AGRISCIENTIA, 2013, VOL. 30 (2): 79-85

Detection and characterization of a *Cucumber mosaic virus* isolate infecting peperina, a species native to Argentina

Rodríguez Pardina P.; M. Ojeda, E. Biderbost and L. Di Feo

SUMMARY

Minthostachys mollis (Kunth.) Griseb., “peperina”, a member of the family Lamiaceae, is an aromatic species used in modern pharmacology and medicine. It is widely distributed in the Andes, from Venezuela and Colombia to Argentina. In Argentina, the main exploitation area of peperina includes the mountain area of the province of Córdoba, where the plant is indiscriminately harvested, leading to an irreversible loss of the germplasm. To preserve this plant as a native resource and regional source of incomes, the species has been domesticated. During this process, noticeable symptoms of yellow mosaic appeared. Biological, serological and molecular studies (RT-PCR, RFLP, cloning and sequencing) determined the presence of subgroup IA of *Cucumber mosaic virus* in domesticated peperina plants. The viral isolate studied is closely related to strain Y, which was reported from Japan. To our knowledge, this is the first report of a virus infecting peperina.

Keywords: *Cucumovirus, Mynthostachys mollis*, RT-PCR sequence

Rodríguez Pardina P.; M. Ojeda, E. Biderbost y L. Di Feo, 2013. Detección y caracterización de un aislamiento de *Cucumber mosaic virus* que infecta peperina, una especie nativa de Argentina. Agriscientia 30 (2): 79-85

RESUMEN

Minthostachys mollis (Kunth.) Griseb., “peperina”, un miembro de la familia Lamiaceae, es una especie aromática que se emplea en la farmacología moderna y en medicina. Está ampliamente distribuida en los Andes, desde Venezuela y Colombia hasta Argentina. En el último país, la principal área de explotación de peperina incluye el área serrana de la provincia de Córdoba, donde la especie es arrancada indiscriminadamente, lo que conlleva una pérdida irreversible de germoplasma. A los fines de preservar este recurso nativo y fuente regional de ingresos, la especie está siendo domesticada. Durante este proceso, se observó la aparición de síntomas de un conspicuo mosaico amarillo, típico de infección viral. Análisis biológicos, serológicos y moleculares (RT-PCR, RFLP, clonado y secuenciación) pusieron de manifiesto la presencia del subgrupo IA de *Cucumber mosaic virus* en las plantas

Fecha de recepción: 25/06/12; fecha de aceptación: 22/10/13

domesticadas de peperina. El aislamiento viral estudiado está íntimamente relacionado con la raza Y previamente informada en Japón. Éste es el primer informe de un virus que infecta a la peperina.

Palabras claves: *Cucumovirus, Mynthostachys mollis*, secuencia producto de RT-PCR

P. Rodríguez Pardina, E. Biderbost and L. Di Feo: IPAVE-INTA, Camino 60 Cuadras km 5,5. X5020ICA Córdoba, Argentina. M. Ojeda: Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Av. Valparaíso s/n, Ciudad Universitaria, 5000 Córdoba, Argentina. Correspondence to L. Di Feo: ldifeoar@yahoo.com.ar

INTRODUCTION

Minthostachys mollis (Kunth.) Griseb., locally known as "peperina", is a member of the family Lamiaceae; it is a perennial, aromatic semi-shrub of about 0.3-2.0 m in height. Due to its menthol content, its aroma resembles that of mint. It is widely used in infusions because of its stimulant, antispasmodic, antidiarrhoea, anticholera, antiemetic and digestive properties (Ordóñez *et al.*, 2006). This aromatic plant is also used for liquor and mixed herb production. It has received increasing attention from modern pharmacology and medicine, as plant decoctions and extracted essential oils are tested for pharmacological effects (Ojeda *et al.*, 2004; Ordóñez *et al.*, 2006; Banchio *et al.*, 2007). This species is widely distributed in the Andes, from Venezuela and Colombia to Argentina. In Argentina, it is present in the north-western region (provinces of Salta, Jujuy, Catamarca, Tucumán, and La Rioja) and in the central-western region (San Luis and Córdoba) (Schmidt-Lebuhn *et al.*, 2008). In Córdoba, the plant grows between 700 and 1200 m a.s.l. In this area, the trade of these plants reaches its maximum levels; hence, it is indiscriminately gathered by collectors, who sometimes uproot whole plants without considering the phenological state or the ability of the species to recover. This practice has a strong impact on natural plant resources, modifies the environment, and leads to irrecoverable loss of germplasm through genetic erosion. For these reasons, research is carried out aiming at conservation, domestication and culture improvement of this native species. However, during the domestication process, loss of resistance genes takes place, as demonstrated for example in rice (Hiroaki & Takeshi, 2010).

Peperina plants were asymptomatic at their natural habitat (Biderbost, unpublished data). However, when they were transferred to the experimental plots, conspicuous yellow mosaic symptoms, typical of virus infection, started to appear.

Cucumber mosaic virus (CMV), a member of the family *Bromoviridae*, and the type member of the genus *Cucumovirus*, is one of the most important viruses in the world, infecting more than 1200 species in over 100 families of mono and dicotyledonous plants, including vegetables, ornamentals, legumes and other important crops (Palukaitis & Garcia-Arenal, 2003). The virus is mechanically transmitted by sap and naturally spread by more than 80 aphid species in a non-persistent manner (García-Arenal *et al.*, 2000). The virus genome consists of three positive-sense, single-stranded RNAs (RNA 1, RNA2 and RNA3) (Owen & Palukaitis, 1988). Many CMV strains have been described and classified into subgroups I and II on the basis of serology, nucleic acid hybridization, and restriction fragment length polymorphism (Owen & Palukaitis, 1988; Hu *et al.*, 1995; Finetti Sialer *et al.*, 1999; Szilassy *et al.*, 1999). Subgroup I is further subdivided into IA and IB on the basis of gene sequence and phylogenetic analysis (Roossinck, 2002). The objective of the present work was to identify and characterize the possible viral agents infecting peperina in Argentina.

MATERIALS AND METHODS

Source of inoculum

Eight peperina plants showing conspicuous yellow mosaic symptoms (Figure 1) were collected from a field assay that included clones from different localities in the provinces of Córdoba (Villa Al-

Figure 1. Peperina plants showing conspicuous yellow mosaic symptoms produced by *Cucumber mosaic virus*.

lende, Capilla del Monte, Tala Cañada and Cuesta Blanca), San Luis (Merlo), Tucumán (Tafí del Valle and Escaba), and Catamarca (Balcosna). The plants were transferred to pots and kept under greenhouse conditions for further analysis.

Biological characterization

Six healthy plantlets of peperina, *Chenopodium amaranticolor* Coste and Reyn, *Nicotiana benthamiana* Domin, *N. rustica* L., *N. occidentalis* H-M. Wheeler, *N. glutinosa* L., *Vigna unguiculata* (L.) Walp. and *Solanum lycopersicum* L. were sap inoculated with symptomatic peperina leaf samples using phosphate buffer, pH 7.0 containing 0.1% sodium sulphite (Na_2SO_3). The inoculated plants were maintained under greenhouse conditions until symptom development.

Serological characterization

The symptomatic peperina plants were screened for the presence of *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV), *Potato virus Y* (PVY), *Potato virus X* (PVX), *Tobacco mosaic virus* (TMV), by PTA (Mowat & Dawson, 1987) and for *Tomato ringspot virus* (ToRSV), *Tomato spotted wilt virus* (TSWV) and *Groundnut ringspot virus* (GRSV) by DAS-ELISA (Clark & Adams, 1977). The reactions with 0.75 mg/ml p-nitrophenyl phosphate (ADGIA Inc., Elkhart, IN, USA) in 10% diethanolamine, pH 9.8, were quantified after 60 min at 410 nm (OD410) using a Dynatech MR 700 ELISA plate spectrophotometer (Chantilly, USA). Samples were considered positive when OD410 values were higher than the mean of the healthy controls plus three times the standard deviations (six healthy peperina controls per plate were used). Samples that tested positive with the polyclonal CMV antibody were subsequently analyzed with monoclonal CMV antisera (anti-CMV subgroups I and II ADGIA Inc, Elkhart, IN, USA). Tests were performed as described above, using commercial positive controls (ADGIA Inc., Elkhart, IN, USA).

Molecular characterization. Antigen-capture (AC) RT-PCR and RLFP

For molecular characterization of the CMV isolate, antigen capture followed by reverse transcription-polymerase chain reaction (AC-RT-PCR) was performed using Access RT-PCR System (Promega Corp. Madison, WI, USA) and primers 5´ CP (-) and 3´ CP (+) that amplify an 870 bp fragment, including the capsid protein (CP) gene, for all CMV isolates (Rizos *et al*., 1992). RT-PCR reaction volume was 50 µl and amplification conditions were as follows: 48 °C for 45 min, 94 °C for 4 min (40 cycles of 94 °C for 30 s, 40 °C for 30 s, 68 °C for 1 min) and a final extension of 68 °C for 7 min. Following PCR, 10 µl of the product was electrophoresed in a 1% agarose gel and stained with ethidium bromide. In order to determine the CMV subgroup by restriction fragment length polymorphism (RFLP), 10 µl of the PCR product were digested with *Msp*I in a final volume of 20 µl (Rizos *et al*., 1992). Digestion was carried out at 37 °C for 4 h in the buffers supplied by the manufacturer; products were electrophoresed in 1.5% agarose and stained as above.

The AC-RT- PCR products were cloned using the Topo TA Cloning ® Kit (Invitrogen Corp., San Diego, CA, USA) following the manufacturer's instructions. Three positive clones were purified using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and further sequenced by the Unidad de Genómica, Instituto de Biotecnología-INTA (Argentina).

The nucleotide and deduced amino acid sequences were compared with those of other CMV isolates available in GenBank (www.ncbi.nlm.nih.gov). The accession number and assigned abbreviations of these isolates are listed in Table 1. Database searches were carried out using the Blast*n* algorithm (Altschul *et al.*, 1990). Multiple sequence alignments of nucleotide and deduced amino acid sequences were performed with Clustal W (www.ebi.ac.uk/clustalw). Phylogenetic trees were generated with MEGA program version 4.0 (Kumar *et al.*, 2004), using UPGMA method for nucleotide sequences and Neighbour-joining method for amino acids. The ER strain of *Peanut stunt virus* (acces-

Table 1. *Cucumber mosaic virus* strains used in comparative sequence analyses, with their corresponding subgroup and GenBank accession numbers.

Strain	Subgroup	Origin	Genbank accession number
Fny	IA	USA	D10538
Leg	IA	Japan	D16405
Mf	IA	South Korea	AJ27648
Y	IA	Japan	D12499
Ri-8	IA	Spain	AM183119
O	IA	Japan	D00385
IA	IB	Indonesia	AB042294
Ix	IB	Philippines	U20219
Nt9	IB	Taiwan	D28780
SD	IB	China	AB008777
Tfn	IB	Italy	Y16926
LS	II	USA	AF127976
Ly	II	Australia	AF198103
Q	II	Australia	M21464
S	II	South Africa	U37227
Trk7	II	Hungary	L15336

sion number U15730) was used as an out-group. Tree branches were bootstrapped with 2000 replications. Multiple alignments of the amino acid sequences of the coat protein gene were performed using LASERGENE (DNASTAR Inc. Madison, WI, USA program).

RESULTS

The virus was successfully transmitted by mechanical inoculation to all the inoculated plants. Chlorotic local lesions were observed on *C. amaranticolor* and severe systemic mosaic on *N. rustica*, *N. occidentalis*, *N. glutinosa* and *V. unguiculata*. Shoestring symptoms were observed on *S. lycopersicum.* On the other hand, original symptoms were reproduced in inoculated peperina plants.

All infected peperina plants reacted with the polyclonal CMV antiserum and with the monoclonal antiserum for CMV subgroup I, but not with subgroup II CMV antiserum. The strong reaction with subgroup I antiserum (Table 2), indicated its inclusion in this subgroup

As all eight evaluated plants were positive with subgroup I antiserum, two of them were randomly selected for AC-RT-PCR. The amplification resulted of an approximately 900 bp fragment (Figure 2). After digestion of the amplified products with *Msp*I and electrophoresis, the isolate gave two bands of 540 and 340 bp (Figure 3). This pattern allowed us to classify our local isolate as subgroup I according

Table 2. OD410 values, of different peperina samples, when tested with polyclonal and monoclonal antisera

Sample	OD410 Polyclonal antibody	OD410 monoclonal antibody subgroup I	OD410 monoclonal antibody subgroup II
1	0.875	3.223	0.065
2	0.561	2.374	0.082
3	0.578	2.313	0.032
4	0.653	1.985	0.021
5	0.301	2.681	0.034
6	0.659	3.500	0.032
7	0.749	2.247	0.040
8	0.258	1.614	0.013
Healthy control	0.054	0.040	0.078
Positive control	1.01	1.122	0.971

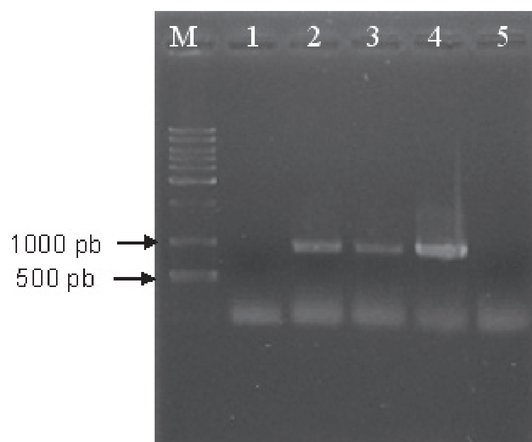

Figure 2 Electrophoretic analysis in 1.5% agarose of the amplified products, obtained with primers 5´ CP (-) and 3´ CP (+).M indicates 1000 bp molecular weight marker. Lane 1 Healthy peperina control; Lane 2 Positive control; Lanes 3-4 Infected peperina samples; Lane 5 Water

to Rizos *et al.* (1992).

The CP ORF length of the isolate was identical to that of previously described CMV strains, consisting of 657 bp, which therefore encoded a coat protein of 218 aa.

Sequence comparisons showed that the three sequenced clones shared 99.7-100% nucleotide sequence identity, indicating that they belong to the same isolate. The *Minthostachys* CMV isolate (CMV-Min) has the highest nucleotide sequence identity (95.2-98.0%) with those isolates belonging to Subgroup IA (Table 3). Comparisons of amino acid identity between the entire CMV-Min CP and other CMV strains revealed a homology of 81.6-83.9% for subgroup II members and of 97.7-99.1%

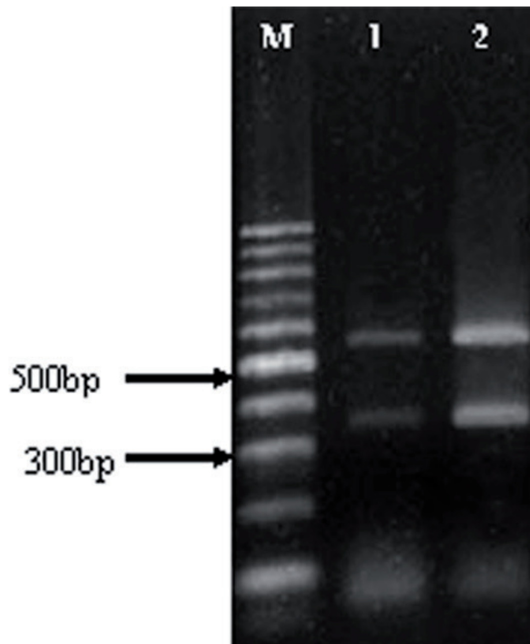

Figure 3 Electrophoretic analysis in 1.5% agarose of the amplified digested with *Msp*I. Gels stained with ethidium bromide. M indicates 100 bp molecular weight marker. Lane 1 and 2: infected peperina samples.

for CMV subgroup IA members, indicating that the studied isolate (CMV-Min) belongs to subgroup IA (Table 3). The highest homologies (99.1%) were found with strains Leg and Y, both of them reported for Japan.

Table 3. Comparisons of percent nucleotide sequence and the deduced amino acids identities between *Minthostachys Cucumber mosaic virus* isolate (CMV-Min) and other CMV strains

Strain	Subgroup	% nucleotide homology	% amino acid identity
Fny	IA	98.0	98.2
Leg	IA	96.9	99.1
Mf	IA	95.8	97.7
Y	IA	97.2	99.1
Ri-8	IA	96.6	98.2
O	IA	95.7	97.7
IA	IB	89.4	97.7
Ix	IB	89.5	96.8
Nt9	IB	91.8	97.7
SD	IB	91.1	98.2
Tfn	IB	91.7	98.2
LS	II	75.2	82.9
Ly	II	74.9	83.4
Q	II	75.4	83.9
S	II	74.7	82.5
Trk7	II	74.6	81.6

The multiple sequence alignment showed the local isolate differs from strain Y in that residue 17 of the capsid protein exhibits proline (P) as the remaining strains, whereas strain Y from Japan has leusine (L) at that site, and at residue 129 the Mint isolates has, as the rest of the strains, a proline while the Y has a serine (S) (Figure 4). It has been reported that substitution of this last amino acid affects symptom expression, inducing chlorosis instead of necrosis in infected tobacco plants (Mochizuki & Ohki, 2011).

The phylogenetic relationship between the nucleotides sequences of CMV-Min and those of other selected isolates is shown in Figure 5. The CMV-Min isolate clustered together with subgroup IA isolates and is closely related to CMV-Y, the strain reported for Japan.

DISCUSSION

The need to preserve peperina as a natural resource of great regional importance led to its domestication. In the natural ecosystem, symptoms were not observed, but during the domestication, symptoms were developed. Serological and molecular characterization confirmed the presence of CMV infecting the species. Studies conducted worldwide have shown that the numerous CMV strains are classified into subgroups I and II (Rizos *et al.,* 1992) on the basis of serology (Hu *et al*., 1995; Ilardi *et al*., 1995), nucleic acid hybridization (Owen & Palukaitis, 1988), gene sequencing (Owen *et al*., 1990; Hu *et al*., 1995; Szilassy *et al*., 1999) and RFLP (Rizos *et al*., 1992; Finetti Sialer *et al*., 1999). In turn, CMV subgroup I is further divided into IA and IB on the basis of sequences and phylogenetic relationships (Palukaitis & Zaitlin, 1997; Roossinck, 2002). Strains IA and II of this virus are distributed worldwide, whereas the strains of IB subgroup are mainly restricted to Asia (Roossinck, 2002; Koundal *et al*., 2011). Previous studies conducted in Argentina showed the presence of both subgroups. In 2003, CMV subgroup I was first mentioned infecting bean crops in northwestern Argentina (Rodríguez Pardina *et al*., 2003). It was further detected in the province of Córdoba, in greenhouse-grown plants of the ornamental Snapdragon (*Antirrhinum majus* L.) (Arneodo *et al*., 2005). However, this isolate was only serologically characterized by those researchers. Later, the presence of subgroup II in peanut crops was reported (de Breuil *et al*., 2005).

The present work is the first report of *Cucumber mosaic virus* infecting peperina in Argentina. The serological and molecular characterizations suggest that the isolate found in this aromatic plant be-

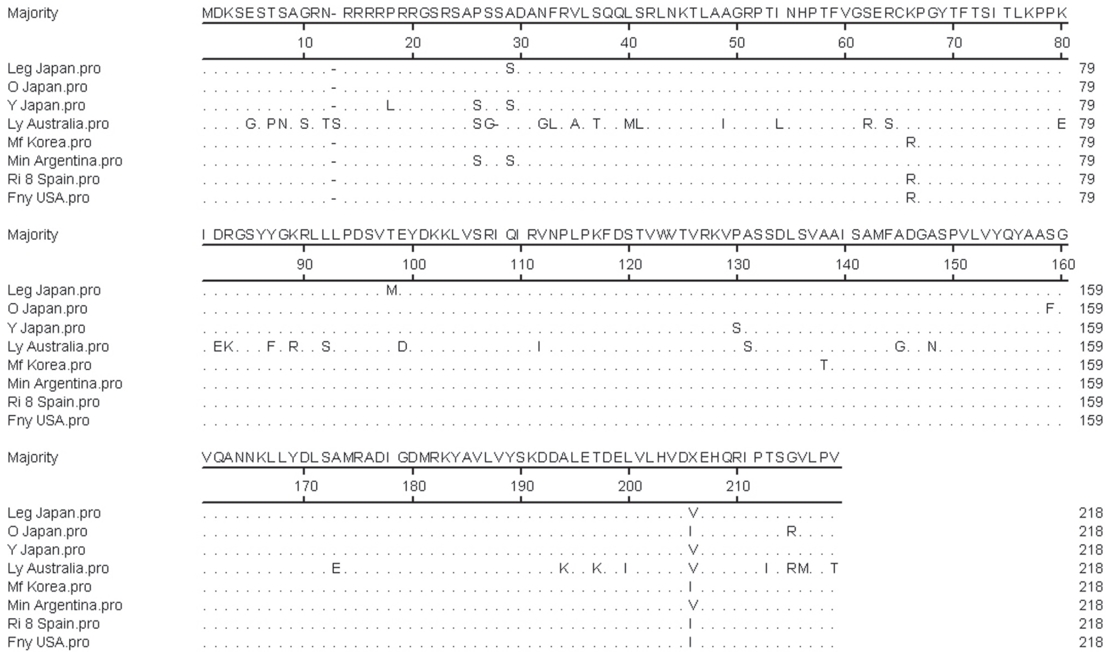

Figure 4. Alignment of the deduced amino acid (aa) sequences of coat protein of the *Minthostachys Cucumber mosaic virus* isolate (CMV-Min) compared with other selected strains. Dots represent identical amino acids in all the isolates studied.

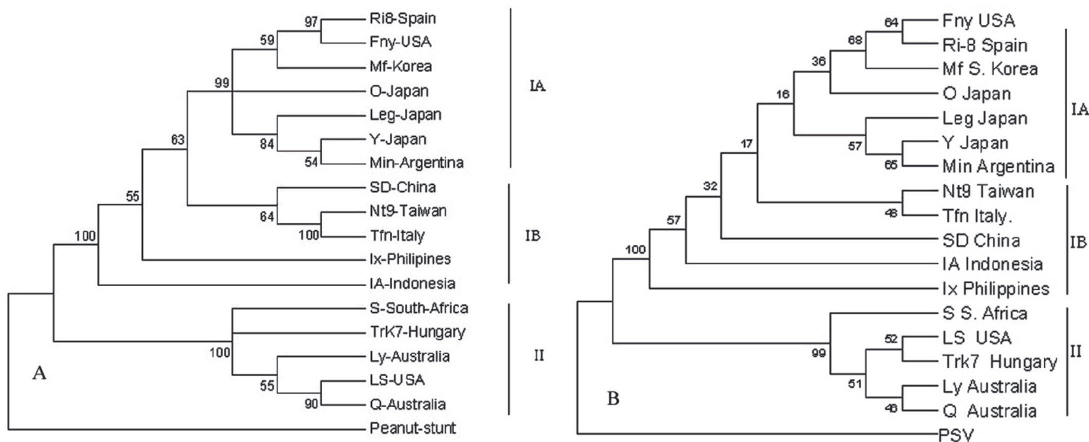

Figure 5. Phylogenetic trees based on the CP nucleotide (A) and amino acid (B) sequences of the *Minthostachys Cucumber mosaic virus* isolate (CMV-Min) and other selected CMV strains. The trees were constructed using UPGMA and neighbour joining methods with MEGA program version 4.0

longs to subgroup IA; the cluster analysis grouped the isolate with strain Y, which was first described in Japan (Nitta *et al.*, 1988). However, the local isolate differs from strain Y in that residue 129 of the capsid protein exhibits proline (P) as the remaining strains, whereas strain Y from Japan has a mutation P x S (proline x serine) at that position. This fact could lead to differences in the expression of symptoms (Mochizuki & Ohki, 2011).

On the other hand it is necessary to consider that peperina can constitute a natural reservoir of CMV.

ACKNOWLEDGMENTS

This work was partially supported by Agencia Córdoba Ciencia. The authors wish to thank Dr. Luis Conci for critical review of the manuscript.

REFERENCES

Altschul, S.F.; W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

Arneodo, J.D.; F.A. Guzmán, C.F. Nome and L.R. Conci, 2005. Ocurrencia de *Cucumber mosaic virus* en *Antirrhinum majus* en Argentina. Fitopatología Brasilera 30: 90.

Banchio, E.; G. Valladares, J. Zygadlo, P.C. Bogino, L.V. Rinaudi and W. Giordano, 2007. Changes in composition of essential oils and volatile emissions of Minthostachys mollis, induced by leaf punctures of Liriomyza huidobrensis. Biochemical Systematics and Ecology 35: 68-74.

Clark, M.F. and A.N. Adams, 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34: 475-483.

de Breuil, S.; F. Giolitti and S. Lenardon, 2005. Detection of *Cucumber mosaic virus* in Peanut (*Arachis hypogaea* L.) in Argentina. Journal of Phytopathology 153: 722-725.

Finetti Sialer, M.M.; F. Cillo, L. Barbarossa and D. Gallitelli, 1999. Diffferentiation of *Cucumber mosaic virus* subgroups by RT-PCR RFLP. Journal of Plant Pathology 81: 145-148.

García-Arenal, F.; F. Escriu, M.A. Aranda, J.L. Alonso-Prados, J.M. Malpica and A. Fraile, 2000. Molecular epidemiology of *Cucumber mosaic virus* and its satellite RNA. Virus Research 71: 1-8.

Hiroaki, S. and I. Takeshi, 2010. Massive gene losses in Asian cultivated rice unveiled by comparative genome analysis. BMC Genomics 11: 121.

Hu, J.S.; H.P. Li, K. Barry, M. Wang and R. Jordan, 1995. Comparison of dot-blot ELISA and RT-PCR assays for detection of two *Cucumber mosaic virus* isolates infecting banana in Hawaii. Plant Disease 79: 902-906.

Ilardi, V.; M. Mazzei, S. Loreti, L. Tomassoli and M. Barba, 1995. Biomolecular and serological methods to identify strains of *Cucumber mosaic virus* on tomato. EPPO Bull 25: 321-327.

Koundal, V.; Q. Haq and S. Praveen, 2011. Characterization, Genetic Diversity, and Evolutionary Link of *Cucumber mosaic virus* Strain New Delhi from India. Biochemical Genetics 49: 25-38.

Kumar, S.; K. Tamura and M. Nei, 2004. MEGA3: Integrated software for molecular evolutionary genetic analysis and sequence alignment. Briefings in Bioinformatics 5: 150-163.

Mochizuki, T. and S.T. Ohki, 2011. Single amino acid substitutions at residue 129 in the coat protein of C*ucumber mosaic virus* affect symptom expression and thylakoid structure. Archives of Virology 156: 881-886.

Mowat, W. P, and S. Dawson. 1987. Detection and identification of plant viruses by ELISA using crude sap extracts and unfractionated antisera. Journal of Virological Methods 15:133-247.

Nitta, N.; C. Masuta, S. Kuwata and Y. Takanami, 1988. Comparative studies on the nucleotide sequence of *Cucumber mosaic virus* RNA3 between Y strain and Q strain. Ann. Phytopathol. Soc. Jpn. 54: 516-522.

Ojeda, M.; A. Arroyo, P. Borgogno, E. Biderbost and M. Balzarini, 2004. Yield of peperina (*Minthostachys mollis* (Kunth.) Griseb.) populations in the year following planting: response to cropping regimen. Spanish Journal of Agricultural Research 2: 393-399.

Ordóñez, A.; S. Baldoncini, G. Berioli, G. Chaves, L. Bled; Y. Massuh, C. Liébana, L. Torres and M. Ojeda, 2006. Domestication of native aromatic plants. Molecular Medicinal Chemistry 11 58-59.

Owen, J. and P. Palukaitis, 1988. Characterization of *Cucumber mosaic virus* I: molecular heterogeneity mapping of RNA 3 in eight CMV strains. Virology 166: 495-502.

Owen, J.; M. Shintaku, P. Aeschleman, S.F. Tahar and P. Palukaitis, 1990. Nucleotide sequence and evolutionary relationships of *Cucumber mosaic virus* (CMV) strains, CMV RNA 3 Journal of General Virology 71: 2243-2249.

Palukaitis, P. and M. Zaitlin, 1997. Replicase-mediated resistance to plant virus diseases. Advances virus research 48: 349-377.

Palukaitis, P., and F. Garcia-Arenal. 2003. Cucumoviruses. Adv. Virus Res. 62:241–323.

Rizos, H.; L.V. Gunn, R.D. Pares and M.R. Gillings, 1992. Differentiation of *Cucumber mosaic virus* isolates using the polymerase chain reaction. Journal of General Virology 73: 2099-2103.

Rodríguez Pardina, P.; G. Truol, J. Arneodo and I.G. Laguna, 2003. Enfermedades virales en cultivos de poroto en el noroeste argentino. IDIA XXI: 125-129.

Roossinck, M.J., 2002. Evolutionary history of *Cucumber mosaic virus* deduced by phylogenetic analyses. Journal of Virology 76: 3382-3387.

Schmidt-Lebuhn, A.N.; J. Fuchs and M. Kessler, 2008. Flow cytometric measurements do not reveal different ploidy levels in *Minthostachys* (Lamiaceae). Plant Systematics & Evolution 27: 123-128.

Szilassy, D.; K. Salánki and E. Balázs, 1999. Molecular evidence for the existence of two distinct subgroups in Cucumber Mosaic Cucumovirus. Virus Genes 18: 221-227.