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Phenol and antioxidant enzymatic activity in root knot nematode, *Meloidogyne incognita* infected tomato plants treated with chitosan nanoparticles

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Abstract
Root knot nematode, *Meloidogyne* spp. causes severe yield losses in vegetable crops. Chitosan is one of the most abundant biopolymer available worldwide next to cellulose. It is known to have anti-nemic, anti-fungal and anti- bacterial properties. Application of chitosan induces systemic resistance in tomato plants against pathogens by increasing phenolic content and antioxidant enzymes *viz.,* Polyphenol oxidase, Superoxide dismutase and phytoalexins. A study was conducted to study the phenol content and activity of antioxidant enzymes, polyphenol oxidase and peroxidase activities in root knot nematode infected tomato plants treated with chiotsan nanoparticles. The antioxidant enzymatic activities were recorded after 25th day of nematode inoculation. It was observed that the phenolic content increased due to the application of chitosan nanoparticle in nematode infected plants (174.32 µg/g of root) compared to 160.37 µg/g of root in untreated plants. Similarly the peroxidase and polyphenol oxidase activity also increased in chitosan nanoparticle treated nematode infected plants. The study revealed that chitosan nano particles protect plants from root knot nematode infection by increasing the phenol content, peroxidase and polyphenol oxidase activity and induce systemic resistance against nemayodes.

Keywords: Chitosan nanoparticles, M. incognita, phenol, peroxidase, polyphenol oxidase

Introduction
Insects, pathogens and nematodes are more prevalent in both agricultural and horticultural crops. Among nematodes, the endoparasitic nematodes like Root knot and Cyst nematodes causes severe yield losses in vegetable crops (Khan and Sharma, 2020) [14]. Plant parasitic nematodes interact with the root pathogens like *Fusarium* spp., *Phytophthora* spp., *Rhizoctonia* spp. and *Pythium* spp and increase disease severity. Several measures are taken to manage the pest, pathogen and nematode attacks. Nanotechnology is one of the recent technologies that can be used to develop several tools for drought, pest and disease resistance (Yanat and Schroen, 2021) [30]. Chitosan biopolymer is reported to possess anti-nemic, anti-fungal and anti- bacterial properties (Goy *et al.,* 2016) [9]. It elicits enormous defense response related to biotic and abiotic stress (Malerba and Cerana, 2015) [16]. Pichyangkura and Chadhawan, 2015 [16, 24], studied the chitosan mechanism in plants and signaling molecules were induced by chitosan involving H_2O_2 via octadecanoid pathway and nitric oxide. Nitric oxide regulated phosphatidic acid through phospholipase and diacyglycerol kinase pathways. Those pathways activated the biotic stress responsive genes. Sathiyabama *et al.,* 2014 [25] reported that phenolic compounds, Poly Phenol Oxidase activites, Super Oxide Dismutase activities and phytoalexins activities in tomato plants increased by chitosan which gives resistance mechanism to *Alternaria solani.* Hidangmayum *et al.* (2019) [11] found that chitin or chitosan specific receptors in plant cell membrane enhance the defense responses. The defense responses includes production of phytoalexins, pathogenesis related proteins (chitinase, β-glucanase, proteinase and induction stress response gene). It induced the signaling molecules in plants such as specific cellular receptor which is transduced by secondary messanger. The secondary messengers includes Reactive Oxygen Species (ROS), H_2O_2, Ca^{2+}, nitric oxide and phytohormones. Chandra *et al.* (2015) [5] reported that chitosan nanoparticles induce plant immunity and defense related enzymes in plants. With this background a study was carried out to assess the phenol content and antioxidant enzyme activity in *M. incognita* infected tomato treated with chitosan nanoparticles.

Materials and Methods

Pure culture maintenance of root knot nematode, *M. incognita*

Roots with conspicuous galls were collected from tomato field, washed gently in tap water and examined under stereo zoom microscope. Egg masses were collected from *M. incognita* infested tomato roots for hatching of infective juveniles (J2). After two days, the infective juveniles were used for inoculation. Fifteen days old tomato seedlings (Shivam hybrid) were planted in pots with sterilized pot mixture (1 part FYM: 2 part red earth: 1 part sand). After the establishment of seedling, J2 were inoculated at the rate of 2 nematode per gram of soil. Egg masses were collected from the tomato roots and allowed to hatch. The infective juveniles were used as an inoculums for the experiment.

Treatment details

1. 1% Chitosan nanoparticles@2ml/plant
2. Nematode (*M. incognita)*
3. 1% Chitosan nanoparticles@2ml/plant + Nematode (1 week after chitosan nanoparticles application)
4. Control.

The experiments were arranged in a Completely Randomized Block design. For each treatment four replications had maintained.

Estimation of antioxidant enzymes

Twenty-five days old tomato seedlings were planted in a sterilized 1kg pot.

⇩

After 1 week transplanting, 2 ml of 1% chitosan nanoparticles was added.

⇩

Root knot nematode was inoculated (2 J2 per g soil).

⇩

Peroxidase, polyphenol oxidase and phenolic content were estimated after 25th day of nematode inoculation.

Assay of total phenol content (Malick and Singh, 1980) [17]:
Five hundred milligram of root sample was taken and cut in to small bits. The test tube with root bits and 5ml of 80% ethanol was kept in hot water bath for 10 minutes. The cooled root sample was macerated again with another 5ml of 80% ethanol. The contents were centrifuged at 5000 rpm for 10 minutes. The supernatant was made up to 25 ml with distilled water. One ml of 20% Sodium carbonate and one ml of Folin reagent were added to one ml of supernatant. After the color development the OD was measured at 660 nm (CAFTA).

$$\textbf{Calculation:} \frac{X \times 25 \times 1000}{1 \times 500} \ \mu g/g \text{ of root sample}$$

Assay of Peroxidase (Wilstatter *et al.*, 1971) [29]:
Five hundred milligram of root sample was collected and grinded with 10 ml of 0.1M phosphate buffer (pH 6.5) and centrifuged@10,000 rpm for 15 minutes. The supernatant was used as an enzyme source. Pyrogallol solution (0.05M) 3ml and 1ml of enzyme extract were taken in a test tube and 0.5ml of 1% hydrogen peroxide solution was added immediately. The change in absorbance was documented at 430 nm for 2 minutes for every 30 seconds interval in spectrophotometer (CAFTA). The difference in the OD value was calculated.

$$\textbf{Calculation:} \frac{X \times 60 \times 10 \times 1000}{1 \times 30 \times 500} \text{ change in the OD at 430 nm/min/g}$$

Assay of Polyphenol oxidase (Mayer *et al.*, 1966) [18].
Five hundred milligram of root sample was macerated with 5ml of ice-cold 0.1 M phosphate buffer (pH 7.0). The above solution was centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected and used as an enzyme extract. To 200µlof enzyme extract, 1.5ml of 0.1M sodium phosphate buffer (pH 6.5) was added. The reaction gets initiated by adding of 200 µl of 0.001 M catechol and the absorbance was recorded at 495 nm for every 30 seconds up to 5 minutes in a spectrophotometer (CAFTA).

$$\textbf{Calculation:} \frac{OD \text{ X } 60X \text{ } 5X1000}{30X \text{ } 500 \text{ X } 0.2} \text{ change in the OD at 495 nm/min/g}$$

Statistical analysis

The data obtained were analyzed statistically using ANOVA and Duncan's Multiple Range Test (DMRT) (Panse and Sukhatme, 1954) [21].

Results and Discussion

In the present study, the phenolic content increased due to the application of chitosan nanoparticles in nematode inoculated plants (174.32 µg/g of root sample). In untreated uninoculated control, the phenolic activity was 160.37 µg/g of root sample. In nematode inoculated plants without chitosan nanoparticles application the phenolic content was 156.75 µg/g of root sample whereas in nematode uninfected tomato plants with chitosan nano particle application the phenol content was 167.15 µg/g of root sample. The activity of phenol increased in the treatment 1% chitosan nanoparticles+ nematode (Fig1). Phenols play significant role in plant development, particularly in biosynthesis of lignin and pigment. They also provide structural integrity to plants (Bhattacharya, 2010) [4].

Phenolic compounds are involved in plant defense and hence provide resistance against nematode attack. Chitosan acts as a potent biotic elicitor, able to induce plant defence responses and to activate different pathways that increase the crop resistance to diseases (Hadwiger, 2013; Katiyar *et al.*, 2014) [10, 13]. Chitosan treatment results in formation of chemical and mechanical barriers and the synthesis of new molecules and enzymes involved in the defence response (Iriti and Faoro, 2009; Falcon and Wegria, 2012) [12, 7]. In some cases, chitosan causes the induction of the hypersensitive response, mainly around the infection site, that leads to the programmed cell death (Vasilev *et al.*, 2009) [28]. This hypersensitive response is followed by systemic response of the plant defence mechanisms.

In a similar study by Asif *et al.*, (2017) [1] it was found that the total phenol content in roots increased when nematode infected egg plants were treated with chitosan. Application of chitosan and *Bursaphelenchus xylophillus* in *Pinus pinaster* increased total phenolic content on 14th days of post inoculation. On 14th after *B. xylophilus* inoculation, the phenolic content (2.3 µg/g of root sample) was enhanced in the treatment chitosan+ *B. xylophilus* compared to untreated inoculated control (1.3 µg/g of root sample) in *Pinus pinaster* seedlings. Chitosan was found to increase the tolerance mechanism in *Pinus* spp. to the pinewood nematode, *Bursaphelenchus xylophillus* with decreased nematode activities and increased anti-oxidant activity (Silva *et al.*, 2021) [27].

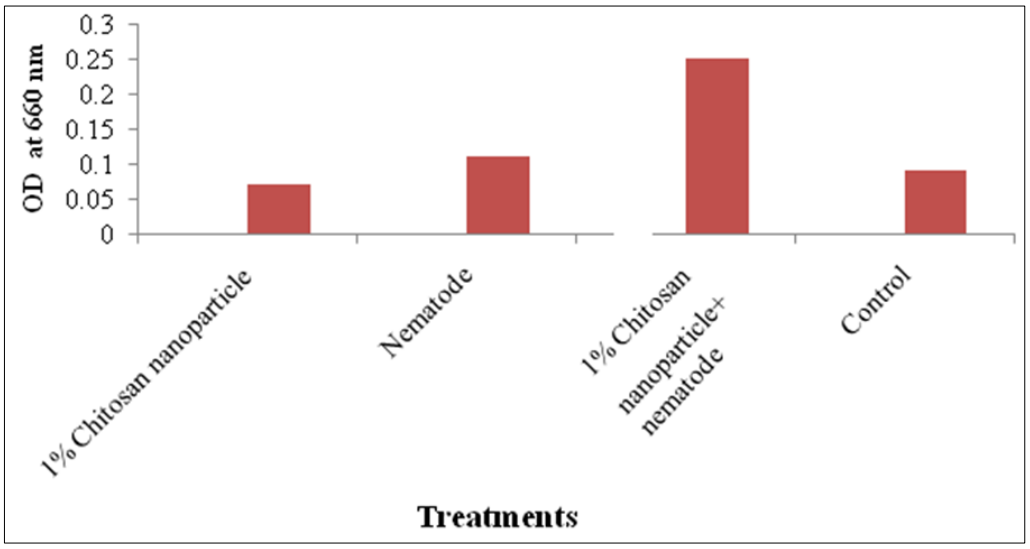

Fig 1: Phenol content in *M. incognita* infected tomato plant treated with chitosan nanoparticles.

In the present study it was observed that application of chitosan nanoparticles and *Meloidogyne incognita* in tomato increased peroxidase enzymatic activity on 25th days of post inoculation (56.11gm^{-1} fresh weight of roots) compared to 33.09 gm^{-1} fresh weight of roots in untreated inoculated control plants. Increased peroxidase activity indicates induction of systemic resistance in plants due to chitosan nanoparticles (Fig 2). ISR leads to synthesis of plant defense enzymes such as peroxidase, polyphenol oxidase, chitinase and phenylalanine ammonia lyase (Osman *et al.*, 2013) [20].

The peroxidase and polyphenol oxidase enzymatic activities were enhanced in high molecular weight chitosan treated *M. javanica* infested tomato plants (Sayd and Mahdy, 2017). Asif *et al.* 2017 [1] studied the peroxidase activities in chitosan treated + *M. incognita* infested egg plant and untreated inoculated control (nematode alone) after 90 days of nematode inoculation. The peroxidase content was 1.96 gm^{-1} fresh weight of roots in chitosan treated nematode infested egg plant compared to all other treatments.

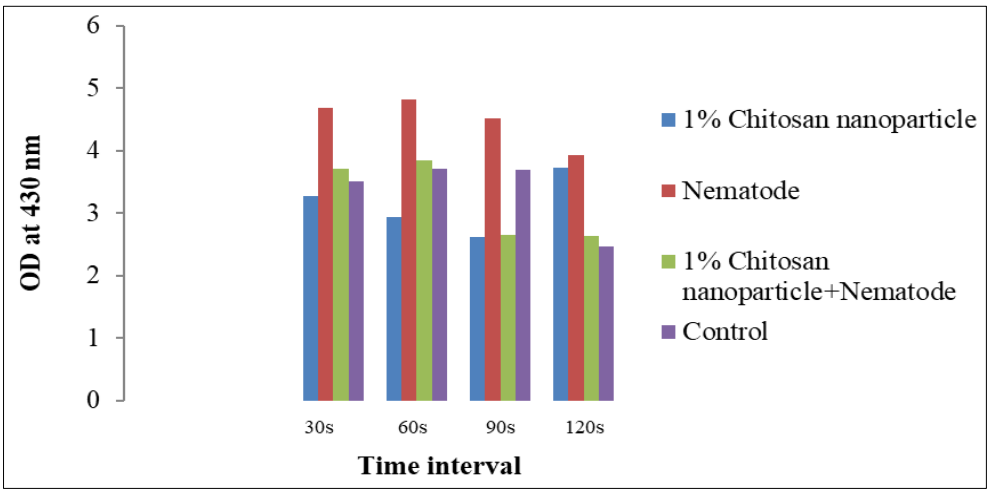

Fig 2: Peroxidase activity in *M. incognita* infected tomato plant treated with chitosan nano particles

Estimation of polyphenol oxidase activity in tomato plants inoculated with *M. incognita* and treated with chitosan nano particles revealed higher activity (1.4 gm^{-1} fresh weight of roots) and was on par with the untreated inoculated control (1.62 gm^{-1} fresh weight of roots) and untreated uninoculated control (1.7gm^{-1} fresh weight of roots). Polyphenoloxidase activity was highest (2.12 gm^{-1} fresh weight of roots) roots treated with chitosan nanoparticles. The activity of polyphenol oxidase increased with increase in time. (Fig3). Polyphenoloxidase plays a major role in resistance mechanism of host plants and catalyses the phenolic compounds (Siddiqui and Husain 1992) [26]. It also synthesizes plant cell wall components like lignin and suberin (Lamport 1986) [15]. Lignification of cell wall leads to form a defense response to pathogens (Gaspar *et al.*, 1982) [8]. Fabriction of chitosan with graphene oxide increased the activitiy PPO by 113.8% in *M. incognita* infested egg plant (Attia *et al.*, 2021) [2]. The maximum poyphenol activity (660% of control) was documented in root dipping of chitosan @2500 ppm + *M. incognita* infested egg plant after 5 days of nematode inoculation (Osman *et al.*, 2013) [20].

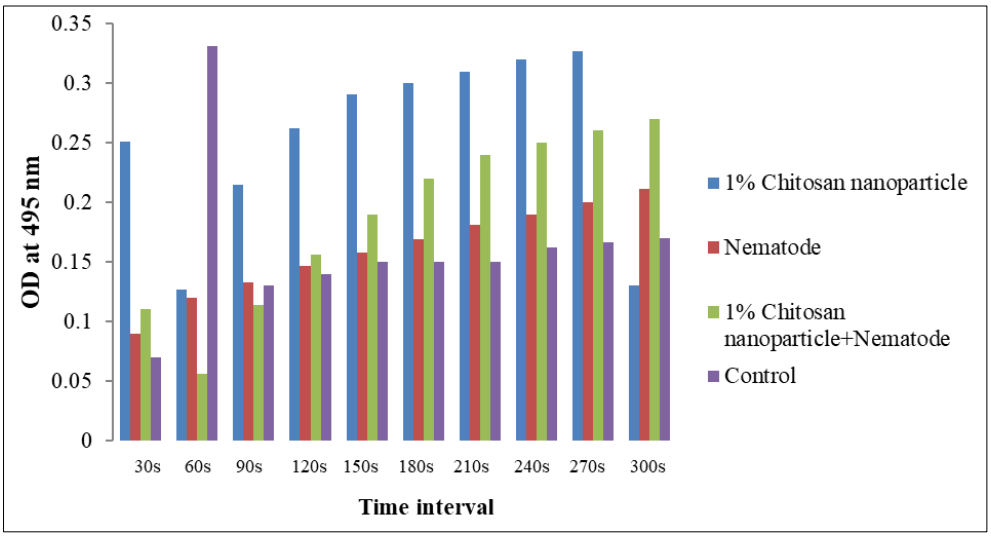

Fig 3: Polyphenoloxidase activity in *M. incognita* infected tomato plant treated with chitosan nanoparticles.

Conclusion

Application of 1% chitosan nanoparticles in root knot nematode *M. incognita* infected tomato was found to induce systemic resistance in plants by way of increasing phenol content and activity of antioxidant enzymes, peroxidase and polyphenol oxidase. Chitosan nano particles can be included as an environment friendly component in integrated nematode management system for sustainable agriculture.

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