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RESEARCH ARTICLE

Bio-efficacy of a chitosan based elicitor on *Alternaria solani* and *Xanthomonas vesicatoria* infections in tomato under tropical conditions

A. Ramkissoon, J. Francis, V. Bowrin, R. Ramjegathesh, A. Ramsubhag & J. Jayaraman

Department of Life Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago

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Armour-Zen; chitosan; *PIN II*; plant defence; tomato foliar pathogens.

Correspondence
J. Jayaraman, Department of Life Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago.
Email: jaya1965@hotmail.com

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Abstract

With increasing problems of decreased efficacy, toxicity and environmental degradation due to the use of chemical fungicides, there is urgent need for developing alternative strategies for managing crop diseases. One approach currently being explored is the application of safe organic-based compounds to prime the defence systems of plants in order to prevent or limit infections by pathogens. Chitosan, a deacylated derivative of chitin, is one of the compounds known to promote plant defence and growth, but the mechanisms by which it is able to do so are still unclear. This study investigated the ability of a commercial chitosan extract (Armour-Zen®) to reduce disease incidence and induce the production of enzymes and expression of marker genes involved in plant defences for two important tomato pathogens, *Alternaria solani* and *Xanthomonas vesicatoria*. Foliar spray applications of the chitosan extract significantly reduced the incidences of both diseases in greenhouse and field grown tomato plants and also displayed a positive effect on growth and yields of tomato fruits. Chitosan-treated plants recorded elevated levels of defence enzymes and upregulation of the *PIN II* marker gene for defence signalling pathways when compared to the control. It was therefore proposed that chitosan would able to provide broad-range protection through induced systemic resistance mechanisms.

Introduction

Alternative crop protection strategies are being sought to not only prevent resistance development by pathogens, but also promote green farming. Studies have been performed using the natural defence mechanisms that plants use to fight off microbial invasions through induced responses involving signal molecules (Kunkel & Brooks, 2002; Grennan, 2006). These signal molecules, or elicitors, generally enhance non-specific plant resistance due to their effect of mimicking a pathogenic attack, thereby priming plant defences before infection (Dangl & Jones, 2001). A diverse range of elicitor molecules have been reported including oligosaccharides, polysaccharides, lipids, glycoproteins, peptides and proteins (Benhamou, 1996; Shibuya & Minami, 2001). The effects of elicitors are mediated by signalling pathways which include the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways. These play major roles, either alone or in combination, in local and systemic induction of defence responses. Chitosan is derived from deacetylation of chitin, a naturally occurring linear polysaccharide which comprises part of the carbohydrate skeleton of fungal cell walls (Kumar, 2000). It acts similarly to a general elicitor by inducing non-host resistance and priming systemic acquired immunity (Badawy & Rabea, 2011). Several studies have demonstrated the plant protection effects of chitosan on various crops. Jayaraj *et al.* (2009) demonstrated decreased incidence of necrotic fungal pathogens in carrots when sprayed with chitosan. Similarly, El-Tantawy (2009) reported decreased disease incidence along with growth promotion and increased yield in tomato.

Tomato is an important food rich in vitamins A and C as well as the antioxidant lycopene but the crops are afflicted by a plethora of diseases, which severely

reduce fruit yield and results in high financial losses to farmers. Early blight caused by the fungal pathogen *Alternaria solani* and bacterial spot/blight caused by *Xanthomonas campestris* pv. *vesicatoria* are the two most economically important tomato diseases worldwide including the Caribbean (Ali *et al.*, 2016). Both agents are responsible for major crop damages each year and account for yield losses of 35–78% per year (Chaerani & Voorrips, 2006). Management of these diseases has become increasingly challenging due to the indiscriminate use of fungicides/bactericides in tomato crop production, which has resulted in the evolution of resistant strains of the pathogens (El-Hendawy *et al.*, 2005). In an effort to control diseases, farmers commonly practice frequent application of chemical fungicides, causing multiple adverse effects to the environment and human health. Tomato is one of the vegetable crops which registers the highest pesticide usage (Jones, 1984) in the Caribbean and other developing countries. In light of these constraints, there is a necessity to identify alternative products for disease management and in that perspective use of organic elicitors is being investigated as a promising approach.

In this study, a commercially available chitosan extract, Armour-Zen® (Botry-Zen Ltd, Dunedin, New Zealand), was used in greenhouse and field trials of tomato to assess its effect on disease incidence and yield. Furthermore, we studied the mechanisms of action by assessing the induction of host-defence systems and activation of defence pathways.

Materials and methods

Plant material, greenhouse and field disease studies

Healthy 2-week-old tomato seedlings (Hybrid 61 variety) were transplanted into pots (20 cm diameter) containing peat moss and garden soil in a ratio of 1:1. The plants were grown in a greenhouse at 25–32°C, 70–85% relative humidity and 600–1000 µmol photons m^{-2} s^{-1} light intensity at a 12 h photoperiod. Plants were fertilized with a 20:20:20 N: P: K water soluble fertilizer. After 25 days of growing in the greenhouse, the plants were sprayed with a commercial formulation of chitosan, Armour-Zen® (Botry-Zen Ltd) at 0.05% v/v concentration at 20 mL per plant. Treatments were repeated at 15 day intervals. A copper based fungicide/bactericide (Kocide, DuPont Crop Protection) (0.2% w/v), salicylic acid (0.01% w/v) and water acted as controls for the experiment.

Six hours after the treatment, the plants were inoculated with conidial suspensions of *Alternaria solani* (1×10^6 spores mL^{-1}) or cell suspensions of *Xanthomonas campestris* pv. *vesicatoria* (1.5×10^8 CFU mL^{-1}). Briefly, the leaves were pre-wounded by slight abrasion using a fine sand paper and the pathogen suspension was sprayed onto the plant until run off. The plants were then incubated in a humid chamber for 48 h to allow infection set. They were then returned to the greenhouse and grown under the conditions described earlier.

The plants were scored for disease severity 40 days after inoculation employing a six point disease rating scale described by Gondal *et al.* (2012). The scale was based on the percentage of leaf area infected (1 = 0%, 2 = 1–10%, 3 = 11–25%, 4 = 26–40%, 5 = 41–55% and 6 = >56%). Percent disease index was calculated as follows:

$$\text{PDI} = \frac{\text{Sum of disease rating of individual leaves}}{\text{Total no. of leaves}} \times \frac{100}{\text{Maximum rating}}$$

Data was represented as percent reduction in disease incidence compared to the untreated control and was calculated using the formula:

$$\%\ \text{Reduction} = \frac{\text{PDI of control} - \text{PDI treatment}}{\text{PDI of control}} \times 100$$

Thirty replicate plants were maintained per treatment and two independent trials were conducted using a completely randomized design.

Four field trials were conducted during 2013 and 2014 in various farmers' fields across Trinidad as two replicated trials in each of the wet and dry seasons. Experimental details of trials are presented in Table 1. Six week old tomato (Hybrid 61) tomato seedlings were transplanted on ridges (1.2 m × 51 cm). Plants were grown under staking system. Standard cultural practices as recommended by the Ministry of Agriculture were followed for land preparation, fertilization, weed management and insect control in each trial. Trial plots were arranged in a completely randomized block design and four treatments namely, Chitosan (Armour-Zen®) treatment (0.5%), SA (0.01%), fungicide (Kocide 0.2%) and water control were applied. Applications of each treatment commenced 10 days after planting and continued every 15 days subsequently. Treatments were replicated thrice and each treatment consisted of 300 plants. Plant height and leaf number were measured at 40 days after planting and the total yields (kg per 100 plants) were calculated at the end of the crop (90 days after transplanting).

Assessment of activity of defence enzymes and quantification of total phenols

To examine the effect of the chitosan extract (Armour-Zen®) on the activity of defence enzymes and total phenolic content, healthy 2-week-old tomato seedlings were grown in the greenhouse under previously described conditions. The plants were arranged in

Table 1 Field trial details

Trial and Location[a]	Season	Treatments
(1) Maloney	Dry (January to April, 2014)	Chitosan (T1), Kocide (T2), Salicylic acid (T3), Water control (T4)
(2) Orange Grove	Dry (February to May, 2014)	Chitosan (T1), Kocide (T2), Salicylic acid (T3), Water control (T4)
(3) Las Lomas	Wet (September to December, 2013)	Chitosan (T1), Kocide (T2), Salicylic acid (T3), Water control (T4)
(4) Orange Grove	Wet (September to December, 2013)	Chitosan (T1), Kocide (T2), Salicylic acid (T3), Water control (T4)

[a]All field locations were in Trinidad

completely randomized design with three replications. Two independent trials were conducted.

Plants were sprayed with a 0.5% solution of Armour-Zen® and leaf samples collected in triplicate and pooled at 0, 6, 12, 24, 48, 72 and 96 h after treatment before storing −80°C. The defence enzymes assayed included phenylalanine ammonia-lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), chitinase (Chi) and β-1,3 glucanase (Glu).

Phenylalanine ammonia-lyase activity was determined following the methodology described by Dickerson *et al.* (1984). Activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1}. Enzyme activity was expressed in fresh weight basis as nmol trans-cinnamic acid min^{-1} mg^{-1} of sample.

Peroxidase activity was carried out as per the procedure described by Hammerschmidt *et al.* (1982). Activity was expressed as the increase in absorbance at 470 nm min^{-1} mg^{-1} of protein. Enzyme extract (0.1 mL) was added to initiate the reaction, which was followed by absorbance measurement at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units min^{-1}. The boiled enzyme was used as a blank.

Polyphenol oxidase activity was determined as per the procedure described by Mayer & Harel (1979). To start the reaction, 200 µL of 0.01 M catechol was added and the activity was expressed as change in absorbance $min^{-1}mg^{-1}$ of protein.

A modified version of the method of Tonon *et al.* (1998) was used to evaluate Chi activity using chitin (Sigma Aldrich, St. Louis, MO, USA) as the substrate. The Chi activity was determined using a spectrophotometer at 585 nm wavelength. N-acetyl glucosamine (GlcNAc) was used as a standard and the enzyme activity was expressed as nmoles GlcNAc equivalents min^{-1} g^{-1} fresh weight.

β-1,3 glucanase activity was assayed using the laminarin-dinitrosalicylic acid method (Pan *et al.*, 1991). The enzyme activity was expressed as µmols glucose released min^{-1} mg^{-1} of sample.

Total phenolic content was estimated using the procedure of Zieslin & Ben-Zaken (1993) using Folin-Ciocalteu reagent with a phenol solution (C_6H_6O) as standard and expressed as µg catechol equivalents g^{-1} tissue.

Defence gene expression analyses

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was used to test the expression of defence pathway marker genes (*PR-1a*, *PIN II* and *ETR-1*) involved in the SA, JA and ET mediated defence pathways, respectively. Healthy 2-week-old tomato seedlings were grown for 5 days in the greenhouse as described before.

The plants were sprayed with a 0.5% solution of chitosan extract. Controls included SA (0.01%), MeJA (0.05%) and water treated plants. Leaf samples were collected in triplicate and pooled 0, 6, 12, 24, 48, 72 and 96 h after treatment. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol and stored at −80°C until needed. Reverse transcription was done using the Multiscribe Reverse Transcriptase Kit (Invitrogen) employing oligo dT primers with 1 µg RNA as starting material in a 20 µL reaction. Complementary-DNA samples were then quantified by real-time PCR using specific primers in an Applied Biosystems 7500 Fast Real Time PCR system (Life Technologies Corp., Carlsbad, CA, USA) and the data analysed using the $2^{-\Delta\Delta C_t}$ method (Schmittgen & Livak, 2008). The details of gene primers are listed in Table 2.

Primers were generated using the Primerdesign tool (NCBI.nlm.nih.gov) and synthesized at Integrated DNA Technologies Inc. (Coralville, IA, USA). The expression levels of all the genes were normalized using the β-actin gene as an internal standard. Primer specificity was confirmed by melting curve analysis and the observation of a single PCR product on 1% agarose gel.

Data analysis

The data were analysed using analysis of variance (ANOVA) using Genstat Discovery Edition 4 (VSN International Ltd. 2013). Significant differences at 5% confidence among means were determined using Fisher's protected LSD.

Table 2 Primers used in the study

Gene	Function	Sequence
***β*- actin**	Used as the normalization gene in this study	**F 5′**-CTCGAGCAGTGTTTCCCAGT- **3′** **R 5′**-GGTGCCTCAGTCAGGAGAAC-**3′**
PR-1a	Acidic *pathogenesis related protein* gene. Salicylic acid-inducible defence gene	**F 5′**- TCCTCCATTTTCGTTGCTTGT-**3′** **R 5′**- TCGTCCCACATCTTCACAGC-**3′**
PIN II	*Proteinase inhibitor II* gene. Jasmonic acid- inducible defence gene	**F 5′**-ACGACGTGTTGCACTGGTTA- **3′** **R 5′**-GCAACCCTCTCCTGCACTAC-**3′**
ETR-cat	*Ethylene response* gene. Essential in the ethylene signal transduction pathways	**F 5′**-TGGTGTCATGTCCTTGCTGG- **3′** **R 5′**-CTGAGTAGCGTGGCTGTGAT-**3′**

Results

Effect on disease incidence, plant growth and yield

Fig. 1 (A, B and C) shows the percent reduction compared to untreated plants for disease incidence of *Alternaria solani* and *Xanthomonas vesicatoria*. In both the greenhouse (1A) and field (1B and 1C) trials, treatments significantly affected the mean disease incidence compared to the control ($P < 0.001$).

For the greenhouse trials there was no significant difference ($P = 0.105$) observed in controlling *A. solani* infections between plants treated with SA (58.73% reduction) and the chitosan extract (64.19% reduction), though both were considerably better in controlling infection when compared to the fungicide (37.22% reduction) as shown in Fig. 1A. Similarly, *X. vesicatoria* infections were greatly reduced in plants treated with chitosan (62.59%), SA (49.64%) and fungicide (29.35%) with each treatment being significantly different.

In both wet and dry seasons, under field conditions the chitosan extract maintained good control of both diseases. Chitosan-treated plants displayed significantly lowered disease incidence of *A. solani* during the dry season, compared to the SA and fungicide treated plants. This effect was not as pronounced in the rainy season trials, as disease control was on average lower than the SA and fungicide controls. Similarly, in both seasons, the chitosan extract displayed the best average reduction of *X. vesicatoria* disease incidence amongst the various treatments. Plants sprayed with chitosan resulted in 45.54% decrease in incidence during the dry season compared to untreated plants and no significant difference was observed between chitosan and the SA treatments. A substantial decrease was detected in the wet season however, as chitosan-treated plants had an average of 66.79% decrease in disease incidence compared to untreated plants.

Plants treated with chitosan extract and SA displayed delayed symptom development and the lesion size was also smaller when compared to water and fungicide controls (data not shown). The chitosan formulation at the concentrations used did not display any phytotoxicity in tomato plants.

Tables 3 and 4 compare the effects of the chitosan extract, SA, fungicide and control on tomato plant growth and yield for both wet and dry season trials. Plants sprayed with SA recorded the highest average plant lengths and leaf numbers in both dry and wet seasons, though it was not significantly higher than chitosan-treated plants. Tomato yields increased significantly in plants treated with chitosan compared to the other treatments. Dry season yields increased approximately 26% and wet season yields by 23% compared to untreated plants. The SA and fungicide did not significantly increase yields compared to untreated plants.

Activity of defence enzymes and total phenolic levels

Tomato plants treated with the chitosan extract, SA and water were assayed for defence enzyme activities (Fig. 2) (Chi (2A), Glu (2B), PO (2C), PAL (2D) and PPO (2E) at 0, 6, 12, 24, 48, 72 and 96 h after treatment) and for total phenolic levels (2F). Comparison of the mean enzyme activity by repeated measures ANOVA showed there were significant differences ($P < 0.001$) between the chitosan and SA treatments and untreated controls. Time × treatment interactions were also significant for enzyme activity ($P < 0.001$).

All enzymes examined displayed a strong induction of activity from as early as 6–12 h after treatment with chitosan formulation. Enzyme activity continued to increase up to 96 h for all the enzymes tested. The levels of enzyme activity were higher in plants treated with chitosan compared to SA, though significant differences were mainly observed after 48 h. Total phenolic content was also significantly higher in chitosan-treated plants from 24 h onwards compared to SA treatments.

Real time gene expression of defence pathway marker genes

To understand the effect of the chitosan extract on the defence pathways involved in induced resistance in

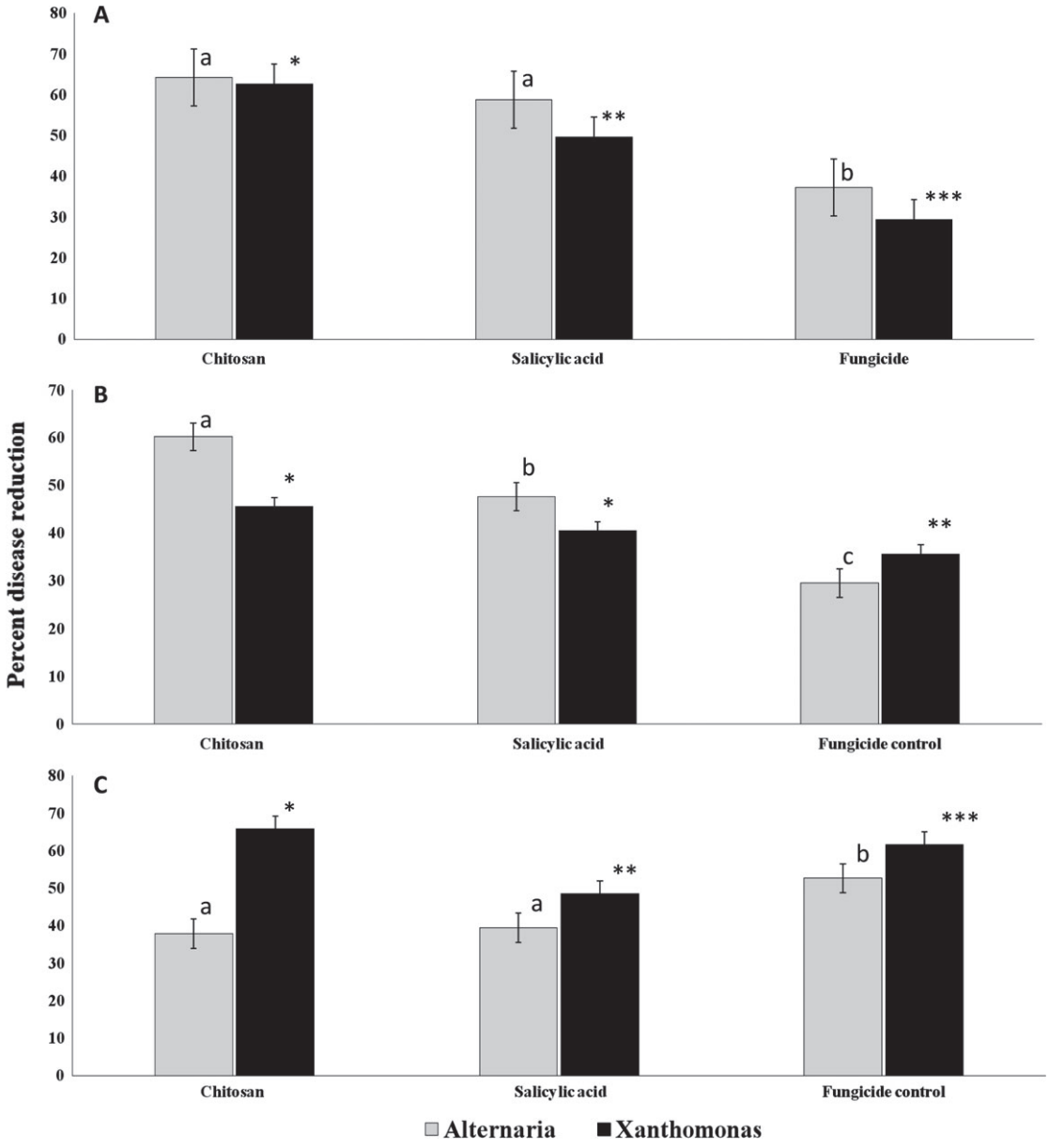

Figure 1 Effect of the chitosan extract, Salicylic acid and fungicide on disease incidence 40 days after infection with *A. solani* and *X. vesicatoria* for (A) greenhouse and (B and C) field trials. Two independent greenhouse trials and four field trials (2 wet and 2 dry season) were conducted. Means ($n = 60$) are represented as the percent reduction of diseases compared to untreated control plants (two experimental trials). Significant differences were observed between treatments with different alphabets (infections with *A. solani*) and asterisks (infection with *X. vesicatoria*) at 5% confidence levels using Fisher's protected LSD.

tomato, real time PCR of defence pathway marker genes (*PR-1a*, *PIN II* and *ETR-1*) was performed. Comparison by repeated measures ANOVA, showed all the genes tested with the chitosan extract, SA control and MeJA control, displayed statistically significant responses to gene expression for treatments ($P < 0.001$), time ($P < 0.001$) and treatment × time ($P < 0.001$) interactions (Fig. 3).

Only plants treated with SA displayed any significant increases in transcript levels of the *PR-1a* gene (marker gene for the SA mediated defence pathway), compared to the other treatments (3A). *PR-1a* gene expression rose exponentially between 0 h and 24 h but then dropped steadily. None of the other treatments caused any significant increase in *PR-1a* gene expression compared to levels of the untreated controls. Both the chitosan extract and MeJA treated plants were observed to have significantly increased transcription of the *PIN II* gene (marker gene of the JA mediated defence pathway) above the

Table 3 Effect of chitosan, salicylic acid, fungicide on plant growth and yield during the dry season in tomato

Treatments	Plant Height (cm)	Leaf Number	Yield (kg 100 plants^{-1})
Chitosan	74.43 ± 2.47 **(a)**	31.83 ± 1.74 **(a)**	571.50 ± 21.95 **(a)**
Salicylic acid	75.78 ± 3.62 **(a)**	32.30 ± 2.03 **(a)**	501.00 ± 15.81 **(b)**
Fungicide control	62.65 ± 6.62 **(b)**	23.37 ± 1.95 **(b)**	457.00 ± 29.63 **(c)**
Water control	58.06 ± 4.20 **(c)**	19.67 ± 3.34 **(c)**	452.00 ± 33.78 **(c)**
P-value	<0.001	<0.001	<0.001
LSD ($P = 0.05$)	3.79	1.83	27.51

Height and leaf number were recorded 40 days after treatments and yield was accumulated throughout the cropping cycle (90 days). Statistical significance was observed between treatments with different alphabets. Data is represented as the mean ($n = 60$) ± SE.

Table 4 Effect of chitosan, salicylic acid, fungicide on plant growth and yield during the wet season in tomato

Treatments	Plant Height (cm)	Leaf number	Yield (kg 100 plants^{-1})
Chitosan	91.54 ± 5.79 **(a)**	35.01 ± 1.55 **(a)**	645.50 ± 19.30 **(a)**
Salicylic acid	98.48 ± 8.01 **(b)**	35.53 ± 2.07 **(a)**	558.00 ± 30.33 **(b)**
Fungicide control	70.69 ± 6.74 **(c)**	25.71 ± 2.74 **(b)**	557.50 ± 53.05 **(b)**
Water control	63.34 ± 5.87 **(d)**	21.63 ± 4.63 **(b)**	524.00 ± 32.27 **(b)**
P-value	<0.001	<0.001	0.029
LSD ($P = 0.05$)	6.95	6.33	42.70

Height and leaf number were recorded 40 days after treatments and yield was accumulated throughout the cropping cycle (90 days). Statistical significance was observed between treatments with different alphabets. Data is represented as the mean ($n = 60$) ± SE.

water control plants (3B). The chitosan extract however maintained a significantly higher expression of this gene above the MeJA control plants after 24 h. The *ETR-1* gene (marker gene for ET mediated defence) was only upregulated significantly after 24 h by the chitosan extract (3C) with a peak at 72 h. No significant change in the expression of this gene was observed in control plants over time.

Discussion

A primary feature of induced resistance to disease is the priming of plant tissues by elicitors that allows rapid deployment of active defence mechanisms against invading pathogens. Elicitors are environmentally friendly chemicals that can induce effective immune responses in plants (Ramirez-Estrada *et al.*, 2016). Chitosan has been shown to be an effective protective agent in agriculture because of its antifungal and elicitor activities (Ramjegathesh & Jayaraman, 2015).

This article demonstrated the ability of a chitosan formulation (Armour-Zen®) to protect tomato plants against *Alternaria solani*-early leaf blight and *Xanthomonas vesicatoria*-leaf spot. There were lower disease severities recorded for both pathogens in the plants treated with the chitosan extract when compared to SA and fungicide and water controls. This assessment was supported via field trials in both the dry and wet seasons conducted in Trinidad. Chitosan also induced activities of several defence enzymes in tomato plants and elicited expression of genes (*PIN II* and *ETR-1*) from several molecular pathways involved in pathogen defence. The combination of these two responses would have made the plants to be more resistant to the pathogens.

We propose that the reduction in disease prevalence was due to the chitosan extract's unique elicitation of physiological and biochemical changes in the plant leading to induced resistance. This effect was proved through the enhanced activities of various enzymes linked to plant defence in response to treatment with the extract and induction of defence pathways as evidenced through upregulation of corresponding marker genes of pathways. Evidence to this claim has been also been put forth by Chen *et al.* (2016), who observed that increased defence enzyme activity was responsible for the *Begonia hemalis* resistance to *Botrytis cinerea* after treatments with chitosan.

A key component of plant defence systems is the activation of various defence enzymes (Chi, Glu, PO, PAL and PPO) that function to limit or eliminate an infectious agent. β-1,3-glucan and chitin, both polymers of N-acetyl glucosamine (GlcNAc), are major components of the carbohydrate skeleton of bacterial and fungal cell walls. These two molecules are natural substrates for the two plant hydrolases – Glu and Chi. These two hydrolytic enzymes are capable of attacking bacterial and fungal pathogens via their cell wall components and as such are proposed to contribute to the antifungal defences of many plant species (Xu *et al.*, 2016). It was observed in this experiment that the activity of both enzymes increased significantly in the chitosan as well as the SA treated plants when compared to the control plants. Peroxidases are key enzymes that hamper pathogen ingress and spread in plants through aiding construction of structural barriers and generation of reactive oxygen species (ROS) and phenols (Taheri & Tarighi, 2012). As PO activity was seen to significantly increase with both elicitors it may indicate that chitosan may increase the level of lignin formation, suberization and the hypersensitive response which involves the oxidative burst and generation of ROS – an early response to pathogen attack or elicitor treatment.

Increase in PAL activity is an important response to pathogenic infection in many species of plants and tends to be closely linked with resistance (Mandal *et al.*, 2013). Phenylalanine ammonia-lyase regulates secondary metabolism in plants and is the first enzyme in the polypropanoid metabolism pathway and results

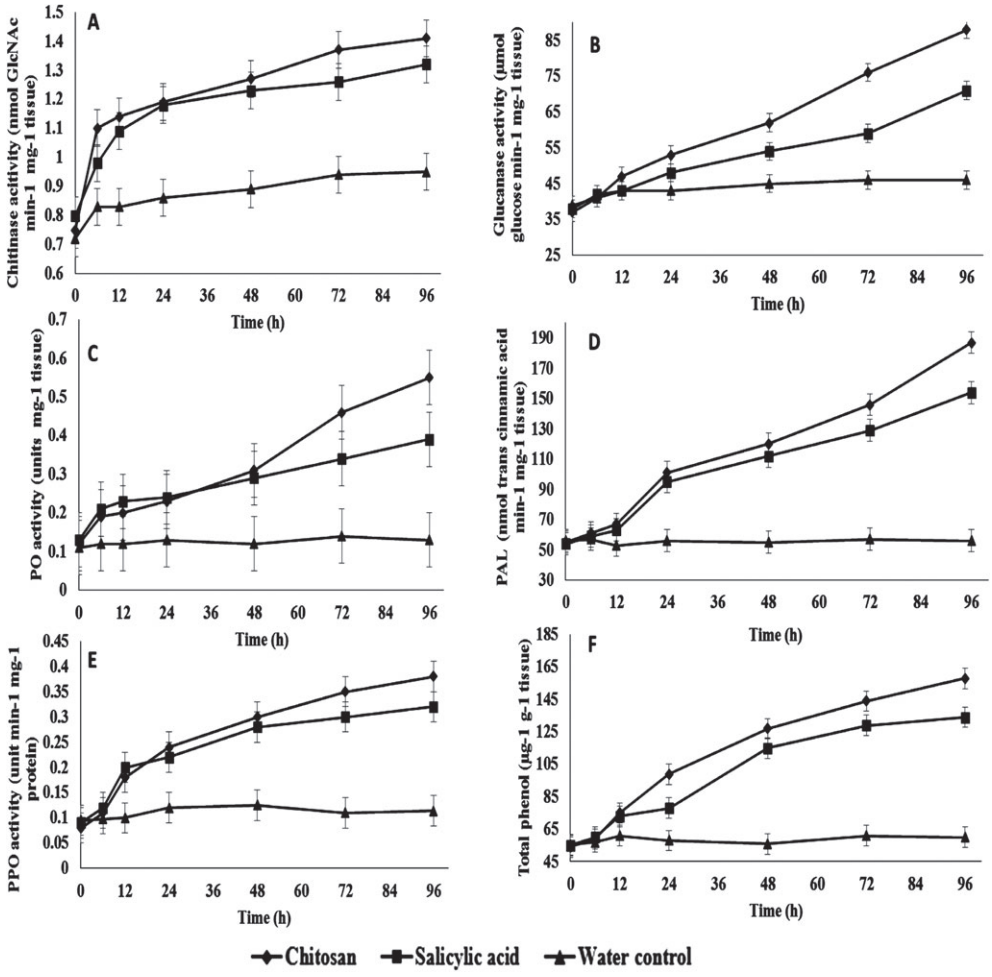

Figure 2 Defence enzyme activities and total phenol levels in tomato plants treated with the chitosan extract, salicylic acid and water. (A) Chitinase activity; (B) Glucanase activity; (C) Phenylalanine ammonia-lyase (PAL) activity; (D) Peroxidase activity; (E) Polyphenol oxidase (PPO); (F) Total phenol content. Tissue samples were taken at 0, 6, 12, 24, 48, 72 and 96 h after treatment. Data points represent mean ± LSD ($n = 6$) (two independent trials). Repeated measures ANOVA was used to test the significance of the means at 5% confidence taking into account both treatment and time effects.

in the production of trans-cinnamic acid and phytoalexins, which in turn results in both structural and enzymatic control of fungal pathogens (Chandra *et al.*, 2007). Enhancement of PAL activities was reported in response to *Ralstonia solanacearum* inoculation in tomato pre-treated with chitosan and SA (Mandal *et al.*, 2013). Increased PAL activity in mandarin fruits was attributed to reduced postharvest infection by *Penicilium digitatum* (Waewthongrak *et al.*, 2015). As evident in the current study, chitosan elicited an increased activity of PAL in tomato leaves.

PPO are copper containing oxidase enzymes that catalyze the oxidation of hydroxyphenols into their antimicrobial quinone derivatives. In tomato, it was found that the wound inducible expression of PPO was regulated by the octadecanoid pathway which responds to various signalling molecules such as SA, JA and ET (Li & Steffens, 2002). This means that both SAR and ISR can trigger increased PPO activity, and the same has been observed in this study as chitosan and SA treated plant leaves had similar PPO activities.

Plant phenolics are the most abundant secondary metabolites of plants and are needed by plants for pigmentation, growth, reproduction and as a set of defensive compounds which offer resistance to pathogens as well as a host of other functions (Dai & Mumper, 2010). As part of the defence mechanisms in plants, phenolics are involved in lignin biosynthesis and accumulation of phytoalexins. Mandal *et al.* (2013) reported that a high quantity of antimicrobial phenolic acids were found in tomato roots and Saavedra *et al.* (2016) reported increased levels of phenol in *Fragaria chiloensis* fruit that

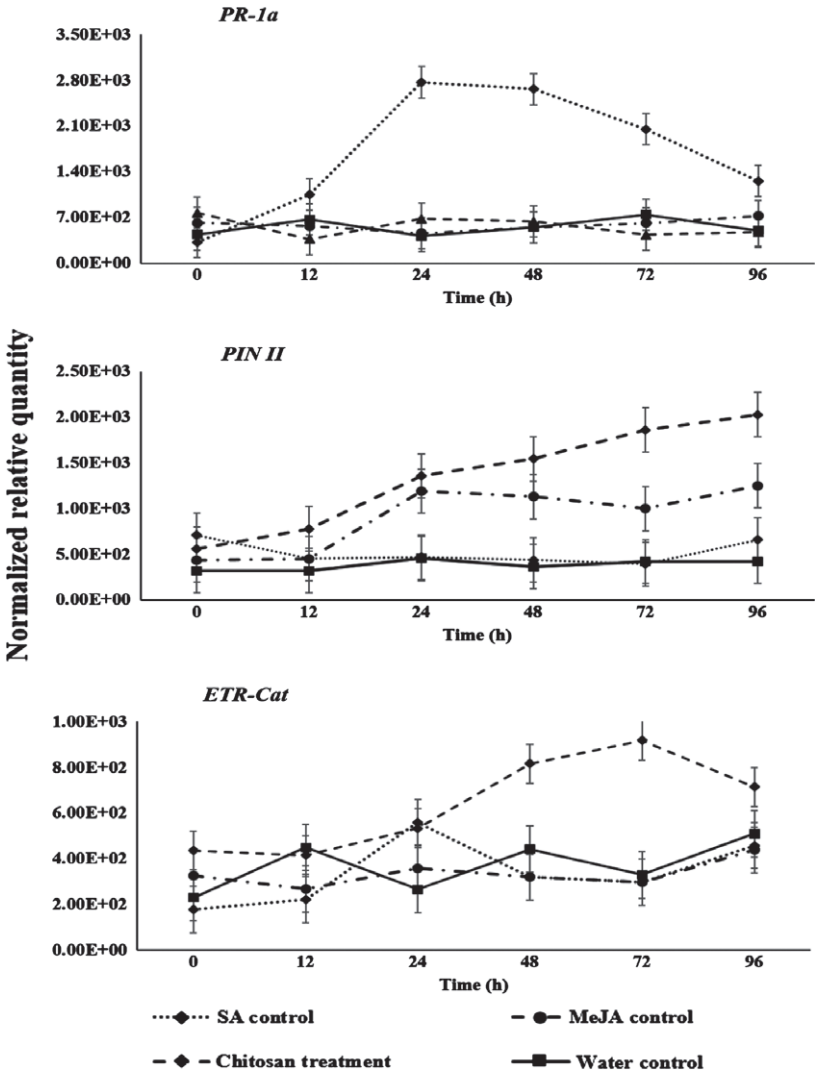

Figure 3 Real time transcription of defence signaling pathway marker genes in tomato plants treated with chitosan, salicylic acid (SA), methyl jasmonate (MeJA) and water. Data points represent the mean ± LSD ($n = 6$). Pooled leaf samples were collected at various time points after treatments and analyzed for gene expression of the (A) *PR-1a*, (B) *PIN II* and (C) *ETR-Cat* genes. Data was normalized using the β-actin gene. Data was analyzed using repeated measures ANOVA to test the significance of the means at 5% confidence taking into account treatment and time.

were treated with chitosan. In this study, chitosan was able to positively influence phenolic levels more than SA and water control.

Chitosan is able to induce plant resistance due to its structural similarity to microbial associated molecular patterns and therefore prime as a nonspecific, long-lasting immunity response possibly by binding to a receptor on the cell membranes of plants (Iriti & Varoni, 2015). An increase of *in-planta* SA level is closely linked to systemic acquired resistance (SAR) and it has been demonstrated that the expression of the *PR-1a* gene also correlates to increased resistance to a broad range of pathogens (Vlot *et al.*, 2009), as observed in certain instances of the current study. The *PIN II* (proteinase inhibitor II) and *ETR-1* (ethylene receptor 1) were used in this study as marker genes for induced systemic resistance (ISR), as it has been reported by numerous studies that both JAs and ET can trigger this type of induced resistance (Niki *et al.*, 1998). Plants treated with chitosan extract in this study, did not induce the expression of the *PR-1* gene, but had a strong inducible effect on the *PIN II* and *ETR-1* genes. This suggests that their effect on tomato plants to the pathogens tested did not involve SA dependent pathways but relied on signaling of either JA or ET or both. Both the SA mediated and JA mediated pathways have been demonstrated to antagonise each other (Kunkel & Brooks, 2002). This

was apparent as plants treated with SA only expressed the *PR-1a* gene but had no effect on the *PIN II* or *ETR-1* genes. The opposite was observed for plants treated with MeJA which strongly induced *PIN II*/*ETR-1* and had no effect on the *PR-1a*.

Apart from its plant protective ability, the chitosan extract was shown to also enhance plant growth and significantly improve yield. The plant growth promoting ability of chitosan has been demonstrated by previous studies (Algam *et al.*, 2010), and has been attributed to increased nutrient uptake, cell division, cell elongation and increased protein biosynthesis (Amin *et al.*, 2007). These factors may explain the significantly greater yield in chitosan-treated plants. There is a direct correlation to plant health and improved yields in crops. Farouk & Ramadan (2012), suggest that the increased photosynthesis and vegetative growth, coupled with the increased translocation of carbon assimilated from source to sink are the main reasons why plants treated with chitosan display improved yields. Our results, indicate that a similar phenomena might be taking place in tomato crops treated with the chitosan extract resulting in a significant improvement in yield.

This study demonstrates the ability of chitosan to be used as an alternative plant protection strategy in cropping systems. A significant reduction in the two most severe foliar diseases of tomato was observed as well as improvements in plant growth and yield. The ability of this extract to reduce disease was attributed to the elicitation of JA/ET mediated ISR which in turn increased the activity of a broad range of defence enzymes and plant phenolics. This research provides good support for the integration of chitosan extracts for sustainable disease management and enhancing yield in tomato. This is especially relevant in the tropics where there is a high use of pesticides and inorganic fertilizers. Further investigations are needed to assess the potential benefits of this extract in other crops grown under tropical conditions.

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References

Algam S.A.E., Xie G., Li B., Yu S., Su T., Larsen J. (2010) Effects of *Paenibacillus* strains and chitosan on plant growth promotion and control of Ralstonia wilt in tomato. *Journal of Plant Pathology*, **92**, 593–600.

Ali N., Ramkissoon A., Ramsubhag A., Jayaraj J. (2016) *Ascophyllum* extract application causes reduction of disease levels in field tomatoes grown in a tropical environment. *Crop Protection*, **83**, 67–75.

Amin A.A., Rashad M., El-Abagy H.M.H. (2007) Physiological effect of indole-3-butyric acid and salicylic acid on growth, yield and chemical constituents of onion plants. *Journal of Applied Sciences Research*, **3**, 1554–1563.

Badawy M.E.I., Rabea E.I. (2011) A Biopolymer chitosan and its derivatives as promising antimicrobial agents against plant pathogens and their applications in crop protection. *International Journal of Carbohydrate Chemistry*, **2011**, 460381.

Benhamou N. (1996) Elicitor-induced plant defence pathways. *Trends in Plant Science*, **1**, 233–240.

Chaerani R., Voorrips R.E. (2006) Tomato early blight (*Alternaria solani*): the pathogen, genetics, and breeding for resistance. *Journal of General Plant Pathology*, **72**, 335–347.

Chandra A., Saxena R., Dubey A., Saxena P. (2007) Change in phenylalanine ammonia lyase activity and isozyme patterns of polyphenol oxidase and peroxidase by salicylic acid leading to enhance resistance in cowpea against *Rhizoctonia solani*. *Acta Physiologia Plantarum*, **29**, 361–367.

Chen Y.-E., Yuan S., Liu H.-M., Chen Z.-Y., Zhang Y.-H., Zhang H.-Y. (2016) A combination of chitosan and chemical fertilizers improves growth and disease resistance in *Begonia* × *hiemalis* Fotsch. *Horticulture, Environment, and Biotechnology*, **57**, 1–10.

Dai J., Mumper R.J. (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, **15**, 7313–7352.

Dangl J.L., Jones J.D.G. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826–833.

Dickerson D.P., Pascholati S.F., Hagerman A.E., Butler L.G., Nicholson R.L. (1984) Phenylalanine ammonia-lyase and hydroxycinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. *Physiological Plant Pathology*, **25**, 111–123.

El-Hendawy H.H., Osman M.E., Sorour N.M. (2005) Biological control of bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* by *Rahnella aquatilis*. *Microbiological Research*, **160**, 343–352.

El-Tantawy E.M. (2009) Behavior of tomato plants as affected by spraying with chitosan and aminofort as natural stimulator substances under application of soil organic amendments. *Pakistan Journal of Biological Sciences*, **12**, 1164–1173.

Farouk S., Ramadan A.A. (2012) Improving growth and yield of cowpea by foliar application of chitosan under water stress. *Egyptian Journal of Biology*, **14**, 14–26.

Gondal A.S., Ijaz M., Riaz K., Khan A.R. (2012) Effect of different doses of fungicide (mancozeb) against Alternaria

leaf blight of tomato in tunnel. *Journal of Plant Pathology & Microbiology*, **3**, 1–3.

Grennan A.K. (2006) Plant response to bacterial pathogens. Overlap between innate and gene-for-gene defense response. *Plant Physiology*, **142**, 809–811.

Hammerschmidt R., Nuckles E.M., Kuć J. (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiological Plant Pathology*, **20**, 73–82.

Iriti M., Varoni E.M. (2015) Chitosan-induced antiviral activity and innate immunity in plants. *Environmental Science and Pollution Research*, **22**, 2935–2944.

Jayaraj J., Rahman M., Wan A., Punja Z.K. (2009) Enhanced resistance to foliar fungal pathogens in carrot by application of elicitors. *Annals of Applied Biology*, **155**, 71–80.

Jones M. T. (1984) Pest Management and the Vegetable Farmer in Trinidad and Tobago. In *Proceedings of the Caribbean Food Crops Society 20thMeeting, St. Croix.* **1**: 173–175.

Kumar M.N.V.R. (2000) A review of chitin and chitosan applications. *Reactive and Functional Polymers*, **46**, 1–27.

Kunkel B.N., Brooks D.M. (2002) Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology*, **5**, 325–331.

Li L., Steffens J.C. (2002) Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta*, **215**, 239–247.

Mandal S., Kar I., Mukherjee A.K., Acharya P. (2013) Elicitor-induced defense responses in *Solanum lycopersicum* against *Ralstonia solanacearum*. *The Scientific World Journal*, **2013**, 561056.

Mayer A.M., Harel E. (1979) Polyphenol oxidases in plants. *Phytochemistry*, **18**, 193–215.

Niki T., Mitsuhara I., Seo S., Ohtsubo N., Ohashi Y. (1998) Antagonistic effects of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant and Cell Physiology*, **39**, 500–507.

Pan S.Q., Ye X.S., Kuć J. (1991) Association of β-1, 3-glucanase activity and isoform pattern with systemic resistance to blue mould in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. *Physiological and Molecular Plant Pathology*, **39**, 25–39.

Ramirez-Estrada K., Vidal-Limon H., Hidalgo D., Moyano E., Golenioswki M., Cusidó R.M., Palazon J. (2016) Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules*, **21**, 182.

Ramjegathesh R., Jayaraman J. (2015) Chitosan for plant disease manangement – prospects and problems. In *Sustainable Crop Disease Management using Natural Products*, pp. 198–212. Eds S. Ganesan, K. Vadivel and J. Jayaraman. Boston, MA, USA: CABI.

Saavedra G.M., Figueroa N.E., Poblete L.A., Cherian S., Figueroa C.R. (2016) Effects of preharvest applications of methyl jasmonate and chitosan on postharvest decay, quality and chemical attributes of *Fragaria chiloensis* fruit. *Food Chemistry*, **190**, 448–453.

Schmittgen T.D., Livak K.J. (2008) Analysing real-time PCR data by the comparative C_T method. *Nature Protocols*, **3**, 1101–1108.

Shibuya N., Minami E. (2001) Oligosaccharide signalling for defence responses in plant. *Physiological and Molecular Plant Pathology*, **59**, 223–233.

Taheri P., Tarighi S. (2012) The Role of Pathogenesis-Related proteins in the tomato–*Rhizoctonia solani* interaction. *Journal of Botany*, **2012**, 137037.

Tonon C., Andreu A., Aued M.E., Van Damme M., Huarte M., Daleo G.R. (1998) Defence reactions in two potato cultivars following infection with two races of *Phytophthora infestans*. *Potato Research*, **41**, 319–325.

Vlot A.C., Dempsey D'M.A., Klessig D.F. (2009) Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology*, **47**, 177–206.

Waewthongrak W., Pisuchpen S., Leelasuphakul W. (2015) Effect of *Bacillus subtilis* and chitosan applications on green mold (*Penicilium digitatum* Sacc.) decay in citrus fruit. *Postharvest Biology and Technology*, **99**, 44–49.

Xu A., Zhan J.-C., Huang W.-D. (2016) Combined elicitation of chitosan and ultraviolet C enhanced stilbene production and expression of chitinase and β-1,3-glucanase in *Vitis vinifera* cell suspension cultures. *Plant Cell, Tissue and Organ Culture*, **124**, 105–117.

Zieslin N., Ben-Zaken R. (1993) Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. *Plant Physiology and Biochemistry*, **31**, 333–339.