Eur J Plant Pathol (2011) 130:165–172
DOI 10.1007/s10658-011-9742-1

Evidence of *olive mild mosaic virus* transmission by *Olpidium brassicae*

Carla M. R. Varanda · Marta S. M. R. Silva · Maria do Rosário F. Félix · Maria Ivone E. Clara

Accepted: 10 January 2011 / Published online: 26 January 2011

Abstract Transmission of three strains of OMMV by an *Olpidium* sp. was evaluated and compared. The three strains were 1) an OMMV wild type (WT) recovered from olive trees, 2) an OMMV variant (L11) obtained after 15 serial passages of single local lesions induced in *Chenopodium murale* plants, and 3) a construct OMMV/OMMVL11 in which the coat protein (CP) gene replaced that of the wild type. A single-sporangial culture derived from Chinese cabbage (*Brassica pekinensis)* used as a bait plant grown in soil of an olive orchard, was identified as *Olpidium brassicae* based on the size and sequence of the generated amplicon in PCR specific tests. Each of the three virus strains was soil transmitted to cabbage roots in the absence of the fungus at similar rates of 30 to 40%. Separate plant inoculation by *O. brassicae* zoospores incubated with each viral strain resulted in enhanced transmission of OMMV, reaching 86% of infection whereas that of the other two strains remained practically unaffected at *ca*. 34%. Binding assays showed that the amount of virus bound to zoospores, estimated spectrophotometrically, was 7% in the case of OMMV, and practically nil in the case of the other two viral strains. Substitution of the coat protein (CP) gene of OMMV by that of the OMMV L11 strain, drastically reduced viral transmissibility in the presence of zoospores to the level of that observed in their absence. Our data shows that OMMV soil transmission is greatly enhanced by *O. brassicae* zoospores and that the viral CP plays a significant role in this process, most likely by facilitating virus binding and later entrance into the host plant roots.

Keywords Coat protein · Fungal transmission · OMMV mutants

C. M. R. Varanda · M. S. M. R. Silva · M. d. F. Félix · M. I. E. Clara
Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora,
7002-554 Évora, Portugal

C. M. R. Varanda (✉) · M. S. M. R. Silva · M. d. F. Félix · M. I. E. Clara
Departamento de Fitotecnia, Universidade de Évora,
7002-554 Évora, Portugal
e-mail: carlavaranda@uevora.pt

Introduction

Olive mild mosaic virus (OMMV) is a recent member of genus *Necrovirus*, family *Tombusviridae* originally isolated from olive trees in Portugal (Cardoso et al. 2005). New sequence data submitted to Genbank (accession numbers EF 201608, EF 201607, EF 201606 and EF 201605) reveals that it also infects tulips, and is the causal agent of Augusta disease (Pham et al. unpublished), which was previously ascribed to Tobacco necrosis virus (TNV) (Kassanis 1949). The virus has a diameter of *ca*. 28 nm and a single stranded positive-sense RNA genome, 3683 nts long, containing 5 open reading frames (ORF).

OMMV is considered a recombinant between two other necroviruses, namely *Olive latent virus 1* (OLV-1) and *Tobacco necrosis virus D* (TNV-D) as its RNA dependent RNA polymerase (RdRp) shares a high aminoacid (aa) identity with that of OLV-1 and its coat protein (CP) has a high identity with that of TNV-D (Cardoso et al. 2005).

Due to these characteristics, identifications of TNV-D done in the past, based either on serology or polymerase chain reaction (PCR) amplification of CP gene are not reliable. A specific reverse-transcription polymerase chain reaction (RT-PCR) assay was recently developed using primers designed to amplify a genomic sequence, sized 934 nts, encompassing part of the CP and the RdRp genes, thus allowing unequivocal diagnosis of OMMV (Varanda et al. 2010). Application of this test to an olive orchard that had revealed in the past 31% necrovirus infections, showed that 28% of trees were infected by OMMV (Varanda et al. 2010). On the other hand, the same field showed the widespread presence of *Olpidium* like species, as evaluated by microscopic observation of resistant spores with typical morphology on the roots of bait plants grown in that soil. These findings, and prior information gathered from the literature that TNV (taken in a broad sense) is soil transmitted through zoospores of *Olpidium brassicae* (Temmink et al. 1970), led us to investigate the possibility of OMMV being transmitted in a similar manner.

In this work we applied a PCR-based test developed by Herrera-Vásquez et al. (2009) that allows the discrimination of *O. brassicae* from *O. virulentus* through the size of the generated amplicons. *Olpidium* species present in the olive orchard soil were characterized and used to test their capacity to transmit OMMV, a variant designated as OMMV L11 and a construct OMMV/OMMV L11.

Materials and methods

Viruses

An infectious transcript of OMMV cDNA clone obtained from the OMMV type strain, originated from *Olea europaea* L. (Cardoso et al. 2005). An OMMV variant, designated OMMV L11, was obtained by mechanically passaging the above infectious transcript by 15 serial inoculation of single local lesions induced in *C. murale* plants. A mutant was obtained by substituting the OMMV CP gene by that of OMMV L11, designated as OMMV/OMMVL11. All three strains were used in fungus mediated transmission assays.

Construction of OMMV/OMMVL11

RNA of OMMV L11 was extracted using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA copies of the CP coding region were obtained by RT-PCR. The plus sense primer (OMMVcoat5′: 5′ GACATTTACTATAACACC 3′) corresponds to OMMV nucleotide sequence 2613 to 2630 and lies 6 nucleotides upstream of the CP ORF. The minus sense primer (OMMVcoat3′: 5′ AAGGGTAGATATGTGGGCG 3′) corresponds to the complementary OMMV nucleotide sequence 3471 to 3489 and lies 25 nucleotides downstream of the CP ORF. The sequence of OMMVL11 CP was determined. The OMMV L11 CP RT-PCR product was ligated into pGEM easy vector (Promega). DNA from this clone was digested with XagI and HPaI, which cut at unique sites flanking the CP ORF. The fragment of *ca*. 811 nt was ligated into similarly digested pUC18OMMV (a full length OMMV cDNA clone) to produce pUC18OMMV/OMMVL11. The sequence of the entire transferred region of the OMMV L11 CP gene was determined (data not shown). Plasmid DNA of pUC18OMMV/OMMVL11, under control of the bacteriophage T7 promotor, was linearized by digestion with SmaI restriction endonuclease and further purified by extraction with QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. In vitro transcription was achieved using Ribomax™ Large Scale RNA Production System—T7 (Promega). For that, about 3.5 μg of linear plasmidic DNA were used in a transcription reaction in the presence of T7 RNA Polymerase according to the manufacturer's instructions. DNA template was removed by digestion with DNase (1 U μg^{-1} of template DNA) and the transcript was purified by extraction with phenol: chloroform (5:1) acid equilibrated (pH 4.7) (Sigma) and ethanol precipitated. About 2 μg of the synthesized RNA was mechanically inoculated directly onto one leaf of *C. murale* host plant to evaluate infectivity. Symptoms were recorded and infected tissues were used to inoculate healthy

plants for further virus purification to use in fungus transmission assays.

Virus purification

OMMV, OMMV L11 and OMMV/OMMVL11 were purified from infected *C. murale* leaves. The leaves were ground in cold 0.1 M sodium phosphate buffer (1:3 w/v), filtered, clarified in the presence of organic solvents, concentrated by differential centrifugation and further purified by ultracentrifugation through sucrose density gradient columns (Zhang et al. 1993). The single light scattering virus band was recovered and concentrated by ultracentrifugation. Virus concentration was determined at 260 nm in a UV/Vis spectrophotometer DU 530 Life Sciences (Beckman) and the extinction coefficient used was $E^{1\%}_{260} = 5.0$.

Olpidium spp. recovery and molecular identification

Olpidium spp. were baited using Chinese cabbage plants sown in soil from an olive orchard located in Mirandela (northeast of Portugal) where OMMV had been found in a high percentage of olive trees (Varanda et al. 2010). Seven weeks later, plants were carefully removed, the roots washed, immersed into an aqueous 10% KOH solution and autoclaved at 121°C for 15 min. Roots were stained overnight in 1% trypan blue lactoglycerol (1 water: 1 glycerol: 1 lactic acid), de-stained in an aqueous 50% glycerol solution for 18 h, essentially as described by Phillip and Hayman (1970) and observed under a light microscope for the presence of fungal resting spores with typical morphology.

To obtain a single sporangial culture, *Olpidium*-infected cabbage roots, were homogenized in chilled 0.5 M glycine–NaOH (pH 7.6) in a blender for 30 s, the resulting extract was filtered and a drop of the filtrate was observed under a light microscope (Lin et al. 1970). A single mature sporangium was collected and serially transferred 5 times to small drops of cold sterile-water to eliminate possible contaminating zoospores of a different origin. The single isolated sporangium was placed in a 30 ml plastic pot containing a mixture of sterile sand and vermiculite where 5 day old cabbage seedlings were growing and maintained in a growth chamber at 16–22°C with a 16 h photoperiod for 1 month.

To collect zoospores, infected cabbage plants were kept without watering for 3 days prior to placing the roots in distilled water for 30 min. The resulting spore suspension was filtered through Whatman No 4 filter paper, the filtrate was centrifuged at $1000g$ for 10 min, the pellet resuspended in sterile distilled water and the zoospore yield determined with a Fuchs-Rosenthal counting chamber. *Ca*. 5×10^6 zoospores were lysed and total DNA was directly extracted using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

A primer specific for *O. virulentus* (OLPvirF) and a primer specific for *O. brassicae* (OLPbraF) were used together with one common reverse primer (OLPR) (Herrera-Vásquez et al. 2009) in a multiplex PCR assay. DNA of *O. brassicae* and *O. virulentus* used as positive controls in PCR assays were kindly supplied by Herrera-Vasquez. DNA amplification was performed using 10 ng of DNA, 2.5 units of DreamTaq DNA Polymerase (Fermentas) and 0.2 μM of each primer, in a MyCycler Thermal Cycler (BioRad) programmed for a 5 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. Amplified products were analyzed by electrophoresis in 1% agarose gel in 1x TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA, pH 8.4) at 80 V for approximately 1 h and stained with 0.2 μg ml^{-1} ethidium bromide.

Virus transmission by Olpidium species

Five μg of each purified OMMV, OMMV L11 and OMMV/OMMVL11 were added to 50 ml of 50 mM glycine-NaOH (pH 7.6) containing 1×10^5 zoospores mL^{-1} or to glycine solution alone, as a control. After a 20 min period to allow virus acquisition, 1 ml of the suspension was poured into 100 ml pots containing 5 day old cabbage seedlings growing in sterile sand. Six days later, plants were taken, the roots washed carefully for 10 min with a 1% SDS aqueous suspension and then with running tap water for 3 h. Cabbage roots were tested by DAS-ELISA (double antibody sandwich-enzyme linked immunosorbent assay) (Clark and Adams 1977) for the presence of virus. Absorbance readings, at 405 nm after about 2 h, greater than twofold over the average of negative controls were considered positive. Similar experiments were conducted in the

absence of fungal zoospores. Twenty pots, containing 10 plants each, were used in each experiment, which was repeated 5 times, involving a total of 100 pots.

Virus binding assays

100 μg of purified OMMV, OMMV L11 and OMMV/OMMVL11 were separately incubated with 1×10^6 zoospores ml^{-1} in 10 ml 50 mM glycine-NaOH (pH 7.6) for 20 min. Zoospores were then pelleted by centrifugation at 2800 g for 7 min. Supernatant containing unbound virus was further centrifuged at 117000 g for 3 h, the pellet was resuspended in 30 μl of 0.02 M sodium phosphate buffer and the amount of virus in the pellet was estimated spectrophotometrically, as indicated above. Experimental controls were carried out as above except that no zoospores were included.

Results

Virus yield and infectivity

Three days after inoculation of *C. murale* plants with 2 μg of RNA of each OMMV, OMMV L11 and OMMV/OMMVL11, the plants began to develop local lesions (Fig. 1). Typically 100 g of tissue infected with each virus strain yielded approximately the same amount, i.e. *ca*. 0.3 mg of purified virus.

Molecular identification of *O. brassicae*

Cabbage roots inoculated with a single sporangial culture showed abundant typical stellate resting spores (Fig. 2) that under appropriate conditions released zoospores showing a rapid and jerky movement. Visualization in gel of a PCR generated amplicon of 579 bp identifies *O. virulentus* whereas an amplicon of 204 bp reveals the presence of *O. brassicae* (Herrera-Vásquez et al. 2009). Multiplex PCR assays using primers specific for *O. brassicae* and for *O. virulentus*, produced a single amplified product of *ca*. 204 bp, thus confirming the identification of the fungal isolate, here evaluated as virus vector, as *O. brassicae* (Fig. 3).

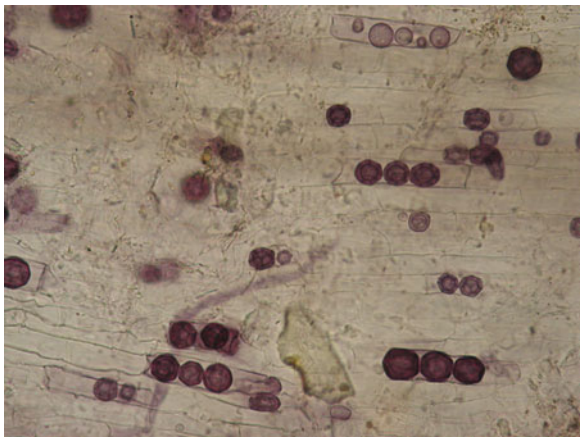

Fig. 2 Root cells of cabbage plants, revealing typical stellate resting spores of O. brassicae (x400)

OMMV WT CP and OMMVL11 CP sequencing and alignment

The sequences of OMMV WT CP and OMMVL11 CP were determined and the alignment revealed 2 amino acid differences, a change from Asparagine to Tyrosine in result of a change of an adenine to timine in position 3200 nt and a change from Alanine to Threonine in result of a change of a guanine to adenine in position 3281 nt of OMMV genome (Fig. 4).

Fig. 1 C. murale plants showing local lesions 3 days after inoculation with RNA from: **a** - OMMV, **b** - OMMVL11 and **c** - OMMV/OMMVL11

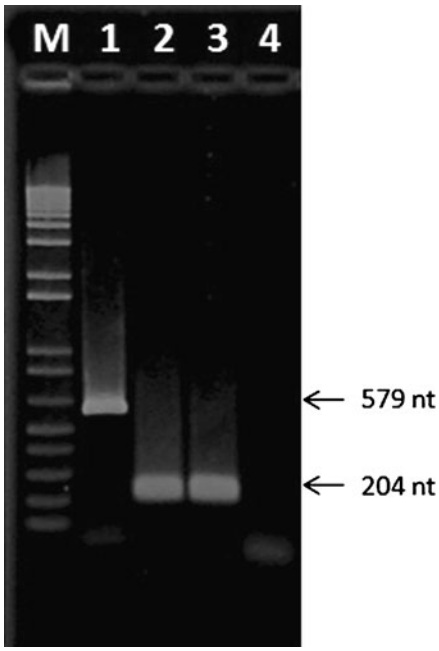

Fig. 3 Products of multiplex PCR assay applied to DNA extracted from zoospores of an isolate of Olpidium sp. using specific primers for O. brassicae and for O. virulentus, separated on a 1% agarose gel. Lane M: 1 kb plus DNA ladder (Invitrogen); Lane 1: DNA from O. virulentus; Lane 2: DNA from O. brassicae; Lane 3: DNA of Olpidium species recovered from soil of olive orchard; Lane 4: water used as negative control. Size of generated amplicons are indicated on the right

Virus transmission by *O. brassicae*

Cabbage roots inoculated with each virus, alone or previously incubated with zoospores of *O. brassicae*, were pooled from each pot after 5 days and tested for the presence of virus by DAS-ELISA. Results showed that each virus could infect plant roots in the absence of the fungus at similar levels i.e. 30 to 40%. Furthermore, the presence of the fungus did not affect the transmissibility level of OMMV L11 or OMMV/OMMVL11, which remained at *ca.* 30%. On the other hand, OMMV transmission in the presence of the fungus more than doubled, increasing from 39% to 87% (Table 1).

Virus binding assays

Assays were carried out to determine if the reduced ability of OMMV L11 and OMMV/OMMVL11 particles to be transmitted by *O. brassicae* zoospores was due to a binding deficiency.

It was found that only *ca.* 7 μg out of the 100 μg of OMMV initially incubated with zoospores became associated with them as the remaining 73 μg were found free in the supernatant after low speed centrifugation of the virus-zoospore mix. On the other hand, OMMV L11 and OMMV/OMMVL11 concentrations found in the supernatant were similar to the initial amount used in the incubation step, indicating a lack of significant adsorption to the spores. In experiments where no spores were used, the amount of virus found in the supernatant was identical to that added initially, as was expected.

Discussion

In this study the possibility of OMMV being soil-transmitted by *Olpidium* sp. was examined, in view of

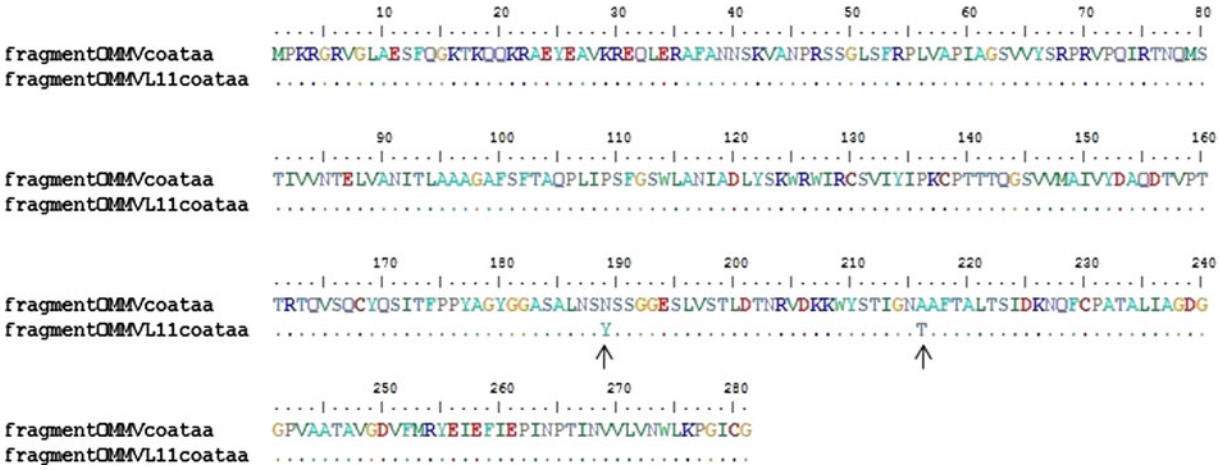

Fig. 4 OMMV CP and OMMVL11 CP aminoacid sequence alignment. Mutations are marked with arrows

Table 1 Soil transmissibility of OMMV, OMMV L11, and OMMV/OMMV L11 to cabbage roots in the presence of O. brassicae zoospores

	Transmission efficiency (%)[a]					
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Average
OMMV	35	35	45	40	40	39
OMMV + zoospores [b]	80	90	90	85	90	87
OMMV L11	30	35	30	30	35	32
OMMV L11 + zoospores [b]	35	30	30	30	30	31
OMMV/OMMVL11	30	30	25	30	30	29
OMMV/OMMVL11 + zoospores [b]	30	30	35	30	30	31

Virus root infection was determined by DAS-ELISA

[a] Percentage of pots containing infected plants. Twenty pots for each treatment were used

[b] Virus was incubated with zoospores for 15 min prior to adding to the plants in each pot

the fact that both are common in certain olive orchards and that OMMV CP has a high aa identity to that of TNV-D, which appears to be fungally transmitted (Temmink et al. 1970).

The *Olpidium* sp. isolate used here was identified on the basis of morphology and by a molecular method. Microscopic examination of cabbage roots previously inoculated with the single sporangial culture of *Olpidium* sp., placed it as either *O. brassicae* or *O. virulentus* based on the morphology of the resting spores (Fig. 2) as well as on the rapid and jerky movement of the zoospores (Lange and Insunza 1977). Differentiation of these two species is only possible at a molecular level. Application of a multiplex PCR based test, using specific primers (Herrera-Vásquez et al. 2009) for each of the two *Olpidium* species, clearly identified the fungus captured by *B. pekinensis* in the olive orchard soil as *O. brassicae*.

This work shows that OMMV is naturally soil-transmitted *per se* to roots of host plants but the rate of transmission rises dramatically, more than two fold from an average rate of 39% to 87%, when the virus is previously incubated with a suspension of *O. brassicae* zoospores. The high level of transmission obtained in the absence of the fungus is probably due to the high inoculum level used. In nature, it is not very likely to have such a high amount of viruses near the roots and under such conditions the virus is probably even more vector-dependent. The binding assays revealed that only *ca*. 7 μg of the OMMV particles were bound to the zoospores, which could indicate that zoospores receptors may have reached saturation and therefore were not able to bind more viruses (Kakani et al. 2003), thus demonstrating that a very small proportion of bound particles accounts for a high increase of virus transmission.

By comparison, the transmission of an infectious OMMV L11 variant did not increase in the presence of the fungus, and was practically unaffected. In a similar way when the infectious construct OMMV/OMMVL11 was tested, transmissibility remained almost unaltered. This confirms that with successive mechanical passages, plant viruses frequently lose their fungal transmissibility (Campbell 1996). OMMV L11 and OMMV/OMMVL11 did not bind *O. brassicae* zoospores at a detectable level suggesting that the lack of increased fungal transmissibility is due to the failure of viral capsids to recognize or stably bind to zoospores. This reinforces the suggestion that the specificity of transmission lies at the level of *O. brassicae* recognition and binding (Temmink et al. 1970; Robbins et al. 1997). OMMV, OMMV L11 and OMMV/OMMVL11 particles proved to be highly infectious and symptoms consisted of local necrotic lesions that usually appeared 2 to 3 days after inoculation. Since the only difference between the construct and OMMV lies in the CP, it is plausible to assume that the loss of fungal transmissibility lies in alterations of certain domains of that peptide. This agrees with previous studies showing that the CP of plant viruses plays an important role in transmission and particular amino acids within the protein are essential for this process (Brown et al. 1995; Campbell 1996; Gray 1996; Gray and Rochon 1999; Pirone and Blanc 1996; Van den Heuvel et al.

1999). It was also demonstrated by the reciprocal exchange of the CP gene of *Cucumber necrosis virus* (CNV) and that of a non-transmissible tombusvirus, the cherry strain of *Tomato bushy stunt virus* (TBSV-Ch). Particles containing a modified TBSV-Ch genome with the CNV CP gene were efficiently transmitted and those containing the TBSV-Ch CP gene were not (McLean et al. 1994). Similar observations were obtained when Robbins et al. (1997) performed the reciprocal exchange of the CP gene of CNV and that of a non-transmissible mutant (LL5). In this study the mutated CP gene resulted in loss of recognition of the vector either by causing conformational changes on the virus particle affecting an efficient binding to the vector receptors (Kakani et al. 2004) or by altering putative attachment sites of the viral subunits thus, failing to bind specific receptors in the zoospore outer membrane. An identical situation was recorded with several animal viruses, such as poliovirus, foot-and-mouth disease virus and influenza virus (Fry et al. 1999; Rossman 1994; Skehel and Wiley 2000). Failure of the virus to stably interact with a receptor, perhaps by altering specific ionic interactions with a component of the zoospore membrane can also be an explanation (Robbins et al. 1997).

This is the first report of OMMV transmission by *Olpidium brassicae*. This study contributes to a better understanding of the features involved in fungal virus transmission, about which little is known, and has important implications in the management of OMMV associated diseases thus contributing to more successful control of the virus.

Acknowledgments The authors would like to thank Ron Reade (Agriculture and Agri-Food Canada) for many helpful discussions, to José Ángel Herrera-Vásquez (Universidad Politécnica de Valencia, Spain) for providing DNA from *O. brassicae* and *O. virulentus* to use as controls, to Maria Mário Azedo for her technical assistance and to Alan Phillips (Universidade Nova de Lisboa) for critical reading of the manuscript and helpful advice. Carla Marisa R. Varanda is recipient of a PhD fellowship from Fundação para a Ciência e a Tecnologia (FCT), SFRH/BD/29398/2006 and Marta Silva is recipient of a FCT fellowship, UUE/ICAAM/BII/04 A/2009.

References

Brown, D. J. F., Robertson, W. M., & Trudgill, D. L. (1995). Transmission of viruses by plant nematodes. *Annual Review of Phytopathology, 33*, 223–249.

Campbell, R. N. (1996). Fungal transmission of plant viruses. *Annual Review of Phytopathology, 34*, 87–108.

Cardoso, J. M. S., Félix, M. R., Clara, M. I. E., & Oliveira, S. (2005). The complete genome sequence of a new necrovirus isolated from *Olea europaea* L. *Archives of Virology, 150*, 815–823.

Clark, M. F., & Adams, A. N. (1977). Characteristics of microplate method of enzyme-linked immunosorbent assay for detection of plant-viruses. *The Journal of General Virology, 34*, 475–483.

Fry, E. E., Lea, S. M., Jackson, T., Newman, J. W. I., Ellard, R. M., Blakemore, W. E., et al. (1999). The structure of a foot-and-mouth disease virus-oligosaccharide receptor complex. *The EMBO Journal, 18*, 543–554.

Gray, S. M. (1996). Plant virus protein involved in natural vector transmission. *Trends in Microbiology, 4*, 259–264.

Gray, S. M., & Rochon, D. M. (1999). Vectors of plant viruses. In A. Granoff & R. Webster (Eds.), *Encyclopedia of virology, vol. 1* (pp. 1899–1910). London: Academic Press.

Herrera-Vásquez, J. A., Cebrián, M. C., Alfaro-Fernández, A., Córdoba-Sellés, M. C., & Jordá, C. (2009). Multiplex PCR assay for the simultaneous detection and differentiation of *Olpidium bornovanus*, *O. brassicae*, and *O. virulentus*. *Mycological Research, 113*, 602–610.

Kakani, K., Robbins, M., & Rochon, D. (2003). Evidence that binding of cucumber necrosis virus to vector zoospores involves recognition of oligosaccharides. *Journal of Virology, 77*, 3922–3928.

Kakani, K., Reade, R., & Rochon, D. (2004). Evidence that vector transmission of a plant virus requires conformational change in virus particles. *Journal of Molecular Biology, 338*, 507–517.

Kassanis, B. (1949). A necrotic disease of forced tulips caused by tobacco necrosis viruses. *The Annals of Applied Biology, 36*, 14–17.

Lange, L., & Insunza, V. (1977). Root-inhabiting *Olpidium* species: the O.radicale complex. *Transactions of the British Mycological Society, 69*, 377–384.

Lin, M. T., Campbell, R. N., Smith, P. R., & Temmink, J. H. M. (1970). Lettuce big-vein virus transmission by single-sporangium isolates of *Olpidium brassicae*. *Phytopathology, 60*, 1630–1634.

McLean, M. A., Campbell, R. N., Hamilton, R. I., & Rochon, D. M. (1994). Involvement of the cucumber necrosis virus coat protein in the specificity of fungus transmission by *Olpidium bornovanus*. *Virology, 204*, 840–842.

Robbins, M. A., Reade, R. D., & Rochon, D. M. (1997). A cucumber necrosis virus variant deficient in fungal transmissibility contains an altered coat protein shell domain. *Virology, 234*, 138–146.

Rossman, M. G. (1994). Viral cell recognition and entry. *Protein Science, 3*, 1712–1725.

Skehel, J. J., & Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza virus hemagglutinin. *Annual Review of Biochemistry, 69*, 531–569.

Temmink, J. H. M., Campbell, R. N., & Smith, P. R. (1970). Specificity and site of in vitro acquisition of tobacco necrosis virus by zoospores of *Olpidium brassicae*. *The Journal of General Virology, 9*, 201–213.

Pham, K. T. K., Lemmers, M. E. C., van Doorn, J., & Derks, A. F. L. M. (unpublished). Revision of taxonomy of the virus causing Augusta disease in tulips in the Netherlands.

Phillip, J. M., & Hayman, D. S. (1970). Improved procedure for clearing roots and staining parasitic and vesicular-arbuscula mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society, 55*, 158.

Pirone, T. P., & Blanc, S. (1996). Helper dependent vector transmission of plant viruses. *Annual Review of Phytopathology, 34*, 227–247.

Van den Heuvel, J. F. J. M., Hogenhout, S. A., & van der Wilk, F. (1999). Recognition and receptors in virus transmission by arthropods. *Trends in Microbiology, 7*, 71–76.

Varanda, C., Cardoso, J. M. S., Félix, M. R., Oliveira, S., & Clara, M. I. (2010). Multiplex RT-PCR for detection and identification of three necroviruses that infect olive trees. *European Journal of Plant Pathology, 127*, 161–164.

Zhang, L., French, R., & Langenberg, W. G. (1993). Molecular-cloning and sequencing of the coat protein gene of a Nebraskan isolate of Tobacco necrosis virus - the deduced coat protein-sequence has only moderate homology with those of strain-A and strain-D. *Archives of Virology, 132*, 291–305.