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Original Article

Nano-encapsulated Ajwain essential oil elicits resistance against early blight in tomatoes (Solanum lycopersicum L.)

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Abstract

Early blight, caused by Alternaria alternata, poses a significant threat to tomato production worldwide. This study investigates the potential of nano-encapsulated ajwain (Trachyspermum copticum) essential oil, delivered via chitosan nanoparticles, to induce systemic resistance in tomato plants against early blight. Oxidative stress, measured by malondialdehyde content, was significantly reduced in plants treated with nano-encapsulated Ajwain essential oil compared to controls. Furthermore, the activity of antioxidant enzymes (SOD, CAT, and POD) was significantly elevated in treated plants, indicating an enhanced defense response. The nano-encapsulated essential oil demonstrated superior efficacy in controlling early blight symptoms. These results suggest that chitosan nanoparticle-mediated delivery of ajwain essential oil is a promising, environmentally friendly strategy for enhancing tomato resistance to early blight.

Keywords: Nano-encapsulation, Elicitor, Chitosan, Herbal extract, Oxidative stress.

1. Introduction

Early blight caused by *Alternaria sp.* is a devastating disease of tomatoes (*Solanum lycopersicum* L.) in tropical and subtropical regions, leading to significant yield losses (up to 79% in some regions) [1]. The pathogen produces distinctive "bullseye" patterned leaf spots and can also cause stem lesions and fruit rot on tomato (*Solanum lycopersicum* L.). Despite the name "early," foliar symptoms usually occur on older leaves. *Alternaria alernata* infects stems, leaves and fruits of tomato. As the disease progresses, symptoms may migrate to the plant stem and fruit. Stem lesions are dark, slightly sunken and concentric in shape. Basal girdling and death of seedlings may occur, a symptom known as collar rot. The life cycle starts with the fungus overwintering in crop residues or wild members of the family *Solanaceae*, such as black nightshade. In the spring, conidia are produced. Multicellular conidia are splashed by water or by wind onto an uninfected plant [2].

Some common practices for controlling the disease are removing the plant residues from the field, drip irrigation, mulching, crop rotation, resistant plants and chemical control. Recently, it has been demonstrated that plant hormones including salicylic acid (SA), jasmonic acid (JA) and ethylene along with some elicitors like plant extracts, chitosan and their nano-formulated compounds also can induce resistance against fungal diseases and can be considered as alternative control method [3].

Ramkissoon *et al*. reported that foliar spraying of chitosan on tomato plants elevated the defense mechanism of plants against *Alternaria solani* and *Xanthomonas vesicatoria* through induced systemic resistance [4]. Chandra *et al*. used chitosan nanoparticles (CNP) to induce plant defense in *Camellia sinensis* and found that antioxidant enzyme activity, phenolic content and NO (nitric oxide) increased in response to CNP together with enhanced expression rate of some defense genes [5]. Ravikumar *et al*. tested 39 plant aqueous extracts against *Alternaria solani* and found that seven extracts would able to inhibit mycelia growth above 20% [6].

Due to some drawbacks such as chemical and physical

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instabilities of essential oils, researchers have proposed an encapsulation technique to overcome these challenges and increase the efficiency of extract usage [7, 8]. El-Mohamedy *et al*. successfully tested the chitosan nanoparticles against 18 pathogen fungi in tomato, potato and green bean [9]. Chitosan can be used as a drug delivery system in the field of sustainable agriculture [3].

Today, one of the subjects that is of high importance is the application of nanotechnology in the targeted delivery of drugs. The chitosan-based nanosystems have improved drug bioavailability and reduced toxicity by creating new drug delivery systems [10]. This polymer is a good choice for the creation of chitosan-based nanomaterials due to their excellent physical properties [11].

Accordingly, the objective of this study was to determine the effects of chitosan, essential oil and their nano and encapsulated forms on controlling the tomato leaf spot disease caused by *Alternaria alernata* from the point of view of evaluating the antioxidant defense system.

2. Materials and Methods

In order to investigate the effects of essential oil, chitosan and their derivate on the control of tomato leaf spot pathogen (*Alternaria alernata*) an experiment was carried out based on a completely randomized design with three replications. Treatments were Ajwain (*Trachyspermum copticum)* essential oil, chitosan, chitosan nanoparticles, encapsulated essential oil with chitosan nanoparticles and water as control.

Tomato (*Solanum lycopersicum*) certified seeds (imperial cultivar) were sterilized using 5% sodium hypochlorite and planted in sterilized clay loamy soil in an isolated standard greenhouse (16/8 h photoperiod, 25/17 °C temperature and 65% humidity). Plants were inoculated with the pathogen seven weeks after planting and elicitors were sprayed on plants 24 h after inoculation. Each plot consists of three pots (25 cm diameter) including four plants in the pot. Samples were taken at 0, 24, 48, 96 and 192 h after spraying of elicitors and frozen with nitrogen and kept at -80 until enzyme measurements.

2.1. Preparation of essential oil

Five different plant essential oils were purchased from Barij Essence Co, Iran and used in a preliminary experiment to select the best one in controlling the pathogen and use for main experiment and encapsulation purposes. These essential oils included mint (*Mentha arvensis*, with a concentration of 200, 400 and 1000 ppm), peppermint (*Mentha pipperia*, with concentrations of 100 and 400 ppm), eucalyptus (with concentrations of 100, 400 and 1000 ppm), rosemary (*Salvia Rosmarinus*, with the concentration of 100, 400 and 1000 ppm) and Ajwain (*Trachyspermum copticum,* with the concentration of 200, 400 and 1000 ppm). Based on the measurement of inhibition diameter results of preliminary experiment showed that Ajwain essential oil (200 ppm) had the highest effect on the inhibition of pathogen growth which was not statistically different with 400 and 1000 ppm of Ajwain essential oil, so Ajwain 200 was selected for using in main experiment and encapsulation (data were not shown).

2.2. Chitosan and chitosan nanoparticles preparation

A 0.5 g of chitosan was dissolved in 100 ml of 0.1% acetic acid and kept until use. Chitosan nanoparticles were prepared according to Qi *et al*. [12]. Briefly, aqueous tripolyphosphate solution (0.25%, w/v) was added to chitosan under continuous stirring. The produced nanoparticles were collected by centrifugation at 9000 rpm.

2.3. Encapsulation of essential oils using chitosan nanoparticles

Ajwain essential oil was encapsulated within chitosan nanoparticles. Briefly, Tween-80 (10:1 w/v relative to the essential oil) was added to the chitosan solution under vigorous stirring. Ajwain essential oil was then added dropwise to the chitosan/Tween-80 solution with continuous vigorous stirring. Subsequently, a buffer solution (pH 5, 1:1 v/v) was added, followed by 0.5 ml of 0.25% tripolyphosphate (TPP, pH 5). The mixture was stirred for an additional 10 minutes and then sonicated at 60 W for 1 minute.

Determination of particle size, polydispersity index and zeta potential of nanocapsules was executed by Zetasizer apparatus model PSS0012-22 (Malvern, UK). The analysis was done at a scattering angle of 90° [13].

2.4. Enzyme extraction and activity measurement

One gram of the frozen leaf sample was homogenized in mortar with 5 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM dithiotreitol and 2% polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15,000 g for 25 min and the supernatant was used for SOD, CAT, and POD assay.

The activity of SOD (EC 1.15.1.1) was determined according to Beyer and Fridovich [14]. In small glass tubes, 20 µL of enzyme supernatant were added to 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 µM nitro blue tetrazolium (NBT), and 0.025% triton-X100. The reaction was started under fluorescent light for 10 minutes by adding 10 µL of riboflavin solution. The absorbance of the solution was measured at 560 nm for both blank and control. SOD activity was expressed as unit per min g^{-1} FW.

The activity of CAT (EC 1.11.1.6) was assayed according to Chance and Maehly [15]. A 1.5 mL reaction mixture containing 30 µL water, 50 µL 1M Tris-HCl buffer (pH 8.0), 5 mM EDTA and 900 µL 10 mM H2O2 was added to 20 µL of enzyme supernatant. The decrease in the absorbance at 240 nm was recorded for 60 seconds. CAT activity was expressed as units per min g^{-1} FW.

The activity of POD (EC 1.11.1.7) was determined by a spectrophotometer at 470 nm according to Yamane *et al*. [16] in a 3 mL reaction mixture containing 1.5 mL 0.1 M potassium phosphate buffer (pH 7.0), 600 µL 10 mM guaiacol, 800 µL 4 mM H2O2 and 100 µL crude enzyme. POD activity was expressed as units per min g^{-1} FW.

Malondialdehyde (MDA) content of leaf samples was measured as described by Stewart and Bewley in a colorimetric method [17]. Samples were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA) and centrifuged. Then, 0.5 ml of supernatant was mixed with 2 ml of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 95˚C for 30 minutes. The samples were centrifuged at 10,000 g for 10 minutes. The absorbance of the supernatant was read at 532 and 600 nm. The amount of MDA was calculated from the extinction coefficient of 155 mM-1 cm-1 and expressed as nM g^{-1} FW.

2.5. Statistical analysis

Data were subjected to analysis of variance (ANOVA) after normality test (Smirnov-Kolmogorov test using SPSS16) by SAS9.1 software and means were compared with LSD 5%.

3. Results

3.1. Determination of particle size and zeta potential of nanoparticles

The stability of colloidal nanosystems is strongly related to the average particle size, size distribution (polydispersity index), and zeta potential. Dynamic Light Scattering (DLS) analysis revealed average particle sizes of 120 nm and 150 nm, and polydispersity indices of 0.18 and 0.23, for unloaded and Ajwain essential oil-loaded chitosan nanoparticles, respectively. This narrow size distribution and small particle size may enhance nanoparticle penetration into plant or fungal cell walls and contribute to system stability by minimizing Ostwald ripening. The average zeta potential was +26 ± 0.5 mV for unloaded chitosan nanoparticles and +23 ± 2 mV for essential oil-loaded nanoparticles. Zeta potential values exceeding +20 mV generally indicate good colloidal stability due to electrostatic repulsion. Overall, these DLS results suggest that the prepared Ajwain essential oil-loaded chitosan nanoparticles exhibit acceptable stability, consistent with experimental observations.

3.2. Chitosan nanoparticles morphology

Figure 1 shows the SEM-derived morphology details of the essential oil-loaded chitosan nanoparticles. As shown in this Figure, chitosan nanoparticles have a spherical shape with average size 84.38 ± 13.90 nm. These homogeneous nanoparticles are clearly in accordance with the data obtained from DLS.

3.3. Activity of antioxidant enzymes

The activity of antioxidant enzymes in tomato leaves was significantly affected by treatments and fungi inoculation. SOD activity increased over time in all treatments and the highest activity (204 units min^{-1} g^{-1} FW) was observed in encapsulated ajwain essential oil with chitosan nanoparticles at 120 h after inoculation which was 7.5 fold greater than water control at 120 h (Fig. 2).

Catalase activity increased over time in all treatments and the highest value (437 units min^{-1} g^{-1} FW) was recorded in encapsulated essential oil in chitosan nanoparticles at 120 h after inoculation (Fig. 3).

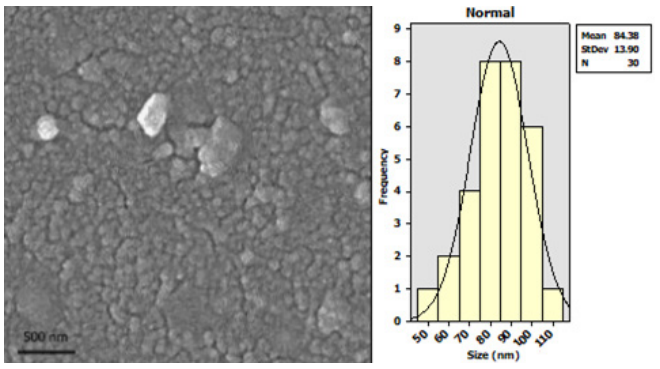

Fig. 1. SEM imaging of the as prepared essential oil-loaded chitosan nanoparticles.

Peroxidase activity also increased over time in all treatments, but there is no significant difference between 24 and 48 h in water control. The highest peroxidase activity (383 units min^{-1} g^{-1} FW) was related to the encapsulated essential oil with chitosan nanoparticles at 120 h (Fig. 4).

MDA content increased in water control over time and reached the highest (7.6 nM g^{-1} FW) at 120 h. There were no significant differences among treatments in MDA content at 24 h except for encapsulated treatment. The lowest MDA content (1.2 nM g^{-1} FW) was observed in

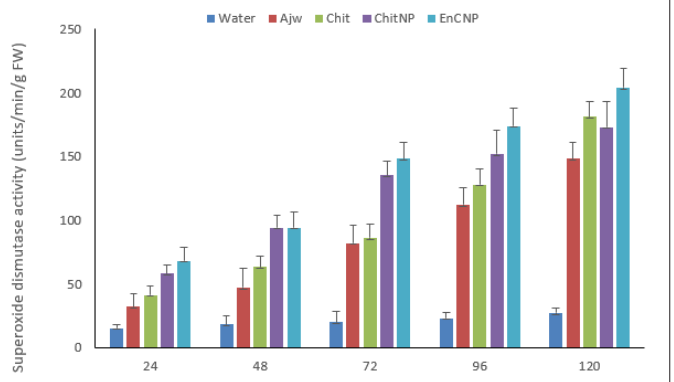

Fig. 2. Effect of treatments on the activity of superoxide dismutase in tomato leaves inoculted with *Alternaria alternata* after 24-120 h post inoculation (each column is the mean of three replicates. Ajw, Ajwain essence, Chit, Chitosan, ChitNP, chitosan na.

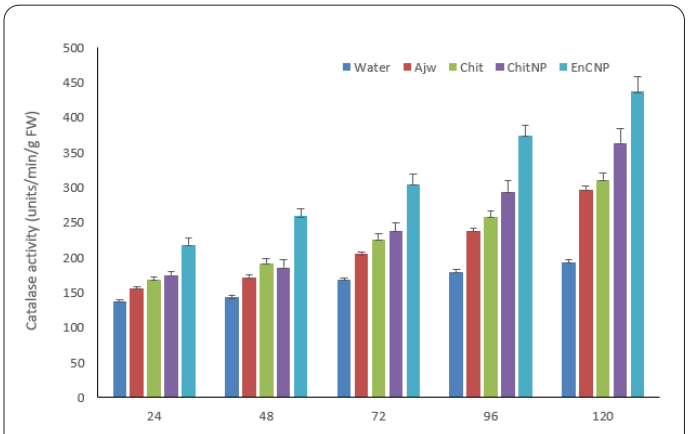

Fig. 3. Effect of treatments on the activity of catalse in tomato leaves inoculted with *Alternaria alternata* after 24-120 h post inoculation (each column is the mean of three replicates. Ajw, Ajwain essence, Chit, Chitosan, ChitNP, chitosan nanoparticles.

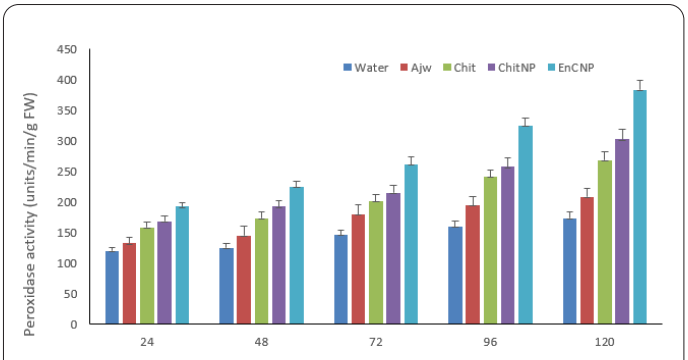

Fig. 4. Effect of treatments on the activity of peroxidase in tomato leaves inoculted with *Alternaria alternata* after 24-120 h post inoculation (each column is the mean of three replicates. Ajw, Ajwain essence, Chit, Chitosan, ChitNP, chitosan nanoparticle.

encapsulated treatment at 120 h (Fig. 5). The results for MDA content were in accordance with antioxidant enzyme activity so that the highest activity of enzymes coincided with the lowest content of MDA.

3.4. Effect of different treatments on disease symptoms in vivo

Two weeks after inoculation with the *Alternaria alernata* and the application of treatments, the investigation of the symptoms showed that: In all the treatments tested except the inoculation with the *Alternaria alernata*, the spots did not show much spread. However, in the treatment that was only inoculated with the *Alternaria alernata*, the spots were extensive and severe symptoms were observed (Fig. 6).

4. Discussion

Biotic stresses especially caused by fungal pathogens initially target the plant cell membrane phospholipids and destroy the membrane integrity. The consequence of this event is the production of oxygen free radicals named reactive oxygen species (ROS) which actively react with membrane phospholipids and degrade them to toxic agents like aldehydes, di-acids, malon dialdehyde, etc. Degradation of membrane integrity finally leads to cell death [18]. Antioxidant enzymes are the most important defense mechanism of plants against ROS and detoxify the radicals and protect the membranes [19].

Inducing resistance to disease by elicitors is a useful technique for developing rapid defense against invading pathogens [4]. Elicitors as an environmentally friendly chemical can affect positively on defense responses in plants [20]. Pérez-MartínezI et al. [21] showed a significant increase in the activity of Antioxidant enzymes by resistance induction by mycorrhiza fungi in infected tomatoes by Alternaria solan [21].

Sathiyabama. et al [22] indicated that chitosan can be applied as foliar spray to protect tomato plants from early blight disease pathogens and to increase the yield [22]. Chitosan and its derivatives are known to be potent inducers, and increased activity of antioxidant enzymes has been demonstrated as a result of chitosan treatment [23].

Chitosan and its nanoparticles have proven to be effective against fungal pathogens. Nano-sized chitosan is more effective due to its high surface area to volume ratio and penetrates cells rapidly [9]. Sathiyabama, and Manikandan [24] indicated that the Copper-chitosan nanoparticle plays a dual role in enhancing defense enzymes as well as protecting finger millet plants from blast fungus [24]. Also, Chandra *et al*. [5] reported that antioxidant enzymes activity in *Camelia sinensis* leaves increased 24 h after application of chitosan and nano chitosan particles, so that the increasing amount in SOD and CAT by nanoformulation was 8 and 9% greater than chitosan, respectively [5]. The increased activity of antioxidant enzymes due to application of elicitors can develop the required defense from oxidative stress associated with pathogenic invasion [3].

A nanoparticle loaded with essential oils of plants seems to be more effective against microbes [7].

Some plant extracts (essential oils or essences derived from roots, leaves, stems or seeds) contain natural products that are toxic to pathogens [25] and affect fungal growth, and multiplication or enhance plant systemic resistance [26]. Scientists believe that plant extracts and other elicitors likely act as secondary messengers in enhancing plant resistance mechanism [27] which activating antioxidant enzymes and inhibition of membrane degradation could be part of resistance and might lead to induced systemic resistance (ISR).

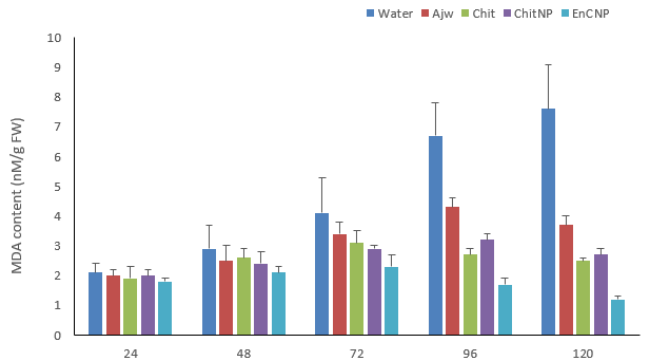

Fig. 5. Effect of treatments on malon dialdehyde (MDA) content in tomato leaves inoculted with *Alternaria alternata* after 24-120 h post inoculation (each column is the mean of three replicates. Ajw, Ajwain essence, Chit, Chitosan, ChitNP, chitosan nanopart.

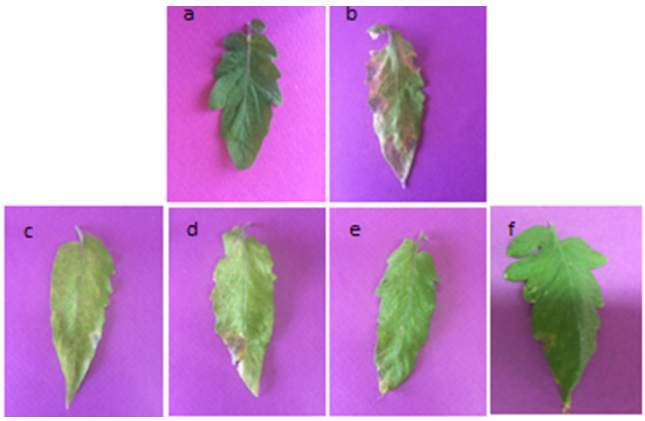

Fig. 2. Effect of treatments on symptoms of disease: a) water, b) fungi of *Alternaria alernata,* c) Ajwian, d) chitosan, e) chitosan nano paricle and f) chitosan nano particle loaded with essential oil.

Increasing the amount of polyphenol oxidase and peroxidase in a plant treated with several plant extracts in tomato plants inoculated with Xanthomonas campestris pv. Vesicatoria has been shown by Abo-Elyousr et al. [28] and increasing the amount of oxidase, peroxidase and chitinase in wheat leaf rust has been shown by Draz et al. [29].

In this study, the activity of antioxidant enzymes in tomato leaves was significantly affected by fungal treatments and inoculation. In all treatments tested, enzyme activity, POX SOD and CAT, important antioxidant enzymes involved in ROS inhibition, were significantly higher than the control treatment and the highest increase was observed in the treatment of chitosan nanoparticles containing essential oil.

In conclusion, the role of elicitors in plant defense against pathogens is well known and researchers are attending to increase the efficacy of elicitors. Using nanoparticles or encapsulation of essential oils in nanoparticles could be the answer for these efforts because of their high prepotency in plant or pathogen cells. Results of this work also showed that encapsulated Ajwain essential oil in chitosan nanoparticles was the superior treatment for inhi-

bition of oxidative stress in tomato plants after infection with *Alternaria alernata.*

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