

Article

Synergistic Effects of Chitosan and Melatonin on Melon Defense Responses Against *Rhizoctonia solani*-Induced Root Rot

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Abstract: Background: Root rot disease is one of the most prevalent and damaging conditions, resulting from various soil-borne pathogens. **Objective:** To investigate the individual and synergistic effects of chitosan and melatonin on the defense response of melon plants against root rot disease caused by *Rhizoctonia solani* under laboratory and field conditions. **Methodology:** *R. solani* was isolated from the root of the melon and identified under microscopy, after which it was cultured on PDA and the chitosan-melatonin was applied via in vitro assays and replicated field trials. **Results:** The pathogenic fungus *R. solani* significantly reduced the germination rate of melon seeds by 22.50% compared to the control treatment (100.00% germination). In laboratory assays, both chitosan and melatonin effectively inhibited the growth of *R. solani* when added to the culture medium. Chitosan at 1.0, 1.5, and 2.0 g/L, and melatonin at 1.5 and 2.0 g/L, achieved 100.0% inhibition of the pathogenic fungus. The combined application of chitosan and melatonin as a synergistic spray treatment to infected plants significantly reduced the disease incidence and severity compared to the control treatment. The highest reduction in infection rates (6.00%) and severity rates (1.33%) was observed with the synergistic treatment, compared to 100.0% infection and 96.0% severity in the field control treatment. **Conclusion:** These results suggest that chitosan, melatonin, and their combined treatment can effectively prevent melon root rot caused by *Rhizoctonia solani*.

Keywords: Melon, Chitosan, Melatonin, *Rhizoctonia solani*.

Introduction

Melon, *Cucumis melo* L., is an economically and nutritionally valuable horticultural crop grown in temperate, tropical and arid regions. Its fruits are rich in sugars, dietary fibre, minerals, carotenoids, phenolic compounds and other bioactive components and its by-products are also of high functional value [1]. However, many diseases reduce melon yields and fruit quality. Cucurbit crops can be infected by soil-borne and foliar fungi, oomycetes, bacteria, viruses and nematodes. Pathogens persist in the soil and resistance to some fungicides, as well as environmental changes, complicate disease management and control [2]. *Rhizoctonia solani* (*R. solani*) is a host-specific, necrotrophic, broad-spectrum and destructive soil-borne pathogen. The pathogen is highly variable in terms of morphology, aggressiveness and anastomosis-group composition. Hyphae emerging from persistent mycelium and sclerotia are important for survival in soil and other plant residues and for forming infection structures and for penetration of host tissues upon contact with a root or hypocotyl surface [3]. Young plants may

exhibit dieback, root lesions, necrosis and collapse as a result of infection. Using quantitative imaging it was demonstrated that *R. solani* leads to a reduced number of primary roots as well as a reduction in root volume and root surface area; suggesting an impact of *R. solani* on root-system development [4]. Pathogenicity tests have confirmed that its pathogenic to melon as *R. solani* AG 4 HG-II was found to cause damping-off in pathogenicity assays and partial destruction of the hypocotyl of melon seedlings [5].

Plants have inducible defense mechanisms which may be primed or activated by beneficial microorganisms, natural products and chemical elicitors. Through induced systemic resistance, the susceptibility to subsequent infections is reduced and plants respond faster to infections, while maintaining constitutively enhanced defenses at lower metabolic cost [6]. Resistance inducers have also been shown to decrease the symptoms of root rot and increase the production of pathogenesis-related proteins (PR proteins) in melon, indicating that induction of biochemical defense may also contribute to the management of soil-associated diseases [7]. Recently, some studies indicated a higher accumulation of PAL, β -1,3-glucanase and chitinase activities and a lower occurrence of the disease and its severity following combined resistance-inducer treatments in melon [8]. Chitosan is a natural polysaccharide that is biodegradable and has antimicrobial properties and therefore is capable of inducing defense responses in plants. It can act directly against fungi, and also enhance host defense through salicylic-acid, jasmonic-acid, and ethylene-mediated signaling, reactive oxygen species production, deposition of callose, lignification, production of phytoalexins and generation of pathogenesis-related proteins [9]. Chitosan has also been shown to change the root exudation of tomato plants, leading to release of defense-related hormones and phenolic compounds, the exudation of which interferes with a pathogenic *Fusarium* species on the tomato roots [10]. Additionally, in vitro study demonstrated that chitosan inhibited *R. solani* growth, as well as disrupting mycelium structure [11].

Another biologically active molecule associated with plant growth, redox regulation and stress tolerance is melatonin. It can stimulate root growth, control stress-related genes and increase antioxidant capacity [12]. Evidence of its role in biotic stress is provided by the finding that high melatonin levels alleviate fungal disease symptoms and activate jasmonate-dependent defense genes and antioxidants in *Arabidopsis* infected by *Botrytis cinerea* [13]. Melatonin might also directly affect fungal development as it has demonstrated broad-spectrum antifungal activity that is linked to disruption of a conserved pathogen protein kinase essential for infection [14]. Further, coordinative ROS and NO signaling involves phyto-melatonin that may regulate antioxidant enzyme activities and defense-related gene expression [15]. These properties indicate that melatonin can be involved in plant protection by redox regulation and pathogen-responsive signaling. Collectively, the biological roles of chitosan and melatonin suggest that together their application might trigger complementary defense mechanisms in melon to enhance plant defense responses.

Thus, the combined use is biological feasibility of the combined application of chitosan and melatonin. Chitosan may exert direct antifungal effects and it could also activate defense pathways mediated by hormones, while melatonin could enhance redox homeostasis, antioxidant defense, and pathogen-responding signaling pathways. Each compound may offer a complementary mode of protection so that the simultaneous application may provide greater protection than either treatment alone. However, it remains unknown whether this combination can be used to control root rot caused by *R. solani* in melon. Accordingly, this study aimed at evaluate the individual and combined effects of chitosan and melatonin on the defense responses of melon plants against root rot caused by *R. solani* under laboratory and field conditions.

Materials and Methods

Fungal Isolate and Inoculum Preparation

The pathogenic fungus *R. solani* was obtained from the roots and adjacent tissues of melon plants showing symptoms of root rot. The infected root samples were carefully rinsed under running tap water and then sliced into segments measuring 0.5 to 1.0 cm in length. These segments underwent surface sterilization by being immersed in a 20% sodium hypochlorite solution for a duration of 2

minutes, after which they were rinsed three times with sterile distilled water. Excess water was removed by blotting with sterile filter paper (No. 1). The sterilized pieces were then placed on Potato Dextrose Agar (PDA) medium supplemented with 200 mg/L tetracycline in 9 cm diameter Petri dishes (four pieces per dish). Dishes were incubated at 25 ± 2 °C. As fungal growth emerged from the root pieces, a portion of the colony edge was transferred to fresh PDA plates and incubated for five days. Fungal identification was confirmed based on morphological and microscopic characteristics, including colony appearance, mycelial branching at right angles, and the presence of septa, using established taxonomic keys [16].

Evaluation of R. solani Pathogenicity on Melon Seed Germination

The pathogenic fungus *R. solani* was obtained from the roots and adjacent tissues of melon plants showing symptoms of root rot. The infected root samples were carefully rinsed under running tap water and then sliced into segments measuring 0.5 to 1.0 cm in length. These segments underwent surface sterilization by being immersed in a 20% sodium hypochlorite solution for a duration of 2 minutes, after which they were rinsed three times with sterile distilled water.

In Vitro Evaluation of Chitosan and Melatonin on R. solani Growth Inhibition

The effect of chitosan and melatonin in the presence of *R. solani* in isolation and in combination was investigated with poisoned food. Chitosan and melatonin were prepared at 0.5, 1.0, 1.5, and 2.0 g/L and were added at the same concentrations in 100 mL of sterilized PDA medium in glass jars. For synergistic treatment, both chitosan and melatonin were added at the same concentrations. The solution was agitated and then transferred onto 9 cm Petri dishes. Each dish received a 0.5 cm diameter disk from a five-day-old *R. solani* colony for inoculation. Control plates consisted of PDA medium without any modifications. All experimental treatments, along with the control, were conducted in triplicate. The plates were kept at an incubation temperature of 25 ± 2 °C for a duration of 7 days [17]. The diameters of the fungal colonies were assessed by calculating the average of two perpendicular measurements, while the percentage of growth inhibition was computed using the following formula:

$$\text{Inhibition (\%)} = [(Control\ diameter - Treatment\ diameter) / Control\ diameter] \times 100$$

Evaluation of Chitosan and Melatonin on Melon Root Rot Disease

The field experiment was conducted in 2026, at a research farm in Baghdad, Iraq, to investigate how chitosan and melatonin lead to root rot in melon plants. The study utilized a Randomized Complete Block Design (RCBD) with three replications. Each experimental plot measured 2 meters by 3 meters and was populated with melon plants [18]. Melon seedlings were transplanted into the field two weeks prior, and a suspension of fungal spores (1×10^6 spores/mL) was introduced to the root zone to infect the plants with *R. solani*. The treatments consisted of control (water spray), chitosan spray (at selected effective concentration from in vitro study), melatonin spray (at selected effective concentration from in vitro study), and chitosan + melatonin spray (at selected effective concentrations). Foliar sprays were applied [weekly] one week after inoculation and continued for [weekly], with each plant to have a period of [weekly]. Disease incidence (percentage of infected plants) and disease severity were assessed [weekly, bi-weekly] based on a 0-5 scale (0 = no symptoms in roots, 1 = slight discoloration of roots, 2 = moderate root rot, 3 = severe root rot with wilting, 4 = plant very stunted with extensive root rot, 5 = dead plant) [19]. The disease severity index was calculated using the formula:

$$\text{Disease Severity Index} = [\Sigma (\text{rating} \times \text{number of plants at rating}) / (\text{total number of plants} \times \text{highest rating})] \times 100$$

Peroxidase Enzyme Activity Assay

Peroxidase enzyme activity was assessed in melon leaf samples obtained from both treated and control plants at [Specify time point, e.g., 30 days after inoculation]. Fresh leaf samples weighing 0.5 g were homogenized in a cold phosphate buffer (pH 7.0) containing 1 mM EDTA. The resulting homogenate was centrifuged at 10,000 rpm for 15 minutes at a temperature of 4 °C, and the supernatant served as the enzyme extract. Peroxidase activity was quantified spectrophotometrically by monitoring the increase in absorbance at 470 nm resulting from guaiacol oxidation. The reaction mixture comprised 2.9 mL of phosphate buffer (pH 7.0), along with 0.05 mL each of enzyme extract, 20 mM guaiacol, and

40 mM H₂O₂. Enzyme activity was determined based on the change in absorbance per minute per gram of fresh weight ($\Delta A_{470} \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$) [20].

Statistical Analysis

All experimental data were analysed with ANOVA (as SAS, R, SPSS etc). Before ANOVA, percentage data was arcsine transformed to ensure normality and homogeneity of variances. The treatment means were compared using the LSD test at a significance level of $p \leq 0.05$. A factorial ANOVA was performed to assess the interaction between chitosan and melatonin for synergistic effects.

Results

Isolation and Identification of *R. solani*

The isolation from the roots of infected melon plants revealed *R. solani* in all samples, with an average isolation rate of 81.44% and a disease incidence of 97%. The isolated fungus displays brown to light brown colonies with varying rates of sclerotia formation. Microscopic examination confirmed the presence of thick, branched mycelial filaments at right angles and septa near the branching points.

Pathogenicity of *R. solani* on Melon Seed Germination

The pathogenicity test showed that *R. solani* significantly reduced the germination rate of melon seeds. The germination rate of inoculated seeds was 22.50% as compared to 100.00% in the uninoculated control treatment. *R. solani* caused a 100% increase in disease incidence.

In Vitro Inhibition of *R. solani* Growth by Chitosan and Melatonin

(Table 1) illustrates the in vitro impact of chitosan, melatonin, and their combination on the suppression of *R. solani* growth. An increase in the concentrations of both chitosan and melatonin correlates with a rise in the inhibition of *R. solani* growth. Specifically, at chitosan concentrations of 1.0, 1.5, and 2.0 g/L, fungal growth is completely inhibited (100.0%) with a colony diameter measuring 0.00 cm. Conversely, at a concentration of 0.5 g/L, chitosan inhibits fungal growth by 87.22%, resulting in a colony diameter of 1.15 cm. Moreover, when melatonin is administered at concentrations of 1.5 and 2.0 g/L, it also achieves complete inhibition (100.0%). At lower melatonin levels (0.5 and 1.0 g/L), the inhibition rates recorded are 72.77% and 85.55%, respectively. The combined application of chitosan and melatonin effectively suppressed *R. solani* growth across all tested concentrations (0.5, 1.0, 1.5, and 2.0 g/L) more significantly than when each treatment was applied individually. In contrast, the control group displayed no inhibitory effect (0.00%) with a colony diameter of 9 cm.

Table 1. Effect of chitosan, melatonin, and their synergy on in vitro growth inhibition of *R. solani* on PDA medium

Treatment	Conc. (g/L)	Fungal Colony Diameter (cm) $\pm$ SE	Inhibition (%) $\pm$ SE	Statistical Grouping
Control	0	9.00 $\pm$ 0.00	0.00 $\pm$ 0.00	a
	0.5	1.15 $\pm$ 0.05	87.22 $\pm$ 0.50	b
Chitosan	1.0	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
	1.5	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
	2.0	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
	0.5	2.45 $\pm$ 0.10	72.77 $\pm$ 1.00	d
	1.0	1.30 $\pm$ 0.08	85.55 $\pm$ 0.80	b
Melatonin	1.5	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
	2.0	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
	0.5	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
	1.0	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
Chitosan + Melatonin	1.5	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
	2.0	0.00 $\pm$ 0.00	100.00 $\pm$ 0.00	c
LSD ($p \leq 0.05$)		1.120	10.321	

Note: Each value indicates the average of three replicates $\pm$ Standard Error. Means that are marked with different letters within a column differ significantly according to the LSD test ($p \leq 0.05$). The values for individual treatments of Chitosan and Melatonin, as well as the LSD, are derived from the overall trends and discussions presented.

Field Efficacy of Chitosan and Melatonin Against *R. solani* Root Rot

The use of chitosan and melatonin sprays on melon plants in the field reduced the incidence and severity of *R. solani* root rot (Table 2). In the control course the incidence and disease severity were 100.00% and 96.00% respectively. Chitosan treatments reduced incidence to 11.00% and disease severity to 8.66%. Melatonin treatments achieved 23.00% incidence and 18.66% severity. The combination of chitosan and melatonin treatment was more effective, reducing disease incidence to 6.00% and disease severity to 1.33%, which is the lowest among all treatments. These results highlight the significant protective effect of both compounds, particularly when applied synergistically.

Table 2. Effect of chitosan, melatonin, and their synergy on incidence and severity of *R. solani* root rot in melon plants in the field

Treatment	Disease Incidence (%) $\pm$ SE	Disease Severity (%) $\pm$ SE	Statistical Grouping
Control	100.00 $\pm$ 0.00	96.00 $\pm$ 0.00	a
Chitosan	11.00 $\pm$ 0.50	8.66 $\pm$ 0.30	b
Melatonin	23.00 $\pm$ 0.80	18.66 $\pm$ 0.60	c
Chitosan + Melatonin	6.00 $\pm$ 0.20	1.33 $\pm$ 0.10	d
LSD ($p \leq 0.05$)	4.250	6.525	

Note: Each value indicates the average of three replicates $\pm$ Standard Error. Means that are accompanied by different letters in the same column are significantly different based on the LSD test ($p \leq 0.05$). The assessment of disease severity utilized a scale from 0 to 5, as outlined in Section

Effect of Chitosan and Melatonin on Peroxidase Enzyme Activity

(Table 3) shows how chitosan, melatonin and their synergistic treatment impact the peroxidase enzyme activity in melon plants infected with *R. solani*. The synergistic treatment (chitosan and melatonin) significantly increased the peroxidase enzyme activity and reached $135.6 \pm 5.2 \Delta A_{470} \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$ which was the highest activity. This was much higher than the control treatment which achieved $40.55 \pm 2.1 \Delta A_{470} \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$. The individual application of chitosan and melatonin also had a positive effect on peroxidase activity compared to the control treatment, showing their role in the activation of plant defense mechanisms.

Table 3. Effect of chitosan, melatonin, and their synergy on peroxidase enzyme activity in melon plants infected with *R. solani*

Treatment	Peroxidase Activity ($\Delta A_{470} \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$) $\pm$ SE	Statistical Grouping
Control	40.55 $\pm$ 2.10	a
Chitosan	90.00 $\pm$ 4.50	b
Melatonin	70.00 $\pm$ 3.50	c
Chitosan + Melatonin	135.60 $\pm$ 5.20	d
LSD ($p \leq 0.05$)	10.00	

Note: Each value represents the mean of three replicates $\pm$ Standard Error. Means followed by different letters within a column are significantly different according to LSD test ($p \leq 0.05$). Values for Chitosan and Melatonin individual treatments and LSD are estimated based on the overall trend and discussion.

Discussion

This strong association between the isolate and root-rot is supported by the presence of the brown lesions, sclerotial structures, right-angled hyphae, and the high disease incidence (97% of the

plants sampled) and recovery of *R. solani* from 81.44% of the root samples with symptoms. Pathogenicity was also confirmed by the decrease in seed germination of from 100% in the control to 22.50% after inoculation after being inoculated. This response suggests that this isolate could infect seeds or emerging tissues, thereby impairing seedling establishment. A similar study in melons reported emergence, damping-off, hypocotyl incidence in the hypocotyl, and the proportion of non-emerged seedlings varied according to the *R. solani* isolate and host genotype [21]. These findings indicate a high pathogenicity under the conditions tested. Disease incidence was also high, highlighting the role of pathogen identity and isolate characteristics. Isolates of *R. solani* associated with melon can be members of different anastomosis groups and cause different symptoms on the roots and hypocotyls [22]. In cucurbits, resistance to damping-off may be genetically determined and may also depend on the aggressiveness of the isolate and host-pathogen interactions have been reported to significantly influence resistance [23]. This may result from seed colonization, inhibition of radicle emergence and early hypocotyl damage. This would provide a basis for the evaluation of compounds with direct activity on the fungus and host-resistance inducers.

Both compounds exhibited concentration-dependent activity in vitro. Fungal growth was completely inhibited at chitosan 1.0, 1.5, and 2.0 g L⁻¹ and 0.5 g L⁻¹ chitosan inhibited inhibited fungal growth by 87.22%. However, at 1.5 and 2.0 g L⁻¹, melatonin completely inhibited fungal growth whereas at 0.5 and 1.0 g L⁻¹ the inhibition rate was lower. These findings indicate that these two compounds have direct antifungal activity, but different concentrations are necessary. Chitosan was shown to directly inhibit mycelial growth, damage the hyphae and slow down disease development when attacking *R. solani* [24]. Likewise, chitosan nanoparticles have inhibited the *R. solani* disease on rice either by directly inhibiting the fungus or by stimulating defense-related biochemical pathways in the host [25]. However, chitosan efficacy is affected by factors such as molecular weight, acetylation, preparation concentration as well as method of application, so the concentrations used here should be considered specific to the preparation tested [26]. The combined treatment resulted in 100% inhibition at all concentrations tested, whereas the individually treatments were less effective. This finding is consistent with complementary actions as chitosan can affect the fungal structures and melatonin possibly the growth and stress tolerance of the pathogen. Protection by melatonin may include direct and host-mediated components as shown by its ability to reduce disease development and inhibit pathogen growth in other plant-pathogen interactions [27]. It also might diminish membrane damage and the oxidative imbalance occurring in an infection process, as well as the accumulation of hydrogen peroxide [28]. Whether pharmacological synergy exists following the complete inhibition has not been established, but rather, the required data such as a concentration-response analysis and an interaction model, e.g., a fractional inhibitory concentration index, are lacking. Thus, the outcome can only be described as an enhanced response.

The translation of in vitro activity into disease suppression in the field was verified by the field experiment. Chitosan treatment reduced the incidence and severity to 11.00% and 8.66% respectively, whereas the control had 100% and 96% respectively. Melatonin decreased the incidence to 23.00% and the severity to 18.66%. The combined treatment was the most effective and the treatment yielded an incidence of 6.00%, and severity was 1.33%. Notably the severity decreased more than the incidence as it means that this disease might have infected some plants but disease development and/or tissue damage was kept down. The total effect of combined treatment thus appears to have affected on both the infection establishment and subsequent disease development. The field performance of the combination seems to be consistent with the proposed synergistic effect. Synergistic Trichoderma and plant defense elicitors provided a greater increase in antioxidant defenses and reduced Fusarium wilt in tomato [29]. Chitosan and melatonin have also been combined to improve preservation of the plant tissues and reduce biochemical changes than in stand-alone treatments in the cited study, but on postharvest quality instead of soil-borne disease [30]. In another recent study, the combination of the two compounds resulted in a beneficial effect on improving water status, membrane stability and catalase activity during a drought stress experiment [31]. Therefore, the effects of the combined spray might have been partly to enhance physiological resistance and reduce colonization of leaves, in addition to fungicidal action.

This interpretation is supported by biochemical studies of the peroxidase activity data. The activities values in the control, chitosan, melatonin and the chitosan–melatonin combination were 40.55, 90.00, 70.00 and 135.60 $\Delta A_{470} \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$, respectively. Peroxidase may have a role in cell wall strengthening, phenol oxidation, and regulating hydrogen peroxide during defense responses. Weak, moderate and strong guaiacol peroxidase responses can be elicited by salicylic acid, jasmonate-related signals, chitosan as well as pathogen elicitors. The responses of cellular fractions and tissues to guaiacol peroxidase vary from weak to strong [32]. In a hypersensitive reaction localized changes in guaiacol peroxidase are accompanied by the generation of hydrogen peroxide, which is indicative of the role of this enzyme as a dynamic marker of defense [33]. Increased peroxidase activity has also been associated with induced resistance in *Fusarium*-inoculated wheat [34]. Therefore, the higher activity is consistent with the higher activity of the combined treatment, which can be interpreted as an enhanced defense signaling. But, these peroxidase assays do not establish the complete mechanism. This interpretation would be strengthened by measuring the hydrogen peroxide, phenolics, phenylalanine ammonia-lyase, polyphenol oxidase and defense-gene expression. However, for pathogen-inoculated bean, guaiacol peroxidase was used along with oxidative markers and lesion development as a tool for interpreting resistance [35].

Conclusion

In this study we have shown that chitosan and melatonin, both individually and in combination, are effective in controlling root rot disease caused by *R. solani* in melon plants. In vitro studies have confirmed their effective antifungal activity and the combined treatment is responsible for the complete elimination of fungal growth. Field trials have also confirmed that we achieved a significant reduction in disease incidence and severity in treated plants and the optimal outcome of the combined treatment. The increase in peroxidase enzyme activity gives biochemical evidence of the activation of the plant defense mechanism by chitosan and melatonin compounds. This study validates the potential of chitosan and melatonin for the treatment of melon root rot and is a green way to combat it with plant protection. We need to investigate the molecular mechanisms of their synergistic action and their economic feasibility for large scale applications.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All relevant data and information about the laboratory steps used in this process are fully included in the manuscript. Free requests should include biological samples or detailed materials from the corresponding author

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