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REGULAR ARTICLE

Effect of the application of inducers on soursop fruit (*Annona muricata* L.): postharvest disease control, physiological behaviour and activation of defense systems

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ABSTRACT

The application of chitosan (Chi) alone or in combination with methyl jasmonate (MJ) and salicylic acid (SA) in soursop infected with *C. gloeosporioides* and *R. stolonifer in vivo* as well as quality parameters (pH, total soluble solids, firmness and titratable acidity), respiration rate, ethylene production and defense related enzyme activities were investigated. Diseases caused by phytopathogenic fungi at postharvest stage are a serious problem throughout the world. The pathogens evaluated in this study were isolated from orchards in Nayarit, Mexico. The coating application of 1.0% Chi - 0.1 mM MJ - 6 mM SA on fruit inoculated with *C. gloeosporioides* and 0.5% Chi- 0.1 mM MJ on fruit inoculated with *R. stolonifer* were more effective to reduce the disease incidence and the extension of the lesion on soursop rather than the application of Chi, MJ or SA alone. In order to maintain quality parameters Chi and inducers were applied on fruits. During the storage time quality parameters of fruit were maintained. The respiration rate and ethylene production decreased with the application of the treatments combination. Higher activities of PPO (polyphenol oxidase), and POD (peroxidase) were evidenced with the combination of treatments compare to control. The application of chitosan, methyl jasmonate and salicylic acid is a suitable method for postharvest disease control of soursop during storage time as well as to maintain the fruit quality.

Keywords: *Annona muricata*; Anthracnose; Induced resistance; Respiration rate; Soft rot

INTRODUCTION

The production of Soursop (*Annona muricata* L.) contributes to the economic growth of the state of Nayarit, Mexico (SAGARPA, 2017). It is prized for its very pleasant as well as other sensorial characteristics like odor and taste (Shashirekha et al., 2008). It is a perishable tropical fruit, susceptible to fungal infection, two of the most important are: *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*. Worldwide, high economical losses in fruit in vegetables production are reported mainly by infections of phytopathogenic fungi. Traditionally, synthetic chemical fungicides are used as the principal method for postharvest diseases control of fruit or vegetables. However, the increasing cost of fungicides, handling hazards, and the presence of chemical residues on food and environment, as well as health issues promote the research of other alternatives to fungicides application. (Adaskaveg et al., 2002). Chitosan (poly-β-(1,4)N-acetyl-d-glucosamine) posses interesting characteristics like antimicrobial activity, its natural origin, and can induce defense responses in plant tissue (Terry and Joyce, 2004). In a previous study, in fruit treated with chitosan the enzyme activities of POD, PPO and PA were reported as a defense responses. Jasmonic acid and methyl jasmonate (volatile methyl ester) are known as regulators (endogenous) which play an important role not only in the regulation in response to stress but also in the development and plant growth. Defense genes as well as the induction of resistance of host against phytopathogens can be achieved by the exogenous application of methyl jasmonate (Adaskaveg et al., 2002). Another important compound wich plays an important role in the resistance

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against pathogens is salicylic acid (phenolic compound) due to the production of pathogenicity-related proteins (PR proteins) (Adaskaveg et al., 2002). To our knowledge, there are a few reports about the efficacy of chitosan in combination with methyl jasmonate and salicylic acid in the control of decay produced by *C. gloeosporioides* and *R. stolonifer* in soursop. The goal of this project was to investigate the effect of chitosan´s application alone or in combinations with methyl jasmonate and salicylic acid on postharvest disease control and the preservation of fruit quality as well as the evaluation of the enzymes involved in host defense.

MATERIALS AND METHODS

Plant material and treatments application

The soursop fruit were harvested at physiological maturity from orchards in the municipality of Compostela, Nayarit, Mexico. The fruit were selected on the basis of uniformity in size, without defects and mechanical injuries. Fruit were washed with water, disinfected with a sodium hypochlorite (2%) solution for 1 min, then left to dry at room temperature in a biosafety hood. Nine treatments were prepared: 1) Sterile distilled water (Control); 2) 1.0% Chi; 3) 1.0% Chi – 0.01 mM MJ; 4) 1.0% Chi – 2 mM SA; 5) 1.0% Chi – 0.1 mM MJ – 6 mM SA; 6) 0.5% Chi; 7) 0.5% Chi - 0.1 mM MJ; 8) 0.5% Chi – 6 mM SA; 9) 0.5% Chi – 0.01 mM MJ – 2 mM SA. Individually, fruit were dipped by immersion during 60 s. Fruit were air dried for 4 h and stored at 25°C, with high relative humidity (90-95%) for 8 d. Measurements of respiration rate were made daily and the physicochemical evaluations were performed at 8 d. For the phytopathological evaluation test and enzymatic activity determination, only the best treatments were applied: 1) Sterile distilled water (Control); 2) Control – Pathogen; 3) 1.0% Chi – 0.1 mM MJ – 6 mM SA; 4) 1.0% Chi – 0.1 mM MJ – 6 mM SA - Pathogen; 5) 0.5% Chi - 0.1 mM MJ; 6) 0.5% Chi – 0.1 mM MJ - Pathogen. Artificial fruit inoculation was made as follows: 40 µL of the conidial suspension (10^6 spores mL^{-1}) of *C. gloeosporioides* and *R. stolonifer* were inoculated using a BD ultra fine syringe (Becton, Dickinson and Company, Franklin, Lakes, NJ USA) into the soursop fruit at a depth of 3 mm. Thereafter, fruit were maintained during 30 min at room temperature in a biosafety hood and finally treatments were applied by immersion.

Physicochemical evaluations

Firmness was performed using a TA.XT. Plus Texture Analyser (Stable Micro Systems, Hamilton, MA) with a 2 mm diameter punch, using the penetration test at three points along the fruit with the husk (ends and middle). The results were expressed in Newton (N).

The physicochemical parameters were performed by method AOAC, (2005). The total soluble solids (SST) were determined by a digital refractometer (Abbé HI 96801, Hanna instruments, Rhode Island, USA). pH was determined using a potentiometer Hach SensIONTM pH3 (Hanna Instruments, Maharashtra, India). The titratable acidity was determined by titration with a standard solution of sodium hydroxide (0.1 N). The calculations were reported as a percentage of malic acid.

Ethylene production (EP) and respiration rate (RR)

The RR was estimated daily using the method of Tovar et al. (2001) with some modifications. Five fruit from each treatment were used. Briefly, each fruit was placed inside of a 378 mL plastic bottle, hermetically closed with a septum and stored at room temperature (25ºC). After 1 h, 1 mL samples of air from the headspace were collected and injected into a gas chromatograph (HP 6890, Santa Clara, USA). After collecting the gas samples, the bottles were opened every day. The FID detector was used for ethylene and the TCD detector for CO_2. The injector port and the detectors were kept at 250°C; the oven temperature was kept at 50°C for 30 s and then heated at 30°C min^{-1} to 80°C. The air and H_2 flows were 400 mL min^{-1} and 30 mL min^{-1}, respectively. RR was expressed in $mLCO_2kg^{-1}h^{-1}$ and EP in $\mu Lethylenekg^{-1}h^{-1}$.

Phytopathological evaluation

Changes on weight loss, was performed as follows: the weight of each fruit was recorded initially and during storage using a digital balance (Torrey BL 3100, Torrey Solutions and quality©, México). Weight loss was expressed as percentage loss of the initial weight. Five fruit were used per treatment.

The disease development of anthracnose and soft rot on the fruit were evaluated at 8 d after inoculation. The severity of the infection and incidence rate were calculated using the equations proposed by Hernández-Lauzardo et al. (2007).

Enzyme activity of polyphenol oxidase (PPO), peroxidase (POD) and phenylalanine ammonium lyase (PAL)

Evaluations of these enzymes were performed in the pericarp of soursop fruits with and without inoculation of the pathogens, which were treated with 1.0 % Chi - 0.1 mM MJ - 6 mM SA for the pathogen of *C. gloeosporioides* and 0.5% Chi - 0.1 mM MJ for *R. stolonifer*. The control treatment was only immersed in sterile distilled water. The enzyme activities were performed at 0, 6, 12, 24, 48 and 72 h after each treatment.

The crude extract for PAL, POD and PPO was obtained by the method of Wang et al.(2011). POD, PPO and PAL activity were measured according to protocol of Tian et

al. (2006), with some modifications. The enzyme activity of POD was determined by the increase in absorbance at 470 nm each 10 s for 90 s, PPO activity was measured at 420 nm every 20 s for 3 min and PAL activity was determined by the absorbance change at 290 nm. The enzymes activity was analyzed in a spectrophotometer (JENWAY 6705, Beacon Road, UK). Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as standards. The activity was expressed in U mg protein^{-1}.

Statistical analysis

Phytopathological evaluation consisted of 25 fruit per treatment with four replicates. The physicochemical evaluations, measurements of respiration rate and enzyme activity were made on five fruit per treatment with four replicates. Each experiment was repeated at least twice, using a completely randomized block design. Data were subjected to analysis of variance (ANOVA) and a Tukey test ($p \leq 0.05$) was used for the comparison of means.

RESULTS AND DISCUSSION

Physicochemical evaluations

The firmness gradually decreased during the storage period. However, the fruit treated with 1.0% Chi - 0.1 mM MJ - 6 mM SA and 0.5% Chi - 0.1 mM MJ had significantly ($p \leq 0.05$) higher values (10.90-11.17 N) compared to control (Table 1, 2). The firmness decreases according to the maturation progress due to changes occurring at the cell wall, whit the hydrolysis of pectic compounds by enzymes like cellulase, β-galactosidase, pectilmethylesterase and polygalacturonase, which in turn degrade high molecular weight polymers such as cellulose and hemicellulose (Lima et al., 2006). Salvador et al. (2003) also reported that with the application of chitosan (1.25%), the firmness in mandarin fruit was acceptable up to 7 d of storage.

TSS, pH and titratable acidity in soursop fruit treated with chitosan alone or combination with methyl jasmonate and salicylic acid present a similar behaviour to the control without showing significant differences (Table 1, 2). A gradual increase in TSS and pH was observed, titratable acidity decreased as the storage progressed. These results are in agreement with the reported by Hernández-Ibáñez et al. (2013) obtained an increase in TSS in a range of 10 to 18 % and a pH of 4.46 to 5.12, while the total acidity decrease in ranging from 0.75 to 0.23 %, in chitosan-treated banana fruit as storage days progressed. The banana fruits do not modify the quality paraments by application of inducers. The behaviour of TSS is associated with fruit maturity, during this process occurs the synthesis of sugars, which is the hydrolysis of starch to simpler carbohydrates of the disaccharide and monosaccharide types (glucose, sucrose and fructose), mediated by the action of enzymes such as α and β amylases (Park et al., 2006).

Table 1: Evaluation of firmness, TSS, pH and titratable acidity during storage of soursop fruits treated with 1.0% chitosan alone and in combination with MJ and SA without inoculation of *C. gloeosporioides*

Analysis	Days	Control	1.0% Chi	1.0% Chi - 0.01 mM MJ	1.0% Chi – 2 mM SA	1.0% Chi - 0.1 mM MJ - 6 mM SA
Firmness (N)	0	24.12±0.93	23.81±0.50	25.64±0.81	23.07±0.60	24.67±0.63
	8	0.62±0.05^{d}	8.52±0.44^{b}	7.61±0.64^{bc}	6.96±0.56^{c}	10.90±0.23^{a}
TSS (%)	0	9.73±0.17	9.78±0.09	10.53±0.15	10.38±0.05	9.88±0.09
	8	18.98±0.02	18.90±0.16	18.90±0.03	19.10±0.16	19.00±0.14
pH	0	3.53±0.02	3.57±0.08	3.60±0.06	3.63±0.04	3.62±0.03
	8	4.05±0.00	4.06±0.01	4.06±0.02	4.05±0.03	4.02±0.02
Titratable acidity (%)	0	1.05±0.04	1.05±0.02	1.04±0.01	1.04±0.04	1.05±0.02
	8	0.29±0.03	0.31±0.03	0.31±0.02	0.28±0.02	0.30±0.03

Data are mean±standard deviation. The values with different superscripts in the same row are significantly different (Tukey's honestly significant difference; $p \leq 0.05$)

Table 2: Evaluation of firmness, TSS, pH and titratable acidity during storage of soursop fruits treated with 0.5% chitosan alone and in combination with MJ and SA without inoculation of *R. stolonifer*

Analysis	Days	Control	0.5% Chi	0.5% Chi - 0.1 mM MJ	0.5% Chi – 6 mM SA	0.5% Chi - 0.01 mM MJ - 2 mM SA
Firmness (N)	0	24.12±0.93	22.80±0.83	23.74±0.45	23.13±0.58	22.12±0.68
	8	0.62±0.05^{d}	7.38±0.49^{bc}	11.17±0.26^{a}	8.21±0.38^{b}	7.38±0.58^{bc}
TSS (%)	0	9.73±0.17	10.10±0.18	9.90±0.29	10.48±0.22	9.65±0.23
	8	18.98±0.02	19.10±0.31	19.00±0.24	18.90±0.20	18.80±0.29
pH	0	3.53±0.02	3.68±0.03	3.58±0.05	3.60±0.08	3.68±0.02
	8	4.15±0.00	4.11±0.03	4.15±0.03	4.14±0.03	4.10±0.01
Titratable acidity (%)	0	1.05±0.04	1.03±0.04	1.02±0.01	1.04±0.02	1.02±0.03
	8	0.28±0.03	0.29±0.01	0.28±0.02	0.30±0.01	0.28±0.03

Data are mean±standard deviation. The values with different superscripts in the same row are significantly different (Tukey's honestly significant difference; $p \leq 0.05$)

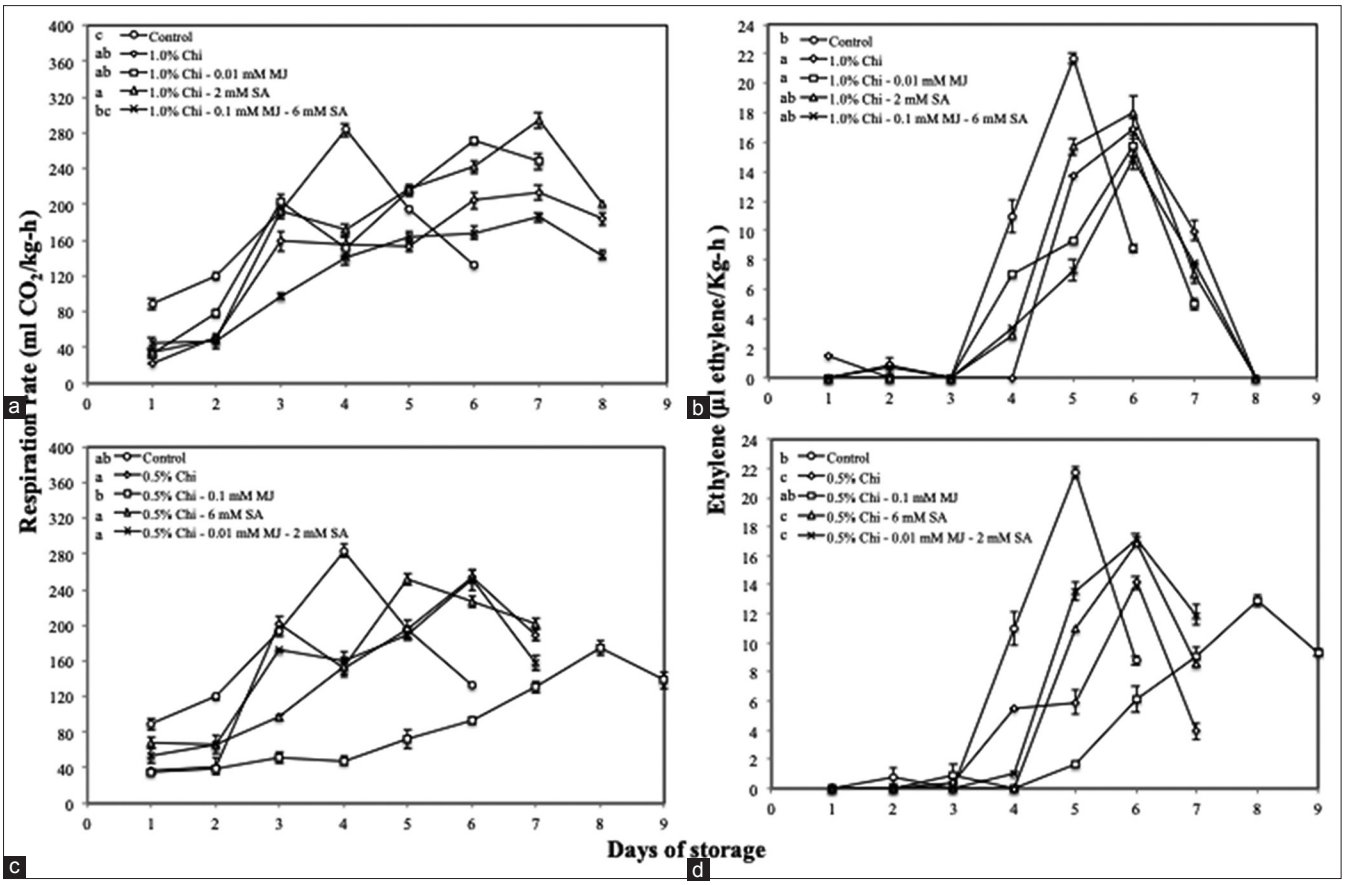

Fig 1. Effect of chitosan, salicylic acid and methyl jasmonate on soursop fruit without inoculation of pathogens, stored at 25ºC for 8 d. (a, b) Respiration rate of fruit treated with 1.0 and 0.5% Chi in combination with SA and MJ; (c, d) Ethylene production of fruit treated with 1.0 y 0.5% Chi in combination with SA and MJ. For each treatment, mean values not followed by the same lowercase letter are significantly different ($p \leq 0.05$). Vertical bars indicate the standard error of the means.

Ethylene production (EP) and respiration rate (RR)

In Fig. 1a, treatment with 1.0 % Chi - 0.1 mM MJ - 6 mM SA, presented a lower RR during storage, the maximum value was 185.29 mL CO_2 $kg^{-1}h^{-1}$ at 7th day. In Fig. 1b, the application of 0.5 % Chi - 0.1 mm MJ presented a lower RR with a climacteric peak of 174.37 mL CO_2 $kg^{-1}h^{-1}$ at 8th day. Control fruit presented a maximum value of 283.42 mL CO_2 $kg^{-1}h^{-1}$ at 4th day. In Fig. 1c observed that EP was lower with the application of Chi 1.0 % - 0.1 mM MJ - 6 mM SA at 6th day (14.86 µL ethylene $kg^{-1}h^{-1}$) with respect to other treatments. In Fig. 1d, the treatment with Chi 0.5 % - 0.1 mM MJ showed a maximum value at 8 d of storage (12.92 µL ethylene $kg^{-1}h^{-1}$), obtaining the lower EP compared to the other treatments. In control fruit, EP was higher during the storage days with a maximum value of 21.71 µL ethylene $kg^{-1}h^{-1}$at 5th day. A significant ($p \leq 0.05$) difference in RR and EP between treatments was observed.

These results are in agreement with Arce-Ortiz et al. (2016) observed that applying chitosan at 1.5% in combination with whey protein (3%) presented a lower respiration rate and climacteric peak appeared at 9th day in banana fruit. The respiration is a process in the degradation and synthesis of metabolites in the fruit. The inhibition of ethylene production in coated fruit may be due to the oxygen barrier exerted by edible films, which results in the reduction of the activity of the enzyme amino-cyclopropane carboxyl oxidase (ACC-oxidase), dependent on oxygen and precursor of ethylene synthesis (Márquez et al., 2009).

Phytopathological evaluation

The weight loss was observed in Fig. 2a. Fruit treated with 1.0 % Chi - 0.1 mM MJ - 6 mM SA with and without inoculation of *C. gloeosporioides* showed a lower weight loss (8.94 and 8.42%), control (with and without inoculation) obtained greater weight loss (17.34 and 20.04%). Fruit treated with 0.5% Chi - 0.1 mM MJ with and without inoculation of *R. stolonifer* presented a lower weight loss (8.20 and 8.06%). Control fruit showed a loss of weight of 21.31 and 18.34%, respectively (Fig. 2b). A significant ($p \leq 0.05$) difference on weight loss between treatments was observed.

The results showed a 0% disease incidence and severity of infection for *C. gloeosporioides* and *R.*

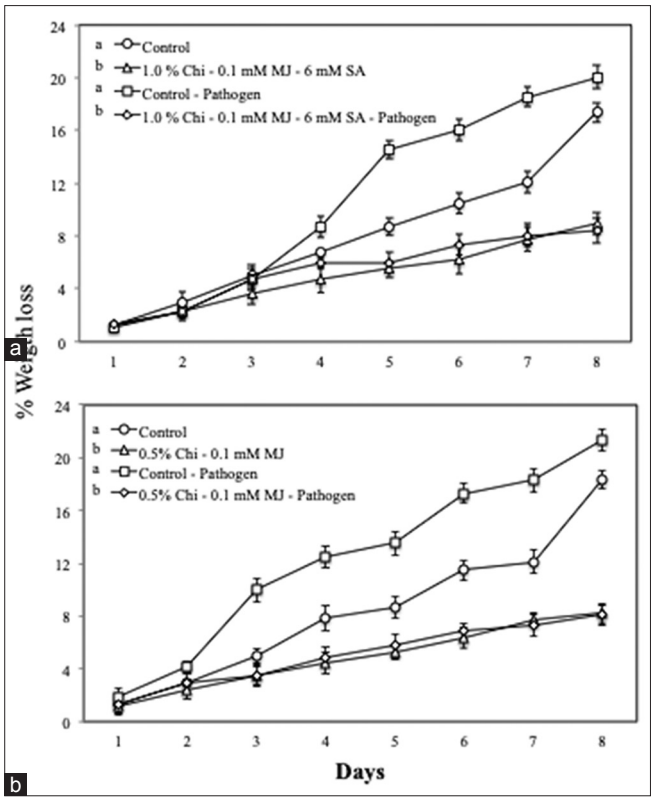

Fig 2. Weight loss of soursop fruit stored at 25°C for 8 d. (a) Fruit treated with 1.0% Chi – 0.1 mM MJ – 6 mM SA with and without inoculation of *C. gloeosporioides*; (b) Application treatment 0.5% Chi – 0.1 mM MJ with and without inoculation of *R. Stolonifer* in the fruit. For each treatment, mean values not followed by the same lowercase letter are significantly different ($p \leq 0.05$). Vertical bars indicate the standard error of the means.

stolonifer with and without inoculation artificial applying 1.0 % Chi - 0.1 mM MJ - 6 mM SA (Fig. 3a) and 0.5% Chi - 0.1 mM MJ (Fig. 3b), respectively. Control fruit presented a 100% of disease incidence and severity of infection at 8th day.These results are in agreement with the investigation by Berumen-Varela et al. (2015), where total postharvest protections against anthracnose disease and lower weight loss in mango fruit treated with chitosan (1.0 and 1.5%) were obtained. Chen et al. (2014) report that using chitosan (0.1%) and methyl jasmonate (500 mL L^{-1}) a 50% disease incidence was obtained. Romanazzi et al. (2016) reports that arrays of defense mechanisms are activated in the fruit when they are exposed to biotic or abiotic stress, including chitosan application. The decrease of weight loss in fruit is due to the fact that chitosan forms a coating on surface´s fruit, acting as a physical barrier, avoiding the moisture losses (Romanazzi et al., 2016).

Enzyme activity of PPO, POD and PAL

In Fig. 4a,c, treatment of 1.0% Chi - 0.1 mM MJ - 6 mM SA with and without inoculation of *C. gloeosporioides* showed higher activity of POD and PAL at 24 h after the inoculation. The best treatment for PPO activity was 1.0% Chi - 0.1 mM MJ - 6 mM SA inoculated with *C. gloeosporioides* observing an increase of the enzyme during storage (Fig.4b), with a higher enzymatic activity at 12 h after inoculation. In Fig 4d, the treatments that induced the highest enzymatic activity of POD during storage were 0.5 % Chi - 0.1 mM MJ with and without

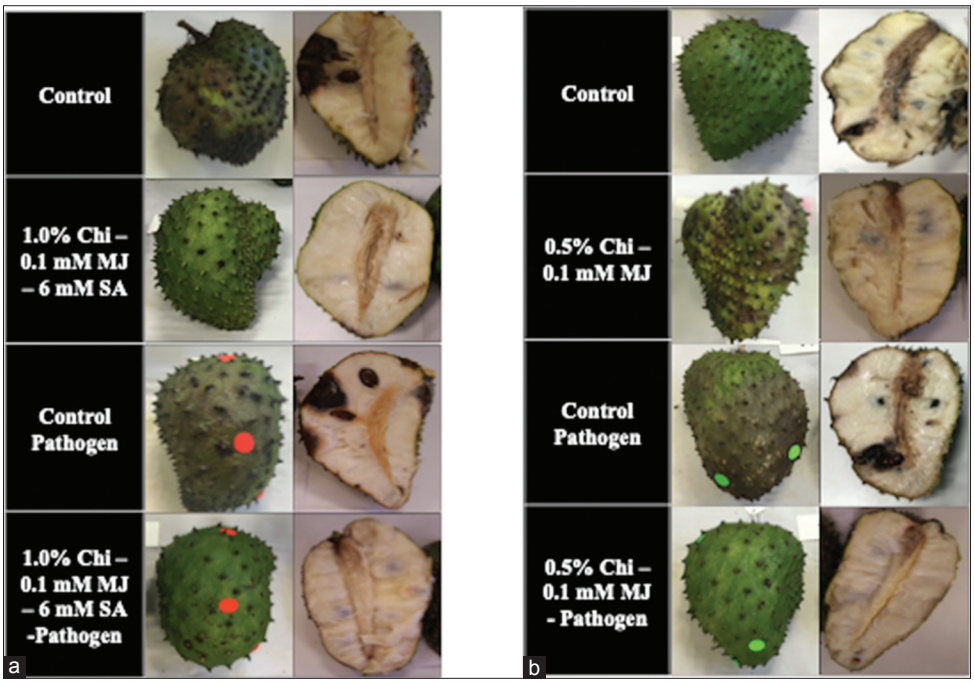

Fig 3. Disease incidence and severity of infection in soursop fruit treated with the combination of chitosan, methyl jasmonate and salicylic acid stored at 25 ºC for 8 d. (a) 1.0 % Chi - 0.1 mM MJ - 6 mM SA with and without inoculation of *C. gloeosporioides*; (b) 0.5 % Chi - 0.1 mM MJ with and without inoculation of *R. stolonifer.*

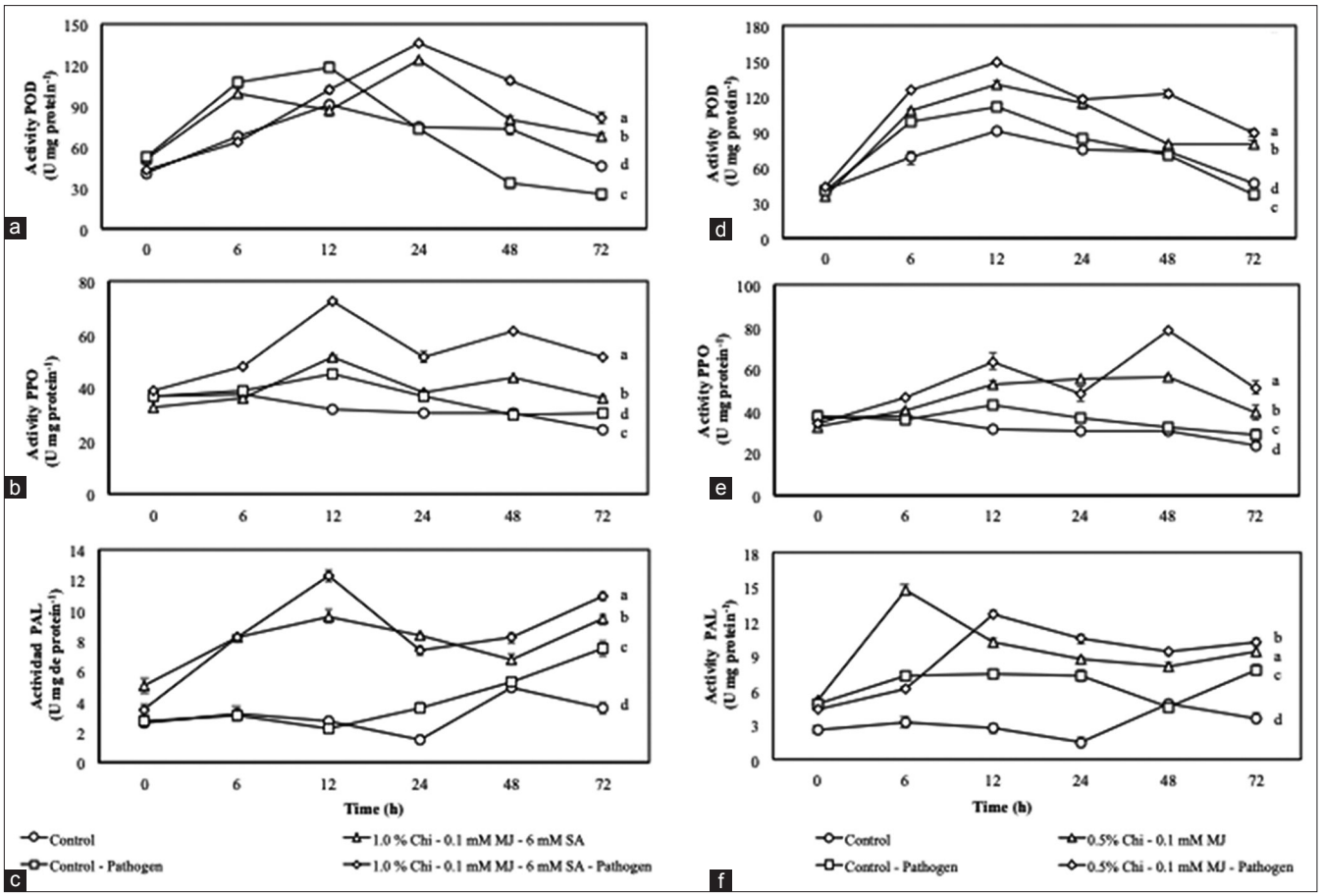

Fig 4. Enzymatic activity in soursop fruit treated with chitosan, salicylic acid and methyl jasmonate with and without inoculation of pathogens, stored at 25°C for 8 d. (a, b, c) Enzymatic activity of POD, PPO and PAL in fruit treated 1.0% Chi – 0.1 mM MJ – 6 mM SA with and without inoculation of *C. gloeosporioides*; (d, e, f) Enzymatic activity of POD, PPO and PAL in fruit treated 0.5% Chi – 0.1 mM MJ with and without inoculation of *R. stolonifer*. For each treatment, mean values not followed by the same lowercase letter are significantly different ($p \leq 0.05$). Vertical bars indicate the standard error of the means.

inoculation of *R. stolonifer*, showed maximum activity at 12 h. The treatment 0.5 % Chi - 0.1 mM MJ inoculated with *R. stolonifer* induced an increase in the PPO activity at 48 h after inoculation (Fig 4e). In Fig. 4f, the treatment 0.5 % Chi - 0.1 mM MJ with and without inoculation of *R. stolonifer* induced highest PAL activity at 6 and 12 h, respectively. There are statistically significant differences between treatments for each enzyme activity ($p \leq 0.05$).

Chen et al. (2014) showed that at 96 h after inoculation of *Alternaria alternata* in cherry tomato fruit, the activity of PPO treated with 0.1% chitosan and 500 mL L^{-1} of methyl jasmonate was higher in comparison with other treatments. Sun et al. (2013) applied methyl jasmonate showing an increase in PAL and POD activity in banana fruit at 60 h after *Fusarium oxysporum* inoculation. POD and PPO enzymes catalyse the formation of different phenolic antimicrobial substances such as quinones that are toxic to pathogens and participate in lignin biosynthesis (Asghari and Rashid, 2015). Phenolic metabolism is regulated by the action of PAL, enzyme that activate the phenylpropanoid pathway, that is directly involved in the synthesis of phenolic compounds with structural and defense functions, including lignins, phenolic acids, phytoalexins, flavonoids and salicylic acid (Chen et al., 2014).

CONCLUSION

The application of the combination of chitosan, methyl jasmonate and salicylic acid as coating can be a smart choice and a suitable alternative for postharvest control of *C. gloeosporioides* and *R. stolonifer* infections in soursop without affecting quality parameters as well as delaying the maturation process.

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Authors' contributions

ARG: Collection of experimental data and writing of manuscript. RRGE: review of the manuscript. EFM: respiration rate analysis. SPMC: statistical analysis. PGM: supervision of the study.

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