EU imports of roses). Other major importers were the UK (10%), Germany (6%), and Spain (5%). There is a minor trade of cut flowers with the USA (200 kg in 2016; Eurostat, 2019), and there is no trade with Canada. The trade with India increased from 300,000 to 900,000 kg in 2012–2016, mainly exported to the Netherlands and the UK (EPPO, 2018).

The possibility of RRV being introduced by cut flowers is unlikely, though not impossible if infected plants and vectors were found in an exporters production site. Cut flower shelf-life is around 2 weeks. Cut flowers are mostly used indoors, which reduces the risk of mites moving outdoors to transmit the virus in gardens. However, when the cut flowers are disposed of outdoors, for example in compost, mites may still be able to reach garden roses and transmit the virus.

4.9 | RRV impact on the US industry and environment

Rose rosette virus has led to a significant decline of garden roses and urban landscapes of cities in the USA (Laney *et al.*, 2011). The outbreak of RRV has been particularly evident in Tulsa and Oklahoma City (Oklahoma) and has affected the rose industry in other states (authors' personal observations). RRV infects randomly in the field with other viruses, creating new combinations of mixed infections

in a large number of rose varieties and hybrids (Peña-Zuñiga *et al*., 2017), and threatens to decimate the US rose industry (Byrne *et al*., 2015).

In the USA about 35% of the rose sales are specifically used by the landscape industry. Recently, this market has reduced the use of roses by about 10% per year due to RRV and associated virus complexes. There are approximately 2,000 businesses that produce garden roses to sell in the USA. These growers produced 36.6 million garden-rose bushes in 2014 generating sales worth $203.5 million (£165.5 million), creating approximately $777 million (£632 million) for the US economy (Pemberton *et al*., 2018). The overall losses caused by RRV to present are being estimated and the official magnitude of the economic loss caused by the RRD is yet to be determined (communication with rose stakeholders at technical meetings).

4.10 | Potential impact in Europe

For RRV introduction and establishment in Europe, it will require the introduction of its vector *P. fructiphilus*. The economic impact is expected to be high. Breeders, nurseries, retailers of garden and pot roses, and landscapes would be affected. Rose plants with symptoms would be unmarketable and eradication measures, which include destruction of plants in a range of 100 m even if they remain symptomless, will damage the economy of this sector (EPPO, 2018). The cost associated with replacement of rose plants in private and public landscaping will be high and the rose industry will be seriously affected by the introduction of alternative ornamentals into both the garden and landscape industry.

Bulgaria and Turkey are the largest producers of rose oil worldwide, which relies primarily on species like *R. damascena*, which is reported to be an RRV host (EPPO, 2018). In Bulgaria, the rose oil industry provides labour for c. 65,000 people, mostly seasonal workers (Kovacheva *et al*., 2010). In Turkey, 8,200 families grew oil roses in 2005 (Gunes, 2005).

The environment is also expected to be affected by RRV. In Europe there are several wild species known to be susceptible to the virus, for example *R. canina* and *R. rubiginosa* (EPPO, 2018). Roses are used for hedges, game cover, slope stabilization, and erosion control. Invertebrates that rely on *Rosa* spp. would also be affected, for example the gall-forming wasp *Diplolepis spinosissimae*. This insect causes the so-called robin's pincushion. A negative impact on pollinators is expected, as there are species that feed on roses. Pollinators have alternative sources available, but some have a specific relationship with these plants (Tuffen, 2016).

The introduction of RRV to Europe would also cause serious social impact, from affecting the mental and physical health benefits associated with gardening (Soga *et al*., 2017) to the loss of employment and income in the nursery industry and other associated sectors such as tourism, which rely heavily on public gardens and attractive urban landscapes. The availability of rose products with cultural importance like jam, rosehips, rose water, rose petals, or flower buds is likely to be reduced.

Rose germplasm repositories and unique European rose germplasm collections will be threatened, such as the "Europa-Rosarium Sangerhausen" (Germany), which is the largest rose collection in the world and plays an important role as a source of budwood and support for research.

5 | CONCLUSIONS

Roses have a significant cultural value for a number of European countries (EPPO, 2018), and is a valuable flower crop worldwide affected by a range of pathogens. RRV is a devastating mite-transmitted virus that could potentially be introduced into Europe. The first finding of RRV outside North America has triggered interest and raised concern. The introduction pathway for RRV to India remains unknown. In view of the intercontinental distance between RRV-infected countries and Europe, the virus or the vector is unlikely to be introduced by natural spread, but other pathways of entry are possible.

Creating awareness plays a critical role in preventing RRV establishment. Thus, European governments should inform stakeholders and interested parties, including members of the public, about this virus, the disease that it causes, and the economic consequences. Simple tips to follow include spacing of plants to prevent mites crawling from plant to plant or implementing good hygiene measures to avoid spread (e.g., clean equipment before pruning, cleaning clothes). Breeders, nurseries, and botanic gardens should be informed and made aware about RRV and routine checks should be performed during the year. In the event of an outbreak, a prompt notification to the authorities to allow a regulatory response must be quick.

Controls within rose trading countries are key to prevent the introduction of RRV. Early detection and surveillance programmes are necessary, because plants can have long latent periods, during which the mite vector can spread the virus. Regular inspection throughout the growing season with destruction of plants with symptoms appears to be the most effective control measure for RRV at present. Visual diagnosis of RRV requires serological or molecular confirmation during the early stages of infection (Figure 5). Several diagnostic methods have been developed and incorporating these into early detection strategies is essential to intercept the virus and vector before it is able to establish. The different diagnostic methods available enable techniques to be chosen depending on the resources available for each laboratory (Babu *et al*., 2018). All the techniques available are useful for detecting an outbreak, within the limit of detection and capacities of the assay. RT-qPCR is a good option, due to its high sensitivity compared with RT-PCR or ELISA. HTS has a potential as a front-line diagnostic tool, in particular for screening multiple virus infections in propagation material, but further research work is in process. RRV testing must be rapid to target symptomless infections because these are common. In the case of an outbreak, an eradication and tracing programme should be followed. First, suspect plants are to be

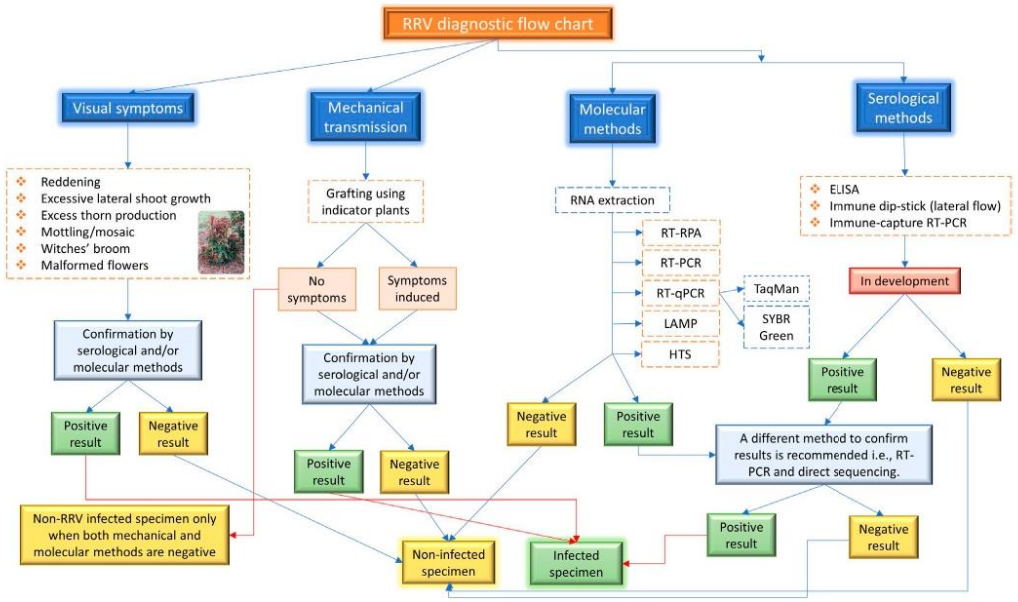

FIGURE 5 Flow chart representation of a purposed decision scheme for detection and the identification of *Rose rosette virus*

tested to confirm the presence of RRV, and if positive, infested and adjacent plants (including the roots) are to be destroyed. Inspect for presence of the vector and forbid movement of rose plants from the site of the RRV outbreak. Precautions should be taken to avoid spreading the vector during the response (e.g., bagging plants before any manipulation to avoid dispersing the vector). In addition, nearby host plants must be treated with acaricides. If occurring in a glasshouse, the whole glasshouse should be disinfested (EPPO, 2018).

Following an outbreak, delimiting surveys of the area surrounding the infected plants are needed, including visual surveys. Trace-forward and -back analysis should be conducted to identify possible areas where infected plants might be present. Surveys of *Rosa* plants in late spring and summer should be performed each year and for at least 2 years after the outbreak of the first infection due to the long incubation period of RRV. Similar surveys should be carried out for vector infestations before declaring the outbreak eradicated. No *Rosa* spp. should be moved out of risk demarcated areas until the eradication is declared successful (EPPO, 2018).

Another measure to limit the spread of RRV is to import roses from an RRV and *P. fructiphilus* pest-free area (PFA), as England and Wales (UK) have now declared (Department for Environment, Food and Rural Affairs, 2019). Other considerations should be taken, for example the packing conditions to prevent infestation by *P. fructiphilus* during transport, and pre- or postentry quarantine period for at least one growing season. This should include visual inspection for RRV and *P. fructiphilus* and molecular testing for RRV.

5.1 | Future directions

Further research is needed to identify other possible RRV vectors or transmission pathways, as well as to improve understanding of RRV variability and diversity. The susceptibility of cut rose varieties needs to be assessed, as they could play an important role in the spread of RRV and its vector if infection can occur. Resistant varieties adapted to European hardiness zones need to be developed and released to reduce the impact and spread of RRV in advance. An effective educational programme is required to inform the general public and create awareness regarding RRV, all of which will help to develop a quick response in case of an RRV outbreak.

ACKNOWLEDGEMENTS

We thank Dr Gary R. Bauchan for sharing the scanning electron microscopy images of *Phyllocoptes fructiphilus* and *Eriophyes eremus*, and Dr Alan McLeod and Dr Melanie Tuffen for their help with the use of Eurostat.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Vazquez-Iglesias I, Ochoa-Corona FM, Tang J, et al. Facing *Rose rosette virus*: A risk to European rose cultivation. *Plant Pathol*. 2020;00:1–15. https://doi.org/10.1111/ppa.13255

B.2 High Throughput Sequencing and RT-qPCR assay Reveal the Presence of Rose Cryptic Virus-1 in the United Kingdom.

Journal of Plant Pathology
https://doi.org/10.1007/s42161-019-00307-5

SHORT COMMUNICATION

High throughput sequencing and RT-qPCR assay reveal the presence of rose cryptic virus-1 in the United Kingdom

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Received: 8 November 2018 / Accepted: 6 April 2019

Abstract
Rose cryptic virus-1 (RoCV1) also known as *Rosa multiflora* cryptic virus is a partitivirus affecting roses, one of the most important ornamental crops worldwide. RoCV1 has previously been reported in the US, Canada and New Zealand, and has now been identified in the United Kingdom for the first time. Using High Throughput Sequencing (HTS) RoCV1 sequences were found in two samples collected in 2007 and 2012. This discovery led to the development of a RT-qPCR (TaqMan) assay for the detection of this virus. As part of a rose virus survey in the UK, 251 samples were analysed using the newly developed RoCV1 RT-qPCR test, following ELISA analysis for other common rose viruses. The results of the RT-qPCR test were confirmed using published conventional PCR primers and Sanger sequencing of amplified products. Results suggest that RoCV1 could have been infecting roses in the UK since at least 2007, with a large number of recently collected samples (43%) found to be infected. Cryptoviruses are not thought to cause direct economic losses in their plant host, although it is not clear what impact they might have in mixed infections.

Keywords RoCV1 · TaqMan · HTS · *Rosa* · Diagnostic methods

Roses are one of the most important ornamental crops worldwide, they are grown for their desirable aesthetics and scent for gardens and landscaping, and their use in industrial products (Dobhal et al. 2016). The garden industry contributes £9 billion to the UK economy every year. The UK's Department for Environment, Food & Rural Affairs (Defra) valued ornamental plant production at £1.1 billion in 2015, with an estimated disease loss of £630 million within UK ornamental plant production of which £40 million could be attributed specifically to viral diseases (Department for Environment 2016; Little 2016).

A number of viruses have been reported affecting roses in the UK including arabis mosaic virus (ArMV), prunus necrotic ringspot virus (PNRSV) and strawberry latent ringspot virus (SLRSV) (Thomas 1984). Rose mosaic disease, one of the most common diseases in roses, is caused by single or mixed infections of these viruses, and others such as apple mosaic virus (ApMV). Symptoms include chlorotic line patterns, ring spots, mottles in leaves, yellow net and yellow mosaic. Infected plants are less vigorous and more likely to die during winter (Horst et al. 1983).

Rose cryptic virus-1 (RoCV1) also known as *Rosa multiflora* cryptic virus (Martin and Tzanetakis 2008) is a partitivirus, related to other species in the genus *Alphacryptovirus*, but not yet classified within this genus (King et al. 2012).

Partitivirus genomes are generally composed of two monocistronic dsRNA segments of 1.4–3.0 kbp, which are encapsidated in isometric particles about 30–40 nm in diameter. The larger RNA encodes a viral RNA-dependent RNA polymerase (RdRp), and the smaller one encodes a coat

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42161-019-00307-5) contains supplementary material, which is available to authorized users.

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Published online: 06 May 2019

protein (CP) (King et al. 2012). However, RoCV1 has been shown to contain a third dsRNA segment (Sabanadzovic and Abou Ghanem-Sabanadzovic 2008), the origin of which is not clear, though several hypotheses have been published: (i) it may represent a co-infection by two different partitiviruses, (ii) the virus may possess two different versions of dsRNA2, or (iii) the third dsRNA element may represent a satellite virus (Nibert et al. 2014).

Cryptoviruses are reported to be widespread in nature and have escaped detection for many years because most of them cause no visible symptoms or, in a few situations, very mild symptoms (Hull 2014). RoCV1 has been found in asymptomatic plants as well as plants with mottling, leaf spots and necrosis (Milleza et al. 2013), as well as in mixed infections with rose spring dwarf-associated virus (Salem et al. 2008) or rose rosette emaravirus (Martin and Tzanetakis 2008).

In common with other partitiviruses there are no known natural vectors for RoCV1, it cannot move from cell-to-cell but can be transmitted by pollen and seed (Boccardo et al. 1987).

It was first reported in the US and it is thought to be widespread there, being reported from several locations (Martin and Tzanetakis 2008; Sabanadzovic and Abou Ghanem-Sabanadzovic 2008; Lockhart et al. 2011). Subsequently it has been reported in New Zealand, being the most prevalent virus infecting roses (Milleza et al. 2013), and in Canada (James et al. 2015). This is the first report describing the detection of RoCV1 in rose in the United Kingdom.

Rose samples were submitted to the plant clinic at Fera Science Ltd. (York, UK) in 2007 (LS11S16) and 2012 (LS13S9), suspected of having a viral infection based on symptoms: distortion on new growth, and wrinkled and streaked leaves. ELISA analysis was performed for a range of viruses using commercially available kits following the manufacturer's instructions, as follows: arabis mosaic virus (ArMV; Leibniz Institute DSMZ GmbH, Germany); prunus necrotic ringspot virus (PNRSV; Loewe Biochemica GmbH, Germany); apple mosaic virus (ApMV; Loewe Biochemica GmbH, Germany); strawberry latent ringspot virus (SLRSV; BIOREBA AG, Switzerland); and impatiens necrotic spot virus (INSV; BIOREBA AG, Switzerland). In each case a negative control corresponding to the same host plant species as the test sample was used. The result was considered positive when the optical density (OD) value at 405 nm after 1 h for a given sample was greater than 3× the mean OD value of the corresponding negative control. Each sample was tested in duplicate. Sample LS11S16 gave a positive result for ApMV and sample LS13S9 gave a positive result for SLRSV.

Subsequently, High Throughput Sequencing (HTS) using nucleic acid extracted from samples LS11S16 and LS13S9 was performed. Ribosome depleted indexed sequencing libraries were prepared from extracted RNA using the ScriptSeq complete plant leaf kit (Illumina, USA) which were then sequenced on an Illumina MiSeq instrument using a V3, 2 × 300 cycle run kit (Illumina, USA). Sequence reads were trimmed for quality using Sickle (Najoshi 2011), assembled using Trinity (Grabherr et al. 2011) and then compared to the GenBank nr and nt databases using BLAST+ (Camacho et al. 2009). Reads of viral origin were inspected using MEGAN community edition (Huson et al. 2016). Sequences of RoCV1 were detected in both samples: for LS11S16, the total number of reads was 654,746 and 1893 mapped to RoCV1 (GenBank Accession Nos. MK075821; MK075822; MK075823; MK075824; MK075825). For LS13S9, the total number of reads was 317,946 and 62 mapped to RoCV1 (MK075826; MK075827; MK075828).

As part of a survey of rose viruses in the UK, 251 leaf samples were collected in autumn 2017 and spring/early summer 2018 from roses in the Royal Horticultural Society gardens at Harlow Carr (Harrogate, North Yorkshire, UK) and Wisley (Woking, Surrey, UK). In addition, samples of rose leaves were taken from a nursery in the Midlands, a public garden near London, the Royal National Rose Society Garden (St Albans, UK) and from samples submitted to the plant clinic at Fera Science Ltd. (York, UK).

ELISA analysis was performed for the same viruses as described above, and also tested for the following: alfalfa mosaic virus (AMV; BIOREBA AG, Switzerland); raspberry ringspot virus (RpRSV; Leibniz Institute DSMZ GmbH, Germany); cucumber mosaic virus (CMV; Agdia Inc., US); tomato spotted wilt virus (TSWV; Leibniz Institute DSMZ GmbH, Germany); and tobacco ringspot virus (TRSV; Agdia Inc., US).

ELISA results showed that six samples were infected with SLRSV (2.4%); ten samples were infected with ArMV (4%); and three samples with both viruses (1.2%).

To further investigate the incidence of RoCV1 in the UK, primers and a probe for the specific detection of RoCV1 by RT-qPCR (TaqMan) were designed (RoCV1–2-Fw-5′-TGATCGACCAAAGTTGCAACC-3′/RoCV1–2-Rv-5′-GAAGATAAGACAATGCAGTCA CTTTCTT-3′/RoCV1–2-Pe-5′-FAM-ATTCGGACTGAATTTGCTA-MGBNFQ-3′) using Primer Express 3.0.1 (Applied Biosystems) in regions conserved within the RoCV1 genome (dsRNA1 segment) and divergent from other species. Sequences used for assay design included those generated using HTS, and sequences obtained from GenBank® of target and non-target viruses (NC_010346; KM598758.1; JX492318.1; EU413666.1; EU350962.1; EU024675.1; DQ093961.2).

Samples collected for the survey were analysed using the new assay. Nucleic acid was extracted from the leaf samples using a magnetic bead-based extraction method and the

KingFisher® mL platform (Thermo Scientific). Nucleic acid samples were also tested using an RT-qPCR simplex assay designed to the plant COX (Weller et al. 2000) and subsequently modified (Hughes et al. 2006). RT-qPCR was performed on previously extracted RNA in 96 well plates on either an ABI Prism 7500 or Viia7 (Applied Biosystems). Reactions contained iTaq One-step (2x), iScript™ reverse transcriptase (Bio Rad), 300 nM of each primer (RoCV1–2-Fw and RoCV1–2 Rv), 100 nM of probe (RoCV1–2-Pe) and 1 μl of extracted RNA (concentration as extracted) in a final reaction volume of 20 μl. The cycling conditions used were: 10 min at 50 °C, 2 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Negative controls where water replaced template were included in all runs and all samples were tested in duplicate. Results were scored positive if a C_T value <40 was recorded.

Previously published primers for the dsRNA2 segment (Sabanadzovic and Abou Ghanem-Sabanadzovic 2008) were used to confirm the results obtained by RT-qPCR. A two-step RT-PCR was performed on a single sample, 7B3, which tested positive for RoCV1 using RT-qPCR. Generation of cDNA was performed using SuperScript™ II Reverse Transcriptase (Thermo Scientific) following the manufacturer's instructions. cDNA (10% by volume) was added in a total volume of 30 μl, containing dNTP mix, 5x Phusion ® HF Buffer, Thermo Scientific® Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) and primers (300 nM**).** Cycling was done using a C1000™ Thermal Cycler (Bio-Rad) as follows: 30 min at 40 °C, 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C, a final extension step of 72 °C for 10 min was included.

Using agarose gel electrophoresis, a PCR product of the expected size (610 bp) was obtained and PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen, Germany), following the manufacturer's instructions before being sequenced (Eurofins Genomics, Germany) using both PCR primers (GenBank Accession No. MK075829).

The resulting sequence was compared to published nucleic acid data (99% identity; GenBank Accession Nos. KM598759.1; EU024677.1; EU350963.1; EU413667.1) and amino acid data (89–99% identity; ABY60413.1; ABV89764.1; YP_001686787.1), confirming that the sample 7B3 was infected with RoCV1.

The remaining samples were then tested for RoCV1 using the RT-qPCR, the results showed 43% (75 samples of 251, including 7B3) were positive (C_T between 17.4 and 38.4). Of these, two samples (0.8%) were found to be co-infected with RoCV1 and SLRSV, eight (3.2%) with RoCV1 and ArMV and two (0.8%) with all three viruses. All the samples tested positive with the plant COX assay previously, confirming the success of the nucleic acid extraction.

Following conventional PCR, products from three RoCV1 positive samples (4B3, 13A3, 16B1) were sent for Sanger sequencing (GenBank Accession No. MK075830; MK075831; MK075832). Comparison of the nucleic acid sequences with published data confirmed the presence of RoCV1 with a 95%–100% identity.

Furthermore, eight samples (11C, 16A1, 19A3, 21A1, 69A2, 74A2, 89 and 99) with high Ct values for RT-qPCR were analysed by conventional PCR. For three of these samples PCR gave a band of the expected size (610 bp) while the remaining gave negative results. To investigate if these samples were false-positive results following RT-qPCR or false negatives following testing using conventional PCR, the DNA amplified using the RT-qPCR from the eight samples were cloned using a pGEM®-T Easy Vector System (Promega), following the manufacturer's recommended protocols. Ligation of the insert was performed, bacterial cultures grown overnight after transformation, clones containing inserts were identified, and plasmid DNA was purified. Following sequencing of the plasmid inserts, comparisons were made with published sequence for RoCV1. The results showed that the sequence of all inserts have a high sequence identity (80–100%) to GenBank accessions KM598758.1; JX492318.1; EU413666.1; EU024675.1; AFQ36917.1; YP_001686786.1 and ABV89762.1. The results led us to conclude that the samples were infected, giving positive results with the RT-qPCR and negative results with the conventional RT-PCR most likely due to lower sensitivity.

ELISA analysis of the samples in the UK rose survey demonstrated the presence of SLRSV and ArMV, both previously described in the UK (Thomas 1980). Whilst the symptoms were frequently as described in the literature, SLRSV-infected plants (Fig. 1a) and plants with mixed infections (SLRSV + ArMV) display a chlorotic mosaic whilst ArMV-infected plants have yellow vein clearing symptoms (Fig. 1b). Nevertheless, not all the samples followed this pattern, some samples infected with ArMV had chlorotic mosaic symptoms typical of SLRSV infections. Further analysis is required to confirm these results.

Based on the provenance of samples, these results suggest that RoCV1 has been in the UK at least since 2007. Following the genus demarcation criteria from the International Committee on Taxonomy of Viruses (Vainio et al. 2018), < 24% amino acid sequence identity within the RdRP gene indicates a new species. Analysis of the sequence of samples LS11S16 and LS13S9, show that the amino acid sequences for RNA1 (RdRP protein) share between 98.85–100% identity with published sequences.

RoCV1 coat protein nucleic acid sequences (dsRNA2) from this work (GenBank Accession No. MK075822; MK075824; MK075827; MK075829; MK075830;

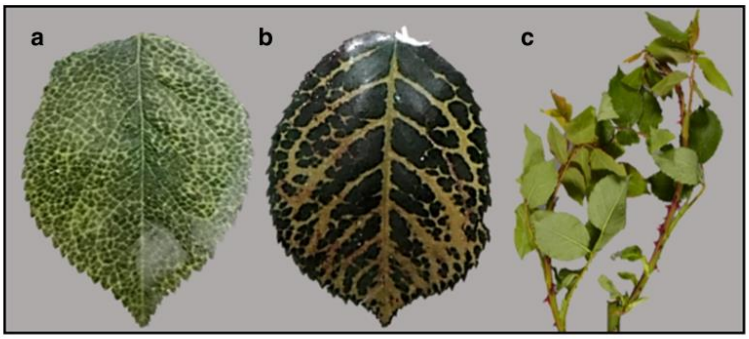

Fig. 1 Rose leaves showing rose mosaic disease symptoms, infected with (**a**) SLRSV and RoCV1 (**b**) ArMV and RoCV1 and (**c**) an asymptomatic rose stem infected with RoCV1

MK075831; MK075832) showed little difference (<0.5%) between isolates. For samples LS11S16 and LS13S9 dsRNA 3 was detected (MK075823; MK075825; MK075828). Comparison of the nucleic acid sequence showed a 99% of identity with published sequences.

RoCV1 has been reported in the US (Martin and Tzanetakis 2008; Sabanadzovic and Abou Ghanem-Sabanadzovic 2008; Lockhart et al. 2011), Canada (James et al. 2015), and New Zealand where it was the most prevalent virus in roses with a 48% incidence (Milleza et al. 2013). By using HTS we have shown the presence of RoCV1 in the UK for the first time, follow on testing using RT-qPCR (TaqMan) has shown that a large percentage (43%) of the samples tested were infected with RoCV1. There is no known natural vector for RoCV1; it is assumed to be pollen and seed transmitted like other cryptoviruses. Therefore, it is probable that RoCV1 has spread between the US, New Zealand, Canada and the UK during commercial trade of planting material.

In New Zealand, infected roses were associated with mottling, leaf spots and necrosis and also one with flower break but it was also found in asymptomatic samples (Milleza et al. 2013). In this study, several samples were asymptomatic, others had vein banding, mottling or leaf distortion symptoms. It is possible that these symptoms could be caused by another, as yet unidentified virus (or pathogen), by herbicide damage or adverse cultural conditions.

A sample with symptoms similar to those caused by rose rosette virus was positive for RoCV1; further studies will be undertaken to investigate if those symptoms were due to mixed infections with other, as yet undescribed viruses. We also found roses with symptoms of rose mosaic disease which were infected with RoCV1, where ELISA analysis indicated that they were co-infected with ArMV, SLRSV or all three viruses. It is unknown whether the symptoms caused by ArMV or SLRSV were impacted by co-infection with RoCV1, although we found samples with mosaic symptoms which were not infected with RoCV1. Cryptoviruses are not thought to cause direct economic losses in their plant host, although it is not clear what impact they have in mixed infections.

Acknowledgements This work was carried out as part of a joint studentship funded by the Fera Science Ltd.- Department for Environment, Food and Rural Affairs (DEFRA) long-term service agreement (LTSA), the Royal Horticultural Society, and Newcastle University.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. It is original and has not been submitted or published elsewhere.

Informed consent Informed consent was obtained from all individual participants included in the study. All the authors have seen and approved the manuscript, and all have taken a valid role through either study design, data generation or manuscript preparation.

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B.3 First Report of Rose Spring Dwarf-associated Virus in *Rosa* spp. in United Kingdom.

New Disease Reports **(2020) 42, 13. http://dx.doi.org/10.5197/j.2044-0588.2020.042.013**

New Disease Reports

First report of Rose spring dwarf-associated virus in *Rosa* spp. in United Kingdom

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Received: *28 Sep 2020.* ***Published:*** *13 Oct 2020.* ***Keywords:*** *plant diagnostics, HTS*

In July 2019, a sample of *Rosa* spp.was submitted to Fera Science Ltd. via the Royal Horticultural Society (RHS) gardening advice service. The sample (ID220) was sent in following the appearance of unknown symptoms including mottling, yellow/white patching, thin texture and a pink colour in the leaves.

RNA was extracted using a CTAB method adapted from Adams *et al.* (2009), with the 4M LiCl incubation performed overnight at 4°C. The sample was tested for common rose viruses using RT-qPCR (Table 1). A positive result was achieved for Rose cryptic virus 1. Subsequently, the sample was analysed by high throughput sequencing (HTS) using a TruSeq Stranded Total RNA Library Prep Plant kit (Illumina Inc., USA) for library preparation. A MiSeq instrument and a MiSeq Reagent Kit v3 (600-cycle) (Illumina Inc.) were used to run the library. The run generated 569,452 reads for the sample, and data was analysed as described by Fox *et al.* (2019). Three fragments of rose spring dwarf-associated virus (RSDaV) were identified (234, 251 and 229 bp; GenBank Accession Nos. MT993839-MT993841). A BLAST+ search found sequences with high sequence identity in both nucleotide (92.11-93.59% identity, EU024678.1) and amino acid comparisons (94.34-100%, YP_001949737.1; YP_001949736.1; YP_001949738.1). RT-PCR amplification using specific primers (Salem *et al.*, 2008) was performed to confirm the result, and a product of the expected size (418 bp) was obtained.

To assess the spread of RSDaV in the UK, 171 roses were analysed using the RT-PCR assay. Samples were collected as part of a survey of rose viruses in the UK and both asymptomatic and symptomatic leaf samples, consistent with virus infections symptoms (mottling, yellow veining, distortion, and ringspots) were included. Only one sample (ID140) resulted positive for RSDaV, and no symptoms were identified. Previous analysis showed this sample was positive for *Arabis mosaic virus* by ELISA and RT-qPCR.

The RT-PCR product (418 bp) from both RSDaV-positive samples (ID220, 140) were sequenced, and nucleotide comparisons showed a 98.51-99.02% identity with sequences in GenBank (HM236366.1; HM2363641; HM236362.1; HM236364.1). Amino acid comparison showed a 98.51-100% identity with previously published sequences (ADK78852.1; ADK78851.1).

RSDaV has previously been found in the USA (Salem *et al.*, 2008), Chile (Rivera & Engel, 2010), and New Zealand (Milleza *et al.*, 2013). This is the first report of RSDaV in Europe. Further samples (4) were submitted to the RHS and Fera Science Ltd. Plant Clinic, showing the previously described unknown symptoms. They were tested by RT-PCR and also sequenced by HTS and tested negative for RSDaV. The cause of these symptoms is not believed to be of viral origin.

Acknowledgements

Authors would like to thank Dr Maher Al Rwahnih for providing a positive control. The work was funded through the Defra-Fera Long Term Service Agreement, the RHS and Newcastle University.

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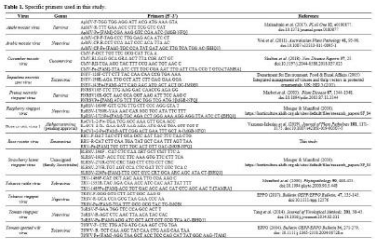

Figure 1

To cite this report: Vazquez-Iglesias , Scrace J, McGreig S, Pufal H, Robinson R, Clover GRG, Adams IP, Boonham N, Fox A, 2020. First report of Rose spring dwarf-associated virus in *Rosa* spp. in United Kingdom. *New Disease Reports* **42**, 13. http://dx.doi.org/10.5197/j.2044-0588.2020.042.013

 This report was published on-line at www.ndrs.org.uk where high quality versions of the figures can be found.

Appendix C Supplementary Material of Included Publications

C.1 Facing *Rose rosette virus:* A Risk to European Rose Cultivation

Table C.1- Primers and probes sequences designed for different diagnostic techniques available for rose rosette virus (RRV).

Code	Name	Diagnostic method	Sequence (5′-3′)	Fragment	Amplicon (bp)	Length (bp)	Tm	GC%	Any[a]	3′[b]	Reference
RRV	RRVFor	RT-PCR	CAGAATGAACCATAGATGTC	RNA1	375	20	51.05	40.00	4.00	2.00	Laney *et al*. (2011)
	RRVRev		AATGGTCTGCTCGAGATT			18	53.15	44.44	6.00	1.00	
RRV2	RRV2F	RT-PCRA and RT-qPCR	TGCTATAAGTCTCATTGGAAGAGAAA	RNA3	104	26	56.70	35.00	6.00	2.00	Dobhal *et al*. (2016)
	RRV2R		CCTATAGCTTCATCATTCCTCTTTG			25	56.60	40.00	6.00	0.00	
	RRV probe-2		TGCTAGAGACATTGGTACAACAAGCAA			27	62.00	41.00	4.00	0.00	
RPA-267	RPA-267F	RPA	TGAAGCTGCTCCTTGATTTCCAGGGACCTA	RNA3	267	30	71.00	50.00	6.00	2.00	Babu *et al*. (2017a)
	RPA-267R		AGCACATCCAACACTCTTGCAGCCGATAC			30	71.00	50.00	5.00	1.00	
	RPA-267 exo P2		GTAAGGTGCTAGACTAAAATTGTTGGGACTT [FAM-dT] G [A-THF]A [BHQdT] CTCTGAAGTAAAAGG			51	63.80	37.30	5.00	3.00	
RPA-131	RPA-131F	RPA	GATGTACATGCACCACAGACAGTTGCAGTAG	RNA2	131	31	69.00	48.40	8.00	2.00	Babu *et al*. (2017b)
	RPA-131R		GATGGAGCCGTTGAATGCTTAGCAGATCTCA			31	71.00	48.40	7.00	2.00	
RPA-267	RPA-267F	RPA	TGAAGCTGCTCCTTGATTTCCAGGGACCTA	RNA3	267	30	71.00	50.00	6.00	2.00	Babu *et al*. (2017b)
	RPA-267R		AAGCACATCCAACACTCTTGCAGCCGATAC			30	71.00	50.00	5.00	1.00	
RPA-321	RPA-321F	RPA	CCTCTATCAGCAGCTAAAGCAGGAGCAAAG	RNA3	321	30	69.00	50.00	7.00	2.00	Babu *et al*. (2017b)
	RPA-321R		GTATGAGCTCTATCCAGCTGAAGTGTTGGC			30	69.00	50.00	6.00	2.00	
RRV_2-1	RRV_2-1For	RT-qPCR	CCACAGACAGTTGCAGTAGTT	RNA2	117	21	51.60	48.00	4.00	0.00	Babu *et al*. (2016)
	RRV_2-1Rev		TGGAGCCGTTGAATGCTTAG			20	57.60	50.00	3.00	3.00	
	RRV_2-1Probe		FAM-ACAGCTGAAGCCATCATGAACCTT-BHQ-1			24	62.10	46.00	6.00	4.00	
RRV_2-2	RRV_2-2For	RT-qPCR	CCATTGCAGGTTGTTGCATT	RNA2	100	20	58.10	45.00	4.00	2.00	BAbu *et al*. (2016)
	RRV_2-2Rev/		TTGGCTCTACCCTTTCTTTCC			21	56.30	48.00	2.00	0.00	
	RRV_2-2Probe		FAM-TGAACAAGGGTGGACCATTCCACA-BHQ1			24	66.20	50.00	6.00	4.00	
RRV_3-2	RRV_3-2For	RT-qPCR	ACACTCTTGCAGCTGATACTG	RNA3	117	21	51.90	48.00	6.00	2.00	Babu *et al*. (2016)
	RRV_3-2Rev		CTGGGTCCAATTCTGAACTCTC			22	56.50	50.00	5.00	1.00	

	RRV_3-2Probe		FAM-AGCTTCGGGTCCTCAAGTTGACAA-BHQ1			24	64.10	50.00	7.00	5.00	
RRV_3-5	RRV_3-5For/	RT-qPCR	CTGATACTGTTATCATCGGAGCTG	RNA3	94	24	56.80	46.00	7.00	2.00	Babu *et al*. (2016)
	RRV_3-5Rev		TCTGAACTCTCAGGCTTCACTA			22	53.20	45.00	7.00	3.00	
	RRV_3-5Probe		FAM-AGCTTCGGGTCCTCAAGTTGACAA-BHQ1			24	64.10	50.00	7.00	5.00	
RRV	RRVF	RT-PCR	GCACATCCAACACTCTTGCAGC	RNA3	271	22	62.83	54.55	5.00	3.00	Di Bello *et al*. (2018)
	RRVR		CTTATTTGAAGCTGCTCCTTGATTTCC			27	61.11	40.74	5.00	0.00	
RRV-3	RRV-3F	RT-qPCR	CAGGAGCAAAGTTCTTGATCAG	RNA3	103	22	55.50	45.00	6.00	4.00	Joe Tang, personal communication
	RRV-3R		GCATATGTTGAACTTGCTAGAGA			23	53.00	39.00	6.00	2.00	
	RRV-P		CCTATAGCTTCATCATTCCTCTTTG			25	74.00	40.00	6.00	0.00	
P4	RRVP4-F3	LAMP	ATTGTTGGCTCAGGGGAA	RNA4	193	18	54.10	50.00	3.00	0.00	Salazar-Aguirre *et al*. (2016)
	RRVP4-B3		ATCCAGCTGTAGATTGAGTT			20	50.80	40.00	6.00	0.00	
	RRVP4-FIP		ACGAATTGTTGGAAATTTGGATCAAGCTTAATCTTGATCTTATGGGAAC			49	63.70	34.70	-	-	
	RRVP4-BIP		CAGGCTCACTTGATTTTGCAACTGCACCCATCCTAGTATCAGG			43	66.90	48.80	-	-	
P3	RRVP3-F3	LAMP	AGAAGCCTTCGAAGATCG	RNA3	194	18	51.70	50.00	8.00	3.00	Salazar-Aguirre *et al*. (2016)
	RRVP3-B3		AATCTCTGAAGTAAAAGGTGTAG			23	50.00	34.80	3.00	0.00	
	RRVP3-FIP		CGAAGCTTCTGATCAGCTCCGAAAATCCTGGAACAAGCACA			41	67.30	48.80	-	-	
	RRVP3-BIP		GGTCCTCAAGTTGACAAATGTTCAGTTCAATATAAACTGGGTCCAATT			48	64.50	37.50	-	-	

[a]The self-complementarity score of the oligo (tendency of oligo to form secondary structure) calculated by Primer3.

[b]3’ self-complementarity of the oligo (tendency to form primer-dimer with itself) calculated by Primer3.

C.2 High Throughput Sequencing and RT-qPCR Assay Reveal the Presence of Rose Cryptic Virus-1 in the United Kingdom.

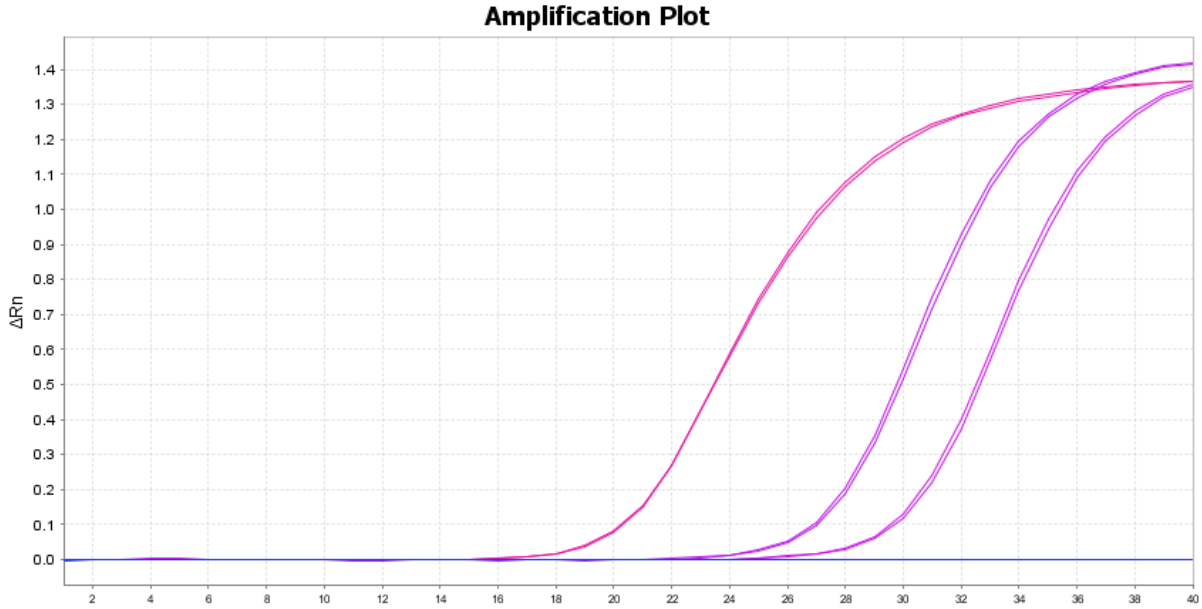

Figure C.2- A graph showing ΔRn vs PCR cycle number for some samples tested using the RT-qPCR RCV1 assay. The results show (in pink) amplification plots for sample 7B3 (C_T of 20.47/20.42). The results for two different dilutions of the RoCV1 positive control are shown in (in purple), with C_T values of 27.1/26.96 and 29.86/29.72 respectively. In addition, the negative control (in blue) is shown, giving a negative result.

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