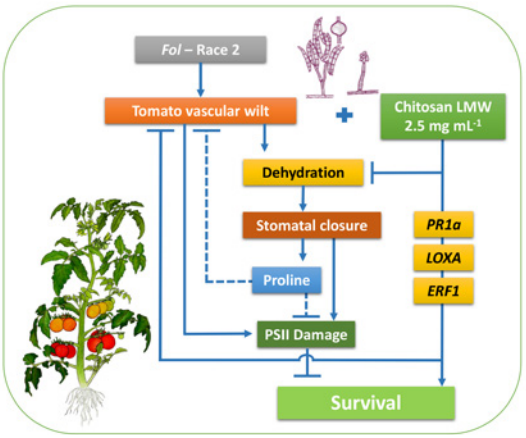
Doi: https://doi.org/10.17584/rcch.2021v15i3.12822

Protection of tomato plants against *Fusarium oxysporum* f. sp. *lycopersici* induced by chitosan

Protección de plantas de tomate frente a *Fusarium oxysporum* f. sp. *lycopersici* inducida por quitosán



Model that describes the induction of resistance by chitosan in the tomato-*Fol* pathosystem.

Source: elaborated by the authors

SANDRA L. CARMONA[1]
ANDREA VILLARREAL-NAVARRETE[1]
DIANA BURBANO-DAVID[1]
MAGDA GÓMEZ-MARROQUÍN[1]
ESPERANZA TORRES-ROJAS[2]
MAURICIO SOTO-SUÁREZ[1, 3]

ABSTRACT

Physiological processes of plants infected by vascular pathogens are mainly affected by vascular bundle obstruction, decreasing the absorption of water and nutrients and gas exchange by stomatal closure, and inducing oxidative cascades and PSII alterations. Chitosan, a derivative of chitin present in the cell wall of some organisms including fungi, induces plant defense responses, activating systemic resistance. In this study, three chitosan molecules (low, medium and high molecular weight) at different concentrations (0.5, 1, 1.5, 2, 2.5 and 3 mg mL^{-1}) were assessed by *in vitro* tests against *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). Concentrations higher than 1 mg mL^{-1} were found to inhibit significantly the mycelial growth of *Fol*, with 95.8% of inhibition using chitosan with high molecular weight (3 mg mL^{-1}). For *in planta* assays, chitosan treatment (low molecular weight 2.5 mg mL^{-1}) showed significantly lower incidence and severity of wilting disease symptoms, 70 and 91%, respectively, compared to healthy plants used as a negative control. The effect of chitosan on the physiological and molecular responses of tomato plants infected with *Fol* was studied, evaluating the maximum potential quantum efficiency of PSII photochemistry (Fv/Fm), photochemical efficiency of PSII (Y(II)), stomatal conductance (g_s), relative water content (RWC), proline content, photosynthetic pigments, dry mass, and differential gene expression (*PAL, LOXA, ERF1,* and *PR1*) of defense markers. A reduction of 70% in the incidence and 91% in the severity of the disease was achieved in plants treated with chitosan, mitigating the damage caused by *Fol* on Fv/Fm, Y(II), and chlorophyll contents by 23, 36, and 47%, respectively. Less impact

[1] Corporación Colombiana de Investigación Agropecuaria (Agrosavia), Centro de Investigación Tibaitatá, Mosquera (Colombia). ORCID Carmona, S.L.: 0000-0001-7348-3566; ORCID Villarreal-Navarrete, A.: 0000-0001-9580-6936; ORCID Burbano-David, D.: 0000-0003-0109-3088; ORCID Gómez-Marroquín, M.: 0000-0002-7487-0805; ORCID Soto-Suárez, M.: 0000-0002-2392-2839
[2] Universidad Nacional de Colombia, Facultad de Ciencias Agrarias, Bogota (Colombia). ORCID Torres-Rojas, E.: 0000-0002-3682-0372
[3] Corresponding author. msoto@agrosavia.co

was observed on g_s, RWC, and dry mass (55, 11, and 26%, respectively). Chitosan-treated and *Fol*-infected plants over-expressed *PR1a* gene suggesting a priming-associated response. These results demonstrate the high potential of chitosan to protect tomato plants against *Fol* by regulating physiological and molecular responses in tomato plants.

Additional key words: vascular wilt; resistance induction; priming; photosynthesis; differential gene expression.

RESUMEN

Los procesos fisiológicos de plantas infectadas por patógenos vasculares, se afectan principalmente por el taponamiento de los haces vasculares, disminuyendo la absorción de agua y nutrientes, el intercambio de gases por cierre estomático e induciendo cascadas oxidativas y alteraciones en el PSII. El quitosán induce la señalización y defensa de la planta, activando la resistencia sistémica. Se determinó la capacidad de inhibición de crecimiento *in vitro* de *Fol* de tres moléculas de quitosán (bajo, medio y alto peso molecular) a diferentes concentraciones (0.5, 1; 1,5; 2; 2,5 y 3 mg mL^{-1}). Las concentraciones mayores a 1 mg mL^{-1} inhibieron el crecimiento de *Fol*, alcanzando 95,8% de inhibición con quitosán de alto peso molecular (3 mg mL^{-1}). En plantas, el quitosán (bajo peso molecular 2,5 mg mL^{-1}) redujo en 70% la incidencia y en 91% la severidad de la enfermedad. Se evaluó el efecto del quitosán sobre la respuesta fisiológica de plantas de tomate infectadas por *Fol*, observando la Fv/Fm, Y(II), conductancia estomática (g_s), CRA, contenido de prolina, pigmentos fotosintéticos, masa seca y expresión diferencial de genes (*PAL, LOXA, ERF1* y *PR1*) relacionados con la defensa. Se obtuvo una reducción de 70% en la incidencia y 91% en la severidad de la enfermedad en plantas tratadas con quitosán, mitigando el daño del patógeno sobre la Fv/Fm, Y(II) y contenidos de clorofila a respectivamente en 23, 36 y 47%. Se observó menor impacto (55, 11 y 26% respectivamente) sobre la g_s, CRA y masa seca. Las plantas tratadas con quitosán e infectadas con *Fol*, expresaron una respuesta de defensa inducida por la sobreexpresión de los genes de defensa, principalmente el *PR1a*, en una respuesta asociada a *priming*. Esto comprueba la capacidad inductora de resistencia del quitosán en plantas de tomate, a través del efecto protector sobre procesos fisiológicos.

Palabras clave adicionales: marchitez vascular; inducción de resistencia; *priming*; fotosíntesis; expresión diferencial de genes.

Received: 09-05-2021 **Accepted:** 19-06-2021 **Published:** 30-07-2021

INTRODUCTION

Fusarium oxysporum is the causative agent of vascular wilt and root rot in plants and is part of a complex of more than 120 *formae speciales* (ff. spp.) that differ in their ability to infect certain hosts (Dean *et al.,* 2012). *F. oxysporum* f. sp. *lycopersici* (*Fol*) causes significant economic losses in tomato production (Solanki *et al*., 2015). This pathogen can produce resilient spores called chlamydospores that may persist in the soil for more than 20 years (Michielse and Rep, 2009; Dean *et al.,* 2012).

Fol colonizes the elements of the xylem and causes the formation of gums and tylose, obstructing and increasing resistance in rising water, and decreasing the xylem and leaf water potential (Chekali *et al.,* 2011; Yadeta and Thomma 2013; Srinivas *et al.,* 2019). Consequently, stomatal closure increases, and CO_2 absorption is reduced in detriment of photosynthetic activity, generating decreases in the quantum efficiency of photosystem II (PSII). These disorders end up affecting the biomass accumulation capacity of the plant and, overall, resemble those caused by water stress (Lorenzini *et al.,* 1997; Nogués *et al.,* 2002; Yadeta and Thomma, 2013).

Light photon reception and electron transport must be regulated to maintain energy production and consumption balance. In high radiation conditions,

excess energy must be dissipated to avoid photodamage to the chlorophyll reaction centers (PSI and PSII). Thus, the energy perceived by the PSII can be absorbed and redirected towards photochemical processes such as photolysis and synthesis of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate ($NADPH^+$); this process is called photochemical quenching (qP). Meanwhile, excess energy is harmlessly dissipated as heat (non-photochemical quenching) or fluorescence, to avoid damage to the leaf. In this way, chlorophyll fluorescence is one of the best indicators for detecting early stress in plants since damage to PSII is avoided, and photosynthetic activity is preserved through energy dissipation as an acclimatization mechanism (Pérez-Bueno *et al.,* 2019).

The chlorophyll fluorescence parameter most broadly evaluated is the maximum potential quantum efficiency of PSII photochemistry (Fv/Fm); the stability of Fv/Fm value indicates the absence of stress. The photochemical efficiency of PSII (Y(II)) reveals the amount of energy being used in the photochemical phase of photosynthesis (Melgarejo *et al.,* 2010; Kardile *et al.,* 2019).

Resistance inducers can be synthetic substances, components derived from plants or microorganisms, as well as Microbial or Pathogen Associated Molecular Patterns (MAMPs or PAMPs) that induce a resistance response in the plant, triggering PTI (PAMP Triggered Immunity) (Vidhyasekaran, 2016). These resistance inducers trigger signaling through hormonal pathways (Salicylic Acid: SA, Jasmonic Acid: JA, and Ethylene: ET), changes in calcium concentrations, ubiquitin-dependent protein degradation, activation of G-proteins, and phosphorylation of Mitogen-Activated Protein Kinase (MAPKs), among others. Then transcription factors or epigenetic modifications regulate the transcriptional activity of Pathogenesis-Related (PR) genes that encode antimicrobial proteins and antioxidant substances to counteract the oxidative cascades triggered during the defense response (Walters *et al.,* 2013; Andersen *et al*., 2018).

One of the most studied resistance inducers in plants is chitosan (Orzali *et al.,* 2017), a derivative of chitin present in the cell wall of some fungi, yeasts, green algae, insects, and crustaceans. Chitosan is a linear polymer comprising partially deacetylated N-acetyl glucosamine (GlcNAc) subunits (Orzali *et al.,* 2017). If more than 50% of the GlcNAc residues are deacetylated at position 2 the heteropolymer is referred to as chitosan (Kappel *et al.,* 2020).

Two mechanisms have been documented by which chitosan protects plants against different phytopathogenic fungi: the first one is the direct action by inhibiting mycelial growth, sporulation, and germination of conidia, mainly through membrane destabilization and cell wall weakening by interrupting the β-1,3 glucan synthesis (Kumaraswamy *et al.,* 2018). The second mechanism is the induction of resistance in plants as a consequence of biochemical and physiological changes such as the production of Reactive Oxygen Species (ROS), the formation of callose deposits, and tissue strengthening (lignification and suberization) (Xing *et al.,* 2015; Vidhyasekaran, 2016).

Chitosan is widely used worldwide in agriculture through commercial formulations (Walters *et al*., 2005; Ávila-Orozco *et al*., 2017). This work aimed to evaluate the effect of chitosan treatment on tomato plants at the physiological and molecular parameters during their infection with *Fol*.

MATERIALS AND METHODS

Biological material. This work was carried out at Corporación Colombiana de Investigación Agropecuaria, Agrosavia (Mosquera, Cundinamarca). The *Fol*59 strain identified as *F. oxysporum* f. sp. *lycopersici* (*Fol*) Race 2 (Carmona *et al*., 2020) was used.

Chonto type tomato seeds of the Santa Cruz Kada variety (Impulsemillas®) were used, superficially disinfected with 2% sodium hypochlorite for 10 min, followed by 70% ethanol and sterile distilled water washes. The seeds were germinated on sterile peat. The seedbeds were maintained for 30 d as done on a commercial scale.

Chitosan preparation and *in vitro* inhibition of *Fol*59 growth by chitosan. A stock solution of 10 mg mL^{-1} of chitosan was prepared, adding 2 g of chitosan (Sigma-Aldrich®) in 200 mL of acidified water (1% acetic acid), and the pH was adjusted to 5.6 (Hernández-Lauzardo *et al*., 2008).

Three molecules of chitosan (Sigma - Aldrich®) of different molecular weight (low molecular weight – LMW, medium molecular weight – MMW, high

molecular weight – HMW) were used. Physicochemical characteristics of chitosan are described in table 1. PDA culture medium was prepared and supplemented with seven concentrations of chitosan (0.5, 1, 1.5, 2, 2.5, 3, and 4 mg mL^{-1}) and a commercial product based on poly-(D) glucosamine, that was used according to the manufacturer's recommendations. Unsupplemented PDA (pH 5.6) was used as a negative control.

A 5 mm disk was removed from the margin of 14-d-old *Fol*59 culture and transferred in the center of a Petri dish with a culture medium supplemented with each chitosan treatment. Five technical replicates were done, and the whole experiment was repeated three independent times (biological replicates). After incubation for 7 d at 25°C, the equation of You *et al*. (2016) was used to calculate the IPRG (inhibition percentage (%) on radial growth): *IPRG = (C2-C1/C2)*100, w*here *C2* is the mycelial growth area of *Fol* in the control treatment, and *C1* is the mycelial growth area with chitosan treatment.

Effect of chitosan on tomato vascular wilt. Thirty-day-old tomato plants were treated with 10 mL of chitosan applied to the soil at different concentrations of each molecular weight considered, 24 h before transplantation. During the transplant process, plants were inoculated through the root immersion method (Jelinski *et al*., 2017), using a *Fol*59 suspension of $1{\cdot}10^6$ conidia/mL. Plants immersed in sterile water were used as absolute control, and *Fol*59-infected plants untreated with chitosan were used as pathogenic control. Nutritional management was based in recommendations from a soil analysis, adding 1 g of 15-15-15 (NPK) per plant.

After transplanting, plants were kept at 30°C/d and 20°C/night, with 54% relative humidity under 12 h photoperiod with a light intensity of 90 μmol m^{-2} s^{-1}. The variables incidence, severity, and area under the disease progress curve (AUDPC) were evaluated from the onset of symptoms to 14 days after inoculation (DAI). The severity of the disease was established using a modified visual scale (Rongai *et al.,* 2017) from 0 to 5, where 0 is a healthy plant, and 5 is a dead plant. AUDPC was calculated using the equation: $AUDPC=\sum_i Y_i + Y_{i+1} /2* (t_{i+1} - t_i)$, where Y corresponds to the percentage of the disease, whether expressed as incidence or severity, while t is the time elapsed in days (Pedroza-Sandoval and Samaniego, 2009).

Physiological changes in tomato plants during the interaction with *Fol* and chitosan. For this test, the following treatments were evaluated: (i) Absolute control (plants treated with water), (ii) Chitosan (not infected with *Fol*59), (iii) Chtsn + *Fol* (application of chitosan, and after 24 h infection with *Fol*59), and (iv) Pathogen (Infection with *Fol*59). The concentrations of chitosan used in this experiment were selected according to the *in vitro* and *in planta* assays. Chitosan applications were made 24 h before inoculating the plants with *Fol*59.

Photochemical efficiency, stomatal conductance, and relative water content. The parameters Fv/Fm and Y(II) were evaluated. In the first case, a miniPAM II modulated fluorometer (Walz Germany®) was used. Stomatal conductance was measured at 3, 6, 9, 12, and 15 DAI employing an SC-1 porometer (Decagon®, USA) and the results were expressed as cm s^{-1}. Relative water content (RWC) was calculated using the equation by Melgarejo *et al.* (2010): $RWC=(mf-ms/mt-ms)*100$, where *mf* corresponds to fresh mass, *ms* is dry mass, and *mt* is full turgor mass, and the results were expressed as percentage of RWC at each day.

Proline. Proline content was established using the protocol taken from Bates *et al.* (1973). Briefly, proline

Table 1. Characteristics of the chitosan-based products used in *in vitro* and *in vivo* assays.

Product	LMW	MMW	HMW	Commercial product
Presentation	Fine powder	Small flakes	Fine powder	Soluble concentrate
Viscosity (cp-centipoise)	20-100	200-800	800-2,000	200-2,000
Degree of deacetylation (%)	75-85	75-85	>75	Non indicated
Molecular weight (kDa)	50-190	190-310	310-375	Non indicated
Manufacturer	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Bioagro

LMW, low molecular weight; MMW, medium molecular weight; HMW, high molecular weight

from 100 mg grounded leaf tissue was extracted using sulfosalicylic acid (3%), glacial acetic acid, acid ninhydrin and toluene. The absorbance was read at 520 nm employing a microplate spectrophotometer (Biotek®, USES). Proline was calculated using the equation given after calibrating a standard curve from 0-70 μg mL^{-1} of a proline standard solution: $y = 0.0097x + 0.0398$, with an $R^2 = 0.98$; the results were expressed in μg g^{-1} of fresh weight.

Photosynthetic pigments. Carotenoid and chlorophyll *a* and *b* contents were calculated in μg mL^{-1} after extraction using the protocol described by Rojas-Tapias *et al*. (2012). Briefly, pigments were extracted from 10 mg of fresh leaf tissue incubating on 1 mL of dimethyl sulfoxide (DMSO) for 2 h at 96°C. Absorbance was detected in a microplate spectrophotometer (Biotek®, USA) using wavelengths of 649 nm and 665 nm for chlorophylls, and 480 nm for carotenoids. The concentration was calculated according to the equations indicated by Wellburn (1994) as follows: C_a=12.19A_{665}-3.45A_{649}; C_b=21.99A_{649}-5.32A_{665}; C_{x+c}=(1,000A_{480}-2.14C_a-70.16$_b$)/220, with C_a is chlorophyll *a*, C_b is chlorophyll *b*, and C_{x+c} corresponds to carotenoids.

Dry mass. Complete plants (stem, leaves and roots) of 15 DAI were dried in an oven at 60°C for 48 h to establish the accumulation of dry mass. Results were expressed as g of dry mass per plant at 15 DAI.

Effect of chitosan on the expression of defense marker genes in tomato plants infected with *Fol*. Plants were first treated with chitosan, and 24 h after this treatment, the same plants were infected with *Fol*59, then after 48 h of *Fol*59 inoculation (72 h after chitosan treatment), the foliar part of the plants was collected, and quickly frozen in liquid nitrogen. Subsequently, total RNA was extracted using the protocol by Yockteng *et al.* (2013). The following treatments were assessed: (i) Control (plants treated with water), (ii) Chitosan (not infected with *Fol*), (iii) Chtsn+*Fol* (application of chitosan and subsequent infection with *Fol*59), and (iv) Pathogen (Infection with *Fol*59).

The iScript™ cDNA Synthesis Kit (Bio-Rad®) was used for cDNA synthesis, and the quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the iScript™ One-Step RT-PCR Kit (Bio-Rad®), marking with SYBR® Green (Bio-Rad®), following the manufacturers' recommendations. Defense-related genes involved in salicylic acid (SA) signaling (*PAL-PHENYLALANINE AMMONIA LYASE* and *PR1a- PATHOGEN RESPONSE1*), and Jasmonic Acid (JA) signaling (*LOXA- LIPOXYGENASE A*), and the Ethylene-Response gene (ET) (*ERF1-ETHYLENE RESPONSE FACTOR1*) were evaluated. The tomato gene *EF1a* (elongation factor) was used to normalize the expression. Each reaction was performed in triplicate with two biological replicates. ΔCT values were compared, as described by Soto-Suárez *et al.* (2017). The sequence for all primers used in this work are in table 2.

Experimental design and data analysis. All experiments were performed separately at least twice, according to a complete randomized blocks design with a one-plant experimental unit. Measurements were taken every 3 d until 14 DAI for AUDPC analysis and 15 DAI for physiological parameters. The non-destructive samplings had ten plants, and the

Table 2. Primers used in this study for qRT-PCR analysis.

Primer name	Sequence	Gene and signalling pathway	Reference
EF1a_F	GATTGGTGGTATTGGAACTGTC	*EF1a*, normalizing gene. Tomato elongation factor	Martínez-Medina *et al*. (2013)
EF1a_R	AGCTTCGTGGTGCATCTC		
ERF1_F	GAGGGGTCCTTGGTCTCTACTC	*ERF1*, response factor to ethylene and jasmonic acid	Huang *et al*. (2004)
ERF1_R	ACAGCAGCTGGAGATAATCCAT		
LOXA_F	GAAAAACCCCGATAAGGCAT	*LOXA*, Lipoxygenase A, induced by MeJA (Methyl Jasmonate)	León-Morcillo (2012)
LOXA_R	AGGAGACTCTCGTTGTCCGA		
PAL_F	CGTTATGCTCTCCGAACATC	*PAL*, Phenyl alanine ammonia lyase, SA biosynthesis	Martínez-Medina *et al*. (2013)
PAL_R	GAAGTTGCCACCATGTAAGG		
PR1a_F	GTGGGATCGGATTGATATCCT	*PR1a*, Inducible by salicylic acid	Martínez-Medina *et al*. (2013)
PR1a_R	CCTAAGCCACGATACCATGAA		

destructive samplings included five plants on each sampling day. Physiological variables were measured on the terminal leaflet of the third fully expanded leaf of each plant. The Statistix 8.0 software was used for data analysis. The normality of the data distribution was verified, and subsequently, a Kruskal-Wallis non-parametric test with $\alpha = 0.05$ was performed.

RESULTS

Effect of chitosan on *Fol* mycelial growth

All the molecules evaluated reduced the radial growth of *Fol*59 at a concentration of 1.5 mg mL^{-1} (Fig. 1). As the concentration of the chitosan in the culture medium increased, mycelial growth inhibition was higher (Tab. 3). Of the three molecules (LMW: low molecular weight, MMW: medium molecular weight, and HMW: high molecular weight), the LMW chitosan inhibited the growth of *Fol*59 at the lowest concentrations compared to the control (0.5 and 1 mg mL^{-1}) (Fig. 2).

Low concentrations of MMW, HMW and commercial chitosan promoted the growth of *Fol* (Tab. 1), and that fact can be related to the hormesis phenomenon, which is defined as the response of organisms to a toxic component, which in low concentrations produces a beneficial effect. This response is related to a

Table 3. Inhibition percentage on radial growth of Fol59 after 7 d of chitosan application at different concentrations and molecular weights.

Concentration (mg mL^{-1})	LMW	MMW	HMW
0.5	22.7±3.1 de	-21.3±5.3 d	-20.4±3.8 e
1	24.8±3.4 de	11.4±4.8 cd	5.6±3.9 de
1.5	65.5±1.5 cd	45.6±2.3 bcd	53.6±1.6 cde
2	81.5±0.7 bcd	72.1±1.9 abc	76.6±1.9 bcd
2.5	89.9±0.6 abc	83.8±0.8 ab	90.1±0.5 abc
3	92.4±0.4 ab	90.7±0.4 a	95.8±0.2 a
4	94.0±0.3 a	92.5±0.7 a	94.9±0.2 ab
Commercial	-24.5±5.1 e	-24.5±5.1 d	-24.5±5.1 e

± indicate the standard error (n=15). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis ($\alpha = 0.05$; $P = 0.0000$; df = 59; F = 66.4).

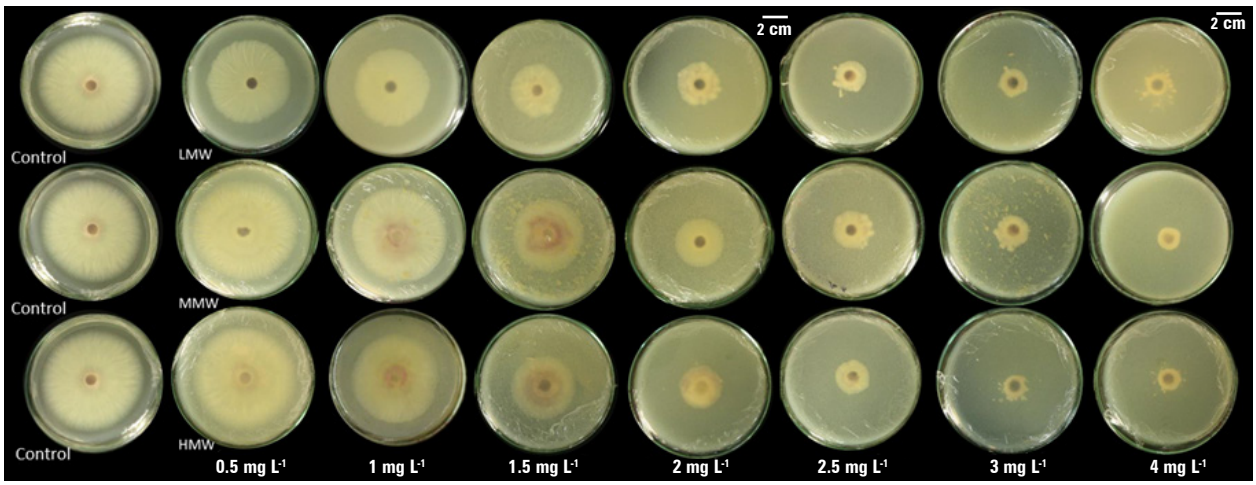


Figure 1. Different chitosan molecules in increasing concentrations inhibit *Fol*59 mycelial growth under in vitro conditions at seven days after incubation at 25°C.

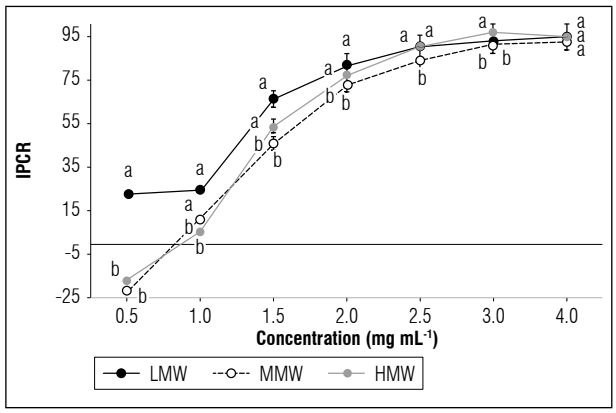


Figure 2. Different chitosan molecules in increasing concentrations inhibit *Fol*59 growth under *in vitro* conditions at 7 DAI (α= 0.05; *P* value=0.0000 df=59; F=66.4). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis (α = 0.05). Bars indicate the standard error (*n*=15).

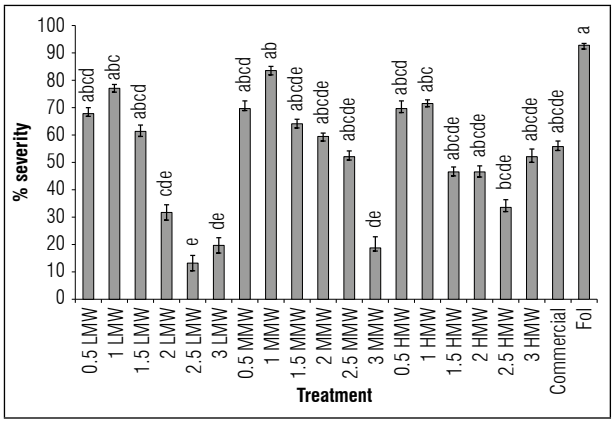


Figure 3. Results of the effect of chitosan severity of the disease 14 DAI in terms of percentage. The data are means of 40 replicates. Bars indicate the standard error (*n*=20). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis (α = 0.05).

metabolic overcompensation, triggered by an adaptive response to stress induced by a determined substance (Vargas-Hernandez *et al*., 2017).

Effects of chitosan on the reduction of vascular wilt caused by *Fol* in tomato plants

Disease severity in plants treated with LMW and MMW chitosan were significantly lower with the 2.5 and 3 mg mL^{-1} treatments (Fig. 3, α = 0.05; *P* = 0.0000; F = 10.2; df = 159) in comparison to the pathogen treatment. Regarding the efficacy of chitosan supplementation on the severity, the highest value reached was 91% (LMW: 2.5 mg mL^{-1}), followed by 85% (LMW: 3 mg mL^{-1}) and 78% (MMW: 3 mg mL^{-1}) (Tab. 4). In agreement with what was found *in vitro*, the low concentrations of chitosan were not able to effectively decrease the expression of the disease.

Table 4. Effect of chitosan on the AUDPC of incidence and severity, and the efficacy on incidence and severity in infected plants with *Fol*59.

Molecule	Concentration (mg mL^{-1})	Incidence		Severity	
		AUDPC	% efficacy	AUDPC	% efficacy
LMW	0.5	390 ±4.1 abcdef	5.0 ±3.9 bc	196 ±3.5 abcdef	20.6 ±3.3 bcd
	1	670 ±8.8 abc	0.0 ±0.0 c	318 ±4.3 abc	0.1 ±9.0 cd
	1.5	360 ±5.2 abcdef	20.0 ±5.0 abc	181 ±3.9 abcdef	33.3 ±3.6 abcd
	2	210 ±5.2 def	45.0 ±4.3 abc	84 ±3.8 def	61.2 ±2.8 abc
	2.5	80 ±6.0 f	70.0 ±5.5 a	28 ±3.8 f	90.9 ±1.2 a
	3	113 ±13.7 ef	67.5 ±2.2 a	45 ±7.1 ef	85.0 ±1.3 ab
MMW	0.5	583 ±7.1 abc	7.5 ±5.1 bc	284 ±5.7 abcd	7.9 ±3.0 cd
	1	715 ±3.0 ab	5.0 ±5.9 bc	371 ±2.9 ab	-17.0 ±8.2 d
	1.5	498 ±15.7 abcdef	12.5 ±4.8 abc	223 ±11.1 abcdef	14.6 ±6.1 abcd
	2	530 ±3.9 abcd	10.0 ±4.5 abc	237 ±3.3 abcde	26.2 ±2.4 abcd
	2.5	341 ±9.2 bcdef	15.0 ±5.6 abc	155 ±6.3 bcdef	43.0 ±3.7 abcd
	3	291 ±17.9 bcdef	50.6 ±2.1 ab	79 ±8.1 def	78.4 ±1.7 ab

to be continued

Molecule	Concentration (mg mL^{-1})	Incidence		Severity	
		AUDPC	% efficacy	AUDPC	% efficacy
HMW	0.5	503 ±6.1 abcde	12.5 ±4.8 abc	296 ±6.5 abcd	7.4 ±5.3 cd
	1	695 ±7.0 abc	5.0 ±3.9 bc	353 ±4.4 abc	-2.8 ±7.7 cd
	1.5	378 ±8.8 abcdef	17.5 ±3.7 abc	155 ±5.7 bcdef	51.5 ±3.0 abcd
	2	303 ±11.0 bcdef	22.5 ±5.7 abc	132 ±9.2 bcdef	58.6 ±2.8 abcd
	2.5	265 ±6.6 cdef	45.0 ±4.4 abc	120 ±5.1 cdef	60.3 ±3.8 acd
	3	363 ±6.1 abcdef	32.5 ±5.9 abc	180 ±6.1 abcdef	55.9 ±4.1 abcd
Commercial		350 ±7.3 bcdef	20.0 ±4.5 abc	164 ±5.8 abcdef	37.0 ±2.4 abcd
Pathogen		915 ±4.3 a	0 ±0.0 bc	504 ±3.8 a	0 ±0.0 cd
Control		0 ±0.0 f	100.0 ±0.0 a	0 ±0 f	100.0 ±0 a

± corresponds to standard error (n=40). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis (α = 0.05).

The results showed the protective effect of chitosan against vascular wilt (Fig. 4), and the best performance of the LMW (2.5 mg mL^{-1}) treatment; therefore, it was selected for the following tests.

Effect of chitosan on physiological parameters of tomato plants infected with *Fol*

The results on Fv/Fm and Y(II) indicate a reduction in photosynthetic processes due to the infection by *Fol*59 (Fig. 5). To investigate if chitosan can mitigate the early tomato stress conditions caused by *Fol*59, we measured photosynthesis efficiency (Fv/Fm). In this experiment, the Fv/Fm decreased in the infected plants from 9 DAI, observing a decrease of up to 70% at 15 DAI in plants inoculated with *Fol*59 compared to uninfected control plants (Fig. 5A). Contrastingly, in the Chtsn + *Fol* treatment (plants applied with chitosan 24 h before being infected with *Fol*), the Fv/Fm was 7.5% lower than the absolute control (control). In plants infected only with *Fol* (pathogenic treatment), the Fv/Fm were significantly different (P = 0.000; F = 15.3; df = 79).

Y(II) parameter decreased at 6 DAI in the *Fol* treatment, although with a slight rebound on day nine after inoculation (Fig. 5B). Subsequently, the Y(II) value for the plants infected with *Fol*59 decreased significantly (P = 0.000; F = 18; df = 79). Lower Y(II) values represent decreases in the energy flow destined for the ATP and NADPH production. Plants previously treated with chitosan and then infected with *Fol*59 (Chtsn + *Fol*) showed a lower decrease in Y(II) values (5%) compared to plants only infected with *Fol*59 (41%). The results obtained in Fv/Fm and Y(II) suggest an important role of the treatment with chitosan, mitigating the damage by *Fol* on the light phase of tomato photosynthesis.

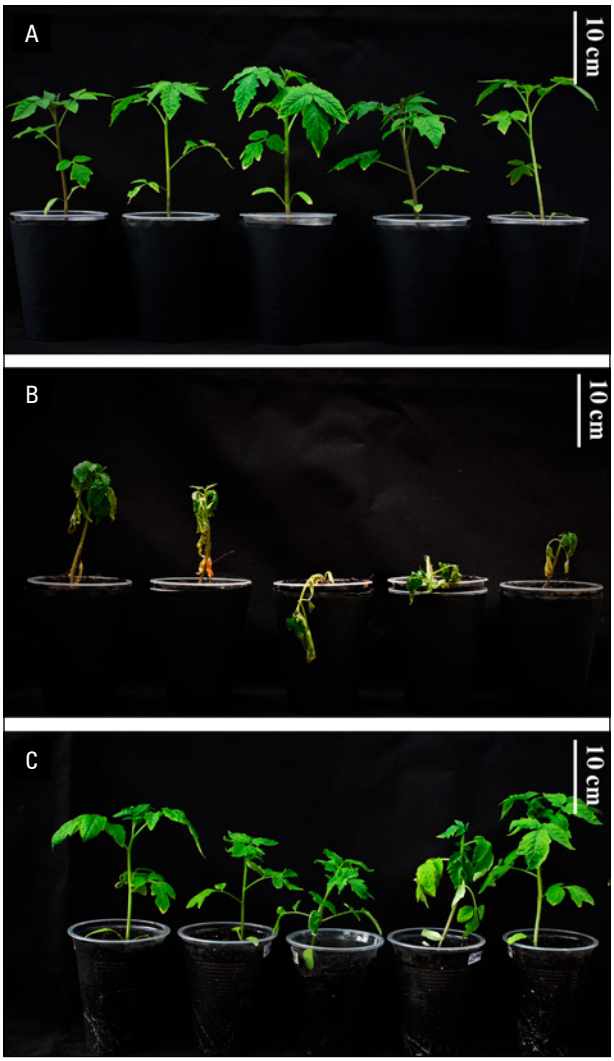


Figure 4. Visual symptoms of vascular wilt 14 DAI. A) Absolute control; B) Pathogen control (infected with *Fol*); C) Plants treated with chitosan (low molecular weight: 2.5 mg mL^{-1}) and inoculated with *Fol*59. Source: elaborated by the authors.

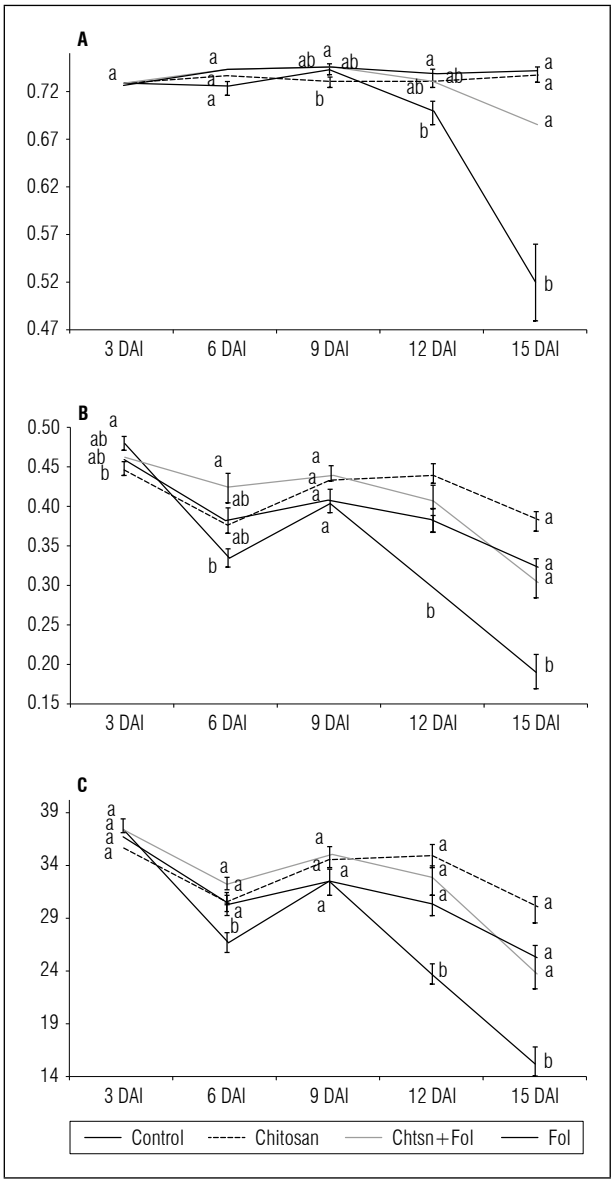


Figure 5. A) Fv/Fm progress for 15 d; B) Y(II) progress for 15 d; C) Electron Transfer Rate (ETR). Control (plants treated with water); Chitosan (not infected with *Fol*59), Chtsn + *Fol* (application of chitosan and subsequent infection with *Fol*59); *Fol* (Infected with *Fol*59). Bars correspond to the standard error (*n*=20). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis (α = 0.05).

Dry mass

The accumulation of biomass in the plant under stress conditions depends on the adequate use of energy for direct metabolic and photosynthetic processes, constituting an indicator of plant health (Fig. 6). To gain an insight into the effect of the chitosan application on tomato plant health infected with *Fol*59, the accumulation of biomass was evaluated.

Our results indicate that 15 DAI, *Fol*59 infection significantly affects the biomass accumulation in plants, decreasing from 36 and 62% in the Chtsn + *Fol* and *Fol* treatments, respectively, compared to the control (P = 0.000; F = 20; df = 79). The two treatments with *Fol*59 inoculated plants did not differ significantly between them.

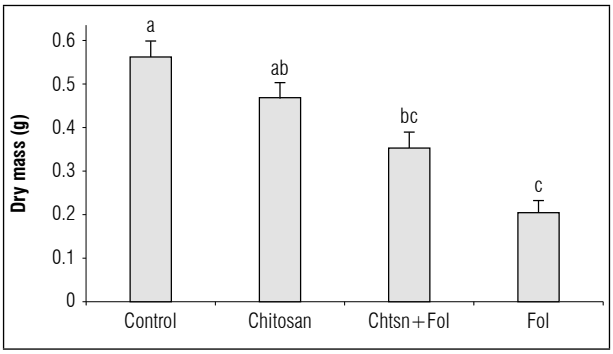


Figure 6. Dry mass accumulation 15 DAI in tomato plants with different treatments. Control (plants treated with water); Chitosan (not infected with *Fol*59), Chtsn + *Fol* (application of chitosan and subsequent infection with *Fol*59); *Fol* (infected with *Fol*59). Bars correspond to the standard error (*n*=20). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis (α = 0.05).

Stomatal conductance

One of the most well-known alterations produced during *Fol* infection is the difficulty by the plant in absorbing water and nutrients (Yadeta and Thomma, 2013), inducing stomatal closure. At 9 DAI, a 40% decrease in g_s in the *Fol* treatment compared to the Chtsn + *Fol* treatment (Tab. 6) is explained by the proliferation of the pathogen in the xylem. This trend was maintained until 15 DAI, where the g_s was 87% lower in the *Fol* treatment plants compared to the control treatment; meanwhile, in the Chtsn + *Fol* treatment, the reduction was 52% compared to the control. Despite not detecting significant differences between the *Fol* and Chtsn + *Fol* treatments, it seems that chitosan-treated plants show less hydric stress and less stomatal closure than the plants only infected with *Fol*59.

Relative water content (RWC)

The RWC remained stable in the first phase of the infection (6 DAI) and began to decrease at 9 DAI in plants infected with *Fol*59 (Tab. 6). It is important to note that after 9 DAI, the RWC was 10% higher in the chitosan treatment compared to the absolute control, suggesting that an optimization of the water use may be induced by chitosan. However, there were no significant differences between the inoculated treatments; therefore, in the Chtsn + *Fol* treatment plants, this effect does not seem sufficient to mitigate the water limitations caused by the pathogen.

Proline content

At 15 DAI, the proline content in the plants infected with *Fol*59 was nine times higher than the control, and in the Chtsn + *Fol* treatment, the values were six times higher compared to the control. The proline values were significantly higher in the treatments inoculated with *Fol*59 compared to those not inoculated (Tab. 5, $P = 0.000$; $F = 10.4$; $df = 39$).

Table 5. Proline content measured in the different treatments.

Treatment	Proline	
Control: plants treated with water	6.1	±0.69 b
Chitosan: not infected with *Fol*59	4.8	±0.72 b
Chitosan+*Fol*: application of chitosan and subsequent infection with *Fol*59	38.6	±8.35 a
Pathogen: infected with *Fol*59	54.4	±9.02 a

± corresponds to standard error ($n=10$). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis ($\alpha = 0.05$).

Chlorophyll content

The chlorophyll content is shown in the table 6 and was stable until 12 DAI. From 15 DAI in the plants of the pathogenic treatment, chlorophylls a and b showed a decrease of 39% to the control; meanwhile, in the Chtsn + *Fol* treatment, the contents remained stable without significant differences compared to the control (Tab. 6). The carotenoid content was not

Table 6. Physiological variables evaluated.

Treatment	Fv/Fm	qP	Y(II)	g_s	RWC (%)	Chlorophyll *a*	Chlorophyll *b*	Carotenoids
Day 3								
Control	0.729 ±0.003	0.682 ±0.014	0.463 ±0.008	- -	62.1 ±4.4	5.392 ±0.104	1.677 ±0.031	0.948 ±0.022
Chitosan	0.724 ±0.003	0.679 ±0.011	0.449 ±0.008	- -	58.4 ±2.6	4.711 ±0.147	1.528 ±0.043	0.811 ±0.025
Chitosan+*Fol*	0.725 ±0.004	0.710 ±0.011	0.469 ±0.008	- -	59.2 ±3.1	3.989 ±0.154	1.318 ±0.039	0.765 ±0.021
Pathogen	0.728 ±0.003	0.700 ±0.013	0.480 ±0.008	- -	61.5 ±2.9	4.836 ±0.078	1.584 ±0.024	0.783 ±0.016
Day 6								
Control	0.726 ±0.010	0.645 ±0.019	0.381 ±0.017	- -	77.4 ±7.2	5.899 ±0.079	1.959 ±0.027	0.768 ±0.010
Chitosan	0.739 ±0.003	0.644 ±0.014	0.375 ±0.008	- -	62.6 ±2.8	4.539 ±0.107	1.538 ±0.035	0.588 ±0.017
Chitosan+*Fol*	0.744 ±0.003	0.681 ±0.013	0.423 ±0.019	- -	65.8 ±4.2	6.170 ±0.061	2.102 ±0.018	0.858 ±0.014
Pathogen	0.725 ±0.010	0.610 ±0.014	0.336 ±0.012	- -	62.7 ±3.7	6.827 ±0.058	2.361 ±0.019	0.881 ±0.014
Day 9								
Control	0.743 ±0.004	0.663 ±0.017	0.407 ±0.014	878.81 ±44.55	70.2 ±4.6	5.038 ±0.114	1.759 ±0.040	0.672 ±0.019
Chitosan	0.732 ±0.003	0.683 ±0.027	0.431 ±0.012	928.36 ±69.43	77.7 ±2.8	4.225 ±0.068	1.491 ±0.022	0.547 ±0.016
Chitosan+*Fol*	0.746 ±0.003	0.694 ±0.010	0.441 ±0.011	525.02 ±86.27	57.0 ±3.1	6.122 ±0.098	2.211 ±0.034	0.795 ±0.015
Pathogen	0.744 ±0.004	0.624 ±0.021	0.405 ±0.016	315.01 ±110.83	51.7 ±2.7	5.988 ±0.122	2.128 ±0.039	0.837 ±0.020
Day 12								
Control	0.739 ±0.004	0.603 ±0.016	0.383 ±0.015	664.31 ±84.79	85.6 ±2.6	4.959 ±0.161	1.751 ±0.042	0.613 ±0.020
Chitosan	0.736 ±0.004	0.688 ±0.013	0.438 ±0.013	789.26 ±66.69	91.5 ±3.9	4.599 ±0.058	1.664 ±0.017	0.574 ±0.013
Chitosan+*Fol*	0.731 ±0.004	0.630 ±0.024	0.407 ±0.019	231.50 ±64.42	71.3 ±3.4	4.131 ±0.122	1.493 ±0.036	0.512 ±0.016
Pathogen	0.698 ±0.012	0.548 ±0.015	0.299 ±0.012	39.73 ±4.79	61.4 ±6.4	3.236 ±0.113	1.204 ±0.039	0.372 ±0.014
Day 15								
Control	0.742 ±0.003	0.519 ±0.015	0.322 ±0.012	272.57 ±30.97	74.6 ±5.1	6.002 ±0.154	2.182 ±0.055	0.661 ±0.020
Chitosan	0.738 ±0.003	0.617 ±0.015	0.383 ±0.013	290.84 ±28.00	81.0 ±4.5	6.880 ±0.147	2.479 ±0.048	0.827 ±0.031
Chitosan+*Fol*	0.687 ±0.027	0.537 ±0.021	0.304 ±0.020	131.91 ±20.54	67.1 ±5.5	6.500 ±0.150	2.264 ±0.050	0.884 ±0.027
Pathogen	0.519 ±0.039	0.453 ±0.032	0.190 ±0.021	33.09 ±3.56	59.2 ±5.1	3.674 ±0.199	1.332 ±0.061	0.594 ±0.039

Control, plants treated with water; Chitosan: not infected with *Fol*59; Chitosan+*Fol*: application of chitosan and subsequent infection with *Fol*59; Pathogen: infected with *Fol*59.

altered in any of the treatments during the experiment. The decrease in chlorophyll concentrations is consistent with the appearance of chlorosis in plants only infected with *Fol*.

Effect of chitosan on the expression of defense marker genes in tomato plants infected with *Fol*

The differential expression of the *PR1a, ERF1, PAL,* and *LOXA* genes in leaves and stems was evaluated after 72 h of chitosan application and 48 h after infection with *Fol*59 (Fig. 7). As an interesting result, in the chitosan treatment (without *Fol*59 inoculation), the *ERF1, LOXA,* and *PAL* genes were not differentially expressed compared to the control, while the *PR1a* gene was expressed 3.6 times more compared to the control.

For the Chtsn + Fol treatment, all genes were differentially expressed compared to the control. The *PAL*, *ERF1*, *LOXA*, and *PR1a* genes were expressed 1.8, 8.2, 12.2, and 14.2 times more, respectively, compared to the control plants. This result suggests that 72 h after applying chitosan and 48 h after *Fol59* inoculation, detection of the pathogen activates a systemic response that extends to the aerial part of the plant, even when the infection has not spread to that level. On the other hand, in the *Fol* treatment, the *PR1a* gene did not differ from the control (0.9 times more than the control), while the *ERF1*, *LOXA*, and *PAL* genes were expressed 2.2, 4.9, and 1.3 times more than the control.

DISCUSSION

The high *in vitro* growth inhibition of chitosan on *Fol*59 is probably due to the antimicrobial mechanisms of chitosan on fungi, affecting mainly cell walls by inhibition in glucan biosynthesis and destabilization of cell membranes (Xing *et al.,* 2015;

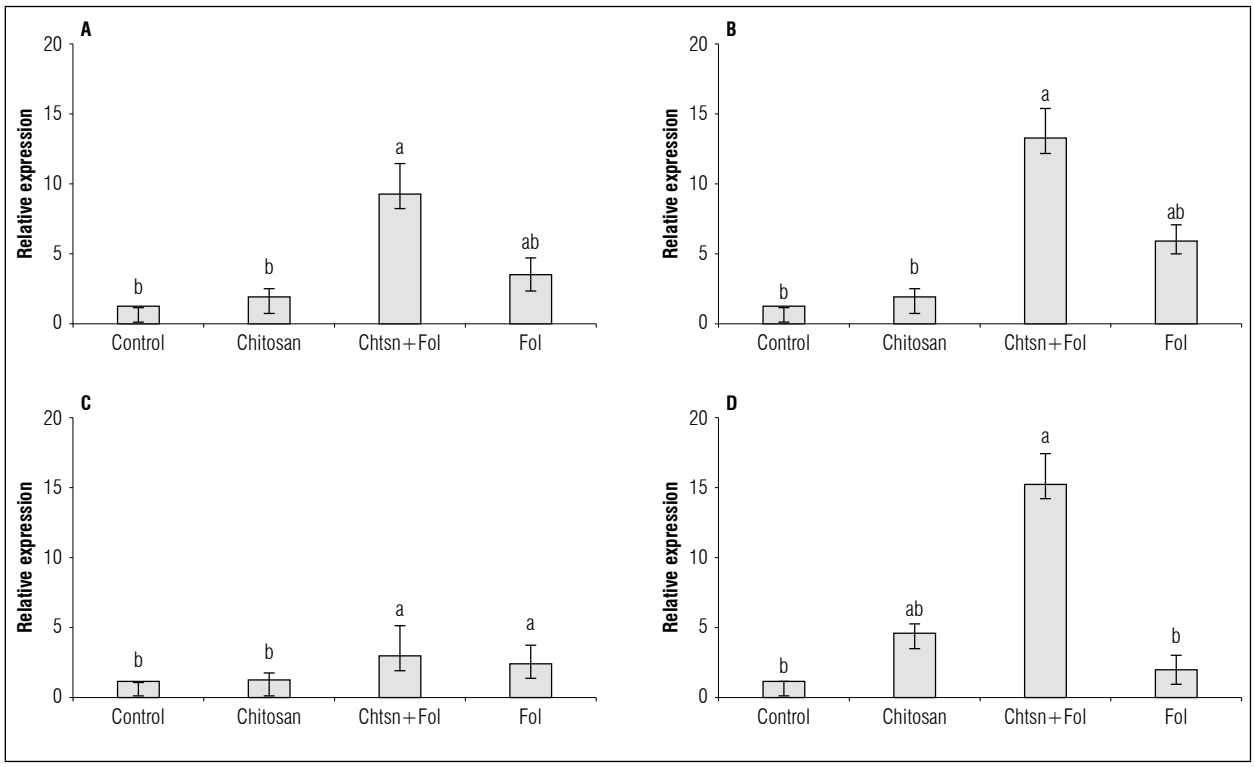


Figure 7. Differential expression of the defense genes A) *ERF1* (ET/JA) (*P* = 0.0002; F = 16.1; df = 17); B) *LOXA* (JA) (*P* = 0.0000; F = 30.3; df = 17); C) *PAL* (SA) (*P* = 0.0002; F = 16.1; df = 17), and D) *PR1a* (SA) (*P* = 0.0221; F = 4.96; df = 17) in tomato plants subjected to different treatments. Standardized data with respect to the control (= 1). Control (plants treated with water); Chitosan (not infected with *Fol59*), Chtsn + *Fol* (application of chitosan and subsequent infection with *Fol59*); *Fol* (infected with *Fol59*). Bars correspond to the standard error (*n*=6). Equal letters indicate that there are no statistically significant differences according to Kruskal-Wallis (α = 0.05).

Kumaraswamy *et al.,* 2018). In our research, chitosan exerts antifungal activity *in vitro*. El-Mohamedy *et al*. (2019) and Soliman and El-Mohamedy (2017) found that HMW and LMW chitosan effectively inhibited the growth of fungal pathogens and that this inhibition increased depending on the concentration. Besides, the results indicated that growth and sporulation decreased significantly when the LMW molecule was used compared to the HMW molecule.

The efficacy of LMW chitosan was consistent *in vitro* and *in planta* assays and, in accordance with other studies for plant pathogens, including *F. oxysporum* (Tikhonov *et al.,* 2006; Zhang *et al.,* 2017), highlighting the physicochemical properties of chitosan and its ability to induce resistance in plants against phytopathogens (Orzali *et al.,* 2017). The process is presumed to occur by binding the chitosan molecule to the plant cell membrane, initiating signaling by hydrogen peroxide (H_2O_2) and nitric oxide (NO) in photosynthetic plastids (Kauss *et al.,* 1989), triggering antioxidant substance synthesis and abscisic acid (ABA), which ends in the induction of stomatal closure, gene activation and other responses related to biotic stress (Zhang, 2004).

Fv/Fm has been widely used as a stress indicator in plants, and its values decrease according to the severity of the stress. This parameter represents a measure of the photon absorption capacity by PSII intended to reduce plastoquinone A, and is a sensitive indicator of plant photosynthesis, whose optimal values are around 0.83 in stress-free plants (standard for a wide variety of plant species) (Maxwell and Johnson, 2000). Decreases in Fv/Fm values are a clear indicator of severe stress that is causing damage to the photosynthetic apparatus (Goltsev *et al.,* 2016). Plants inoculated with *F. oxysporum,* with a severity level of 92%, (Wagner *et al.,* 2006) reported a decrease in Fv/Fm to 0.343, highlighting the relationship between this variable and disease stress. In this work, the fact of not finding significant differences in the Fv/Fm in untreated plants (absolute control) compared to plants only treated with chitosan, reinforces the potential of using chitosan as an alternative *Fol* control without the appearance of phytotoxic effects.

In this work, the reduction in Fv/Fm occurred after 9 DAI, long after the first symptoms appeared (6 DAI), suggesting that in the initial stages of infection, no irreversible damage occurs in the PSII and that this happens as a consequence of the xylem obstruction by the pathogen and a decrease in stomatal conductance. Segarra *et al*. (2010) described a decrease of 24% in Fv'/Fm' after 31 d of inoculation with *Fol*.

Furthermore, according to our results, *Fol* seriously compromises the photochemical processes of photosynthesis through the energy flow. This result is similar to the one reported by Nogués *et al*. (2002), where *Fol* infection decreased by 50% the Y(II) in tomato plants. Besides, decreases in Fv/Fm and electron transfer rate (ETR, Supplementary file 1: Fig. 3) were found in the current study, leading to a decrease in ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity. In this sense, Pshibytko *et al*. (2006) attributed this result to a decrease in electron transport due to decreases in the acceptor flux in quinone A (QA) of the PSII.

The symptoms caused by *Fol* infection have previously been related to disorders caused by water stress (Duniway, 1971); for example, decreases in the photosynthetic rate of infected plants have been correlated with the consequences of the decrease in g_s, such as the water state and gas exchange (CO_2 absorption) (Lorenzini *et al.,* 1997; Nogués *et al.,* 2002). Segarra *et al*. (2010) reported that one of the first responses of tomato plants in diseases caused by *Fusarium* is stomatal closure; with this reaction, the plant seeks to keep its water state stable under different conditions; however, if these conditions are prolonged, alterations occur in other processes. Our study suggests that in the treatments where the plants were inoculated with *Fol*, water limitations occurred due to vascular obstructions, so the plants responded by closing their stomata, avoiding water loss.

The increase in the concentration of proline in the tissues 15 DAI seems to be more an indicator of severe stress (Alsamir *et al*., 2017). In agreement with the g_s and RWC parameters, the increase in proline content occurred as a response to water shortage to maintain an osmotic adjustment in the leaves. Thus, the reductions in g_s and RWC found in *Fol* infected tomato plants cause a decrease in energy flow observed in the fluorescence variables during the development of vascular wilt (Pshibytko *et al*., 2006).

The maintenance of chlorophylls integrity by chitosan suggests that after its application, protection mechanisms of the apparatus and photosynthetic pigments are activated in the plant. Similar results were obtained in other studies where the photosynthetic performance (30-60%) and the concentrations of chlorophylls and carotenoids were higher (30-74%)

in plants that were treated with chitosan compared to their controls (Dzung *et al.,* 2011; Van *et al.,* 2013). According to our results, the chitosan application activates protective mechanisms such as the accumulation of proline, contributing in part to the protective capacity of membranes and proteins (Soliman and El-Mohamedy, 2017). All the defense processes of the plant require the use of energy so that in addition to the direct alterations of the pathogen in the plant (i.e., water flow, CO_2 uptake and assimilation, oxidative stress, and photosynthetic damage) (Yadeta and Thomma, 2013), the metabolic cost of defense due to the diversion of resources causes a significant decrease in biomass accumulation (Huot *et al.,* 2014).

The results obtained in the expression of defense genes are congruent with those reported in other works where the expression of various defense genes occurs after 72 h of treatment with chitosan and other resistance inducers in tomato plants (Jamiołkowska, 2020). These results were related to a lower expression of the disease caused by *R. solanacearum* and *Fol*, indicating that chitosan induces priming of plant defenses (Zehra *et al.,* 2017).

The *PR1a* protein has been reported to have antimicrobial properties against different pathogens and it is synthesized as part of the SAR response in plants, associated with the accumulation of SA (Jia *et al.,* 2016). In other pathosystems such as tomato-*Fusarium andiyazi*, chitosan activates the *PR1* and *SOD* genes, indicating SA-mediated defense and antioxidant response co-occur (Chun and Chandrasekaran, 2019); similarly, in the kiwi-*Pseudomonas syringae* pv. *actinidiae* pathosystem, the application of chitosan induced 3.5 times the expression of the *PR1* gene when compared to the control (Beatrice *et al.,* 2017). Knowing that *Fol* is a hemibiotrophic pathogen, the defense response of the plant is initially directed towards the biotrophic phase of the pathogen (mediated by the SA pathway) (Chowdhury *et al.,* 2017). The gene expression results indicate that chitosan presumably induces the plant to strengthen its immune system in presence of pathogens in a primming-like response, highlighting the potential of chitosan as a preventive, biocompatible, and non-toxic strategy for disease management (Maluin and Hussein, 2020).

CONCLUSION

The disease control capacity expressed by chitosan LMW (2.5 mg mL^{-1}), under favorable conditions for the establishment of the pathogen, reveals that this molecule has a high control potential against *Fol*. The physicochemical characteristics of chitosan and its biodegradability, together with the results presented here, making chitosan an excellent candidate for its use in sustainable disease management and clean agricultural production. This study validates chitosan as a strong candidate to produce an agroecologically sustainable bioproduct, for controlling *Fol* on tomato crops from specific microenvironments in Colombia.

Conflict of interests: The manuscript was prepared and reviewed with the participation of the authors, who declare that there exists no conflict of interest that puts at risk the validity of the presented results.

BIBLIOGRAPHIC REFERENCES

Alsamir, M., T. Mahmood, N. Ahmad, and R. Trethowan. 2017. Distribution of organic metabolites after *Fusarium* wilt incidence in tomato (*Solanum esculentum* L.). Aust. J. Crop Sci. 11(9), 1123-1129. Doi: 10.21475/ajcs.17.11.09.pne536

Andersen, E., S. Ali, E. Byamukama, Y. Yen, and M.P. Nepal. 2018. Disease resistance mechanisms in plants. Genes 9(7), 339. Doi: 10.3390/genes9070339

Ávila-Orozco, F.D., L.M. León-Gallón, M.I. Pinzón-Fandiño, A. Londoño-Orozco, and J.A. Gutiérrez-Cifuentes. 2017. Residualidad de fitosanitarios en tomate y uchuva cultivados en Quindío (Colombia). Cienc. Tecnol. Agropecu. 18(3), 571-582. Doi: 10.21930/rcta.vol18_num3_art:745

Bates, L.S., R.P. Waldren, and I.D. Teare. 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205-207. Doi: 10.1007/BF00018060

Carmona, S.L., D. Burbano-David, M.R. Gómez, W. Lopez, N. Ceballos, J. Castaño-Zapata, J. Simbaqueba, and M. Soto-Suárez. 2020. Characterization of pathogenic and nonpathogenic *Fusarium oxysporum* isolates associated with commercial tomato crops in the Andean region of Colombia. Pathogens 9,70. Doi: 10.3390/pathogens9010070

Chekali, S., S. Gargouri, T. Paulitz, J.M. Nicol, M. Rezgui, and B. Nasraoui. 2011. Effects of *Fusarium culmorum* and water stress on durum wheat in Tunisia. Crop Prot. 30(6), 718-725. Doi: 10.1016/j.cropro.2011.01.007

Chowdhury, S., A. Basu, and S. Kundu. 2017. Biotrophy-necrotrophy switch in pathogen evoke differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. Sci. Rep. 7, 17251. Doi: 10.1038/s41598-≠017-17248-7

Chun, S.C. and M. Chandrasekaran. 2019. Chitosan and chitosan nanoparticles induced expression of pathogenesis-related proteins genes enhances biotic stress

tolerance in tomato. Int. J. Biol. Macromol. 125, 948-954. Doi: 10.1016/j.ijbiomac.2018.12.167

Beatrice, C., J.M.H. Linthorst, F. Cinzia, and R. Luca. 2017. Enhancement of *PR1* and *PR5* gene expressions by chitosan treatment in kiwifruit plants inoculated with *Pseudomonas syringae* pv. *actinidiae*. Eur. J. Plant Pathol. 147, 163-179. Doi: 10.1007/s10658-016-1080-x

Dean, R., J.A.L. Van Kan, Z.A. Pretorius, K.E. Hammond-Kosack, A. Di Pietro, P.D. Spanu, J.J. Rudd, M. Dickman, R. Kahmann, J. Ellis, and G.D. Foster. 2012. The top 10 fungal pathogens in molecular plant pathology. Mol. Plant Pathol. 13, 414-430. Doi: 10.1111/j.1364-3703.2011.00783.x

Duniway, J.M. 1971. Water relations of *Fusarium* wilt in tomato. Physiol. Plant Pathol. 1(4), 537-546. Doi: 10.1016/0048-4059(71)90015-4

Dzung, N.A., V.T.P. Khanh, and T.T. Dzung. 2011. Research on impact of chitosan oligomers on biophysical characteristics, growth, development and drought resistance of coffee. Carbohydr. Polym. 84(2), 751-755. Doi: 10.1016/j.carbpol.2010.07.066

El-Mohamedy, R.S.R., M.E.A. El-Aziz, and S. Kamel. 2019. Antifungal activity of chitosan nanoparticles against some plant pathogenic fungi *in vitro*. AgricEngInt: CIGR J. 21(4), 201-209.

Goltsev, V.N., H.M. Kalaji, M. Paunov, W. Bąba, T. Horaczek, J. Mojski, H. Kociel, and S.I. Allakhverdiev. 2016. Variable chlorophyll fluorescence and its use for assessing physiological condition of plant photosynthetic apparatus. Russ. J. Plant Physiol. 63, 869-893. Doi: 10.1134/S1021443716050058

Hernández-Lauzardo, A., S. Bautista-Baños, M.G. Velázquez-del Valle, M.G. Méndez-Montealvo, M.M. Sánchez-Rivera, and L.A. Bello-Pérez. 2008. Antifungal effects of chitosan with different molecular weights on in vitro development of *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. Carbohydr. Polym. 73(4), 541-547. Doi: 10.1016/j.carbpol.2007.12.020

Huang, Z., Z. Zhang, X. Zhang, H. Zhang, D. Huang, and R. Huang. 2004. Tomato TERF1 modulates ethylene response and enhances osmotic stress tolerance by activating expression of downstream genes. FEBS Lett. 573(1-3), 110-116. Doi: 10.1016/j.febslet.2004.07.064

Huot, B., J. Yao, B.L. Montgomery, and S.Y. He. 2014. Growth-defense tradeoffs in plants: A balancing act to optimize fitness. Mol. Plant 7(8), 1267-1287. Doi: 10.1093/mp/ssu049

Jamiołkowska, A. 2020. Natural compounds as elicitors of plant resistance against diseases and new biocontrol strategies. Agronomy 10(2), 173. Doi: 10.3390/agronomy10020173

Jelinski, N.A., K. Broz, W. Jonkers, L.J. Ma, and H.C. Kistler. 2017. Effector gene suites in some soil isolates of *Fusarium oxysporum* are not sufficient predictors of vascular wilt in tomato. Phytopathology 107(7), 842-851. Doi: 10.1094/PHYTO-12-16-0437-R

Jia, X., Q. Meng, H. Zeng, W. Wang, and H. Yin. 2016. Chitosan oligosaccharide induces resistance to *Tobacco mosaic virus* in *Arabidopsis* via the salicylic acid-mediated signalling pathway. Sci. Rep. 6, 26144. Doi: 10.1038/srep26144

Kappel, L., M. Münsterkötter, G. Sipos, C. Escobar Rodriguez, and S. Gruber. 2020. Chitin and chitosan remodeling defines vegetative development and *Trichoderma* biocontrol. PLoS Pathog. 16(2), e1008320. Doi: 10.1371/journal.ppat.1008320

Kardile, P.B., B.B. Dharbale, and M.V. Ugale. 2019. Chlorophyll fluorescence: a tool to study plant physiology and abiotic stress in plants. Indian J. Agric. Allied Sci. 5(2), 29-35.

Kauss, H., W. Jeblick, and A. Domard. 1989. The degrees of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*. Planta 178, 385-392. Doi: 10.1007/BF00391866

Kumaraswamy, R.V., S. Kumari, R.C. Choudhary, A. Pal, R. Raliya, P. Biswas, and V. Saharan. 2018. Engineered chitosan based nanomaterials: Bioactivity, mechanisms and perspectives in plant protection and growth. Int. J. Biol. Macromol. 113, 494-506. Doi: 10.1016/j.ijbiomac.2018.02.130

León-Morcillo, R.J., J.A. Martín-Rodríguez, H. Vierheilig, J.A. Ocampo, and J.M. García-Garrido. 2012. Late activation of the 9-oxylipin pathway during arbuscular mycorrhiza formation in tomato and its regulation by jasmonate signalling. J. Exp. Bot. 63(10), 3545-3558. Doi: 10.1093/jxb/ers010

Lorenzini, G., L. Guidi, C. Nali, S. Ciompi, and G.F. Soldatini. 1997. Photosynthetic response of tomato plants to vascular wilt diseases. Plant Sci. 124(2), 143-152. Doi: 10.1016/S0168-9452(97)04600-1

Maluin, F.N. and M.Z. Hussein. 2020. Chitosan-based agronanochemicals as a sustainable alternative in crop protection. Molecules 25(7), 1611. Doi: 10.3390/molecules25071611

Martínez-Medina, A., I. Fernández, M.J. Sánchez-Guzmán, S.C. Jung, J.A. Pascual, and M.J. Pozo. 2013. Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. Front. Plant Sci. 4, 206. Doi: 10.3389/fpls.2013.00206

Maxwell, K. and G.N. Johnson. 2000. Chlorophyll a fluorescence - a practical guide. J. Exp. Bot. 51(345), 659-668. Doi: 10.1093/jxb/51.345.659

Melgarejo, L.M. (ed.). 2010. Experimentos en fisiología vegetal. Universidad Nacional de Colombia, Bogota.

Michielse, C.B. and M. Rep. 2009. Pathogen profile update: *Fusarium oxysporum*. Mol. Plant Pathol. 10, 311-324. Doi: 10.1111/j.1364-3703.2009.00538.x

Nogués, S., L. Cotxarrera, L. Alegre, and M.I. Trillas. 2002. Limitations to photosynthesis in tomato leaves

induced by *Fusarium* wilt. New Phytol. 154(2), 461-470. Doi: 10.1046/j.1469-8137.2002.00379.x

Orzali, L., B. Corsi, C. Forni, and L. Riccioni. 2017. Chitosan in agriculture : A new challenge for managing plant disease. In: Shalaby, E. (ed.). Biological activities and application of marine polysaccharides. IntechOpen. Doi: 10.5772/66840

Pedroza-Sandoval, A. and J.A. Samaniego. 2009. Analisis del area bajo la curva del progreso de las enfermedades (ABCPE) en patosistemas agrícolas. pp. 179-189. In: Bautista, N., L. Soto, and R. Pérez (eds.). Tópicos selectos de estadística aplicados a la fitosanidad. Colegio de postgraduados. Mexico, DF.

Pérez-Bueno, M.L., M. Pineda, and M. Barón. 2019. Phenotyping plant responses to biotic stress by chlorophyll fluorescence imaging. Front. Plant Sci. 10, 1135. Doi: 10.3389/fpls.2019.01135

Pshibytko, N.L., L.A. Zenevich, and L.F. Kabashnikova. 2006. Changes in the photosynthetic apparatus during *Fusarium* wilt of tomato. Russ. J. Plant Physiol. 53, 25-31. Doi: 10.1134/S1021443706010031

Rojas-Tapias, D., A. Moreno-Galván, S. Pardo-Díaz, M. Obando, D. Rivera, and R. Bonilla. 2012. Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (*Zea mays*). Appl. Soil Ecol. 61, 264-272. Doi: 10.1016/j.apsoil.2012.01.006

Rongai, D., P. Pulcini, B. Pesce, and F. Milano. 2017. Antifungal activity of pomegranate peel extract against *Fusarium* wilt of tomato. Eur. J. Plant Pathol. 147, 229-238. Doi: 10.1007/s10658-016-0994-7

Segarra, G., E. Casanova, M. Avilés, and I. Trillas. 2010. *Trichoderma asperellum* strain T34 controls fusarium wilt disease in tomato plants in soilless culture through competition for iron. Microb. Ecol. 59, 141-149. Doi: 10.1007/s00248-009-9545-5

Solanki, V.H., R.M. Patel, and K.G. Patel. 2015. Biochemical alteration in tomato varieties resistant and susceptible to *Fusarium oxysporum* f. sp. *lycopersici*. J. Cell Tissue Res. 15(2), 5029-5036.

Soliman, M.H. and R.S.R. El-Mohamedy. 2017. Induction of defense-related physiological and antioxidant enzyme response against powdery mildew disease in okra (*Abelmoschus esculentus* L.) plant by using chitosan and potassium salts. Mycobiology 45(4), 409-420. Doi: 10.5941/MYCO.2017.45.4.409

Soto-Suárez, M., P. Baldrich, D. Weigel, I. Rubio-Somoza, and B.S. Segundo. 2017. The *Arabidopsis* miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. Sci. Rep. 7, 44898. Doi: 10.1038/srep44898

Srinivas, C., D.N. Devi, K.N. Murthy, C.D. Mohan, T.R. Lakshmeesha, B.P. Singh, N.K. Kalagatur, S.R. Niranjana, A. Hashem, A.A. Alqarawi, B. Tabassum, E.F-Abd-Allah, S.C. Nayaka, and R.K. Srivastava. 2019. *Fusarium oxysporum* f. sp. *lycopersici* causal agent of vascular wilt disease of tomato: Biology to diversity – A review. Saudi J. Biol. Sci. 26(7), 1315-1324. Doi: 10.1016/j.sjbs.2019.06.002

Tikhonov, V.E., I.A. Yamskov, J. Palma-Guerrero, H.-B. Jansson, L.V. Lopez-Llorca, J. Salinas, D.V. Gerasimenko, I.D. Avdienko, and V.P. Varlamov. 2006. Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl) succinoyl/-derivatives. Carbohydr. Polym. 64(1), 66-72. Doi: 10.1016/j.carbpol.2005.10.021

Van, N.S., D.H. Minh, and N.D. Anh. 2013. Study on chitosan nanoparticles on biophysical characteristics and growth of *Robusta coffee* in green house. Biocatal. Agric. Biotechnol. 2(4), 289-294. Doi: 10.1016/J.BCAB.2013.06.001

Vargas-Hernandez, M., I. Macias-Bobadilla, R.G. Guevara-Gonzalez, S.J. Romero-Gomez, E. Rico-Garcia, R.V. Ocampo-Velazquez, L.L. Alvarez-Arquieta, and I. Torres-Pacheco. 2017. Plant hormesis management with biostimulants of biotic origin in agriculture. Front. Plant Sci. 8, 1762. Doi: 10.3389/fpls.2017.01762

Vidhyasekaran, P. 2016. Switching on plant innate immunity signaling systems - Bioengineering and molecular manipulation of PAMP-PIMP-PRR signaling complex. Springer, Cham, Switzerland. Doi 10.1007/978-3-319-26118-8

Wagner, A., W. Michalek, and A. Jamiolkowska. 2006. Chlorophyll fluorescence measurements as indicators of fusariosis severity in tomato plants. Agron. Res. 4(Special Issue), 461-464.

Walters, D., D. Walsh, A. Newton, and G. Lyon. 2005. Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. Phytopathology 95(12), 1368-1373. Doi: 10.1094/PHYTO-95-1368

Walters, D.R., J. Ratsep, and N.D. Havis. 2013. Controlling crop diseases using induced resistance: Challenges for the future. J. Exp. Bot. 64(5), 1263-1280. Doi: 10.1093/jxb/ert026

Wellburn, A.R. 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant Physiol. 144(3), 307-313. Doi: 10.1016/S0176-1617(11)81192-2

Xing, K., X. Zhu, X. Peng, and S. Qin. 2015. Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review. Agron. Sustain. Dev. 35, 569-588. Doi: 10.1007/s13593-014-0252-3

Yadeta, K.A. and B.P.H. Thomma. 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. Front. Plant Sci. 4, 97. Doi: 10.3389/fpls.2013.00097

Yockteng, R., A.M.R. Almeida, S. Yee, T. Andre, C. Hill, and C.D. Specht. 2013. A method for extracting high-quality RNA from diverse plants for next-generation

sequencing and gene expression analyses. Appl. Plant Sci. 1(12), 1300070. Doi: 10.3732/apps.1300070

You, J., J. Zhang, M. Wu, L. Yang, W. Chen, and G. Li. 2016. Multiple criteria-based screening of *Trichoderma* isolates for biological control of *Botrytis cinerea* on tomato. Biol. Control 101, 31-38. Doi: 10.1016/j.biocontrol.2016.06.006

Zehra, A., M. Meena, M.K. Dubey, M. Aamir, and R.S. Upadhyay. 2017. Synergistic effects of plant defense elicitors and *Trichoderma harzianum* on enhanced induction of antioxidant defense system in tomato against *Fusarium* wilt disease. Bot. Stud. 58, 44. Doi: 10.1186/s40529-017-0198-2

Zhang, A.X., P. Zou, and P. Li. 2017. Biostimulant activity of chitooligomers: Effect of different degrees of acetylation and polymerization on wheat seedlings under salt stress. Int. J. Agric. Biosyst. Eng. 4(12), 1.

Zhang, W., C. Qin, J. Zhao, and X. Wang. 2004. Phospholipase Dα1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc. Natl. Acad. Sci. USA 101(25), 9508-9513. Doi: 10.1073/pnas.0402112101