

Oxidative Stress Defence Response of *Carica papaya* Challenged by Nitric Oxide, *Papaya meleira virus* and *Saccharomyces cerevisiae*

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Abstract: Seedlings of papaya (*Carica papaya*), cultivar Golden, were exposed to a nitric oxide donor, sodium nitroprusside (SNP), *Papaya meleira virus* (PMeV) and yeast (*Saccharomyces cerevisiae*, as a model fungal elicitor). The aim was to investigate the response of *C. papaya* to infection and the role of nitric oxide in this process. SNP alone led to a short lived (6 h) burst of peroxidase activity and a relatively low level thereafter, although higher than the control, whilst the virus PMeV caused a sustained increase for at least 30 days. In contrast, both SNP and PMeV treatments increased superoxide dismutase activity for at least 30 days. In general, concomitant addition of PMeV and SNP produced the highest increases in peroxidase and superoxide dismutase activity over the 30 day time course. Levels of phenolics were elevated after addition of SNP and histochemistry confirmed increased peroxidase and superoxide dismutase activity, especially around the phloem cell walls, suggestive of lignification. Carbohydrate content was also elevated after SNP treatment, in particular saccharose. Treatment with yeast produced increased peroxidase activity and phenolic and carbohydrate content in the plant tissues. These results demonstrate some of the defences elicited in *Carica papaya* in response to infection, and the role of nitric oxide in this process. The apparent systemic acquired resistance displayed suggests that artificial initiation of this process might be a useful future technique against PMeV in the horticultural industry.

Keywords: Antioxidant, carbohydrate, *Carica papaya*, nitric oxide, *Papaya meleira virus*, peroxidase, superoxide dismutase.

INTRODUCTION

Papaya (*Carica papaya*) is a major fruit crop in many tropical parts of the world. It has been ranked first amongst 38 common fruits based on its accordance to the United States Recommended Daily Allowance for many vitamins and consumption of papaya has been recommended for preventing vitamin A deficiency [1] which causes childhood blindness in many tropical and subtropical countries.

Unfortunately, papaya is susceptible to multiple pathogens. Susceptibility to *Papaya ringspot virus* has been largely contained by genetic modification [2], but there are other important challenges to papaya cultivation. A significant viral disease of papaya is "sticky disease" or "meleira", which can cause loss of up to 100% of the crop in a plantation. It is caused by a virus, *Papaya meleira virus* (PMeV), a double stranded RNA virus of approximately 12 kbp [3]. So far PMeV has only been found in the lactifers of papaya [4], an environment normally hostile to micro-organisms. PMeV induces potassium phosphate accumulation in the lactifer, changing the osmotic balance and

increasing the water content, whilst reducing the content of sugars and proteins [5]. This leads to the exudation of a watery latex solution from fruit and leaves which oxidises in air creating a black sticky mass on the plant surface, giving the disease its name. As well as the unpleasant appearance, changes in the texture and flavour of the fruit render it unsaleable. Control of PMeV infection is by identification of infected plants by symptoms and molecular diagnosis and removal of these plants as there are no resistant varieties [6]. It is important therefore to understand and eventually enhance the natural response of papaya plants to viral attack.

A major defence mechanism in plants is the "hypersensitive response" (HR) whereby cells infected with pathogens, and often surrounding cells as well, are instructed to self-destruct by the host plant [7]. This is thought to deny nutrients to the invading pathogen. It is proposed that in plants the process is initiated by a reaction between nitric oxide (NO) and hydrogen peroxide (H₂O₂), which is formed by superoxide (O₂⁻) dismutation by superoxide dismutase (SOD) during the HR [8].

Many studies have correlated a burst of NO production with plant defence responses against pathogens. This process has been demonstrated in fungal [9], bacterial [10] and viral [11] infections and NO accumulation usually reaches a peak 4 to 6 h after pathogen recognition. Administration of NO donors such as sodium nitroprusside (SNP) induces the expression of defence genes such as phenylalanine lyase

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(PAL) [10,11] and also SOD [13]. As well as an involvement in the hypersensitive response, it has been demonstrated that NO has the capacity to induce post translational modifications in plant proteins such as nitrosylation, nitration and oxidation [12], and these alterations have the potential to alter the activity of regulatory proteins [14,15].

Complimenting the sizeable production of NO and reactive oxygen species (ROS) such as O_2^- , plants possess a number of antioxidant defences. Peroxidases, together with catalases, are the major H_2O_2 degrading enzymes in plant cells [16], although the reaction of peroxidases with H_2O_2 is a process which can lead to lipid peroxidation and thus membrane damage [17]. Several authors have found a relationship between peroxidase levels and resistance to fungal [18-20] or viral [21,22] pathogens.

As well as oxidative responses, we also determined to investigate other components of the plant defence system. Increases in carbohydrate titres are a general plant response to stress [23]. However, more specific links to different stresses can be made and carbohydrate levels have been shown to increase in response to infection by studies since the 1930s, (for example [24-26]). A link of carbohydrates with oxidative stress also seems clear as carbohydrates have been shown to provide protection against ROS [27], induce oxidative stress defences [28] and are essential in the production of many anti-oxidants [29]. Plant phenolics are also an important part of plant protection against fungi and bacteria [30], and titres are often elevated following infection [31, 32]. Plant phenolics are also effective against viruses, and are induced by virus infection [33] which has led to a number of studies of the possibility of their use against human viruses (reviewed in [34]).

In summary, we proposed to study the response of *Carica papaya* to pathogen attack in general, and to the virus PMeV in particular, and determine any role of nitric oxide in these processes.

MATERIALS AND METHODS

Vegetative Material

The seedlings of cultivar Golden used in the experiments were obtained from the experimental farms of INCAPER (Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural), located in the state of Espírito Santo, Brazil. Seeds were planted in pots in a mixture of compost to sand of 3:1 (w:w). Seedlings were irrigated 3 times per week. At four months, seedlings were transferred to a glasshouse with mean temperature of 20 °C and a photoperiod of 12 h. At this stage, seedlings were divided into experimental groups.

Experimental Plan

Seedlings were treated with the NO donor sodium nitroprusside alone or in combination with *Saccharomyces cerevisiae* or PMeV. Seedlings were initially treated with the NO donor sodium nitroprusside or *Saccharomyces cerevisiae* at 72, 120 and 168 h to determine the time course and magnitude of responses to NO donation and pathogen challenge. The second tranche of studies focused specifically on interactions of NO and the virus PMeV, over a more

comprehensive time scale from 3 min to 30 days. It has been reported that mechanical perturbation from spraying can cause changes in gene expression in *C. papaya* [35] and to assess this, and differentiate it from other treatments, controls of untreated and water sprayed plants were included.

Treatment with Nitric Oxide (NO)

Cuttings were sprayed with 5 ml of 10 mM sodium nitroprusside solution (SNP), [36] or the same volume of distilled water as a control. Sodium nitroprusside solution was used as it is the most widely accepted experimental donor of NO [37].

Treatment with Yeast *Saccharomyces Cerevisiae*

A fresh suspension of 200 g l^{-1} yeast (Fleishmann Ltda, Sao Paulo, Brazil) was prepared. The solution was autoclaved for 30 min at 121 °C (1 atm) and the plants were sprayed with 10 ml of solution.

Treatment with Papaya Meleira Virus (PMeV)

Latex was collected from infected plants and the presence of PMeV confirmed by gel electrophoresis [6]. This was used as the source of PMeV inoculate in the treatments. Seedlings of cultivar Golden, 4 months of age, were injected with 20 μ l of latex from an infected plant in the leaf petiole. Five replicates were used for each treatment.

Total Protein Content

The protein content of the seedlings was determined according to the method of Lowry [38] with absorbance measured at 660nm (FEMTO 482, spectrophotometer, FEMTO, Sao Paulo, Brazil). For preparation of the crude extract 0.3 g of leaves were crushed in liquid nitrogen and resuspended in 4 ml of 0.01M phosphate buffer (pH 6.0) at 4 °C. The suspension was centrifuged at 12,000 rpm for 25 min at 4 °C and the supernatant collected. To 1 ml of crude extract was added 5 ml of copper reagent (48 ml of sodium carbonate solution (3% in 0.1M NaOH), 1 ml sodium potassium tartarate solution (4%) and 1 ml Copper sulphate solution (2%)). After 10 min, 500 μ l of phenol reagent (1 volume of Folin-Ciocalteu reagent and 2 volumes of distilled water) was added and incubated for 10 min. Absorbance was measured at 660 nm. To determine the concentration a standard curve was prepared using aliquots of bovine serum albumin (25, 50, 100, & 150 μ l of 1 mg ml^{-1}).

Peroxidase Activity

Peroxidase activity was determined using a previously published method [39] with some modifications. Leaves (0.3 g) were crushed in liquid nitrogen and resuspended in 4 ml of extraction buffer (phosphate buffer, 0.01M, pH 6.0) at 4 °C. The suspension was centrifuged at 12,000 rpm for 25 min at 4 °C and the supernatant was collected. To 60 μ l of supernatant were added 0.9 ml of reaction buffer (0.15 μ l of 30% H_2O_2 , 0.13 μ l of guaiacol in 50 ml extraction buffer) and 40 μ l of extraction buffer. Absorbance was read at 470nm and activity expressed as Abs $min^{-1} mg^{-1}$ protein.

Determination of the Activity of Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined using a previously published method [40]. Frozen leaves (0.3 g) were homogenised with polyvinylpyrrolidone (PVPP) in a solution of 50 mM phosphate buffer, pH 7.5, 1 mM EDTA- Na_2 , 50 mM NaCl and 1 mM ascorbic acid. The homogenate was centrifuged at 11,000 rpm (30 min, 4 °C). To the supernatant was added 0.5 ml of 0.54 mM EDTA- Na_2 , 0.8 ml of phosphate potassium buffer (0.1 M, pH 7.0), 0.5 ml of 0.13 mM methionine, 0.5 ml of 0.44 mM p-nitroblue tetrazolium (NBT) and 0.2 ml of 1 mM riboflavin. The resultant solution was exposed to fluorescent light (80 W) for 20 min.

Extracts prepared following the same procedure were kept in the dark. The absorbance of the solution was measured by spectrophotometer ($\lambda=560\text{nm}$) for both types of extract (illuminated and not illuminated). SOD activity was determined by comparison of the difference between the two absorbances, reflecting inhibition of NBT reduction by the enzymatic activity of SOD. Enzymatic activity was expressed in $\text{Abs min}^{-1} \text{mg}^{-1}$ of protein.

Microscopy

Leaf stems were examined histochemically for the presence of superoxide (O_2^-) and Hydrogen peroxide (H_2O_2) following treatment (time points 3, 15, 30, 45, 60, 180 and 360 min). Results were recorded digitally using a Leica optical microscope with Moticam 2000 2.0 pixel camera and Motic Plus Soft Imaging software.

i) Detection of Superoxide (O_2^-)

Detection followed the histochemical method of [41] with some modification. Traverse sections of the leaf stem were cut by hand free and deposited in Petri plates containing a solution of 0.5 mM nitroblue tetrazolium (NBT), pH 7.4, for 12 h. Control samples were maintained in deionised water. All sections were destained in 96% boiling ethanol for 20 min and mounted on microscope slides in 50 % glycerol.

ii) Detection of Hydrogen Peroxide (H_2O_2)

Detection again followed the method of [41] with some modification. Traverse sections of the leaf stem were cut by hand free and deposited in Petri plates containing a solution of 5 mM diaminobenzidine HCL (DAB), pH 3.8 for 12 h. Control samples were maintained in deionised water. All sections were destained in 96% boiling ethanol for 20 min and mounted on microscope slides in 50 % glycerol.

Phenolic Composition

Analysis of total phenolic content used methanol as an extraction agent and Folin and Ciocalteu reagent to determine concentration [42].

To extract phenolics, 0.3 g of leaves were ground in liquid nitrogen and the powder resuspended in 4 ml 50% methanol. The suspension was incubated at 80 °C for 1.5 h and then centrifuged at 12,000 rpm for 15 min. The supernatant was collected and analysed for free phenolics.

The pellet was resuspended in 2 ml 0.5 M NaOH and incubated for 24 h for saponification of bound phenolics in the cell wall. The reaction was neutralised with 0.5ml 2M hydrochloric acid and the mixture centrifuged at 15,000 rpm for 15 min. The supernatant was recovered.

To 150 μl of supernatant (either free or bound phenolics) was added 3 ml of 2% sodium carbonate and 150 μl of Folin Ciocateu reagent (diluted 1:1 in water). Absorbance was read by spectrophotometer (Fento 482, USA) at 750 nm. A standard curve was prepared using chlorogenic acid solutions and the concentration of phenolics was expressed as chlorogenic acid equivalents per mg of fresh tissue.

Saccharose, Fructose and Total Carbohydrate Content

Soluble sugars, fructose and saccharose were measured using the antrona reagent method [43].

i) Preparation of Crude Extract

One gram of leaves was immersed in 10 ml of 80% ethanol for 3 min and then homogenised and incubated at 80°C in a water bath for 30 min. After incubation samples were centrifuged at 10,000 rpm for 10 min. The supernatant was extracted and the process was repeated on the pellet two more times. After three ethanol extractions the supernatants were mixed and stored.

The pellet was resuspended in 15 ml of water and this suspension was incubated at 60 °C in a water bath for 30 min and then centrifuged as above. The supernatant was removed and the process was repeated. The supernatants (ethanol and aqueous fractions) were mixed and placed in a rotoevaporator at 60 °C and a rotation speed of 7000rpm until reaching a volume of 5 ml. The crude extract (CE) was kept at -20 °C until analysed.

ii) Total Carbohydrate Content

Total soluble carbohydrate content was determined using 50 μl of CE, 200 μl of water and 2.5 ml of 0.2% antrona solution (76 ml sulphuric acid, 24 ml water and 0.2 g antrona). The samples were incubated at 100 °C in a water bath for 10 min and then kept in darkness for 20 min. Absorbance was measured at 620 nm. The absorbance was compared to standard curve of glucose (0.5, 1.0, 1.5 and 2.0 mg ml^{-1}).

iii) Fructose Content

To 50 μl of CE was added 250 μl water and 2.5 ml 0.2% antrona reagent. The samples were mixed using a vortexer and incubated at 37 °C for 45 min. Absorbance was measured at 620 nm and compared at a glucose standard curve (0.5 to 2.0 mg ml^{-1}).

iv) Saccharose Content

To 100 μl of crude extract was added 250 μl water, 100 μl 5.4M potassium hydroxide and 3 ml 0.2% antrona reagent. Absorbance was read at 620nm and compared at a standard curve of saccharose (0.5 to 2.0 mg ml^{-1}).

Statistical Analysis

Each experiment used three replicates, and was repeated on two further occasions. Comparisons were performed

using an ANOVA, with Tukey test at 5% probability for variation over time, or two way ANOVA for comparison of treatments.

RESULTS

Peroxidase

Sodium nitroprusside (SNP) increased peroxidase activity significantly after 120 h (Table 1). Application of yeast produced a similar, though slightly faster, increase in enzyme activity, significant after 72 h.

On a shorter timescale after treatment with SNP an immediate increase in peroxidase activity was observed and this was sustained for 6 h (Fig. 1). Time points up to 6 h and time points beyond 6 h formed two groups statistically, significantly different to each other, but not within groups ($p < 0.05$), except for a secondary peak at 72 h which was not significantly different to either group. Peroxidase activity did not change significantly over this time course for either control or water treatments ($p < 0.05$). Overall, peroxidase activity was significantly different in the SNP treated group compared to either control or water treatment ($p < 0.001$). It should be noted however that activity following water treatment was also significantly different to the control ($p < 0.001$).

Peroxidase activity after PMeV inoculation formed two significantly different time point groups, before and after 6 h ($p < 0.05$) (Fig. 2). Activity rose by approximately 10 fold after 6 h and this level was maintained for at least 30 days. A very similar pattern was found with PMeV inoculation concomitant with SNP treatment, although the 6 h time point was intermediate between the two groups. This is in contrast

to the activity of peroxidase following treatment with SNP alone (Fig. 1), where the same 6 h cut off point was observed, but with a decrease rather than increase in activity.

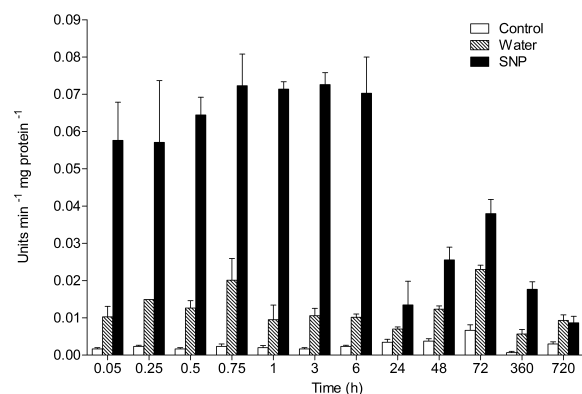


Fig. (1). Peroxidase (POD) activity after treatment with SNP. Time after treatment represented by letters, (A) 3 min; (B) 15 min; (C) 30 min; (D) 45 min; (E) 1 h; (F) 3 h; (G) 6 h; (H) 24 h; (I) 48 h; (J) 72 h; (K) 15 days; (L) 30 days. The bars represent standard error of three replicates. Controls (white), treatment with water alone (grey), treatment with SNP (black).

To determine the location of peroxidase activity increase, histochemical studies were undertaken. Histochemical staining of transverse leaf stem sections for hydrogen peroxide showed differences in intensity between treated and untreated plants (Fig. 3). Staining was more intense in SNP treated plants, especially in the phloem (Fig. 3b, d, f, j, l), and was more intense over the first hour. Differences in intensity of staining were observed over six h.

Table 1. Peroxidase Activity and Mean Concentration of Free and Bound Phenolics after Treatment with Water, SNP or Yeast Extract

Time (hr)	Treatment	Peroxidase Activity (abs min ⁻¹ g ⁻¹ Fresh Tissue)	Phenolics (mg g ⁻¹ Fresh Tissue)	
			Free	Bound
0	Water	0.187 +/- 0.033 a	157.5 +/- 1.4 a	120.3 +/- 2.5 a
	SNP	0.217 +/- 0.017 a	167.2 +/- 1.7 a	129.5 +/- 2.8 a
	Yeast	0.255 +/- 0.059 a	164.6 +/- 2.7 a	133.6 +/- 2.4a
72	Water	0.173 +/- 0.064 a	137.9 +/- 11.0 a	119.5 +/- 2.8 a
	SNP	0.398 +/- 0.034 a	225.5 +/- 12.5 b	126.3 +/- 4.5 a
	Yeast	0.561 +/- 0.096 b	191.9 +/- 12.7 b	161.2 +/- 5.2 b
120	Water	0.410 +/- 0.069 a	160.3 +/- 6.7 a	116.5 +/- 4.0 a
	SNP	0.786 +/- 0.041 b	214.0 +/- 7.4 b	127.1 +/- 4.9 a
	Yeast	1.080 +/- 0.182 b	199.4 +/- 11.8 b	141.2 +/- 1.8 b
168	Water	0.305 +/- 0.074 a	123.6 +/- 4.3 a	127.2 +/- 4.4 a
	SNP	0.839 +/- 0.067 b	215.6 +/- 6.4 b	137.1 +/- 6.8 a
	Yeast	1.426 +/- 0.093 b	133.8 +/- 10.4 a	140.9 +/- 4.1 a

Within each vertical group of three measurements (Water, SNP, Yeast) numbers with the same letter are not significantly different by Tukey test (5% level of probability).

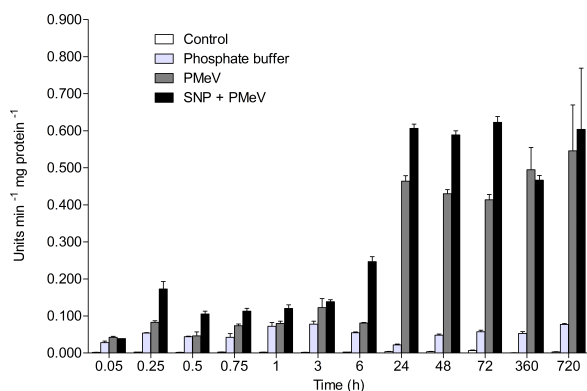


Fig. (2). Peroxidase (POD) activity after treatment with PMeV or SNP. Time after treatment represented by letters, (A) 3 min; (B) 15 min; (C) 30 min; (D) 45 min; (E) 1 h; (F) 3 h; (G) 6 h; (H) 24 h; (I) 48 h; (J) 72 h; (K) 15 days; (L) 30 days. The bars represent standard error of three replicates. Controls (white), injection with phosphate buffer (light grey), injection with PMeV (dark grey), injection with PMeV and SNP treatment (black).

Superoxide Dismutase

Treatment with the NO donor sodium nitroprusside (SNP) led to an increase in SOD enzyme activity. Enzyme activity peaked almost immediately after treatment and maintained a high level up to 30 days post-treatment (Fig. 4). There was however variation in activity during the time course of the experiment, and significant difference between time points ($p < 0.05$), although no clear pattern could be discerned. In neither control nor water treatments was a significant change in SOD activity observed over time ($p < 0.05$).

Overall, SOD activity was considerably higher following SNP treatment compared to water or control treatments and was significantly different to both ($p < 0.001$). As with peroxidase activity, it should be noted that SOD activity was also significantly different between water treated and control groups over this time course ($p < 0.001$).

To discern the effect of PMeV on superoxide dismutase activity plants were treated with both virus and NO. Overall, PMeV inoculation with or without SNP increased SOD expression (Fig. 5). SOD activity following PMeV treatment alone did not change significantly over time after the initial peak ($p < 0.05$), and overall activity was significantly different to all other groups, being greater than the controls or phosphate buffer treated group, but less than PMeV with SNP ($p < 0.001$). Indeed, all four treatment groups were significantly different to each other over the 30 days of the experiment ($p < 0.001$). SOD activity following PMeV with SNP was the highest measured at each time point, and was significantly different from each treatment overall ($p < 0.001$). However, considerable variation was observed over time, with a trend towards decrease of activity towards the end of the time course. An increase in SOD enzyme activity was observed at all time points from simple injection of phosphate buffer alone, compared to the control, suggesting that mechanical injury triggered a response (Fig. 5).

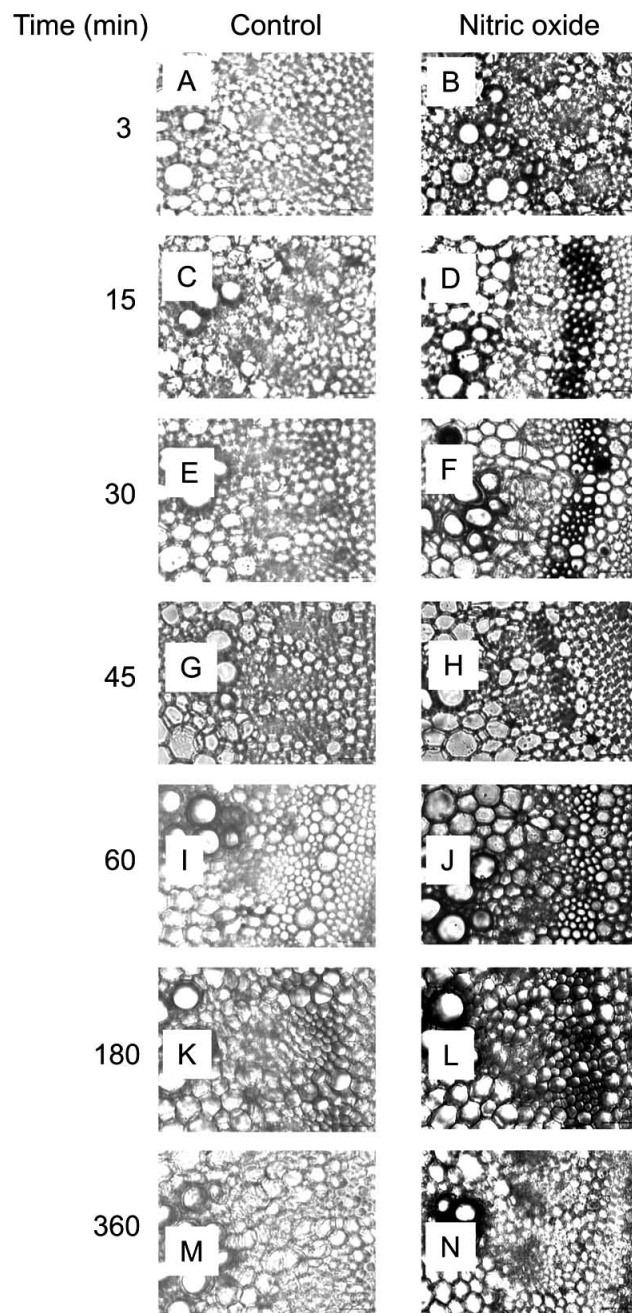


Fig. (3). Histochemistry of transverse sections of leaf stem after treatment with SNP. Stained with diaminobenzidine HCl (DAB). Time post treatment represented by letter. Pictures A, C, E, G, I, K & M represent control, water treated, plants at times 3, 15, 30, 45, 60, 180 and 360 min after treatment respectively, while B, D, F, H, J, L & N represent plants treated with SNP at the same times. Bar = 100 μ m.

To identify the area of the leaf in which SOD enzyme activity takes place, leaves were analyzed for formation of superoxide radicals using histochemistry. Radicals appeared to be located mostly in the phloem and xylem, with lesser activity in the epidermis and subepidermis (Fig. 6d, h, i).

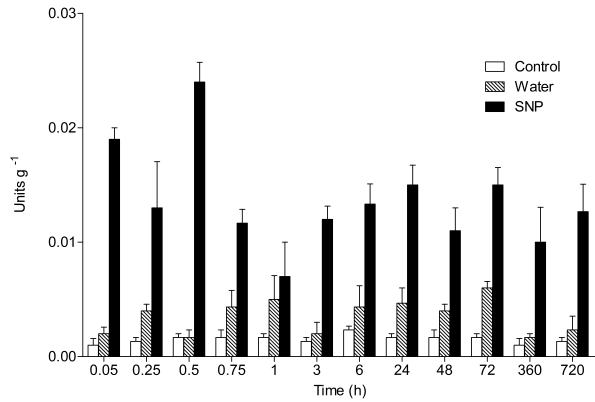


Fig. (4). Activity of superoxide dismutase after treatment with SNP. Cultivar Golden. Time points represented by letters, (A) 3 min, (B) 15 min, (C) 30 min, (D) 45 min, (E) 1 h, (F) 3 h, (G) 6 h, (H) 24 h, (I) 48 h, (J) 72 h, (K) 15 days, (L) 30 days. Bars represent standard error after 3 replicates. White columns are controls, grey are seedlings treated with water, and black are seedlings treated with SNP.

Sizeable differences were observed in the intensity of staining, being consistently greater in plants treated with SNP (Fig. 6a,c,e). A relationship was observed between histochemistry and enzymatic activity. This is illustrated by the histochemistry 30 min after SNP treatment (Fig. 6f), in which dark staining for superoxide in the area of xylem and phloem corresponds to a peak in SOD activity.

Phenolics

NO increased the titre of free phenolics at all time points, increasing by 63, 36 and 74% over controls after 72, 120 and 168 h respectively (Table 1). There was no significant change in the level of bound phenolics.

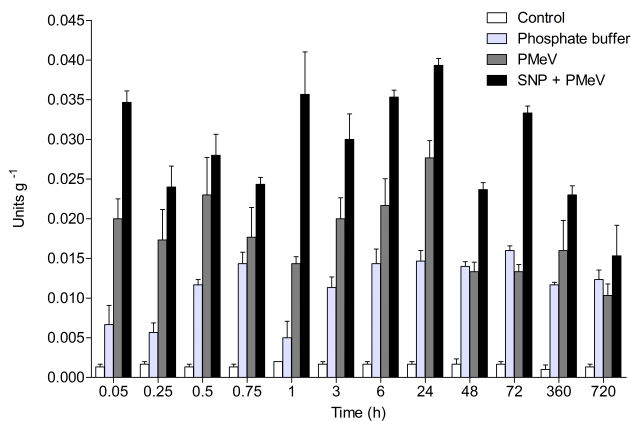


Fig. (5). Activity of superoxide dismutase (SOD) at various times post treatment with PMeV or SNP. Times represented by letters. (A) 3 min; (B) 15 min; (C) 30 min; (D) 45 min; (E) 1 h; (F) 3 h; (G) 6 h; (H) 24 h; (I) 48 h; (J) 72 h; (K) 15 days (L) 30 days. Bars represent standard errors of three replicates. Treatments represented by colours. White (control), light grey (injection with phosphate buffer), dark grey (injection with PMeV) and black (SNP + PMeV injection).

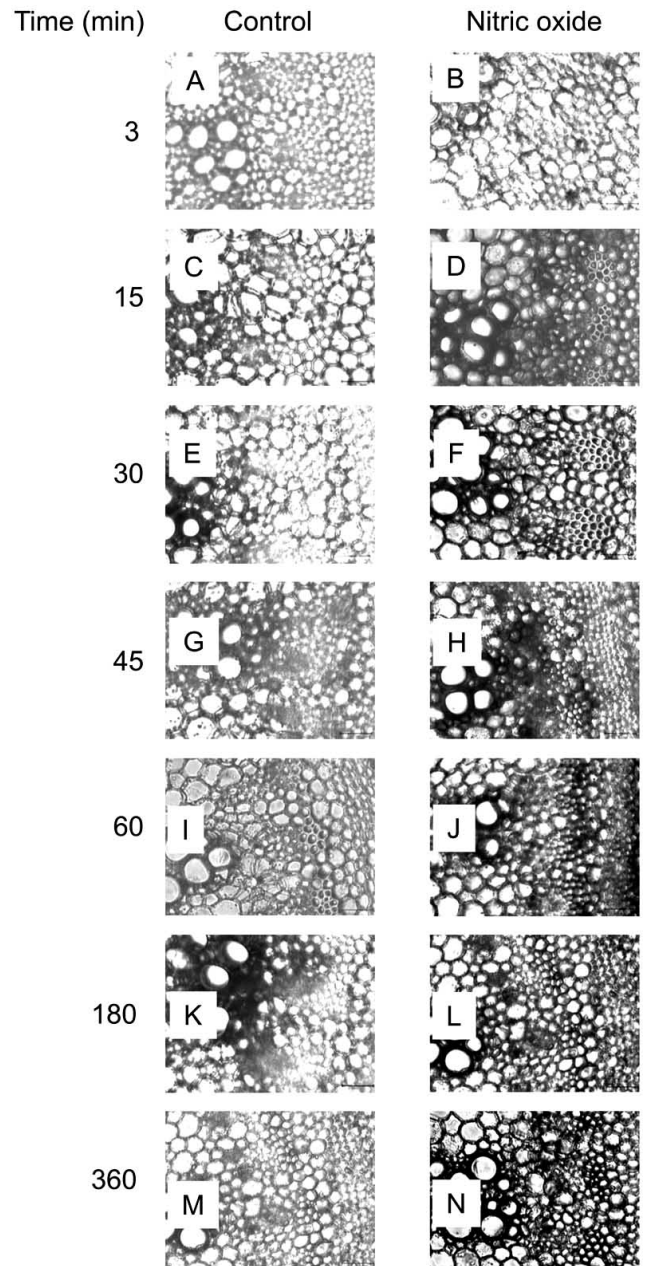


Fig. (6). Microscopy of transverse sections of leaf stem of papaya, cv. Golden following treatment with SNP. Stained with nitroblue tetrazolium. Pictures A, C, E, G, I, K & M represent control, water treated, plants at times 3, 15, 30, 45, 60, 180 and 360 min after treatment respectively. Pictures B, D, F, H, J, L, & N represent plants treated with SNP at the same time periods. Bar = 100 μm.

The greatest difference between yeast treatment and controls was recorded 72 hrs after treatment (Table 1). The concentration of free phenolics after 72 hrs was 39% greater than the control, whilst the level of bound phenolics was 35% greater. Both phenolic types were not significantly different to control levels after 168 hrs.

Concentration of Carbohydrates

Addition of SNP did not have a significant effect on total carbohydrate levels or fructose, but saccharose content was significantly higher after 24 and 48 h (Table 2).

Total carbohydrate content increased significantly 24 h after treatment with yeast but by 48 h was not significantly different to the control. Fructose content did not change, but saccharose content increased significantly after 24 h and was maintained until at least 48 h.

DISCUSSION

Papaya meleira virus (PMeV) is a significant disease of the important crop papaya (*Carica papaya*). To investigate a reported involvement of nitric oxide (NO) in plant defence signalling we challenged papaya seedlings with a NO donor sodium nitroprusside (SNP), PMeV, and a model fungus (*Saccharomyces cerevisiae*). The responses of peroxidases, superoxide dismutases, phenolics and carbohydrates were determined. Treatments using SNP and yeast took the form of a spray and so it was considered necessary to include controls of both untreated and water treated plants. Mechanical perturbation has been shown to increase peroxidase activity in plants [44] and water spraying can change expression of some genes in *C. papaya* [35]. We found significant increases in peroxidase and superoxide dismutase activities in water sprayed plants compared to those without treatment, suggesting that both these enzymes respond to thigmomastic stress in *C. papaya*.

Peroxidases, together with catalases, are the major H₂O₂ degrading enzymes in plant cells [16]. We observed peroxidase activity to increase following addition of both yeast and PMeV to *Carica papaya*. Peroxidase activity after PMeV inoculation, with or without SNP, formed two significantly different time point groups, increasing by approximately 10 fold after 6 h and this level was maintained for at least 30 days. A similar increase was found following yeast exposure with an increase in activity found from 3 until at least 7 days. This is in contrast to the activity of

peroxidase following treatment with the nitric oxide donor (SNP) alone, where the same 6 h cut off point was observed, but with a decrease rather than increase in activity although levels remained higher than the control. This suggests that NO can induce an increase in activity, but continued high enzyme activity is dependent on the presence of a pathogen.

Several authors have found a relationship between peroxidase levels and resistance to disease and it has been known for some time that a combination of peroxidases and hydrogen peroxide can be fatal to fungi [18]. Peroxidase activity is increased in plants infected with fungus [20], and peroxidase levels in maize rose faster post infection in varieties of maize resistant to *Exserohilum turcicum* [19]. Increase in peroxidase activity is also a response to viral infection, and has been reported in tobacco [45], peaches and apricots [21] and beans [22]. The relationship of NO to oxidative stress is complex. Beyond an effect on cell signalling, nitric oxide can cause direct oxidative damage by reacting with superoxide (O₂⁻) to form peroxynitrite (ONOO⁻), a powerful oxidant which can react with DNA, lipids and proteins under physiological conditions, leading to cellular damage and cytotoxicity [46]. Conversely, NO can also act as an antioxidant [47]. The effect of NO in any given circumstances appears to depend to some extent on the cellular ratio to reactive oxygen species (ROS) especially in terms of apoptosis [10]. It is in this matrix that the patterns of peroxidase activity we observed following SNP application to *C. papaya* with or without PMeV, of an initial surge followed by a considerable decrease or increase in activity after 6 h, need to be seen. The secondary increase after 6hrs is similar to the time scale found by [48] for an increase in gene expression in soybean plants following infection with *Phytophthora sojae*, which suggests that the pathogen related increase after 6hrs can be attributed to new gene expression.

Another class of antioxidant enzymes, superoxide dismutases (SODs) were also investigated. SODs are a group of metalloenzymes that catalyse the dismutation of superoxide radicals (O₂⁻) to H₂O₂ and O₂ [49]. Treatment

Table 2. Mean Concentration of Total Carbohydrates, Fructose and Saccharose in Cultivar Golden treated with Water, SNP and Yeast Extract

Time (hr)	Treatment	Total Carbohydrate (mg g ⁻¹ Fresh Tissue)	Fructose (mg g ⁻¹ Fresh Tissue)	Saccharose (mg g ⁻¹ Fresh Tissue)
0	Water	0.115 +/- 0.006 a	0.042 +/- 0.002 a	0.044 +/- 0.002 a
	SNP	0.109 +/- 0.004 a	0.044 +/- 0.002 a	0.041 +/- 0.002 a
	Yeast	0.107 +/- 0.011a	0.045 +/- 0.002 a	0.044 +/- 0.003 a
24	Water	0.112 +/- 0.007 a	0.045 +/- 0.002 a	0.046 +/- 0.004 c
	SNP	0.144 +/- 0.012 ab	0.045 +/- 0.002 a	0.068 +/- 0.002 b
	Yeast	0.167 +/- 0.005 b	0.055 +/- 0.002 a	0.087 +/- 0.003 a
48	Water	0.131 +/- 0.009 a	0.046 +/- 0.002 a	0.056 +/- 0.005 b
	SNP	0.166 +/- 0.020 a	0.046 +/- 0.002 a	0.071 +/- 0.003 a
	Yeast	0.138 +/- 0.009 a	0.042 +/- 0.002 a	0.081 +/- 0.003 a

Within each vertical group of three measurements (Water, SNP, Yeast) numbers with the same letter are not significantly different by Tukey test (5% level of probability).

with SNP led to an increase in total SOD enzyme activity, peaking almost immediately after treatment and remaining at a high level for up to 30 days post treatment, whilst no change was observed in controls or water treated. ($p < 0.05$). Overall, PMeV inoculation also increased SOD activity and did not change significantly over time after the initial peak. SOD activity following PMeV with SNP was the highest measured at each time point, and was significantly different from each treatment overall ($p < 0.05$). This is consistent with previous reports that show that virus infection can induce SOD activity [50,51].

To identify the area of the leaf in which SOD enzyme activity takes place following NO exposure, leaves were analyzed for formation of superoxide radicals using histochemistry. Activity appeared to be located mostly in the phloem and xylem, with lesser activity in the epidermis and subepidermis and was consistently greater in plants treated with SNP. A relationship was observed between histochemistry and measured enzymatic activity. Recently a role for SOD in the regulation of H_2O_2 has been described [52] and thus SOD activity at the cell walls may be related to lignification.

Phenolics are an important part of plant defences against fungi and bacteria [30]. Although papaya fruit have a relatively low level of phenolics compared to other fruit [53], we observed a significant response. Levels of free phenolics were elevated in response to NO challenge, but bound phenolics were not significantly altered. Both free and bound phenolics were elevated after 72 and 120 h in response to yeast. An increase in phenolic levels in response to a fungal elicitation was expected from previous studies [54,55]. Plant phenolics are also effective against viruses, and are induced by virus infection [33] which has led to a number of studies of the possibility of their use against human viruses (reviewed in [56]). It is therefore interesting in the context of PMeV that SNP increased phenolic levels in papaya. Phenolics can also be potent antioxidants [57] and so it is unclear whether a rise in phenolic levels in response to NO would be an antioxidant defence, or the result of NO signalling of another challenge such as viral infection. Of course the two are not incompatible.

Another important involvement of phenolics with resistance is in the formation of lignin, in conjunction with peroxidases, to create a physical barrier to limit fungus [58,59] or virus [21] invasion. This also renders the wall more water resistant and thus less accessible to cell wall-degrading enzymes [60]. *Carica papaya* has an intermediate numbers of lignin synthetic genes, fewer than poplar, but more than *A. thaliana* [61]. Histochemical staining of transverse leaf stem sections for hydrogen peroxide showed differences in intensity between control plants and those treated with the NO donor SNP. Staining was more intense in SNP treated plants, especially in the phloem and was more intense over the first hour with activity maintained for at least 6 h. It has been reported that class III peroxidases, those involved in lignification, are concentrated in the cell wall [62], and so this, like the elevated SOD levels described above, would be consistent with an increase in lignification in response to nitric oxide.

We observed a significant increase in saccharose content after yeast inoculation, and a more transient increase in total carbohydrate content. This is in accordance with other studies which have found increases in carbohydrate titres to be a general plant response to stress [23] and fungal infection [25]. Regarding viruses, carbohydrates appear to have a role in plant defences, and it has been known since the 1930s that accumulation of starch precedes the presence of virus symptoms [24] and, for example, marrows infected with *Zucchini yellow mosaic virus* show increased sucrose titres [26]. Following SNP treatment we also observed a significant increase in saccharose content, and a more transient increase in total carbohydrate content. A link of carbohydrates with defences against oxidative stress has been observed in other studies [27,28] and carbohydrates are essential in the production of many anti-oxidant defences ([29]).

It has been known for some time that plants can acquire a long term systemic immunity to the original, and other, pathogens, known as Systemic acquired resistance (SAR) [63]. It has been known since the 1960s that viruses are SAR inducers [64] and that resistance to viruses can be induced [33, 65]. The potential applicability of this technique to papaya has already been shown using acibenzolar-S-methyl as an inducer [64]. We examined the response of various biological parameters in response to yeast and PMeV inoculation. Application of yeast led to enhanced peroxidase activity in the plants for at least 7 days, whilst we found that injection of the virus PMeV enhanced peroxidase activity for over 30 days. This activity was especially strong in the cells wall, and is presumably associated with increased lignification. These results would appear to suggest that long lasting defence responses can in deed be induced by pathogens in *Carica papaya*, including PMeV.

CONCLUSIONS

Induction of resistance to PMeV in *Carica papaya* would appear to present an attractive opportunity for disease control, given the lack of resistant varieties available. Systemic resistance has already been successfully induced in other plants against viruses, and because no transgenic techniques are employed it should be more commercially acceptable. However, it should be borne in mind that some concern has been expressed about the applicability of induced resistance in the field compared to the laboratory or glasshouse, as plants may already be at or near their limit from pre-existing stresses [67,68]. Another consideration is that the applicability of this technique is likely to be cultivar dependent, as has been found in tomatoes [69].

There appears to be an important role for NO in defence induction in *C. papaya*, as the NO donor SNP not only induced a long lasting (30 day) increase in peroxidase titre, but also the production of both phenolics and carbohydrates, both of which are antioxidant defences and important in plant defence against viruses. Additionally a rise in both bound phenolics and wall-associated peroxidases was observed following SNP addition which was still evident 7 days after treatment, suggesting an increase in wall lignification, another viral defence. An involvement of NO in the SAR response appears to be supported by the

literature. When tobacco plants are treated on one leaf with NO donors, the whole plant develops enhanced resistance to TMV up to 13 days later, whilst addition of NOS inhibitors reduces considerably tobacco SAR [70].

Many plant diseases, especially viruses, have no cure as yet. Induction of natural plant resistance has potential to fill this gap, and the involvement of NO in this process appears to be critical, although further work is needed to completely establish the pathways involved in the response.

LIST OF ABBREVIATIONS

H ₂ O ₂	=	Hydrogen peroxide
HR	=	Hypersensitive response
NO	=	Nitric oxide
PMeV	=	<i>Papaya meleira virus</i>
ROS	=	Reactive oxygen species
SNP	=	Sodium nitroprusside
SOD	=	Superoxide dismutase

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CONFLICT OF INTEREST

None declared.

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