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RESEARCH ARTICLE

Molecular responses of *Phytophthora capsici*-challenged cucumber (*Cucumis sativus* L.) plants as influenced by resistance inducer application

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ABSTRACT: Phytophthora species are considered as the major cause of several plant diseases resulting in huge yield losses in agricultural crops. Despite years of effort to develop Phytophthora resistance varieties, there is no reports of a resistant cucumber variety. In this study, the effect of concomitant application of potassium phosphite (KPhi) and chitosan on some physiological and molecular responses of *Phytophthora capsici*-challenged cucumber plants were investigated. Cucumber plants were treated with KPhi and/or Chitosan at different concentrations and were then inoculated with zoospores of *P. capsici* and leaf samples were collected at different time courses. Results showed that Guaiacol peroxidase (GPOD) enzymatic activity surged immediately at first and second days after pathogen inoculation with a peak in plants treated with 4 gL^{-1} KPhi 2 days after inoculation. Compared to GPOD, the highest superoxide dismutase (SOD) activity was observed in the same treatment but later at 5 days after inoculation. It was indicated that the activity of antioxidant enzymes was greatly influenced by application of either KPhi or chitosan while their activity was not remarkably enhanced in control plants. qPCR analysis revealed that the highest increase in glutathione peroxidase (*gpx*) gene expression was achieved in plants concomitantly treated with 4 gL^{-1} KPhi and 200 mgL^{-1} chitosan 5 days after inoculation. The findings of this study provide novel information regarding inducing mechanisms of KPhi and chitosan which may be effective in mitigating disease severity.

KEYWORDS: Resistance inducer, Cucumber, Potassium phosphite, Chitosan, Defense response

INTRODUCTION

Higher plants have developed various defense mechanisms enabling them to survive unfavorable conditions including biotic and biotic stresses [29]. Pathogenic organisms attack plants in all developmental stages and cause huge yield losses which indirectly increases the production costs. Extensive application of chemical pesticides is one of the most focused controlling measures to reduce the adverse effects of pathogen attack. This continuous application of pesticides resulted in several environmental problems and health issues [14]. During the past decade alternative strategies have been developed to fulfil the increasing demand for an environment-friendly methodology guarantying a healthy final product. Cultural practices, biological control and resistance inducer application are gaining more attention as low cost environmentally safe approaches. In most of these strategies, pathogen penetration and extension are limited by plant natural defense systems [27]. This complicated defense machinery has been evolved during the evolutionary processes in higher plants and have been the subject of several research programs. Compared to other organisms, plants exploit a multidisciplinary

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defense system that inhibits pathogen penetration as well as reducing their adverse effects after penetration. Systemic Acquired Resistance (SAR), Hypersensitive reaction (HR) and accumulation of a wide range of defensive biochemical are among the most important plant defense mechanisms [20]. Plant strengtheners or resistance inducers are chemical, biological and physical factors that efficiently induce plant defense cascade. In this way, plants will be ready before pathogen attack and can inhibit pathogen infection more efficiently. This has triggered formulation and application of various compounds to be used instead of chemical pesticides [1, 14].

Chitosan, a polycationic biopolymer consisting of glucosamine and N-acetylglucosamine units, is found in large amounts in nature and is one of the most prevalent biological inducers which increases plants tolerance to different biotic stresses. This substance is found in insects, and most of fungi, yeast and has been proved as generally regarded as safe compound with no environmental hazards and is not toxic to mammals [2]. The effect of chitosan on plant fungal pathogens have been reported by various research groups [25]. These investigations have revealed that when applied before pathogen infection, chitosan is more effective compared to cases it was applied after the pathogen was developed in plant tissues. The efficiency of chitosan on disease suppression is dependent to the application dose and the virulence of the pathogen [2]. Moret et al. [18] investigated the controlling effects of chitosan against cucumber powdery mildew and reported that chitosan induced plant defense responses through overexpression of defense enzymes i.e., chitinases, chitosanases and beta-glucanases as well as increased lignin and callose deposition.

For decades phosphite salts have been used both as fertilizers and antifungal agent [9]. This group of compounds generally enhance plant growth and have been reported to be able to control a wide range of plant pathogenic organisms [8]. Rakha et al. studied the alleviative effect of potassium phosphite on *Phytophthora*-challenged cucumber plants. It was revealed that all studied doses reduced the disease damage [21]. The application of plant resistance inducers like chitosan and phosphite salts not only provides a reliable control of plant pathogens but also improve consumer health through reduced use of chemical pesticides.

Cucumber is used both as vegetable and as fruit and generally is prone to infection by various pathogens, hence extensive application of pesticides is unavoidable. This imposes a potential health problem for consumers. Substituting chemical pesticides with safe compound like potassium phosphite and chitosan would be very important in terms of public health and environmental conservation.

In this study the effect of potassium phosphite and chitosan application on *Phytophthora capsica*-challenged cucumber plants was investigated. The findings of this study would be so helpful in designing precise controlling measures for application of these compounds in disease control in cucumber production.

MATERIALS AND METHODS

Plant growth

Cucumber seeds from a greenhouse cultivar, Sultan were cultured on a sterilized soil mixture consisting equal volumes of peat and perlite. The plants were grown under controlled condition with a 16:8 (light: dark) photoperiod, 70% relative humidity and temperature of 23–27 °C and were fertigated regularly.

Pathogen culture and plant inoculation

The *Phytophthora capsici* isolate was kindly provided by Prof. Z. Banihashemi from his culture collection (Culture number PH-2-19-92) at Shiraz University, Shiraz, Iran. A zoospore suspension was prepared from *P. capsici* according to the method of Matheron et al. [15] with some modifications. Fungal mycelia were grown on V8 juice agar (HiMedia Laboratories, Mumbai, India) and then 5 blocks with a 6 mm diameter from 5 days old cultures were transferred to new plates and were flooded with non-sterile soli extract. The plates were incubated at room temperature for 5 days and were then incubated at 4°C for 30 minutes to induce zoospore release. The suspension was examined under light microscope and the zoospores were counted using a hemocytometer. The suspension concentration was finally adjusted to 5×10^6 zoospores per milliliter.

Potassium phosphite Preparation

Potassium phosphite stock solution was prepared according to the method of Mofidnakhaei et al. [16]. Briefly, phosphorous acid (AppliChem, Darmstadt, Germany) was partially neutralized with potassium

hydroxide (by gradual mixing of phosphorus acid and potassium hydroxide solution) and the pH was adjusted to 6.3.

Chitosan preparation

Low molecular weight chitosan was purchased from Sigma-Aldrich and the stock solution was prepared according to a previously described method [22] with some modifications. Briefly, different concentrations of chitosan powder were dissolved in 10% acetic acid and insoluble fractions were discarded after a 20 min centrifugation at 8000 g. The pH of the solution was then adjusted to 5.8 with sodium acetate.

Total protein extraction

Crude leaf proteins were extracted as described previously [16]. About 500 mg of leaf tissue was powdered with liquid nitrogen and homogenized in 5 ml of cold phosphate buffer (0.1 M phosphate buffer (pH 7.5) plus 0.5 mM EDTA). The mixtures were transferred to new tubes and were centrifuged at 20000 g for 15 min at 4 ℃ and the supernatants were used for enzyme activity assays.

Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was analyzed through a modified Nitro blue tetrazolium method as described previously [17]. Three tubes with the same reaction mixes including 1.5 ml of 50 mM phosphate buffer, 0.3 ml of 130 mM MeOH, 0.3 ml of 750 mM nitro blue tetrazolium (NBT), 0.3 ml of 100 mM EDTA-Na2 and 0.5 ml of water. The enzyme extract (0.05 ml) was added to the first tube and then exposed to 4000 lux light for 15 min at 25℃. The second tube was also exposed to the above mentioned light conditions, while the third tube was stored in the dark. One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition in NBT reduction at 560 nm wavelength using a spectrophotometer (Biochrom WPA Biowave II).

Guaiacol peroxidase activity assay

Guaiacol peroxidase (GPOD) activity was measured by the oxidation of Guaiacol in the presence of H_2O_2 according to the modified method described previously [23]. The reaction mix comprised 1700 µl phosphate buffer (100 mM), 600 µl Guaiacol (20 mM) and 600 µl of the enzyme extract. The reaction was started by adding 100 µl of 10 mM hydrogen peroxide to the mixture. The increase in absorbance at 470 nm was recorded using a spectrophotometer (Biochrom WPA Biowave II).

Table 1. Genes and related primers used for qPCR

Genes	Amplicon size	Primers
gpx XM_004143867	101 bp	gttcttcgcttatcgcctcgt tcctttagcatccttgacggtg
actin XM_011659465.1	155 bp	gattctggtgatggtgtgagtc tcggcagtggtggtgaacat

Total RNA isolation and cDNA synthesis

Total RNA was extracted from leaf samples using RNAx-plus solution (CinnaGen Inc, Iran) and were then treated with DNase I to eliminate possible genomic DNA contaminants. The first-strand cDNA was synthesized using 2 µg of total RNA, oligo(dT) primers, RevrtAid reverse transcriptase and RiboLock RNase Inhibitor (Thermo Scientific, USA) according to manufacturer`s instructions.

Glutathione peroxidase gene expression analysis

The Real-time PCR primers for *gpx* gene were designed using Oligo 7 software and ACTIN gene was used as reference gene for expression data normalization (Table 1). One tenth dilutions of cDNA samples were used as templates and Real-time PCR reactions were performed using a Maxima SYBR Green/ROX qPCR Master Mix kit (Thermo Scientific, USA) in a Bio-Rad CFX-96 instrument (Bio-Rad, USA) The following program was used for the reactions: 3 min at 95 ℃, denaturation at 95 ℃ for 25 s, annealing at 60 ℃ for 20 s and extension at 72 ℃ for 25 s for 40 cycles. The specificity of the PCR was confirmed by melting curve analysis of the products and the size of the products was checked on 2% agarose gel. Relative fold expression of *gpx* gene was calculated by $2^{-\Delta\Delta CT}$ method using Bio-Rad CFX Manager software (BioRad, USA).

Data analysis

This study was s set up as a completely randomized design (CRD) with three replications in factorial arrangement with 3 factors including, potassium phosphite concentrations, Chitosan Concentrations and pathogen inoculation. Duncan's multiple range test was used for mean comparison at 1%. All statistical analyses

were performed using SAS 9.1 software (SAS Institute, Cary, NC).

RESULTS

The results of the present study indicated that the immune system of the *Phytophthora capsici*-challenged cucumber plants was remarkably induced by application of either chitosan and potassium phosphite. The activity of reactive oxygen species (ROS) scavenging enzymes as well defense gene expression was significantly enhanced.

Guaiacol peroxidase enzyme activity

The results revealed that the highest GPOD activity (30.19 U/mg protein) was recorded in plants treated with 4 grL^{-1} KPhi at 2^{nd} day post inoculation and the lowest enzyme activity (0.001 U/mg protein) was observed 4 days after inoculation in KPhi-treated plants inoculated with *P. capsici* and also in plants pretreated with 200 mgL^{-1}chitosan before being inoculated with *P. capsici* (Figure 2). It can be inferred from the figure that GPOD activity is extinguished in all treatments with the time. It is also clear that GPOD activity was not significantly in control plants even at 1 and 2 days after inoculation, while plants in other treatments showed fluctuations in enzyme activity.

Superoxide dismutase enzyme activity

It was revealed that the highest SOD activity (53.27 U/mg protein) was observed in cucumber plants treated with 4 grL^{-1} KPhi 5 days after treatment. On the other hand, the lowest activity of SOD (1.04 U/mg protein) was recorded in plants solely inoculated with *P. capsici* 2 days after inoculation (Figure 1).

As it is shown in the figure, SOD activity was not changed in control plants with the time. Compared to control plants, in plants treated with either KPhi and chitosan, SOD activity was decreased at first and was then increased gradually in 4 and 5 days after application. The SOD activity was dramatically decreased in plants inoculated with *P. capsici* at 2 days after inoculation, while this decrease was not observed form *P. capsici*-challenged plants pretreated with KPhi, Chitosan or both.

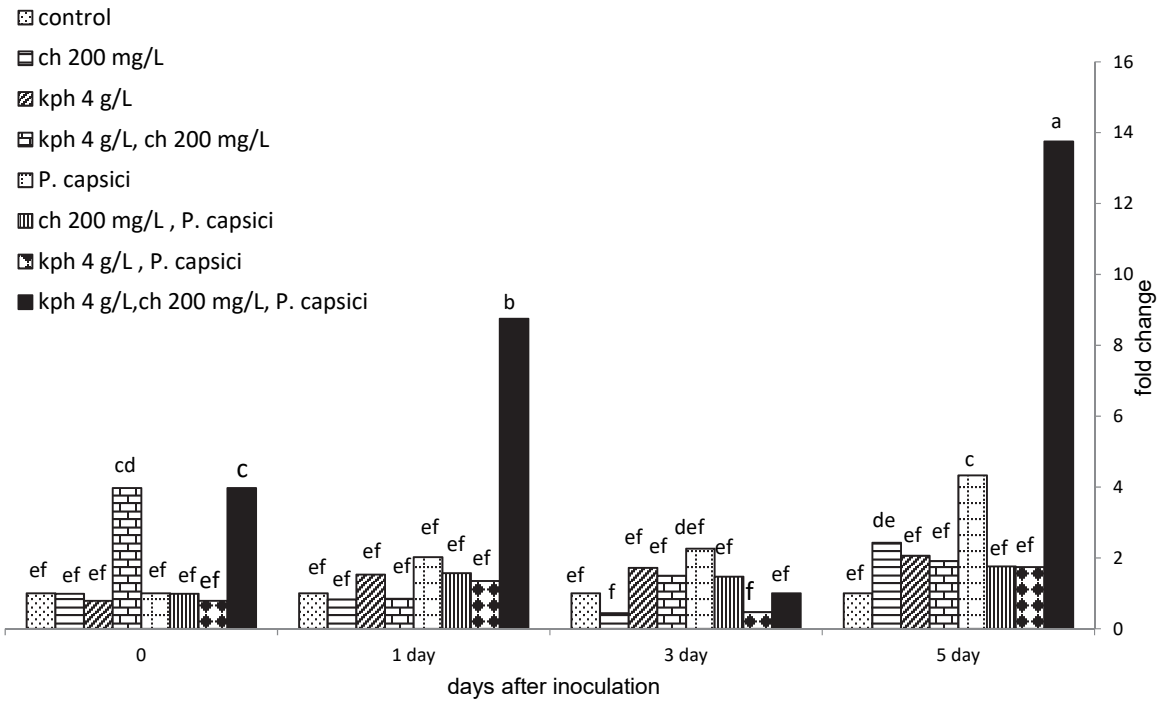

Figure 3. Relative gene expression profile of *GPX (Cucumis sativus* glutathione peroxidase) gene over a time course from 1 day to 5 days after inoculation in *P. capsici*-challenged cucumber plants pretreated with potassium phosphite and chitosan.

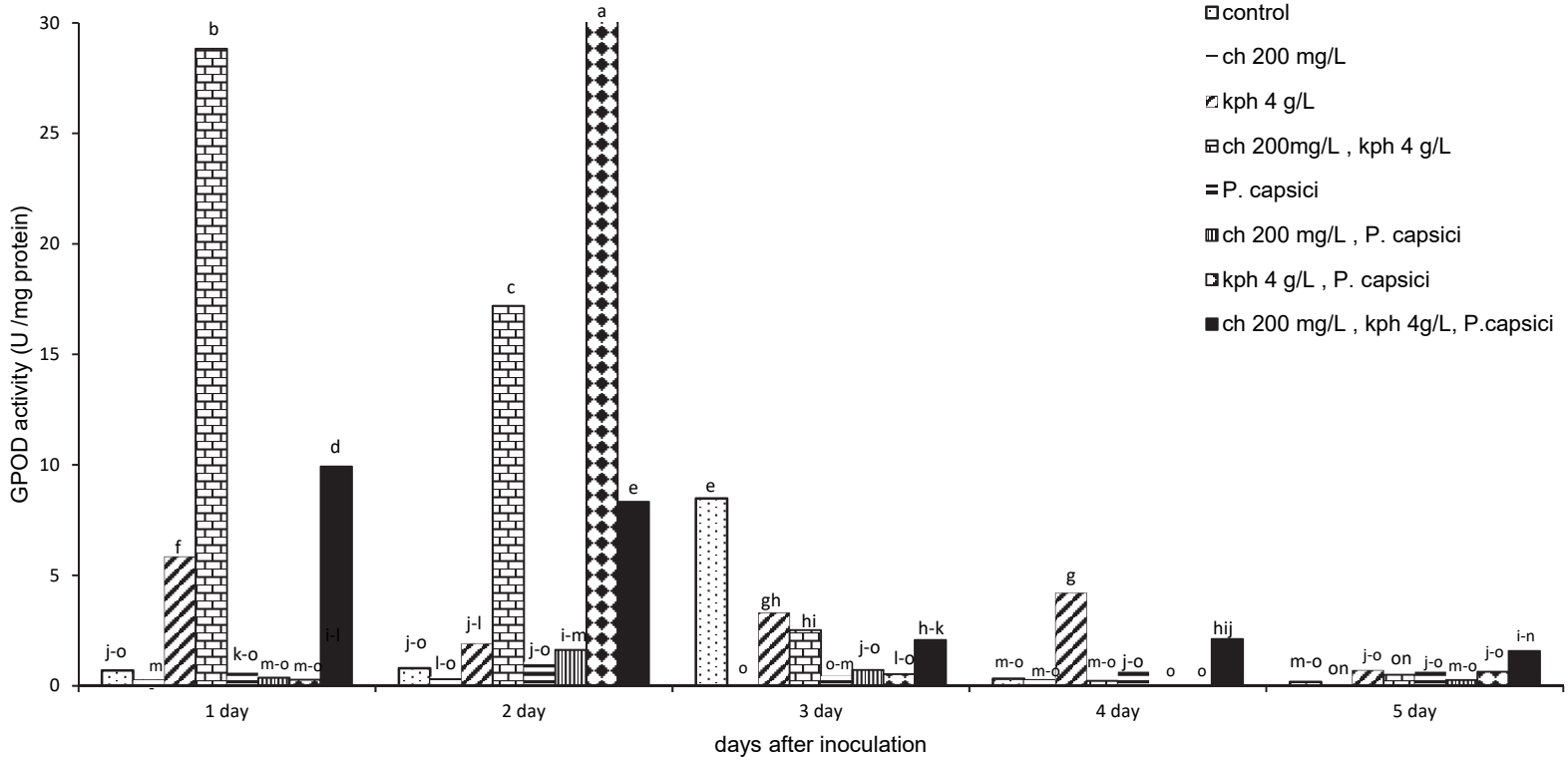

Figure 1. Alterations is the activity of Guaiacol peroxidase (GPOD) enzyme activity over a time course from 1 to 5 days after inoculation in *P. capsici*-challenged cucumber plants pretreated with potassium phosphite and chitosan

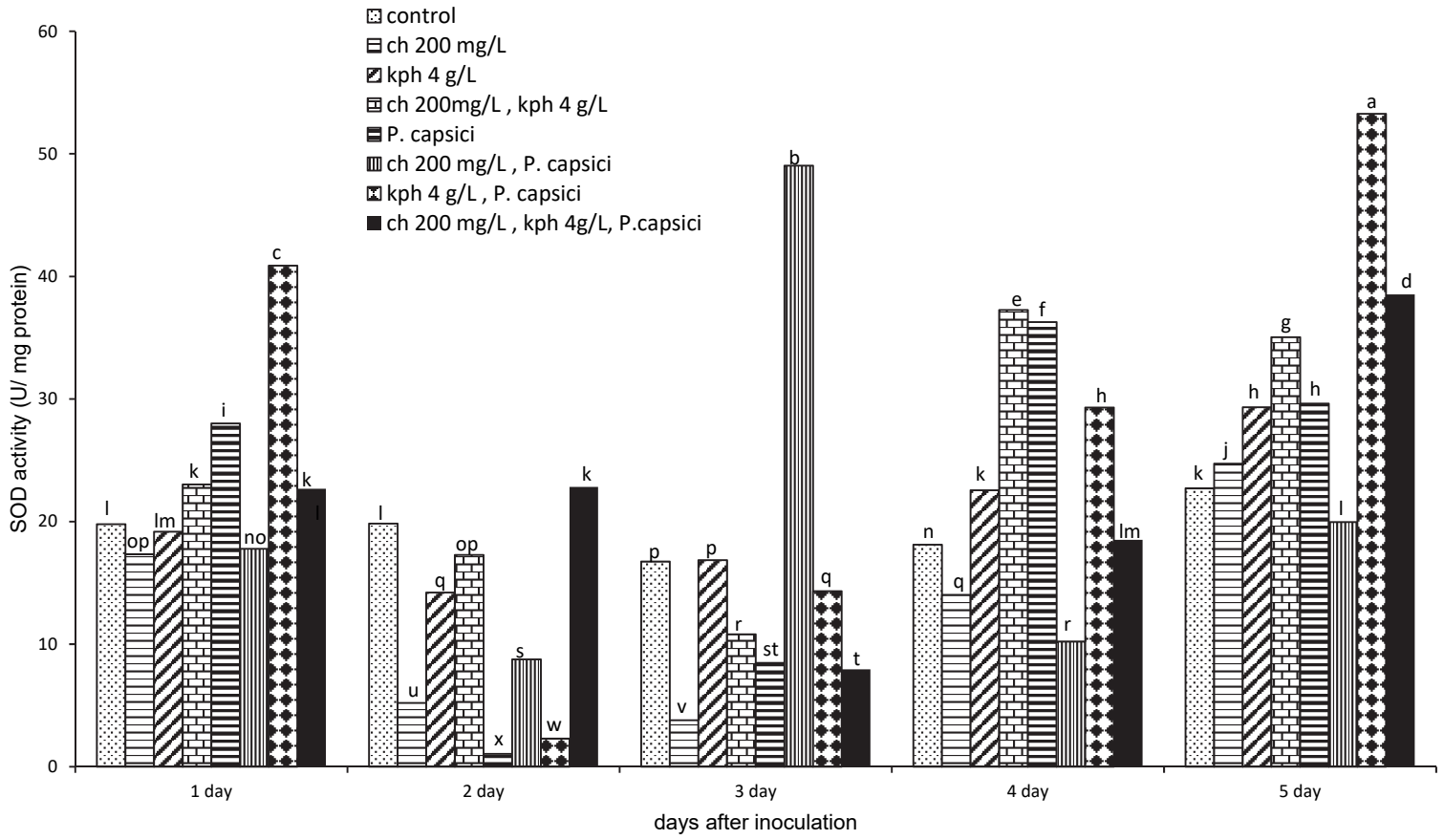

Figure 2. Changes is the activity of superoxide dismutase (SOD) enzyme over a time course from 1 to 5 days after inoculation in *P. capsici*-challenged cucumber plants pretreated with potassium phosphite and chitosan

Glutathione peroxidase gene expression

Gene expression analysis revealed that the expression of *gpx* gene was significantly changed in different treatments (Figure 3). It was shown that the highest expression rate of *gpx* was recorded in plants concomitantly treated with 4 grL^{-1} KPhi and 200 mgL^{-1} chitosan followed by *P. capsici* inoculation 5 days after inoculation. This increase was observed in the same treatment 1 day after inoculation. As it can be seen in the figure, it seems that compared to KPhi, Chitosan treatment increased the *gpx* expression more efficiently either when it is applied singly or in concomitant application with KPhi.

DISCUSSION

Plants respond to various biotic and abiotic stresses through a complicated defense machinery which enables them to survive under unfavorable conditions. Pathogenesis-related proteins (PRPs) are a diverse group of functional proteins which are overexpressed in plants upon infection by various pathogens [13, 24]. Additionally, to encounter the invading pathogens, plants produce a wide array of secondary metabolites which limit the pathogen expansion in infected tissues [17].

The overall results of the present study indicated that the application of Potassium phosphite (KPhi) and chitosan would be a promising measure for controlling the adverse effects of *P. capsici* on cucumber seedlings. Antioxidant enzymes including Guaiacol peroxidase (GPOD) and superoxide dismutase (SOD) were analyzed as indicators of plant defense response. These enzymes like other antioxidant enzymes, e.g., catalase, peroxidase, polyphenol oxidase and ascorbate peroxidase are important as primary plant defense responses through reactive oxygen scavenging and neutralizing the free oxygen radicals [12]. In adverse pathogen infection the reactive oxygen species increase to a high extent and these antioxidant systems would not be able to remove these harmful compounds leading to increased cell damage and cell death [10].

Our results showed that the highest SOD and GPOD activities were recorded in cucumber plants treated with 4 grL-1 KPhi before being inoculated with *P. capsici*. The highest SOD activity was observed on the 5th day after inoculation while the GPOD peak activity was observed at 3 days after inoculation. In an investigation the controlling effect of KPhi on *P. capsici* was studied in pepper and tomato plants. It was revealed that the KPhi-treated plants exhibited lower disease symptoms compared to control plants and there was a significant difference between treated and untreated plants. It was suggested that phosphite salts could be used as controlling measures against Oomycetes [7]. In another study the effects of Potassium phosphite on the zoospore production in *Phytophthora* sp. Was investigated. It was reported that although KPhi significantly reduced the production of zoospores, it did not completely extinguish the disease development [30].

Or results are also in accordance with the findings of Mofidnakhaei et al. who reported that a pretreatment with KPhi resulted in a higher disease resistance in cucumber plants inoculated with *Pythium ultimum* through increased activity of antioxidant enzymes in plant tissues. [16]. It was reported that in KPhi-treated Arabidopsis thaliana plants inoculated with *P. cinnamomi*, a higher resistance was observed compared to control plants. It was reported that besides increased ROS accumulation, KPhi caused increased cell wall lignification in plant tissues [5].

In the present study the activity of both GPOD and SOD, which are essential enzymes in removing hydrogen peroxide from cells, was significantly increased in all treatments. In the other hand, as the highest enzyme activities were recorded in KPhi-treated plants inoculated with *P. capsici*, it would be concluded that KPhi induced overexpression of this defense enzymes leading to increased disease resistance. Our enzymatic records are in accordance with the findings of Machinandiarena et al. (2012). They studied KPhi-treated potato plants under *P. infestans* attack and realized that KPhi inhibits pathogen development through limiting the production of superoxide and hydrogen peroxide [14].

It has been believed that Low molecular weight chitosan can cross bacterial cell wall and interfere with its growth through colloidization of the cell components. This will drastically decrease the bacterial growth and eventually leads to disturbed physiological functions and bacterial death [31]. Furthermore, it is an accepted idea that chitosan can penetrate the nuclei of microorganisms and disturb the mRNA and protein synthesis through irreversible binding to DNA molecules [26]. In an experiment oligochitosan molecules were applied on Arabidopsis plants infected with tobacco mosaic virus (TMV). It was revealed that oligochitosan improved Arabidopsis resistance against TMV by activating the Salicylic Acid (SA) pathway [11].

It was reported that treatment with water-soluble low-molecular-chitosan significantly decreased the late blight symptoms in potato plants [28]. In a study the inhibitory effects of chitosan on the resistance of barley seedling against powdery mildew was investigated. It was shown that chitosan induced oxidative burst and phenolic compound deposition in treated leaves, resulting in an unfavorable condition for pathogen development. Furthermore, a higher level and the more homogeneous diffusion of H2O2 in the treated leaf tissues were reported [6].

In our study it was indicated that chitosan application, singly or in combination with KPhi, increased t defense responses in *P. capsici*-challenged cucumber plants. Several investigations have reported the significant increase in transcription rate of plant *GPX* genes under various biotic or abiotic stress conditions such as cold, drought, herbivory and pathogen attack [19].

In the present study the highest expression rate of *GPX* gene was observed in cucumber plants concomitantly treated with KPhi and chitosan under *P. capsici* stress which indicates the inducing activity of these compounds in the plants. In an investigation it was revealed that two *GPX* genes in Arabidopsis plants play a key role in response to pathogen attack and activating the immune responses, therefore they might be involved in defense against virulent pathogen infection [3]. It was previously shown that in *Gossypium hirsutum* plants grown under different stresses, the expression rate of *GPX* gene was significantly increased in all biotic and abiotic stresses which indicated its importance in plant defense machinery [4]. In our study the expression of *GPX* was surged significantly at 5 days after inoculation. It seems that the defensive function of the enzyme is needed at the first hours after infection to limit the pathogen development.

CONCLUSION

The overall results of the present study showed that pretreatment of cucumber plants with 4 gr L-1 KPhi significantly increased the activity of defense enzymes i.e., guaiacol peroxidase (GPOD) and superoxide dismutase (SOD) which may lead to increased tolerance to *Phytophthora capsici*. On the other hand, concomitant application of KPhi and chitosan on *P. capsici*-inoculated plant showed the highest impact on *GPX* gene expression. In was also revealed that 4 grL-1 KPhi caused the highest increase in defense enzyme activities and the highest activities of GPOD and SOD were recorded at 3 and 5 days after inoculation, respectively. These findings would be successfully applied for determining the optimum treatment program for controlling *P. capsici* damages in cucumber plants.

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پاسخ‌های مولکولی گیاهان خیار (*Cucumis sativus* L.) تیمار شده با القاگرهای مقاومت در برابر تنش فایتوفتورا کپسیسی (*Phytophthora capsici*)

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چکیده

گونه های *Phytophthora* عامل بیماری های متعددی در گونه های گیاهی گیاهی هستندکه موجب افت عملکرد تولید در محصولات کشاورزی می شوند. علیرغم سالها تلاش برای ایجاد گونه های مقاوم به *Phytophthora*، هیچ گزارشی از ارقام مقاوم خیار وجود ندارد. در این مطالعه اثر مصرف همزمان فسفیت پتاسیم (KPhi) و کیتوزان بر برخی از پاسخ های فیزیولوژیک و مولکولی گیاهان خیار تحت تیمار *Phytophthora capsici* مورد بررسی قرار گرفت. گیاهان خیار با غلظت های مختلفی از KPhi و کیتوزان تیمار و پس از آن با زئوسپورهای *P. capsici* تلقیح شدند و نمونه های برگی در دوره های زمانی متفاوت جمع آوری گردید. نتایج نشان داد فعالیت آنزیم گایاکول پراکسیداز (GPOD) بلافاصله در روز اول و دوم پس از تلقیح با پاتوژن افزایش یافته بطوری که بیشترین فعالیت را در گیاهان تیمار شده با فسفیت پتاسیم (4 gr/l) بعد از گذشت دو روز از تلقیح نشان داد. در مقایسه با آنزیم گایاکول پراکسیداز، بیش‌ترین فعالیت سوپراکسید دیسموتاز (SOD) در همان تیمار اما بعد از گذشت ۵ روز از تلقیح مشاهده شد. بنابراین مشخص شد که فعالیت آنزیم‌های آنتی اکسیدان در اثر تیمار با فسفیت پتاسیم یا کیتوزان به شدت تحت تاثیر قرار گرفته، درحالی که فعالیت آنها در گیاهان شاهد افزایش قابل ملاحظه‌ای نیافته است. تجزیه و تحلیل qPCR نشان داد که بیش‌ترین افزایش بیان ژن گلوتاتیون پراکسیداز (GPX) گیاهان تیمار شده با فسفیت پتاسیم (4 gr/l) و کیتوزان (200 mg/l) بعد از گذشت ۵ روز از انجام تلقیح با پاتوژن حاصل شده است. یافته های این مطالعه اطلاعات جدیدی در مورد مکانیسم‌های القاء فسفیت پتاسیم و کیتوزان ارائه می‌دهد که می‌تواند در کاهش شدت بیماری موثر باشد.

کلمات کلیدی: القاگر مقاومت، خیار، فسفیت پتاسیم، کیتوزان، پاسخ دفاعی