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Relevance of resistance pathways in tomato plants infected with *Meloidogyne incognita* and treated with chitosan nanospheres

R Mouniga[1*], B Anita Bellie[2], A Lakshmanan[3], A Shanthi[4], G Karthikeyan[5]
[1] Department of Nematology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
[2] Professor and Head, Department of Nematology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
[3] Department of Nanoscience and technology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
[4] Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
[5] Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
Corresponding Author: R Mouniga
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Abstract
Resistance in plants is influenced by resistance genes. Those genes give the defense signals against insect, disease and nematodes. Hypersensitive reaction is happened at the nematode feeding site. Chitosan is considered as the best biopolymer next to cellulose. It possesses anti-fungal, anti-bacterial and anti-nemic properties. Chitosan indirectly enhances the resistance pathways such as Jasmonic acid and salicylic acid. Nanotechnology plays a major role for the formulation of chitosan nanospheres. Ionic gelation method is commonly used to develop a chitosan nanospheres. Those nanoparticles easily increase the production of phytoalexins and Reactive Oxygen Species in plants. Several treatments were taken for GCMS analysis such as Nematode (*M. incognita)*, Chitosan nanospheres 1% @2ml/plant + Nematode (1 week after chitosan nanospheres application) and Control. The two compounds *viz.*, 9- Octadecenoic acid (Z)-2, 2- (acetyloxy)-1 [(acetyloxy) methyl] ethyl ester and 6-bromohexanoic acid of jasmonate pathway were present in chitosan nanospheres treated tomato plants but absent in untreated plants.

Keywords: *Bromohexanoic acid, Chitosan nanospheres, Octadecenoic acid, tomato*

Introduction

Insect, pest and pathogens are known to cause a damages in both agricultural and horticultural crops. Among the above biotic stress, nematode is the most important one to cause some yield losses in various crops. They are known to cause a 19% of yield losses in agricultural crops. Among the nematodes, endoparasites, Root knot nematode, *Meloidogyne incognita* are known to cause a more economic damage in tomato. It causes 11-35% yield losses in tomatoes. Nematicides are available in few numbers in a worldwide due to their toxic effects on environment. Resistance in plants is defined as the heritable ability of plants create a defense mechanism from other enemies such as insect, disease and nematodes (Painter, 1951 [20]; Mitchell *et al.,* 2016 [16]). Nematode resistance is characterized by host plant cell death at the feeding site of nematodes. Chitosan is considered as the important biopolymer available in earth next to cellulose. Yeast, fungi, insects, nematodes, bacteria, and crustaceous shells are all sources of chitosan (Canella and Garcia, 2001). Composed of α, 1-4 linked D-glucosamine (GlcN) and N-acetyl D-glucosamine (GlcNAc), it is a polysaccharide with a variable composition of two monomers (Brine and Austin, 1981). As compared to the polymer cellulose (1.25%), it has a higher nitrogen content (6.89%) (Rabea *et al.,* 2003). High levels of biocompatibility, bioactivity, biodegradability, selective permeability, antibacterial activity, gel and film formation, chelation ability, and absorptive capacity are all attributes of chitosan (Synowiecki & Al-Khateeb, 2003). Chitosan has extensive application in a variety of sectors, including pharmacology, medicine, food biotechnology, cosmetics and agriculture (Hamed *et al.,* 2016)**.** It possesses anti-bacterial (Fei *et al.*, 2006), anti-viral, anti-fungal (Rabea *et al.*, 2003) and nematicidal properties (El-Sayed and Mahdy, 2015). Chitosan depolarized the plasma membrane of tomato root cells that leads to secrete the hormones, lipid signaling and defense compounds. According to War *et al.* (2011) [28], SA and JA are connected to how plants react to stress. Chitosan can therefore trigger plant defenses in root exudates (Benhamou, 1998) [3]. Chitosan enhanced the Induced Systemic Resistance (ISR) and reduced the root knot nematode population by the production of Salicylic acid (Vasyukova *et al.*, 2001 [27]). According to Vasilev *et al*. (2011), chitosan causes a hypersensitive reaction at the pathogen or nematode infection site, which results in programmed cell death. By demonstrating elicitor activity and causing both local and systemic resistance, chitosan gives plants resistance. Plant defense mechanisms against microbial infections, such as the production of lignin, callus formation, phytoalexin accumulation, pathogen-related (PR) proteins, and proteinase inhibitors, are triggered by chitosan. As a biotic elicitor, low molecular weight chitosan can trigger defense responses in plants and trigger several pathways that boost crop disease resistance. Phytoalexins and pathogenesis-related proteins (chitinase, β-glucanase, proteinase, and induction stress response gene) are produced as part of the defense responses. In plants, it triggers signalling molecules like a particular cellular receptor that is transduced by a secondary messenger. Reactive oxygen species (ROS), H2O2, Ca^{2+}, nitric oxide, and phytohormones were among the secondary messengers (Hidangmayum *et al.*, 2019) [11]. In many dicot plant species, chitosan promotes the production of callose, proteinase inhibitors, and phytoalexins (Orzali *et al.*, 2014) [19]. The defense-related pathways triggered a massive defense response to both biotic and abiotic stress by activating the genes that respond to biotic stress (Malerba *et al.*, 2012). Pichyangkura and Chadchawan (2015) [14, 21] investigated the mechanism of chitosan in plants and found that chitosan induced signaling moleculesinvolving H2O2 via nitric oxide and the octadecanoid pathway. Through the phospholipase and diacyglycerol kinase pathways, nitric oxide controlled phosphatidic acid. Peroxidases, chitinases, and phenylalanine ammonia lyase are among the important enzymatic defense-related pathways that it controls (Orazil *et al.*, 2014). According to Sathiyabama *et al*. (2014) [25], the application of chitosan to tomato plants boosted their levels of phenolic compounds, polyphenol oxidase activity, superoxide dismutase activity and phytoalexins, which in turn caused a systemic resistance mechanism against *Alternaria solani*. Chitosan treatment of chilli seeds increased their shelf life, storage quality, and germination index (Chookhongkha *et al.*, 2012) [5]. Plant reactions to chitosan treatment result in the formation of mechanical and chemical barriers as well as the synthesis of defense-related enzymes (Falcón-Rodríguez *et al.*, 2011). Numerous secondary metabolites that are important for defense responses are synthesized by chitosan. Phenolic substances including lignin, callose, phytoalexins, and PR proteins (pathogenesis-related proteins) are examples of secondary metabolites. By building up antimicrobial chemicals, it stimulates the plant immune system and is essential for causing systemic resistance. Nanotechnology play an important role in the formation of formulation. Several methods employed to synthesis nanoparticles *viz.*, physical, chemical and biological methods. Chitosan nanoparticles has been synthesized by ionic gelation, micro-emulsion, poly-electrode complexes, solvent diffusion methods. Chitosan nanoparticle increased plant defense processes: - activate ROS and Systemic Acquired Resistance, accumulation of phytoalexins, elicitate the receptors, produces the Pathogenesis Related Protein (PR), synthesis of jasmonic acid and salicylic acid pathway and activate Molecular Associated Pathogen Kinases (MAP) (Acosta *et al.*, 2009) [1]. Chitosan induces a hypersensitive response at the infection site of a pathogen or nematode that leads to cause programmed cell death (Mouniga and anita, 2025) [17]. With these background, chitosan nanospheres treated and nematode infected tomato plants were taken for GCMS analysis.

Materials and methods

Pure culture of Root knot nematode, *M. incognita*

Roots with galls were collected, washed gently with tap water and examined under stereo-zoom microscope. Egg masses were collected from *M. incognita* infected 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 sterilized pot mixture (1 part FYM: 2 part red earth: 1 part FYM) in five kg pots. After the establishment of seedling, J2 were inoculated at 2 nematodes per gram of soil in each pot. Egg masses were collected from the tomato roots after 45 days of inoculation. The infective juveniles were used as an inoculum for different experiments.

Formulation of chitosan nanospheres:

Based on sodium tripolyphosphate ionic gelation method, the chitosan nanospheres were formulated (Marei *et al.*, 2018) [15].

GCMS analysis of nematode infected tomato plant roots treated with chitosan nanospheres:

Fifteen days old tomato seedlings were planted in a sterilized pot mixture. After 1 week of transplantation, 1% of chitosan nanospheres were incorporated to soil followed by inoculation of egg masses of root knot nematode, *M. incognita.* After 25th day of inoculation, tomato plants were uprooted and the secondary metabolites were identified with the help of Gas Chromotography- Mass Spectrophometry (GCMS).

Treatment details

- T1- Nematode (*M. incognita)*.
- T2- Chitosan nanospheres 1% @2ml/plant + Nematode (1 week after chitosan nanospheres application).
- T3-Control.

Preparation of sample

The roots were properly cleaned and sliced into small pieces after being collected after each treatment. The chopped roots were shade-dried for 24 hours before being crushed into a fine powder using liquid nitrogen (N2).

After being ground into a powder, the roots were used in later processes.

Preparation of methanolic extract

Ten grams of root powder from each treatment were extracted with methanol using the hot percolation method over a 24-hour period using a Soxhlet apparatus. The extract was extracted and filtered using a 0.2µ bacteriological syringe filter. The extract was concentrated using a rotary evaporator.

Gas Chromatography- Mass Spectroscopy analysis

The GC-MS analysis of the methanolic extract was carried out using a Thermo Scientific DSQII qudrape mass spectrometer in combination with a Thermo Scientific Trace Ultra Chromatography System (Thermo Fischer Scientific, Austria). The column's measurements are IG-SQC, 0.25µm film thickness, 25 mm diameter, and 15 m length. The carrier gas is helium at 1.0/min. The starting column temperature was increased from 50° C (1 minute) to 150° C at a rate of 25° C per minute. The used syringe has a 2.5 ml capacity and a needle length of 65 mm. While the vials were in the sonicator at 30° C for one minute, the injection speed was consistently kept at 20 ml/min. The sample was introduced into the GC-MS using the Triplus RSH head space auto sampler.

Results

GCMS analysis of nematode infected tomato plant roots treated with chitosannanospheres

GCMS analysis of tomato roots was carried out to identify the resistance inducing compounds due to the application of chitosan nanospheres. Variations in compounds in nematode infected roots treated with chitosan nanospheres treatment and without treatment were recorded after twenty five days and compared with healthy tomato roots. Compounds such as phenol, 2,4-Di-tert-butylphenol, linoleic acid ethyl ester, ascorbic acid, N-acetyl-L-glutamic acid, oleic acid an 2-((4-Methylpentyloxy) carbonyl) benzoic acid were present in both healthy and nematode infected roots with or without chitosan nanospheres treatment. The compounds *viz.,* 9-Octadecenoic acid (Z)-2, 2- -(acetyloxy)-1 [(acetyloxy) methyl] ethyl ester and 6-bromohexanoic acid were observed in nematode infected roots treated with chitosan nanospheres but absent in healthy and nematode infected roots without treatment (Table1). These compounds are reported to be involved in Jasmonic acid synthesis pathway which is one of the important resistancesinducing mechanism.

GCMS analysis of chitosan nanospheres and *M. incognita* untreated tomato root:

GCMS analysis of roots treated with chitosan nano formulation was done to identify the metabolites responsible for inducing systemic resistance against nematode infection. In this study, it was observed that two compounds *viz*., 9-Octadecenoic acid (Z)-2, 2- -(acetyloxy)-1 [(acetyloxy)methyl] ethyl ester and 6-bromohexanoic acid of jasmonate pathway were present in chitosan nanospheres treated tomato plants but absent in untreated plants (Table 1).

Discussion

Resistance inducing metabolites

GCMS analysis of roots treated with chitosan nano formulation was done to identify the metabolites responsible for inducing systemic resistance against nematode infection. In this study, it was observed that two compounds *viz.*, 9-Octadecenoic acid (Z)-2, 2- -(acetyloxy)-1- [(acetyloxy) methyl] ethyl ester and 6-bromohexanoic acid of jasmonate pathway were present in chitosan nanospheres treated tomato plants but absent in untreated plants. The 13-allene oxide synthase (13-AOSs) in plastids produce octadecanoids and jasmonates (Wasternack, 2007) [29]. In the peroxisomes region, OPC6 produces hexaonic acid (Ruan *et al.*, 2019) [23]. Systemin, a particular inducer of proteinase inhibitors (PIs), mediates the response to insect attack and mechanical wounding (Ryan *et al.*, 1998) [24]. Jasmonate signalling pathway increases the levels of nematode toxic chemicals such as phytoectosteroids, flavonoids, and proteinase inhibitors. Chitosan acts as a potent biotic elicitor, able to induce plant defense responses and activate different pathways that increase the crop resistance to diseases (Hadwiger, 2013; Katiyar *et al.*, 2015) [10, 13]. The LeAoc and LeJA3 genes are upregulated in the jasmonate signalling pathway, providing a resistance strategy to the tomato root knot nematode (Fan *et al.*, 2014).

Conclusion

In the chitosan nanospheres treated *M. incognita* infested tomato plants, compounds 9 Octadecenoic acid (Z)-, 2-(acetyloxy)-1- [(acetyloxy) methyl] ethyl ester, and 6 Bromohexanoic acid which are precursors to the jasmonate signalling pathway were identified. These compounds will be developed in the future as nematicide, which cause plants to develop defense systems against diseases and pests.

Table 1: GCMS analysis of chitosan nanospheres treated tomato roots

Treatments	Compounds	Retention time	Properties	References
M. incognita	Phenol	4.61	During biotic stress condition, the phenolic compound is accumulated. Defense reaction against root knot nematode.	(Giebel, 1974) [9]
	N-Acetyl-L-glutamic acid	7.79	Indirectly triggered the secondary messengers in plants	(Qiu *et al.*, 2020) [22]
	2,4-Di-tert-butylphenol	13.09	Nematicidal properties	Google patent
	Oleic acid	16.87	Mediated defense related pathways and also gave a resistance to root knot nematode in groundnut	(Muitia *et al.*, 2006); (Kachroo, 2008) [12, 18]
	2-((4-Methylpentyloxy) carbonyl) benzoic acid	21.88	Benzoic acid triggered the salicylic acid pathway	(Tamaoki, 2008) [26]
	Ascorbic acid 2,6-dihexadecanoate	22.27	Damages Reactive Oxygen Species and protect the plants from pathogen or nematode attack	(Bobukari, 2018); (Arrigoni *et al.*, 1979) [2]
	Linoleic acid ethyl ester	24.80	Precursor for jasmonic acid signaling pathway leads to Induced systemic resistance	(Acosta *et al.*, 2009) [1]
Chitosan nanospheres (1%) + *M. incognita*	9-Octadecenoic acid (Z)-, 2-(acetyloxy)-1- [(acetyloxy) methyl] ethyl ester	3.02	Involved in Jasmonic acid signaling pathway	Ruan *et al.*, 2019) [23]; (Gheyson and mitchum, 2018)
	Ascorbic acid 2,6-dihexadecanoate	3.02	Damages Reactive Oxygen Species and protect the plants from pathogen or nematode attack	(Bobukari, 2018); (Arrigoni *et al.*, 1978)
	6- Bromohexanoic acid	3.59	Involved in Jasmonic acid signaling pathway	Ruan *et al.*, 2019) [23]; (Gheyson and mitchum, 2018)
	Phenol	4.67	During abiotic stress condition, the phenolic compound is accumulated. Defense reaction against root knot nematode.	(Giebel, 1961)
	N-Acetyl-L-glutamic acid	7.85	Indirectly triggered the secondary messengers in plants	(Qiu *et al.*, 2020) [22]
	Oleic acid	9.05	Mediated defense related pathways and also gave a resistance to root knot nematode in groundnut	(Muitia *et al.*, 2006) [18]; (Kachroo, 2008) [12]
	Linoleic acid ethyl ester	9.05	Precursor for jasmonic acid signaling pathway leads to Induced systemic resistance	(Acosta *et al.*, 2009) [1]
	2,4-Di-tert-butyl phenol	13.16	Nematicidal properties	Google patent
Control	Oleic acid	3.29	Mediated defense related pathways and also gave a resistance to root knot nematode in groundnut	(Muitia *et al.*, 2006); (Kachroo, 2008) [12, 18]
	2-((4-Methyl pentyloxy) arbonyl) benzoic acid, 4 Ethylbenzoic acid	3.48	Benzoic acid triggered the salicylic acid pathway	(Tamaoki, 2008) [26]
	Phenol	4.68	During abiotic stress condition, the phenolic compound	(Giebel, 1961)

			is accumulated. Defense reaction against root knot nematode.	
	N-Acetyl-L-glutamic acid	7.86	Indirectly triggered the secondary messengers in plants	(Qiu *et al.*, 2020) [22]
	2,4-Di-tert-butylphenol	13.17	Nematicidal properties	Google patent
	Ascorbic acid 2,6-dihexadecanoate	22.03	Damages Reactive Oxygen Species and protect the plants from pathogen or nematode attack	(Bobukari, 2018); (Arrigoni *et al.*, 1978)
	Linoleic acid ethyl ester	24.83	JA pathway- ISR	(Acosta *et al.*, 2009) [1]

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