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Effects of the *Papaya meleira virus* on papaya latex structure and composition

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Abstract Spontaneous latex exudation is the main symptom of papaya sticky (meleira) disease caused by the *Papaya meleira virus* (PMeV), a double-stranded RNA (dsRNA) virus. This paper describes different effects of PMeV on papaya latex. Latex samples were subjected to different histochemical tests to evaluate their chemical composition. Additionally, the integrity of the latex particles was assessed by transmission and scanning electron microscopy analysis. Biochemical and micro- and macro-element measurements were performed. PMeV dsRNA extraction was performed to evaluate the interaction of the virus with the latex particles. Sticky diseased latex was positive for alkaloid biosynthesis and showed an accumulation of calcium oxalate crystals. PMeV also increased H_2O_2 synthesis within sticky diseased laticifers. The protein, sugar and water levels were altered, probably due to chemical changes. The morphology of the latex particles was further altered; PMeV particles seemed to be bound to the latex particles. The alkaloid and H_2O_2 biosynthesis in the papaya laticifers indicate a papaya defense response against PMeV. However, such efforts failed, as the virus affected the plant latex. The effects described here suggest some advantages of the infection process, including facilitating the movement of the virus within the papaya plant.

Keywords *Carica papaya* · Latex · Laticifers · Meleira · *Papaya meleira virus* · Papaya sticky disease

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Introduction

Papaya (*Carica papaya* L.) laticifers are a series of ramified and interconnected cells forming an array of complex tubes throughout the papaya plant (Esaú 1976). When mature, they are mainly composed of large latex vesicles. Papaya latex is a fluid with a milky appearance that contains about 85% water. An insoluble particulate fraction, whose composition is still practically unknown, makes up 25% of the dry matter. The soluble fraction, on the other hand, contains both the usual ingredients such as carbohydrates (~10%), salts (~10%) and lipids (~5%), and representative biomolecules such as glutathione, cysteine proteinases (~30%) and several other proteins (~10%) (El Moussaoui et al. 2001). Latex from other species contains carbohydrates, organic acids, alkaloids, oils, terpenes, resins, rubber and other compounds (Calvin 1987; Hunter 1994). Until now, latex composition was suspected to inhibit microorganisms from colonizing the laticifers, as only a few examples of such an infection had been observed. Bacterial colonization in bunchy top-affected papaya laticifers has been demonstrated (Davis et al. 1996), and flagellate protozoan (*Phytomonas* sp.) are well-known microorganisms located in the *Euphorbiaceae* laticifers (Da Cunha et al. 2000). However, *Papaya meleira virus* (PMeV) is a unique plant virus among those observed in laticifers described so far.

First reported by Rodrigues et al. (1989), the 'papaya sticky disease' or 'meleira' was characterized by a spontaneous fluid and translucent latex exudation from fruits and leaves. After atmospheric exposure, the latex oxidizes, resulting in small necrotic lesions on the edges of young leaves and, subsequently, stickiness on the plant organs (Ventura et al. 2001). Using electron microscopy, spherical 50 nm virus-like particles were observed only in diseased papaya laticifers (Kitajima et al. 1993). Additionally, 12 kbp double-stranded RNA (dsRNA) molecules were extracted from infected latex in abundance, suggesting a viral disease etiology (Kitajima et al. 1993; Rodrigues et al. 2005). This hypothesis was confirmed by Zambolim et al. (2003), who purified the virus and demonstrated Koch's postulates by the development of disease symptoms in healthy plants after inoculation with purified particles. Koch's postulates demonstrated that the dsRNA originated from the virus and proved that it was associated with the disease agent (Ventura et al. 2001, 2004; Zambolim et al. 2003). Recently, specific PMeV primers were designed based on viral dsRNA nucleotide sequences. Through RT-PCR, a 669-nucleotide fragment was amplified and found to be very similar to other viral RNA-dependent RNA polymerases after sequencing (Araújo et al. 2007). Despite the reports presented on the papaya sticky disease, the effects of the virus on plant laticifers are not yet understood. Here, we provide evidence that the PMeV alters the latex structure and composition by influencing the physiology of the laticifers.

Materials and methods

Plant material and latex collection

Leaves and unripe fruits were collected from a total of three healthy and three sticky diseased papaya (cv. Golden). Plants of 26 months old were collected from the INCAPER Experimental Field in Espírito Santo State, Brazil. Diseased plants were identified in the field by the typical sticky disease symptoms, which were later confirmed by PMeV molecular diagnosis (Rodrigues et al. 2005; Araújo et al. 2007). Papaya plants showed the most common symptom of meleira by exudation of fluid latex from fruits that oxidizes and becomes dark. Also, it was observed the exudation of latex from edges of young leaves in the top of the plant that provokes small light-brown necrotic lesions on the leaf tips (Ventura et al. 2004). Samples of plant latex were monitored for PCR detection of the viral dsRNA. The latex from fruits of the plants with meleira presented a clear watery aspect, due to its lower viscosity and lack of coagulation that darkens with greater facility than that of healthy fruits.

Latex was obtained by tapping the fruits using a steel razor blade. The samples were collected in 1.5 ml microtubes containing a 0.1 M citrate buffer at pH 5.0 1:1 (v:v) for molecular extraction of viral dsRNA. Crude latex aliquots were used for micro- and macro-elemental testing and biochemical measurements, as well as for histochemical tests. All latex samples were maintained at −20°C before use. For electron microscopy, the latex samples were kept at 25°C for 2 h in a solution containing 2.5% glutaraldehyde and 4.0% paraformaldehyde in a 0.05 M cacodylate buffer at pH 7.4 (1:1 v:v), followed by storage at 4°C.

Latex histochemical tests

Healthy latex (HL) and sticky diseased latex (SDL) (100 µl) were deposited on microscope slides separately. The following reagents were used at 25°C at a 1:1 (v:v) ratio. Reducing sugars were stained red after heating them together with Fheling's reagent for 10 s (0.8 M cupric sulfate, 2.5 M potassium sodium tartrate; Purvis et al. 1964). Alkaloids were stained dark-green using Dragendorff's reagent for 1 h (0.1 M bismuth nitrate, 5.5 M hydrochloric acid, 0.01 M potassium iodate; Kraus and Arduin 1997). Proteins were stained blue using a coomassie brilliant blue solution for 10 min (0.025% coomassie brilliant blue, 40% methanol, 7% acetic acid; Kraus and Arduin 1997). Starch grains were stained dark-brown using Lugol's reagent for 5 min (0.09 M potassium iodate, 0.01 M iode; Jensen 1962). Lipids were stained yellow using a Sudan III solution for 20 min (0.01 M Sudan III, 80% ethanol; Jensen 1962). Phenols were stained dark-green using a ferric chloride solution for 2 min (0.37 M hexahydrate ferric chloride, 0.02 M sodium carbonate; Johansen 1940). The oxalate chemical nature of inclusions in papaya latex was investigated using a modification of a previously described method (Yasue 1969). The crystals were then separately subjected to 10% hydrochloric acid and 10% acetic acid for 20 min. According to Yasue's method, the crystals are assumed to be composed of oxalate when they readily dissolve in hydrochloric acid without observable effervescence, while only partially dissolving in acetic acid. Unstained latex was used as a control. The microscope slides were recorded and analyzed using an Axiophoro ZEISS light microscope coupled with an Analysis Sis Link/Oxford Zeiss system. For each sample, 25 fields (1 mm^2 each) were analyzed.

In situ detection of H_2O_2

Oxalate has been shown to be a substrate for oxidative enzymes during H_2O_2 production (Lane et al. 1993, 1994). In order to assess the correlation between calcium oxalate

and H_2O_2 in papaya laticifers, H_2O_2 was investigated in papaya tissues. For this purpose, a DAB-uptake method based on the plant's peroxidase activity was used. DAB is used as a substrate during the peroxidase reaction, and locally produces a reddish-brown precipitant (Orozco-Cárdenas and Ryan 1999; Ma et al. 2008). Laticifers were easily identified within leaf stalk tissues, recognized as articulated and anastomosed cells between the xylem and phloem tissues (Fig. 3). These anatomical features had already been fully described (Esaú 1976).

The production of H_2O_2 was detected by approximately 10 mm cuts in leaves, stalks and fruits. Transverse and longitudinal cuts were made by hand using a razor blade. The samples were then incubated for 12 h in 2.5 mM 3,3′-diaminobenzine (DAB)-HCl at pH 3.8 (Sigma, USA). Control samples were immersed in deionized water. Samples were decolorized in hot 96% ethanol for 20 min. They were then placed in 50% glycerol and observed using light microscopy as described elsewhere. H_2O_2 was seen as a reddish-brown coloration (Orozco-Cárdenas and Ryan 1999; Ma et al. 2008).

Protein and sugar assays

The protein content was determined according to the procedure described by Lowry et al. (1951), with modifications. Briefly, latex diluted in ultra-pure water (1:1 v:v) was homogenized using a vortex. An aliquot (1 ml) of the diluted latex was added to 5 ml of cupper reagent (48 ml of 3% sodium carbonate diluted in 0.1 M sodium hydroxide; 1 ml of 4% sodium and potassium tartrate; 1 ml of 2% copper sulphate). After 10 min, 500 µl of water-diluted Folin-Ciocalteu reagent (1:2 v:v) was added to the mixture. The samples remained at 25°C during 10 min and had the absorbance measured at 660 nm. The protein content was determined using a BSA standard curve.

The papaya latex was diluted in water and mixed using a vortex in order to make a more homogeneous latex suspension. Pure water (Moutim et al. 1999) or an aqueous solution (Azarkan et al. 2006) was previously used for the collection of papaya latex. This procedure allowed the disaggregation of the latex particles (instead of solubilize the latex particles) turning the samples more fluid and easy to process.

The total sugar concentration was determined by an anthrone reaction as previously described by Scott and Melvin (1953), with modifications. Shortly, the latex diluted in ultra-pure water (1:6 v:v) was centrifuged 11,000 g for 5 min and had the supernatant collected. A sample volume (500 µl) was added to 2.5 ml of 0.02% anthrone solution (1 g of anthrone in 50 ml of distilled water, making it to volume (500 ml) with concentrated sulfuric acid). The samples were incubated at 100°C during 10 min. They remained at 25°C in the dark and had their absorbance measured at 620 nm. The sugar content was determined using a glucose standard curve.

Quantification of micro- and macro-elements

A quantitative measurement of the nitrogen (N) content was carried out by Kjeldahl's method (Stuart 1936). Phosphorous (P), potassium (K), calcium (Ca), iron (Fe), zinc (Zn), manganese (Mn) and magnesium (Mg) contents were determined by nitric–perchloric acid digestion (Miller 1997) followed by concentration measurements using an atomic-absorption spectrometer (AAS) (Varian AA 240 FS, Victoria, Australia). The P concentration was determined at 725 nm using a spectrophotometer (Celm E 225 D, São Paulo, Brazil).

Dry mass quantification and pH measurements

The mass of 5 ml fresh latex was measured before and after heating at 70°C for 5 h. The supernatant of latex, diluted in water (1:2 v:v) and centrifuged at 8,000 g for 10 min at 25°C, was used for pH measurement.

Electron microscopy analysis

Latex stored as previously described was centrifuged at 8,000 g for 5 min at 25°C, after which the solid phase was post-fixed in 1.0% osmium tetroxide (OsO_4) for 1 h at 25°C and dehydrated in a graded series of 30, 50, 70, 90 and 100% (v:v) acetone, 30 min for each step. The dehydrated samples were then embedded with epoxy resin. Ultra-thin sections were investigated using a ZEISS 900 transmission electron microscope (TEM). For the scanning electron microscopy (SEM) studies, the latex was critical point dried in CO_2 after fixation and dehydration, mounted onto carbon stubs and coated with 20 nm gold. The samples were then examined using a ZEISS 962 microscope. SEM and area-restricted X-ray analysis by energy dispersive spectrometry (EDS) were carried out using an SEM instrument with an attached energy-dispersive X-ray analytical system containing a lithium-drifted silicon detector. SEM analysis was carried out using an acceleration voltage of 20 kV.

Interaction between the PMeV and the latex polymers

Healthy and SDL samples (1.0 ml each), previously diluted in a citrate buffer at pH 5.0, were centrifuged at 11,000 g for 15 min at 4°C. The resulting pellet, composed of coagulated latex particles, was washed twice using 1.0 ml cold ultra-pure water followed by centrifugation at 11,000 g for 15 min at 4°C. The solid phase was

resuspended in 100 µl ultra-pure water. Then, both the liquid and solid phases were subjected to nucleic acid extraction using 1 V 2:1 v:v phenol/chlorophorm. The nucleic acids were precipitated using 2 V cold-ethanol and 0.1 V 3 M sodium acetate at pH 5.2 in liquid nitrogen for 30 min. The pelleted nucleic acids were separated using 1% agarose gels stained with 10 ng ml^{-1} ethidium bromide (Sigma, USA), and were observed under UV light using the Eagle-Eye photo equipment (Rodrigues et al. 2005).

Statistical analysis

The plants in the field were arranged in a completely randomized design. The crop maintenance was similar to standard commercial production, and inoculated plants showed the most common symptom of meleira (Ventura et al. 2004). All experiments were performed with three biological samples and three technical replicates.

The data obtained from the chemical and biochemical measurements on the latex were analyzed by ANOVA using the SAEG software (Release 4.0, UFV, MG, Brazil). The Tukey test at $P = 0.05$ was used to determine statistical significance.

Results

Latex histochemical tests

Direct observation of the latex using bright-field microscopy revealed the occurrence of structures with irregular shape composed of latex particles (Fig. 1l, m). In order to determine their composition, latex samples were subjected to different reagents. Both sticky diseased papaya latex and healthy papaya latex were stained for phenols, proteins, reduced sugars (Fig. 1) and lipids (Fig. 2). SDL was stained for proteins at a lower intensity than HL (Fig. 1c, d). Reducing sugars showed an intense brown coloration uniformly distributed on HL particles (Fig. 1e). On the other hand, SDL had a diffuse light brown coloration, occasionally delimiting vesicle-like structures (Fig. 1f). Only SDL was stained for alkaloids, suggesting a papaya response to PMeV infection (Fig. 1g, h).

Starch grains were not observed in any sample (Fig. 1i, j). When starch grains are present they can be identified as individualized structures instead of having a disperse coloration (as shown in Fig. 1i, j). The samples were analyzed in different microscopic magnification and such structures could not be observed. The occurrence of starch grains in papaya latex was somewhat expected. However, the negative result might be associated with the intrinsic latex amidase activity. It was reported that after tapping the

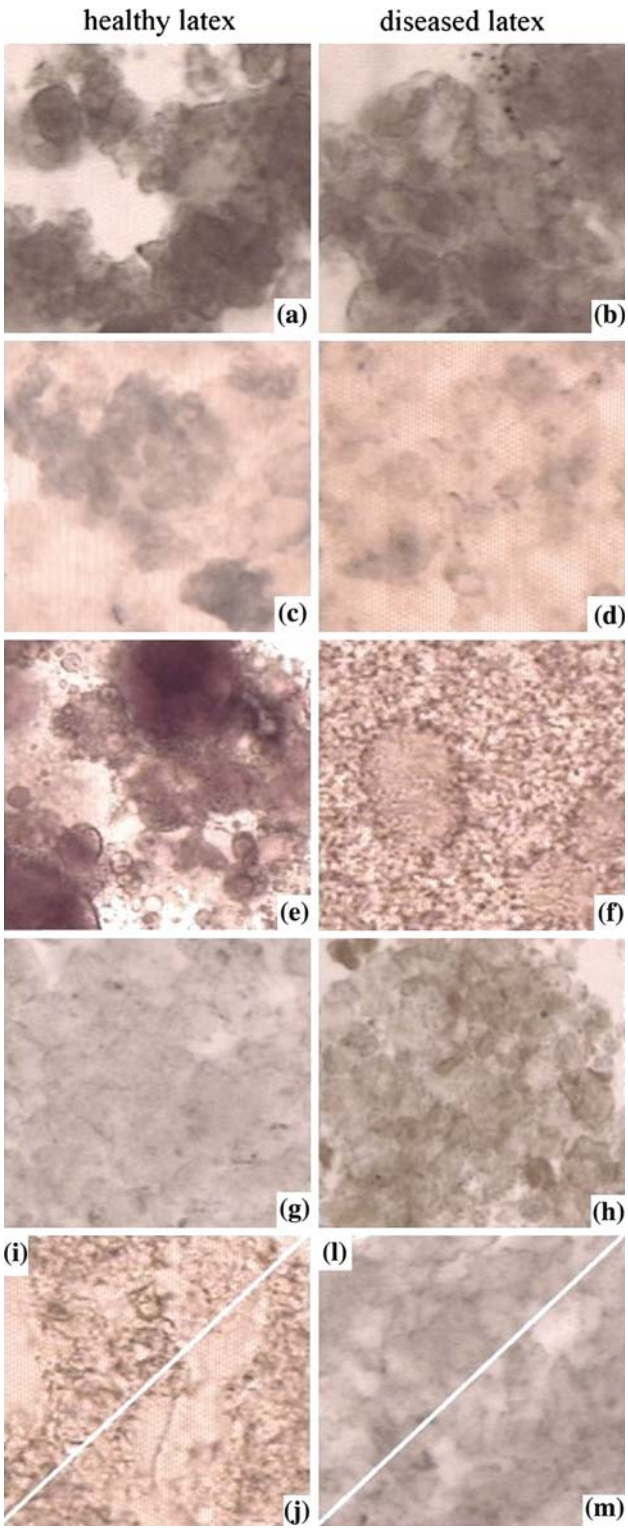

Fig. 1 Histochemical tests of papaya latex. Latex samples collected from healthy (**a**, **c**, **e**, **g**, **i**, **l**) and diseased (**b**, **d**, **f**, **h**, **j**, **m**) plants were subjected to non-permanent coloration of different compounds such as phenols (**a**, **b**), proteins (**c**, **d**), reduced sugars (**e**, **f**) and alkaloids (**g**, **h**). Starch grains were not observed (**i**, **j**). Visualization by contrast of the gray tones background with that of unstained control latex (**l**, **m**)

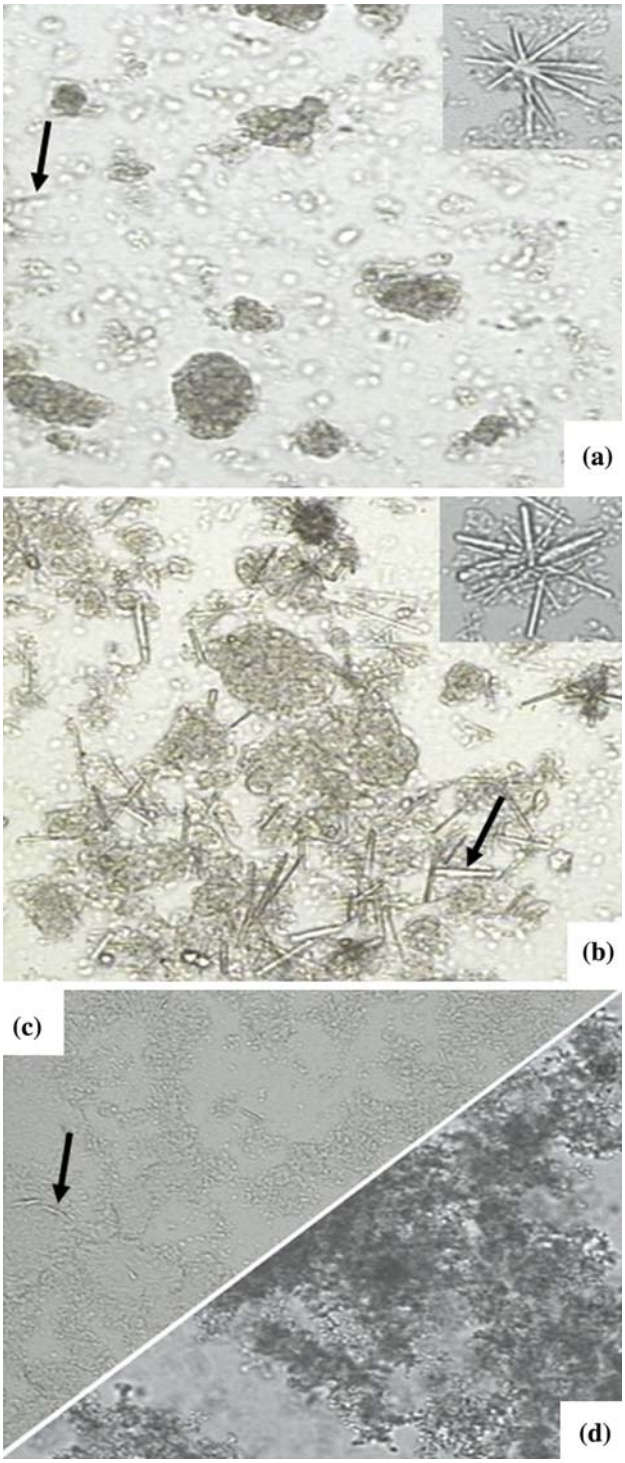

Fig. 2 Histochemical tests of papaya latex for lipids. Latex samples collected from healthy (**a**) and diseased (**b**) plants were stained using a Sudan III reagent. The *dark background* on latex particles compared with unstained latex (Fig. 1l, m) indicates the presence of lipids. After staining with Sudan III, some crystals could be observed (indicated by *arrows*). Agglomerates of crystals (**a**, **b** insets) were observed in both diseased and healthy latex. They were subjected to acetic (**c**) or hydrochloric (**d**) acids

papaya latex shows amidase activity that is maintained for about 15 min (Moutim et al. 1999). Perhaps, in our work, the period between tapping and the collection of the latex was long enough to cause the starch degradation that could explain for the observed result.

Interestingly enough, after the SDL and HL samples were treated with the Sudan III solution staining for lipids, raphyd-like crystals were observed (Fig. 2a, b). Moreover, no additional treatment was needed to observe these crystals, which were discernible even at the lowest microscopic magnification. The crystals were remarkably more pronounced in SDL (Fig. 2b) and were also seen forming clusters, which seemed to be associated with latex particles (Fig. 2). In order to reveal the chemical nature of the crystals, the samples were separately subjected to hydrochloric and acetic acid treatments. They completely dissolved in hydrochloric acid without any effervescence but only partially dissolved in acetic acid (Fig. 2c, d), indicating that they were composed of oxalate (Yasue 1969). An elemental analysis of the crystals by energy-dispersive X-ray spectroscopy confirmed their oxalate composition (data not shown).

In situ detection of H_2O_2

Papaya laticifers were brown after DAB treatment (Fig. 3), identifying the papaya laticifers as H_2O_2 production sites. Furthermore, the brown color was more intense in diseased samples (Fig. 3c, d), suggesting an H_2O_2 accumulation during PMeV infection. In addition, H_2O_2 was highly localized only in the phloem companion cells from diseased tissues (Fig. 3), suggesting a specific papaya response to PMeV. Similar results were obtained from fruit tissues (data not shown).

Biochemical analysis

Latex from sticky diseased papaya is known to be more fluid and translucent than its healthy counterpart (Ventura et al. 2001, 2004). In order to understand what factor(s) could be related to this symptom, we measured the biochemical contents of the samples. As seen in Table 1, SDL contained a large amount of water (87.33%) when compared to HL (81.03%). Additionally, proteins and sugars were more concentrated in HL, with concentrations of 0.17 and 0.44 mg ml^{-1}, respectively, compared to 0.13 and 0.18 mg ml^{-1} in SDL samples. There was no significant difference in pH, as similar values were obtained from diseased (pH 6.13) and healthy (pH 5.68) samples.

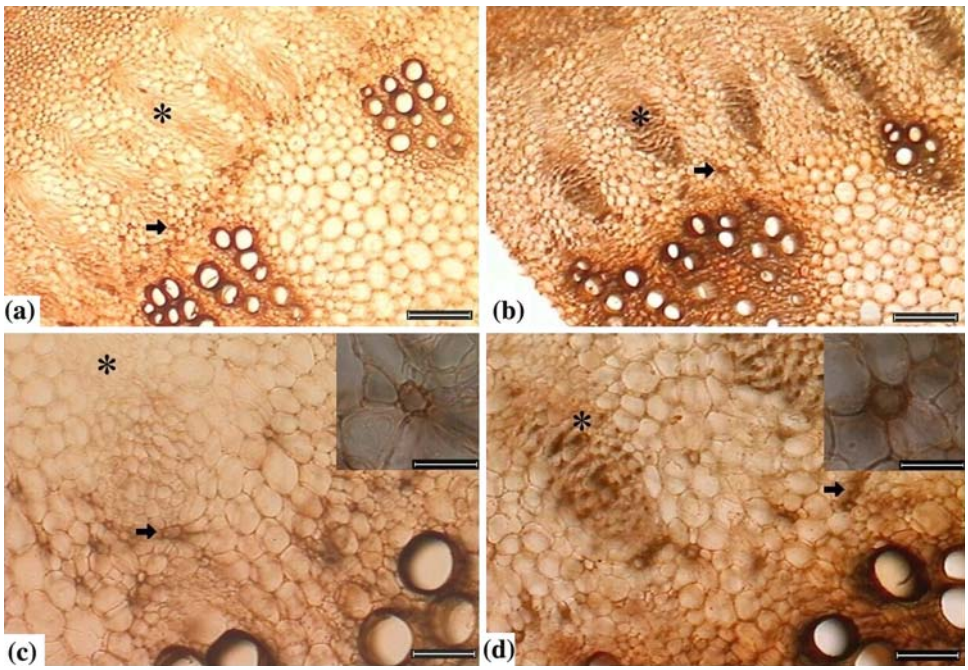

Fig. 3 In situ detection of H_2O_2 in papaya. Petioles from healthy (**a**, **c**) and diseased (**b**, **d**) plants were subjected to a DAB reagent. The presence of H_2O_2 was seen in the laticifers (indicated by *arrows*) from both healthy and diseased samples. A more intense coloration was observed in the diseased laticifers (insets in **c** and **d**). The *asterisk* indicates the phloem tissues. *Scale bars* are 0.1 mm in **a** and **b** and 1.0 mm in **c** and **d**

Table 1 Concentration of biochemical and chemical elements from healthy and diseased papaya latex samples

Parameters	Papaya latex	
	Healthy plants (HL)	Diseased plants (SDL)
Water content (%)	81.03b	87.33a
pH	5.68a	6.13a
Sugars (mg ml^{-1})	0.44a	0.18b
Proteins (mg ml^{-1})	0.17a	0.13b
Phosphorus (% DW)	0.30b	0.33a
Potassium (% DW)	0.59b	0.77a
Calcium (% DW)	0.71a	0.35b
Magnesium (% DW)	0.81a	0.78a
Sulfur (% DW)	1.63a	1.71a
Iron (μg g^{-1} DW)	20,000a	20,000a
Zinc (μg g^{-1} DW)	7,000a	6,000a

Values on a line followed by the same letter are not significantly different according Tukey's Studentized range test ($P = 0.05$). These values were obtained from dry weight (DW) samples

Measurements of chemical elements

Considering the SDL's increased water content, we postulated that the infected laticifers might undergo some osmotic alteration, resulting in an uptake of water. To search for such osmotic constituents, micro- and macro-element measurements were performed by atomic-absorption spectroscopy. The percentages of potassium (K), phosphorus (P), iron (Fe), zinc (Zn), manganese (Mn), calcium (Ca), magnesium (Mg) and sulphur (S) were determined from HL and SDL dry mass samples. K and P constituted 0.77 and 0.33% of SDL dry mass, respectively, compared to 0.59 and 0.30% of HL dry mass. HL contained 0.71% Ca, compared to 0.35% in the SDL. None of the other tested elements varied significantly between HL and SDL (Table 1).

Scanning electron microscopy analysis

In addition to the chemical latex constituents, the coagulation process has also been associated with latex fluidity. It has been shown that the papaya latex protein profile is completely altered during coagulation, leading to an activation of several enzymes (Moutim et al. 1999). However, the exact coagulation mechanism is not yet known. Recently, Wititsuwannakul et al. (2008a, b, c) described the purification and characterization of three proteins (*Hevea* latex lectin-like protein, HLL; rubber-particle HLL biding protein, RP-HLLBP; C-serum lectin biding protein, CS-HLLBP) involved in the rubber tree (*Hevea brasiliensis*) latex coagulation process. According to the described model, the RP-HLLBP glycoprotein binds to HLL that is exposed on lutoid membranes when the laticifers are tapped. This causes the aggregation of adjacent rubber particles and the formation of the latex coagulum. The coagulation intensity is reduced by CS-HLLBP, that competes with RP-HLLBP by the HLL linkage (Wititsuwannakul et al. 2008a, b, c). Therefore, the surface of latex particles is important to the coagulation process.

In order to analyze the morphology of the papaya latex particles, SEM was used. Analysis of SEM micrographs showed that PMeV infection notably reduced the

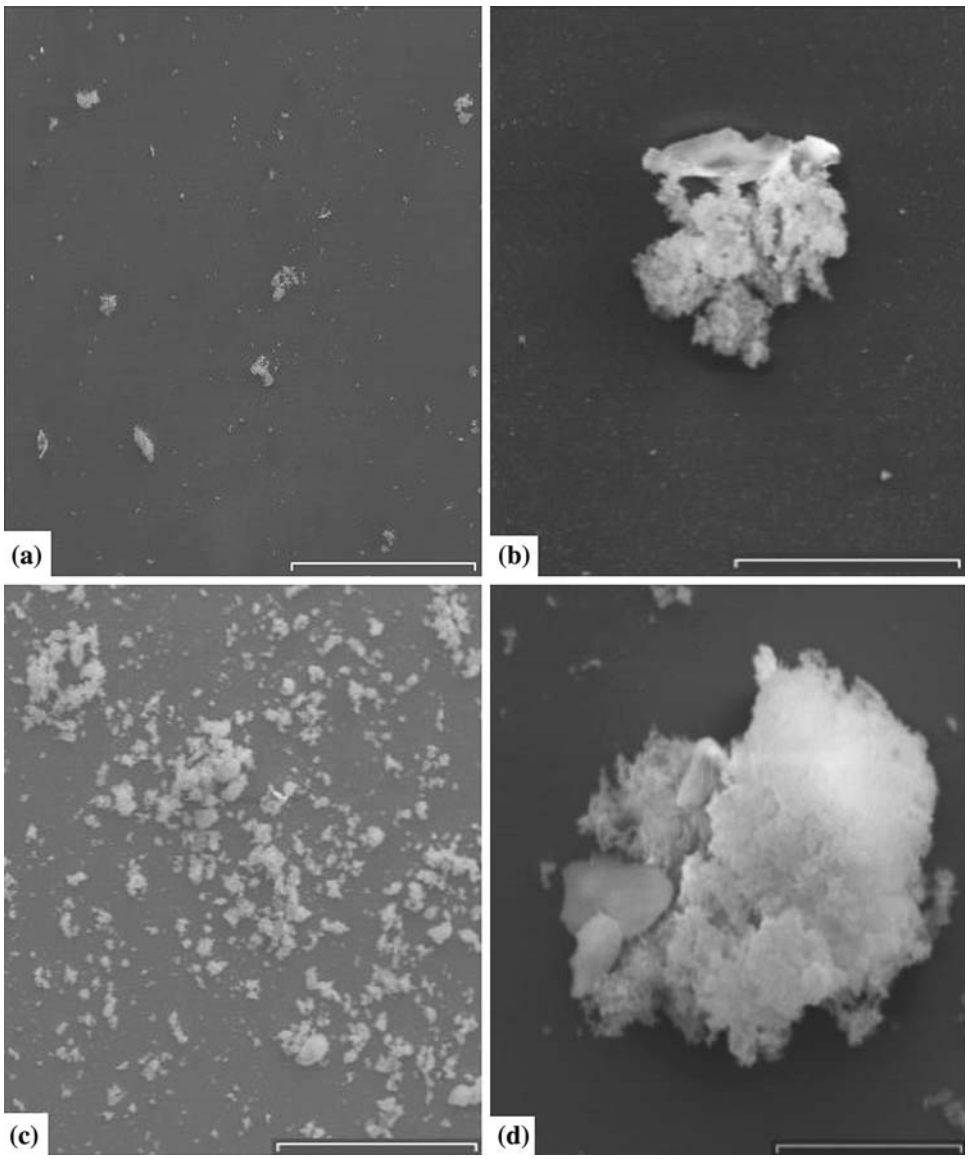

Fig. 4 Papaya latex analysis by scanning electron microscopy (SEM). Latex samples collected from diseased (**a**, **b**) and healthy (**c**, **d**) plants were studied by SEM, which revealed the morphology of the latex polymers. *Scale bars* are 500 μm in **a** and **c** and 20 μm in **b** and **d**

amount of papaya latex particles (Fig. 4a, c). Comparing Fig. 4b (SDL) and d (HL) we may also assume that the latex particles from diseased plants had their morphology altered and present a more loose structure. A similar pattern was observed by Da Cunha et al. (2000) analyzing the latex of *Euphorbiaceae* plants infected by *Phytomonas*.

Interaction between the PMeV and the latex particles

TEM was conduced in order to verify the interaction between the virus and the latex particles. The micrograph presented on Fig. 5 clearly shows the virus particles preferentially gathering at the grain boundaries. The PMeV was localized on and linked to the latex polymers. To confirm this hypothesis, we tested different treatments (pH 1.0, 8.0, 2–8 M urea, 50–300 MPa high hydrostatic pressure, 1–10% SDS detergent solution and ultrasonic treatment) for their ability to release the PMeV dsRNA from the latex solid phase (latex particles) to the latex liquid phase. However, none of the treatments was able to release the PMeV dsRNA from the latex particles (unpublished results). The inability of the detergent treatment to liberate the PMeV dsRNA suggests that such interaction can not involve the particle membrane fatty acids. By contrast, it is important to note that during the TEM sample preparation, the latex liquid phase was completely eliminated through successive washes. Moreover, the resistance to other severe treatments indicates that only strongly attached elements could remain on the latex particles. Taken together, these data suggest that the PMeV is entrapped in the papaya latex particles and the infection provoked by the PMeV alters the laticifer's physiology compromising the perfect latex particles formation.

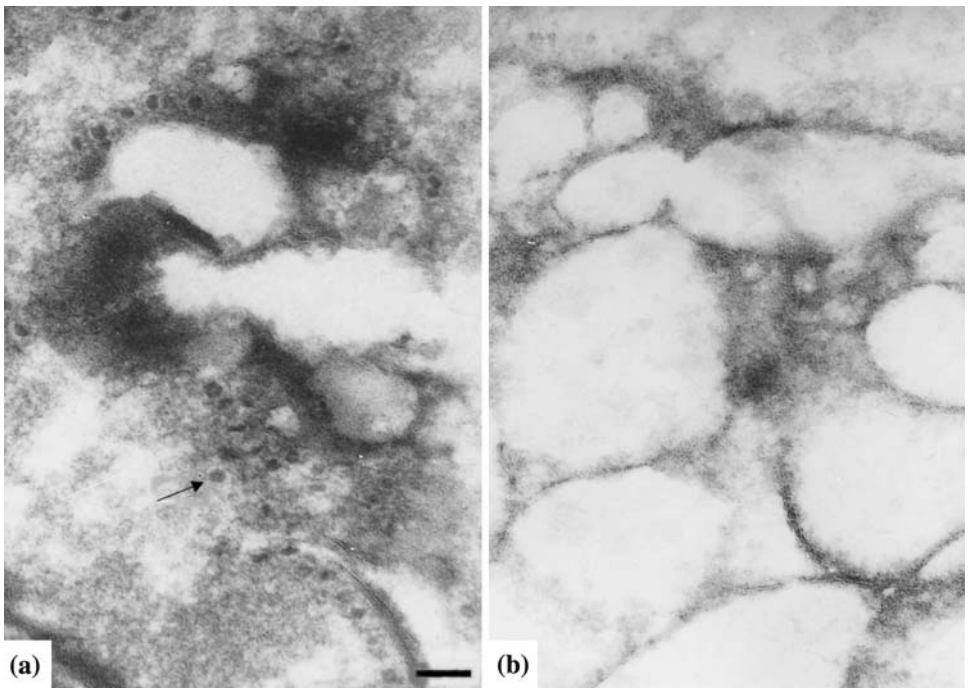

Fig. 5 Papaya latex analysis by transmission electron microscopy (TEM). Diseased (**a**) and healthy (**b**) latex samples were studied by TEM, which revealed PMeV particles (indicated by *arrow*) on the polymers. The *scale bar* is 200 nm

To confirm the close association of PMeV particles with the latex particles, both the SDL liquid and solid phases were subjected to nucleic acid extraction, and the approximately 12-kbp nucleic acid band seen in the agarose gel was correlated with the presence of the PMeV as previously published (Ventura et al. 2004; Zambolim et al. 2003; Rodrigues et al. 2005). In this work, the dsRNA was clearly observed only on the solid phase, indicating that the virus is linked to the papaya latex particles (Fig. 6). Similar results were obtained from plants infected for different periods of time (data not shown). Therefore, the results suggest that the PMeV is linked to the latex particles during different viral life cycle stages.

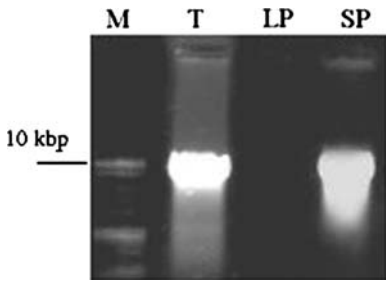

Fig. 6 Nucleic acid extraction from the solid and liquid latex phases. Latex from diseased papaya was centrifuged, and both liquid (*LP*) and solid (*SP*) phases were subjected to nucleic acid extraction. The total latex (*T*) with nucleic acids extracted was used as a control. The samples were separated by a 1% agarose gel using a 1 kbp DNA molecular marker (*M*)

Discussion

After the papaya sticky disease was described (Rodrigues et al. 1989), studies were published investigating the interaction between the PMeV and the papaya plant. Initially, it was described a spontaneous latex exudation from fruits and leaves in the field (Rodrigues et al. 1989; Ventura et al. 2001, 2004). After the PMeV particles were found only in papaya laticifers and not in other cell types (Kitajima et al. 1993), the sticky disease symptoms were supposed to be a direct effect of the virus. However, the details of these effects have remained unknown until now.

Effects of the PMeV on latex composition and polymers structures

This work subjected papaya latex to different biochemical tests aiming to detect possible PMeV-induced changes. The HL was observed to have a sugar concentration about twice as high as that of the SDL (Table 1). This data was confirmed by latex histochemical tests, in which reducing sugars were stained at a lower level in diseased samples (Fig. 1e, f). It is noteworthy that the concentration difference is not directly correlated to an increase of water, as shown in Table 1. Similar effects were also observed in pumpkin (*Cucurbita pepo* cv. Eskandarani) leaves infected with *Zucchini yellow mosaic virus* (ZYMV), in which a decrease of carbohydrate, protein and pigment levels was observed (Radwan et al. 2007). Plant viruses have been shown to affect the host's carbohydrate metabolism, altering, for example, photosynthesis and sugar transport

(Sajnani et al. 2007; Kronberg et al. 2007). The flow of sugar sap from photosynthesizing leaves to sink tissues is compromised, as the plant reduces the plasmodesmata pore diameter in order to limit the mobility of the virus. As a result, sugars accumulate in the photosynthetic tissues, diminishing their drain to other tissues (Rinne et al. 2005). In contrast, sugar fluxes across the plasma membranes of the laticifers could not be altered. When the protoplasts of the laticifers were subjected to ethylene treatment simulating stress conditions, their sugar uptake was maintained (Bouteau et al. 1991). Therefore, these results suggest a critical influence of PMeV on papaya carbohydrate metabolism. Instead of being restricted to laticifers, the reduction of sugar concentration could reflect a systemic effect of PMeV in the papaya plant. Consequently, the arrival of sugars from photosynthetic cells in laticifers can be impaired as an indirect effect of the papaya defense response against the PMeV.

The sticky appearance of the diseased papaya latex is not caused by an increase in the sugar content. In fact, we observed a reduced level of total sugar in the diseased samples (Table 1). Indeed, the sticky aspect shall be related to the oxidative state of the latex. Our data showed an increased level of H_2O_2 in the infected laticifers cells (Fig. 3). At the same time, when the latex is exposed to the atmosphere, their components can react with the molecular oxygen. This process appears to be accelerated by high temperature as we usually observe much intense symptoms (including sticky appearance) in diseased papaya growing in the field during the hottest Brazilian year's mouths (from January to March) and when the latex is heated, the diseased samples became dark brown while its healthy counterpart remains white (data not shown). The latex cysteine-proteases (in the sulphydril group) are good target molecules to be oxidized. Nowadays, the level of these and other papaya latex proteins, as well as their oxidation state, are in progress in our laboratory.

The reduction in the amount of latex particles (Fig. 4) could be related to the supposed PMeV-induced papaya carbohydrate translocation changes. We observed lipids, phenols, alkaloids, sugars and oxalate crystals occurring in the latex (Figs. 1, 2). Nevertheless, papaya latex is known to be mainly composed of proteins (Bravo et al. 1994; Moutim et al. 1999), and as observed for other species, polyisoprene molecules (Hunter 1994). The reduced amount of sugar in the papaya laticifers could down-regulate the biosynthesis of both proteins and polyisoprene molecules. Indeed, a smaller amount of protein was observed in the SDL than in the HL after the protein assay (Table 1) and the histochemical test (Fig. 1). The sugar content has an equivalent effect on isoprene biosynthesis (Chaykin et al. 1958), a highly active pathway in the laticifers of the rubber tree plant (Chow et al. 2007). For this plant, the process uses elevated amounts of sugar that are efficiently imported to the laticifers through a cell plasma membrane H^+-sugar symport system (Bouteau et al. 1999).

In addition to the reduced biosynthesis of latex particles, the polymer shape was also altered, leading to the assumption that they had been disabled (Figs. 4, 5). In rubber trees, it has been demonstrated that, after tapping, latex coagulation was associated with adjacent polymer grains linking together (Wititsuwannakul et al. 2008a, b, c). The authors showed that HLL created multivalent bridges between rubber-particles through their binding to glycosilated (*N*-Acetyl-D-glucosamin, GluNAC) receptors (RP-HLLBP) located on the surfaces of the rubber-particles. The binding between HLL and GluNAC was Ca^{2+}-dependent (Gidrol et al. 1994; Wititsuwannakul et al. 2008a). If this model also applies to papaya, the reduced amount of sugar and the morphological latex-particle changes could compromise the latex coagulation, perhaps compromising the perfect contact between individual grains. The PMeV also altered the levels of sugars and Ca^{2+}, two additional important coagulation factors in papaya latex. The histochemical tests showed that not only the amount but also the distribution pattern of reducing sugars on the latex particles was changed (Fig. 1e, f). The water-insoluble calcium salt is the most commonly occurring oxalate in nature, and calcium oxalate crystals were previously reported in latex from different plant species (Hunter 1994). The virus increased the amount of oxalate crystals in the latex (Fig. 2), and oxalate calcium salt is important in regulating the calcium levels in higher plants (Borchert 1986). In addition, the SDL had only about half as much Ca^{2+} as healthy samples (Table 1). The combination of these effects could contribute to the increased latex fluidity typical of sticky diseased papaya.

Effects of the PMeV on papaya laticifers defense responses and osmotic balance

Oxalate has been associated with different biological processes, although its precise mechanism of action is not completely understood. Recent studies suggest that oxalate impinges on plant signaling. Oxalate oxidase activity was first reported in barley extract, in which the conversion of oxalate into CO_2 and H_2O_2 was demonstrated (Lane 2002). Therefore, oxalate-mediated signaling could involve the production of H_2O_2. After subjecting papaya to in situ H_2O_2 detection, laticifers were indicated as H_2O_2-producing sites (Fig. 3), and PMeV-infected papaya tissue showed an increase in H_2O_2 production (Fig. 3c, d). Thus, the accumulation of calcium oxalate crystals in sticky diseased papaya latex suggests that the oxalate is participating in the increased H_2O_2 production. This data is positively correlated to the contribution to plant stress response by the

laticifers. Expressed sequence tags (ESTs) from rubber tree latex (Chow et al. 2007) and the proteomic analysis of *Chelidonium majus* latex (Nawrot et al. 2007) indicated the presence of highly expressed proteins with the ability to stress-response or defense against pathogens. Similarly, laticifers are known to participate during the synthesis and accumulation of secondary metabolic compounds, for example phenols and alkaloids (Hunter 1994; Samanani et al. 2006). This extensive group of molecules is related to abiotic and biotic stress responses. In this study, an accumulation of alkaloids was detected only in SDL samples (Fig. 1h), suggesting an antiviral papaya response. Consistent with this idea, An et al. (2001) proved the inhibitory activity of *Cynanchum komarovii* alkaloids against the *Tobacco mosaic virus* (TMV).

An accumulation of K^+ was observed in the SDL (Table 1). In general, K^+ serves several important functions in plant cells, such as neutralizing electrical anionic groups, controlling membrane polarization and participating in osmoregulation (Lebaudy et al. 2007). Inward-rectifying K^+ channels were previously identified in guard cells (Schroeder et al. 1984), but can be found in several types of plant cells, including laticifers (Bouteau et al. 1996, 1999). Alternatively, K^+ uptake could be related to the accumulation of calcium oxalate crystals. *Sclerotinia sclerotiorum* (Lib.) fungi are known to produce considerable amounts of oxalate in infected tissues as a virulence factor (Ferrar and Walker 1993). In this plant-fungi infection model, oxalate induces starch degradation in guard cells, followed by K^+ accumulation (Guimarães and Stotz 2004). The osmotically active solutes lead to an increase of water uptake. The effect is likely to occur in papaya laticifers as well, since increased water content was observed in the SDL (Table 1). The authors further reported a swelling and, in some cases, a bursting of protoplasts in fungi-infected plants. Under normal conditions, the exudation of papaya latex requires tissue tapping, so the spontaneous latex exudation from sticky diseased papaya (Rodrigues et al. 1989; Ventura et al. 2001, 2004) indicates that the plant laticifers could be bursting as well.

The biological significance of the swelling and consequent cell rupture of the sticky diseased papaya laticifers could be related to the movement strategy of the virus. A recent review points to laticifers as an alternative plant tube system (Pickard 2008). It emphasizes the rupture and subsequent laticifer drainage as a special transport mechanism. As the PMeV is located only in the papaya laticifers (Kitajima et al. 1993), the results on latex fluidity and exudation presented in this work suggest that the virus uses host laticifers to move itself through the plant. Electron microscopy (Fig. 5) and molecular data (Fig. 6) show the virus in close association to latex particles, and thus support this idea. This association would allow the virus to flow more efficiently, since the PMeV could use this mechanism to move quickly from an inoculation site to non-infected cells.

This paper presents an important characterization of the effects of the PMeV on papaya latex. Overall, the presented results provide new insights on the interaction between the papaya and the PMeV, which could prove helpful when trying to understand and control the papaya sticky disease.

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