Note

Natural infection of several *Coffea* species and hybrids and *Psilanthus ebracteolatus* by the Coffee ringspot virus (CoRSV)

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ABSTRACT: Coffee ringspot is a minor coffee disease caused by the nuclear type of *Brevipalpus* mite-transmitted virus, Coffee ringspot virus (CoRSV). Recently outbreaks of the disease in some growing regions of the state of Minas Gerais, Brazil, were registered with qualitative and quantitative yield losses. *Coffea arabica* was the only species registered as natural host. A survey was made on a germplasm collection of *Coffea* and related species kept at the Centro de Café "Alcides Carvalho", Instituto Agronômico, Campinas, state of São Paulo (SP), Brazil, to assess natural susceptibility of *Coffee* species, other than *C. arabica* and some interspecific hybrids of *Coffea* as well as other non-*Coffea* plant species to the Coffee ringspot virus (CoRSV). The following plants were found with ringspot symptoms on their leaves and/or fruits besides *C. arabica* L.: *C. kapakata* (IAC 4511), *C. dewevrei* cv. Excelsa, *C. canephora* cv. Robusta, hybrid derivative of the *C. arabica* × *C. racemosa* (IAC1195-5-6-2), *C. arabica* × *C. dewerei* (Piatã IAC 387), Híbrido de Timor CIFC 832/1 (derivative from a natural crossing between *C. arabica* × *C. canephora*) and *C. racemosa*. Also *Psilanthus ebracteolatus*, a species close to the genus *Coffee* was also found with ringspot lesions on their leaves. All these plants were also found infested by *Brevipalpus* mites identified as *B. phoenicis*. Infection of these plants by CoRSV was confirmed by the observation of characteristic cytopathic effects in the tissues of the lesion and by RT-PCR using a pair of primer specific for CoRSV. Only with *C. racemosa* RT-PCR failed to amplify the CoRSV genome. The susceptibility of *P. ebracteolatus* to CoRSV adds new dimension regarding its controversial taxonomic position.
Keywords: *Coffea arabica*, C. *kapakata*, C. *racemosa*, C. *dewevrei*, Híbrido de Timor

Introduction

Ringspot symptoms on leaves (Figure 1 A) and berries of coffee (*Coffea arabica* L.) were first observed in the State of São Paulo, Brazil, and named "mancha anular" (Coffee ringspot) (Bitancourt, 1938). The disease has been observed in several regions of Brazil (Chagas et al., 2003; Kitajima and Chagas, 2009) and outside Brazil, confirmed only in Costa Rica (Rodrigues et al., 2002). The viral nature of the Coffee ringspot was inferred by electron microscopy which revealed the presence of short rod-like particles in the nucleus and cytoplasm and a characteristic electron lucent inclusion (viroplasma) in the nucleus (Kitajima and Costa, 1972; Chagas, 1980), its transmission by the tenuipalpid mite *Brevipalpus phoenicis* (Geijskes) (Acari: Tenuipalpidae) (Chagas, 1973), and mechanical transmission to *Chenopodium quinoa* Willd., *C. amaranticolor* Coste & Reyn. and *Gomphrena globosa* L. (Chagas et al., 1981). The causal virus was named Coffee ringspot virus (CoRSV). Coffee ringspot has been a minor disease in coffee plantations, but in some localities of the state of Minas Gerais, it caused significant yield loss (Chagas et al., 2003; Kitajima and Chagas, 2009) and affected the quality of the beverage (Boari et al., 2006). The virus was purified (Boari et al., 2004) and part of its genome was sequenced (GenBank accession GQ 979998). So far, CoRSV has been reported only in *C. arabica*. On the other hand, coffee is undergoing intense breeding program to produce plants with better agronomic properties not only selecting new lines of *C. arabica* but also through crossing with other *Coffee* species. Thus a survey was made on different *Coffee* species and hybrids of the germplasm bank maintained at the Centro de Café "Alcides Carvalho", of the Instituto Agronômico, Campinas, SP, Brazil, to detect cases of natural infection of some of these plants by the CoRSV. This article reports the finding of different species of *Coffea* and hybrids as well as a non-*Coffea* plant susceptible to CoRSV.

Materials and Methods

Coffee germplasm are kept either under slate wood roof nursery or in the field at the above mentioned germplasm bank (22°52'20" S, 47°04'44" W), where no chemical control of diseases or pests is made. Visual inspections were made throughout this collection and samples were taken whenever leaves and/or fruits showed ringspot-like symptoms. Samples were kept in plastic bags for later analysis.

Presence of mites was assessed by visual inspection under binocular. When present, they were fixed in ethanol 90% and mounted for scanning electron microscopy after air-dried

for species identification. Some mites were transferred to a leaf of *C. arabica* and left few hours. The leaf was instantly frozen by contact with a block of stainless steel at liquid nitrogen temperature and subsequently fixed in the vapor of osmium tetroxide, air dried, sputter coated before examination in a Zeiss 940A DSM scanning electron microscope.

Tissues from lesions on the leaves and fruits were fixed in a modified Karnovsky solution (2.5% glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 7.2) for at least 1-2 h at room temperature and post-fixed in a 1% solution of osmium tetroxide in the same buffer for 1 h. After dehydration in an increasing series of concentration of acetone, fixed tissues were infiltrated and embedded in the Spurr's low viscosicty epoxy resin (Kitajima and Nome, 1999). Blocks were sectioned in a Leica UTC ultramicrotome equipped with a diamond knife. After staining with 3% uranyl acetate and Reynold's lead citrate, the ultrathin sections were examined under a Zeiss EM 900 transmission electron microscope and the images registered digitally.

For the confirmation of virus presence, total RNA was extracted from symptomatic leaf tissues and health leaf tissues (negative control), according to Gibbs and Mackenzie (1997) and used for RT-PCR to detect CoRSV. RNA concentration and purity were estimated by spectrophotometry and denaturing agarose gel electrophoresis (1% agarose, 6.7% formaldehyde, MOPS 10X [200 mM MOPS, 5 mM sodium

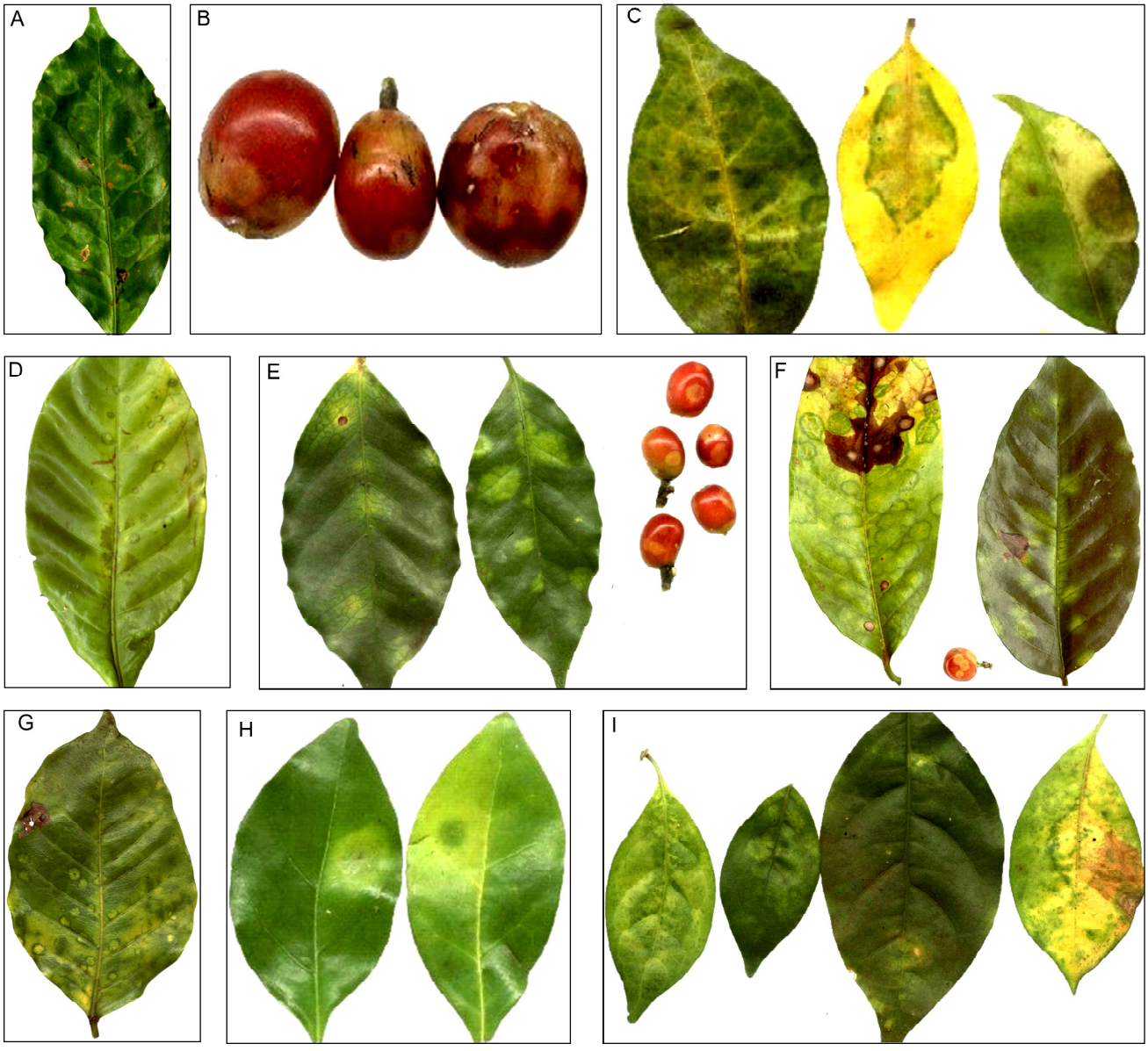

Figure 1 – A-I. Ringspot symptoms in *Coffea* species and hybrids and *Psilanthus ebracteolatus*. A- Leaf of *C. arabica*. B. Berries of *C. canephora* cv. Robusta. C. leaves of *C. kapakata*. D. Leaf of *C. dewevrei* cv. Excelsa. E. Leaves and berries of the Hybrid *C. arabica* × *C. racemosa*. F. Leaf and berry from Híbrido de Timor (derivative from a natural hybrid of *C. arabica* × *C. canephora*). G. Leaf of the hybrid Piatã (*C. arabica* × *C. dewevrei*). H. Leaves of *C. racemosa*. I. Leaves of *P. ebracteolata*, a close relative of the genus *Coffea*.

acetate, 10 mM EDTA and DEPC-treated H_2O]). Two hundred U of M-MLV reverse transcriptase (Invitrogen), 1.5 μL of 50 mM $MgCl_2$, 100 ng of total RNA and 100 ng of random primers (3 μg μL^{-1}) were used for RT reaction. Samples were denatured at 95°C for 10 min and placed into ice. Then, 4 μL of 5X buffer were added along with 1 μL dNTP mix (10 mM), 0.5 μL (2 mM) DTT, 15 U RNAse inhibitor (Invitrogen), and sterile Milli-Q water to a 20 μL final volume. The reaction was incubated at 37°C for 2 h. All PCR amplifications were conducted in a PTC 100 (MJ Research, Waltham, MA) thermocycler. The amplification reactions consisted of 2.5 mM $MgCl_2$, 10 mM dNTP mix (Invitrogen), 100 ng of each especific primer: CoRSVF (5' - GGACCATGAGACAGGAGGTG - 3') and CoRSVR (5' CTCTGCCAGTCCTCAATGTG - 3'), 2 μL of cDNA used as template, 1 U of *Taq* DNA polymerase (Invitrogen), and sterile Milli-Q water for a final volume of 25 μL. These primers were designed based on the sequence of the virus polymerase gene, the sequence of which is deposited in the GenBank (accession GQ 979998). An initial denaturing cycle at 94°C for 2 min was followed by 32 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 40 sec. A final 5 min extension was added. An aliquot of eight microliters of the PCR product were run in a 1% agarose gel.

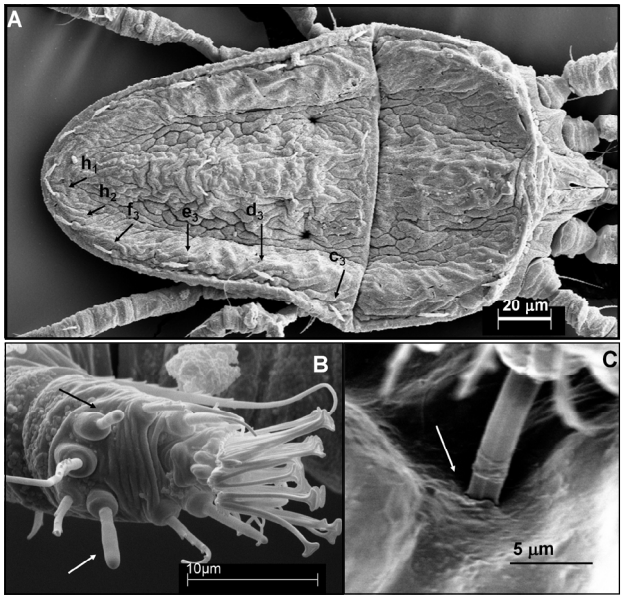

Figure 2 – Scannning electron micrographs of the flat mite *Brevipalpus phoenicis* (Acari: Tenuipalpidae) naturally infesting *Coffea* species and hybrids and *Psilanthus ebracteolata* plants showing ringspot symptoms on the leaves and fruits. A. Low magnification, dorsal view of an individual collected from the Híbrido de Timor. The six dorsal setae (c_3, d_3, e_3, f_3, h_2, h_1) are indicated by arrows. B. Detail of the end of the second pair of legs, revealing the presence of two solenidia (arrows) from a *B. phoenicis* collected from symptomatic *C. dewevrei* cv. Excelsa. C. *B. phoenicis* feeding on a leaf of *C. arabica*. Note the stylet penetrating through the leaf epidermis (arrow).

Results and Discussion

All sampled plants (several *Coffea* species and hybrids and the related species *P. ebracteolatus*) were infested by the flat mite identified as *Brevipalpus phoenicis*. Scanning electron microscopy (Figure 2) revealed the presence of two solenidia at the end of the second pair of legs (Figure 2 B) and six dorsal setae in the opistosome (Figure 2 A) which identify these mites as *B. phoenicis* (Welbourn et al., 2003). In frozen leaves, some *B. phoenicis* individuals were preserved in feeding position with the stylet perforating the epidermal layer (Figure 2 C). There are preliminary data indicating that CoRSV multiplies in the vector (E.W. Kitajima, *unpublished data*), showing that the virus vector relationship is of circulative/replicative type.

The following plants were found with ringspot symptoms on their leaves and/or fruits: *C. kapakata* (IAC 4511) (A. Chev.) Bridson (Figure 1 C), *C. dewevrei* (De Wild. and T. Duran) Lebrun cv. Excelsa (Figure 1 D), *C. canephora* Pierre ex. A. Froehner cv. Robusta (Linden) A. Chev. (Figure 1 B), hybrid derivative of the *C. arabica x C. racemosa* Lour. (IAC1195-5-6-2) (Figure 1 E), *C. arabica x C. dewevrei* (Piatã IAC 387) (Figure 1 G), Híbrido de Timor CIFC 832/1 (derivative from the natural crossing between *C. arabica* x *C. canephora*) (Figure 1 F), *C. racemosa* (Figure 1 H) e *P. ebracteolatus* Hiern. (IAC 3461-7) (Figure 1 I).

Transmission electron microscopy detected the previously described cytopathic effects induced by CoRSV (Kitajima and Costa, 1972; Chagas, 1980) in the cells of the leaf and fruit lesions. Several nuclei of the parenchymal and epidermal cells contained an electron lucent inclusion in the nucleus, referred to as viroplasma. Short, rodlike particles ca. 40 nm wide and 100-110 nm long were present in the nucleus, either in the nucleoplasm and/or within the viroplasma. These particles were also present in the cytoplasm, commonly associated with the membranes of the endoplasmic reticulum (Figure 3 A-J). This intracellular behavior of CoRSV is typical of the so-called nuclear type of *Brevipalpus*-transmitted virus (Kitajima et al., 2003). The best studied virus of this group is *Orchid fleck virus* (OFV). Its genome was completely sequenced revealing to be bipartite (ca. 6 kb each) and negative sense ssRNA with organization similar to that of rhabdoviruses and a new genus *Dichorhabdovirus* was proposed (Kondo et al., 2006). Though distinct from OFV, CoRSV has biological and molecular similarities with OFV (Chagas et al., 2003; Kondo et al., 2003) and may belong to the same genus.

RT-PCR assays using primers specific for CoRSV consistently amplified genomic fragments of the expected size in all, except one (*C. racemosa*) symptomatic samples (Figure 4). These results clearly reveal that these *Coffea* species and hybrids were naturally infected by CoRSV. The virus must have been transmitted by the mite *B. phoenicis* present in all symptomatic plants, and known as the vector for CoRSV (Chagas, 1973). A plant of *C. racemosa* was found also with leaves exhibiting ringspot symptoms (Figure 2 H). Electron microscopy demonstrated the presence of cytopathic effect typical for CoRSV (Figure 3 H) but RT-PCR failed to amplify the viral genome in at lest three attempts. The cause of this discrepancy is being investigated.

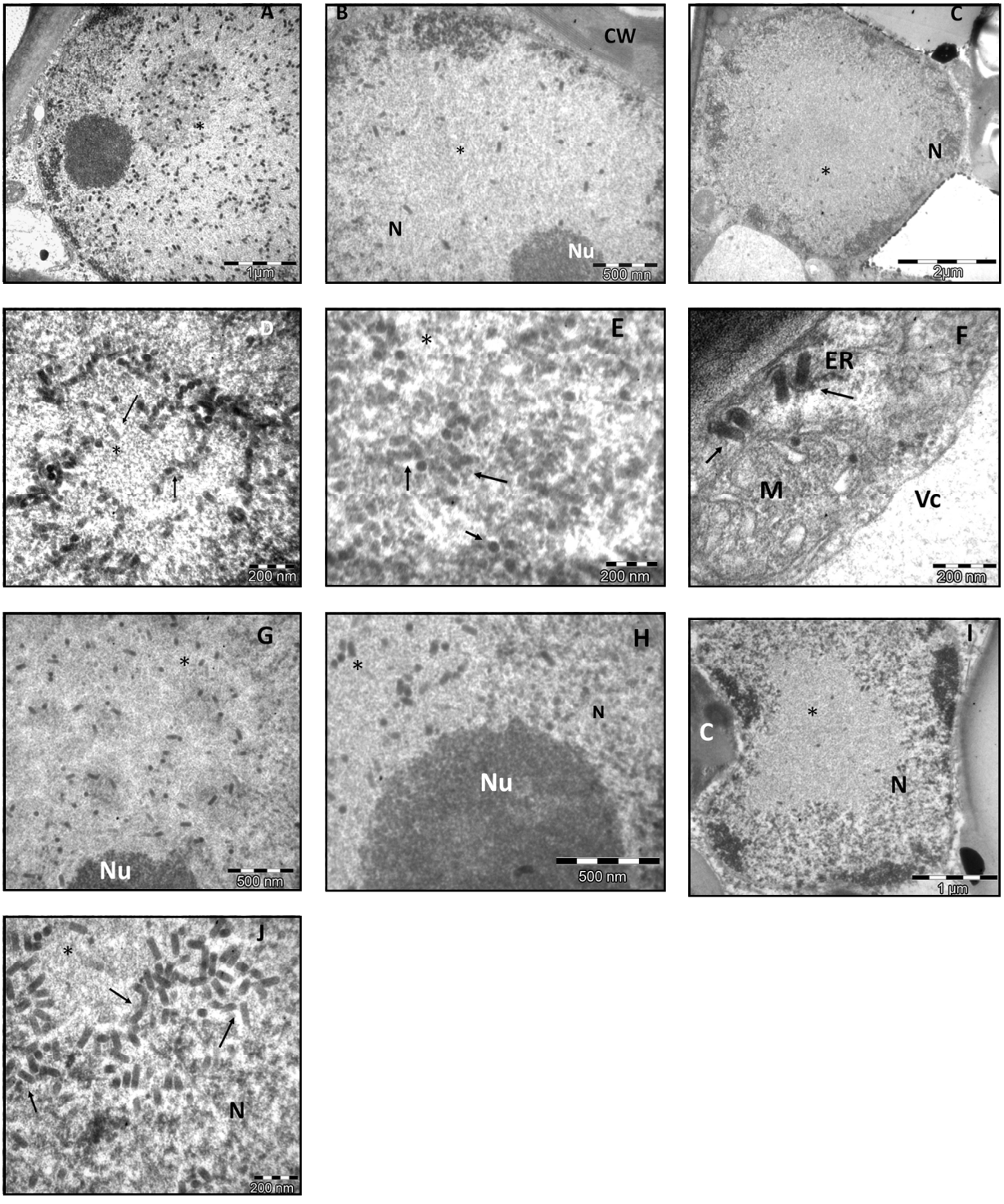

Figure 3 – Transmission electron micrographs of thin sections of parenchymal cells from leaves or fruits with ringspot symptoms. A-C. Low magnification images showing the characteristic intranuclear viroplasma (*), respectively in leaf parenchymal cells of the hybrid *Coffea arabica* × *C. racemosa* (A), *C. dewevrei* cv. Excelsa (B) and from fruit cell of *C. canephora* cv. Robusta (C). D. A detail of the nucleus of a parenchymal cell from the hybrid Piatã *C. arabica* × *C. dewevrei*. A group of presumed virions (arrows) appear interspersed within the viroplasma. E. Similar to D, but from a nucleus of *C. kapakat*a; F. A detail of the cytoplasm of a leaf parenchyma cells of *C. dewevrei* cv. Excelsa showing rodlike virions (arrows) arranged perpendicularly onto membranes of the endoplasmic reticulum (ER). G. Presumed CoRSV virions scattered within a nuclear viroplasma in a cell of leaf parenchyma of the Híbrido de Timor (*C. arabica* × *C. canephora*). H. Similar to G, but in a leaf cell of *C. racemosa*. I. Low magnification image of a nucleus from leaf parenchyma cell of *Psilanthus ebracteolatus.* An electron lucent viroplasma is present within the nucleus. J. Detail of presumed virions (arrows) scattered within the viroplasma in a leaf cell of *P. ebracteolata*. Key for the abbreviations: C- chloroplast, CW- cell wall, M- mitochondrion; N- nucleus, Nu- nucleolus, Vc- vacuole.

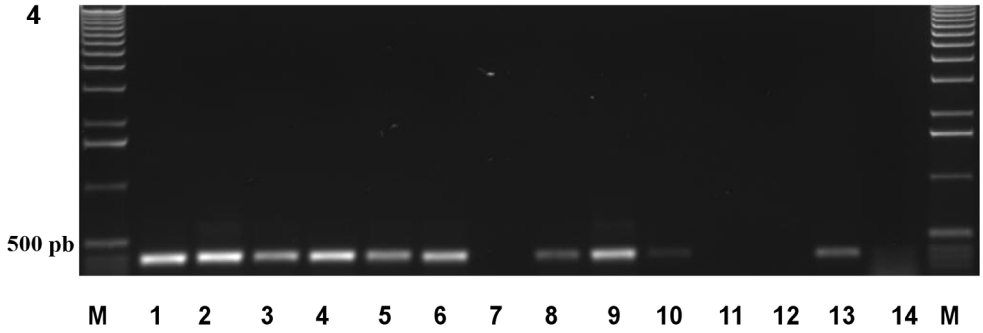

Figure 4 – Agarose gel (1%) profile showing the results of the RT-PCR amplification of genomic material of Coffee ringspot virus (CoRSV) present in the tissues of the ringspot lesions of leaves and/or fruits of some different *Coffea* species and hybrids and in *Psilanthus ebracteolatus*. Lanes: M. Molecular weight marker "Ladder 1 Kb Plus"; 1 and 2: *P. ebracteolatus* leaves ; 3 and 4: hybrid *C. arabica* × *C. racemosa*- leaves; 5 and 6: likewise, fruits with lesions ; 7 and 8: *C. arabica* (HW 17/12)- leaves with ringspot; 9 and 10 – Híbrido de Timor- leaves; 11 and 12 – likewise, fruits; 13: Leaves of *C. arabica* with ringspot, positive control; 14: Leaves from healthy *C. arabica* (negative control).

P. ebracteolatus is distributed in the African continent (Ghana, Ivory Coast, Nigeria, Cameroon). It was first described by Hiern in 1877 but was suggested to be a member of the genus *Coffea* (*C. ebracteolata*) by Brennan in 1953. Experimental hybrids were obtained between *C. arabica* and *P. ebracteolatus* (Couturon et al., 1998). Recent studies applying cytological and molecular techniques did not clearly resolve the taxonomic relationship between the genera *Coffea* and *Psilanthus* (Lombello and Pinto-Maglio, 2003; Davis et al., 2006; Maurin et al., 2007) though Kumar et al. (2008) showed diversity between Indian species of *Psilanthus* and West and Central African species of *Coffea* using RAPD and ISSR assays. Though several non-Rubiaceae plant species have been described as experimental host for CoRSV (Chagas et al., 2003; Kitajima and Chagas, 2009), *P. ebracteolatus* is the only non-*Coffea* natural host for this virus reported so far. This susceptibility to a virus affecting naturally many *Coffea* species may add a new factor for the discussion of the taxonomical position of the genus *Psilanthus*.

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