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Chitosan coatings reduce fruit fly (*Anastrepha obliqua*) infestation and development of the fungus *Colletotrichum gloeosporioides* in Manila mangoes

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Abstract

Background. Mangoes are tropical fruits appreciated worldwide but are extremely perishable, susceptible to decay, pest infestation and fungal diseases. Using the flavorful and highly valued 'Manila' cultivar, we examined the effect of second-generation chitosan coatings on shelf-life, phenolic compound variation, phytohormones, pest infestation by fruit flies (*Anastrepha obliqua*) and anthracnose disease caused by the fungus *Colletotrichum gloeosporioides*.

Results. We observed almost total annihilation of *A. obliqua* eggs with 10 and 20 g L^{-1} chitosan in diluted acetic acid and a 5- to 6-fold reduction in anthracnose damage. Treatment with 20 g L^{-1} chitosan also extended shelf-life. External (skin) and internal (pulp) discoloration process were delayed. Fruit firmness was higher than control and acetic acid treatments, and total soluble solids (TSS) were lower in chitosan-treated fruit. Targeted and non-targeted metabolomics analyses on chitosan-coated fruit identified some phenolic compounds related to the tannin pathway. In addition, abscisic acid (ABA) and jasmonic acid (JA) in the peel were down-regulated in chitosan coated mango peels. Both, phytohormones and phenolic content, may explain mangoes reduced susceptibility to anthracnose development and *A. obliqua* egg eclosion or larval development.

Conclusion. We conclude that chitosan coatings represent effective postharvest treatment that significantly reduces anthracnose disease, inhibits *A. obliqua* egg eclosion, and significantly extends 'Manila' mango shelf-life, a key factor currently inhibiting large-scale commercialization of this precious fruit.

Keywords: *Anastrepha obliqua*; ‘Manila’ mango, chitosan; postharvest treatments; phenolic compounds

1. Introduction

Mangoes (*Mangifera indica* L.) are appreciated worldwide for their juicy flesh, tropical flavor, pleasant odor and nutritional properties.[1] Commercialization of 'Manila' mangoes, the most flavorful and valued cultivar in Mexico and some other countries, is limited due to their extremely short postharvest shelf-life, pathogen susceptibility such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., the causative agent of anthracnose[2], and infestation by tephritid fruit fly larvae.[3] Mango susceptibility to pathogens and fruit flies varies markedly among cultivars and largely depends on fruit characteristics such as the cuticle structure[4], resin duct density, sap content[5], tannin content and fruit ripeness stage.[6] Compared to other popular cultivars, such as 'Tommy Atkins' and 'Ataulfo', 'Manila' mangoes are highly susceptible to fruit fly attack.[5,7,8]

Fruit flies represent a quarantine risk for many importing countries and cause significant economic damage in their countries of origin.[3] In Mexico, 'Manila' mangoes are mainly infested by the West Indian fruit fly *Anastrepha obliqua* (Macquart) and to a lesser extent by the Mexican fruit fly *Anastrepha ludens* Loew (Diptera: Tephritidae).[5,8] Due to its high reproductive potential and widespread distribution, *A. obliqua* represents a pervasive threat to 'Manila' mango production and export.[7,8] In addition, 'Manila' mangoes are highly susceptible to the effect of anthracnose. Anthracnose appears on mango fruit and foliage as irregular necrotic lesions.[9] In physiologically mature-green fruit, the infection remains dormant until the ripening process begins, thereby complicating the classification of healthy and

infected fruit during harvest. Anthracnose also renders the fruit unmarketable due to extensive aesthetic damage to the exocarp.[9]

Larval disinfestation and antifungal treatments for mangoes in Mexico include hot water immersion treatment (HWT), and gamma irradiation as well as the intensive use of pesticides and fungicides.[10] In thin-skinned mango cultivars such as 'Manila', hot water immersion can result in skin scalding, lenticel damage, cavities, starchy areas in the pulp and an unwanted delay in the ripening process.[11] Therefore, the search for alternatives to prolong postharvest shelf-life and kill fruit fly eggs and larvae inside the fruit has been a priority for growers eager to gain access to export markets. A promising option, tested in other mango cultivars and several additional types of fruit, is the use of chitosan coatings.[12,13]

Chitosan is an aminopolysaccharide composed of β-1,4-linked D-glucosamine units and variable number of *N*-acetylated glucosamine residues, representing one of the few cationic biopolymers in nature.[14,15] This compound has been widely used in plant protection and when applied as a fruit coating, has exhibited insecticidal effects against Mexican fruit fly larvae[13] and antimicrobial effects against a range of pathogenic fungi and bacteria.[16,17] Chitosan can also act as an elicitor molecule and thereby stimulate plant immune responses and phytoalexin production.[18,19] As a result, chitosan treatment has consistently been reported to extend shelf-life and reduce decay in different types of fruit.[14,20]

The objectives of our study were three-fold: investigate the potential of the structurally well-defined second generation chitosan coatings (with a known degree of acetylation [DA] and polymerization [DP], and with known molecular structure-function relationships)[21] on 1) 'Manila' mango postharvest shelf-life particularly in terms of fruit maturation, physical and chemical traits, 2) inhibition of *A. obliqua* larval development, and 3) *C. gloeosporioides* growth reduction. Given the mode of action of the type of chitosan coating used, our general hypothesis was that both fruit fly egg eclosion/larval development, as well as fungal growth (*G. gloeosporioides*) would be inhibited. We predicted that the expression of defense-related phenolic compounds associated with the tannin pathway would enhance the resistance of 'Manila' mangoes to the pest/pathogen and extend the fruits' shelf-life.

2. Material and Methods

2.1 Chemical reagents

Microcrystalline chitosan (Code No. 651; prepared by heterogeneous alkaline de-*N*-acetylation of shrimp shell chitin, degree of acetylation 20%, as determined by ^{1}H-NMR; the degree of polymerization ~400 and polydispersity index 2.17, as determined by HPSEC-RID-MALLS) was obtained thanks to a generous gift from Dr. Dominque Gillet (Mahtani Chitosan, Veraval, India) and was used without any further purification.

2.2 Fruit collection

Mangoes were harvested directly from organic 'Manila' orchards in Apazapan, Veracruz, Mexico (N 19°19' 3.55", W 96°42' 54.09" and N 19°20' 30.69", W 96°45' 54.57"). All fruit were selected based on their mature-green colour, size, shape, and absence of visible fungal infection or insect damage. Fruit maturity was determined in the field by selecting fully developed mangoes (based on shoulder development, green-bright skin color and the presence/absence of bloom on the skin surface).[22] Fruit was transported to the laboratory, gently washed with tap water and dried. Experiments were set up the same day fruit was harvested.

2.3 Chitosan solutions, fruit-coating process, and storage conditions

The preparation of 10 and 20 g L^{-1} chitosan solutions was achieved by mixing 11 or 22 g of chitosan powder with 28 mL or 56 mL, respectively, of 100 mL L^{-1} acetic acid (JT Baker, Phillipsburg, NJ, US), and then adding distilled water until a 1 L solution was reached. For comparative purposes, we added blank treatment solutions containing only 2.8 mL L^{-1} and 5.6 mL L^{-1} acetic acid diluted in distilled water and an absolute control (distilled water). All solutions were stored at 4 °C for a maximum of three days until use. The coating process entailed dipping each mango for 60 s in a 1 L beaker filled with distilled water, acetic acid or chitosan solutions. To obtain a homogenous coating at environmental conditions, which is commonly used in non – industrial farming systems, harvested fruits were stored in a dark room to prevent heating caused by direct sunlight. We allowed the fruit

coating to dry for 24 h at 26 ± 1 °C, 67 ± 5% RH, and stored coated and uncoated mangoes under the same conditions throughout the experiment.

2.4 Experiment 1. Effect of chitosan coating on postharvest shelf-life and ripening process

Groups of green, field-collected 'Manila' mangoes were coated with 20 g L^{-1} chitosan solution, acetic acid solution without chitosan, or distilled water by directly dipping the fruit in the corresponding solutions, as described above. Based on preliminary observations of chitosan coatings indicating that the latter delay ripening, the effects of chitosan on fruit characteristics were determined at five time points (one day after fruit coatings had dried, 5, 9, 13 and 17 d after application of treatments) using three fruit from each treatment (approximately 45 total fruits) to determine ripeness and decay degree, weight loss, fruit firmness, sugar content, and metabolomics changes according to previous reports.

2.4.1 Fruit coloration and decay

Internal ripening was assessed by the pulp coloration development according to the CIE classification[23]. A color scale from one to six was established for peel and pulp (see Fig. 1A) from untreated fruit and compared to digital images of treated mangoes using a Canon SX10IS PC1304 10 Mpx camera. All fruit slices were photographed directly from above the sample at a distance of 25 cm in an arena illuminated by LED lamps (Minilight led tubes 10 W 90-260 V 6500 K). The red-

blue-green (RBG) space code of 1600–1200 pixel JPEG digital images were transformed to CIE-L*a*b* using Easy RGB (2010) to determine the L* value (lightness), and a* and b* rectangular chroma coordinates. We determined differences among values of a* and b* chroma as internal color change following Camacho et al.[24]

2.4.2 Determination of fruit weight loss

Fruit weight loss was assessed at each time point by using a digital scale to a precision of one mg. Results were reported as the proportion of weight loss following AOAC standards and were calculated based on the first weight measurement.[25]

2.4.3 Determination of fruit firmness and TSS

Fruit firmness was determined using a penetrometer FT 011–Firmness Pressure Tester (lbf) fitted to a 10 mm diameter plunger (QA Supplies, Virginia). The fruit area was peeled on two fruit parts (peduncle and lateral points) before firmness was recorded. Lbf data per fruit were averaged and transformed into Newtons.[25] Total soluble solids (TSS), such as glucose, fructose, sucrose and other soluble carbohydrates were determined using a digital Atago Pal-1 0-53% Digital Pocket Refractometer (g kg^{-1}) testing two drops of the squeezed fruit part (peduncle and side).[25]

2.5 Targeted and non-targeted metabolomics analysis in mango peel with and without chitosan coating using liquid chromatography and mass spectrometry

2.5.1 Phenolic targeted metabolomics analysis in mango peel and pulp by liquid chromatography and tandem mass spectrometry (LC-ESI-MS/MS)

The representative peel chemical fingerprint (15 mangoes per treatment, three mangoes for each time point) was determined using two different approaches. First, we ran a targeted metabolomic analysis aimed at phenolics using fruit stemming from the most representative treatments (distilled water, 5.6 mL L^{-1} acetic acid and 20 g L^{-1} chitosan) and representing four sampling times (1, 5, 9 and 13 d). Dissected peel and pulp were prepared for methanol – based extraction, frozen at -80 °C and lyophilized in a FreeZone® Benchtop Shell Freezer. The lyophilized powder was mixed in 1:1 ratio with diatomaceous earth and extracted in a DIONEX-ASE 350 extractor using as solvent methanol HPLC grade purchased from Sigma-Aldrich (St. Louis, MO, US). Methanol, acetonitrile, water, and formic acid used for analysis of phytochemicals were LC-MS grade and were purchased from Sigma-Aldrich (St. Louis, MO, US). The extraction was accomplished in one extract cycle using 60 °C heat for five min at steady 1500 psi and five min of static time. The rinse volume was 30% of used solvent and nitrogen was used for 90 s as the purge gas. The remaining solvent was evaporated in a laboratory hood. The targeted metabolomics analysis method used was outlined in Monribot-Villanueva et al.[26] In brief, metabolomics analyses were performed by using 200 mg of pulp extracts and 15 mg of peel extracts. Extracts were dissolved in 1 mL methanol with 1 mL L^{-1} formic acid, agitated for 3 min and filtered through a 2 µm

PTFE syringe filter. Specific phenolics were identified and quantified using an UPLC system (Agilent 1290) coupled to QqQ mass spectrometer (Agilent 6460).

The column used was an Agilent, Zorbax SB-C18, 2.1 x 50 mm, 1.8 µm with a working temperature of 40 °C. The mobile phase consisted of (A) water and (B) acetonitrile, both with 1 mL L^{-1} of formic acid. The gradient conditions of the mobile phases were: 0–40 min, linear gradient from 10 to 400 mL L^{-1} of B; 40–42 min, from 400 to 900 mL L^{-1} of B; 42–44 min, isocratic at 900 mL L^{-1} of B; 44–46 min, linear gradient from 900 to 10 mL L^{-1} of B (total run time 47 min). The flow rate was 0.1 mL min^{-1} and the injection volume was 2 µL. The method used was a dynamic Multiple Reaction Monitoring (dMRM) with electrospray ionization (ESI) in positive and negative modes. The dMRM transitions for each compound were searched in public databases as Metlin and experimentally corroborated in our laboratory. The precursor and product ions were considered as qualifier ions and the product ion was considered as the quantifier ion. Nitrogen was used as collision gas for fragmentation with a fragmentation voltage of 100 V and cell accelerator voltage of 7 V. The source of gas temperature flow was 300 °C and 5 L min^{-1}. The nebulizer pressure was 3.16 kg cm^{-2} and the sheath gas temperature and flow were 250 °C and 11 L min^{-1}, respectively. The capillary and nozzle voltages (positive/negative) were 3500 and 500 V, respectively. Quantification was possible by establishing a calibration curve for each compound. Gallic acid, penta-O-galloyl-β-D-glucose, mangiferin, (+)-catechin, quercetin-3-D-galactoside, quercetin, and quercetin-3-glucoside standards were purchased from Sigma-

Aldrich (St. Louis, MO, US). 4-coumaric acid and luteolin-7-O-glucoside standards were purchased from Extrasynthese (Lyon, France). The determination coefficient was 0.99 or higher for each compound. The data were obtained using the Agilent Mass Hunter Workstation software (B.06.00). The transitions used were 4-coumaric acid 165.05 > 147.04, mangiferin 423 > 302.8, (+)-catechin 291 > 138.9, quercetin 302.9 > 153.1, quercetin-3-D-galactoside 465 > 302.9, quercetin-3-glucoside 465 > 303 and luteolin-7-O-glucoside 449 > 287. The concentration of specific phenolics was expressed in μg g^{-1} on a dry weight basis (DW).

2.5.2 Non-targeted metabolomics analysis by LC-ESI-HRMS

A non-targeted metabolomics analysis was performed with the same extracts used for the targeted analysis by means of an UPLC system (Waters™, Class I) coupled to a QToF high resolution mass spectrometer (Waters™, Synapt G2-Si HDMI) as previously reported by Monribot-Villanueva et al.[26] Chromatography was performed in an Acquity BEH column (1.7 μm, 2.1 x 50 mm) with column and sample temperatures of 40 °C and 15 °C, respectively. The mobile phase consisted of (A) water and (B) acetonitrile, both with 1 mL L^{-1} formic acid (Sigma). The gradient conditions of the mobile phases were 0–13 min, linear gradient from 10 to 800 mL L^{-1} of B; 13–14 min, isocratic at 800 mL L^{-1} of B; 14–15 min, linear gradient from 800 to 10 mL L^{-1} of B (total run time 20 min). The flow rate was 0.3 mL min^{-1}, and 1 μL of the extract was injected. Mass spectrometric analysis was performed with an electrospray ionization source in the positive mode with a

capillary, sampling cone and source offset voltage of 3000, 40 and 80 V, respectively. The source temperature was 100 °C and the desolvation temperature was 20 °C. The desolvation gas flow was 600 L h^{-1} and the nebulizer pressure was 6.5 bar. Leucine-enkephalin was used as the lock mass (556.2771, $[M+H]^+$). The conditions used for MS analysis were: mass range 50-1200 Da, function 1 CE, 6 V, function 2 CER 10–30 V, scan time 0.5 s. The data were acquired and processed with MassLynx (Waters, version 4.1), MarkerLynx (Waters, version 4.1) and Metaboanalyst software to identify discriminant chemical biomarkers. Fold change analysis and volcano plot were performed to identify those features (retention time-m/z) with the highest fold change values. Tentative identification was performed by comparing the mass spectra with those reported in the public databases Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage), Foodb (https://foodb.ca/) and Massbank (https://massbank.eu/MassBank/). The maximum error allowed was 5 ppm. Principal component analysis (PCA), heatmap with hierarchical clustering and fold change analyses (volcano plots) were performed using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca).

2.5.3 Chemical markers identification and quantification by LC-ESI-HRMS

A second targeted metabolomics analysis was run to quantify some chemical markers identified by the non-targeted analysis. Jasmonic acid (JA) and abscisic acid (ABA) are two phytohormones that are closely related to defense traits in

plants against microorganisms and insects. Both are also involved in the fruit ripening process.

JA and ABA were identified and quantified in an UPLC chromatographic Class I system (Waters™) coupled to a Synapt G2-Si HDMi mass spectrometer. JA was purchased from Cayman Chemical Company (Michigan, USA) and ABA from Sigma-Aldrich (St. Louis, MO, US). Chromatography was performed in an Acquity BEH column (1.7 µm, 2.1 x 50 mm) with column and sample temperatures of 40 and 15 °C, respectively. The mobile phase consisted of (A) water and (B) acetonitrile, both with 1 mL L^{-1} of formic acid (Sigma). The gradient conditions of the mobile phases were 0–1 min, isocratic at 10 mL L^{-1} of B; 1–15 min, linear gradient from 10 to 950 mL L^{-1} of B; 15–16 min, isocratic at 950 mL L^{-1} of B; 16–17 min, linear gradient from 950 to 10 mL L^{-1} of B (total run time 20 min). The flow rate was 0.1 mL min^{-1}, and 1 µL of the extract was injected. Mass spectrometric analysis was performed with an ESI source in negative mode with a capillary, sampling cone and source offset voltage of 3000, 40 and 80 V, respectively. The source temperature was 100 °C and the desolvation temperature was 20 °C. The desolvation gas flow was 600 L h^{-1} and the nebulizer pressure was 6.5 bar. Leucine-enkephalin was used as the lock mass (554.2615, $[M-H]^-$). The mass range conditions used for MS analysis were 50–1200 Da. The transitions used were 263.1 > 153.1 and 209.1 > 165.1 for ABA and JA, respectively. The data were acquired and processed with MassLynx (version 4.1) and TargetLynx (Version 4.1). Calibration curves were constructed with ten concentration points

(0.5, 1, 2, 4, 6, 8, 10, 14, 16 and 18 µM) using the authentic commercial standards. Each point was injected twice, and the corresponding areas were considered to generate the calibration curve. A 2nd order curve regression with a coefficient of determination of 0.99 was used. The concentration is expressed in µg g^{-1} on a dry weight basis (DW). The identification and quantification of ellagic acid was performed as described in section 2.5.1 (Phenolic targeted metabolomics). The transition of ellagic acid was 301 > 145, and the analytical standard was purchased from Sigma-Aldrich (St. Louis, MO, US).

2.6 Experiment 2. Effect of chitosan coating on A. obliqua *infestation*

Anastrepha obliqua was obtained from field-collected 'Criollo' mangoes in Apazapan, Veracruz at an altitude of 299 m (N 19°19' 5.56", W 96°43' 0.32") and maintained the following methods outlined in Guillén et al.[5] Manila mangoes were transported to the laboratory, rinsed with tap water, and exposed for a 24 h period to gravid *A. obliqua* females (15–20 d of age) at a ratio 1:2, fruit: females inside 30 x 30 x 30 cm Plexiglas cages. After 24 h, fly-exposed mangoes were dipped for 60 s into one of five solutions (treatments): (i) distilled water, (ii) 2.8 mL L^{-1} acetic acid, (iii) 5.6 mL L^{-1} acetic acid, (iv) 10 g L^{-1} chitosan, and (v) 20 g L^{-1} chitosan. Tests were replicated five times (n = 50, 10 fruit/treatment). Treated fruit were placed on a plastic cup and allowed to dry for 24 h at 27 ± 1 °C, 75 ± 5% RH, and a 12:12 h light/dark photoperiod. After drying, each fruit was transferred to a 1 L plastic container with a fine layer of sterile vermiculite, covered with nylon mesh and

stored for 21 d under the same conditions. Infestation level was determined as the number of larvae plus pupae per fruit after a 21 d-period. All fruit were dissected under a stereomicroscope, and larvae and pupae were counted and placed in 250 mL plastic containers until emergence.

2.7 Experiment 3. Effect of chitosan on anthracnose disease

2.7.1 Isolation and identification of fungus pathogen

Isolates of *C. gloeosporioides* were obtained from field-collected, infected 'Manila' mangoes by transferring small portions of symptomatic tissue to potato-dextrose agar (PDA) in Petri plates. The fungus was grown at ambient temperature (22 ± 2 °C) and once mycelial growth was observed, the colonies were transferred to PDA plates to obtain pure cultures. Identification of the fungus as *C. gloeosporioides* was performed by examination of conidia morphological structures and by molecular identification (gene sequencing) using the ITS region (ITS1-5.8S-ITS2) as a molecular marker.[27] The sequence obtained (515 bp) was aligned using blastn algorithm in two databases: GenBank and UNITE. In both databases, the best hit for the query sequence was *C. gloeosporioides* with a coverage and sequence identity (nt) of 99%. A spore suspension of 10^6 spores mL^{-1} was prepared by transferring the sporodochium to a sterile plastic tube containing 30 mL-distilled water and one drop of Tween 80. The concentration of spores was determined by counting in a Neubauer chamber with a depth of 0.1 mm.

Harvested fruit were taken to the laboratory, rinsed with water, and 20 fruit were artificially inoculated with 5 µL (5000 spores average) of 1 x 10^6 spores mL^{-1} spore suspension on both equatorial sides of each mango using a 25 x 5/8" syringe needle (Sensimedical®, Mexico). The suspension was injected gently at a depth of 2 mm between the peel and pulp. Inoculated fruits were stored for 6 h at 27 °C, 50% RH and then subjected to one of the following five treatments (i) distilled water, (ii) 2.8 mL L^{-1} acetic acid, (iii) 5.6 mL L^{-1} acetic acid, (iv) 10 g L^{-1} chitosan and (v) 20 g L^{-1} chitosan, all applied to four fruit per treatment (n = 20). Fruits were placed inside 150 mL plastic cups in turn placed inside 1 L sterile plastic containers covered with a nylon gauze to avoid insects from entering, and stored for 8 d at 27 ± 1 °C, 75 ± 5% RH. Plastic containers were disinfected daily using 700 mL L^{-1} ethanol to avoid nonspecific fungal growth due to high humidity.

Lesion development was determined at 24 h intervals by taking photographs alongside a 30 cm scale using a Canon SX10IS PC1304 (Tokyo, Japan) 10 Mpx camera. The area of each lesion was subsequently determined using Nikon imaging software (NIS-Element, AR 3.2) and expressed in square millimeters (lesion surface).

2.8 Statistical analyses

All experiments were run using a completely randomized design. The effects of chitosan on ripeness degree, firmness, total soluble solids were determined by

analysis of covariance. In all cases, time was considered as the continuous predictor. Weight loss, specific phenolics and infestation rate were analyzed by means of a one-way analysis of variance (ANOVA). When data did not fit normality and homoscedasticity, data were rank transformed (e.g., firmness, total soluble solids, fruit infestation), or arcsine transformed (e.g., weight loss) or a generalized linear model with a Poisson error distribution was used (e.g., ripening process). When the response variable was measured over a time-period on the same fruit, a repeated measures analysis of variance was performed (e.g., development of fungal lesions). Differences among means at each time point were compared by using Fisher´s Least Significant Difference (LSD) test at $\alpha = 0.05$. All data were analyzed using Statistica Version 10.[28]

3. Results

3.1 Effect of chitosan coatings on postharvest shelf-life and maturation

3.1.1 Ripeness process and color change

Color changes of mangoes are represented in Figure 1. The internal ripening process was clear through changes in the color of the pulp that transitioned from light-yellow to orange (Table 1). Control treatments (water and acetic acid coatings) decreased faster in L^* and b^* values, the consequence of browning. Control mangoes exposed to water exhibited a light-yellow color at 1-d post-treatment, which changed to yellow-orange on day five, light orange on day nine, and deep orange on day 13 (Table 1). The same pattern over time was observed

in mangoes treated with the acetic acid solution (5.6 mL L^{-1}). In sharp contrast, the pulp of chitosan-treated did not develop an orange coloration during the 17 d storage period as shown by *L**, *a** and *b** values that varied the least (Table 1) (GLM, ANCOVA, $\chi^2 = 5.31$, df = 2, $P = 0.07$, Poisson, log link function). At the end of the storage period (17 d), water- and acetic acid-treated mangoes exhibited a severe decrease in the pulp lightness (Δ*L** 15.27 [control]/12.34 [acetic acid]), in contrast to chitosan-coated fruit that only exhibited a slight decrease (Δ*L** 0.06). Green to reddish internal coloration sharply increased in water, and acetic acid-treated mangoes (Δ*a** 25.97 [control]/16.17 [acetic acid]) and merely remained equal in chitosan-coated fruit (Δ*a** -0.25). A similar pattern was observed in the case of *b**, as water- and acetic acid-treated mangoes exhibited the highest increase (Δ*b** 7.91 [control]/8.58 [acetic acid]) and chitosan-coated fruit maintained the internal yellowish coloration (Δ*b** 1.66). The marked decrease of *L** and increase in *a** value indicate a change from light yellow to deep orange and the marked decrease in *b** values was related to the brownish color change, typical of decaying fruit.

3.1.2 Weight loss

Weight loss values differed significantly among treatments (ANOVA, $F_{2,26} = 3.81$, $P = 0.036$). The highest weight loss was observed in water-treated mangoes, which lost more than 16% of their initial weight during the 17-d storage period. Mangoes exposed to acetic acid and treated with chitosan lost almost 12% of their initial weight (Fig. 1B).

3.1.3 Fruit firmness

Fruit exposed to all treatments exhibited a significant decrease in firmness and reached similar values after 17 d of storage (ANCOVA, treatment, $F_{1,41} = 13.75$, $P < 0.001$; time, $F_{1,41} = 41.8$, $P < 0.001$). Firmness values fluctuated over time in the chitosan treatment (Fig. 1C) and decreased markedly in the sample taken at 17 d. Mangoes from the water and acetic acid treatments exhibited the lowest firmness values (Fig. 1C).

3.1.4 Total soluble solids (TSS)

TSS content differed significantly among treatments and increased throughout the maturation process (ANCOVA, treatment, $F_{2,41} = 7.33$, $P < 0.001$, time, $F_{1,41} = 37.72$, $P < 0.001$). Chitosan-coated mangoes exhibited significantly lower total soluble solids (g kg^{-1}) than water control and acetic acid treatments. This trend was maintained throughout the maturation process (Fig. 1D).

3.1.5 Targeted and non-targeted metabolomics analyses of chitosan-treated mangoes using LC-ESI-MS

Phenolic-targeted metabolomics analyses were performed for samples of four sampling points (Table 2). A total of seven different compounds were identified including: 4-coumaric acid, (+)-catechin, mangiferin, quercetin, quercetin 3-O-galactoside, quercetin 3-O-glucoside and luteolin 7-O-glucoside. The most

abundant compounds were quercetin glycosides and the xanthonoid mangiferin. Only 4-coumaric acid and quercetin exhibited higher concentrations in chitosan-treated mango peels at one-day post-treatment (Table 2). Although the maturation process was markedly retarded in chitosan-coated mangoes, the presence of these phenolic compounds decreased in the same manner as in control mangoes (Table 2).

Since the main effect of chitosan coatings were observed in the first two sampling times (Fig. 1A-D), we performed an non-targeted metabolomics analysis of mango peels treated with acetic acid and chitosan at 1 and 5 d after treatment to identify chemical markers that can be up-regulated in chitosan-coated mangoes (Fig. 2A and B). PC1 to PC3 of the principal component analysis (PCA) based on chemical composition explained 84.9% of the variance. In the PCA, chitosan samples were closer compared to acetic acid samples (Fig. 2A), whereas the opposite grouping tendency was observed in the hierarchical cluster analysis depicted in the heatmap based on the total sample metabolome, where samples are mainly grouped based on sampling times (Fig. 2B). To identify chemical compounds up-regulated in chitosan-coated mangoes, fold change analyses were run, and volcano plots were constructed (Fig. 3A and 3B). Mangiferic acid, kaempferol rhamnoside, ellagic acid, mangiferin gallate and ellagic acid rhamnoside were up-regulated one day after chitosan coatings (Fig. 3A), while theogallin, trigalloyl glucopyranose, dihydrophaseic acid, and the aromatic amino acids tryptophan and tyrosine did so five days after treatment (Fig. 3B).

Confirmation of some chemical markers was achieved using a second targeted metabolomics analysis. Ellagic acid was quantified at a higher concentration in chitosan-coated samples one-day post-treatment (Table 3). Dihydrophaseic acid is a catabolite of abscisic acid (ABA), given the plausible role of phytohormones as chemical markers, we quantified ABA and jasmonic acid. Both phytohormones were downregulated in chitosan-coated mango peels on the fifth day post-treatment (Table 3).

3.2 Assessment of A. obliqua *infestation rate*

Chitosan treatments resulted in a dramatic reduction in the number of *A. obliqua* larvae that developed in treated mangoes compared to the control treatments (i.e., water) or acetic acid; ANOVA, $F_{4,20} = 17.66$, $P < 0.001$) (Fig. 4A). The mean number of larvae and pupae found in chitosan-coated mangoes ranged from 0.1 ± 0.01 insects/fruit in the 20 g L^{-1} chitosan treatment to 1 ± 0.5 insects/fruit in the 10 g L^{-1} chitosan treatment. In contrast, in control fruit treated with water or acetic acid, we recorded much higher levels of infestation, ranging from 15.6 to 28.2 insects/fruit (Fig. 4A).

The emergence of adults from recovered pupae also differed significantly among treatments (ANOVA, $F_{4,20} = 16.39$, $P < 0.001$). Adult emergence was zero in mangoes treated with 20 g L^{-1} chitosan and almost zero (0.5 ± 0.32) in the 10 g L^{-1} chitosan treatment. In sharp contrast, the fruit treated with acetic acid at 2.8 mL

L^{-1} or 5.6 mL L^{-1}, or with water only, yielded many more adults (9.7 ± 2.3, 7.2 ± 1.5, and 12.7 ± 5 insects/fruit, respectively) (Fig. 4B).

3.3 Effect of chitosan on anthracnose disease

The severity of lesions caused by the fungus varied significantly depending on treatment (repeated measures ANOVA, treatment, $F_{4,15} = 9.34$, $P < 0.001$, time, $F_{7,105} = 70.03$, $P < 0.001$; time*treatment $F_{28,105} = 9.35$, $P < 0.001$) (Fig. 5). Lesions on chitosan-treated mangoes were significantly smaller (60.99 ± 20.38 mm^2 [10 g L^{-1}], 36.76 ± 15.94 mm^2 [20 g L^{-1}]) at 8 d post-treatment compared with those observed in mangoes treated with the corresponding acetic acid concentrations that developed ca. five- to six-fold (350.53 ± 26.74 mm^2 [5.6 mL L^{-1}], 168.12 ± 26.68 mm^2 [2.8 mL L^{-1}]) larger lesions during the observation period. Fruit immersed in distilled water exhibited a similar lesion development pattern as fruit coated with the lower concentration of acetic acid (349.82 ± 53.92 mm^2) (Fig. 5).

4. Discussion

Our study yielded several relevant new insights into the positive role that chitosan coatings can play as a postharvest treatment in highly valued mangoes with short shelf lives, in reducing both fruit fly infestation and *C. gloeosporioides* damage. We also show that chitosan coatings significantly slowed the rate of external and internal ripening as well as fruit weight loss, in addition to maintaining peel firmness[13] and lowering total soluble solids (TSS), likely as a result of the reduced

gas exchange caused by the chitosan film as shown in our preliminary tests (Aluja unpublished data) and in previous studies.[20,29] Chitosan-coating resulted in lower TSS content compared to control treatments.[30] Chitosan-induced reduction in respiration could lead to slower metabolic rates resulting in lower amounts of hydrolyzed TSS.[31] Our results agree with observations of reduced TSS obtained from chitosan-coated bananas and mangoes cv Alphonso.[32] Also, in previous studies with Tainong mangoes, chitosan treatment modulated firmness in a concentration- and molecular weight-dependent manner when compared with untreated fruit.[20]

Phytohormones are important compounds that control different physiological processes, including fruit ripening.[33] ABA is involved in abiotic stress response, while JA is mainly involved in plant defense against pathogens and herbivores.[34] Besides, both phytohormones are involved in fruit ripening and their concentrations increase during the onset of this process.[35,36] The fact that ABA and JA were downregulated in chitosan-coated mango peels at 5-d post-treatment suggests that chitosan treatments delay the ripening process in mangoes.

Regarding our targeted metabolomics analysis aimed at phenolic compounds, only 4-coumaric acid, quercetin and ellagic acid transiently exhibited higher concentrations in chitosan-coated mango peels when compared to non-treated mangoes. In addition, our non-targeted metabolomics analysis involving chitosan-coated mangoes revealed higher concentrations of ellagic acid, ellagic acid

rhamnoside and trigalloyl glucopyranose, compounds known to be involved in the biosynthetic pathway of tannins.[37,38] Notably, there was a ca. 2000-fold concentration increase in trigalloyl glucopyranose in chitosan-treated mangoes when compared to mangoes treated with acetic acid (Supplementary Spreadsheet, [SS1]). The presence of an elevated concentration of phenolic compounds involved in the tannin pathway suggests that tannins may be partly responsible for the beneficial effects of chitosan treatments in mangoes. As plant phenolic compounds, tannins are known to reduce the growth of fungal pathogens[39] and inhibit fruit fly development.[40] Tannins have shown to be noxious to fruit fly development and are found in high concentrations in unripe mangoes, which are also less suitable for immature fruit fly development.[6] The effect of chitosan-coatings on the chemical composition of mangoes could partially explain the inhibition of *A. obliqua* egg eclosion and immature development. We also surmise that eggs and larvae could also have been harmed by an inefficient gaseous exchange, caused by the chitosan-coatings, as recorded in preliminary studies conducted by our group.

Chitosan coatings also had a significant and welcome effect in reducing the size of *C. gloeosporioides* lesions.[39] Therefore, chitosan treatments could greatly benefit 'Manila' mango growers by extending postharvest-shelf life by at least a week, possibly as much as 12 days. Positive effects of chitosan coatings on *C. gloeosporioides* and *Alternaria alternata* had previously been reported in the

mango cultivars 'Ataulfo' and 'Tommy Atkins' in Mexico[12,41] Alphonso in India[30] and Pakistan[42], and Nam Dok Mai in Thailand.[20]

4. Conclusions

We conclude that chitosan coatings (DA ~20% and DP ~400) can efficiently extend shelf-life, reduce *C. gloeosporioides* growth and inhibit *A. obliqua* development in the case of the highly flavorful and valued Manila mango cultivar, probably by enhancing the production of phenolic compounds such as ellagic acid. Further studies are needed to refine and combine this highly effective pest and disease management approach with other methods, as fruit ripening was retarded. This side effect would represent a potential problem for the commercialization of chitosan-treated fruit; however it may easily be overcome by using ethylene or other plant growth regulators (e.g., ethephon) that can enhance and uniformize the ripening processes in mangoes shortly before they are placed in shelves in stores.[43] Additional research is also required to determine exactly which tannins are biologically active against fruit fly eggs and first instar larvae at the concentrations present in chitosan-treated fruit, as this information could be used in breeding programs aimed at producing fruit fly-resistant cultivars.[8]

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Conflict of interest. All authors declare that they do not have any conflict of interest.

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Figure legends

Figure 1. (A) Color chart of 'Manila' mango peel and pulp discoloration process that depicts six ripening stages (Peel: 1 = green, 2 = green with yellow areas, 3 = breaking yellow, 4 = yellow with some green areas, 5 = yellow, 6 = orange; and Pulp: 1 = light yellow, 2 = yellow, 3 = bright yellow, 4 = yellow-orange, 5 = light orange, 6 = orange) and image of RGB space code conversion. Chitosan effect on **(B)** weight loss (%), **(C)** peel firmness (N) and **(D)** total soluble solids (TSS) (g kg^{-1}) of 'Manila' mangoes coated with distilled water (control), 5.6 mL L^{-1} acetic acid and 20 g L^{-1} chitosan (n = 3) and stored under 26 ± 1 °C, 67 ± 5% RH conditions. Bars and lines represent means and standard errors.

Figure 2. (A) Principal component analysis grouping based on chemical composition of 'Manila' mangoes after 1 and 5 days of 20 g L^{-1} chitosan (T1_Chitosan and T5_Chitosan) and 5.6 mL L^{-1} acetic acid (T1_Acetic and T5_Acetic) treatments. **(B)** Heatmap and hierarchical cluster analysis based on the whole metabolomes of mangoes after 1 and 5 days of 20 g L^{-1} chitosan (T1_Chitosan and T5_Chitosan) and 5.6 mL L^{-1} acetic acid (T1_Acetic and T5_Acetic) treatments.

Figure 3. (A) T1 and **(B)** T5 Chitosan (20 g L^{-1}) vs Acetic acid (5.6 mL L^{-1}) Volcano plots. Each point corresponds to a different m/z value. Red points indicate up-regulation (right) and down-regulation (left). Putative names are indicated. Detailed information on identification is shown in the Supplementary Spreadsheet (SS1).

Figure 4. Number (mean ± SE) of **(A)** pupae and larvae/fruit and **(B)** emerged adults of *A. obliqua* in 'Manila' mangoes coated with distilled water, 2.8 mL L^{-1} acetic acid, 5.6 mL L^{-1} acetic acid, 10 g L^{-1} chitosan and 20 g L^{-1} chitosan after 21 d of storage (n = 5). Pupae were kept in the laboratory at 26 ± 1 °C, 67 ± 5% RH.

Figure 5. Lesion development (mm^2) (mean ± SE) of 'Manila' mangoes inoculated with *C. gloeosporioides* that were kept in a laboratory with controlled ambient conditions at 26 ± 1 °C and 67 ± 5% RH. Fruit was coated after artificial inoculation with spore suspension. Post-inoculation treatments were distilled water, 2.8 mL L^{-1} acetic acid, 5.6 mL L^{-1} acetic acid, 10 g L^{-1} chitosan and 20 g L^{-1} chitosan followed by 8 d of storage (n = 4).

Figure 1A-D

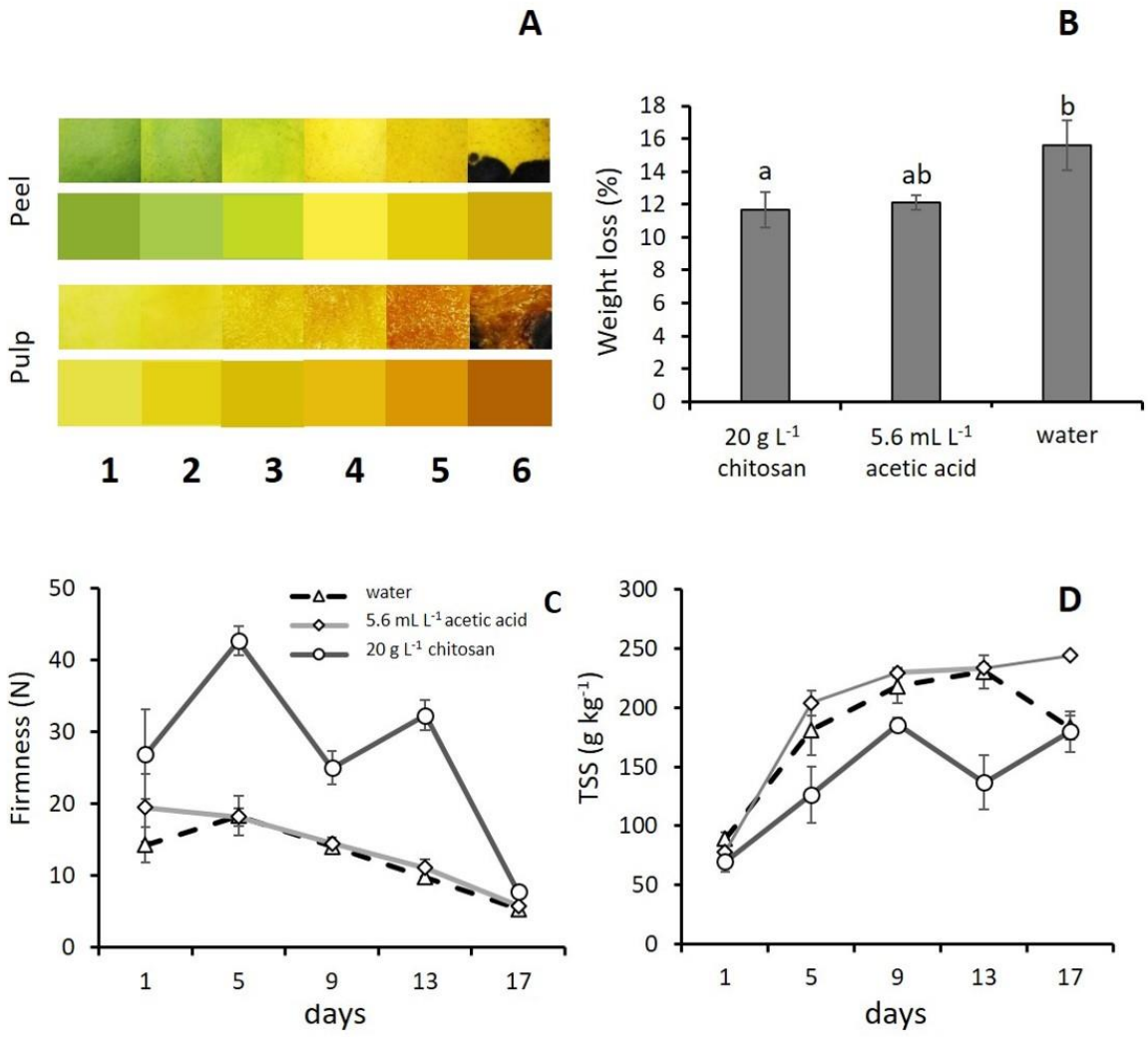
A
B
Peel
Pulp
1
2
3
4
5
6
Weight loss (%)
a
ab
b
20 g L-1 chitosan
5.6 mL L-1 acetic acid
water
C
D
water
5.6 mL L-1 acetic acid
20 g L-1 chitosan
Firmness (N)
TSS (g kg-1)
days
days

Figure 2A & B

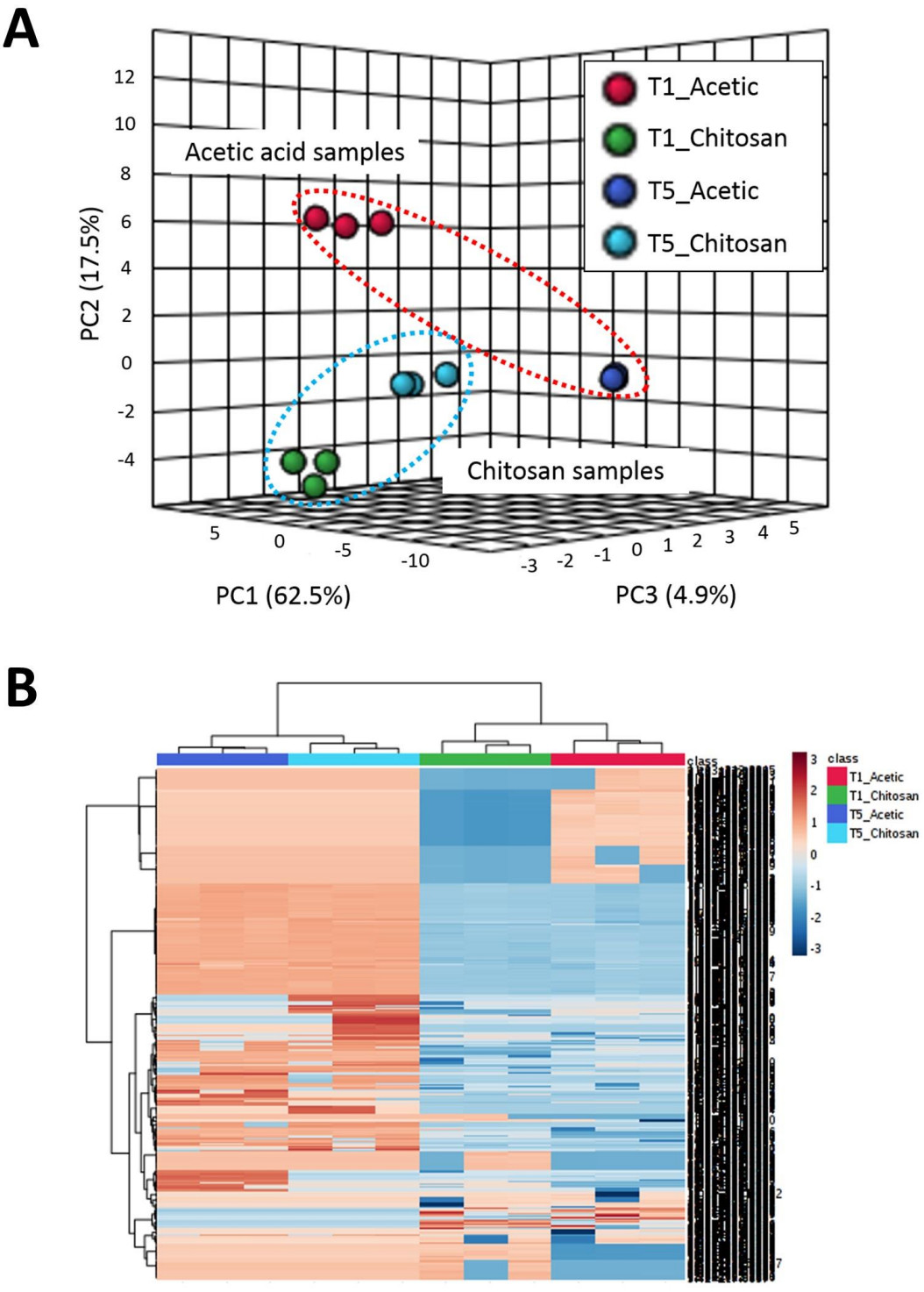
A
T1_Acetic
T1_Chitosan
T5_Acetic
T5_Chitosan
Acetic acid samples
Chitosan samples
PC2 (17.5%)
PC1 (62.5%)
PC3 (4.9%)
B
class
T1_Acetic
T1_Chitosan
T5_Acetic
T5_Chitosan

Figure 3A & B

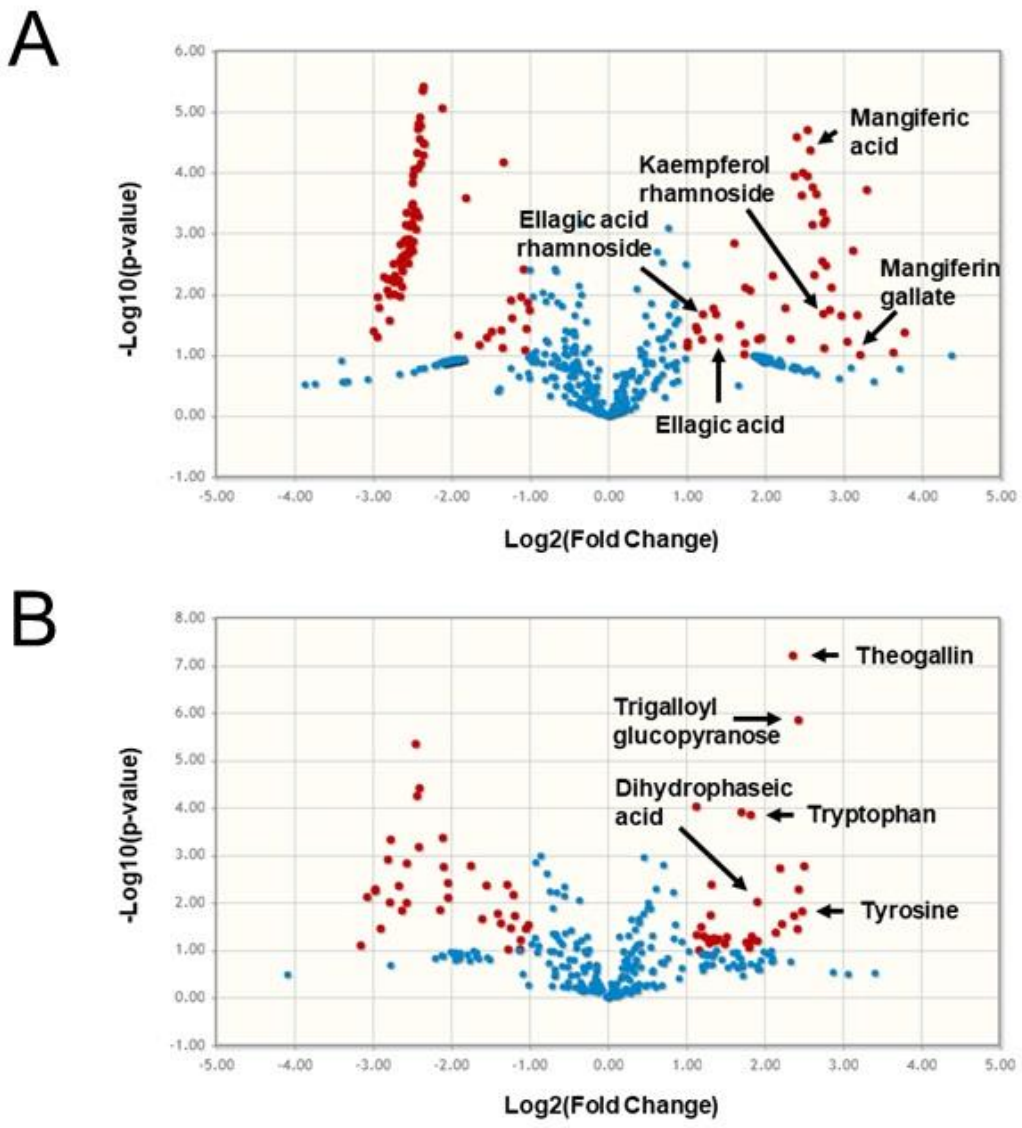
A
-Log10(p-value)
Log2(Fold Change)
Mangiferic acid
Kaempferol rhamnoside
Ellagic acid rhamnoside
Mangiferin gallate
Ellagic acid
B
-Log10(p-value)
Log2(Fold Change)
Theogallin
Trigalloyl glucopyranose
Dihydrophaseic acid
Tryptophan
Tyrosine

Figure 4A & B

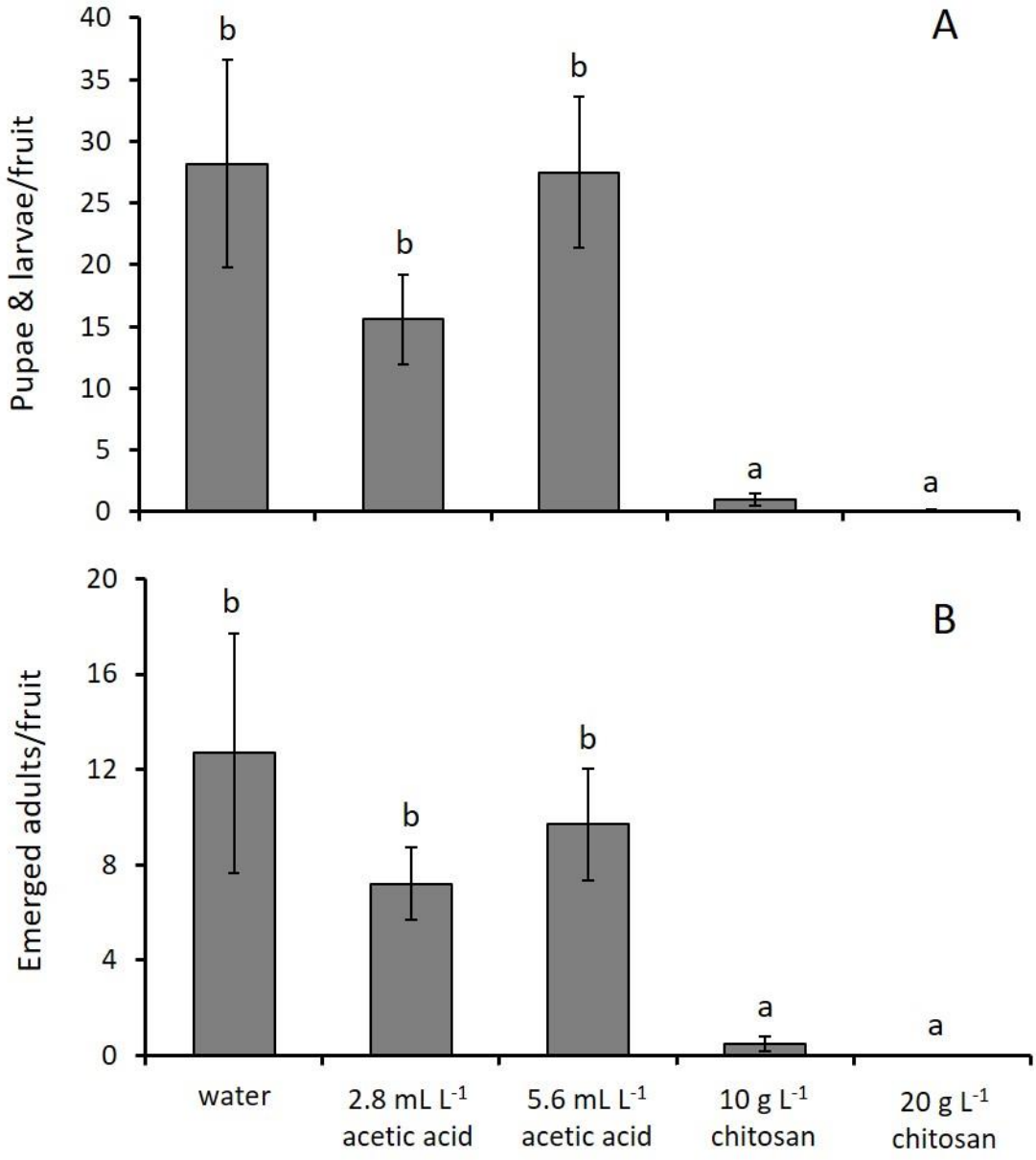
A
Pupae & larvae/fruit
40
35
30
25
20
15
10
5
0
b
b
b
a
a
B
Emerged adults/fruit
20
16
12
8
4
0
b
b
b
a
a
water
2.8 mL L-1 acetic acid
5.6 mL L-1 acetic acid
10 g L-1 chitosan
20 g L-1 chitosan

Figure 5.

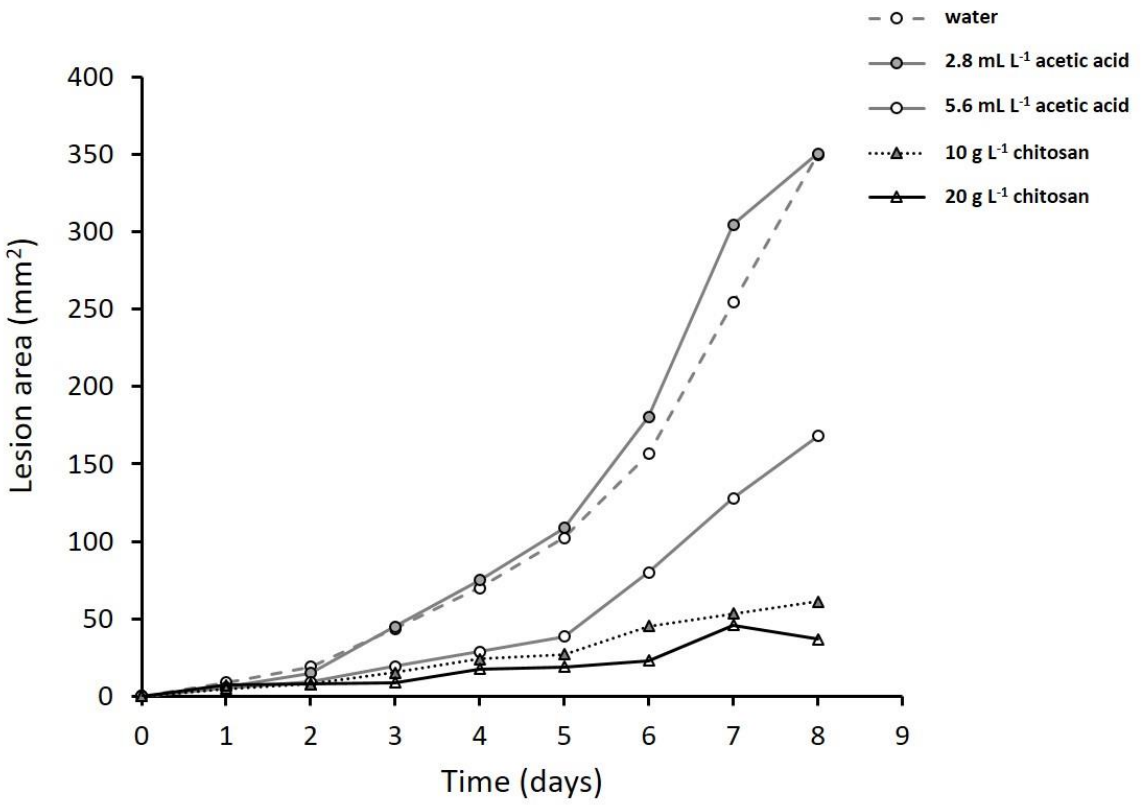
water
2.8 mL L-1 acetic acid
5.6 mL L-1 acetic acid
10 g L-1 chitosan
20 g L-1 chitosan
Lesion area (mm2)
Time (days)
0
50
100
150
200
250
300
350
400
0
1
2
3
4
5
6
7
8
9

Table 1. Changes in lightness/L*, green-red axis/a*, blue-yellow axis/b* colorimetric parameters of distilled water, 5.6 mL L^{-1} acetic acid and 20 g L^{-1} chitosan-coated 'Manila' mangoes internal ripening during storage (26 ± 1 °C, 67 ± 5% RH) * (n = 3)

Treatment	Color space	Day 1	Day 5	Day 9	Day 13	Day 17
Water	*L**	86.31	72.10	71.57	59.69	71.04
	*a**	-15.16	10.49	8.31	28.14	10.81
	*b**	82.02	75.35	74.73	66.08	74.11
acetic acid	*L**	86.63	83.16	73.10	74.29	74.29
	*a**	-15.51	-5.96	8.38	0.66	0.66
	*b**	84.95	83.38	76.03	76.37	76.37
chitosan	*L**	85.10	86.61	82.90	85.00	85.04
	*a**	-14.09	-13.40	-10.71	-14.34	-14.34
	*b**	81.52	74.51	81.57	79.86	79.86

* CIE L*a*b* (CIE, 1998)

Table 2. Concentration of specific phenolic compounds (µg g^{-1} dry matter) in 'Manila' mangoes immersed in water, 5.6 mL L^{-1} acetic acid and 20 g L^{-1} chitosan solutions (n = 3)

Phenolic compounds	Time point (d)	water	acetic acid	chitosan	*P* value
4-Coumaric acid	1	0.26 ± 0.01 (a)	0.06 ± 0.10 (b)	0.74 ± 0.18 (c)	***P* < 0.001**
	5	0.00	0.00	0.00	NA
	9	0.00	0.00	0.00	NA
	13	0.00	0.00	0.00	NA
(+)-Catechin	1	19.65 ± 16.05 (a)	3.56 ± 6.17 (a)	0.00 ± 0.00 (a)	*P* = 0.11
	5	17.88 ± 7.49 (a)	31.93 ± 21.99 (a)	12.50 ± 9.94 (a)	*P* = 0.31
	9	9.26 ± 14.71 (a)	36.10 ± 24.33 (a)	5.79 ± 6.82 (a)	*P* = 0.13
	13	28.26 ± 15.35 (a)	27.80 ± 24.45 (a)	3.76 ± 3.27 (a)	*P* = 0.20
Mangiferin	1	198.94 ± 129.02 (a)	92.16 ± 60.46 (a)	87.46 ± 37.99 (a)	*P* = 0.26
	5	169.15 ± 67.60 (a)	152.97 ± 150.73 (a)	196.90 ± 84.55 (a)	*P* = 0.88
	9	17.14 ± 17.48 (a)	98.24 ± 57.06 (b)	54.01 ± 26.07 (a)	*P* = 0.01
	13	219.36 ± 279.74 (a)	83.20 ± 82.91 (a)	71.84 ± 48.44 (a)	*P* = 0.54
Quercetin	1	7.40 ± 2.74 (a)	0.00 ± 0.00 (a)	28.39 ± 2.91 (b)	***P* < 0.001**
	5	0.80 ± 1.38 (a)	0.51 ± 0.89 (a)	0.00 ± 0.00 (a)	*P* = 0.61
	9	6.20 ± 10.73 (a)	1.35 ± 2.33 (a)	6.02 ± 7.47 (a)	*P* = 0.70
	13	0.57 ± 0.99 (a)	1.28 ± 2.21 (a)	0.00 ± 0.80 (a)	*P* = 0.56
Quercetin 3 - O - galactoside	1	215.12 ± 35.54 (a)	187.21 ± 111.05 (a)	250.78 ± 15.20 (a)	*P* = 0.54
	5	150.14 ± 7.50 (a)	197.71 ± 123.98 (a)	133.19 ± 81.30 (a)	*P* = 0.66
	9	79.81 ± 34.33 (a)	180.64 ± 82.72 (a)	96.52 ± 28.17 (a)	*P* = 0.13
	13	83.48 ± 25.50 (a)	51.12 ± 44.87 (a)	114.05 ± 26.40 (a)	*P* = 0.15
Quercetin 3 - O - glucoside	1	167.98 ± 41.00 (a)	119.41 ± 86.33 (a)	164.38 ± 21.30 (a)	*P* = 0.54
	5	108.09 ± 6.30 (a)	132.28 ± 80.34 (a)	111.21 ± 76.80 (a)	*P* = 0.54
	9	55.99 ± 35.57 (a)	133.80 ± 62.41 (a)	62.96 ± 14.38 (a)	*P* = 0.13
	13	88.75 ± 16.66 (a)	60.29 ± 58.74 (a)	76.48 ± 8.57 (a)	*P* = 0.64
Luteolin-7-O-glucoside	1	2.50 ± 1.79 (a)	1.34 ± 2.32 (a)	1.85 ± 1.39 (a)	*P* = 0.28
	5	0.00 ± 0.00 (a)	0.00 ± 0.00 (a)	0.72 ± 1.19 (a)	*P* = 0.15
	9	0.00 ± 0.00 (a)	0.43 ± 0.74 (b)	0.00 ± 0.00 (a)	*P* < 0.05
	13	0.00	0.00	0.00	NA

Mean (± SD) followed by the same letters in a row are not significantly different by Fisher´s Least Significant Difference (LSD). Each of three replicates had three individually analyzed samples.

NA: Not applicable.

Table 3. Mean (± SD) concentration of abscisic acid, jasmonic acid and ellagic acid (µg/g dry matter) in the peel of 'Manila' mangoes (n = 3) coated with 5.6 mL L^{-1} acetic acid and 20 g L^{-1} chitosan. Fruit were stored 13 days at 26.16 °C and 37.29% RH ± 1. Results were obtained one and five days after fruit was coated with chitosan.

Treatment	Day 1	Day 5
Compound		
Acetic acid		
Absicic acid	0 ± 0 (aA)	34.71 ± 8.68 (bA)
Jasmonic acid	0 ± 0 (aA)	13.51 ± 6.44 (bA)
Ellagic acid	4,493.71 ± 210.44 (aA)	0 ± 0 (bA)
Chitosan		
Absicic acid	0 ± 0 (aA)	7.12 ± 4.42 (bB)
Jasmonic acid	0 ± 0 (aA)	0.69 ± 0.64 (bB)
Ellagic acid	31,806.12 ± 21,077.48 (aB)	0 ± 0 (bA)

Means (± SD) followed by the same lowercase letters represent significant difference between time points ($P < 0.05$). Different letters in uppercase represent significant difference among treatments ($P < 0.05$) by Fisher´s Least Significant Difference (LSD). Each of three replicates had three individually analyzed samples.