



Introduction of Defence Response in Papaya (*Carica papaya*) Against PRSV Through Organic and Inorganic Chemicals as Inducers

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Abstract. *The use of molecules that promote plant defence mechanisms turn out to be an alternative in disease management in agriculturally important crops contributing to the reduction of pesticide use. The activation of the defence responses in plants constitute a promising tool for the control of diseases in conventional agriculture. The results of the effect of a Chamomile aqueous extract as organic inducer and a sulphur-based fungicide as inorganic chemical inducer, as well as their combination, on the induction of resistance-related PR1 and MPK1 genes are presented in this work. In vivo results show a deleterious effect of the organic inducer in papaya plants. However, this effect decreased when combined with the sulphur-based compound, which also reduced the severity of symptoms caused by PRSV. Our results indicate that the combined treatment generates a similar response in plants to that produced by Salicylic Acid in the induction of PR1 expression.*

Keywords: *agrochemicals, chamomile, aqueous extract, Papaya ringspot virus, sulfur, polythion*

1. Introduction

Crops are affected by the effects of pathogens and diseases which lead to economic losses of up to 100% of yield in some cases. Such losses are commonly reduced by the use of pesticides that act on vectors. However, in addition to targeting the causal agent of the disease, pesticides also affect crops, beneficial microorganisms and the health of farmers and consumers [1]. Furthermore, plant growth and development are often affected by different abiotic and biotic conditions, to which they respond by activating a cascade of genes encoding different effectors, receptors and signalling and protection molecules such as pathogenesis-related proteins (PRs), which protect the plant from future infections. PRs are associated with the development of systemic acquired resistance (SAR) or hypersensitive response (HR) against subsequent infections caused by fungi, bacteria or viruses. Generally, SAR provides broad resistance to different pathogens [2]. Pathogenesis-related proteins (PRs), which are the downstream components of systemic acquired resistance in plants and are often used for the defence state of plants, are produced in response to an attack by pathogens [3]. This response includes the accumulation of signal molecules of salicylic acid (SA) throughout the plant and the consequent expression of defence genes, so that plants expressing SAR are more resistant to subsequent attacks by virulent pathogens [4]. In response to viral attack, plants produce a variety of antiviral agents including PR proteins which can function as virus suppressors [5]. Members of the pathogenesis-related protein 1 (PR1) family were first identified in the 1970s from tobacco plants infected with *Tobacco mosaic virus* (TMV) [6].

On the other hand, the mitogen-activated protein kinase (MAPK) cascade is a highly conserved signalling transduction module that transduces extracellular stimuli into intracellular responses in plants [7]. Plant MAPK cascades play pivotal roles in signalling plant defence against pathogen attack including oomycetes, fungi, bacteria and viruses. In example MAPKs are activated by Tobacco mosaic virus (TMV) [8].

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Plant resistance inducers (PRIs) are agents that lead to better protection to pathogen attacks by inducing the plant's own defence mechanisms, called induced resistance (IR). Resistance inducers can be chemical compounds as well as plant extracts or microorganisms. Resistance inducers are effective against different pathogens including viruses, bacteria and fungi, their effect can be local or systemic triggering SAR [9].

In viruses, pioneering work in SAR was developed with Tobacco Mosaic Virus when salicylic acid application was shown to trigger distal resistance to the virus [10].

Among the strategies to reduce the use of pesticides and the damage they cause to the environment, the use of plant resistance inducers (PRIs) or elicitors is considered as a potential option to face the phytosanitary problems of conventional agricultural practices and to reduce the environmental problems generated by the use of pesticides. These agents include different chemical or biological stimulants that can activate defence by exogenous application. Exogenous application of PRIs aims to bring the plant's defence system to an induced or prepared state, resulting in a stronger or faster induction of defence response over subsequent biotic or abiotic stress [11].

The treatment of plants with resistance inducers is an alternative that has been implemented against disease control in different pathosystems [12]; however, there is no information in papaya plants against PRSV. PRSV not only constitutes a risk to papaya, but also affects *Cucurbitaceae* in tropical and subtropical regions by reducing fruit production, fruit quality and sugar levels by 50% or more. In Norte de Santander - Colombia, the papaya crop has increased with respect to the acreage and output in tons. In the year 2007, there were 117 hectares with a production of 1897t, while by 2018, 136 ha were planted yielding 2013 t [13]. The slow increase in the production could be due to the presence of PRSV in the region, among other factors [14], and therefore the strategies used to increase the resistance against PRSV in papaya could contribute to an increase in the production. In this study we analysed the effect of three treatments including a Chamomile (*Matricaria chamomilla*) aqueous extract in the induction of the expression of two genes associated with resistance (MPK1 and PR1) in seedlings of papaya infected mechanically with PRSV at 3- and 7-days post-inoculation, with follow-up to the expression of symptoms and development of the plant until the 30 dpi. *Matricaria chamomilla* belongs to the group of medicinal plants with different active compounds among which are sesquiterpenes, flavonoids, coumarins and polyacetylenes, as the most important constituents and more than 120 chemical constituents have been identified in the flower as secondary metabolites, for which different biological activities have been described [15]. Statistical analysis in this study indicated that there was a significant effect on the induction of expression of genes associated with resistance, in particular PR1; however, in the case of treatment with the Chamomile aqueous extract, deleterious effects were shown in papaya plants, causing the death even of plants not inoculated with the virus, suggesting that the death was not due to the effect of PRSV infection.

2. Materials and methods

2.1. Papaya plants

The papaya seedbed, Maradol variety, was made in polyethylene bags of 20 x 14 cm, under controlled conditions at 32°C, 12 h light and 12 h darkness in a Weisstechnik® (Loughborough, UK) growth chamber, model SGC120-T. Five different papaya seedlings were sprayed with each treatment using a random block design. Schematic experiment timeline is shown in Figure 1.

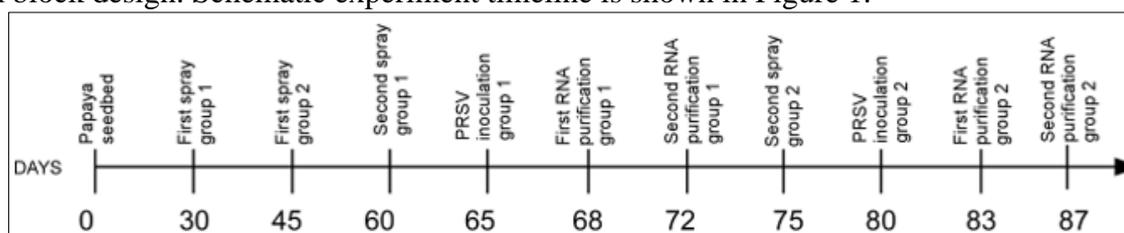


Figure 1. Experimental timeline of events. Experiment timeline from study initiation (Day 0) to termination (Day 87)



Papaya RNA extraction was performed to monitor the expression of the genes of interest. Three plants from each treatment were selected for analysis by semi-quantitative RT-PCR. The positive control consisted of plants inoculated with the virus and sprayed with water.

2.2. Statistical analysis

A randomized complete block design was used, where each block was composed of five treatments: (T1), Salicylic Acid (T2), POLYTHION® SC (Arysta Lifescience). (T3) Chamomile aqueous extract (*Matricaria chamomilla*) (T4), Chamomile aqueous extract + POLYTHION, (T5) water. Each treatment was applied to 5 plants with three replicates for a total of 75 experimental units per group. The experiment was performed twice with a difference of 2 weeks among them, (rep 1 and rep 2). Three plants from each treatment were selected for gene expression induction analyses. The statistical analysis was performed using the SAS System for PC version 9.0 (SAS Institute. 2017). An analysis of variance (ANOVA, F distribution) was performed to determine significant differences between means in the ANOVA, using Tukey and Duncan's multiple comparison tests at probability level (5%).

2.2. Description of the treatments

A total of 5 treatments were used for this study

T1. Salicylic acid. A 0.5 mM solution was prepared for spraying papaya plants

T2. POLYTHION® SC (Arysta Lifescience, Tokyo, Japan). This fungicide for agricultural use was tested as inorganic inducer of plant defence. The active component of the fungicide is sulphur. In 250 mL of distilled water, 250 µL of the fungicide was diluted according to the manufacturer's instruction.

T3. Chamomile aqueous extract. Chamomile was tested as organic inducer of plant defence. This was prepared with 500 g of fresh *Matricaria chamomilla* L. in 4 L of water. The preparation was placed in a plastic container and kept in the dark with daily agitation for 10 days. The Chamomile aqueous extract was passed through filter paper and stored in hermetically sealed glass bottles at 4°C until the time of application in a 1:10 dilution.

T4. Chamomile aqueous extract + POLYTHION® SC 500 mL of the Chamomile aqueous extract plus 500 µL of the fungicide POLYTHION® SC were mixed and used as a treatment. The mixture was stirred until it was homogenized and applied by spraying to each of the seedlings under treatment. POLYTHION has a composition of 720g/L of sulphur.

T5. Distilled water. Mock

2.3. Viral Inoculum preparation

Mechanical inoculation of PRSV using carborundum as an abrasive was carried out in healthy papaya plants previously sprayed with the treatments as described above. A diseased plant of papaya verified by RT-PCR was taken as the source of inoculum. The plant was collected in Villa del Rosario, Norte de Santander, Colombia, where studies with PRSV have been previously carried out [14, 16] The viral inoculum was prepared by macerating approximately 100 mg of infected leaf in 1 mL of inoculation buffer (10 mM of phosphate buffer, pH 7).

2.4. RNA extraction

RNA extraction. Papaya leaves PRSV inoculated and sprayed with the different treatments were used for RNA extractions using the Trizol reagent (Invitrogen, Waltham, MA USA) following the manufacturer's specifications. RNA was stored in 15 µL aliquots and kept at -70°C until it was used as template for RT-PCR.

2.5. Semi quantitative RT-PCR

Total RNA was used as a template in RT-PCR reactions to amplify the PR1, PK1 and ubiquitin genes of papaya and the capsid protein (CP) gene of PRSV using the specific oligonucleotides showed in Table 1.

Table 1. Oligonucleotides used to amplify different genes in papaya plants

Gene	Primer sequence (5'-3')	Ref.	Size (pb)	T°
<i>PRI</i>	FWD TCTCCGCCGTGAACATGTGGGTTAGCG	[17]	200	67°C
	Rev GTATGGCTTCTCGTTCACATAATTCCC			
<i>MPKI</i>	Fwd GATCCGTCAAAGAGGATTAGTGTCTCTG	[17]	200	58°C
	Rev TCAGAGCTCAGTGTTAAGGTTGAAGC			
<i>PRSV-CP</i>	Fwd AAGATAATGCTAGTGACGGAAATGATGTG	(This study)	266	54°C
	Rev TCTTCACTCCCTCGTACCATTCTCAAAT			
<i>Ubq</i>	Fwd GTTGATTTTTGCTGGGAAGC	[18]	200	62°C
	Rev GATCTTGGCCTTCACGTTGT			

For reverse transcription, the RNA and the respective 3' oligo were denatured at 70°C with 10U of MMLV reverse transcriptase (Promega, Madison, WI USA) and incubated at 37°C for one hour. Each resulting cDNA was amplified by PCR using 10 µL of the corresponding RT reaction, 2.5U of Taq polymerase (Invitrogen, Waltham, MA USA), dNTPs set to 625 µM and 0.5 pmol/µL of each of the 3' and 5' primers to a final volume of 50 µL. PCR amplifications were performed in a LABOCON GE4852T thermal cycler (Leicester, UK). 10 µL aliquots of the RT-PCR product were loaded into 1 % agarose gels in a TAE buffer electrophoresis chamber at a constant voltage of 100 volts and displayed with GelRed® (Biotium, Fremont, CA USA). Relative quantification of the amplification of each gene was done by comparing the intensity of the bands to the nanogram concentration of the Biotium (Toronto, Canada) Hyperladder™ 50 bp marker using the 1D analysis option of the Doc-ItLS software of the UVP (Upland, CA USA) gel documentation system.

3. Results and discussions

3.1. Effect of treatments on papaya seedlings and molecular detection of PRSV

Each of the treatments was sprayed on 5 different papaya plants each time (rep 1 and rep 2) under the same conditions and were monitored daily to determine its effect on the plant and disease symptoms. Figure 2 shows the condition of the papaya plants when the first spraying was done with each one of the treatments.

**Figure 2.** Papaya plants at day 30 after initial spraying with different treatments

After 7 days of virus inoculation, papaya plants presented no visible symptoms of PRSV; however, to verify that plants had been efficiently inoculated, a RT-PCR was conducted to amplify a segment of the CP gene (Figure 3). Amplified amplicons identity was verified by double sequencing.

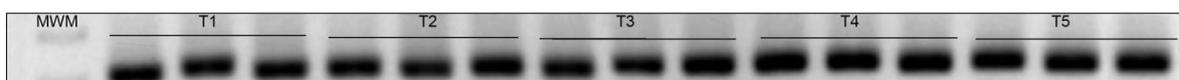


Figure 3. CP gene of PRSV (266 bp) in mechanically inoculated papaya seedlings. All plants tested were positive for PRSV by RT-PCR

3.2. RT-PCR and transcript quantification of PR1 and MPK1 genes

Each PCR amplification product (Figure 4) for each of the genes was quantified semi-quantitatively in triplicate for statistical analysis.

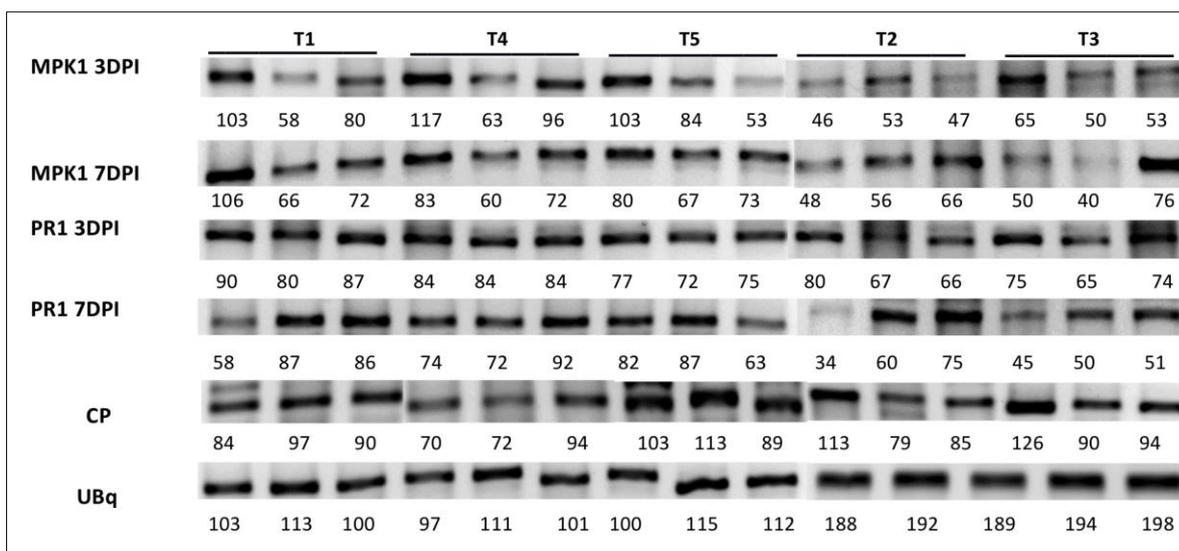


Figure 4. Amplification of PR1 and MPK1 genes in triplicate at 3 and 7 dpi

In example the figure shows the results for rep 1. Each band was quantified as described in methods and used to determine the difference in expression of the PR1 and MPK1 genes. The CP amplification products of PRSV are included. UBq was included as a reference sample loading control. (T1), *Salicylic acid* (T2), POLYTHION (T3) Chamomile aqueous extract (T4), Chamomile aqueous extract + POLYTHION (T5) water.

The values of intensities of each band for PR1 and MPK1 transcripts at 3 and 7 dpi, according to the different treatments, are summarized in Table 2.

Table 2. Relative intensity values of PR1 and MPK1 expression at 3 and 7 dpi using the ladder bands as point of reference. The intensity of green indicates greater expression of the gene. Red intensity indicates lower gene expression. The values are the average of six replicates per day per treatment (three from rep 1 and three from rep 2). These values were taken for semi-quantitative quantification of the gene expression and statistical analysis

	Treatment	T1		T2		T3		T4		T5	
Gene		3dpi	7 dpi	3dpi	7dpi	3dpi	7dpi	3dpi	7dpi	3dpi	7dpi
PR1	r1	90	58	80	34	75	45	84	74	77	82
	r2	80	87	67	60	65	50	84	72	72	87
	r3	87	86	66	75	74	51	84	92	75	63

MPK1	r1	103	106	46	48	65	50	117	83	103	80
	r2	58	66	53	56	50	40	63	60	84	67
	r3	80	72	47	66	53	76	96	72	53	73

3.3. Statistical analysis

The values showed in Table 2 were used to perform the analysis of variance to determine if there were significant differences between the treatments in the putative induction of the expression of the PR1 and MPK1 genes, as well as the differences in expression at 3 and 7 dpi. The ANOVA results suggest a best induction of treatments in the expression of PR1 ($P > F = 0.0001$) in comparison to MPK1 ($P > F = 0.0047$). Multiple comparison tests indicated no significant differences among the treatments to induce the expression of the MPK1 gene. On the other side T4 (Chamomile aqueous extract + POLYTHION) and T1 (Salicylic Acid) produced a best response for PR1 gene induction. Likewise, greater induction of PR1 expression was found at 3 dpi ($P > F = 0.0063$). On the other hand, no statistically significant differences were found in the levels of expression of MPK1 at 3 dpi and 7 dpi.

3.4. Effect of treatments on plant development and disease symptoms

Papaya plants sprayed with treatments and inoculated with PRSV were monitored for 30 days to evaluate the development of the disease. Figure 5 shows the papaya plants at 7 and 30 dpi, respectively.



Figure 5. Effect of treatments on the morphology of papaya plants. At the top are the plants at 7 dpi and at the bottom at 30 dpi. (A), Salicylic Acid (B), POLYTHION (C) Chamomile aqueous extract (D), Chamomile aqueous extract + POLYTHION (E) water

The Chamomile aqueous extract (T3) caused a deleterious effect in papaya plants at 30 dpi yielding the death of 3 plants, including control plants that were not inoculated with the virus (Table 3).

Table 3. Severity of PRSV symptoms in papaya plants sprayed with different treatments. The higher +, the higher the severity of PRSV disease. * Control plants were not inoculated with the virus, but sprayed with the corresponding treatment

Treatment	Plant #1	Plant #2	Plant #3	Plant #4 Control*	Plant #5 Control*
T1: Salicylic acid	+++	+	+	0	0
T2: POLYTHION	+	+	+	0	0
T3: Chamomile aqueous extract	died	+++	+++	died	died
T4: Chamomile aqueous extract + POLYTHION	+++	++	+	0	0
T5: Water (mock)	+++	+++	died	0	0

Interestingly, plants treated with Chamomile aqueous extract (T3) + POLYTHION (T4), developed no symptoms of deleterious effect caused by T3. On the other hand, at 30 dpi the plants sprayed with POLYTHION (T2), showed less deleterious effect, and presented attenuated symptoms caused by PRSV disease. However, T2 was not the treatment that induced a higher expression of MPK1 or PR1. Papaya plants sprayed with T2, had a similar response to plants treated with salicylic acid (T1).



The deleterious effects observed in the Chamomile aqueous extract could be attributed to compounds such as ammonia, ethylene oxide, organic acids, acetic acid, propionic acid, phenols, or other salts [15] as well as synergistic effects of compounds [19]. However, the harmful effect and PRSV symptoms drops when aqueous extract was mixed with sulphur-based POLYTHION. Plant defence against viruses is mainly due the presence of secondary metabolites such as terpenes, phenolic compounds and nitrogen and sulphur containing compounds [20]. The resistance induction observed in papaya plants could be due to the sulphur as the active ingredient of POLYTHION, however, plants treated only with POLYTHION showed lower induction of the expression of PR1 and MPK1 genes, compared to the combination of Chamomile aqueous extract with POLYTHION.

Treatment 4 (Chamomile aqueous extract + POLYTHION), induced the greatest response in the expression of the PR1 gene at 3 dpi, with no statistical difference to the induction caused by the treatment based on salicylic acid. Salicylic acid was used as positive control because induce resistance to viruses and other pathogens [21], it is a key plant defence regulator that primarily mediates responses to biotrophic pathogens [22], and is essential for the induction of systemic acquired resistance (SAR) [23]. Nonetheless little is known about the signal transduction pathway involved in plant-virus interactions, although it is known that expression of defence-related genes in compatible host plants may share a common signalling pathway with incompatible interactions [24]. SA can induce resistance to viral replication, cell-to-cell movement, and systemic movement. But which step of the infection cycle is inhibited depends upon the virus-host combination [25].

Resistance inducers in plants could help to reduce the use of pesticides, but their performance in the field is still not satisfactory because it is not well known how to integrate them into crop protection practices [11]. Although induced resistance can be activated in the field in the absence of pathogens, there is concern among farmers about their direct use on crops because of perceived crop losses and less effective control compared to traditional chemicals. In addition, confidence in resistance-inducing biotic or abiotic products is low among farmers for a number of reasons including the need for several applications or the application of different inducers to achieve the effect of the agrochemical [26]. However, the discovery of compounds that at very low percentages are capable of activating resistance in plants stimulates research in this area and produces fundamental knowledge about local and systemic defence mechanisms against diseases [27]. Treatment with Chamomile aqueous extract to induce resistance against PRSV caused deleterious effect in papaya plants, so it doesn't seem like a recommended alternative to PRSV control by itself. However, that harmful effect decreased when the aqueous extract was combined with POLYTHION, triggering an effect that drops the PRSV symptoms and favoured the expression of the PR1 gene in a similar way to that produced by salicylic acid.

Achieving sustainable crop production to feed an ever-growing population is a challenge today, but it is necessary to reduce adverse effects on the environment from agricultural activities. Pesticide reduction is critical to environmental conservation and can be reduced by adopting novel protection strategies [28]. The treatment of crop plants with resistance inducers is an alternative that has been implemented against disease control in different pathologies [29-32], including diseases caused by viruses [33-35]. For PRSV the literature is scarce, despite the fact that this constitutes the greatest obstacle in the production of papaya, being responsible for considerable losses in the crops. The disease caused by PRSV has been controlled mainly through biotechnological approaches with the generation of genetically modified plants [36]. However, there are studies of resistance induction to PRSV, in which it has been suggested that the severity and its accumulation in cucumber leaves, are greatly reduced when they have been preliminarily treated with silica nanoparticles (SiO₂) or treatments with the growth promoting fungus *Penicillium simplicissimum* [37].

4. Conclusions

POLYTHION, an agrochemical compound based on sulphur reduced the severity of symptoms caused by PRSV in papaya plants; however, the compound by itself did not produce greater expression of resistance related genes. Chamomile aqueous extract + POLYTHION treatment generated a response



in plants similar to that produced by salicylic acid in the induction of PR1 expression. Given the phytotoxic effect of the aqueous chamomile aqueous extract, it is not recommended to be used as an organic inducer of resistance in papaya plants.

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