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IDENTIFICATION OF THE MAIN DESTRUCTIVE PLANT-VIRUS-DISEASES OF HORTICULTURAL CROPS IN SICILY AND DEVELOPMENT OF NEW DIAGNOSTIC TECHNIQUES

PLANT PATHOLOGY

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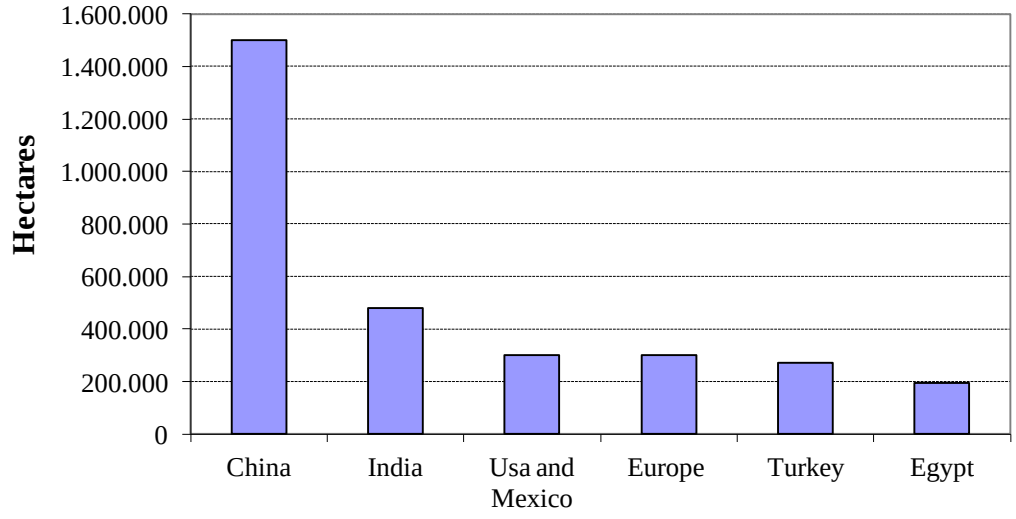
1. INTRODUCTION

1.1 VEGETABLE CROPS: ECONOMIC IMPORTANCE

Vegetable crops represent an important economic segment of the agricultural production worldwide, as well as in Italy and Sicily. In particular, tomato (*Solanum lycopersicum* L.) is one of the most common vegetable species in the world and it is characterized by a double use, since it can be consumed fresh and in its derivatives.

Tomato is cultivated on about 4.6 million hectares in the world (FAO, 2009) and has three major production areas: the Far East, the Mediterranean Basin and North America. Cultivation of tomato is most widespread in Asian countries: China grows less than 1.5 million hectares, followed by India with 480,000 hectares and Turkey with 270,000. Egypt is the first among African countries with 194,000 hectares, while the U.S.A. and Mexico have over 300,000 hectares in total. In Europe, Russia has the largest cultivated area with about 160,000 hectares (Table 1).

Table 1 – Global overwiev of tomato production areas.



Italy is the leader in the European side of the Mediterranean area with about 100,000 hectares (including crops cultivated both in open field and in greenhouse), followed by Spain with 55,000 hectares. Tomato cultivation in European Union counts up to more than 300,000 hectares. From 2009 to 2011 (FAO, 2011) areas intended for tomato are decreased and consequently the production has been contracted by about 15%, from 6,878,161 tons in 2009 to 5,950,215 tons in 2011. Specifically, in 2009 in Italy tomato cultivated in greenhouse has occupied an area of 84,325 hectares with a production of 54,709,551 tons, whereas tomato in open field occupied an area of 19,453 hectares with a production of 1,411,006 tons (ISTAT, 2009).

As regards Sicilian horticulture, tomato, along with pepper, represents even today, a good and important source of income for many farms, although the economic crisis hit many economic and productive sectors. In fact, the tomato in the year 2009 has occupied a total area of approximately 12,700 hectares (of which 5,360 were in the greenhouse), that is more than 10% of the total tomato area planted in Italy, with a production of 233,500 tons (ISTAT, 2009). Provinces where this crop is most grown are: Agrigento, Palermo, Ragusa and Caltanissetta.

In Sicily, furthermore, it is grown about 25% of the pepper (*Capsicum annuum*) produced in Italy, with an area of 3,800 hectares (both in greenhouses and open field), and a total production of about 800,000 tons (ISTAT, 2009). The provinces where the pepper cultivation is most common are Ragusa, Syracuse, Agrigento and Caltanissetta.

1.2 TOMATO

Cultivated tomato belongs to *Solanaceae* family, it is an herbaceous annual dicotyledonous, hardy perennial in favorable climatic conditions (as in the origin area). It originates in the Central and South America and in the southern part of North America.

The date of its introduction in Europe is 1540 supposedly, when the Spaniard Hernàn Cortès brought some specimens, but its cultivation and its diffusion is dated around the second half of the seventeenth century. It arrived in Italy in

1596, but only later, in the south of the country, the production of tomato took place thanks to the favorable climatic conditions and especially to further selections and grafts, that allowed to obtain berries red colored and no longer gold.

The root system is both fibrous and taproot, highly branched and may go deep depending on the origin of the plant, but most of the roots remain confined within the first 30 cm of soil. The habit of the plant was originally expanded, creepy and very long, but the selection and the genetic improvement produced gathered and erect forms. The stem is initially erect and later on decumbent so that it needs tutors. The nodes are swollen and the leaves branch out from them, as well as the lateral shoots and the elongated internodes on which the flowers are inserted. The leaves are alternate and composed of 7-11 leaflets. The fruit is a large berry, red at ripening stage presenting different sizes and shapes depending on the variety.

1.3 PEPPER

Pepper belongs to *Solanaceae* family. It is native to the Central America and Mexico and currently it is cultivated throughout the world. It was already known in Mexico since 5500 a.C. as a cultivated plant and it was the only species used by the native Peruvians and Mexicans. An Italian explorer "Cristoforo Colombo" brought the pepper in Europe from America in 1493. Diffused in Europe by Spanish, it had an instant success since it well adapted to the climatic conditions of the old continent, especially in all southern regions, Africa and Asia.

Pepper *(Capsicum annum)* is a short-lived perennial shrub with a shallow root system that extends in depth only in loose and sandy soils. It has an angular, smooth and slightly woody stem with dichotomous branches, whose development is regulated by genotype and growing conditions of the plants. The leaves are alternate, glabrous with entire margin. The flowers appear in the axils of leaves or branches, they are hermaphrodites, with a white corolla supporting 5 to 7 petals and pale yellow stamens. The flowers may be solitary or grouped in clusters.

The fruit is a berry, green at the beginning, becoming red or yellow of different tones until the physiological maturity (sometimes even purple or cream). The

shape and size can be very various: truncated prism, with three or four lobes, conical-elongated or rounded. The interior is divided in lodges by incomplete septa and placentas. The interior of the fruit in the pedicle area is occupied by a spongy placental tissue in which are inserted the seeds.

1.4 MOST IMPORTANT HORTICULTURAL DISEASES

Vegetable crop cultivations in open field and in greenhouse is nowadays carried out in monoculture conditions, because of the continuous and uninterrupted demand made by the processing industry and consumers. That strongly favors the establishment and the increase in virulence of numerous pathogens.

Today, the heightened incidence of many endemic diseases, as well as the emergence of new ones caused by fungi, bacteria, phytoplasmas and viruses, worsen the crisis of the horticultural sector worldwide and also in Italy.

The increased incidence of diseases related to widespread crops, such as tomatoes and peppers, not only affects the agricultural producers, but it is also a socio-economic issue since it affects both the safety of food and the environment protection.

Tomato and pepper are subject to various disorders, of both abiotic and biotic origin, including some of those that are of greater importance, such as some tracheomycosis, that affect the root system and the collar such as: *Fusarium oxysporum, Verticillium dahliae, Rhizoctonia solani, Pythium, Pyrenocheata lycopersici*, and others that affect the epigeal part of the plant, such as: *Phytophthora infestans, Alternaria solani, Cladosporium fulvum, Septoria lycopersici, Xanthomonas vesicatoria, Pseudomonas corrugata, Botrytis cinerea*.

Among the most common and dangerous pathogens that can cause almost the total loss of production, with consequent serious economic losses for producers, there are different viruses, such as: *Pepino mosaic virus* (PepMV), Tomato yellow leaf curl disease (TYLCD), *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), *Tomato mosaic virus* (ToMV), *Tomato chlorosis virus* (ToCV), *Tomato torrado virus* (ToTV), *Tomato infectious chlorosis virus* (TICV), *Broad bean wilt virus 1* (BBWV-1) and many others.

1.5 VIRAL DISEASES

Viruses are entities composed of a nucleic acid (RNA or DNA), single or double strain, encapsidated by a protein called capsid. Due to the extremely small size, they can be observed only with transmission electron microscope, they reproduce only in living cells and have the ability to cause diseases. They are obligate parasites that multiply within plant cells using its metabolic systems. Viruses cause many diseases in all living organisms such as fungi, mycoplasmas, bacteria, invertebrate, vertebrate and plants.

According to recent data from the International Committee on Taxonomy of Viruses (ICTV, 2012), the number of viral species described to date is about 2,600.

Almost 50% of known viruses cause damage to plants. The virions (virus particles) spread quickly within the plant (systemic infection), causing morphological changes (symptoms) significant on almost all vegetative organs. Moreover, some viruses are able to overcome the physiological barriers present in the reproductive organs, causing production of infected seed.

A virus can infect a single species or more different species as well as each species can be infected by one or more viruses simultaneously.

Viruses can cause damage to the leaves, branches, roots, fruits, seeds and flowers of plants and cause significant economic damage, such as the reduction of yield and fruit quality. The severity of a virus disease may vary depending on the region, crop variety and environmental conditions, which may be different from one season to another.

Viruses are the most common pathogens in cultivated plants for three basic reasons:

1. they cannot be controlled by pesticides used against the enemies of plants;
2. they have different ways of transmission and therefore can easily reach their hosts;
3. they evolve with extreme ease, and then in time always manage to infect new hosts.

Viruses are transmitted by: seed, pollen, vegetative propagation, contact, working tools, insects, mites, nematodes, fungi and plants (cuscuta).

Viruses are the cause of serious concern for the impact that they have on the crop productions. The virus can be defined as "endemic", when their event is regular in a specific area, but with implications contained in the natural, and "epidemic" when the spread is sudden and severe defined by a short time and space.

Insect vectors are the most common means of transmission of plant viruses. In particular aphids, leafhoppers, whiteflies and thrips acquire viruses from infected plants, believe them with different ways and time and finally transmit them to the other plants (hosts). The relationship virus/vector is specific.

In areas where climatic conditions allow the continuous cultivation of horticultural crops, every plant represents the most dangerous source of infection for subsequent ones. Virus transfer from a plant to another is possible during the whole period of overlap of crop cycles. Particularly important for this purpose are also wide spread of weeds such as: *Galinsoga parviflora*, *Portulaca oleracea*, *Solanum* spp., *Amaranthus* spp., etc.

From the information just described, it is deduced that the control of viral diseases is very difficult and complex, since virus diseases and virus-like diseases as is well known cannot be controlled by chemical molecules. However, the adoption of appropriate prophylactic measures mainly designed to reduce the inoculum load in a specific area, as well as the establishment of genotypes tolerant and/or resistant enables to limit the losses caused by these pathogens. Effective strategy to contrast these pathogens has been shown the adoption of adequate farming practices and cultivation techniques.

Therefore, the control of viral diseases is mainly based on the adoption of preventive methods, among which the most important is the use of virus-free plant material for new plants cultivation and cleaning of growing environments.

Recently, the production costs are always higher, unfortunately, forcing some operators to produce the seedlings for transplantation in the field by the use of cuttings. This practice often causes serious production and economic losses

because take cuttings from infected plants results in obtaining infected plants to 100%.

Vector struggle, cut down spread of disease, but certainly does not negate the risk of infection in most of the combinations virus/vector/plant. Among the preventive measures to be put in place, particularly in areas already infected, are ascribed: the adoption of large rotations, the construction of greenhouses (when possible) close uncultivated areas; elimination (selective control) of spontaneous and weed that can be a source of *inoculum*, the eradication of infected plants to reduce the amount of the *inoculum*.

The necessary conditions to implement a successful defense strategy are:

- Identification of virus or viruses that infect vegetable crops and knowledge of those responsible of transmission;
- knowledge of climatic conditions;
- knowledge of cultivation techniques adopted.

The control of the virus infection is based almost exclusively on chemical control of vector populations by insecticide treatments for groped to curb transmission. Reduction of vector populations at levels that reduce viral transmission, however, is a rather difficult task because it requires a very low tolerance thresholds of the vectors, especially for field crops where vectors move easily from one field to another. In addition, the massive and continuous use of insecticides for vector control in areas of intensive farming causes serious environmental damage, such as the elimination of any natural enemies of the vectors, and produces the development of resistant vector populations to the active substances available for its control, including those of recent introduction.

Vector control in greenhouse can be implemented, even resorting to the use of dense mesh networks designed to protect all openings of the structure, preventive measures to avoid contact between the vector and the plant.

Among the means of mechanical type is also to be remembered the use of plastic films that filter ultraviolet light (UV). The principle that governs the control of viruses is based on the interference of the visual capacity of the insect, which results in a lowering of their population in relation to the difficulty of

displacement for the absence of UV light, as well as the difficulty in detecting the host plants. The mulching made with polyethylene films (PE) colored with reflective aluminum seem to be found useful to slow down vector infestations. Among the physical means, treatment of seeds with moist heat is especially effective for those viruses which are located in the most superficial of these organs.

The knowledge of the viruses' epidemiology is useful to properly manage the crops. In fact, knowing the periods during which the incidence of infection is highest is possible to determine the most appropriate time to perform transplants, and consequently containing the infection. Many weeds contain both viruses that their vectors, so their eradication represents a priority to any control strategy. The use of genetic improvement programs based on the introduction, in cultivated plants, of resistance genes from wild progenitors, is another strategy for the fight against the most common viruses.

Finally, it is should be specified that today are available powerful and rapid methods of control based on early diagnosis carried out by means of the use of molecular methods with greater sensitivity, requiring a much shorter time than the techniques of traditional diagnosis, with considerable savings, time and economic resources.

Another problem that should not be underestimate, especially for Sicily, for the geographical location, located in the middle of the Mediterranean basin is the appearance of new disease due to the continuous movement of genetic materials from Northern Africa to our island.

Emerging diseases pose a serious problem for crops worldwide (Anderson *et al.*, 2004; Hannssen *et al.*, 2010) and reliable epidemiological surveys on major viruses and virus-like agents, as well as potential emerging viruses, are a critical factor for the development of effective control strategies. In fact, a new pathogen may be introduced in a given area without immediately giving rise to the disease, which can be developed later as a result of concomitant climate changes or biological factors. The current climate change models provide for a gradual warming of our planet and an increasing of extreme weather events (Houghton *et*

al., 2001, Rosenzweig *et al.*, 2001). By altering the real distribution of insect vectors, increasing stress of plants or simply creating more favorable environmental factors, global warming may lead to the spread of viral pathogens already present in the environment but until then dammed by limiting environmental conditions (Rosenzweig *et al.*, 2000). Another important factor is the trade among distant geographic areas, which may accidentally lead to the introduction of new pathogens that do not necessarily manifest themselves immediately, but thanks to favorable secondary factors, may later give rise to new diseases. A sensational example is the worldwide spread of the virus TYLCV from areas in the eastern Mediterranean (Lefeuvre *et al.*, 2010). The potential risk of new introductions in the regions of Southern Italy will be increased in the near future by the implementation of the "Free Trade Agreement". Indeed, the Barcelona Declaration (1995) starting in 2010 the creation of a free trade zone for goods in the Mediterranean basin.

Despite some delay in implementing this directive, it is clear the intention to facilitate the exchange of products in this area. In the near future, the territories facing the Mediterranean, including Sicily, will be in the centre of increasingly intense trades that will require significant (and certainly not feasible) controls against the risk of the introduction of pathogens. A third important factor in the emergence of new diseases concerns the cultural practices: an excessive irrigation often leads to an increase in the population of vectors with a consequent increase of pathogens; crop diversification and globalisation introduce new hosts and new viral agents (Anderson *et al* ., 2004).

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2. AIM OF THE WORK

Pathogens, as well as viruses, that can adapt quickly to environmental change often pose the greatest challenge to disease control. (Pearce *et al*. - 2009). The means and dynamics by which any pathogens spreads will depend upon the population structure, intra-variability selection and the level of recombination within the populations. For that reason, understanding the population genetic dynamics of a pathogen and how often they share and spread genetic material is an important component in the development of risk assessment and intervention strategies.

The rapid detection of viruses is essential to prevent the spread and to improve their control. Viruses, are the most abundant parasites infecting bacteria, plants and animals. They have a high socio-economic impact on humans' welfare and on productivity of livestock and agriculture. Viruses also have a great potential for rapid evolution due to the high mutation rates, large population sizes and short generation times (Domingo E., Holland J., - 1997).

Phylodynamics, the synthesis between epidemiology and evolutionary biology, can provide relevant information to understand the evolution of virulence, the emergence of new viral diseases and to design more efficient strategies for disease control (Grenfell B.T., *et al.,* 2004; - Moya A, *et al*., 2004).

In this context there is a clear need to develop rapid diagnostic methods for the early detection of diseases, which allows to adopt the best means of struggle in a context of sustainable agriculture.

For this aim, in this work have been developed three rapid diagnostic techniques for the detection of viruses that cause serious damage to many horticulture crops:

1. RT-PCR Multiplex for the detection of the main RNA viruses that infect tomato and other vegetable crops;
2. RT-PCR multiplex for the detection of viruses of genera *Fabavirus* that affect different vegetable crops;
3. Flow through-hybridization for the detection of viruses of genera *Fabavirus*;

In addition, three different case studies have been subject of investigation in order to better understand the variability, evolution and the dynamic of spread of the main phytoviruses present in Sicily:

1. Study of the population genetic structures and the dynamic of spread of *Pepino mosaic virus* in Sicily (RNA single-strand virus);
2. Study of population genetic and spread of Tomato yellow leaf curl disease in Sicily (DNA circular single strand virus);
3. Study and characterization of *Limium mild mosaic virus* (bipartite RNA single strand virus).

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3. SIMULTANEOUS DETECTION OF THE SEVEN MAIN TOMATO-INFECTING RNA VIRUSES BY TWO MULTIPLEX REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTIONS*

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ABSTRACT – *Cucumber mosaic virus, Tomato spotted wilt virus, Tomato mosaic virus, Tomato chlorosis virus, Pepino mosaic virus, Torrado tomato virus and Tomato infectious chlorosis virus* cause serious damage and significant economic losses in tomato crops worldwide. The early detection of these pathogens is essential for preventing the viruses from spreading and improving their control. In this study, a procedure based on two multiplex RT-PCRs was developed for the sensitive and reliable detection of these seven viruses. Serial dilutions of positive controls were analysed by this methodology, and the results were compared with those obtained by ELISA and singleplex versions of RT-PCR. The multiplex and singleplex RT-PCR assays were able to detect specific targets at the same dilution and were 100 times more sensitive than ELISA. The multiplex versions were able to detect composite samples containing different concentrations of specific targets at ratios from 1:1 to 1:1000. In addition, 45 symptomatic tomato samples collected in different tomato-growing areas of Sicily (Italy) were analysed by multiplex RT-PCR, singleplex RT-PCR and commercially available ELISA tests.

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Similar results were obtained using the RT-PCR techniques, with a higher sensitivity than ELISA, revealing a common occurrence of mixed infections and confirming the presence of these seven virus species in Italy.

Tomato (*Solanum lycopersicon*) is the most important vegetable crop, with a world production of 153 million metric tons and a value of over 558 thousand million dollars in 2009 (FAO, 2009). In the last decade, emerging viral diseases of tomato have been reported worldwide and have caused considerable economical losses (Hanssen *et al*., 2010). In the Mediterranean Basin and other subtropical areas, the following seven RNA viruses have been described as the most important viral pathogens of tomato: (i) Cucumber mosaic virus (CMV), the type member of the genus *Cucumovirus* of the family *Bromoviridae* and the virus having the widest host plant species range in addition to transmission by aphids (Palukatis and Garcia-Arenal, 2003); (ii) Tomato spotted wilt virus (TSWV), the type member of the *Tospovirus* genus of the family *Bunyaviridae* that is transmitted by thrips (Adkins, 2000); (iii) Tomato mosaic virus (ToMV), a member of the genus *Tobamovirus* of the family *Virgaviridae* that infects many solanaceous species, is able to survive for several years in dried plant debris and can be transmitted by seeds or mechanical contact (Broadbent, 1976); (iv) Tomato chlorosis virus (ToCV) and (v) Tomato infectious chlorosis virus (TICV), members of the genus *Crinivirus* of the family *Closteroviridae* having limited host range (Martelli *et al*., 2002) – ToCV is transmitted by *Trialeurodes vaporariorum, Trialeurodes abutilonea* and *Bemisia tabaci* biotypes A and B (Wisler *et al*., 1998), whereas TICV is only transmitted by *T. vaporariorum* (Duffus *et al*., 1996); (vi) Pepino mosaic virus (PepMV), genus *Potexvirus*, family *Alphaflexiviridae*, that has a restricted host range and is transmitted by contact (Jones *et al.*, 1980; Mumford and Metcalfe, 2001); and (vii) Torrado tomato virus (ToTV), the type member of the genus *Torradovirus* of the family *Secoviridae* (Verbeek *et al.*, 2007) that has a restricted host range, including solanaceous species, and is transmitted by whiteflies *B. tabaci and T. vaporariorum* (Amari *et al.*, 2008). The most important strategy to control these viral diseases in tomato is

based on preventing virus introduction and establishment in nurseries and fields and the eradication of infected plants in the early stages of outbreaks. This approach requires surveillance using specific, sensitive and rapid diagnostic methods. Virus detection in tomato plants is traditionally performed by serological tests (DAS-ELISA) using polyclonal antibodies, but the low viral titre often found in nursery plants or during the early stages of virus infection can produce false negative results (Jacobi *et al*., 1998). In addition, there are often no commercially available ELISA tests for the main virus species that infect tomato, as is the case for ToCV, TICV and ToTV. Consequently, there is a current need for the implementation and design of reliable diagnostic methods having high sensitivity and specificity for testing plant material.

In this study, a procedure based on two multiplex RT-PCR assays was developed and successfully tested in plant material for the detection of CMV, TSWV, ToMV, ToCV, PepMV, ToTV and TICV. To design appropriate primers that are compatible with a multiplex reaction, nucleotide sequences of different isolates of each virus species were retrieved from the GenBank database. The sequences were aligned using the ClustalW programme implemented within the software Geneious Pro 5.4.6 (Biomatters, New Zealand). To allow the discrimination of the different virus species by electrophoresis, oligo sequences having similar melting temperatures and flanking genomic regions of different sizes were selected using Primer Express 2.0 (Applied Biosystems, USA) and Vector NTI 9.0 (Invitrogen, USA). The nucleotide sequences, genomic position of the primers and size of the amplicons are provided in Table 1.

Table 1 – Designed primers, nucleotide sequences and size of amplicons for each viral species.

Primer	Virus	Nucleotide sequence 5'–3'	GenBank (acc. number)	Genomic position	Amplicon size
TICV 1	TICV	TTGGCTGTGAGTCAAGGAGGT	FJ815441	5414, RNA 2	136
TICV 2		CTGATTTGATAGCCGATTTCCC		5528, RNA 2	
ToTV 1	ToTV	TGGTGCTCAACAGTGCAATCA	DQ388879	859, RNA 1	189
ToTV 2		CACACTGCATCCACTTCTTCCA		1026, RNA 1	
ToCV 1	ToCV	CATTCCGGCTAATCCTAATCGA	AY903448	4461, RNA 2	101
ToCV 2		CCCTAGTGGAGTGTACCTTCAATTTC		4536, RNA 2	
TSWV 1	TSWV	GCCATGGTCTTCTTCTGATGAA	S48091	161, RNA M	456
TSWV 2		AGTTATTGTCCCCTGACCCTTC		595, RNA M	
CMV 1	CMV	ATTAACCACCCAACCTTTG	D10538	1413, RNA 3	480
CMV 2		TGGGAATGCGTTGGTGCTC		1872, RNA 3	
PepMV 1	PepMV	CATAGTTGTGCACGGAATTGC	AF484251	4503	773
PepMV 2		TTCCGTCTTGATACTGACCA	HQ663892	5256	
PepMV 3		TGCCGTCTTGATATTGGCCA	AF484251	5260	777
ToMV 1	ToMV	GATAATTTGATTGAAGATGAAGCC	AF332868	5644	274
ToMV 2		CTGTACACCTTATAAACATCGCC		5895	

Greenhouse-grown tomato plants infected naturally and artificially by well-characterised viral species were used as the positive controls. These controls were employed to prepare 10-fold serial dilutions using healthy plant extract as diluent and also to obtain specific PCR products that were cloned and used for the optimisation of the multiplex reactions. The plant extracts were prepared by grinding leaves 1:20 (w/v) in PBS buffer, pH 7.2, supplemented with 0.2% DIECA, and 2% (w/v) polyvinyl-pyrrolidone (PVP-10) in individual plastic bags with a net (Bioreba, Switzerland) to avoid contaminations among the samples. Total RNA was purified from approximately 100 mg of plant leaves using the Ultraclean Plant RNA Isolation Kit (MoBio, USA) according to the manufacturer's instructions. One-step singleplex RT-PCRs were performed for each virus using a Mastercycler gradient thermal cycler (Eppendorf, Germany), which allowed the inclusion of a gradient temperature from 50 to 60 ºC to test the optimal annealing temperatures. Optimal RT-PCRs were performed in a 25 µl volume consisting of 10 mM Tris–HCl (pH 8.9), 50 mM KCl, 0.3% Triton X-100 (w/v), 1 µM each primer (TICV1/TICV2 for TICV detection; ToTV1/ToTV2 for ToTV detection; TSWV1/TSWV2 for TSWV detection; CMV1/CMV2 for CMV detection; and ToMV1/ToMV2 for ToMV detection) or 1 µM PepMV3, 0.5 µM PepMV1, 0.5 µM PepMV2 for PepMV detection, 250 µM dNTPs, 0.25 units AMV-RT (Promega), 0.5 units GoTaq DNA polymerase (Promega), and 5 µl of RNA template. The one-step RT-PCR was performed at 45 ºC for 30 min for the cDNA synthesis, followed by 95 ºC for 5 min for denaturation and 40 cycles of amplification (95 ºC for 30 s, 50 ºC for 30 s and 72 ºC for 30 s), with a final step of 72 ºC for 5 min. The expected amplicons were obtained from the virus-infected plants, whereas no amplification products were obtained from the healthy plants. These specific RT-PCR products were inserted into the pGem-T vector (Promega) and cloned into Escherichia coli JM-109. The transformants were selected by ampicillin resistance, and the presence of the fragments was verified by PCR using specific primers. The subsequent plasmid purification was performed using the UltraClean Standard Mini Plasmid Prep Kit (MoBio, USA), and the specific nucleotide sequences of the viral targets were confirmed by sequencing in both

directions using the ABI PRISM DNA 377 sequencer (Perkin-Elmer, Boston, MA, USA). The plasmid concentration was estimated using the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). In an initial attempt, a single multiplex assay for the simultaneous detection of the seven viruses was evaluated. For this purpose, 1 ng of each purified viral target-specific plasmid was used to prepare a mixed template sample containing all of the targets. The multiplex PCR was performed as described above for the one-step RT-PCR, but the reverse transcription step was eliminated due to the DNA nature of the composite template. The amplicons were visualised on 2% agarose gels after staining with ethidium bromide, and problems in the simultaneous detection of the amplicons were observed. Specifically, the primers for ToTV and TICV appeared to be incompatible, showing an inhibition in the amplification of the ToTV and TICV targets when their specific primers were included in the same reaction cocktail. In addition, to evaluate the theoretical sensitivity of the multiplex PCR, tenfold serial dilutions of each prepared viral target plasmid were prepared, ranging from 1 to 10^{-4} ng. The results showed sensitivities that were from 10 to 100-fold less than the singleplex versions. To overcome these drawbacks and to discriminate among the seven viruses, the initial multiplex PCR was divided into two multiplex assays: the first multiplex PCR included the simultaneous detection of PepMV, TSWV, ToTV and ToCV viruses, and the other multiplex allowed the detection of CMV, ToMV and TICV. Optimisation was performed with plasmid targets (Fig. 1).

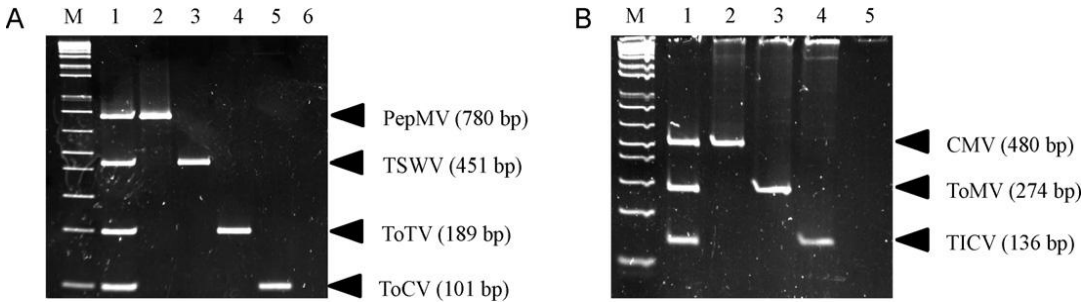

Figure 1. **Panel (A)** – Multiplex PCR for the detection of PepMV, TSWV, ToTV and ToCV target plasmids; lane M, molecular weight marker 1 kB plus (Invitrogen); lane 1, simultaneous detection of PepMV, TSWV, ToTV and ToCV; lane 2, specific detection of PepMV; lane 3, specific detection of TSWV; lane 4, specific detection of ToTV; lane 5, specific detection of ToCV; lane 6, negative control of a virus-free tomato plant. **Panel (B)** – Multiplex PCR for the detection of CMV, ToMV, TICV; lane M, molecular weight marker 1 kB plus (Invitrogen); lane 1, simultaneous detection of CMV, ToMV and TICV; lane 2, specific detection of CMV; lane 3, specific detection of ToMV; lane 4, specific detection of TICV; lane 5, negative control of a virus-free tomato plant.

The reaction cocktail for each multiplex assay was the same as that described above but included only the specific primers for the corresponding virus. The theoretical sensitivity using the dilutions of the specific target plasmids was the same as that obtained by the singleplex assays. Subsequently, 10-fold serial dilutions of infected tomato plant extracts diluted into healthy plant extracts were used to compare ELISA, singleplex RT-PCR and the two multiplex RT-PCRs for PepMV, TSWV, CMV and ToMV. However, due to the lack of commercially available ToTV, ToCV, TICV ELISA tests, the comparisons of these viruses were performed only between the two RT-PCR versions. In all cases, the multiplex and singleplex RT-PCRs were able to detect specific targets up to the same dilution and were at least 100 times more sensitive than ELISA in those cases in which serological tests were available. To determine the reliability of the viral detection in mixed infections and the interference of amplifications due to relative concentration issues, 28 samples were prepared, combining four or three viral target plasmids of the different viruses by changing the concentration of one target (1 ng to 10^{-4} ng) and maintaining the same concentration (1 ng) for the other targets. The PCR analyses of these samples showed that any plasmid target was

detectable in a relative ratio of 1:1000 with respect to the other plasmid targets present in the sample (Fig. 2).

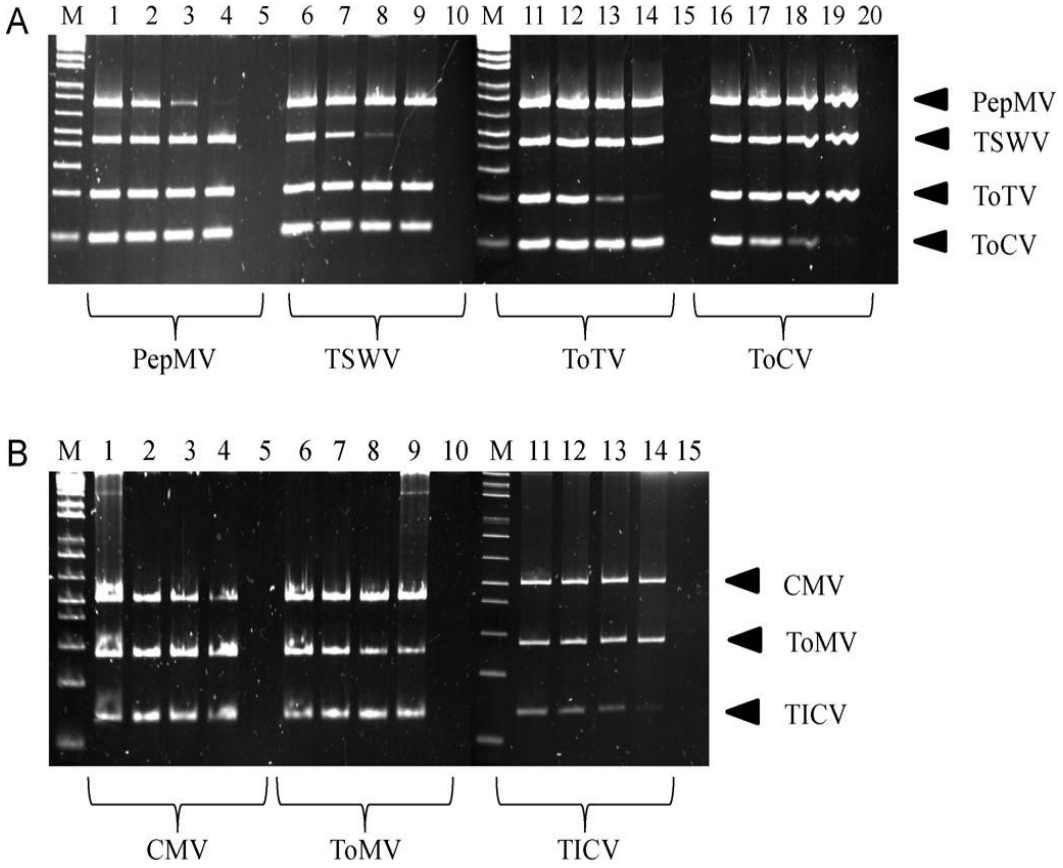

Figure 2 – Panel (A) – Multiplex PCR for the detection of PepMV, TSWV, ToTV and ToCV target plasmids; lane M, molecular weight marker 1 kB plus (Invitrogen); lanes 1–4, 10-fold serial dilutions of PepMV target (from 1 ng to 10−4 ng), maintaining the TSWV, ToTV and ToCV target concentrations at 1 ng; lane 5, negative control of a virus-free tomato plant; lanes 6–9, 10-fold serial dilutions of TSWV target (from 1 ng to 10−4 ng), maintaining the PepMV, ToTV and ToCV target concentrations at 1 ng; lane 10, negative control of a virus-free tomato plant; lanes 11–14, 10-fold serial dilutions of ToTV target (from 1 ng to 10−4 ng), maintaining the PepMV, TSWV and ToCV target concentrations at 1 ng; lane 15, negative control of a virus-free tomato plant; lanes 16–19, 10-fold serial dilutions of ToCV target (from 1 ng to 10−4 ng), maintaining the PepMV, TSWV and ToTV target concentrations at 1 ng; lane 20, negative control of a virus-free tomato plant. **Panel (B)** – Multiplex PCR for the detection of CMV, ToMV, TICV; lane M, molecular weight marker 1 kB plus (Invitrogen); lanes 1–4, 10-fold serial dilutions of CMV target (from 1 ng to 10−4 ng), maintaining the ToMV and TICV target concentration at 1 ng; lane 5, negative control of a virus-free tomato plant; lanes 6–9, 10-fold serial dilutions of ToMV target (from 1 ng to 10−4 ng), maintaining the CMV and TICV target concentrations at 1 ng; lane 10, negative control of a virus-free tomato plant; lanes 11–14, 10-fold serial dilutions of TICV target (from 1 ng to 10−4 ng), maintaining the CMV and ToMV target concentrations at 1 ng; lane 15, negative control of a virus-free tomato plant.

In addition, these two multiplex RT-PCRs were used to analyse 45 tomato samples collected from greenhouses and fields in different provinces of Sicily in 2011 (Fig. 3), and the results were compared with the results of ELISA (except for ToCV, TICV and ToTV) and singleplex RT-PCRs described in the literature: PepMV (Mumford and Metcalfe, 2001), TSWV (Mumford *et al.*, 1994), ToTV (Pospieszny *et al.*, 2007), ToCV (Louro *et al.*, 2000), CMV (Lin *et al.*, 2004), ToMV (Kumar *et al.*, 2011) and TICV (Vaira *et al.*, 2002).

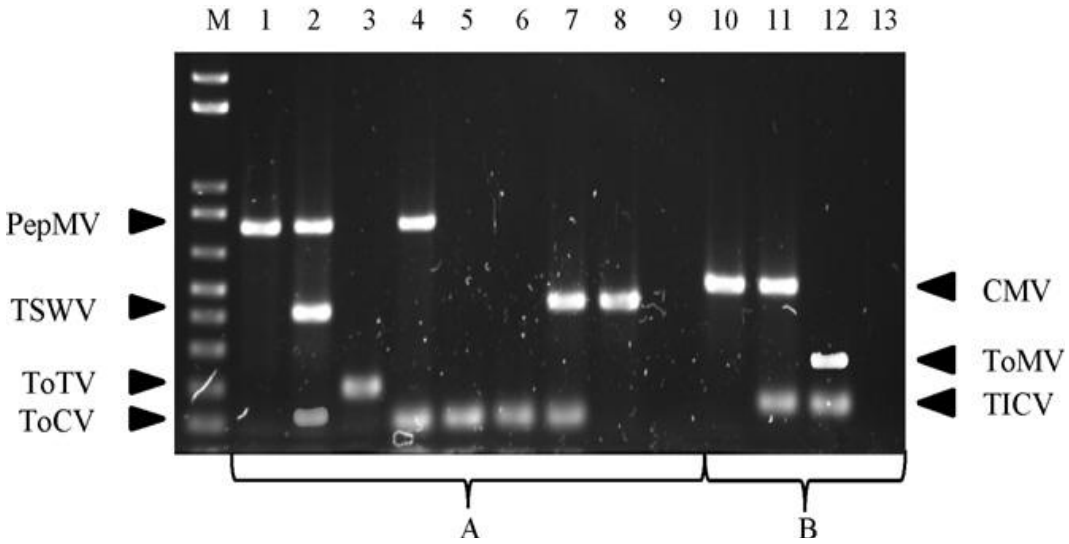

Figure 3 – Multiplex RT-PCR results from the analysis of field tomato plants collected in Sicily; lane M, molecular weight marker 1 kB plus (Invitrogen). Panel (A) Multiplex RT-PCR for detection of PepMV, TSWV, ToTV and ToCV; lane 1, detection of a single infection by PepMV; lane 2, detection of a mixed infection by PepMV, TSWV and ToCV; lane 3, detection of a single infection by ToTV; lane 4, detection of a mixed infection by PepMV and ToCV; lanes 5 and 6, detection of single infections by ToCV; lane 7, detection of a mixed infection by TSWV and ToCV; lane 8, detection of a single infection by TSWV; lane 9, negative control of a virus-free tomato plant. Panel (B) Multiplex RT-PCR for detection of CMV, ToMV and TICV; lane 10, detection of a single infection by CMV; lane 11, detection of mixed infection by CMV and TICV; lane 12, detection of a mixed infection by ToMV and TICV; lane 13, negative control of a virus-free tomato plant.

The plant extracts were prepared using a similar methodology as that employed for the infected tomato plants used as the controls. The same crude plant extracts were used for the ELISA tests and for the purification of total RNA. The results are shown in Table 2; we obtained the same diagnostic for all of the plants using both PCR techniques. In the case of the ELISA tests (by which only four viruses

could be analysed), we obtained the same results as the RT-PCR methods, with the exception of one sample that tested negative for PepMV by ELISA and positive by RT-PCR versions. The most prevalent virus was ToCV, which was detected in 32 plants, fol-lowed by TSWV in 25 plants, CMV in 20 plants, PepMV in 17 plants, ToMV in 12 plants, TICV in 6 plants and ToTV in 2 plants. Three tomato samples were not infected by any virus. Single infections were present in 8 plants, whereas 10 plants were infected by two different viruses, 13 plants by three viruses, 8 plants by four viruses and 3 plants by five viruses (Table 2).

Table 2 – Analysis of 45 tomato plants collected in Sicily using multiplex RT-PCR, singleplex RT-PCR and ELISA..

Geographic origin	Multiplex RT-PCR							DAS-ELISA (1) and RT-PCR singleplex (2)						
	PepMV	TSWV	ToTV	ToCV	CMV	ToMV	TICV	PepMV(1,2)	TSWV(1,2)	ToTV(2)	ToCV(2)	CMV(1,2)	ToMV(1,2)	TICV(2)
Ragusa Province	+	-	+	-	-	+	+	+,+	-,-	+	-	-,-	+,+	+
	+	-	+	+	-	+	+	+,+	-,-	+	+	-,-	+,+	+
	+	-	-	+	+	-	+	+,+	-,-	-	+	+,+	-,-	+
	+	+	-	+	+	-	+	-,+	+,+	-	+	+.+	-,-	+
	-	+	-	+	-	-	-	-,-	+,+	-	+	-,-	-,-	-
	+	-	-	+	-	-	-	+,+	-,-	-	+	-,-	-,-	-
	-	-	-	+	-	-	-	-,-	-,-	-	+	-,-	-,-	-
	-	-	-	-	-	-	-	-,-	-,-	-	-	-,-	-,-	-
	+	-	-	+	-	-	-	+,+	-,-	-	+	-,-	-,-	-
	-	-	-	+	-	-	+	-,-	-,-	-	+	-,-	-,-	+
	-	-	-	+	-	-	-	-,-	-,-	-	+	-,-	-,-	-
	+	+	-	+	-	-	-	+,+	+,+	-	+	-,-	-,-	-
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	+	-	-	-	-	-	-	+,+	-,-	-	-	-,-	-,-	-
	-	-	-	+	-	-	+	-,-	-,-	-	+	-,-	-,-	+
Agrigento Province	+	-	-	+	+	+	-	+,+	-,-	-	+	+,+	+,+	-
	+	-	-	+	+	+	-	+,+	-,-	-	+	+,+	+,+	-
	+	+	-	-	+	+	-	+,+	+,+	-	-	+,+	+,+	-
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	-	+	-	-	+	+	-	-,-	+,+	-	-	+	+,+	-
Trapani Province	-	-	-	+	+	-	-	-,-	-,-	-	+	+,+	-,-	-
	-	+	-	+	+	-	-	-,-	+,+	-	+	+,+	-,-	-
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These data show that mixed infections with several viruses are very frequent. The interactions between viruses co-infecting the same plant could have an important impact in epidemics and could increase the damage in crops if synergisms arise (Syller, 2012). Testing an additional number of plants should confirm these results for the distribution of certain viruses, which was different in each province in Italy. Although ToCV was spread throughout Sicily, ToTV and TICV were only found in the Ragusa Province. In addition, CMV and TSWV were more prevalent in Agrigento and Trapani. These different prevalences and infections in each region could be explained in part by the tomato genotypes, vectors and climatic conditions and in part by the different agronomical practices, as tomatoes in Ragusa Province are cultivated mainly under greenhouse conditions and in open fields in Agrigento and Trapani. The procedure developed in this study based on two multiplex RT-PCR assays allows for the rapid identification of the main RNA viruses that infect tomato and for the analysis of mixed infections, thus saving time, reagents and costs. This methodology could be included in phytosanitary protocols for early detection of these viruses in nurseries or in certification programs.

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4. HIGH "*SPATIO-TEMPORAL*" DISPLACEMENT OF *PEPINO MOSAIC VIRUS* IN TOMATO CROP IN SICILY*

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ABSTRACT – *Pepino mosaic virus* (PepMV) is a highly infectious *Potexvirus* and a major disease of greenhouse tomato (*Solanum lycopersicon*) crops worldwide. In Sicily, the first outbreak of PepMV has been detected in a single greenhouse in the year 2005 and has been quickly eradicated. After this first report, *Pepino mosaic virus* has not been detected in Sicily until the end of 2008, where the number of infected greenhouse suggest that this time a control of the disease could be difficult. The purpose of the present study has been to assessed the dispersion and the genetic diversity of PepMV from its first outbreak in Sicily and to compare it to other PepMV isolates from other parts of the world in order to know what factors are determinant for the evolution and epidemiology of this virus. 1,800 samples from symptomatic and asymptomatic plants have been collected in the provinces of Ragusa, Syracuse, Caltanissetta, Agrigento, Trapani and Palermo. Phylogentic analisys showed that all the Italian isolates of PepMV have been closely related with the isolate Chile Ch2 whereas no correlation has

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been found between genetic relationship and geographic location or collection year.

INTRODUCTION

Tomato (*Solanum lycopersicon* L.) is the most important vegetable crop, with a worldwide production of about 135,000,000 MT and a value of over 558 thousand million dollars in 2009 (FAO, 2009). China represents the first producer of tomatoes (about 484 MT) followed by India (about 168 MT) and United States (about 125 MT), while, Italy together with Spain are the largest producers of tomatoes in Europe (about 219 MT and 386 MT respectively). Sicily (island located in the Mediterranean basin) produces 7.8% of the total national production (FAO, 2012). In the last decade, emerging viral diseases of tomato have been reported worldwide and have caused considerable economical losses (Hanssen *et al*., 2010). Especially in Sicily tomato represents one of the most economically important crop, but several viral diseases such as Tomato yellow leaf curl complex disease (TYLCD) (Accotto *et al*., 2003; Davino *et al*., 2006, 2009, 2012), *Tomato infectious chlorosis virus* (TICV), *Tomato mosaic virus* (ToMV), *Tomato Chlorosis virus* (ToCV), *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV) (Panno *et al.,* 2012) and most recently *Pepino mosaic virus* (PepMV) (Davino *et al.,* 2005, 2008; Tiberini, 2011) and Tomato torrado virus (ToTV) (Davino *et al.,* 2010) affect considerably the tomato crop production. PepMV has been first described in Peru from pepino plants (*Solanum muricatum* L.) by Jones *et al.,* in 1980. PepMV appeared for the first time in Europe, specifically in Netherlands and United Kingdom, into a tomato crop greenhouse (Van der Vlugt *et al*., 2000; Mumford and Metcalfe, 2001). Subsequently this, PepMV has been first reported in many European and non-European countries, such as Germany (Lesemann *et al*., 2000), Italy (Roggero *et al*., 2001, Davino *et al.*, 2008, Tiberini *et al.,* 2011), Belgium (Mumford and Metcalfe, 2001), Spain (Jordà *et al*., 2001), Canada (French *et al*., 2001), France (Cotillon *et al*., 2002), United States (French *et al*., 2001; Maroon-Lango *et al*., 2005), and Austria

(Verhoeven *et al*., 2003). In addition, PepMV has the capability to infect some asymptomatic wild plants and herbs collected around affected tomato glasshouses in Spain (Jordà *et al*., 2001) and in Italy (Davino *et al.,* 2008; Tiberini *et al.,* 2011). Especially in the last five years, in Sicily, *Pepino mosaic virus* represents the most destructive tomato crops disease. PepMV symptoms are highly variable, infact, infection in tomato include chlorosis, leaves necrotic spots, mosaic, marbling on the stems and fruits. However, the variability of symptoms expression can be determined by mixed infection with other viruses, environmental conditions, different tomato cultivars and the virus strain (Davino *et al*., 2008; Gomez *et al.*, 2009; Hanssen and Thomma, 2010; Hasiow-Jaroszewska *et al*., 2010; Soler-Aleixandre *et al*., 2005; Van der Vlugt *et al*, 2009; Fakhro *et al.,* 2011). Pepino mosaic virus spreads easily within greenhouses through manual handling, contamination pot to pot, propagation by grafting, and bumblebees pollination. Moreover, in some tomato cultivars has been reported also the transmission by seed (Cordoba *et al*., 2007; Hanssen *et al*., 2010) as well as by whitefly *Trialeurodes vaporariorum* (Noel *et al.,* 2013).

PepMV is a filamentous virus of the genus *Potexvirus*, belonging to the *Flexiviridae* family. Its genome is a single-stranded, positive-sense, monopartite RNA of approximately 6,400 nucleotides (nt) long with a polyadenylated 3' end. The genome consisting of five open reading frames (ORFs). ORF1 encodes the putative viral replicase containing the methyltransferase, helicase, and RNA-dependent RNA (RdRp) polymerase domains. ORFs 2, 3 and 4 contain the triple gene block (TGBs) essential for cell-to-cell movement and ORF5 encodes the coat protein (Aguilar *et al*., 2002; Cotillon *et al*., 2002; Hasiow-Jaroszewska *et al*., 2008a) and two uncodifing region named UTR 5' and UTR 3'. To date PepMV has been classified into four main genotypes: European isolates (EU), North American isolates (US1 and US2), Chilean isolates (CH2) and Peruvian isolates (LP) (Hanssen and Thomma, 2010).

In Sicily, the first outbreak of PepMV has been detected in a single greenhouse in the year 2005 and has been quickly eradicated (Davino *et al.,* 2005). After this first report, *Pepino mosaic virus* has not been detected in Sicily until the end of

2008, where the number of infected greenhouse suggest that this time a control of the disease could be difficult.

The purpose of the present study has been to assess the dispersion and the genetic diversity of PepMV from its first outbreak in Sicily and compare it to other PepMV isolates from other parts of the world in order to know what factors are determinant for the evolution and epidemiology of this virus.

MATERIAL AND METHODS

In Sicily, during the years 2011-2013, the effective presence and dispersion of PepMV has been investigated. During this period 1,800 samples from symptomatic and asymptomatic plants have been collected in the provinces of Ragusa, Syracuse, Caltanissetta, Agrigento, Trapani and Palermo that represent the most suitable areas for tomato production (Table 1).

Table 1 – Numbers of infected per collected samples in Sicily during the years 2011-2013

Province	**N. of infected plants per tested plants**		
	January 2011	**January 2012**	**January 2013**
Agrigento	0/100	29/100	42/100
Caltanissetta	0/100	28/100	68/100
Palermo	0/100	34/100	43/100
Ragusa	78/100	85/100	88/100
Syracuse	0/100	14/100	65/100
Trapani	0/100	32/100	70/100
TOTAL	**78**	**222**	**376**

Every year have been collected 100 samples per province (600 per year) as reported in Table 1. All the samples collected have been splitted in two sub-samples, useful to carry out the serological analysis and the consequential molecular analysis. Samples have been analyzed by DAS-ELISA using PepMV polyclonal antibodies from Loewe-Phytodiagnostica Biochemica (Sauerlach,

Germany) and samples showing positive signals were used to inferred biological characterization.

Portions of PepMV-infected leaves were used for mechanical transmission tests on herbaceous plants. Crude extracts have been made by grinding symptomatic leaf tissue in 0.03M cold phosphate buffer pH 7.0 containing 3% (w/v) polyethylene glycol (PEG, mol. wt 6,000). Plants of *Chenopodium quinoa*, *Nicotiana benthamiana*, *N. tabaci*, *N. clevelandii*, *Datura* spp., *L. esculentum*, *Capsicum annuum*, *Petunia* hybrida and *Ocymum basilicum* have been inoculated. The subjected plants to treatment (three per species) have been grown in an aphid-proof glasshouse in sterilized soil, with a photoperiod characterized by 14 h of light and a target air temperature set at 28-20 ℃ day/night. Symptoms have been weekly recorded and presence of PepMV has been checked by DAS-ELISA one month after inoculation in inoculated and uninoculated leaves.

The 1,800 samples has been tested by DAS-ELISA and 10% of positive samples has been used for sub-sequential molecular analysis.

Total RNA has been extracted from young leaves. For each sample, approximately 100 mg of leaf tissue has been ground in an 1.5 ml Eppendorf tube with 500 µl extraction buffer (200 mM Tris pH 8.5; 1.5% SDS; 300 mM LiCl; 1% sodium deoxycholate; 1% Igepal CA-630; 10 mM EDTA), the mixture has been incubated at 65 ºC for 10 min, and 500 µl of potassium acetate with a pH value of 6.5 has been added and incubated on ice for 10 min. After a centrifugation of 10 min 13 rcf, 650 µl of supernatant has been transferred into a new tube and an equal volume of cold isopropanol has been added, and the mixture has been incubated for 1 hour at –80 ºC. After a centrifugation of 10 min at 13 rcf, the pellet has been washed with 70% ethanol and resuspended in 50 µl of diethylpyrocarbonate-treated water. The products obtained have been used as a template for RT-PCR with the following primers: PepMV-TGB 5'-CACACCAGAAGTGCTTAAAGCA-3'and PepMV-UTR 5' CTCTGATTAAGTTTCGAGTG-3', which included the coat protein gene (Mumford and Metcalfe, 2001). After this, within-isolate PepMV population structure has been assessed by single-strand conformation polymorphism (SSCP)

analysis. SSCP has been performed using RT-PCR products as previously described in Sambade *et al.,* 2002, in a non-denaturing 8% (w/v) polyacrylamide gel adjusting condition at 200 V at 4 ^{o}C for 7 h.

All samples showed simple patterns (characterized by two bands corresponding to the two DNA strands), indicating that intra-isolate populations have been composed only by a predominant genetic variant (haplotype) have been sequenced in both direction using an ABI PRIMS 3100 DNA sequence analyzed (Applied Biosystem).

The obtained sequences have been trimmed to remove the contaminating TGB and UTR sequences, leaving only the 714 nt long putative CP gene. Nucleotide sequences of the coat protein genes of 130 PepMV isolates from different countries have been retrieved from GenBank (accession numbers are indicated in Fig. 3) and have been used to inferred any subsequent Phylogenetic analysis.

Multiple nucleotide sequence alignment has been performed with the algorithm CLUSTAL W version 2.0 (Larkin *et al*., 2007). To estimate the number of nucleotide substitutions that have occurred, it is necessary to use a mathematical model considering the nucleotide frequencies and the instantaneous rate change among them. The substitution model that best fit these sequence data, with the lowest Bayesian information criterion, has been calculated. Phylogenetic relationships have been inferred by the maximum-likelihood method (Nei and Kumar, 2000) with 1000 bootstrap replicates to estimate the statistical significance of each node (Efron *et al.,* 1996). All of these analyses have been performed using MEGA 5 program (Tamura *et al*., 2011).

Subsequently, nucleotide distance or nucleotide sequence diversity of the CP-PepMV gene was estimated within and between different countries or geographical regions, that have been considered as subpopulations.

To assess the genetic differentiation and the gene flow level between subpopulations, three permutation-based statistical tests: Ks*, Z* and Snn (Hudson *et al*., 1992; Hudson 2000) and the statistic Fst (Weir and Cockerham, 1984) have been used. All of these tests are implemented in the DnaSP 5.0 program (Librado and Rozas, 2009).

To study the role of natural selection at the molecular level, the rate of synonymous substitutions per synonymous site (dS) and the rate of nonsynonymous substitutions per nonsynonymous site (dN) have been analyzed separately. It is assumed that, generally, in a protein, only nonsynonymous changes (producing amino acid changes) are subjected to selection, as they can alter the protein function or structure. The difference between dN and dS provides information on the sense and intensity of selection. Thus dN>dS indicates positive or adaptive selection, dN<dS indicates negative or purifying selection, and dN≈dS indicates neutral evolution; the larger the difference between dN and dS, the larger the selection pressure. These values have been estimated by the Pamilo-Bianchi-Li method (Pamilo and Bianchi, 1993), implemented in the program MEGA 5.0

Selection at individual codons has been statistically tested by the fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) methods available from the DATAMONKEY server (http://www. datamonkey.org). Only concordant results (using both methods) have been considered.

RESULTS

The year and the collection site of the samples randomly collected (symptomatic and asymptomatic tomato plants – Fig. n. 1) has been reported in the Table 1.

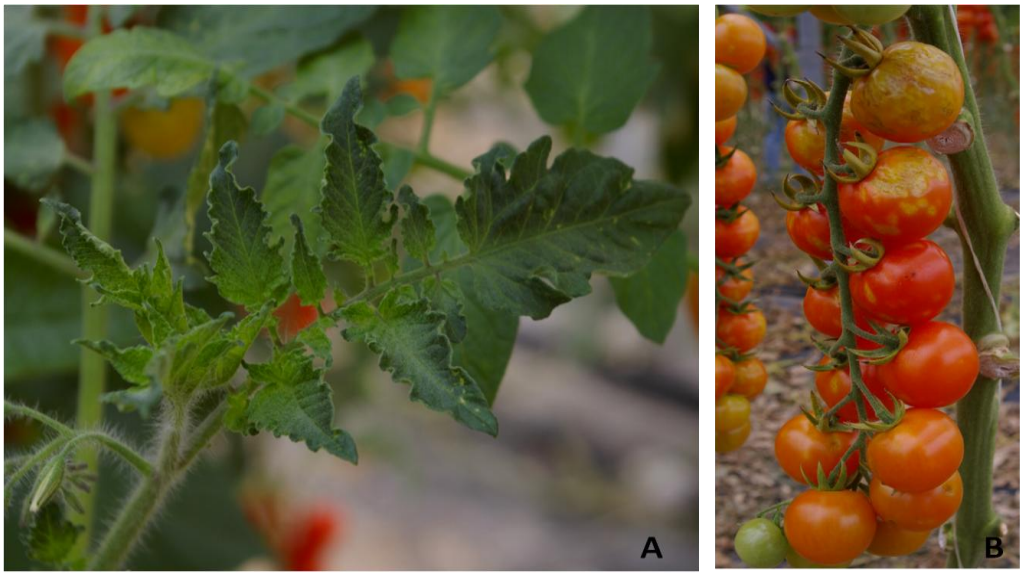

Figure 1 – Different symptoms of PepMV on Tomato plants.

From the 1,800 samples analyzed by DAS-ELISA, 676 showed positive signals. Output analysis, showed a number of 676 positive samples (37.5 %). The Table 1 reports the infected plants number per year and per province. It is interesting to note that from 2011 to 2013 the disease showed an increasing trend, in fact the infected plants percentage has been around 13, 37, and 63% in the years 2011, 2012 and 2013, respectively. Mechanical inoculations made it possible to transmit PepMV from 8 of the 9 symptomatic herbaceous plants, since all experiments concerning *C. annuum* have been negative (Table 2).

The table 2, reports the indicator plants reactions and the post inoculation syndrome showed after 1, 2, 3 and 4 weeks post inoculation.

Table 2 – Host range and symptoms caused by sicilian isolates of PepMV on different herbaceous species.

Host	I weak	II weak	III weak	IV weak
C. quinoa	-	-	-	-
N. benthamiana	lbl	ld - b	ld - b	ld - b
N. tabaci	-	-	-	mml
N. clevelandi	-	-	-	-
Datura spp.	lblyl	byl-lsd	byl-lsd	byl-lsd-mm
Tomato	-	-	fbyl	byl
C. annuum	-	-	-	-
Petunia hybrida	-	md	md	md
Ocymum basilicum	-	-	y	y

Low bubbling level (lbl); Leaves deformation (ld); Bubbling (b); Mosaic on mature leaves (mml); Low bubbling level on the basal part of the young leaves (lblyl); Bubbling on the young leaves (byl); Leaves slow development (lsd); Mild mosaic (mm); Few bubbles on the young leaves (fbyl); Mild deformation (md); Intervenial yellowing (y).

Experimental host range showed more visible differences especially on *Datura* spp. and *N. benthamiana*. This two species showed already symptoms just from the end of during the first week post inoculation, while *L. escultentum* which is the primary host where PepMV has been collected showed symptoms only at the third week post inoculation.

It is important to note that *Ocymum basilicum* and *Petunia* hybrida showed symptoms at the third and fourth week post inoculation (Fig. 2). To our knowledgement *Ocymum basilicum* has been reported as a host of PepMV on in 2008 (Davino *et al*., 2009), while *Petunia* hybrida is the first time that has been reported as a host of PepMV. All the results of host range have been confirmed by a subsequently DAS-ELISA test.

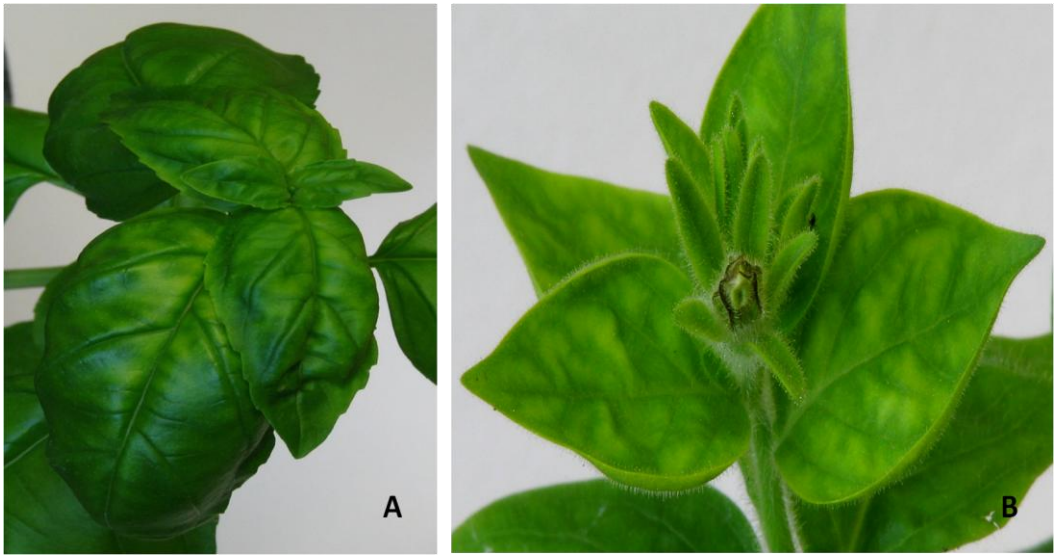

Figure 2 - Different symptoms of PepMV on *Ocymum basilicum* (A) and *Petunia* hybrida (B).

From the 68 samples utilized for molecular analysis, corresponding to 10% of the total samples, resulted positive to the DAS-ELISA. All samples yielded the expected 844 bp fragment corresponding to CP gene and partial TGB block and partial UTR. No amplification has been obtained from tomato plants grown in glasshouse and utilized as negative control. Subsequently, have been carried out SSCP analysis of the 844bp product. All samples showed simple patterns, characterized by two bands corresponding to the two DNA strands, indicating that intra-isolate populations have been composed only by a predominant genetic variant (haplotype), as a consequence the 68 RT-PCR products have been sequenced in both directions. The sequences obtained have been trimmed to remove the contaminating TGB and UTR sequences, leaving only the 714 nt long putative CP-PepMV gene. The sequences were deposited in the GenBank database.

CLUSTAL W algorithm (Larkin *et al.*, 2007) has been used to inferred to construct multiple nucleotide sequences alignment. The substitution model that best fit these sequence data with the lowest Bayesian information criterion was the Tamura Nei model TN93 (Tamura, 1992) assuming variable substitution rates among nucleotide site of α=0.79; Phylogenetic relationship has been inferred by the maximum linkelihood method (Nei and Kumar, 2000). Phylogentic analisys

showed that all the Italian isolates of PepMV have been closely related with the isolate Chile Ch2 (Fig. 3), whereas no correlation has been found between genetic relationship and geographic location or collection year.

Figure 3 – Phylogenetic relationship between Sicilian isolates and isolates retrived from other contries. Phylogentic trees has benne constructed with maximum linkelihood model with a bootstraps of 1,000 replications.

Within each phylogenetic PepMV subgroup the nucleotide distance or diversity between groups related to Italy have been 0.095, 0.121, 0.192, 0.246, 0.081, 0.340 with Belgium group, Poland group, Spain group, New World group France group

and Netherlands group, respectively. These data suggest a moderate GeneFlow between France and Belgium to Italy. The nucleotide sequence diversity to Italy (intra-group) has been 0.010, this suggest that PepMV spread rapidly in the six provinces observed (Table 3).

Three permutation-based statistical tests has been calculated, Ks*, Z* and Snn (Hudson *et al*., 1992; Hudson 2000) and the statistic Fst (Weir and Cockerham, 1984) to assess the genetic differentiation and the gene flow level between subpopulation. In most cases the Ks*, Z* and Snn tests have been significant, suggesting genetic differentiation between subpopulations. In Table 3 only non-significant P-values of Ks*, Z* and Snn has been reported.

PepMV coat protein gene showed the dN and dS values of 0.003 and 0.029, respectively. These values confirm a negative selection due to functional and structural constraints, which occurs in proteins having attained a high adaptation level. The dN/dS ratio has been 0.103. Selection at individual codons was statistically tested by the fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) methods available from the DATAMONKEY server. Regarding the selection at individual codons, only concordant results, using both methods (FEL, and SLAC methods), have been considered. This analysis showed one positively selected site located in aa in position 13 (Fig. 4), which could be caused by a PepMV adaptation.

Table 3 – Nucleotide diversity and genetic differentiation of PepMV in different geographic subpopulations.

Subpo.[a]	N.[b]	Belgium	Poland	Spain	New World	Italy	France	Nentherland
Belgium	4	0,173±0,018[c]	0,145±0,015[d]	0,181±0,018[d]	0,225±0,020[d]	0,095±0,010[d]	0,128±0,013[d]	0,257±0,027[d]
Poland	3	-0,362Fst 0,636Snn 0,614Kst 0,428Z	0,23±0,022[c]	0,179±0,018[d]	0,217±0,018[d]	0,121±0,012[d]	0,143±0,014[d]	0,230±0,023[d]
Spain	92	0,040 Fst 0,138Snn 0,137Kst 0,184Z	-0,132Fst 0,901Snn 0,741Kst 0,895Z	0,177±0,017[c]	0,200±0,017[d]	0,192±0,019[d]	0,183±0,018[d]	0,161±0,016[d]
New World	8	0,148Fst 0,306Snn 0,203Kst 0,104Z	0,003Fst 0,612Snn 0,571Kst 0,287Z	0,088 Fst --- --- ---	0,200±0,018[c]	0,246±0,022[d]	0,229±0,020[d]	0,160±0,014[d]
Italy	27	0,072Fst --- --- ---	0,022Fst --- --- ---	0,502 Fst --- --- ---	0,516Fst --- --- ---	0,010±0,002[c]	0,081±0,008[d]	0,340±0,035[d]
France	10	-0,112Fst --- 0,095Kst ---	-0,201Fst 0,137Snn 0,129Kst 0,074Z	0,191 Fst --- --- ---	0,266Fst --- --- ---	0,197Fst --- --- ---	0,012±0,013[c]	0,268±0,027[d]
Nentherland	4	0,649Fst 0,053Snn --- ---	0,481Fst 0,296Snn 0,125Kst 0,140Z	0.435 Fst 0,936Snn --- ---	0,417Fst --- --- ---	0,966Fst --- --- ---	0,755Fst --- --- ---	0,004±0,002[c]

[a] Subpopulation according geographical origin.

[b] Number of PepMV isolates.

[c] Nucleotide diversity ± standard error within group.

[d] Nucleotide diversity between different groups; The Fst values and the results of the Ks*, Z* and Snn tests to estimate genetic differentiation and gene flow are shown. Only non-significant P-values are reported.

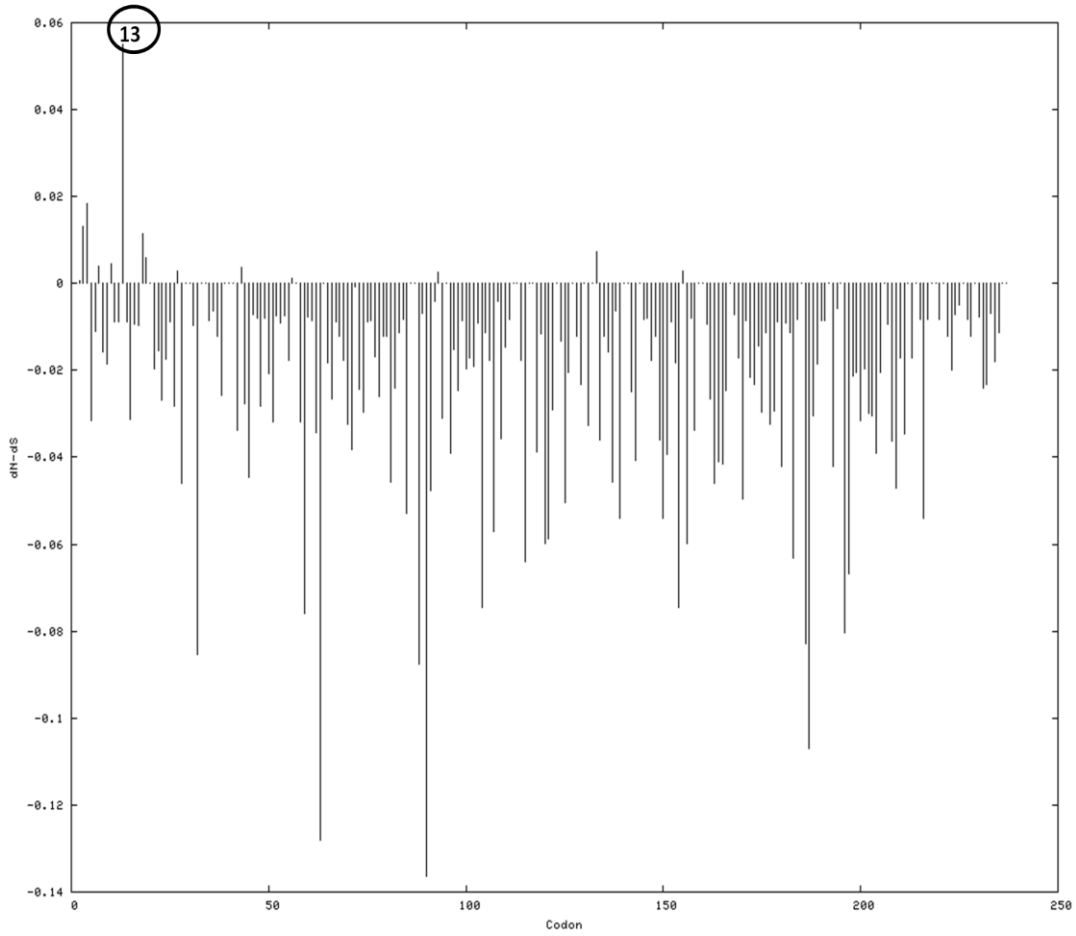

Figure 4 – Codon or amino acid sites of the coat protein gene of Pepino mosaci vurus (PepMV) under positive or negative selection. The Y-axis represents normalized dN-dS (nonsynonymous substitutions minus synonymous substitution), and the X-axis represents codon positions. Numbers at the spikes indicate codon sites with dN-dS values statistically significantly different from zero according to the fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) tests.

DISCUSSION

Greenhouse tomato is an economically important crop in Sicily, with more than 5,000 hectares only in Ragusa province. Tomato crops are commonly infected by several viruses, including TYLCV, TYLCSV and ToCV (Accotto *et al.*, 2001; Accotto *et al.*, 2003; Davino *et al.*, 2006). For this reason it is very important monitoring the territory to avoid the introduction and spread of new pathogens on the EPPO Alert Lists, such as PepMV. Our results provide evidence that this

Potexvirus, hither to only discovered in diseased Sardinian tomatoes (Roggero *et al*., 2001), is spreading to other Italian regions, causing severe symptoms also in combination with other viruses. The unusual disorder found in the Sicilian greenhouse tomatoes was associated to PepMV. The symptomatology described is 'unusual', if it is considered that in tomato plants the most prominent effects of PepMV are the color alterations on the fruits (marbling), while at the date no symptoms of spot-pitting in tomato fruits has never been described in literature.

From an epidemiological point of view, periodic inspections of tomato grower crops, clearly indicate imported non-commercial seed as the origin of PepMV spread. In fact, a high percentage (50%) of leaf symptoms was first observed only in the greenhouses where tomato plants from these seeds were introduced. Later, PepMV infected tomato plantlets were found in almost all the greenhouses in the same area. The virus spread was probably due to mechanical operations, soil movement and/or mechanical agricultural operations carried out by the tomato growers. The host range differs from that reported by Salomone and Roggero (2002) for PepMV in Sardinia which did not infect *Petunia* hybrida and *Ocimum basicilum*. PepMV-Sicily seemed more similar to the Spanish isolate. (Jordà *et al.*, 2001).

Phylogenetic analysis using the MEGA program generated a phylogenetic tree showing that the PepMV isolate in Sicily is different from those found in middle and northern Europe, yet very similar to the Ch2 strain present only in a few regions of the world and exactly in subtropical ones. These results show that probably all isolates of PepMV discovered in Europe do not have a common origin.

From an epidemiological point of view, aspects of the long-distance dissemination of PepMV should be considered, like the trade in living plantlets, seeds, contaminated pots. More restrictive controls are required to avoid PepMV spreading to other Italian regions as Apulia and Campania where tomato crops are widely spread.

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5. RECOMBINATION PROFILES BETWEEN TOMATO YELLOW LEAF CURL VIRUS AND TOMATO YELLOW LEAF CURL SARDINIA VIRUS IN LABORATORY AND FIELD CONDITIONS: EVOLUTIONARY AND TAXONOMIC IMPLICATIONS*

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ABSTRACT – Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus have co-existed in Italian tomato crops since 2002 and have reached equilibrium, with plants hosting molecules of both species plus their recombinants being the most frequent case. Recombination events are studied in field samples, as well as in experimental co-infections, when recombinants were detected as early as 45 days following inoculation. In both conditions, recombination breakpoints were essentially absent in regions corresponding to ORFs V2, CP and C4, whereas density was highest in the 39-terminal portion of ORF C3, next to the region where the two transcription units co-terminate. The vast majority of breakpoints were mapped at antisense ORFs, supporting speculation that the rolling-circle replication mechanism, and the existence of sense and antisense ORFs on the circular genome, may result in clashes between replication and transcription complexes.

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Recombination is known to be an important driving force in plant virus evolution (Garcìa-Arenal *et al*., 2001; Nagy, 2008). Some plant viruses have attracted particular interest in this connection, the family *Geminiviridae*, comprising viruses with small DNA genomes, being one of the most studied. Within the genus *Begomovirus*, several species have been described that clearly result from recombination events (Fauquet *et al*., 2005; Garcìa-Andrès *et al.*, 2007b; Monci *et al*., 2002; Padidam *et al*., 1999; Zhou *et al*., 1997). Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl Sardinia virus (TYLCSV) are two begomoviruses that, throughout the past two decades, have spread to most Mediterranean countries, and cause serious problems particularly to tomato crops: in Italy, Spain and Portugal they are frequently detected, with some tomato plants coinfected by both viruses. The presence of both viruses in the same nuclei, shown by Morilla *et al.* (2004), constitutes a favourable environment for recombination. In Italy, TYLCSV and TYLCV are known to have co-existed since 2002 (Accotto *et al*., 2003), when TYLCV was detected in Sicily, where TYLCSV had been present at least since 1989. TYLCV quickly invaded the area colonized by TYLCSV, and its incidence, often in mixed infections with TYLCSV, became important (Davino *et al*., 2006). Intriguingly, when some years later the same samples were reanalysed, recombinant molecules were detected in field tomatoes collected in 2002 (Davino *et al*., 2008), indicating that recombination actually occurred almost immediately after the second virus arrived. To clarify this question, we here examine recombination events, in natural field conditions and in experimental infections, and compare distribution and location of recombination breakpoints along the viral genomes. Over 4 years (2006–2009) 100 samples per year were collected in Ragusa Province (Sicily) from tomato protected crops showing yellow leaf curl symptoms. Total DNA was extracted from 0.2 g of tissue as described elsewhere (Noris *et al*., 1994) and resuspended in 500 *m*l of TE buffer. Multiplex PCR (Davino *et al*., 2008) was used for amplification, followed by digestion with Psp1406I (AclI) restriction enzyme (Fermentas) and electrophoresis on 2.5% agarose gels. Control reactions used artificial mixtures of DNA extracted from plants infected with TYLCV and

TYLCSV (not shown) to rule out the possibility that recombinants detected in field samples could be artefacts generated during PCR. The pattern obtained (Fig. 1) indicated no evidence of displacement of one viral species by the other, and showed that TYLCSV-, TYLCV- and recombinant-type molecules were all detectable even in 2009, 7 years after the change in virus population due to the spread of TYLCV.

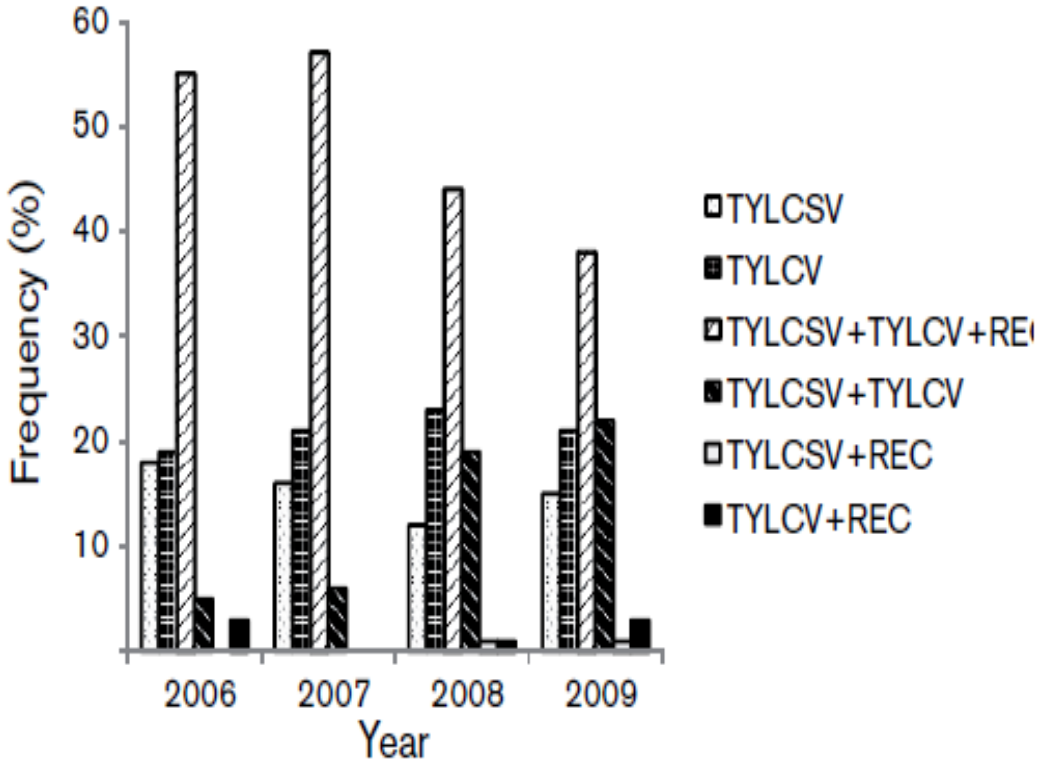

Figure 1 – Distribution of TYLCSV, TYLCV and their recombinants in samples collected in Sicily (100 per year) during 2006–2009.

It is noteworthy that, throughout the period considered, the most common pattern was co-infection with molecules of the three kinds, and that recombinant molecules were never found alone in any plant. Furthermore, each parent virus maintained its own share in single infections: the overall pattern appeared stable

in a sort of equilibrium, neither virus species nor recombinants showing selective advantage. In our survey all recombinants had TYLCSV; sequences in the 39 half of the IR, or in ORF V2, and TYLCV sequences in the 59 half of the IR, as reported in other studies (Davino *et al*., 2008, 2009; Garcìa-Andrès *et al*., 2007a). Twenty samples were selected randomly from those in which recombination events had been detected (five from each year) and used to specifically amplify a 2260 nt long genomic segment from the recombinant molecules, representing more than 80% of the viral genome. Amplifications were run using a primer designed on the TYLCSV-Sar-[IT:Sar:88] V2 sequence (TY224+, 59-CTAGTTGAAGAAACCTACGAACC-39) and another on the TYLCV-IL-[IT:Sic:04] C1 sequence (TY2482-, 59-CCACGAGAATGGGGAACCA-39). The amplified DNAs were cloned into the pCR2.1-TOPO vector (TOPO-TA Cloning kit; Invitrogen), following the manufacturer's instructions, and five clones were sequenced from each plant sample. Sequences were analysed with Vector NTI software (Invitrogen), using PLOTSIMILARITY to align them with known TYLCSV and TYLCV sequences and to map the recombination breakpoints. These breakpoints were not randomly distributed along the viral genome: some portions thereof, e.g. the V2/V1 and the C4 ORFs, were essentially free of breakpoints (cold-spots), with the vast majority of breakpoints being found in the region comprising ORFs C2, C3 and the 39-terminal part of C1 (Fig. 2a and Table S1, available in JGV Online).

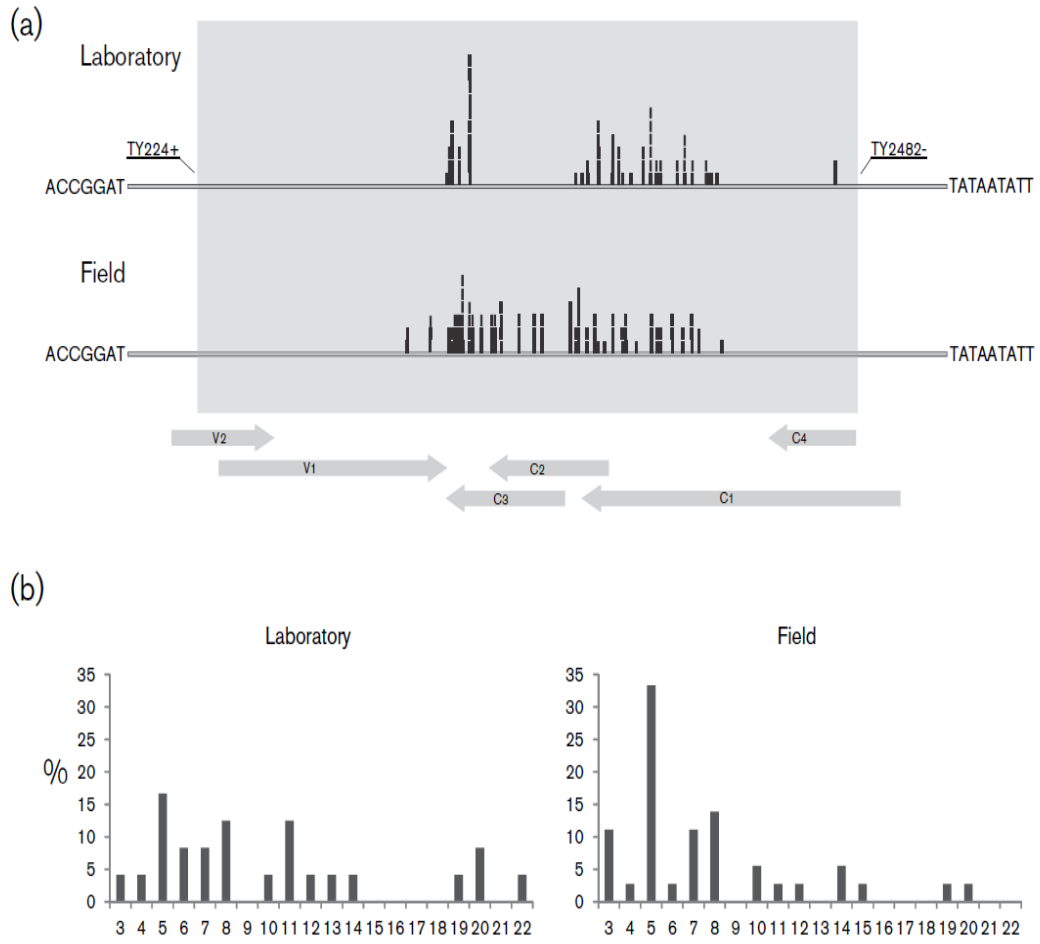

Figure 2 - (a) – Hotspot profiles of recombinant TYLCSV/TYLCV sequences obtained in laboratory and field conditions, with genomic organization represented below. Each rod represents a recombination breakpoint; multiple breakpoints at the same genomic position are piled. The grey rectangle delimits the genomic region studied. The coordinates of breakpoints on the genome of TYLCSV-Sar [IT:Sar:88] are in Table S1. **(b)** – Correlation between the length of common nucleotide stretches between TYLCSV and TYLCV, and number of independent breakpoints detected (vertical axis, per cent).

Within this region, a hotspot in the 39 terminus of C3 was detected. This distribution is a snapshot of the situation in field conditions, but it is not known when the recombination events were produced, nor whether they were fixed in the population: the events could have occurred shortly before sampling, or a long time beforehand, possibly even involving transmission steps by whitefly vectors. To

test whether diverse recombination events could be generated in a short time frame, experiments under laboratory conditions were set up. Ten tomato plants were co-inoculated with infectious clones of TYLCSV-Sar- [IT:Sar:88] (GenBank accession no. X61153, Kheyr-Pour et al., 1991) and TYLCV-IL-[IT:Sic:04] (GenBank accession no. DQ144621, Davino *et al*., 2006). Beginning 10 days post-inoculation (p.i.), plants were checked for systemic infection by the two viruses and by possible recombinants, using the multiplex PCR approach described above. At 24 days p.i., TYLCSV and TYLCV were detected in young leaves of all plants, and at 45 days p.i., recombination events were detected in three of ten plants. At 60 days p.i., when TYLCSV, TYLCV and recombinants were detected in all plants, young tissue was used to extract DNA and amplify recombinant fragments, as described above, with primers TY224+ and TY2482-; 50 clones of the amplified DNAs (five from each plant) were sequenced. The distribution of breakpoints showed a recombination pattern having similarities and differences with field samples (Fig. 2a). No recombination was detected in the CP (V1) ORF, while a hotspot was mapped at the 39 terminus of ORF C3, with a peak at position 1157, followed by a region without breakpoints until position 1518 overlapping between C2/C3 ORFs. This cold-spot was not detected in field samples. Following this region, numerous (44 events) and diverse breakpoints were detected until position 1992. The final portion examined (1992–2482) displayed few breakpoints, located near position 2390. The finding that most breakpoints map at antisense ORFs supports the speculation that, as a consequence of the rolling-circle replication mechanism and the existence of sense and antisense ORFs, clashes between replication and transcription complexes may occur (Lefeuvre *et al.,* 2009). The local degree of sequence similarity is one of the factors that impacts on the efficiency of homologous recombination (Baird *et al.,* 2006). We analysed this feature in our samples, and found that in field conditions (Fig. 2b) 75% of breakpoints were located next to short common stretches (3–8 nt) and only 5.5% next to long stretches (19 nt or more). In laboratory conditions, 54.2% of breakpoints were located next to short common stretches (3–8 nt) and 16.7% next to long stretches (19 or more). We are

unable to offer any reasonable explanation for these results, but a preference for short common sequences in breakpoints was also detected by Martin *et al.* (2011b) in artificial co-inoculations; in their case the most frequent length was 5–12 nt, which compares relatively well with our laboratory data. Whether the relative abundance of short common stretches in field versus laboratory experiments is due to selection deserves future study. Very few laboratory experiments on induction and analysis of recombination have been reported in monopartite *Begomoviruses*. Co-inoculation in tomato of TYLCSVES[ES:Mur1 : 92] and TYLCV-Mld[ES:72 : 97], two different strains of the same virus species used in this study, lead to the detection of recombinants after 130 days (with recombination breakpoints being analysed at 400 days p.i.) (Garcìa-Andrès *et al.*, 2007b), while in our study recombinants were first detected at 45 days p.i., and analysis was at 60 days p.i. (Garcìa-Andrès *et al*., 2007b) detected breakpoints only in the central portion of the genome, approximately 700 nt between the 39-end of ORF V1 and the 39-proximal portion of ORF C1. The hotspot found in the region where ORFs V1 and C3 co-terminate, corresponding to the termini of the two transcription units of the genome, was also present in our field and laboratory samples, and is probably the most frequent recombination hotspot in *Begomoviruses*, apart from that in the stem–loop containing the origin of replication (Lefeuvre *et al*., 2009). Another analysis of breakpoints resulting from artificial co-infections with tomato *Begomoviruses* was recently reported (Martin *et al.,* 2011b); the two viruses coinoculated in tomato plants were TYLCV-Mld [RE:02] and Tomato leaf curl Comoros virus – Mayotte (ToLCKMVYT [YT:Dem:03]) sharing about 82% of sequence similarity. Plants were analysed 4 months after inoculation and, apart from the usual hotspot around the origin of replication, only one other hotspot was found, situated within ORF C4; this hotspot was not present in our case. Curiously, the hotspot found by Garcìa-Andrès *et al*., (2007b) and ourselves in the region where the two ORFs V1 and C3 co-terminate, was considered a cold-spot in Martin's study.

To our knowledge, this is the first attempt to compare recombination profiles in natural and artificial co-infections of two geminiviruses, and this deserves some

consideration. If new recombinants arise at sites where the two parents share a short stretch of nucleotides, as short as 3 nt in our case, then one would expect a large number of different breakpoints in laboratory specimens, where selection had little time to act. In parallel, when studying field samples, one would expect to detect breakpoints mostly in molecules that have maintained (or improved) their fitness. However, although we detected hotspots in both conditions, some genomic regions showed no breakpoints in the laboratory samples, while some were found in the field samples in ORF CP and in the C2/C3 overlapping region (Fig. 2a). One should then conclude that the number of recombination events that can be generated is extremely high, provided time is sufficient (see field data, from samples collected during 4 years), and that most of the resulting variants may continue to exist in an extremely diverse population of viral molecules, whether viable or defective. In the field, whitefly vectors can spread virus variants generated in a single plant to many others, with the result that one plant will eventually host virus variants generated elsewhere. Conversely, in laboratory experiments, where time (60 days) and plants (10) are limited, and vectors lacking, there is less chance of finding diversity. In this view, selection would presumably not play a major role in determining the breakpoints found. Finally, it must be remembered that what are detected are simply recombination breakpoints, not full, infectious, transmissible and fully viable viruses. Recombination in RNA and DNA viruses, in addition to genetic mutations, and to genome reassortment in the case of multicomponent viruses, contributes greatly to virus genome variability (Martin *et al.*, 2011a; Nagy, 2008). In the case of the two virus species examined, only four TYLCSV/TYLCV recombinant viruses have been extensively characterized from field samples thus far, two from Italy (Davino *et al.*, 2009) and two from Spain (Garcìa-Andrès *et al*., 2006; Monci *et al.*, 2002). Their ability to infect plants systemically, induce disease, and be transmitted by the natural whitefly vector, has been demonstrated using infectious clones. It should be stressed that, where the viability of TYLCV/TYLCSV recombinants has been demonstrated, mapping of breakpoints simply gives indications on genomic recombination sites, but does not predict which

recombinant viruses will be viable and eventually spread in field conditions. Despite fears of new epidemics involving the four well-characterized TYLCV/TYLCSV recombinants, their relevance appears limited thus far: only for Tomato yellow leaf curl Malaga virus (TYLCMalV) has an epidemic been reported, in the bean, in Almeria (Spain) in 2000 (Monci *et al*., 2002). No recent data of field surveys highlighting the importance of TYLCV/TYLCSV recombinants in causing epidemics are available. This can be taken as an indication that the fitness of viable recombinant virus variants, generated continuously in mixed infections between similar *Begomoviruses*, is not comparable to that of the parent viruses, at least not in the host plant where they are generated (Sànchez-Campos *et al*., 2002). Our results also show that co-infection with two *Begomoviruses* sharing a certain degree of similarity (above 70% in our case) results in the rapid emergence of a huge number of recombinant variants. Even were it possible to study most of them in depth, and remove defective ones from the list, the final results would still be a multitude of fully viable 'entities'. How should they be classified and named? Are the criteria used thus far for species demarcation in *Begomoviruses* (89% sequence similarity) still valid? Application of this criterion has resulted in the creation of new species (TYLCMalV in Monci *et al*., 2002; TYLCAxV in Garcìa-Andrès *et al*., 2006) for 'entities' that are obviously recombinants between two well-defined viruses. Also, application of the 89% threshold to the case of a multitude of recombinant viral entities deriving from two parents, A and B, would lead to some variants being named strains (or isolates, if very close) of parent species A, others of parent species B, and others as belonging to a different virus species. The resulting taxonomic chaos, with different names for very close viral entities, would not help studies on evolution and phylogeny and, moreover, such a classification of recombinants would not indicate their true origins. Since conventional phylogenetic trees do not provide a reliable picture of the evolutionary relationships between viruses in which recombination has played a major role, a more convenient 'split tree' has been used (Huson & Bryant, 2006). This representation highlights the origins of recombinant viruses, as observed for the

two isolates from Italy: the two recombinant viruses described in Sicily (Davino *et al.*, 2009), TYLCAxV-Sic1-[IT:Sic2/2 : 04] and TYLCAxV-Sic2-[IT:Sic2/5 : 04], are correctly positioned between parent viruses isolated in the same country (Fig. 3). Since genetic variation occurs discontinuously along the genome, classification of recombinants is particularly challenging. The difficulty in demarcating *Begomovirus* species when recombination creates new viral entities has been recognized (Fauquet *et al.*, 2005), but unfortunately the International Committee on Taxonomy of Viruses has not yet found valid alternatives.

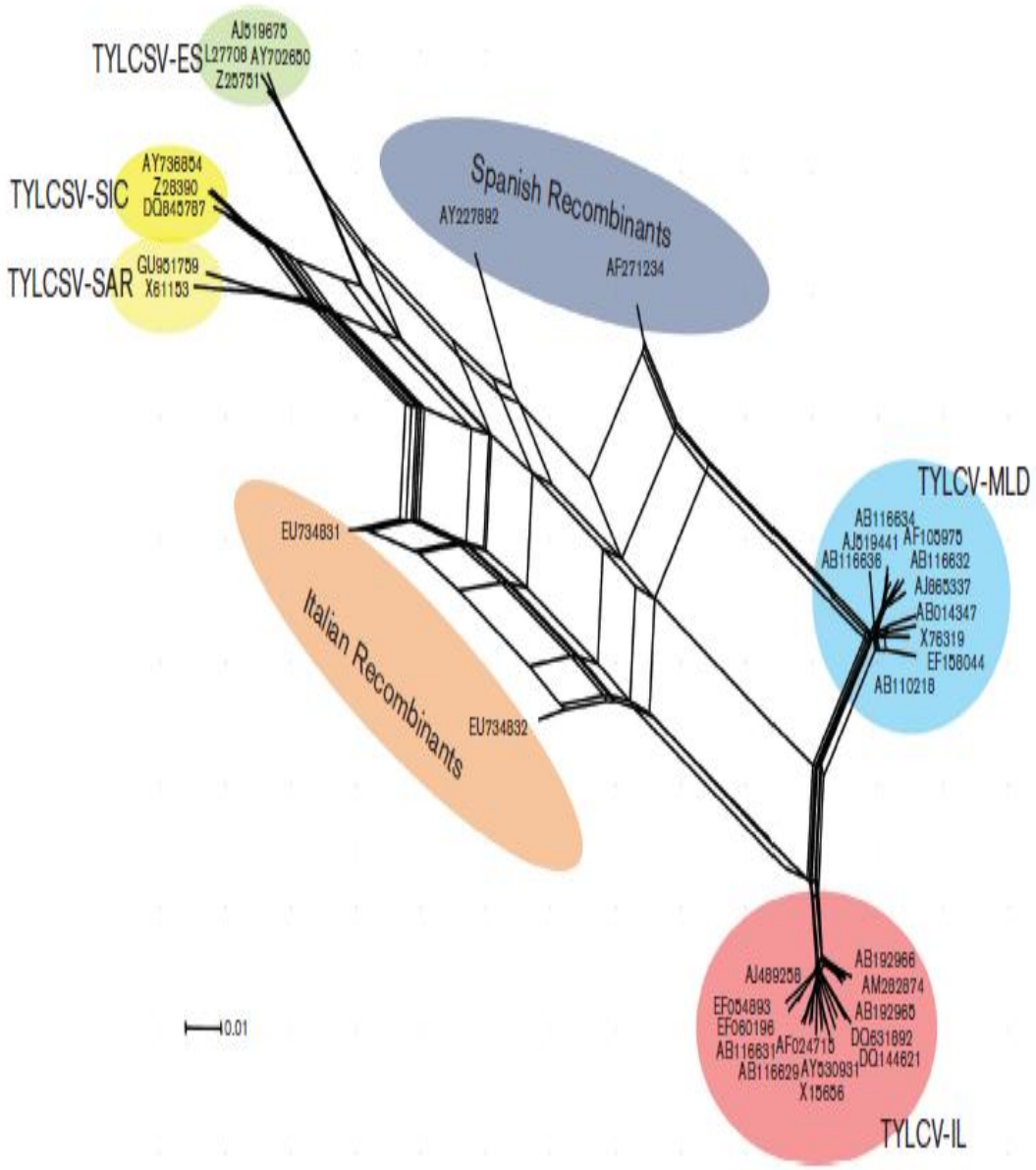

Figure 3 – Phylogenetic network, generated by SplitsTree 4.12.6, of complete genomes of virus isolates causing the tomato yellow leaf curl disease constructed by split-decomposition analysis to visualize reticulated evolutionary relationships produced by recombination. Alignments were obtained using CLUSTAL W. Distance transformation was calculated with the neighbour-net algorithm. GenBank accession numbers of full-length genomes are reported in the figure. Isolates found in Italy are in bold.

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6. THE COMPLETE GENOME SEQUENCE OF *LAMIUM MILD MOSAIC VIRUS*, A MEMBER OF THE GENUS *FABAVIRUS*[*]

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ABSTRACT – *Lamium mild mosaic virus* (LMMV) is the only one of the five members of the genus *Fabavirus* for which there are no nucleotide sequence data. In this study, the complete genome sequence of LMMV was determined and compared with the available complete genome sequences of other members of the genus *Fabavirus*. The genome was the largest of the genus but maintained the typical organization, with RNA 1 of 6080 nucleotides (nt), RNA 2 of 4065 nt, and an unusually long 30 untranslated region in RNA 2 of 603 nt. Phylogenetic analysis of the amino acid sequences of the protease-polymerase (Pro-Pol) region and the two coat proteins confirmed that LMMV belongs to a distinct species within the genus *Fabavirus*.

The genus *Fabavirus*, subfamily *Comovirinae*, family *Secoviridae* [21] consists of five viral species: *Broad bean wilt virus 1* (BBWV-1), *Broad bean wilt virus 2* (BBWV-2), *Gentian mosaic virus* (GeMV), *Cucurbit mild mosaic virus* (CuMMV) and *Lamium mild mosaic virus* (LMMV). Fabaviruses infect a wide range of hosts, including dicotyledonous plants and some families of monocotyledonous plants, and they are transmitted by aphids in a non-persistent

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manner [14]. Members of the genus *Fabavirus* have icosahedral particles and a genome composed of two single-stranded positive-sense RNAs with a genome-linked protein (VPg) covalently attached at the 50 end and a poly A tail at the 30 end. Each RNA encodes a polyprotein that is processed posttranslationally to yield specific proteins. The RNA 1 polyprotein is processed into five proteins involved in genome replication and expression: cofactor of the proteinase (Co-Pro), putative helicase (Hel), viral protein genome-linked (VPg), proteinase (Pro), and RNA-dependent RNA polymerase (Pol). The RNA 2 polyprotein is processed into the movement protein (MP), the large coat protein (LCP), and the small coat protein (SCP). LMMV was found in 1953 in the United Kingdom in Lamium album and other plants of the family *Lamiaceae* that showed mild mosaic or no symptoms [15]. This was the only finding reported for this virus, and presently there is only one isolate available. After *Broad bean wilt virus 1*, *Lamium mild mosaic virus* was the second species established in the genus *Fabavirus* based on host range and serological reactions [13]. Subsequently, another species, *Broad bean wilt virus 2*, was created based on serology [23]. Determination of partial nucleotide (nt) sequences of several BBWV-1 and BBWV-2 isolates confirmed that they were members of two separate species [7]. The other two species of the genus *Fabavirus*, *Gentian mosaic virus* and *Cucurbit mild mosaic virus*, were defined mainly based on nt sequences [2, 9]. Presently, there are complete or almost complete genomic sequences of two BBWV-1 isolates [3, 8], 16 BBWV-2 isolates [5, 6, 10–12, 17, 19, 20], three GeMV isolates [9, 24], and one CuMMV isolate [2]. However, no nucleotide sequence has been reported for LMMV. In this study, the complete genomic sequence of LMMV was determined, and the genetic relationships to the other member of the genus *Fabavirus* were analyzed to clarify its taxonomic position.

LMMV isolate PV-0454, originally collected from a Lamium album plant with mild mosaic symptoms in Cambridge (UK) in 1953, was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures). This isolate has been subsequently multiplied by mechanical inoculation in *Nicotiana clevelandii*. Double-stranded RNA (dsRNA) was purified from LMMV-infected *N.*

clevelandii plants [16]. A cDNA library of LMMV was constructed from the dsRNA by RT-PCR using random primers [4] and cloning into pGEM-T Easy Vector (Promega). The nt sequences of a number of cDNA clones were determined in both directions using a 3130XL Genetic Analyzer (Applied Biosystems). These sequences were used to design specific primers encompassing overlapping regions of the complete genome of LMMV. The 5' and 3' terminal sequences were determined using a 5'/3' RACE Kit, 2nd Generation (Roche). For each genomic region, at least three RT-PCR products were obtained and sequenced in both directions to avoid errors associated with RT-PCR and sequencing. Analysis with BLASTx (http://blast.ncbi. nlm.nih.gov/) showed that these sequences had the highest amino acid (aa) sequence identity (~ 50 %) to sequences of the other fabaviruses BBWV-1, BBWV-2, GeMV and CuMMV. The pool of sequences was assembled with the Staden package [1]. The complete nt sequences of LMMV RNA 1 and RNA 2 were deposited in the GenBank database under accession numbers KC590304 and KC590305, respectively.

LMMV had the typical genome organization of members of the genus *Fabavirus* (Fig. 1A).

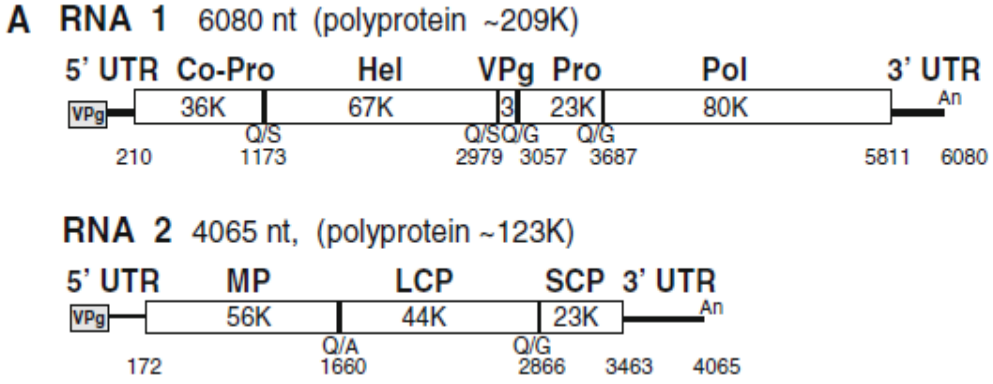

Figure 1 A – Genome organization of LMMV. Horizontal lines indicate untranslated regions; bars represent the polyprotein encoded by long open reading frames (ORFs). The predicted protease cleavage sites are indicated by vertical lines, with the corresponding amino acid sites indicated below them. Numbers below the diagram indicate nucleotide positions of the cleavage sites and the polyprotein start and end.

RNA 1 consisted of 6080 nt, with a 5' untranslated region (5' UTR) of 209 nt and a 30 UTR of 270 nt, excluding the polyA tail. This RNA had a single open reading frame (ORF) of 5601 nt, starting at position 210, encoding a polyprotein of 1866 aa and 209 kDa. By comparison with the sequences of the other Fabaviruses, this polyprotein was predicted to be processed into Co-Pro (321 aa, 36 kDa), Hel (602 aa, 67 kDa), VPg (26 aa, 3 kDa), Pro (210 aa, 23 kDa) and Pol (707 aa, 80 kDa). RNA 2 had 4065 nt, a 5' UTR of 171 nt and the longest 3' UTR (603 nt) of any member of the genus *Fabavirus* (Fig. 1A). This RNA contained one ORF of 3291 nt, starting at position 172, encoding a polyprotein of 1096 aa and 123 kDa, which was predicted to be cleaved into MP (496 aa, 56 kDa), LCP (402 aa, 44 kDa) and SCP (198 aa, 23 kDa).

The complete genome sequence of LMMV was aligned with all of the complete genome sequences available for viral isolates belonging to the genus *Fabavirus* (retrieved from GenBank) using the algorithm CLUSTALW implemented in the program MEGA 5.05 [22]. Nucleotide and amino acid sequence identities for different genomic regions between LMMV and the other fabaviruses were also calculated with the program MEGA 5.05. The aa sequence identity of LMMV to other fabaviruses ranged from 51.1 to 56.0 % for polyprotein 1 and from 45.1 to 49.1 % for polyprotein 2 (Table 1).

However, identity values varied for the different proteins, with the lowest aa sequence identity to BBWV-2 Co-Pro (34.5 %) and the highest aa sequence identity to GeMV VPg (61.5 %). The nt sequence identity was higher in the 5' UTR (~ 75 % with BBWV-1 or BBWV-2 and ~ 50 % with GeMV or CuMMV) than in the 3' UTR (~ 37 % with BBWV-1 or BBWV-2 and ~ 27 % with GeMV or CuMMV). To study the taxonomic position of LMMV, the aa sequences of two genomic regions considered for species demarcation, the conserved Pro-Pol region and both coat proteins [21], of different viral isolates representing the three genera of the subfamily *Comovirinae* – *Fabavirus*, *Comovirus* and *Nepovirus* – were retrieved from GenBank and aligned. The phylogenetic relationships were inferred by the neighbor-joining and maximum-likelihood methods [18] with the Dayhoff model for amino acid substitution and 1000 bootstrap replicates to

estimate the statistical significance of each node, using the program MEGA 5.05. The phylogenic topology was identical in the two genomic regions analyzed, and therefore only the phylogenetic tree of the conserved Pro-Pol region is shown (Fig. 1B).

Table 1 Nucleotide and amino acid sequence identity of different genomic regions of LMMV to those of the other members of the genus *Fabavirus*: BBWV-1, BBWV-2, GeMV and CuMMV

Genome	Region	BBWV-1	BBWV-2	GeMV	CuMMV
RNA 1	5′-UTR	74.2-75.8	75.0-78.0	43.9-47.7	51.5
	PP1	56.4-56.7[a] (51.5-51.9)[b]	55.7-56.8 (52.1-52.8)	56.0-56.3 (52.7-53.2)	58.3 (56.0)
	Co-Pro	47.4-49.0 (37.4-38.5)	45.1-49.0 (34.5-37.9)	46.4-47.8 (35.6-38.6)	52.6 (46.6)
	Hel	58.2-59.3 (54.6-56.0)	58.4-59.6 (55.5-56.4)	57.9-58.5 (55.8-56.4)	59.6 (56.5)
	VPg	59.0-61.5 (50.0-57.7)	50.0-57.7 (44.9-57.7)	64.1-67.9 (61.5-61.5)	55.1 (50.0)
	Pro	51.5-51.7 (46.2-47.6)	50.6-53.1 (43.3-45.2)	51.2-52.7 (45.2-46.2)	58.6 (52.9)
	RNA Pol	57.7-57.8 (53.4-53.5)	56.6-59.0 (54.9-55.9)	57.4-58.5 (55.8-55.9)	59.5 (58.9)
	3′-UTR	39.1-42.0	40.6-47.8	43.5-46.4	37.7
RNA 2	5′-UTR	71.9-75-3	75.3-80.9	36.0-50.6	48.3
	PP2	54.3-54.8 (47.8-49.1)	52.9-54.5 (45.1-47.9)	53.4-54.0 (45.8-47.2)	53.7 (48.9)
	MP	53.1-54.7 (46.6-46.9)	52.5-56.2 (46.6-47.8)	51.8-53.7 (43.5-45.7)	52.5 (49.7)
	LCP	54.7-56.3 (52.5-53.5)	53.2-54.7 (46.0-51.0)	53.8-55.1 (49.8-51.0)	54.6 (50.5)
	SCP	50.6-52.9 (38.1-40.1)	49.2-53.1 (39.5-41.5)	51.0-53.5 (39.5-40.8)	55.6 (44.2)
	3′UTR	36.5-41.3	38.1-46.0	25.4-28.6	28.6

[a] Nucleotide sequence identity values

[b] Amino acid sequence identity values are shown in parentheses

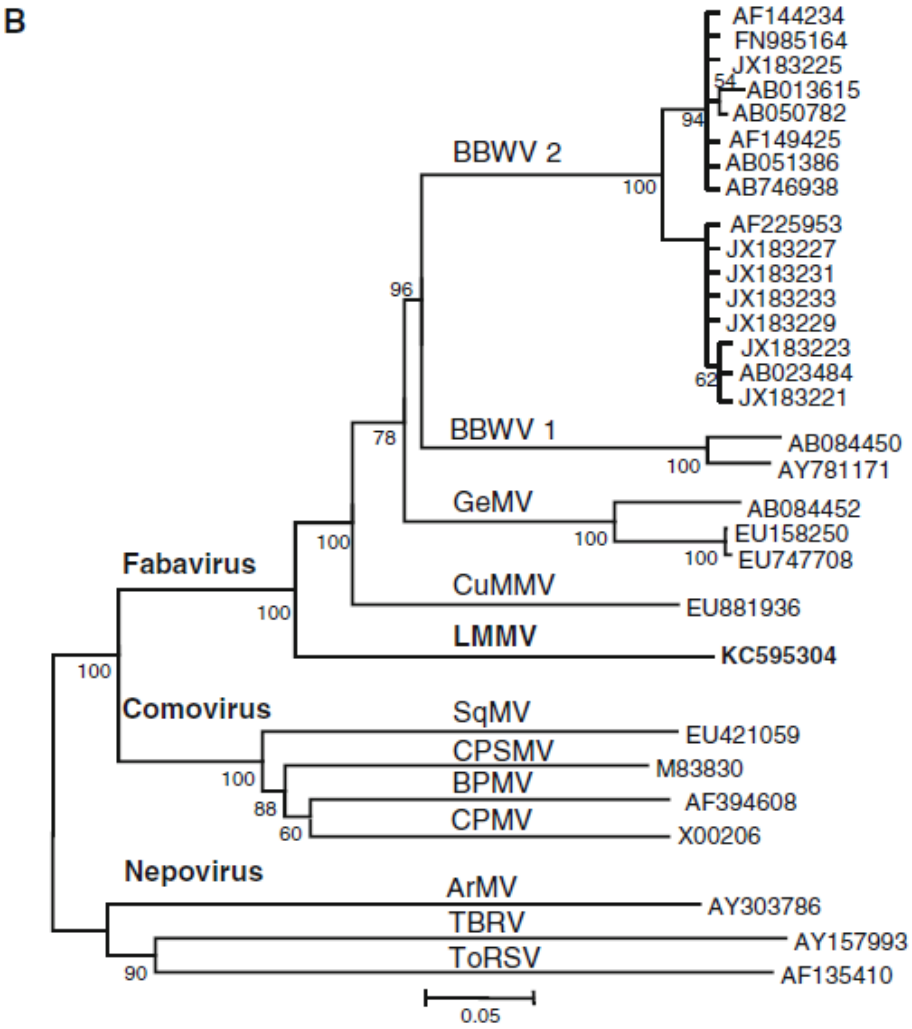

Figure 1B – Phylogenetic relationships of the conserved region Pro-Pol (used as a taxonomic criterion) of members of the genera *Fabavirus*, *Comovirus* and *Nepovirus* of the subfamily *Comovirinae*. The phylogenetic tree includes (I) all viral isolates (whose complete genome sequence is available) belonging to the five species of the genus *Fabavirus*, BBWV-1, BBWV-2, GeMV, CuMMV and LMMV, and (II) for the other two genera, sequences of one isolate of *Squash mosaic virus* (SqMV), *Cowpea severe mosaic virus* (CPSMV), *Bean pod mottle virus* (BPMV), *Cowpea mosaic virus* (CPMV), *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV) and *Tomato ring spot virus*. Nodes with bootstraps values lower than 50 % were condensed.

The phylogenetic tree clearly reflected the taxonomic classification at three levels. First, there were three main clades corresponding to the three genera: *Fabavirus*, *Comovirus* and *Nepovirus*, with aa sequence identities ranging from 33.4 to 47.8 %. In the genus *Fabavirus*, there were five subclades corresponding to the five

species of this genus: *Broad bean wilt virus 2, Broad bean wilt virus 1, Gentian mosaic virus, Cucurbit mild mosaic virus* and *Lamium mild mosaic v*irus, with aa sequence identities ranging from 58.4 to 70.3 %. Finally, aa sequence identities between viral isolates from the same species of the genus *Fabavirus* were higher than 88.8 %. Therefore, this analysis confirmed that LMMV belongs to a separate species in the genus *Fabavirus*.

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7. DETECTION AND IDENTIFICATION OF *FABAVIRUS* SPECIES BY ONE-STEP RT-PCR AND MULTIPLEX RT-PCR*

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ABSTRACT – The genus *Fabavirus* of the family *Secoviridae* comprises a group of poorly characterized viruses. To date, only five species have been described: *Broad bean wilt virus* 1 (BBWV-1), *Broad bean wilt virus* 2 (BBWV-2), *Lamium mild mosaic virus* (LMMV), *Gentian mosaic virus* (GeMV) and *Cucurbit mild mosaic virus* (CuMMV). This article reports the development of two RT-PCR procedures for the detection and identification of *Fabavirus* species: a one-step RT-PCR using a single pair of conserved primers for the detection of all fabaviruses, and a one-step multiplex RT-PCR using species-specific primers for

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the simultaneous detection and identification of the above-mentioned species of the genus *Fabavirus*. These methods were successfully applied to field samples and the results were compared with those obtained by molecular hybridization and ELISA. The combination of the two techniques enables a rapid, sensitive and reliable identification of the five known *Fabavirus* species, as well as the possibility of discovering new species of this genus.

The viruses of the genus *Fabavirus*, subfamily *Comovirinae*, family *Secoviridae* (Sanfaçon *et al.*, 2009), infect a wide range of plants, including economically important vegetable and ornamental crops (e.g. pepper, tomato, bean, broad bean and cucumber) and are transmitted by several aphid species in a non-persistent manner (Ferriol *et al.*, 2013; Lisa and Boccardo 1996). The nucleocapsid structure of fabavirus virions displays icosahedral symmetry, with a diameter of approximately 30 nm and incorporates two coat proteins: a large coat protein (LCP) and a small coat protein (SCP). The genome is composed of two single-stranded, positive-sense genomic RNA molecules (RNA1 and RNA2), whose 3' termini are polyadenylated and encapsidated separately (Sanfaçon *et al.*, 2009). RNA1 encodes a polyprotein that is proteolytically processed into five functional proteins involved in genome replication and expression: protease cofactor (Co-Pro), helicase (Hel), genome-linked viral protein (VPg), protease (Pro) and polymerase (Pol). In turn, RNA2 encodes a polyprotein that is processed into movement protein (MP), large coat protein (LCP) and small coat protein (SCP).
Currently, the genus *Fabavirus* includes five species: *Broad bean wilt virus* 1 (BBWV-1), *Broad bean wilt virus* 2 (BBWV-2), *Lamium mild mosaic virus* (LMMV), *Gentian mosaic virus* (GeMV), and *Cucurbit mild mosaic virus* (CuMMV). BBWV-1 and BBWV-2 are distributed worldwide (Ferrer *et al.*, 2007), while LMMV has been reported in the United Kingdom only (Lisa *et al.*, 1982); GeMV has been found in Japan and China (Kobayashi *et al.*, 2005; Wang *et al.*, 2008), and CuMMV was recently reported in China (Dong *et al.*, 2012). The availability of sensitive and rapid techniques for the detection and

identification of the different *Fabavirus* species is crucial to study their epidemiology and to implement effective disease control strategies. Classically, electron microscopy and biological characterization, based on host range and aphid transmissibility, have been used to ascribe viral isolates to the genus *Fabavirus* (Lisa and Boccardo 1996; Ochoa-Corona *et al*., 2010). However, such methods are not able to identify single fabavirus species, and have the added disadvantages of being cumbersome, expensive and time-consuming. In order to overcome these drawbacks, enzyme-linked immunosorbent assay (ELISA) techniques using polyclonal antibodies were developed (Lisa *et al*. 1982; Kobayashi *et al.*, 2005; Dong *et al*., 2012; Uyemoto and Provvidenti 1974). However, commercial antibodies are available only for BBWV-1 and BBWV-2, and some cross-reactions have been observed between BBWV-2 antibodies and BBWV-1 infected samples (Lisa and Boccardo, 1996). The availability of nucleotide sequences of BBWV-1 (Ferrer *et al*., 2005; Kobayashi *et al*., 1999; Kobayashi *et al.*, 2003), BBWV-2 (Ferrer *et al.*, 2011; Ikegami *et al*., 1998; Ikegami *et al.*, 2000; Koh *et al.*, 2001; Kuroda *et al*., 2000; Lee *et al*., 2000; Nakamura *et al.*, 1998; Qi *et al.*, 2000a; Qi *et al*., 2000b), GeMV (Kobayashi *et al.*, 2005) and CuMMV (Dong *et al.*, 2012) has enabled the development of more sensitive and specific diagnostic methods based on molecular hybridization and reverse transcription, followed by polymerase chain reaction (RT-PCR). These techniques include: 1) molecular hybridization for the detection of BBWV-1 (Ferrer *et al*., 2008); 2) real time RT-PCR for specific detection and quantification of BBWV-1 and BBWV-2 (Ferriol *et al.*, 2011); and 3) simultaneous detection and identification of BBWV-1, BBWV-2 and GeMV using conserved primers (Ferrer *et al*., 2007). Recently, full-length genome sequences of CuMMV and LMMV have been reported, and specific primers for the detection of CuMMV have been developed (Dong *et al.*, 2012; Rangel *et al.*, 2013). However, there are still no broad-spectrum methods available for the detection and identification of all *Fabavirus* species.

The present article reports new methods developed by our research group for: 1) broad-range detection of all members of this genus, including unknown species,

by one-step RT-PCR with a pair of conserved primers; and 2) simultaneous detection and identification of the five currently recognized *Fabavirus* species by multiplex RT-PCR with five species-specific primer pairs. The combination of both of these techniques enables a rapid and sensitive detection and a simultaneous identification of the five species of the genus *Fabavirus* (BBWV-1, BBWV-2, GeMV, CuMMV and LMMV).

Full-length and partial nucleotide sequences of *Fabavirus* isolates were obtained from the GenBank database: BBWV-1 (accession numbers: ab084450, ab084451, af225955, ay781171, ay781172), BBWV-2 (ab011007, ab013615, ab013616, ab018698, ab023484, ab032403, ab050782, ab051386, ab746938, ab746939, af104335, af144234, af149425, af225953, af225954, af228423, aj132844, fn985164, gq202215, hq283389, hq283390, jf704084, jx183221, jx183222, jx183223, jx183224, jx183225, jx183226, jx183227, jx183228, jx183229, jx183230, jx183231, jx183232, jx183233, jx183234, jx575182, kc110085), GeMV (ab084452, ab084453, eu158249, eu719113, eu719114, eu747708), CuMMV (eu881936, eu881937) and LMMV (KC595304, KC595305). Nucleotide sequences were aligned with CLUSTALW (Larkin *et al.,* 2007) and imported into the Geneious Pro 5.4.6 software (Biomatters, New Zealand). Nucleotide identities between sequence pairs were estimated with the MEGA 5.05 program (Tamura *et al.,* 2011), and oligonucleotide primers for RT-PCR were designed using Primer Express 2.0 (Applied Biosystems, USA) and Vector NTI 9.0 (Invitrogen, USA). Based on the aligned sequences, we attempted to design a multiplex RT-PCR with six pairs of primers, one corresponding to a genus-specific conserved genomic region and five species-specific primers for each known *Fabavirus* species. However, computer analysis and RT-PCR assays revealed that none of the primers designed from the genus-specific conserved genomic regions were compatible with the species-specific primers (data not shown). Consequently, two separate procedures were developed: a one-step RT-PCR with conserved primers for a broad-range detection of fabaviruses, and a multiplex RT-PCR for the identification of the five distinct species belonging to this genus. The conserved primers were designed from conserved amino acid

sequences from the RNA2 located at positions ~1000 nt and 1600 nt, respectively (Table 1).

Table 1 – Primers designed from conserved nucleotide regions of the genus *Fabavirus*

Primer	Nucleotide sequence (5' - 3')	Virus[a]	Positions[b]	Size[c]	GenBank[d]
FabaF	AAGGCGTGAYTCIGAYTTYGAYGA	BBWV-1	RNA2: 1062-1501	461	AF225955
		BBWV-2	RNA2: 1138-1607	490	AF225954
FabaR	CTTCCAACACITCYTIYTCCAT	GeMV	RNA2: 999-1453	475	AB084453
		CuMMV	RNA2: 972-1420	470	EU881937
		LMMV	RNA2: 1049-1665	638	KC590305

[a]Viruses of the genus *Fabavirus*: BBWV-1, BBWV-2, GeMV, CuMMV and LMMV.

[b]Genomic position of the primers for each virus.

[c]Predicted size of the amplicons for each virus.

[d]GenBank accession numbers of the sequences used to indicate genomic position and amplicon size.

The primers for multiplex RT-PCR were designed based on: 1) nucleotide sequences specific to each virus, but conserved between isolates; 2) compatibility between primers so that they could be used in the same RT-PCR; and 3) differences in RT-PCR product size from each virus, so that they could be identified by electrophoresis. The design process to obtain specific primers for BBWV-1, BBWV-2 and GeMV was very complex due to the high genetic variability of these viruses (Kobayashi *et al.,* 2005; Wang *et al.,* 2008; Ferrer *et al.,* 2005; Ferrer *et al.,* 2011; Kobayashi *et al.,* 1999) in comparison with most other plant viruses (García-Arenal *et al.,* 2001; Tiberini *et al.,* 2011; Rangel *et al.,* 2011; Davino *et al.,* 2012). The primers were designed to ensure that the expected sizes of the RT-PCR products were approximately 622, 510, 370, 238 and 98 bp for LMMV, BBWV-1, GeMV, BBWV-2 and CuMMV, respectively (Table 2).

Table 2 – Primers designed for simultaneous detection and discrimination of viruses in the genus *Fabavirus* by multiplex RT-PCR

Primer	Nucleotide sequence (5' - 3') [a]	Virus[b]	Position[c]	Size[d]	GenBank[e]
LMMV-4157F LMMV-4755R	AGGAAAGGCCAGATATTTCGAA GACGGAGTAAGCATAACGTACCAG	LMMV	RNA1: 4157 RNA1: 4755	622	KC590304
BBWV1-4758F BBWV1-5245R	TGACACATATGTGGCCATGG CAGATTCTCAAGTTGGTGACAGG	BBWV-1	RNA1: 4758 RNA1: 5245	510	AB084450
GeMV-2497F GeMV-2846R	ACGATAAACCCCTTAGCGG CAAATGAAGCTTCTCCTGCAC	GeMV	RNA1: 2497 RNA1: 2846	370	AB084452
BBWV2-5692F BBWV2-5907R	CAGAGTTCAGTAGTTCCTGCTTATG GGCATTTCAACCCTGCATAATAC	BBWV-2	RNA1: 5692 RNA1: 5907	238	AB013615
CuMMV-419F CuMMV-496R	GAGAAATGATTGTCACTGAGAAGGT CACGGGCACTATAGCATAACC	CuMMV	RNA1: 419 RNA1: 496	98	EU881936

[a]Nucleotide sequences specific for each virus of the genus *Fabavirus* and conserved for the isolates of each virus.

[b]Viruses of the genus *Fabavirus*: BBWV-1, BBWV-2, GeMV, CuMMV and LMMV.

[c]Genomic position of the primers for each virus.

[d]Predicted size of the amplicons for each virus.

[e]GenBank accession numbers of the sequences used to indicate genomic position and amplicon size.

To confirm the specificity of the designed primers to the genus *Fabavirus*, a nucleotide sequence similarity analysis was carried out by BLAST, using GenBank database samples as comparison (Altschul *et al.*, 1990), and adjusting the relevant parameters to search for a short input sequence. To ascertain that our RT-PCR primers do not amplify non-specific products from related viruses, they were analyzed with the Vector NTI 9.0 program (Invitrogen, USA) against sequences of other genera of the family *Secoviridae*: *Cowpea mosaic virus* (genus *Comovirus*), *Tomato torrado virus* (genus *Torradovirus*) and *Tomato ringspot virus* (genus *Nepovirus*). In all cases, the nucleotide identities between the primers and the complete sequences from the other viruses ranged from 40 to 70 %, indicating that our primers are specific for the genus *Fabavirus*.

The viral isolates of the genus *Fabavirus* used in this study are shown in Table 3; these include six genetically diverse BBWV-1 isolates (Ferriol *et al.*, 2013), four

genetically diverse BBWV-2 isolates (Ferrer *et al.*, 2011), one GeMV isolate (Kobayashi *et al*., 2005), one CuMMV isolate (Dong *et al*., 2012) and one LMMV isolate (Rangel *et al.*, 2013). The isolates were maintained by a successive passage in different hosts: *Vicia faba*, *Nicotiana benthamiana*, *N. clevelandii*, and *Chenopodium quinoa*. For each isolate, approximately 100 ng of total RNA, purified with the UltraClean® Plant RNA Isolation Kit (Mobio, Spain), was denatured at 95°C for 5 min, chilled on ice and immediately used for RT-PCR. Broad-range detection of fabaviruses was performed by one-step RT-PCR in a Mastercycler Gradient thermal cycler (Eppendorf, Germany) with the conserved primers FabaF and FabaR (Table 1), using as templates the total RNA extracted from plants infected with BBWV-1 isolate PV132, BBWV-2 isolate IP, GeMV isolate N-1, LMMV isolate PV0454 and CuMMV isolate Beijing (Table 3).

Table 3 – Viral isolates of viruses in the genus *Fabavirus* used for specificity assays.

Virus[a]	Isolate	Original host	Origin	Provider/reference[b]
BBWV-1	PV132	*Spinacia oleracea*	USA (New York)	ATCC
	Ben	*Capsicum annuum*	Spain	Rubio *et al.*, 2002
	PV176	*Nasturtium*	United Kingdom	ATCC
	PV0548	*Vicia faba*	Syria	DSMZ
	480-3	*Capsicum annuum*	Czech Republic	Dr. Cervená
	B41/99	*Capsicum annuum*	Bulgaria	Dr. Kostova
BBWV-2	IP	*Capsicum annuum*	Japan	NIAS
	PV131	*Lactuca sativa*	USA (New York)	ATCC
	94/1996	*Pisum sativum*	South Africa	Dr. A.E.C. Jooste
	PV0550	*Gentiana sp.*	Germany	DSMZ
GeMV	N-1	*Gentiana scabra*	Japan	NIAS
CuMMV	Beijing	*Curcubita moschatta*	China	Dong *et al.*, 2012
LMMV	PV0454	*Lamium orvala*	United Kingdom	DSMZ

[a]BBWV-1 = *Broad bean wilt virus* 1, BBWV-2 = *Broad bean wilt virus* 2, GeMV = *Gentian mosaic virus*, LMMV = *Lamium mild mosaic virus.*

[b]ATCC= American Type Culture Collection; DSMZ= Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), NIAS= National Institute of Agrobiological Sciences of Japan.

RT-PCR was performed in a single step, using a 25 µl reaction volume containing 10 mM Tris–HCl (pH 8.9), 50 mM $MgCl_2$, 0.3% Triton X-100 (w/v), 1 µM of each primer, 250 µM dNTPs, 0.25 units AMV-RT (Promega, Spain) and 0.5 units GoTaq DNA polymerase (Promega, Spain). Cycling was performed through a reverse transcription at 42 ºC for 45 min followed by PCR using a touch-up protocol consisting of 40 cycles: the first five at 94 ºC for 30 s, 40 ºC for 30 s and 72 ºC for 40 s, followed by five cycles at 94 ºC for 30 s, 45 ºC for 30 s and 72 ºC for 40 s, followed by the remaining cycles at 94 ºC for 30 s, 50 ºC for 30 s and 72 ºC for 40 s, with a final step of 5 min at 72 ºC. No amplification products were obtained from healthy broad bean controls. The cDNA fragments amplified from BBWV-1, BBWV-2, GeMV and CuMMV were of a similar size (460-490 bp) and indistinguishable by electrophoresis; whereas the amplicon of LMMV was larger (638 bp) and could be identified by electrophoresis (Fig. 1).

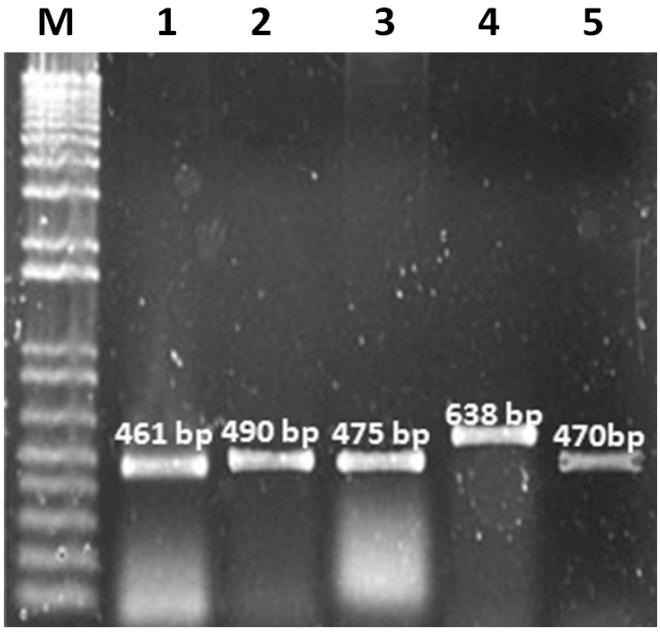

Figure 1 – RT-PCR products of the five species of the genus *Fabavirus* obtained with primers FabaF and FabaR (Table 1). Lane 1: *Broad bean wilt virus 1* (BBWV-1) isolate PV132, Lane 2: *Broad bean wilt virus 2* (BBWV-2) isolate IP, Lane 3: *Gentian mosaic virus* (GeMV) isolate N-1, Lane 4: *Lamium mild mosaic v*irus (LMMV) isolate PV0454 and Lane 5: *Cucurbit mild mosaic virus* (CuMMV) isolate Beijing (Table 3). M= Marker 1KB plus (Invitrogen). 2% agarose gel stained with Ethidium bromide.

RT-PCR with conserved primers has also been reported for a number of plant virus genera or families, e.g. the genus *Torradovirus* (Verbeek *et al.,* 2012), *Nepovirus* (Wei and Clover 2008), *Ilarvirus* (Untiveros *et al*., 2010), *Potexviru*s (van der Vlugt, René AA and Berendsen 2002), *Tobravirus* (Jones *et al.,* 2008) and *Begomovirus* (Davino *et al.,* 2008); the subfamily *Comovirinae* (Maliogka *et al.,* 2004) and the families *Bromoviridae* (Untiveros *et al.,* 2010) and *Luteoviridae* (Chomič *et al.,* 2010).

The simultaneous detection of all the species of the genus *Fabavirus* was performed by multiplex RT-PCR. First, individual RT-PCRs with specific primers were carried out separately for each virus (Table 2) and RNA extracts of virus-infected and healthy (negative control) plants. Primers BBWV1-4758F and BBWV1-5245R were used for BBWV-1 isolate PV132; primers BBWV2-5692F and BBWV2-5907R for BBWV-2 isolate IP; primers GeMV-2497F and GeMV-2846R for GeMV isolate N-1; primers LMMV-4157F and LMMV-4755R for LMMV isolate PV0454; and primers CuMMV-419F and CuMMV-496R for CuMMV isolate Beijing. All RT-PCRs were performed in a final volume of 25 µl, containing 10 mM Tris–HCl (pH 8.9), 50 mM $MgCl_2$, 0.3% Triton X-100 (w/v), 1 µM of each primer, 250 µM dNTPs, 0.25 units AMV-RT (Promega, Spain) and 0.5 units GoTaq DNA polymerase (Promega, Spain). Reactions were performed as described above. RT was performed at 42 ºC for 45 min and PCR involved 40 cycles of 30 s at 94 ºC, 30 s at 60 ºC and 40 s at 72 ºC, followed by a final extension of 5 min at 72 ºC. Electrophoresis showed no amplification products from healthy plants and a single cDNA species of the expected size for each fabavirus species: BBWV-1, BBWV-2, GeMV, CuMMV and LMMV (data not shown).

To assess primer compatibility, the previously obtained cDNA species were cloned in an apCR2.1 – TOPO vector (TOPO-TA Cloning Kit, Invitrogen, Italy). The virus identity of each clone was confirmed by sequencing in both directions, using universal primers T7 and SP6 with an ABI PRISM DNA 377 sequencer (Perkin-Elmer, Boston, MA, USA). The concentration of the cDNA clones was estimated with a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo

Scientific, USA) and adjusted to 1 ng/µl. Subsequently, multiplex PCR with the five primer pairs corresponding to BBWV-1, BBWV-2, GeMV, CuMMV and LMMV was repeated five times, using one of each fabavirus species clones as a template. Moreover, a multiplex PCR was performed with these five pairs of primers and an equimolar mixture of the five fabavirus species clones. When only one fabavirus species was used as template, the multiplex PCR with the five primers amplified only one cDNA species of the expected size, whereas using the mixture as a template yielded five cDNA species easily distinguishable in 2% agarose gel (Fig. 2).

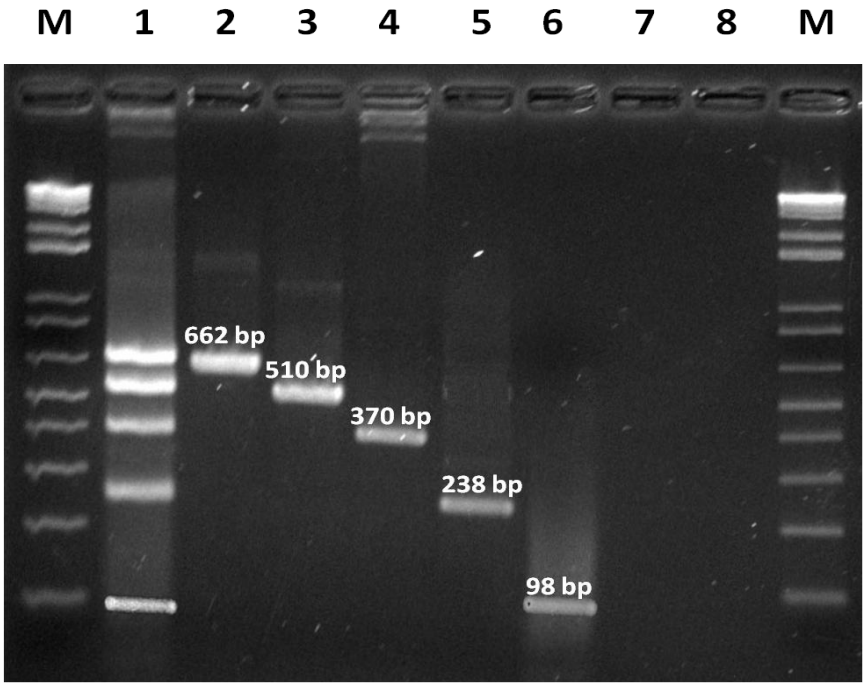

Figure 2 – Multiplex RT-PCRs of the five species of the genus Fabavirus with a mix of five primer pairs (Table 2) using as template cDNA clones. Lane 2: *Lamium mild mosaic virus* (LMMV) isolate PV0454, Lane 3: *Broad bean wilt virus* 1 (BBWV-1) isolate PV132, Lane 4: *Gentian mosaic virus* (GeMV) isolate N-1, Lane 5: *Broad bean wilt virus* 2 (BBWV-2) isolate IP, Lane 6: *Cucurbit mild mosaic virus* (CuMMV) isolate Beijing (Table 3), Lane 7: negative control (broad bean healthy plant), Lane 8: negative control (water) and Lane 1: a mix of these isolates (BBWV-1 + BBWV-2 + GeMV + CuMMV + LMMV). M= Marker 1KB plus (Invitrogen). 2% agarose gel stained with Ethidium bromide.

To determine the sensitivity of detection, tenfold serial dilutions ranging from 1 to 10^{-4} ng with extracts of total RNAs from healthy broad bean plants were prepared for each viral cDNA clone. Combinations of viral cDNA clones of different fabavirus species with distinct proportions were used as template in multiplex PCRs, and amplified in amounts ranging from 1 to 10^{-4} ng. The assay conducted with known amounts of target DNA showed that this method can detect a single *Fabavirus* species even when its quantity is 1000 times lower than that of another one. As a consequence, it is expected that in plants with mixed infections, one *Fabavirus* species can be detected and identified even when it is a minor component of the prevailing *Fabavirus* population.

The multiplex RT-PCR procedure was tested with total RNA extracts from plants infected with isolates of the different *Fabavirus* species, using healthy plants as negative controls. Since no mixed infections involving two or more different *Fabavirus* species were found in the field samples, equimolar mixtures of total RNA extracts containing different fabaviruses were analyzed. All samples showed RT-PCR products of the expected size (Fig. 3), indicating that multiplex RT-PCR can be used for the simultaneous identification of different fabaviruses in plants with single or mixed infections. No amplicons were obtained from healthy tomato, pepper, cucumber and broad bean plants.

Finally, the methods developed were applied to field samples. Twenty pepper plants and five broad bean plants from Spain were collected and tested by the broad-spectrum one-step RT-PCR for the genus *Fabavirus*; moreover, the multiplex RT-PCR with specific primers was used for the identification of the five known *Fabavirus* species. Samples were also analyzed by ELISA and molecular hybridization methods. ELISA was performed with commercial polyclonal antibodies, anti-BBWV-1-IgG and anti-BBWV-2-IgG, raised in rabbit (DSMZ), for the detection of BBWV-1 and BBWV-2. About 0.3 g of plant tissue was blended with 10 volumes of phosphate buffer saline, (PBS) containing 0.05% Tween 20, 2% polyvinyl pyrrolidone (PVP-10000) and 0.2% BSA, using a Polytron homogenizer (Kinematica, Littau, Switzerland). Plate coating, antibody incubation and washings were performed according to the manufacturer's

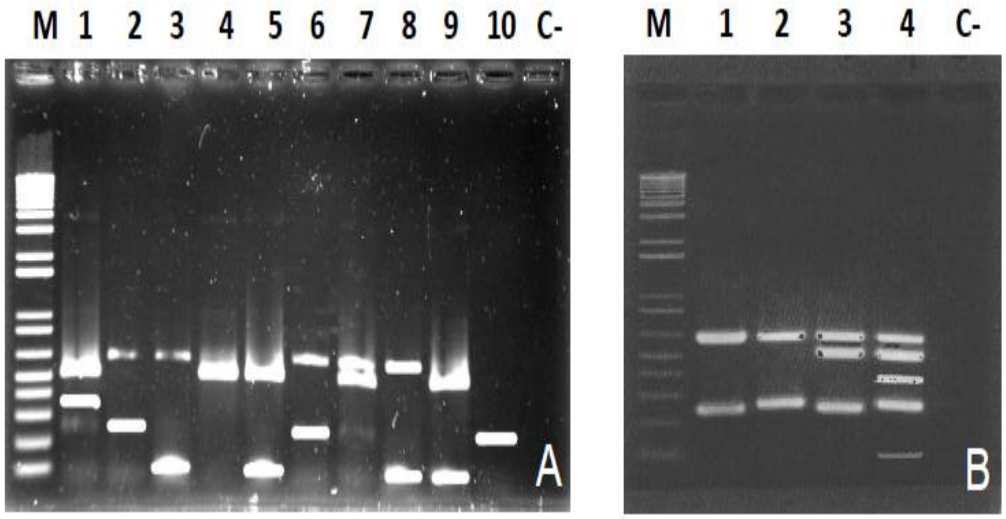

Figure 3 – Multiplex RT-PCRs with a mix of five primer pairs (Table 2) using as template RNA extracts of different fabavirus samples (Table 3). **Panel A** – Lane 1: BBWV-1 isolate Ben + GeMV isolate N-1, Lane 2: BBWV-2 isolate PV131 + LMMV isolate PV0454, Lane 3: LMMV isolate PV0454 + CuMMV isolate Beijing, Lane 4: BBWV-1 isolate PV176, Lane 5: BBWV-1 isolate PV0548 + CuMMV isolate Beijing, Lane 6: BBWV-2 isolate 94/1996 + LMMV isolate PV0454, Lane 7: BBWV-1 isolate 480-3 + LMMV isolate PV0454, Lane 8: LMMV isolate PV0454 + CuMMV isolate Beijing, Lane 9: BBWV-1 isolate B41/99 + CuMMV isolate Beijing, Lane 10: BBWV-2 isolate PV0550, and Lane C-: negative control of non-infected plant (broad bean). M= Marker 1KB plus (Invitrogen). **Panel B –** Lane M: Marker 1KB plus (Invitrogen), Lane 1: LMMV isolate PV0454 + BBWV-2 isolate 94/1996, Lane 2: LMMV isolate PV0454 + BBWV-2 isolate PV131, Lane 3: LMMV isolate PV0454 + BBWV-1 isolate PV176 + BBWV-2 isolate PV131, Lane 4: LMMV isolate PV0454 + BBWV-1 isolate PV176 + GeMV isolate N-1 + BBWV-2 isolate PV131 + CuMMV isolate Beijing, Lane C-: negative control (broad bean healthy plant).

instructions, and optical density (OD) at 405 nm was measured with a Titertek Multiscan_ Plus (Laboratory Systems, Helsinki, Finland) or a BioRad 3550 Microplate reader. Only readings that measured at least three times the mean reading of the negative controls were considered positive. Molecular hybridization was performed at 55 ºC with two digoxigenin-labeled cDNA probes: the A_{Ben} probe, specific and conserved for BBWV-1 isolates, and the A_{PV537} probe, specific

and conserved for BBWV-2 isolates (Ferrer et al. 2008). The total RNA used was extracted with the UltraClean® Plant RNA Isolation Kit (Mobio, Spain) according to the manufacturer's specifications. Eleven pepper plants and three broad bean plants tested positive by one-step RT-PCR using the FabaF and FabaR primers. Only BBWV-1 was detected in all samples by multiplex RT-PCR (data not shown). These results were confirmed by ELISA and molecular hybridization. Even though they can infect economically important crops, fabaviruses have been poorly characterized to date. The absence of diagnostic techniques has also contributed to the lack of knowledge on this group of viruses, and to the consequent underestimation of their impact. The singleplex RT-PCR with conserved primers for the genus *Fabavirus* and the multiplex RT-PCR with species-specific primers reported in this article could be valuable tools for the evaluation of *Fabavirus* distribution worldwide, discovery of new *Fabavirus* species, and implementation of integrated pest management procedures including plant breeding, certification and quarantine programs, eradication, etc.

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8. DETECTION AND IDENTIFICATION OF VIRUSES OF A PLANT VIRUS GENUS BY FLOW-THROUGH HYBRIDIZATION OF TISSUE PRINTS*

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ABSTRACT – Viruses cause important damage in agricultural crops worldwide. Disease management requires reliable, sensitive and specific tools for virus detection and identification. Detection techniques should be of rapid design and application to keep pace with the continuous emergence of new virus diseases. In this work, we developed a procedure based on flow-through hybridization, sensitive enough to detect tissue-prints of virus-infected plants. This is the fastest detection technique, able to analyze 100 samples in less than one hour. We designed digoxigenin-labeled RNA probes with two levels of specificity: A) one probe able to hybridize with all members of the genus *Fabavirus*, being the first described case of a genus-specific probe, and B) probes specific for each viral

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species of this genus. The genetic variability within species was considered for a universal detection of all viral isolates within species to minimize false negatives. The procedure developed here enables a very rapid analysis of numerous samples to identify the known viral species of the genus *Fabavirus* and discover new species of this genus.

INTRODUCTION

Viruses cause considerable economical losses in agriculture every year. Disease control is difficult due to the complex and dynamic epidemiology, with frequent emergence cases and outbreaks, and high evolvability and adaptability of viruses (Acosta *et al.*, 2011). Specific and sensitive diagnostic or detection tools are pivotal for any disease management procedure. These tools should be rapid and easy to design to keep pace with the continuous emergence of new viral diseases. Presently, the three most commonly used techniques are based on detection of proteins: Enzyme Linked Immuno Sorbent Assay (ELISA) and nucleic acids: molecular hybridization and polymerase chain reaction (PCR) for DNA or reverse transcription and PCR (RT-PCR) for RNA. ELISA can analyze many samples simultaneously and has become the most common method for routine detection of plant viruses (Clark *et al.,* 1977). However, nucleic acid detection techniques are much easier to develop and allow control of the level of specificity (family, genus, species and strain or isolate group) based on the nucleotide sequence identity between the target and the probes or primers. RT-PCR or PCR is the most sensitive technique but requires meticulousness to avoid false positives by contamination or false negatives by inhibitors and it is not appropriate for large scale analyses. Molecular hybridization is usually more sensitive than ELISA (Ferrer *et al.,* 2008; Galipienso *et al.,* 2004) and also suited to analyze many samples simultaneously. Additionally, hybridization of tissue-prints saves time and labor by avoiding preparation of plant extracts (Narváez *et al.,* 2000; Rubio *et al.*, 2003).

Conventional hybridization is based on incubating probes with samples fixed in membranes, washing and revealing by a colorimetric or a chemical luminescent

reaction, or using fluorescence or radioactivity. An alternative to passive incubation is flow-through hybridization (US Patent 6,638,760), which directs the flow of the reacting molecules towards the targets immobilized on nylon membranes by applying a negative pressure using a vacuum pump. This dramatically reduces hybridization time, but it has only been used once for plant virus detection (Olmos *et al.,* 2007). However, in this case the sensitivity of the method was insufficient to directly detect nucleic acid extracts from the infected plants, and required an isothermal amplification prior to hybridization.

To develop a procedure based on flow-through hybridization for sensitive detection of plant viruses we used virus isolates of the genus *Fabavirus*. This genus is composed of five viral species (Sanfaçon *et al*., 2009): *Broad bean wilt virus* 1 (BBWV-1), BBWV-2, *Lamium mild mosaic virus* (LMMV), *Gentian mosaic virus* (GeMV), and *Cucurbit mild mosaic virus* (CuMMV). Fabaviruses infects a wide range of plant species causing damage to many agricultural crops worldwide and is rapidly dispersed by aphids (Lisa *et al*., 1996). The genome is composed of two single-stranded positive-sense RNA molecules of about 6 and 4 kb. Both RNAs are translated into single polyprotein precursors, from which functional proteins are derived by proteolytic cleavage. RNA 1 encodes proteins involved in genome replication and expression and RNA 2 encodes the movement protein and two coat proteins (Rangel *et al.,* 2013). Presently, there are commercial polyclonal antibodies to detect BBWV-1 and BBWV-2 by ELISA, although some cross reactions can occur. RT-PCR procedures were developed for detection of BBWV-1 or BBWV-2 (Ferrer *et al.,* 2008; Ferriol *et al.,* 2011), simultaneous detection of BBWV-1, BBWV-2 and GeMV (Ferrer *et al.,* 2007), and after sequence determination of CuMMV (Dong *et al.,* 2012) and LMMV (Rangel *et al.,* 2013), simultaneous detection of the five known fabaviruses (Panno *et al.,* 2014). Molecular hybridization were developed for detection of BBWV-1 or BBWV-2 (Ferrer *et al*., 2008).

In this work, we developed a procedure based on flow-through hybridization of tissue-prints with five probes specific for the five viral species of the genus *Fabavirus* and a general probe which hybridized with all viruses of this genus. To

our knowledge this is the first case of detection of all members of a viral genus by hybridization with a unique probe. The developed procedure enables analysis of a large number of samples in a record time to identify the known species and discover new species of the genus *Fabavirus*.

MATERIAL AND METHODS

VIRUSES AND PLANTS

Ten viral isolates corresponding to the five species of the genus *Fabavirus*: BBWV-1, BBWV-2, GeMV, CuMMV and LMMV and *Tobacco ringspot virus* (TRSV) of the genus *Nepovirus* were used (Table 1).

Table 1 – *Fabavirus* isolates used in this work

Virus	Isolate	Original host	Origin	Provider
BBWV-1	Ben	*Capsicum anuum*	Spain (Castellón)	Collected
	PV0132	*Spinacia oleracea*	USA (New York)	ATCC
	PV0548	*Vicia faba*	Syria	DSMZ
BBWV-2	IP	*Capsicum anuum*	Japan (Hiroshima)	NIAS
	PV0131	*Lactuca sativa*	USA (New York)	ATCC
	L1926	*Verbena sp.*	Netherlands	DSMZ
GeMV	N-1	*Gentiana scabra*	Japan (Nagano)	NIAS
CuMMV	Beijing	*Cucurbita moschata*	China (Beijing)	Collected
LMMV	PV0454	*Lamium orvala*	UK (Cambridge)	DSMZ
TRSV	PV0236	*Phaseolus vulgaris*	USA (Maryland)	DSMZ

ATCC= American Type Culture Collection; DSMZ= Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), NIAS= National Institute of Agrobiological Sciences of Japan.

Some viral isolates were mechanically inoculated to *Vicia faba*, *Chenopodium quinoa*, *Nicotiana megalosiphon*, *N. clevelandii*, *Capsicum annuum*, *Vicia faba* and *Pisum sativum* by grinding in a mortar virus-infected plant material in chilled in 0.01 M phosphate buffer, pH 7.0, and rubbing it with carborundum onto plants with the first true leaves. Plants were grown in an insect-free greenhouse. Twenty

pepper samples from field surveys in Southern Spain on March 2012 were used for preparation of tissue-prints.

RNA EXTRACTION

Total RNAs were extracted from 0.5 - 3 g of fresh or desiccated leaf tissue from virus-infected or healthy plants by using a standard protocol based on phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation (Ferriol *et al.*, 2011). The desiccated tissue was re-hydrated with RNase-free water and homogenized in a power homogenizer TissueLyser (Qiagen) with liquid nitrogen. Then, 500 µl of extraction buffer (Tris-HCL 0.2M, EDTA 0.05M, NaCl 0.2M, SDS 0.2%) and 2.5 µl of 2-β mercaptoethanol were added to the homogenized tissue sample, followed by 5 min incubation in ice. Afterwards, 500 µl of phenol:choloroform:isoamyl alcohol 25:24:1 (v:v:v) were added to the sample following by a centrifugation at 12000 rpm during 10 min at 4 ºC. The aqueous phase was transferred to a fresh tube and mixed with 450 µl of phenol:chloroform:isoamyl alcohol (25:24:1 (v:v:v)) followed by a centrifugation at 12000 rpm during 15 min. Then, the RNA was precipitated by mixing the aqueous phase with 3 volumes of ethanol and 0.1 volume of sodium acetate 3M pH 5.2 and incubating for 30 min at -80 ºC. After a centrifugation at 12000 rpm during 30 min, the pellet containing the RNA was washed with ethanol 70%, eluted in 25 µl of RNase-free water and treated with RNase-free DNase (TurboDNA-free, Ambion). RNA concentration was measured in duplicate with the UV-Vis spectrophotometer nanodrop 1000 (Termo Scientific) and adjusted to approximately 10 ng/µl and stored at –80 ºC until use.

SYNTHESIS OF CDNA: RT-PCR AND CLONING

About 100 ng of total RNAs were denatured by heating at 95 °C for 5 minutes and used as template to synthesize cDNA by RT-PCR amplification with three pair of primers (Table 2).

Table 2 – Probes synthesized in this work.

Probe	Virus	Isolate[a]	Position[b]	Primers[c]
Faba/5'RNA1	BBWV-1	Ben	RNA1 (11-363)	Fab5'R1F / Fab5'R1R
BBWV1/MP	BBWV-1	Ben	RNA2 (1062-1501)	FabaF / Faba R
BBWV2/MP	BBWV-2	IP	RNA2 (1138-1607)	FabaF / Faba R
GeMV/MP	GeMV	N-1	RNA2 (999-1453)	FabaF / Faba R
CuMMV/MP	CuMMV		RNA2 (972-1420)	FabaF / Faba R
LMMV/MP	LMMV	PV0454	RNA2 (1049-1665)	FabaF / Faba R
TRSV/MP	TRSV	PV0236	RNA2	TRSVCPF / TRSVCPR

[a]Isolate used as template for probe synthesis.

[b]Position with respect to nucleotide sequences with Genbank Accessions: AY781171, AY781172, AB018698, AB084453, KC595305, EU881937 and JQ670669

[c]Primer pairs used for probe preparation:

A. Fab5'R1F / Fab5'R1R AAATATTAAAACAAACAGCTTTCGTT/TTCAAAGCTCGTGCCATNTYATTKGC (Ferrer *et al.*, 2007),
B. FabaF / Faba R AAGGCGTGAYTCIGAYTTYGAYGA/CTTCCAACACITCYTIYTCCAT (Panno *et al.*, 2014), and
C. TRSVCPF / TRSVCPR TGTTVTGGGGCCCACATCAGAT/ CCAGTRGCTGCRACAAGCCA (primes designed here).

The denatured RNAs were reverse-transcribed for 1 h at 42 °C in a 20 µl reaction mixture containing First Strand Buffer (Invitrogen), 1mM DTT, 1mM each of dCTP, dATP, dGTP and dTTP, 0.4 µM of an oligo-dT primer ($T_{17}V$), 10 units of RNAaseOUT inhibitor and 100 units of SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen). An aliquot (1/10) of this preparation was added to a 20 µl reaction mixture containing PCR Buffer (Invitrogen), 1.5 mM $MgCl_2$, 1mM of each of the four dNTPs, 0.2 µM of primers of each primer, 5 U of Taq DNA

polymerase (Invitrogen). After an initial denaturizing step at 94 °C for 3 min, PCR was performed using 40 cycles of denaturizing at 94 ºC for 30 s, annealing at 55 ºC for 30 s, and elongation at 72 ºC for 45 s, and a final extension step at 72 ºC for 5 min. RT-PCR products were visualized by electrophoresis in 2% agarose gels and ethidium bromide staining. The resulting RT-PCR products were purified and cloned in pGEMT (Promega). The virus identity of each clone was confirmed by sequencing, using universal primers T7 and SP6 with an ABI PRISM DNA 377 sequencer (Perkin-Elmer).

IN VITRO TRANSCRIPTION: SYNTHESIS OF RNA PROBES AND RNA TEMPLATES

To generate the probes, PCR was performed from cDNA clones with the same forward primers indicated above (Table 2) but with modified versions of the reverse primers including the T7 promote sequence. The resulting PCR products were also purified with GFX PCR DNA kit (GE Healthcare) and used as a template to generate digoxigenin labeled single-strand negative-sense RNA transcripts with the Megascript T7 Kit (Ambion) and the Dig RNA labeling mix (Roche). The transcripts were treated with DNase I RNase free (Roche) and purified with the Rnaid w/Spin Kit (Q-BIO gene). RNA concentration was also measured with the spectrophotometer nanodrop 1000 (Termo Scientific).

To evaluate sensitivity, single-stranded positive-sense RNA transcripts were synthesized as described above but without the Dig Rna labeling mix (Roche) and using as template PCR products obtained from the BBWV-1 cDNA clone with a modified version of the forward primer Fab5'R1F that included the T7-promoter and the reverse primer Fab5'R1R. RNA concentration was measured by the spectrophotometer NanoDrop™ 1000 (Thermo Scientific). Conversion of micrograms of single stranded RNA (ssRNA) to picomoles was performed considering the average molecular weight of a ribonucleotide (340 pg) and the number of bases of the transcript (N_b). The following formula was applied: pmol of ssRNA = µg of ssRNA x (10^6 pg/1µg) x (1 pmol/340 pg) x (1/ N_b). Avogadro's constant (6.023 x 10^{23} molecules/mol) was used to estimate the number of ssRNA copies.

MOLECULAR HYBRIDIZATION

Three types of samples were used as hybridization targets: *in vitro* transcripts, total RNA extracts and tissue-prints. One µl of different dilutions of transcripts or RNA extracts (without denaturing) was spotted on nylon membranes positively charged (Roche). Tissue prints were produced by transversely cutting leaf petioles and gently pressing the nylon membrane. The samples were fixed by irradiating with UV light (250 mJ) in a cross-linking oven.

Conventional hybridization was performed by incubating the membranes in 10 ml of ULTRAhyb buffer (Ambion) at 68 ºC for 1-2 h (prehybridization) and also at 68 ºC overnight after adding 20 ng of probe per each ml of buffer (hybridization). Membranes were washed twice with 2 x SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS at room temperature, and twice more with 0.1 x SSC and 0.1% SDS at 68 ºC; they were equilibrated with 100mM maleic acid, 150mM NaCl pH 7,5, 0.3% (v/v) Tween 20 and blocked with blocking buffer [100mM maleic acid, 150mM NaCl pH 7.5 and 1% (w/v) blocking reagent (Roche)]. Antidigoxigenin-alkaline phosphatase antibodies (Roche) were subsequently added at a concentration of 150 U/ml diluted in blocking buffer, and membranes were washed twice as in the previous steps with 100mM maleic acid, 150mM NaCl, 0.3% (v/v) Tween 20, pH 7.5 and equilibrated for 2 min with 100mM Tris/HCl, 100mM NaCl, pH 9.5. The reaction was revealed using the chemiluminescent substrate CDP-star (Roche) exposing the film for 10 - 30 min at 37 ºC.

Flow-through hybridization was carried out using a Hybrimax device (Hybrivio Limited) which applied a negative pressure using a vacuum pump, so that all solutions flowed through the membrane automatically. The same procedure and conditions were used as described above but using small volumes of these solutions, just enough to cover the membrane. Vacuum pressure reduced each hybridization step to 30-60 s (Table 3).

Table 3 – Comparison between different procedures for analysis of 96 samples

A) Sample preparation

Steps	Dot-blot	Tissue-print
Sample handling	15 min	15 min
Homogenization	5 min	
Phenolization	2 h	
Precipitation	1 h	
Elution	5 min	

B) Hybridization procedure

Steps	Time		Volume of solutions	
	Conventional	Flow-through	Conventional	Flow-through
Prehibridization	1 h	1 min	10 ml	1.25 ml
Hybridization *	overnight	1 min	10 ml (200 ng)	1.25 ml (25 ng)
Washing 4x steps	4x 5 min	4x 1 min	4x 10 ml	4x 1.25 ml
Revealing 2x steps	30 min, 1 h	2x 1 min	2x 10 ml	2x 1.25 ml
Washing 2x steps	2x 5 min	2x 1 min	2x 10 ml	2x 1.25 ml

* Probe quantity is indicated between parentheses.

NUCLEOTIDE SEQUENCE ANALYSES

To evaluate hybridization specificity, nucleotide identity between probes and target (percentage of positions with the same nucleotide) was estimated. Nucleotide sequences of the viral isolates used in this work were retrieved from GenBank which had accession numbers: AB084450-AB084453, AB018698, AB023484, AY78171, AY78172, EU881936, EU881937, JF440076, JQ670669, JX304792, HQ602948, HQ602951, HQ602956, HQ602959, KC595304 and KC595305. The program MEGA 5.05 (Tamura *et al.*, 2011) was used for multiple

sequence alignment with the procedure CLUSTAL W and estimation of p-distance (proportion of distinct nucleotides between two sequences). Nucleotide identity was calculated as (1 – p-distance) x 100.

RESULTS

DEVELOPMENT AND EVALUATION OF A SENSITIVE AND RAPID PROCEDURE FOR VIRUS DETECTION BASED ON FLOW-THROUGH HYBRIDIZATION OF TISSUE-PRINTS

To develop and test a procedure based flow-through hybridization, the RNA probe BBWV-1/MP and RNA extracts of BBWV-1 isolate Ben were used (Table 1 and 2). Among all hybridization temperatures and washing procedures assayed, the best performance was with 68 ºC for hybridization and the most astringent washing conditions (see Material and methods), which gave no background signal in the negative controls of non-infected plants (data not shown). The procedure was much faster and needed much less quantity of reagents than conventional hybridization (Table 3).

To evaluate the sensitivity, ten-fold serial dilutions of BBWV-1 positive-sense RNA transcripts containing from 10^{10} to 10^{7} RNA copies were analyzed by conventional hybridization and flow-through hybridization. Both techniques had a detection limit of 10^{8} RNA molecules (Fig. 1A).

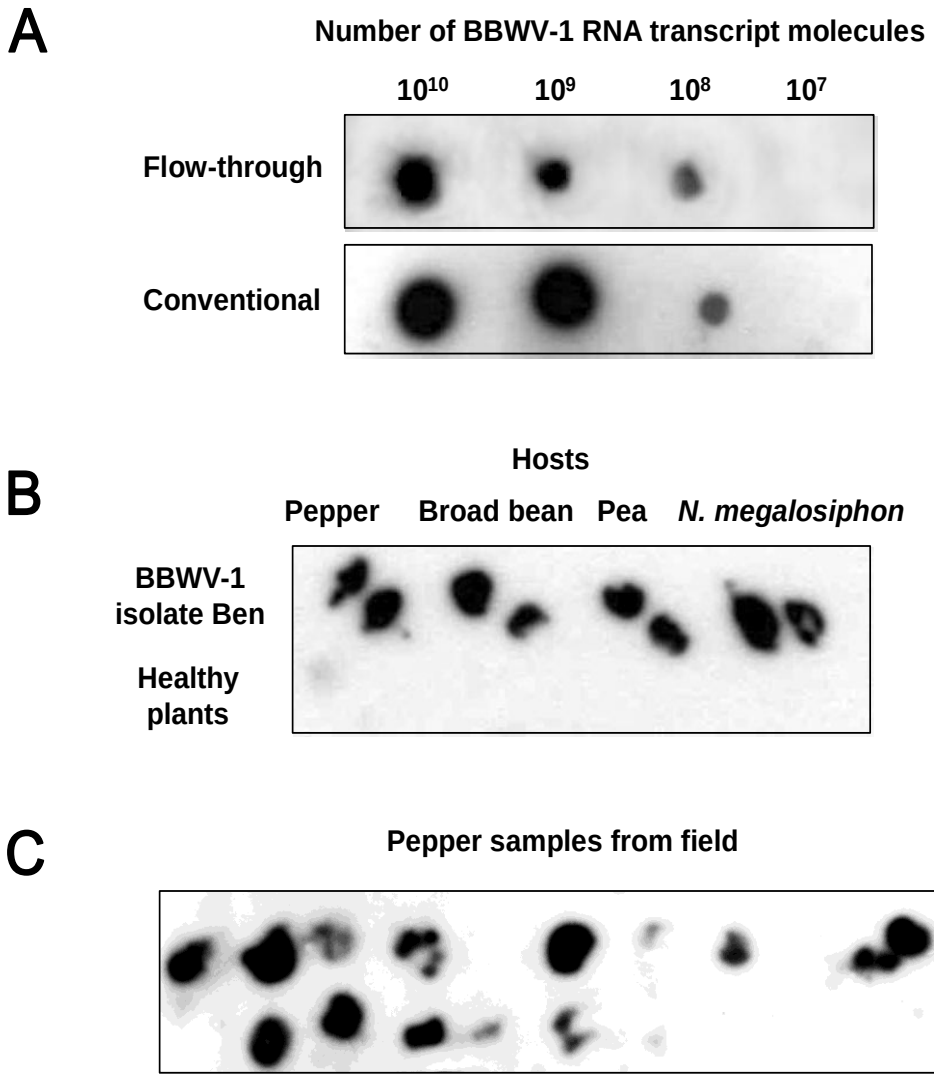

Figure 1 – Molecular hybridization with probe BBWV-1/MP (Table 2): **A)** Flow-through and conventional hybridization of serial dilutions of transcripts of BBWV-1 isolate Ben whose concentrations were adjusted from 10^{10} to 10^{7} RNA molecules. **B)** Flow-through hybridization of tissue prints of different hosts infected with BBWV-1 isolate Ben. **C)** Flow-through hybridization of tissue-prints prepared in pepper fields.

To test if sample processing can be avoided, tissue-prints from plants of pepper (*Capsicum annuum*), broad bean (*Vicia faba*), pea (*Pisum sativum*) and *Nicotiana megalosiphon*, which were non-infected or infected by BBWV-1 isolate Ben, were analyzed by flow-through hybridization. A strong hybridization signal was observed in all BBWV-1 infected plants whereas no signal was observed in tissue prints from healthy plants (Fig. 1B).

Finally, to test if flow-through hybridization is suitable for analysis of field samples, tissue-prints of twenty pepper plants were prepared directly in field from a survey carried out in Southern Spain. Flow-through hybridization showed that eleven plants were infected by BBWV-1 (Fig. 1C). The positive and negative samples were confirmed by conventional dot-blot hybridization, RT-PCR and ELISA (data not showed).

FLOW-THROUGH HYBRIDISATION WITH PROBES WITH DIFFERENT LEVELS OF SPECIFICITY

The genetic variability within and between the different viral species of the genus *Fabavirus* and between this viral genus with other viruses was considered to obtain probes with two levels of specificity: A) a genus-specific probe hybridizing with all viruses of the genus *Fabavirus* and no hybridizing with viruses from other genetically related genera, and B) species-specific probes hybridizing with all isolates within a species and no hybridizing with other related viral species.

Comparison of all completely sequenced genomes of fabaviruses showed several perfect or imperfect repeats of ten nucleotides (AACAGCUUUC) in the 5' untranslated region (UTR) of the two genomic RNAs of the five fabavirus species known so far: BBWV-1, BBWV-2, GeMV, CuMMV and LMMV (Table 4).

Table 4 – Nucleotide sequences of the 5'UTRs of both genomic RNAs of the five species of the genus *Fabavirus* with highlighted nucleotide repeats.

	RNA 1	RNA 2
BBWV-1	UGAUUUAAAAAAUUUUUAAAUCAAACAGC UUUCGUUCGGAAUUAAAACAGCUUUCUUU CAAACAGCUUUCGGAUACUUAAACAGCUU UCA	UGAUUUAAAAAAUUUUUAAAUCAAACAGCU UUCGUUCCGAAAUUUAACAGCUUUCAGAAAC UUUCAAACAGCUUUCA
BBWV-2	UGUUUUAAUAAAAUAUUAAAACAAACAGC UUUCGUUCCGAAAACAGCUUUCAGUUACU AAACAGCUUUCGUUUCGAUUAAACAGCCU UCAAACAAACAGCUUUCAUUUCA	UGUUUUAAUAAAAUAUUAAAACAAACAGCU UUCGUUCCGAAAAACAGCUUUCAAAUUUCAA ACAGCUUUCAG
GeMV	UGUUUAAAUUAAAUAUUUAAACAAACAGC UUUCGUUACUAAAACAGCUUUCGGUUACG AUUCUCUUUCUUCAAUCUUUUCUUUCAAA CAGCUUUCUGAAUACUUUCUUUCUGAAAC GCCUUCUUAAACAGCUUUCU	GUUUAAAUAAAAUAUUUAAACAAACAGCUU UCGUCCGGUUACCCGCUUUCGGUUACUUUUA AACAGCUUUCAGAACAGCUUUCAAAACCCAA ACAGCUUUCAA
CuMMV	UUUAAGAAAUUAAACAACAGCUUUCGUAC CGAAUACAGCUUUCGUGUUUUAAAAGCUU UCAUUUCUGGCGUUUGGCAAGAAUUUGAU UGCUCUCAAAACAGCUUUCGUUCCCAAAAC AGCUUUCGUUCAAACAGCUUUCGUUCCUA AAACAGCUUUCGUU	UUUAAGAAAUUAAACAACAGCUUUCGUACCG GAAAACAGCUUUCGACACAGCUUUCAGUUCU GGCGUUUGGCAAGAAUCUGCAAUAGCUUUCU GAGUGAAUACAGCUUUCA
LMMV	GUUAAAGAAAAAUUCUUUGGCAAACAGCU UUCGUUACUAAAACAGCUUUCGUUCGAUA AAACAGCUUUCGUUACAAACAGCUUUCAG AAAACAGCUUUCA	GUUAAAAUAAAAUAUUAUUUUGACAAAACA GCUUUCGUUACUAAGGUUACAAAACAGCUUU CGUUACCAAACAGCUUUCAAAAAUCUAACAG CUUUCAG

These repeats are not present in other viruses or organisms. They are a hallmark of the genus *Fabavirus* and can be used as a taxonomic criterion.

To test if the nucleotide repeats would enabled hybridization with samples from the five *Fabavirus* species in spite of the low global nucleotide identity (Table 5), the probe Faba/5'RNA1, containing the 5'UTR of RNA 1 of BBWV-1 isolate Ben, was designed (Table 2).

Table 5 – Nucleotide identity between probes and samples.

Species[a]	Isolate	Probes							
		BBWV15' 5'UTR	BBWV15' Cod*	BBWV1MP Cod	BBWV2MP Cod	GeMVMP Cod	CuMMVMP Cod	LMMVMP Cod	TRSVMP Cod
BBWV-1	Ben	100.0	100.0	100.0	64.4	66.0	59.7	60.7	17.7
	PV132	89,5	87.0	83.2	62.3	65.4	63.4	56.5	19.4
	PV-0548	92,6	93.5	95.3	64.9	67.0	60.7	61.8	17.7
BBWV-2	IP	64,2	48.6	64.4	100.0	71.2	65.4	59.2	21.0
	PV131	60,0	52.9	61.8	84.8	69.1	59.7	58.1	19.4
	PV0537	60,0	51.4	63.9	83.8	69.1	58.1	57.6	17.7
GeMV	N-1	40,0	50.7	66.0	71.2	100.0	61.8	59.7	16.1
CuMMV		25,3	44.9	59.7	65.4	61.8	100.0	56.0	17.7
LMMV	PV0454	47,4	44.2	60.7	59.2	59.7	56.0	100.0	14.5
TRSV		27,4	32.6	17.7	21.0	16.1	17.7	14.5	100.0

*Cod: coding region

Positive reactions between probes and samples are shadowed

TRSV of the genus *Nepovirus* was chosen as an outgroup representative as this genus together with the genera *Fabavirus* and *Comovirus* form the subfamily *Comovirinae* (REF). The flow-through hybridization technique with the probe Faba/5'RNA1 gave strong hybridization signals with all viral isolates of BBWV-1, BBWV-2, GeMV, CuMMV and LMMV whereas non-infected plants or plants infected with TRSV gave no signal (Fig. 2).

The strong signal between the probe TRSV/MP and the TRSV sample ensured that the negative reaction between the probe Faba/5'RNA1 and the TRSV samples was due to the low nucleotide identity (Table 4) and not by the bad quality and/or low concentration of RNA extracts. This result showed that this procedure enables universal and specific detection of the genus *Fabavirus*.

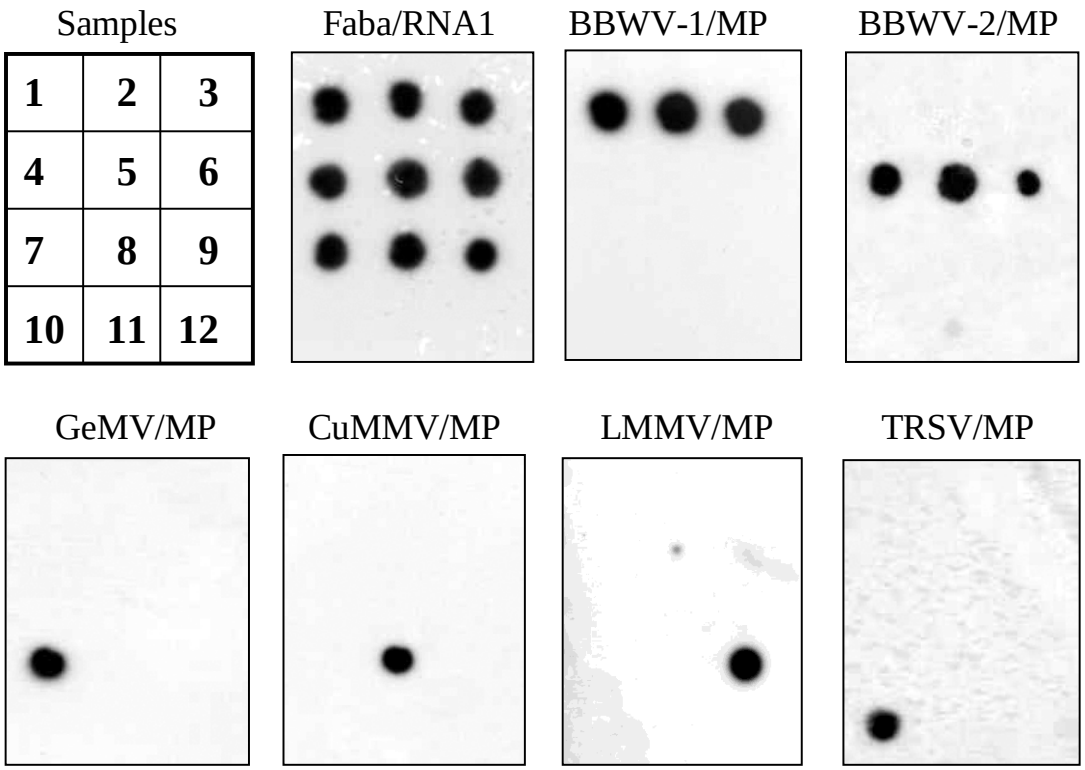

Figure 2 – Flow-through hybridization of RNA samples of BBWV-1 isolates Ben (1), PV132 (2) and PV0548 (3), BBWV-2 isolates IP (4), PV131 (5) and L19260 (6), GeMV isolate N-1 (7), CuMMV isolate Beijing (8), LMMV isolate PV0454 (9), TRSV isolate PV0236 (10), and healthy plants of pepper (11) and broad bean (12) with different probes (Table 2).

Five probes comprising the movement protein coding region were designed for specific detection of BBWV-1, BBWV-2, GeMV, CuMMV and LMMV, (Table 2). For this study we chose three isolates of BBWV-1 and three isolates of BBWV-2 (Table 1) which were genetically very divergent and therefore representative of the genetic spectrum of these two viral species (Ferriol *et al.*, 2013; Ferrer *et al.,* 2011), whereas only one isolate was available for the other three fabaviruses: GeMV, CuMMV and LMMV. Nucleotide identity was 80.0% between isolates from the same viral specie and lower than 75% between isolates from different species (Table 5). The probe BBWV-1/MP gave a strong signal with only the three BBWV-1 isolates and the probe BBWV-2 with only the three BBWV-2 isolates, whereas no signal was obtained with the rest of the samples. These results shows that these probes enables the universal detection of all isolates of each viral species in spite of their high nucleotide diversity and are specific for BBWV-1 and BBWV-2, respectively. The probes GeMV/MP, CuMMV/MP and LMMV/MP reacted only with their corresponding viral species showing also specificity. Therefore, these probes can be used for detection and identification of the five known species of the genus *Fabavirus*.

DISCUSSION

For any strategy for disease control is crucial to have rapid, sensitive and specific methods for detection and identification of pathogens.

A very rapid procedure based on flow-through hybridization without requirement of a previous DNA amplification (Olmos *et al.,* 2007) was developed for the first time for a plant virus, which reduced dramatically the analysis time to about 30 min. This procedure was applied to analyze tissue-prints which also saves time by avoiding RNA extraction and is very convenient for simultaneous analysis of multiple samples. Hybridization of tissue prints is suited for most plant viruses although it has been used only for detection of a few viruses (Navas-Castillo *et al.*, 1999; Galipienso *et al.*, 2004; Rubio *et al.,* 2003; Narváez *et al.,* 2000).

A good sensitivity is very important to minimize false negatives. This was improved by using: a RNA probe for detection of viruses with RNA genomes

since RNA-RNA union is stronger than that with DNA probes, ULTRAhyb buffer (Ambion), and a cheminiluscent development. Our analysis showed that flow-through hybridization with digoxigenin probes had the same sensitivity than conventional hybridization for BBWV-1 and similar to conventional hybridization of other viruses (Galipienso *et al.,* 2005; Galipienso *et al.,* 2004), so this method can be used for most plant viruses.

Specificity is paramount to avoid or minimize false positives in plants infected with other viruses. We considered two levels of specificity: viral species and genus. An added difficulty for specific detection of viruses of the genus *Fabavirus* is the high genetic variability of BBWV-1 and BBWV-2 (Ferriol *et al.,* 2013; Ferrer *et al.,* 2011) in comparison with most plant viruses (García-Arenal *et al.,* 2001). We designed RNA probes comprising the movement protein coding region of the five species of the genus *Fabavirus*. BBWV-1/MP and BBWV-2/MP probes detected genetically divergent isolates of BBWV-1 or BBWV-2 (with nucleotide identity as low as 83.3%), respectively, without reacting with the other *Fabavirus* species. This contrasts with previous results with DNA probes from the same genomic region that discriminated groups of isolates according the BBWV-1 isolates from which the probes were obtained (Ferrer *et al.,* 2008).

Nucleotide analysis showed several repeats (AACAGCUUUC) in the genome of all fabaviruses, not present in other viruses or organisms. They can be considered as a hallmark of the genus *Fabavirus* and used as a taxonomic criterium. Taking advantage of this, we designed a RNA probe comprising the repeats which was able to detect all isolates assayed of the five known *Fabavirus* species. This is the first time that a universal probe for simultaneous detection of all members of a viral genus has been developed, but it has been also achieved for the viroidal genus *Coleviroid* (Jiang *et al.,* 2013). Simultaneous detection of a group of plant viruses can be also performed by conventional hybridization with a combination of probes or polyprobes (Minutillo *et al.,* 2012; Sánchez-Navarro *et al.,* 1999; Herranz *et al.,* 2005; Aparicio *et al.,* 2009). The advantage of using of a universal probe for a taxonomic group of viruses is the possibility of finding new viruses.

The procedure developed here constitutes two milestones: A) the fastest detection technique for plant viruses, and B) the first universal probe for a genus of plant viruses which with the combination of species-specific probes enables the simultaneous identification of known viruses and discovery of new viruses within the genus.

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CONCLUDING REMARKS

Viruses are the fastest evolving entities existing on the Word. Virus–host co-evolution is a continuous process involving both the host immune system and viral escape mechanisms, and is considered to be an important factor in the maintenance of genetic variation in resistance to this group of pathogens (Syller, 2012). One of the most interesting aspects of virus–host co-evolution is the variety of pathways of interactions between the two partners. Especially important aspects are the outcomes of multiple infections in terms of the final fitness exhibited by viral populations selected by competitive interactions. Viral evolution depends on numerous aspect, such as, synergistic interactions between related viruses invading the same cells, error during the process of virus replication, especially for RNA viruses, recombination or pseudo-recombination events that facilitate the emergence of novel virus variants (García- Arenal *et al*., 2003; Malpica *et al*., 2006; Méndez-Lozano *et al*., 2003; Miralles *et al*., 2001; Davino *et al*., 2012). In addition, Elena (2011) indicated that, assuming that sequence similarity may still be significant between two members of the same family, double infection of plant cells constitutes provocative conditions that may yield interspecific recombination or pseudo-recombination or reassortment, and thus the generation of new pathogen species, this theory was amply demonstrated in the work Davino *et al*. (2012), in fact the local degree (correlation between the length of common nucleotide stretches between the two viruses and the number of different breaking point detected) plays a very important role in the appearance of new interspecific hybrids. This is the case study of the dispersion of *Begomoviruses* in Sicily; our results show that co-infection with two *Begomoviruses,* Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl sardinia virus (TYLCSV), family *Geminiviridae*, sharing a certain degree of similarity (above 70% in our case) results in the rapid emergence of a huge number of recombinant variants. Even where it possible to study most of them in depth, and remove defective ones from the list, the final results would still be a multitude of fully viable ‘entities’. How should they be classified and named? Are

the criteria used thus far for species demarcation in *Begomoviruses* (89% sequence similarity) still valid? Application of this criterion has resulted in the creation of new species (TYLCMalV in Monci *et al*., 2002; TYLCAxV in Garcìa-Andrès *et al*., 2006) for 'entities' that are obviously recombinants between two well-defined viruses. Also, application of the 89% threshold to the case of a multitude of recombinant viral entities deriving from two parents, A and B, would lead to some variants being named strains (or isolates, if very close) of parent species A, others of parent species B, and others as belonging to a different virus species. The resulting taxonomic chaos, with different names for very close viral entities, would not help studies on evolution and phylogeny and, moreover, such a classification of recombinants would not indicate their true origins. Since conventional phylogenetic trees do not provide a reliable picture of the evolutionary relationships between viruses in which recombination has played a major role, a more convenient 'split tree' has been used (Huson & Bryant, 2006) (see the figures on the Chapter 5). This representation highlights the origins of recombinant viruses, as observed for the two isolates from Italy: the two recombinant viruses described in Sicily (Davino *et al.*, 2009), TYLCAxV-Sic1-[IT:Sic2/2:04] and TYLCAxV-Sic2-[IT:Sic2/5:04], are correctly positioned between parent viruses isolated in the same country. Since genetic variation occurs discontinuously along the genome, classification of recombinants is particularly challenging. The difficulty in demarcating *Begomovirus* species when recombination creates new viral entities has been recognized (Fauquet *et al.*, 2005), but unfortunately the International Committee on Taxonomy of Viruses has not yet found valid alternatives.

The rate of recombination is an important factor determining the level of genetic diversity within the virus population. Consequently, a low rate reduces genetic diversity, whereas a high rate favors genetic diversity. As speculated by González-Jara *et al*. (2009), high diversity is advantageous to viruses at the beginning of host colonization, but, later on, limiting co-infection would be an advantage for the fittest genomes.

Whether or not spatial separation of related viruses in double (or more) infection occurs commonly in nature and what mechanisms are behind this phenomenon remain largely unknown (Fabre *et al.*, 2009; Roossinck, 2005). In Sicily, in field or in greenhouse, became very common see vegetable crops with multiplex infections. In this context became very important to develop new diagnostic tools able to identifying in rapid manner the new or multiplex infection. With the development of multiplex RT-PCR techniques became easy to discovered multiplex infections in field and in nursery. Our experiments have been demonstrate that in all cases, the multiplex and singleplex RT-PCRs were able to detect specific targets up to the same dilution and were at least 100 times more sensitive than ELISA in those cases in which serological tests were available. In addition, multiplex RT-PCRs were used to analyze 45 tomato samples collected from greenhouses and fields in different provinces of Sicily in 2011 and the results were compared with the results of ELISA (except for ToCV, TICV and ToTV) and singleplex RT-PCRs already described in literature. The most prevalent virus in Sicily was ToCV, which was detected in 32 plants, followed by TSWV in 25 plants, CMV in 20 plants, PepMV in 17 plants, ToMV in 12 plants, TICV in 6 plants and ToTV in 2 plants. Three tomato samples were not infected by any virus. Single infections were present in 8 plants, whereas 10 plants were infected by two different viruses, 13 plants by three viruses, 8 plants by four viruses and 3 plants by five viruses. These data show that mixed infections with several viruses are very frequent. The interactions between viruses co-infecting the same plant could have an important impact in epidemics and could increase the damage in crops if synergisms arise. Testing an additional number of plants should confirm these results for the distribution of certain viruses, which was different in each province in Italy. Although ToCV was spread throughout Sicily, ToTV and TICV were only found in the Ragusa Province. In addition, CMV and TSWV were more prevalent in Agrigento and Trapani. These different prevalence and infections in each region could be explained in part by the tomato genotypes, vectors and climatic conditions and in part by the different agronomical practices, as tomatoes in Ragusa Province are cultivated mainly under greenhouse

conditions and in open fields in Agrigento and Trapani. The procedure developed allows for the rapid identification of the main RNA viruses that infect tomato and for the analysis of mixed infections, thus saving time, reagents and costs. This methodology could be included in phytosanitary protocols for early detection of these viruses in nurseries or in certification programs. Later, our attention was focused on *Pepino mosaic virus* (PepMV) because this pathogen was very destructive for tomato crops in Sicily especially in synergism with ToCV (Davino *et al*., 2008). For this reason 1,800 samples were subsequently collected from all province of Sicily; *Pepino mosaic virus* (PepMV) is a highly infectious *Potexvirus* and a major disease of greenhouse tomato (*Solanum lycopersicum*) crops worldwide. In Sicily, the first outbreak of PepMV has been detected in a single greenhouse in the year 2005 and has been quickly eradicated, but since 2008 this virus began to spread throughout the island. The investigation focused to assess the real dispersion and the genetic diversity of PepMV from its first outbreak in Sicily and to compare it to other PepMV isolates from other parts of the world in order to know what factors are determinant for the evolution and epidemiology of this virus. Phylogenetic analysis using the MEGA 5.0 program generated a phylogenetic tree showing that the PepMV isolate in Sicily is different from those found in middle and northern Europe, yet very similar to the Ch2 strain present only in a few regions of the world and exactly in subtropical ones. These results show that probably all isolates of PepMV discovered in Europe do not have a common origin. From an epidemiological point of view, aspects of the long-distance dissemination of PepMV should be considered, like the trade in living plantlets, seeds, contaminated pots. More restrictive controls are required to avoid PepMV spreading to other Italian regions as Apulia and Campania where tomato crops are widely spread.

Another virus which in the past caused serious problems to horticulture crops in Sicily, especially in pepper plants was *Broad bean wilt virus* 1 (BBWV-1). This virus belongs to the genus *Fabavirus*, for this reason we decide to develop two additional techniques: i) multiplex RT-PCR able to detect all viruses of the genus

Fabavirus and ii) a flow-through hybridization able to detect all viruses of the same genus (*Fabavirus*).

Broad been wilt virus 1 was first report in Sicily since 1980 in pepper plants (Davino *et al*., 1989) and during the subsequently years became a serious problems for the horticulture crops because pepper and tomato are the most cultivable crops in Sicily. Our study reports new methods developed for: 1) broad-range detection of all members of *Fabavirus*, including unknown species, by one-step RT-PCR with a pair of conserved primers; and 2) simultaneous detection and identification of the five currently recognized *Fabavirus* species by multiplex RT-PCR with five species-specific primer pairs. The combination of both of these techniques enables a rapid and sensitive detection and a simultaneous identification of the five species of the genus *Fabavirus* (BBWV-1, BBWV-2, GeMV, CuMMV and LMMV). The absence of diagnostic techniques has also contributed to the lack of knowledge on this group of viruses, and to the consequent underestimation of their impact. The singleplex RT-PCR with conserved primers for the genus *Fabavirus* and the multiplex RT-PCR with species-specific primers reported in this article could be valuable tools for the evaluation of *Fabavirus* distribution worldwide, discovery of new *Fabavirus* species, and implementation of integrated pest management procedures including plant breeding, certification and quarantine programs, eradication, etc. For the first time *Lalium mild mosaic virus* was molecular classified as a member of genus *Fabavirus*.

The second technique developed was the flow-through hybridization for all member of the genus *Fabavirus*, with the probe Faba/5'RNA1 that gave strong hybridization signals with all viral isolates of BBWV-1, BBWV-2, GeMV, CuMMV and LMMV whereas non-infected plants or plants infected with TRSV (outgroup) gave no signal. The procedure developed here constitutes two milestones: A) the fastest detection technique for plant viruses, and B) the first universal probe for a genus of plant viruses which with the combination of species-specific probes enables the simultaneous identification of known viruses and discovery of new viruses within the genus.

In conclusion, the development of new and modern diagnostic techniques, especially at low cost, is particularly useful for sustaining agriculture in the twenty-first century, especially in the future with the opening of the markets with the North African countries bordering the Mediterranean basin will be increasingly important to have tools to make early diagnosis and follow the gene flow of new emerging diseases.

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ANNEX 1 – LIST OF PUBLICATIONS DEVELOPED DURING THE PH.D. PERIOD

INTERNATIONAL PUBBLICATIONS

1. **Panno S**., Ferriol I., Rangel E. A., Olmos A., Cheng-Gui Han, Martinelli F., Rubio L., S.Davino (2014). Detection and identification of Fabavirus species by one-step RT-PCR and multiplex RT-PCR. Journal of Virological Methods 197 (2014) 77– 82.

2. Davino, S; Willemsen, A; **Panno, S**; Davino, M; Catara, A; Elena, FE; Rubio, L. (2013). Emergence and phylodynamics of Citrus tristeza virus in Sicily, Italy. Plos ONE DOI 8, (6), e66700.

3. Rangel, EA; Ferriol, I; **Panno, S**; Davino, S; Olmos, A; Rubio, L. (2013). The complete genome sequence of Lamium mild mosaic virus of the genus Fabavirus. Archives of Virology DOI: 10.1007/s00705-013-1732-5.

4. Bellardi, MG; Cavicchi, L; De Stradis, A; **Panno, S**; Davino, S (2013). Molecular characterization and Phylogenetic analysis of Turnip mosaic virus (TuMV) in Erysimum linifolium L. in Italy. International Research Journal of Plant Science, 4(4):97-102.

5. **Panno S.,** Davino S., Rubio L., Rangel EA., Davino M., Garcia-Hernandez J., Olmos A. (2012). Simultaneous detection of the seven main tomato-infecting RNA viruses by two multiplex reverse transcription polymerase chain reactions. Journal of Virological Methods, 186 (1-2): 152-156.

6. Davino S., **Panno S.,** Davino M., Bellardi M.G. (2012) – Inula viscosa L. a new host of Cucumber mosaic virus. Journal of Plant Pathology 92 (4), 112.

7. Davino S., Miozzi L., **Panno S.,** Rubio L., Davino M., Accotto GP. (2012). Recombination profiles between Tomato yellow leaf curl virus and Tomato yellow leaf curl Sardinia virus in laboratory and field conditions: evolutionary and taxonomic implications. Journal of General Virology, 93 (12): 2712-2717.

8. Davino S., **Panno S.,** Rangel E.A., Davino M., Bellardi M.G., Rubio L. (2011).

Population genetics of Cucumber mosaic virus infecting medicinal, aromatic and ornamental plants from northern Italy. Archives of Virology, 157 (4): 739-745.

NATIONAL PUBBLICATIONS

1. Davino, S; **Panno, S**; Iacono, G; Davino, M. (2012). La Tristezza degli Agrumi. Terra e Vita - Vol. 53/2012: 3-5

ABSTRACT OR CONFERENCES PROCEEDING

1. Rangel E., Ferriol I., **Panno S.,** Martinelli F., Davino S., Olmos A., Chenggui H., Rubio L. (2013). Relaciones filogenéticas en el género *Fabavirus* y su utilidad para el desarollo de mètodos moleculares de diagnóstico, en la mobilidad de ponencia de cartel. XXIII Congreso Venezolano de Fitopatología. – Caracas 3 – 6 Novembre 2013.

2. **Panno S.,** Davino S., Accotto G.P., Davino M., Rubio L. (2012). Recombinación entre los Begomovirus TYLCV y TYLCSV en Italia. In Sociedad Espanola de Fitopatologia. SEF – Malaga 17 – 20 Settembre 2012. P24.

3. Davino S., **Panno S.,** Rangel E.A., Davino M., Bellardi M.G., Rubio L. (2011) - Genetic variation and evolutionary analysis of Cucumber mosaic virus in medicinal, aromatic and ornamental plants from northern Italy. In: Sociedad Espanola de Biologia Evolutiva - SESBE – Madrid, 21-25 Novembre 2011. P13.

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