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Molecular diagnosis of *Papaya meleira* virus (PMeV) from leaf samples of *Carica papaya* L. using conventional and real-time RT-PCR

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A B S T R A C T

Papaya meleira virus (PMeV) is the causal agent of papaya sticky disease. This study describes two methods for molecular diagnosis of PMeV using conventional and real-time PCR. These methods were shown to be more efficient than current methods of viral detection using extraction of PMeV dsRNA and observation of symptoms in the field. The methods described here were used to evaluate the effect of inoculation of papaya plants with purified PMeV dsRNA on the progress of PMeV infection. A single inoculation with PMeV dsRNA was observed to delay the progress of the virus infection by several weeks. The possibility of vertical transmission of PMeV was also investigated. No evidence was found for PMeV transmission through seeds collected from diseased fruit. The implications of these results for the epidemiology of PMeV and the management of papaya sticky disease are discussed.

1. Introduction

Papaya sticky disease, or meleira, is an important and growing problem in papaya (*Carica papaya* L.) cultivation, capable of causing complete loss of the crop. The tissues of papaya contain lactifers which are maintained under high pressure and exude latex upon injury. This latex has high levels of proteases and alkaloids (El Moussaoui et al., 2001) and is believed to be part of the defence mechanisms of the plant. However, papaya sticky diseased plants are characterised by a spontaneously exudation of fluid and translucent latex from the fruit and leaves. The latex oxidises after atmospheric exposure, resulting in small necrotic lesions on the edges of young leaves, and a sticky substance on the fruit (Kitajima et al., 1993; Zambolim et al., 2003) that makes them commercially unacceptable for consumption (Ventura et al., 2004).

The causal agent of papaya sticky disease has been identified as *Papaya meleira* virus (PMeV), a 12 kbp dsRNA virus found as 50 nm spherical particles in infected tissue (Kitajima et al., 1993; Zambolim et al., 2003). Unusually for a plant virus, PMeV appears to reside primarily in lactifers, where it modifies potassium levels and thus the osmotic balance, leading to rupture of cells and latex exudation (Rodrigues et al., 2009c).

As there are as yet no resistant cultivars to PMeV, control is by visual identification of diseased plants and their eradication (roguing) (Ventura et al., 2004). For unknown reasons, visual symptoms of meleira only appear after flowering (Ventura et al., 2004). Even then, diagnosis can be difficult as there is a relatively long asymptomatic incubation period and the early symptoms can be confused with other conditions (Ventura et al., 2004). There is thus a need for alternative diagnostic methods.

Two methods have been published for detection of PMeV by molecular means. The first method (Rodrigues et al., 2005) applies nucleic acids purified from latex to agarose gels and visualises the resultant 12 kbp viral dsRNA band with ethidium bromide. This has the advantage of relatively low cost and rapid turnover, but requires large quantities of latex and a high titer of virus and so is unsuitable for early stages of infection or when little plant material is available. The second method (Araujo et al., 2007) applies conventional reverse transcription PCR (RT-PCR) to latex diluted in ammonium citrate. This requires less latex and is more sensitive, but because the method lacks a nucleic acid purification step, latex proteins remain in the PCR reaction. Papaya latex has a high concentration of proteins, especially proteases (Moutim et al., 1999), which can interfere with the PCR reaction. In addition, a strong link between PMeV and latex particles has been observed (Rodrigues et al., 2009c). Furthermore, dsRNA viruses such as PMeV have the

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capacity, under certain conditions, to transcribe within the capsid envelope (Harrison, 2007). Taken together these factors would reduce the chance of virus detection by PCR.

A more sensitive and qualitative technique for virus detection is real-time RT-PCR (Vincelli and Tisserat, 2008). Measurement by real time PCR is based on the increase in fluorescence of dyes, e.g., SYBR Green, when connected to a double-stranded DNA so that fluorescence recorded at each PCR cycle reflects the number of PCR products generated during amplification (Morrison et al., 1998). This in turn reflects the initial quantity of template in the sample. Real-time RT-PCR has been used successfully to detect low levels of viruses in a number of plants (Lim et al., 2010; Parisi et al., 2011). As well as increased sensitivity, the technique allows simultaneous analysis of large numbers of samples compared to gel based systems, and automated analysis of the results.

A further problem in PMeV control is the limited information about the virus transmission. The common Brazilian whitefly *Trialeurodes variabilis* has been ruled out as a vector (Rodrigues et al., 2009b). It has proved difficult to infect papaya mature trees by mechanical injury short of direct PMeV injection due to exudation of latex and its simultaneous polymerisation on the injured tissues (Rodrigues et al., 2009b). Both commercial and private papaya production are from trees grown from seeds. However, the vertical transmission of PMeV has not yet been evaluated.

A promising development in horticultural virus control is the concept that plants can initiate defence responses based on RNA silencing when challenged by viral RNA. The use of transgenic plants which constituently express viral material has been successfully developed in different crops (Scorza et al., 2001; Tricoli et al., 1995). Alternatively, mechanical inoculation of viral dsRNA has inhibited infection by three virus classes into *Nicotiana benthamiana* (Tenllado et al., 2003) and similar results have been achieved with dsRNA as a spray (Gan et al., 2010).

Our objectives in this study were firstly to develop improved methods for the detection of PMeV by conventional and real-time RT-PCR. The possibility of vertical transmission of the virus was investigated using conventional RT-PCR. The effects of the inoculation of purified PMeV dsRNA in papaya plants were also assessed as a preliminary prospect for papaya sticky disease control.

2. Materials and methods

2.1. Plant material

Samples of papaya (cv. Golden) were collected at the Experimental Farm of the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (INCAPER), Espírito Santo State, Brazil, with the aid of an agricultural technician experienced in the visual diagnosis of papaya sticky disease. For the experiments evaluating different PMeV diagnosis methods, the leaves and latex samples ($n = 32$) were divided into 4 categories: (1) plants ($n = 10$) symptomatic for sticky disease, (2) plants ($n = 8$) with inconclusive diagnosis for sticky disease, (3) plants ($n = 10$) asymptomatic but close to symptomatic plants and (4) plants ($n = 4$) asymptomatic located at 12 rows from symptomatic plants. Latex (300 μL) was sampled from each plant using a sterile razor blade and mixed (1:1, v/v) with 0.1 M citrate buffer pH 5.0 (Rodrigues et al., 2009a). Leaf samples (~2 g) were also collected in 15 mL sterile Falcon tubes. The samples were cooled on ice and the nucleic acids were extracted on the same day and stored at −20 °C.

The experiments evaluating the transmission of PMeV through seeds used seeds collected from diseased fruit. Papaya seeds from Piracicaba, São Paulo State, where there is no evidence of papaya sticky disease, were used as negative controls. The seeds were washed with water and detergent to remove the sarcotesta, dried at 25 °C and disinfected using 70% ethanol for 10 s and 2% sodium hypochlorite for 5 min. After rinsing 3× with distilled water, the seeds (5–8 seeds/plate) were transferred to a150 mm Petri dish containing 180 mL of sterile 1% agar (Suppl. Fig. S1) and incubated at 16 h light/30 °C and 8 h dark/20 °C. 15–20 days after germination, seedlings from originally diseased fruit ($n = 172$) or the negative control ($n = 187$) were collected. The seedlings from each group were pooled and ground together in liquid nitrogen before nucleic acid extraction as described below.

For the experiments involving co-inoculation of papaya seedlings with PMeV and purified PMeV-dsRNA, the plants ($n = 12$) were grown in a greenhouse in 8 kg pots containing fertilised soil and white sand (3:1) for 3–4 months after germination.

2.2. Co-inoculation of papaya with PMeV and purified PMeV dsRNA

Latex from papaya sticky diseased plants was used as a PMeV inoculum source (Kitajima et al., 1993; Rodrigues et al., 2009b; Zambolim et al., 2003). The purified PMeV dsRNA was obtained as previously described (Rodrigues et al., 2005). The plants ($n = 3$) were divided in three groups: (1) Plants simultaneously inoculated with 20 μL of (1:1, v/v) diseased latex/phosphate buffer pH 7.0 and 20 μL of purified PMeV dsRNA (91.7 ng μL^{-1}), (2) plants inoculated with 20 μL phosphate buffer pH 7.0 and (3) plants inoculated with 20 μL of PMeV infected latex, diluted (1:1, v/v) in phosphate buffer pH 7.0. The groups 2 and 3 were used as negative and positive controls, respectively. The inoculations were performed in the leaf petioles using a sterile syringe. Aliquots of latex and leaf were collected from the shoot apex using a sterile blade at 7 day intervals during 71 days, and diluted (1:1, v/v) in sodium citrate buffer pH 5.0 (Rodrigues et al., 2009a). All samples were stored at −80 °C until used.

2.3. Extraction and analysis of papaya nucleic acids

Molecular diagnosis of PMeV-dsRNA from papaya latex was performed as described (Rodrigues et al., 2005). The nucleic acids were extracted from papaya leaf (100 mg) as previously described (Doyle and Doyle, 1990), with modifications. After extraction using organic solvents, the supernatant was precipitated with 600 μL of cold ethanol and 6 μL of 3 M sodium acetate pH 5.2. The samples were incubated at −20 °C overnight, centrifuged 12,000 × *g* for 50 min at 4 °C. The nucleic acids in the pellet were washed with 70% ethanol, dried, and resuspended in water for quantity and purity assessment using a Nanodrop ND 1000 spectrophotometer. The samples were treated with DNase I (RQ1 RNase-Free DNase, Promega) following the manufacturer's instructions. The presence of intact RNA was confirmed by RT-PCR of the actin gene (Suppl. Table S1). The nucleic acids were separated on 1% TBE agarose gels for 1.5 h at 80 V. The gels stained with ethidium bromide (15 ng ml^{-1}) were recorded using an image capture system L-HE-Pix/L-Pix IMAGE (Cotia, Brazil).

2.4. Detection of PMeV by conventional RT-PCR

The primers used in this study are listed in Suppl. Table S1. *PMeVconv* primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) targeting a PMeV sequence homologous to RNA-dependent RNA polymerases (RDRP) reported by (Araujo et al., 2007). In this report, the authors had used the software Gene Runner Version 3.05 (Hastings Software, Inc.) to design a primer, CO5-3′, targeting the PMeV sequence. CO5-3′ was also tested in our study. Actin primers were designed as previously described (Santos, 2005).

Papaya RNA (500 ng) was incubated at 96 °C for 3 min to denature the PMeV dsRNA (Zambolim et al., 2003). cDNA was synthesised using cDNA random hexamers (250 ng μL^{-1}) and M-MLV Reverse Transcriptase kit (Promega) following the manufacturer's instructions. All PCR reactions used GoTaq Flexi DNA Polymerase kit (Promega) following the specific programs in a Mastercycler Thermocycler (Eppendorf, Hauppauge, USA): *PMeVconv* primers, 94 °C for 3 min, 32 cycles at 94 °C for 1 min, 61 °C for 1 min and 72 °C for 1 min, and 72 °C for 7 min, Actin primers, 94 °C for 4 min, 36 cycles at 94 °C for 1 min, 60 °C for 1.5 min and 72 °C for 1 min and 72 °C for 5 min, CO5-3′ primer, 94 °C for 3 min, 30 cycles at 94 °C for 1 min, 57 °C and 72 °C for 2 min and 72 °C for 7 min (Araujo et al., 2007). All reactions were conducted in duplicate and the PCR fragments were separated on 1.0% or 1.5% TBE agarose gels, as described above.

2.5. Diagnosis of PMeV by real-time RT-PCR

The primers used in the real-time RT-PCR reactions, PMeVreal (Suppl. Table S1), were designed using Primer3 again targeting the PMeV RDRP (Araujo et al., 2007). The primers targeting the ribosomal 28S gene were as described (Rojas et al., 2009).

The papaya RNA samples (600 ng) were mixed with 2 μL random hexamers 50 μM, 1 μL 10 mM dNTP mix (Applied Biosystems, Carlsbad, USA) and DEPC water to 12 μL final volume. The samples were incubated for 96 °C for 3 min and the cDNA synthesised using a Super Script III kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Briefly, each sample received 4 μL Super Script 5× buffer, 2 μL DTT (0.1 M), 0.1 μL RNaseOUT (40 U μL^{-1}), 1.3 μL DEPC water and 0.6 μL 5× Super Script III (200 U μL^{-1}) totalling 20 μL. The samples were incubated for 10 min at 25 °C, 50 min at 50 °C and 5 min at 85 °C. The obtained cDNA received 3.5 μL of cDNA, 10 μL of SYBR Green PCR Kit Master Mix (Applied Biosystems, Carlsbad, USA) and 6.5 μL of a solution of 2.8 mM of each primer diluted in 0.01 M Tris pH 8.0. The mixture was incubated at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s using a 7500 Real Time PCR System and the data analysed using a SDS Software System 7500 version 2.0.1 (Applied Biosystems, Carlsbad, USA). Relative abundance was estimated by choosing a threshold of SYBR Green fluorescence during the exponential phase of fluorescence increase. The cycle number at which each sample passes the fluorescence threshold (Ct) was recorded (Bustin et al., 2009). To prevent false positives, a Ct cut off point was determined, beyond which other Ct values could not be regarded as positive (Kokkinos and Clark, 2006).

2.6. Statistical analysis

The data was analysed by Analysis of Variance (ANOVA) and the average of each treatment was compared by Tukey Test ($p \leq 0.05$).

3. Results

3.1. Detection of PMeV in papaya leaves by conventional and real-time RT-PCR

Conventional RT-PCR using the primers PMeVconv amplified a 300 bp fragment from infected leaves (Suppl. Fig. S2). No fragment was amplified from uninfected samples, confirming the specificity of the PMeVconv primers for PMeV diagnosis (Suppl. Fig. S2).

Real time RT-PCR using PMeVreal primers presented above background fluorescence after 22 cycles which continued to grow exponentially until reaching a plateau at cycle 38. The dissociation curves for both PMeVreal and 28S primers were sharp, suggesting no primer dimerisation or non-specific amplification (Ririe et al., 1997). The infected samples produced a Ct value of 22.2 while uninfected samples, used as negative control, were indistinguishable from the background (Table 1). Real-time PCR amplification of 28S ribosomal RNA produced similar Ct values, i.e., 5.53 and 5.89, from both papaya sticky diseased and uninfected leaves, respectively (Table 1).

Table 1
Cycle threshold of 32 samples submitted to PMeV and 28S ribosomal gene amplification.

Symptoms	Sample	Ct	
		28S	PMeV
Asymptomatic (FSP)	1	6.458	Undetectable
	2	6.304	Undetectable
	3	5.773	Undetectable
	4	5.78	Undetectable
Asymptomatic (CSP)	5	5.738	Undetectable
	6	5.738	Undetectable
	7	5.632	Undetectable
	8	5.809	Undetectable
	9	5.887	Undetectable
	10	5.867	Undetectable
	11	5.573	Undetectable
	12	5.749	38.588
	13	5.605	Undetectable
	14	5.795	Undetectable
Symptomatic	15	5.833	29.388
	16	5.874	26.221
	17	6.54	25.791
	18	6.888	27.644
	19	6.862	29.063
	20	5.47	22.202
	21	5.285	26.355
	22	5.543	Undetectable
	23	5.694	Undetectable
	24	5.879	28.665
Inconclusive	25	6,909	Undetectable
	26	6.672	21.735
	27	6.884	Undetectable
	28	6.864	Undetectable
	29	6.316	Undetectable
	30	6.571	Undetectable
	31	6.69	Undetectable
	32	6.831	Undetectable

3.2. Comparison of four methods for diagnosis of PMeV in papaya

The visual diagnosis of 32 papaya plants growing under field conditions was compared to three other PMeV detection methods: (1) extraction of total nucleic acids from latex samples and observation of the 12 kbp dsRNA band on an agarose gel (Rodrigues et al., 2005), (2) conventional RT-PCR using PMeVconv primers and (3) real-time RT-PCR using PMeVreal primers. After visual diagnosis, the plants were divided in symptomatic, asymptomatic and inconclusive. The asymptomatic plants were collected close (CPS) or far (FPS) from symptomatic plants. The presence of the 12 kbp dsRNA band was observed in six samples, numbers 15, 17, 18, 19, 24 and 26 (Fig. 1A). Five of these samples had been defined as symptomatic and one inconclusive. Five symptomatic plants did not present bands (Fig. 1A).

Conventional RT-PCR method using PMeVconv primers detected PMeV in nine leaf samples, eight symptomatic and one inconclusive (Fig. 1B). These included all the plants diagnosed by 12 kbp dsRNA detection, but also symptomatic plants, numbers 16, 20 and 21, demonstrating greater sensitivity. Two symptomatic plants were classified as virus free by 12 kbp dsRNA detection, but no asymptomatic plants were diagnosed as infected.

The Ct values obtained by real-time PCR using the PMeVreal primers are presented in Table 1. The samples positive for PMeV

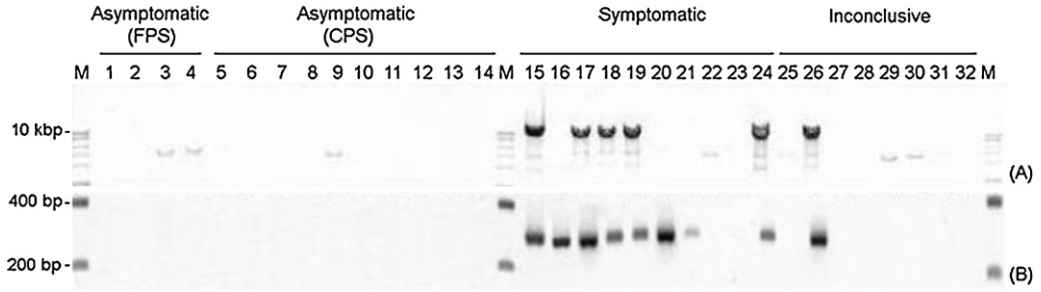

Fig. 1. Comparison of three methods for diagnosis of PMeV in papaya. Papaya plants ($n = 32$) were visually grouped as symptomatic, asymptomatic and inconclusive for papaya sticky disease. Asymptomatic plant samples were harvested far (FPS) or close (CPS) to diseased plants. The presence of PMeV was further assessed by two molecular detection methods, the extraction of PMeV 12 kbp dsRNA from papaya latex (A) and the amplification of PMeV RDRP gene by conventional RT-PCR using PMeVconv primers (B). The amplified fragments were separated by 1% (A) or 1.5% (B) TBE agarose gels, stained with ethidium bromide. M, molecular weight marker.

by real-time RT-PCR were the same that tested positive by conventional RT-PCR (Table 1 and Fig. 1B). Thus, of the ten plants diagnosed as infected by visual symptoms in the field, eight were confirmed by conventional and real-time RT-PCR. All the plants diagnosed as asymptomatic were confirmed negative for PMeV.

3.3. Assessment of PMeV transmission by papaya seeds

Two groups of seedlings 15–20 days after germination were analysed: seedlings ($n = 187$) from seeds collected from healthy papaya fruits, and seedlings ($n = 172$) from seeds collected from diseased fruits. The presence of virus was assessed in pooled RNA samples from each plant group by conventional RT-PCR using PMeVconv primers. Both seedling groups tested negative for PMeV presence (Fig. 2), suggesting the virus is not transmitted by seeds. The amplification of an actin gene, used as a positive control for the RT-PCR reaction, was observed for all samples (Fig. 2).

3.4. Co-inoculation of papaya with PMeV and purified PMeV dsRNA

In order to assess the effect of purified PMeV dsRNA on the progress of PMeV infection in papaya, the plants were co-inoculated with PMeV infected latex and PMeV dsRNA. Plants inoculated with phosphate buffer pH 7.0 or PMeV infected latex were used as negative and positive controls, respectively. The virus load in the plants was estimated using three different molecular diagnosis methods, i.e., extraction of PMeV 12 kbp dsRNA from papaya latex, amplification of PMeV RDRP gene by conventional RT-PCR using PMeVconv primers and real-time RT-PCR using PMeVreal primers. The latex of those plants co-inoculated with purified dsRNA and infected latex presented the PMeV 12 kbp dsRNA band 43 dpi (Fig. 3A). The positive control plants clearly presented dsRNA 29 dpi. These data were confirmed through the analysis of leaf samples by conventional RT-PCR using PMeVconv primers (Fig. 3B) and suggest the co-inoculation of papaya plants with purified PMeV-dsRNA delayed the infection of PMeV. The load of PMeV in co-inoculated samples appeared reduced at 57 dpi (Fig. 3A and B), suggesting that PMeV dsRNA could also elicit general defences in papaya plants, which would result in the inhibition of virus replication.

The seedlings inoculated only with buffer also showed a faint band at 22 and 57 dpi, possibly as a result of a minor infection during the experiment.

The latex samples collected from seedlings were also analysed by conventional RT-PCR using the CO5-3′ primer (Araujo et al., 2007), but all samples tested negative (Suppl. Fig. S3A). In contrast, when nucleic acids were extracted from latex of sticky diseased mature plants, the expected nucleic acid band of 200–300 bp was clearly seen (Suppl. Fig. S3B), verifying that the primers were functional. These results suggest that RT-PCR using CO5-3′ primer is more suitable for the analysis of papaya latex from mature plants with high virus titers.

In order to estimate the PMeV load in the co-inoculated papaya plants, the samples were analysed by real-time RT-PCR using PMeVreal primers. For relative quantification, the data obtained for each sample was normalised against the ribosomal 28S reference gene. Both groups of plants, co-inoculated with purified PMeV dsRNA and diseased latex and positive controls, presented an increasing virus load over time which was clearly less pronounced in the co-inoculated plants (Fig. 4). These data further confirm the delayed PMeV infection in papaya inoculated with purified PMeV dsRNA.

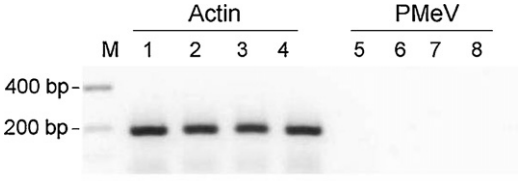

Fig. 2. Evaluation of PMeV transmission through papaya seeds. Papaya seeds ($n = 172$) collected from diseased fruit were germinated in Petri dishes. The seedlings at 15–20 days post germination were pooled and submitted to RNA extraction. Seeds ($n = 187$) from healthy papaya fruit were used as negative control. Duplicate RNA samples from the control (1–2 and 5–6) and from the germinated seeds from diseased fruit (3–4 and 7–8) were analysed by conventional RT-PCR using PMeVconv primers. The amplification of actin gene was used as a RT-PCR reaction control. The nucleic acids were separated on 1.5% TBE agarose gels, stained with ethidium bromide. M, molecular weight marker.

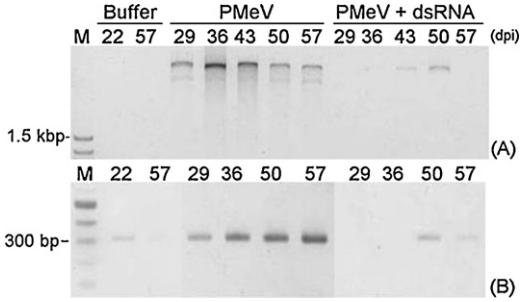

Fig. 3. Assessment of PMeV load in papaya plants co-inoculated with PMeV and purified PMeV dsRNA. The plants were co-inoculated with PMeV infected latex and PMeV dsRNA (PMeV + dsRNA) or inoculated only with phosphate buffer pH 7.0 (Buffer) or PMeV infected latex (PMeV). The latex samples collected from each plant at regular days-post inoculation (dpi) were analysed by extraction of PMeV 12 kbp dsRNA (A) or the amplification of PMeV RDRP gene from papaya leaf by conventional RT-PCR using PMeVconv primers (B). The resultant material was separated by 1% (A) or 1.5% (B) TBE agarose gels, stained with ethidium bromide. M: molecular weight marker.

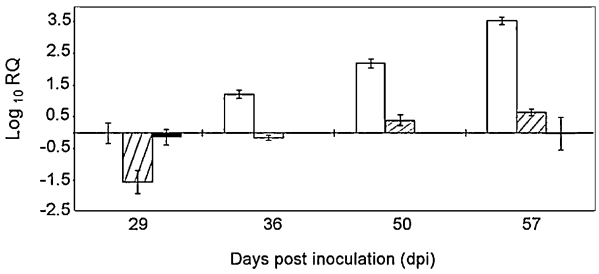

Fig. 4. Assessment of PMeV load in papaya plants co-inoculated with PMeV and purified PMeV dsRNA by real-time RT-PCR. Relative abundance of PMeV in papaya samples collected from plants inoculated with PMeV infected latex (open bars), co-inoculated with PMeV infected latex and PMeV dsRNA (diagonal lines) and inoculated with phosphate buffer (black bars). Samples were collected at different days post inoculation (dpi) and amplified using PMeVreal primer.

The co-inoculated plants tested positive for PMeV at 50 and 29 dpi when analysed by regular RT-PCR (Fig. 3B) or real-time RT-PCR (Fig. 4), respectively. These results indicate the greater sensitivity of the real-time RT-PCR method, as already reported for other viruses (Kokkinos and Clark, 2006).

4. Discussion

The diagnosis of PMeV is of primary importance in the control of papaya sticky disease given that at time of writing there are no commercially available resistant cultivars. Diagnosis is primarily by assessment of symptoms.

Visual diagnosis is relatively quick and cheap, but it has a number of weaknesses as a technique. Unfortunately, for unknown reasons, symptoms only become apparent after flowering (Ventura et al., 2004) and in their earliest stages are open to misinterpretation. Additionally, in the field, the first symptoms of papaya sticky disease are presented only 45 days after inoculation with PMeV infected latex (Ventura et al., 2004). This window of 45 days between virus infection and the onset of symptoms plus the non-appearance of symptoms throughout the period prior to flowering can lead to the presence of asymptomatic plants infected with PMeV. These asymptomatic plants can provide a high concentration of the virus for subsequent transmission, since the PMeV dsRNA is already detectable 15 days after inoculation of the virus in papaya plants (Rodrigues et al., 2005). High air temperature can also affect the appearance of symptoms of papaya sticky disease (Ventura et al., 2004). Therefore, plants can harbor the virus within an orchard, acting as reservoirs for infection.

Two methods have been published for assessment of PMeV infection, i.e., detection of viral 12 kbp dsRNA using agarose gels and ethidium bromide (Rodrigues et al., 2005) and conventional RT-PCR on nucleic acids from crude latex (Araujo et al., 2007). The first method has advantages over other method of being relatively rapid and lower cost. However, various studies have reported a restricted detection of nucleic acids in agarose gels stained with ethidium bromide. The detection limit for a viral dsRNA band is estimated to be 100 ng (Mcfadden et al., 1983). Thus, since the detection of purified PMeV dsRNA does not include any amplification step, the quantity of viral dsRNA extracted must exceed this detection limit. In contrast, 10 fg can be sufficient for detection of viral genome by RT-PCR (Romaine and Schlagnhaufer, 1995). A further consideration is that diagnosis of PMeV by direct application of purified viral dsRNA to an agarose gel can result in false positives for the virus, due to the proximity of molecular weights of PMeV dsRNA (12 kbp) and papaya DNA (~14 kbp).

RT-PCR has been used successfully in the detection of a number of plant viruses (Mekuria et al., 2003; Omunyin et al., 1996) and has been shown to be 10^3–10^4 times more sensitive than immunoassays, an alternative technique (Zhu et al., 2010). The method of PMeV detection by RT-PCR proposed previously (Araujo et al., 2007) uses crude papaya latex diluted in 0.1 M ammonium citrate (1:1, v/v) without prior extraction of nucleic acids as the initial material. The presence of PMeV in the papaya laticifers/latex is well established (Kitajima et al., 1993; Ventura et al., 2004; Zambolim et al., 2003) and therefore latex samples are the primary choice for virus detection. An important point to be considered is the high concentration of proteins present in papaya latex (Moutim et al., 1999) which can result in low yield in RT-PCR. A strong link between viral particles and latex polymer has been observed (Rodrigues et al., 2009c) that can decrease the amount of dsRNA PMeV available for reverse transcriptase reaction. Additionally, there are some stages of development of papaya for which even a small amount (e.g., 5 μL) is unavailable, such as young seedlings.

Seedlings present challenges in the collection of latex. The amount exuded is very small, and is related to turgescence pressure within the lactifer (Moutim et al., 1999). The use of a small amount of latex can be disadvantageous for viral diagnosis due to insufficient nucleic acid for analysis, especially early in an infection (Jarosova and Kundu, 2010), thus generating false negatives.

In this study, latex samples collected from mature papaya, or papaya seedlings previously inoculated with PMeV, were analysed using the method previously described (Araujo et al., 2007). However, only latex from mature plants tested positive. Thus, the analysis of latex by this method does not appear to be suitable for diagnosis of PMeV in seedlings because of the low virus titer. The RT-PCR method developed using the primers PMeVconv did detect PMeV from seedling leaves inoculated with the virus. Using leaf material appears to be preferable for virus detection in papaya seedlings because of the greater purity of material extracted.

The conventional RT-PCR detection method proposed in this paper uses about 100 ng of purified RNA as a template for amplification, a quantity achievable from seedlings. This amount is approximately the same as used for RT-PCR detection of *Prunus necrotic ring spot virus* in almond (Mekuria et al., 2003) and for several strains of *Soybean mosaic virus* (Omunyin et al., 1996). Although conventional RT-PCR was successful in detecting low titers of PMeV in small quantities of material, a method for real-time RT-PCR was also developed. This has the advantage of greater sensitivity, the simultaneous testing of large numbers of samples, automated analysis of the results and reliable quantification (Beuve et al., 2007; Lim et al., 2010).

There are two general approaches for the detection of amplified fragments by real-time RT-PCR, the use of specific and non-specific fluorescent reporters. Both have similar levels of sensitivity (Bustin and Nolan, 2004). However, the use of specific probes such as TaqMan can result in false negatives, especially in RNA viruses. Alternatively, non-specific probes such as DNA intercalating dye SYBR Green, are more reliable, simple and low cost (James and Varga, 2005; Papin et al., 2004). Although very sensitive, real-time RT-PCR requires a cut off point for detection to prevent false positives. The maximum value of Ct that is considered positive for a particular virus varies. While Ct values up to 45 have been accepted as valid for rhinovirus detection (Wadowsky et al., 2010), in general 35 is considered a negative result, for example for detection of *Sweet leaf curl virus* in sweet potato (Kokkinos and Clark, 2006). Thus, it is questionable whether sample 12 can be considered positive with a Ct of 38.58 without the establishment of a standard curve of virus concentration versus cycle threshold. Therefore, we preferred to consider the result of this sample as inconclusive.

Although real-time RT-PCR is more sensitive than conventional RT-PCR, the two methods showed similar results in virus detection in mature plants, perhaps because the diseased plants collected contained a relatively high virus titer within the detection limits of both techniques. Both PCR techniques were more sensitive than

direct observation of the virus 12 kbp dsRNA genome on a gel. Both also have the advantage that they can be applied to all life stages of papaya, and not just to adult plants after flowering. The detection of virus by real-time RT-PCR also allows for the quantification of PMeV which could contribute to future experiments addressing the virus biology.

The question of false positive and negatives is of crucial importance in PMeV control. Of the 10 plants diagnosed by visual symptoms in the field only 8 were confirmed by the two methods of PCR. These false positives can occur as the initial symptom in the field is the necrosis of the leaves tips which can also be caused by the fungus *Stagonosporopsis cariacae* (Syn: *Phoma caricae-papayae*) or damage resulting from insect infestation (Ventura et al., 2004). In terms of false negatives, all of the plants diagnosed as asymptomatic were confirmed by PCR. However, one of the plants diagnosed visually as inconclusive was diagnosed as positive by PCR methods. In practice, plants are not normally removed from cultivation unless positively identified as infected. Plants left in the field can clearly serve as a virus inoculum for other plants.

An additional problem is understanding transmission of papaya sticky disease. With many papaya plants grown from seeds, such plants are also a potential source of infection. It has been unclear whether PMeV can be transmitted from parent to seedling via seeds. This was therefore investigated, as well as developing a method for diagnosis of virus infection in seedlings. No PMeV was detected by conventional RT-PCR using the PMeVconv primer pair on pools of seedlings derived from seeds of healthy ($n = 187$) and infected ($p = 172$) plants, respectively. However, although suggestive, these results do not prove the lack of transmission of PMeV by seeds. The frequency of transmission of PMeV may be very low, requiring a larger number of seedlings to be analysed. The frequency of virus transmission by seeds can vary greatly depending on the virus-host interaction, and may be very small in some cases. Although *Cocoa swollen shoot virus* could be detected in 53 of 98 cocoa seedlings analysed (Quainoo et al., 2008) and *Cucumber mosaic virus* was detected in 27 of 180 seedlings investigated (Yang et al., 1997), *High Plains virus* was detected in maize in only three of 38,473 seedlings analysed (Forster et al., 2001). Similar results were obtained in the transmission of *Maize dwarf mosaic virus*, in which only one of 22,189 seedlings was infected (Mikel et al., 1984). So although virus transmission by seeds cannot be ruled out by this work, it is suggested that if is occurring it is with low frequency.

As has been mentioned, there are no commercially available PMeV resistant papaya cultivars. It has been suggested in the literature that plants can be inoculated with viral dsRNA as a way of preventing or delaying infection and this has been demonstrated in vivo (Tenllado et al., 2003). Thus, our results are in accordance with others in the literature which showed a delay of viral infection by the application of dsRNA sequences (Gan et al., 2010; Tenllado et al., 2003), possibly via post transcriptional gene silencing (Tenllado and Diaz-Ruiz, 2001). Perhaps surprisingly, dsRNA applied exogenously can persist on the leaf despite watering and rain (Tenllado and Diaz-Ruiz, 2001). This principle appears to be worth further consideration as a method for PMeV control in papaya.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2011.12.003.

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