



## Shrimp shells, Chitin and chitosan powders effect on growth of *Lycopersicon esculentum* and their ability to induce resistance against *Fusarium oxysporum* f.sp. *radicis-lycopersici* attack

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### ABSTRACT

The potential of co-products of shrimp shells on the germination parameters, growth, and for inducing defense reactions in tomato (*Lycopersicon esculentum*) plants inoculated with *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) was investigated *in vitro* and *in vivo*. It was found that chitosan at 50mg/l increased the germination percentage by 2.66%; the shrimp shells powder at 50mg/l was best in increasing the length of radical and length and weight of hypocotyl *in vitro*. *In Vivo*, chitosan at 50mg/l increased length, fresh/dry weight of aerial and radicle parts compared to control. An inhibition of FORL growth *in vitro* was obtained by addition of chitosan at 100mg/l. In addition, chitin at 25 mg/l decreased the foliar alteration index by 73.34% compared to control.

**Key words:** Chitin, Chitosan, *Fusarium oxysporum* f.sp. *Lycopersicon esculentum*, *Parapenaeus longirostris*, *Radicis-lycopersici*, Shrimp shells.

### INTRODUCTION

Destined in its entity for human consumption, shrimp generates co-products (heads, shells and tails) as wastes. These wastes contain several substances, which are worth of recovery (lipids, chitin, carotenoid astaxanthin, mineral elements ...) (Heu *et al.*, 2003) and other bioactive compounds (Kim *et al.*, 2008) that are beneficial for both human and animal health.

The chitin is obtained by different methods including the chemical ones (Varun *et al.*, 2017). Shrimp co-products can play an important role in elicitation of plants, inducing a non-host resistance, and prime the plants for systemic acquired resistance (Singh *et al.*, 2018).

In the present study, red shrimp shells (*Parapenaeus longirostris*) were used to extract chitin and chitosan. The objectives of the experiments are to determine the effects of three treatments (shrimp shells, chitin and chitosan powders) on the germination of tomato seeds *in vitro*; and on plant growth parameters of *Lycopersicon esculentum* *in vivo* and, also, on the inhibition of FORL which causes very large yield reductions in tomato.

### MATERIALS AND METHODS

**Preparation of the raw material:** Shrimp shells of *Parapenaeus longirostris* species are collected at the central market of Mehdiya town of Kenitra, and transported to the laboratory in a cooler (4°C). They are washed thoroughly to remove all impurities. The obtained shells were dried at 70°C for 12h, then ground and sieved at -20°C.

### Preparation of chitin and chitosan preparation of chitin

**A. Acid demineralization:** In this step, shrimp shells powders' is suspended in a solution of HCl (2N) with a ratio 1 g: 20ml, and then left to react for 30min with constant stirring at room temperature (Benhabiles *et al.*, 2012). The decalcified product is rinsed until neutrality and then dried for 12h.

**B. Basic deproteinization:** The retentate is introduced into a pyrex flask, in which is added a NaOH (2N) solution with a ratio of 1: 20 (g: ml). The deproteinization takes 2 hours under constant stirring in a water bath at 45°C. (Benhabiles *et al.*, 2012). The residues are then rinsed, filtered and dried for 12 hours.

**C. Discoloration:** A solution of acetone at a ratio of 1g: 10ml is mixed with the powder for 10min. After filtration, it is dried for 2 hours at room temperature. Bleaching is carried out with 0.315% NaOCl at a ratio of 1g: 10ml for 5 min (No *et al.*, 1995). The discolored chitin is washed and filtered.

### Preparation of chitosan

**A. Deacetylation:** This step is carried out using the protocol suggested by Putra and Husni (2013) with some modification. A solution of 50% NaOH at a ratio of 1g: 20ml was mixed with chitin under constant stirring for 8 hours in water bath at 100°C, with a renewal of the NaOH solution every hour.

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### Physico-chemical analysis of shells

**Moisture content:** The shrimp shells were dried at 70°C for 12 hours and the moisture content was determined using the following formula:

$$\text{Moisture content \%} = \frac{\text{Initial weight (g)} - \text{Dry weight}}{\text{Dry weight}} \times 100$$

**Measurement of ash content:** A sample of 2g of the shrimp shells powder was incinerated at 600°C for 6 hours (Kim, 2004), and the ash content was determined by the following formula:

$$\text{Ash content \%} = \frac{\text{weight of ash}}{\text{Original sample weight}} \times 100$$

**Determination of the degree of deacetylation by potentiometric titration:** According to the protocol of Rutherford and Austin (1978), a sample of 0.1g of chitosan and 40ml of NaOH (50%) were stirred for 1 hour and 30 min in water bath at 100°C, after that 25ml of concentrated phosphoric acid was carefully added. The resulting solution is distilled on vigreux colonne. When the distillation flask began to dry, 15ml of hot distilled water was added to the flask. This step was repeated until 250ml of distillate is recovered. A sample of 25ml of distillate was titrated with 0.1N NaOH using phenolphthalein as an indicator. The percentage of acetyl was determined by the following formula:

$$\text{Acetyl \%} = V \times 0.04305/W$$

V: NaOH volume

W: weight of sample

### Germination and growth of *Lycopersicon esculentum*

#### *In vitro* assay

**A. Disinfection of *Lycopersicon esculentum* seeds:** Seeds of the tomato (Rio Grande variety) were disinfected by successive immersion in 1% aqueous NaOCl for 10 minutes (Rao *et al.*, 2006) and then 3 times in sterile distilled water.

**B. Filter paper medium:** Concentrations of 25, 50, and 100mg/l of shrimp shells, chitin and chitosan powders' were macerated in distilled water for 24 hours at room temperature to extract the soluble principles. The solutions' pH was adjusted to 6.0 with 1% NaOH solution.

Seeds (25) placed on the Petri dishes (90mm) containing three discs of filter paper moistened with 4 ml of macerate (Ramanaand *et al.*, 2002). For the control, the sterile distilled water was used. The incubation was carried out at 26±1°C, and a photoperiod of 16h of illumination. After 10 days, the germination parameters was determined.

***In vivo* assay:** Seedlings at the three-leaf stage (Benhamo and Thériault, 1992) were cultivated on pots containing sterilized Maamora soil and then transported in a greenhouse.

The effect of each treatment was determined by a weekly soil amendment for 8 weeks with a volume of 100ml of each treatment (Lafontaine and Benhamou, 1996). For the control, only sterile distilled water was used. Five seedlings were used for each treatment, and the experiment was repeated twice (Benhamou and Thériault, 1992).

### Stimulation of resistance of *Lycopersicon esculentum* seedlings

#### Action on *Fusarium oxysporum* f.sp. *radicis-lycopersici*

***in vitro*:** Discs of 5 mm diameter were cut from a pure culture of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (isolated and identified in the Laboratory of Botany, Biotechnology and Plant Protection, Faculty of Science, Ibn Tofail University, Kenitra) cultured on PDA medium for 7 days. After that, 0.1ml of each treatment was spread on the surface of the PDA medium (Cheah *et al.*, 1997). Finally, discs (5 discs per treatment) were put face down on the surface of the PDA medium plus treatment (repeated five times per treatment) (Fig 1). The antifungal effect was determined by measuring the inhibition percentage of diametric growth using the following formula (Hajji *et al.*, 2016):

$$\text{P.I.C.D} = (\text{Mt} - \text{Me} / \text{Mt}) \times 100$$

P.I.C.D = percentage of inhibition of diametric growth.

Mt=mean diameter of the control colony.

Me=mean diameter of colony exposed to shrimp shells, chitin or chitosan powders'.

***In vivo* assay:** Five discs of 1cm<sup>3</sup> of a 7-days-old pathogenic fungal colony were immersed in 750ml of Potato Dextrose Broth (PDB) for 5 days at 26°C; after that, the final density was adjusted to 3x10<sup>8</sup> (CFU) ml<sup>-1</sup> (Lafontaine and Benhamou, 1996).

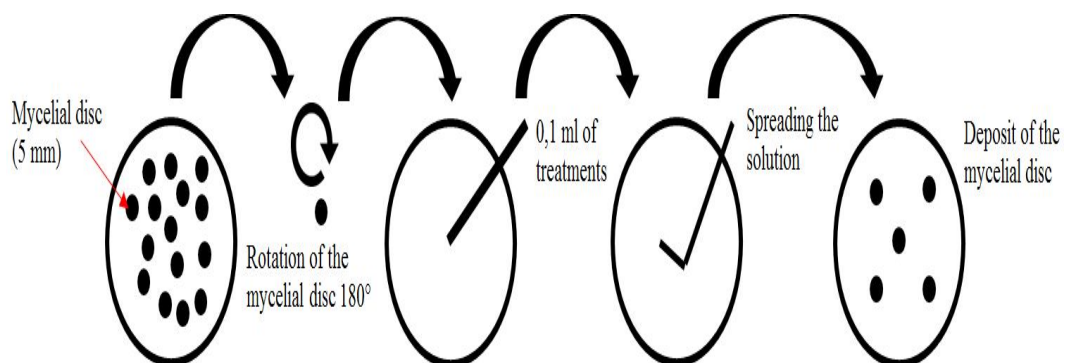


Fig 1: Schematic representation of the *in vitro* test on FORL.

Roots of seedlings at the three-leaf stage (Rio Grande variety) are thoroughly washed with sterile water and treated by soaking for 10min in different concentrations of the three powders' (25, 50 or 100mg/l) with Tween 80 (0.01% v/v) (Benhamou and Thériault, 1992).

After that, each seedling was transplanted into a pot containing sterilized Maamora soil. Next day, the roots were inoculated by soaking for 30 seconds in the inoculum suspension. Five seedlings were used for each treatment, and the experiment was repeated twice (Benhamou and Thériault, 1992).

**Re-isolation of FORL:** The presence of FORL is searched in the roots of the tomato plants. The root system is disinfected by rapid soaking in 90° alcohol, rinsed several times with sterile distilled water, dried and cut into 5mm length fragments. The fragments are deposited in PDA medium. The incubation is carried out for 7 days at 26°C, in the dark.

#### Degree of infection

**A. Alteration of leaves:** The expression of foliar symptoms is estimated by a foliar index using the following rating scale (Douira *et al.*, 1994):

0=healthy appearance leaves.

1=cotyledonary leaves : wilting or yellowing;

2=Drop of the cotyledonary leaf;

3=Wilting or yellowing of the true leaf;

4=Necrosis of the true leaf;

5=Fall of the true leaf;

The sum of the notes relative to the number of leaves is the foliar alteration index. An average index is then calculated for each plant (Douira and Lahlou (1989)).

$$FAI = \frac{[\sum (i \times X_i)]}{6 \times NtF}$$

Where:

FAI: Foliar alteration index;

i: Appearance notes for sheets 0 to 5;

xi: Number of sheets with the note i;

NtF: Total number of sheets.

**Statistical analysis:** The difference between the means of each experiment is analyzed by the TUKEY test at  $\alpha=0.05$  by a statistical software.

## RESULTS AND DISCUSSION

**Moisture and ash content:** The obtained results from the measured parameters (moisture content "55.0764%" and ash content "51.2 %") for the shrimp shells explain the low productivity during the extraction of chitin and chitosan. The demineralization and the deproteinization of 100g of the shrimp shells powder gives 23.93% of chitin, which is comparable to those (24% of chitine) obtained by Benhabiles *et al.*, (2012).

**Degree of deacetylation of chitosan:** Using the formula of Rutherford and Austin (1978), we obtain 1.076% of acetyl; our results were considered high compared to those obtained by Putra and Husni (2013) (7.26% acetyl), even if we used a potentiometric titration, which could be the most reliable and robust of the non-NMR methods (Renata *et al.*, 2012).

#### Action of treatments on the germination and growth of *Lycopersicon esculentum*

**Effect of treatments on the germination of *Lycopersicon esculentum*:** The effect of the three treatments on germination parameters is set out in Table 1.

We found that the seed germination rate of the tomato was affected by the addition of chitosan at 50mg/l, which increased by 2.66% and 1.33% for the other chitosan concentrations (25mg/l and 100mg/l).

As for the hypocotyl and the radicle lengths, the seedlings from the medium containing the shrimp shells powder at 50 mg/l shows the highest values of these parameters. Similarly, the treatments had a favorable effect on the fresh weight of the two parts (hypocotyl and radicle).

**Effect of treatments on the growth of *Lycopersicon esculentum*:** The data for the weekly Maamora soil amendments are shown in Table 2.

Compared to control, a stimulation of length, fresh/dry aerial weight, and fresh/dry root weight are observed

**Table 1:** Germination parameters means of tomato seeds.

Treatments (mg/l)		Germination (%)	Hypocotyl lengths(cm)	Radical lengths(cm)	Hypocotyl fresh weight (g)	Radical fresh weight (g)	Hypocotyl dry weight (g)	Radical dry weight (g)
Control	0	86.67 cde	5.03 abc	07.41 a	0.374 a	0.086 a	0.025 a	0.009 a
Shrimp shells powder	25	86.67 bcde	5.33 abc	09.46 bc	0.612 ef	0.249 b	0.030 cd	0.010 ef
	50	88.00 de	<b>5.65 c</b>	<b>10.52 c</b>	<b>0.632 f</b>	0.251 b	0.030 cd	<b>0.012 g</b>
	100	85.33 bcd	4.97 abc	08.57 ab	0.567 cd	0.234 b	0.029 bc	0.010 f
Chitin	25	82.67 abc	4.64 a	07.61 a	0.619 ef	0.262 c	0.039 e	0.010 cd
	50	80.00 a	5.29 abc	08.75 abc	0.506 b	0.244 b	0.026 ab	0.008 b
	100	84.00 abc	4.93 ab	08.22 ab	0.543 c	0.297 c	0.033 d	0.009 ac
Chitosan	25	88.00 de	5.21 abc	09.46 bc	0.590 de	0.267 c	0.039 e	0.010 de
	50	<b>89.33 e</b>	5.44 bc	09.06 abc	0.541 c	0.097 a	0.029 bcd	0.010 f
	100	88.00 de	5.44 bc	10.43 c	0.568 cd	<b>0.358 d</b>	<b>0.040 e</b>	<b>0.012 g</b>

Means in the same column with the same letter do not differ significantly from each other at  $\alpha=5\%$ .

**Table 2:** Agronomic parameters of plants treated with shrimp shells, chitin and chitosan powders'.

Treatments (mg/l)		Length of aerial part (cm)	length of root part (cm)	Fresh weight of aerial part (g)	Fresh weight of root part (g)	dry weight of aerial part(g)	dry weight of root part(g)
Control	0	16.3a	21.2 a	4.116 a	04.468 a	1.082 a	1.572 a
Shrimp shells	25	19.0 ab	21.8 a	4.170 a	06.730 ab	2.006 ab	3.172 b
	50	29.0 abc	32.0 abc	6.154 ab	08.848 b	2.440 ab	4.946 bc
powder	100	22.4 abc	28.2 ab	5.334 ab	04.876 a	1.826 ab	2.098 a
Chitin	25	27.8 abc	38.0 abc	6.562 ab	03.146 a	2.182 ab	1.612 a
	50	33.2 c	<b>51.2 c</b>	8.938 b	03.108 a	3.732 b	1.286 a
	100	31.2 bc	33.2 abc	7.196 ab	03.656 a	2.454 ab	1.460 a
Chitosan	25	27.2 abc	36.8 abc	7.518 ab	05.288 ab	3.234 b	2.728 a
	50	<b>35.4 c</b>	42.2 bc	<b>9.090 b</b>	<b>10.948 c</b>	<b>3.758 b</b>	<b>5.808 c</b>
	100	30.4 bc	36.0 abc	8.664 b	10.712 c	3.730 b	5.770 c

Means in the same column with the same letter do not differ significantly from each other at  $\alpha=5\%$ .

among plants treated with chitosan at 50mg/l. Similarly, chitin at 50mg/l recorded the maximum value (51.2cm) over the length of the root compared to the control (21.2cm).

It could be deduced that, the improvement of the root system's growth allowed a greater exploration of the soil and, consequently, an improved nutrition, which also results in a better development of the aerial part (size and biomass) (Fig 2).

Our results demonstrate the beneficial effect of the weekly soil amendment by chitosan 50mg/l, 100mg/l and chitin 50mg/l on tomato growth compared to the study of Lafontaine and Benhamou (1996) which demonstrates only the protective effect of chitosan at 37mg/l.

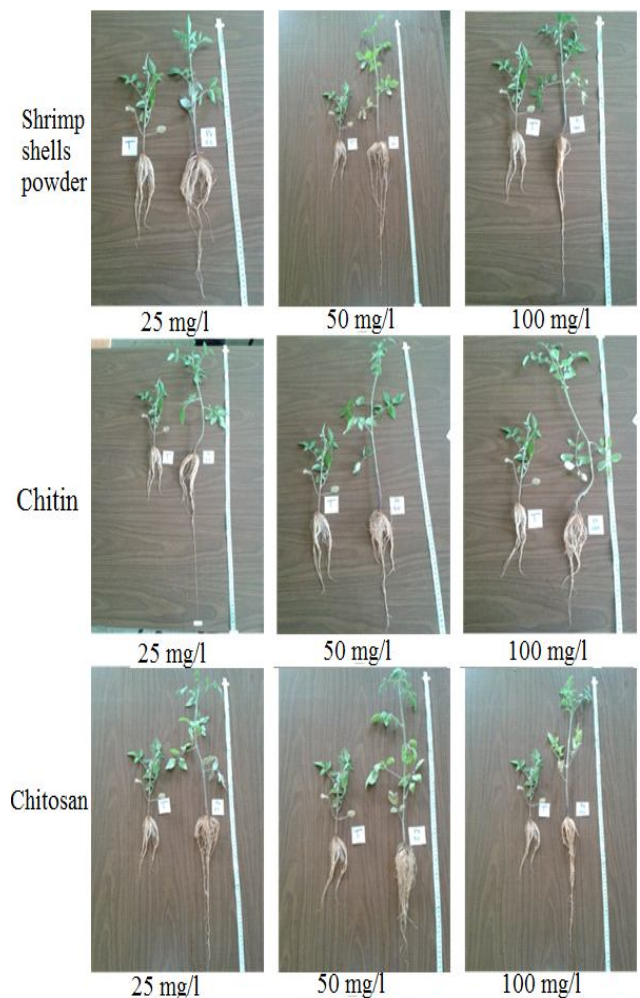
#### Stimulation of resistance

**In vivo inoculation of tomato seedlings with *Fusarium oxysporum* f.sp. *radicis-lycopersici*:** After observation of the typical symptoms of *Fusarium* on the control plants (Fig 3), the experiment is stopped (8 weeks after inoculation). Table 3 reports the average sizes of epicotyl, hypocotyl and means of foliar alteration indices of tomato plants inoculated with FORL.

It was found that the treated plants with chitin (25, 50 and 100mg/l) and chitosan (50 and 100mg/l) give maximum and highly significant means compared to the control.

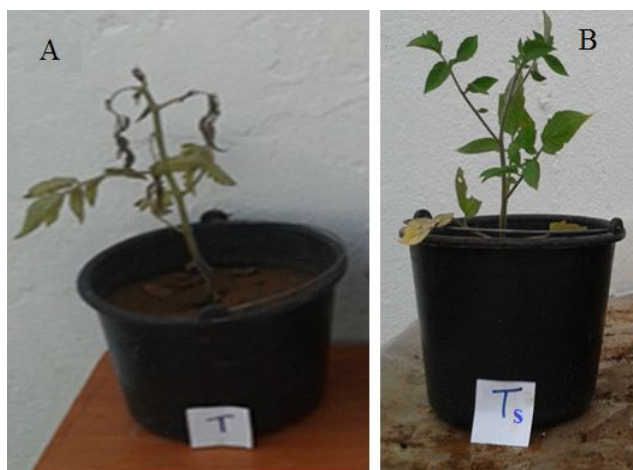
.Based on the foliar alteration index, it could be easily concluded that the addition of treatments to the soil induce a highly significant reduction compared to the control of the symptoms; and the lowest FAI was registered by chitin at 25mg/l (FAI=0,165), contrary to the control with the highest FAI (0.619).

The effect of chitosan on FORL could be explained by the penetration of chitosan into conidia of the pathogen and the ultrastructural changes that might result in disorganization of the cytoplasm and loss of intracellular content in spores than in the mycelium (Palma-Guerrero *et al.*, 2008).



**Fig 2:** Comparison of control plants sizes of tomato and treated plants.





**Fig 3:** Tomato plant show the characteristic symptoms of *FORL* (A), no inoculated plant (B).



**Fig 4:** (A) symptoms of *Fusarium* wilt on the stem of control plants inoculated with *FORL* (B) appearance of pathogen colonies reisolated from the roots of symptomatic plants.

Lafontaine and Benhamou (1996) obtained a mortality reduction of more than 90% by soil amendments with chitosan at 37,5mg/l whereas; in our study, no mortality was observed in the treated plants as compared to 40% mortality in control.

In addition, chitin and its derivatives play an eliciting role on plants, since they stimulate the production of phenylalanine ammonia-lyase and tyrosine ammonia-lyase, which were involved in the synthesis of compounds of the pathogen resistance system (Khan *et al.*, 2003).

On the other hand, Marshall (1976) justified the effect of chitin and its derivatives on the reduction of symptoms caused by *FORL* in tomatoes by the fact that the addition of those substances increased the microflora capable to attack chitinous hyphae by lytic enzymes. This causes lysis of the mycelium.

**In vitro test on *Fusarium oxysporum* f.sp. *radicis-lycopersici*:** Data in Table 4 reports the results of the three

**Table 3:** Size of epicotyl, hypocotyl, and the mean of foliar alteration index.

Treatments (mg/l)		Colony diameter (mm)	Inhibition of diametral growth(%)
Control	0	25.96 a	
Shrimp shells	25	24.58 ab	05,32
powder	50	23.06 abd	11,17
Chitin	100	21.32 bd	17,87
	25	14.62 e	43,68
	50	23.08 abd	11,09
	100	11.28 e	56,55
Chitosan	25	20.24 d	22,03
	50	20.00 d	22,96
	100	07.00 f	73,04

Means in the same column with the same letter do not differ significantly from each other at  $\alpha=5\%$ .

**Table 4:** Diameter and percentage of inhibition of the growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* colonies.

Treatments (mg/l)		epicotyle size (cm)	hyocotyl size (cm)	FAI
Control	0	31.4 a	7.60 a	0.619 a
Shrimp shells	25	44.2 ab	28.2 b	0.208 b
powder	50	35.6 ab	28.8 b	0.202 b
chitin	100	49.0 b	30.8 b	0.215 b
	25	53.6 b	35.0 b	<b>0.165 b</b>
	50	<b>54.6 b</b>	<b>38.4 b</b>	0.184 b
	100	54.0 b	28.4 b	0.173 b
chitosan	25	46.4 ab	32.2 b	0.193 b
	50	52.6 b	32.2 b	0.188 b
	100	51.6 b	25.6 b	0.214 b

Means in the same column with the same letter do not differ significantly from each other at  $\alpha=5\%$ .

treatments on the growth of *FORL* cultivated on PDA medium.

At 25 and 100mg/l of chitin, the inhibition of pathogen colonies reach 43.68% and 56.55%, respectively. However, at the 100mg/l of chitosan, the inhibition of pathogen is maximal (73.04%).

**Re-isolation of *Fusarium oxysporum* f.sp. *radicis-lycopersici*:** After one week of incubation in the dark at 26°C, the colonies of violet color characteristic of the pathogen appear around the root fragments deposited on the surface of the PDA medium (Fig 4).

## CONCLUSION

This study implies that application of shrimp shells, chitin and chitosan powders' to seeds of *Lycopersicon esculentum* *in vitro* and soil application for once a week tend to stimulate germination parameters, growth and significantly increased resistance to fusarium attacks.

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