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NANOPRESERVED TOMATOES FOR IMPROVED POST HARVEST MANAGEMENT

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Abstract
Postharvest management in tomato is crucial for global supply and also to maintain freshness and keeping them in a diseased-free stage for prolonged periods. Nanomaterials have become better choice to preserve the healthiness of the fruit by extending their shelf life and maintaining the pathogen free conditions. In the current study, we have developed harpin (*hrp*-encoded elicitor of the hypersensitive response) loaded chitosan nanoparticles (CSNPs) for improved post-harvest management in tomato. The newly synthesized harpin loaded CSNPs (CSHNPs) were characterized via UV-Vis Spectroscopy, Transmission Electron Microscopy (TEM), powder X-Ray Diffraction (XRD), Atomic Force Microscopy (AFM) confirming the nanoparticle nature of CSNPs and CSHNPs. Furthermore, topical application of CSNPs and CSHNPs on freshly harvested tomatoes revealed the significant increase in the levels of polyphenol oxidase (PPO), peroxidase (POD) and phenolic compounds. From the current studies, it is evident that CSNPs and CSHNPs can potentially help in controlling post-harvest storage problems in tomato and other fresh vegetable fruits.

Keywords: Chitosan, harpin, chitosan loaded nanoparticles, harpin nanoparticles, post-harvest management, tomato.

Introduction:

With the massive applications of nanoparticles in protecting the health of plants, animals, human health care and developing novel products for human welfare, nanotechnology has become buzz of the 21st century (Khodakovskaya et al. 2012). Nanotechnology deals with nano-sized particles and due to the manipulation of the particle size at nano-scale they show profound effect on cell and cellular activities (USEPA 2007). One of the prominent advances in application of nanoparticles is their interaction with plant cell system and thereby inducing their seed germination, plant growth, defense mechanism in plants against various pathogens (Khodakovskaya et al. rev2009; Mahmoodzadeh et al. 2013; Mondal and Mani 2012; Pinto et al.2013).

Plants have evolved a sophisticated defense system against pathogen attack and during such plant-pathogen interactions rapid host cell death (hypersensitive response, HR) is observed to avoid further damage to plant cellular machinery (Lamb and Dixon, 1997). The incompatible interaction

between a host plant carrying a resistance (R) gene and an invading pathogen carrying a corresponding avirulence (Avr) gene often leads to the so-called hypersensitive response (HR), which is characterized by programmed cell death (PCD) at infected sites (Greenberg et al. 1994) and usually associated with an oxidative burst. HR involves a PCD, which is an active process for the cells own demise. Plants cells respond to various biotic chemical signals in their environment including non-self-factors such as cell wall fragments on the surface of a pathogen, self-determinants such as cell wall fragments that are released by a plant in response to an invading pathogen or compounds that are secreted by plant pathogens. Pathogens may produce toxins in various forms that promote disease development and many of which kill plant cells. Alternatively, pathogens may produce compounds such as proteins, small peptides, glycoproteins/peptides, or oligosaccharides that activate mechanisms important in plant defense and are collectively known as elicitors.

One such elicitor is Chitosan which induces PCD and hypersensitive-associated responses in plants. It also induces chromatin condensation and marginalization followed by a destruction of the nuclei and subsequent inter-nucleosomal DNA fragmentation (Hadrami et al. 2010). Harpin, the *hrp*-encoded elicitor of the hypersensitive response, was first isolated from *E. amylovora* (harpinEa;) and later from *P. syringae* pv. *syringae* (harpinPss;) (Wei et al., 1992). Harpins are known to possess multifunctional properties viz., enhanced growth and photosynthesis performance in plants, resistance against broad range of pathogens like viruses, bacteria, fungi and repelling insects. Beside the important biological activities harpins also attract considerable interest due to their potential application as pesticides. Since harpins do not directly interact with the disease-causing organism, pathogens are not expected to develop resistance to harpins. As harpins are biodegradable and have no adverse effects on human health, use of harpins can substantially reduce use of more toxic chemical pesticides (He et al.1993).

Tomato (*Lycoperiscon esculentum*) belongs to the family *Solanaceae* is a highly nutritive vegetable crop. Though it is a temperate crop plant, it is extensively cultivated in the tropical and subtropical regions of the world round the year with an annual production of 12,96,49,883 tons per year and in India with a production of 1, 02, 60,600 tons per year. The total area harvested for tomato cultivation in world is 52, 27,883 hectares with Indian cultivation in 5,71,700 hectares (FAO database, 2014). Tomato is rich in vitamin A and C and fibre, and is also cholesterol free. Tomato contains approximately 20-50 mg of Lycopene/100gm of fruit weight which is the most powerful antioxidant in the carotenoid family and it protects humans from free radicals that degrade many parts of the body and is also known to prevent cancer. Biotic stress caused by virus, bacteria, fungi and insects and abiotic stress due to water scarcity, salt stress and heat stress are the major stress factors hampering the tomato cultivation. Besides these problems, Post harvest diseases and storage are added to the tomato cost in the market. Postharvest decay is a major loss with regard to fruits and vegetables (Janisiewicz and Korsten, 2002). In view of the above, we attempted to develop chitosan nanoparticles (CSNPs) and harpin loaded chitosan nanoparticles (CSHNPs) and characterized them to evaluate their efficacy on post-harvest storage of tomato fruits by studying the elicitation of defense markers, including polyphenoloxidase (PPO), peroxidase (POD), and phenolic compounds.

Materials and Methods:

Low Molecular Weight (LMW) Chitosan (CS) of shrimp origin was purchased from Sigma Aldrich Corporation, Mumbai, India. The viscosity range of Chitosan was 20cps(cubic pascal) under Brookfield viscometery as recorded by manufacturer. CSNPs were produced according to the method developed by Calvo and co-workers (Calvo et al., 1997a and 1997b) with little modifications. For development of Chitosan stock, 1g of Chitosan (LMW) was dissolved in 100ml of 1% acetic acid solution in deionised water by overnight stirring followed by sonication for 2 hr at 55 W. Tripolyphosphate (TPP) salt was dissolved in deionised water at the concentration of 0.7 mg/ml. Different combinations of CS and TPP ratios (3:1, 4:1, 5:1, 6:1 and 7:1) were analyzed at varied pH (3.0 to 7.0). For production of CSNPs with 3:1 ratio of CS to TPP, 210 µl of stock was

diluted to 10 ml deionised water and flush mixed with 10 ml of TPP solution under slow stirring for 45 min with pH maintained at 5.2.

The Hrpz gene (1.02 kb) encoding full length harpin was cloned under Ndel and Xhol sites of pET 28a vector (Novagen) . *E.coli* BL21 (rosettae) cells transformed with pET28a –hrpZ was grown in Luria Bertani(LB) broth with Kanamycin (50 µl/ml) to OD 600nm equivalent to 0.5 and induced with 1 mM IPTG. After 3 hrs of induction, bacterial cells were pelleted, washed and resuspended in 10 mM Sodium Phosphate buffer (pH 7.5) and immediately sonicated (1 min pulse on and 30 sec pulses off, 7 cycles, bandelin MS-72). The sonicate was boiled for 10 min, centrifuged at 14,000rpm for 20 min to remove cell debris and the supernatant was loaded in Ni-NTA column (Sigma Aldrich). Protein was eluted with 200 mM Imidazole in phosphate buffer after washing the column with 20 mM imidazole of the same buffer and then perfectly dialyzed against 10 mM sodium phosphate buffer (pH 7.5). Purity of both the proteins was checked on a 12 % SDS-PAGE. The dialysed protein was concentrated using amicon filter (10 kDa cut off Millipore) and used after estimation by Bradford's method (Bradford 1976).

CSHNPs (CS to TPP ratio 3:1) were produced by first diluting 52.5 µl of Chitosan LMW stock to 2.5 ml. The resultant Chitosan solution was flush mixed under stirring with Harpin solution of volume 1.25 ml, prepared by diluting 1µl of Harpin stock (5 mg/ml) to 1.25 ml in deionised water (3 µl/ml). To this solution 1.25 ml of TPP solution (0.933 mg/ml) was flush mixed with pH maintained at 5.2. (Figure 1).

Transmission Electron Microscopy (TEM) imaging: A drop of sample (both CSNPs and CSHNPs) were placed on a piece of para film, then carbon coated (EM) grid was placed over it for 5-10 min and excess sample was drained with help of filter paper. Grids were washed with drops of distilled water and stained with 2% uranyl acetate, air dried and observed at various magnification under transmission electron microscope (Model: Hitachi H-7500) at RUSKA Lab, College of Veterinary Sciences, Rajendra Nagar, Hyderabad, India.

Atomic Force Microscopy (AFM) imaging: AFM analysis was done using AFM-*Seiko* Instrumentation-3800, Japan, to study surface characteristics and to calculate grain size distribution of nanoparticles. For this, 3 µl of sample was spin coated at 14,000 rpm on glass slide for 60 seconds. Spin coating provided uniform sample film formation and avoided overlapping of sample layers. AFM under non contacting probe mode was used for the purpose.

UV-Visible Spectroscopy: Reagent concentrations were fixed for all the spectroscopic studies. CSNPs, CSHNPs, TPP (0.7 mg.ml), Chitosan (2.1 mg/ml) and Harpin (3.2 µg/ml) samples were analyzed for UV-VIS spectrum (*Cary 100 Bio UV-VIS Spectrophotometer*) in the range 200-800 nm with a 2 nm slit width and a 1 cm path length at intervals of 0.5 nm using water as the baseline reference to obtain preliminary information about the nanoparticle formation and interactions of constituent molecules.

X-Ray Diffraction (XRD): For XRD 100 ml of CSHNP solution was lyophilized overnight after freeze drying in liquid nitrogen. The powdered samples of CSHNP along with chitosan (LMW) powder as control were analyzed for XRD (*PHILIPS PW XRD*) studies.

Attenuated Total Reflectance Infra Red (ATR-IR) Spectroscopy: ATIR (*NICOLET 5700 FT-IR*) analysis was done for all the components of nanoparticle solution and resulting nanoparticle preparations for observing characteristic peaks to reveal the functional groups in the nanoparticles after constituent had interacted.

Effect of CSNPs & CSHNP on Post Harvest Tomato Fruit:

Fruit: Freshly harvested Tomato (*Solanum lycopersicum* cv. PR) fruits raised in green house at mature red stage with no surface injuries or infections were used in the current experiments.

Chemicals: Chitosan, TPP, Harpin stock, Deionised water, Catechol, Guaiacol , H_2O_2 , HCl , Methanol, Sodium Phosphate buffer, Polyvinyl Poly Pyrrolidone.

Test Regimen: Chitosan solution (2.1 mg/ml), Harpin (3.2 mg/ml), TPP (0.7 mg/ml), CSNP, CSHNP, deionised water and unwounded fruit condition taken as test regimen.

Testing on fruit: Tomato fruits were injured (3 mm by 3 mm wound) at the equator by sterile scalpel blade. 15 µl of each seven regimens was injected in the wound space and the fruits were

stored at 25 ^{0}C. Tissue samples were taken from the surrounding area of the wound starting from day "0" (6 hours of post wounding followed by sample treatment) and subsequently followed by day 1, day 2 and day 3. Enzymatic (POD and PPO) and total phenolic content assays were performed as per the standard protocols.

PPO And POD Activity Assay Extract Prepration: PPO and POD were extracted as per the method previously developed by Chen *et al.,* (2000) with little modifications. Isolated tissue (2 gm) from around the wound were homogenized in 10 ml of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of polyvinyl polypyrrolidone (PVPP) and temperature maintained at 4 ^{o}C. Subsequently the homogenate was centrifuged at 15,000xg for 30 min at 4 ^{o}C. Supernatant from these extracts were used for POD and PPO assay.

PPO ASSAY: Activity was determined by adding 0.1 ml of enzyme extract to 3.0 ml of Catechol (500 mM, in 100 mM Sodium Phosphate buffer, (pH 6.4) and increase in absorbance measured immediately at 398 nm.

POD ASSAY: POD activity was determined using Guaiacol as substrate using standard method (Ippolito et al., 2000). 0.1 ml of crude extract was mixed with 2 ml of Guaiacol (8 mM, in 100 mM Sodium Phosphate buffer, (pH 6.4) and incubated for 30 min at 30 ^{o}C followed by addition of 1 ml of H_2O_2 (24 mM). Increase in absorbance was measured immediately at 460 nm.

Extract preparation and Quantification of total phenolic compounds: 1 gm of fruit sample was homogenized with ice cold 1% HCL-methanol solution and centrifuged at 15,000xg for 15 min at 4 ^{o}C following method of Liu et al., 2005. The collected supernatant was measured at 280 nm to estimate changes in total phenolic compounds.

Statistical Methods:

The enzymatic activities and phenolic compounds from five treatment groups that were completed in triplicates at various time points (days) are averaged out in the data and presented in the figures. The data obtained from the above experiments were subjected to statistical tools viz., mean, standard error, and one-way ANOVA and the statistical significance was calculated using Duncan's Multiple Range Test (DMRT) and Holm-Sidak method using Sigma plot software version 12.

Results and Discussion:

Initially several combinations of LMW chitosan and TPP with different ratios and graded pH were analyzed for standardizing the nanoparticle formation. The ratio of LMW CS to TPP ranged from 3:1, 4:1, 5:1, 6:1 and 7:1 with each combination analyzed at constant pH from 3.0, 3.5, 4.0, 4.5, 5.0, 5.2, 5.5, 6.0, 6.5 and 7.0. All the CS:TPP ratios which were tried at extreme ends of pH (3.0 and 7.0) particle disruption and particle aggregation was observed, which was clearly viewed as precipitate. However the combinations of CS:TPP at 3:1 at constant pH of 5.2 resulted in stable nanoparticle formation of desirable size without any of precipitate (Figure 1). When the same ratio of CS:TPP i.e., 3:1 was tried out at graded pH (3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) we observed disruption as well as aggregation of nanoparticles as observed under SEM (data not shown). The disruption and aggregation of CSNPs and CSHNPs may be due to the improper charge distribution on the surface of the particles. All the ratios studied led to nanoparticle formation at pH 5.2 , however the nanoparticle size dramatically increased from 3:1 to 7:1. Hence all the experiments were carried using nanoparticles formed at pH 5.2 with a ratio of 3:1 of LMW CS to TPP. A schematic picture showing Chitosan, TPP and their interactions due to deprotonation and crosslinking leading to formation of Chitosan nanoparticles are shown in Figure 1 (a-e).

Morphology and structure of chitosan nanoparticles and harpin loaded chitosan nanoparticles using TEM studies: TEM images of CSNPs and CSHNPs formed by ionically crosslinking of Chitosan with TPP and harpin loaded with Chitosan TPP in acidic media are shown in Figure 1 G and H. It is interesting to note that harpin loaded chitosan nanoparticles and chitosan nanoparticles displayed spherical to globular structures with an average size of 72 nm formed in acetic medium as shown in Figure 1 (f-g).

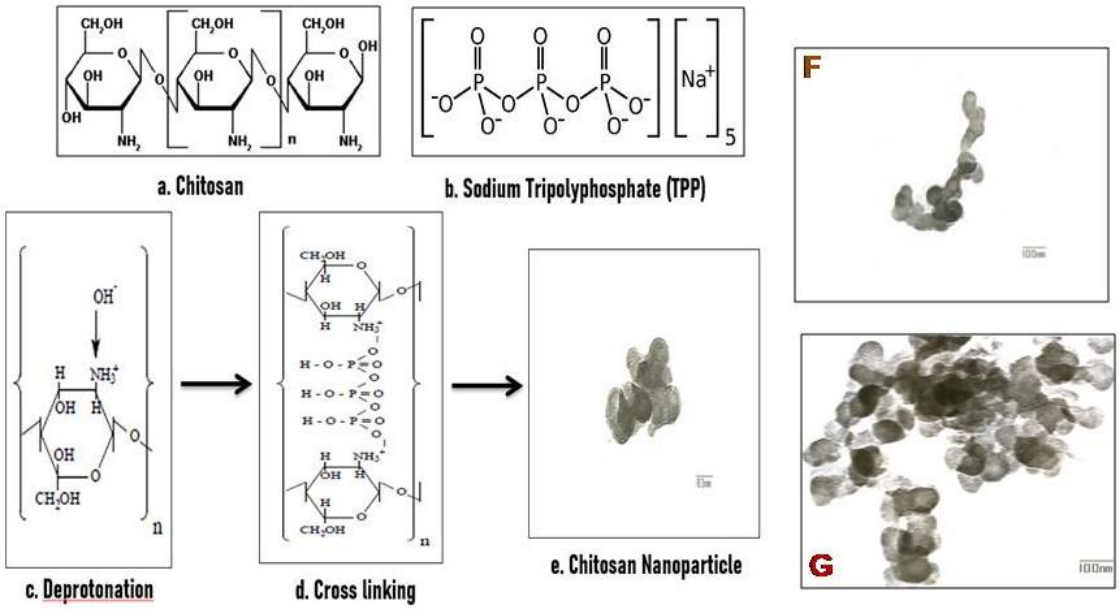

Figure 1: Chemical structures of Chitosan and characterization of chitosan nanoparticles: Chemical structures of Chitosan (a); Sodium Tripolyphohsphate (b); interactions between Chitosan and TPP due to Deprotonation (c); Cross linking (d); formation of chitosan nanoparticle (e); Transmission Electron Microscopy studies of Harpin loaded chitosan nanoparticles (CSNPs) were prepared at pH 4.2 with 3.2 µg/ml of Harpin by ionotropic gelation of Chitosan with Tripolyphosphate anions. Equal amounts of Harpin and Chitosan were premixed before the addition of TPP. (f)- CSNPs and (g)- Harpin loaded CSNPs.

Atomic Force Microscopy studies: The Atomic Force Micrography (AFM) offers the capability of 3D visualization and both qualitative and quantitative information on many physical properties including size, morphology, surface texture and roughness. Statistical information, including size, surface area, and volume distributions, can be determined as well. A wide range of particle sizes can be characterized in the same scan, starting from 1 nanometer. AFM imaging shown that all the CSNP and CSHNP particles are isodiametric and the average grain size of the CSNPs is found to be 62 nm (Figure 2a-c) whereas the CSHNP was 72 nm (Figure 2 d-f). It is interesting to observe that when harpin protein is added into the solution containing chitosan and subsequently TPP addition there was dramatic decrease in particle size. Figure 2 shows the well dispersed particles in both the CSNP and CSHNP spin coated nanofilms.

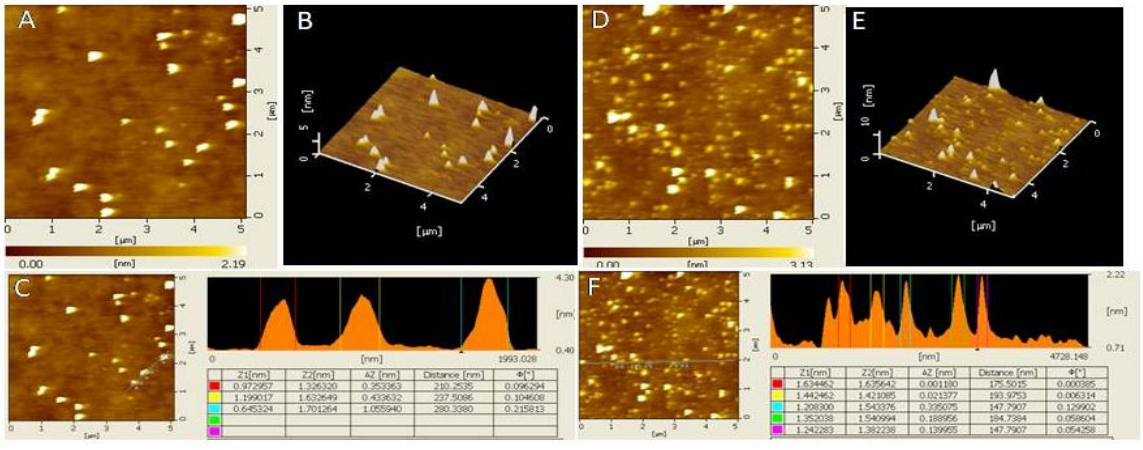

Figure: 2. Atomic force micrographs of chitosan nanoparticles Surface display analysis of chitosan-nanoparticles. CS-NPs were spin coated at 14000 rpm for 60 seconds on a glass slide and observed under atomic force microscope under non contacting mode. The images were acquired at 5 x 5 µM

resolution. Size of the nanoparticles was determined by taking the average of 3 particles. A&D). CS-NPs in 2D view, B&E). CS-NPs in 3D view and C&F). surface morphology analysis of 3 and 5 chitosan nanoparticles has resulted 62nm and 76nm size respectively.

UV-Visible Spectroscopic studies: Chitosan itself is transparent in the UV and visible region, and its optical properties are hard to characterize by spectroscopy methods. However, we have obtained the entire spectrum from 200nm to 800nm and the results obtained are shown in Figure 3a. The interaction of chitosan with TPP and harpin loaded chitosan nanoparticles formation were initially measured using UV-Vis spectroscopy. As seen in figure no detectable absorbance was noted for individual chitosan, TPP and harpin over the chosen wavelength from 200 to 800 nm, however spectral changes with high peak levels were found in CSNPs and CSHNPs (Figure 3a). Similar observations were recorded by Janes et.al., (2001) when chitosan nanoparticles were utilized for delivery system for doxorubicin.

XRD – Studies: The Figure 3b shows the XRD patterns of LMW chitosan and CSHNPs. The XRD pattern of LMW Chitosan at 2θ have shown two strong peaks in the diffractogram at 20.08^o and 11.0^o indicating the high degree of crystallinity of chitosan (Figure 3b)**.** Weak and broad peaks were found in the diffractogram of CSHNPs showing amorphous characteristics of nanoparticles. This structural modification can be related to intermolecular and/or intramolecular network structure of CS, crosslinked to each other by TPP counterions. These interpenetrating polymer chains can imply a certain disarray in chain alignment and consequently a certain decrease in crystallinity of CSHNPs compared to chitosan. XRD implicated greater disarray in chain alignment in the nanoparticles after cross links. Thus, chitosan loaded harpin nanoparticles are complex of polymeric chains cross-linked to each other by TPP counterions.

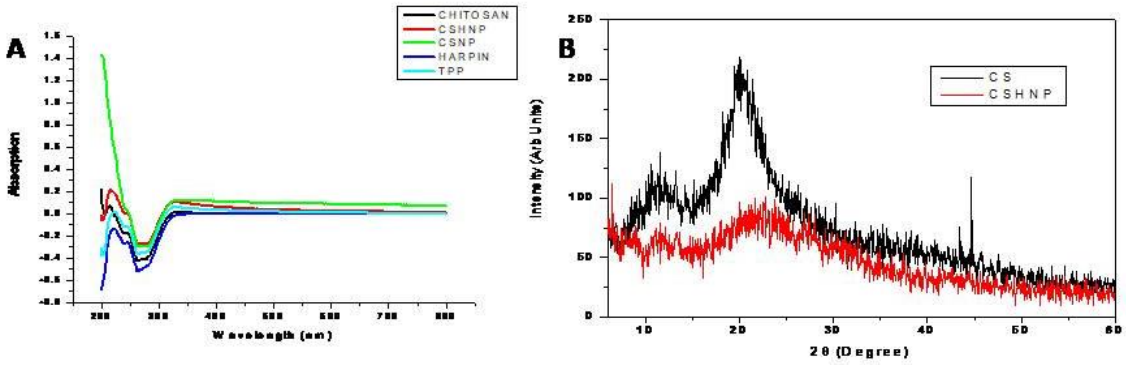

Figure 3: UV-Visible spectra and X-RD patterns of newly synthesized nanoparticles. A) chitosan, harpin loaded chitosan nanoparticles, chitosan nanoparticles, harpin and sodium tripolyphosphate (TPP) and B) XRD patterns of chitosan (black color) and harpin loaded chitosan nanoparticles (red color).

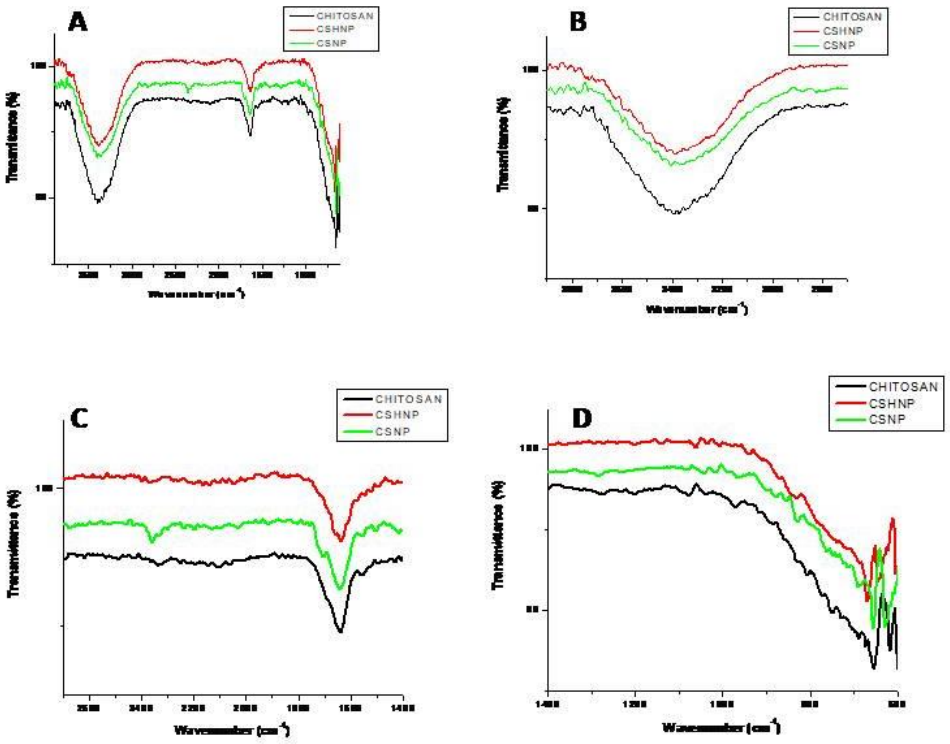

Figure 4: ATR-IR spectra. A). Chitosan, chitosan nanoparticle and harpin loaded chitosan nanoparticle; B)3800 -2800 wavenumber cm^{-1}. C).2600 -1400 wavenumber cm^{-1} and D).1400 -600 Wavenumber cm^{-1}).

ATRIR analysis: ATRIR studies of CS (Chitosan), CSNPs and CSHNPs were done to study the chemical interactions leading to formation of new functional groups during nanoparticle formation. CS, CSNPs and CSHNPs spectra are shown in Figure 4. From the Table 1, the presence of P=O & P-O groups at the frequency 1260 cm^{-1} and 1120 cm^{-1} for CSNP,1200 cm^{-1} AND 1150 cm^{-1} for CSHNP is clearly notable. The band shifts from 1650 cm^{-1} & 1550 cm^{-1} (CS) to 1665 cm^{-1} &1500 cm^{-1} (for CSNP) and 1630 cm^{-1} &1530 cm^{-1} (for CSHNP) indicating interactions between Chitosan, TPP & Harpin.

Enhancement of enzyme activities and phenolics compounds in tomato fruit after treatment with nanoparticles: Tomato fruits treated with Chitosan have shown a significant increase in PPO levels and it was maintained same until 24 hours of treatment. However, from the day 2, there was sudden fall in PPO levels. CSNPs and CSHNPs induced high PPO activity and the induction of the PPO activity was higher than the Chitosan and other treatment (TPP / Harpin/ Water) throughout the 3 days tested (Tables 2&3). It might be due to controlled release of Chitosan as well as harpin from the nanoparticles at the site. Non wounded fruit showed relatively lesser PPO activity among all the samples tested. Similar observations were found with regard to POD activity and total phenolics compounds, where there was a gradient fall in POD levels from Day 0 to Day 2 (Tables 2 &3). There was a cumulative increase in POD activity across all samples on Day 3. CSHNPs took the peak on all sampling days with regards to POD. As per expected harpin loaded Chitosan nanoparticles exhibited highest level of both enzymatic activity as well as total phenolics compounds in the treated tomato fruits followed by CSNP, harpin and Chitosan. Peaks at each day starting from day 0 (6 hr) is observed to be taken by CSHNP despite the fact that each of the enzymes i.e. POD and PPO as well as Phenolics compounds follow a definite characteristic pattern. Elevated concentrations of defense-related proteins such as PPO, POD, lipoxygenase, and protease inhibitors have frequently been well documented in tomato leaf tissue in response to wounding or feeding by arthropod herbivores (Fidantsef et al 1999; Stout et al., 1996). Similarly, Chitosan also has the potential for inducing defense related enzymes (Bautista et al. 2006) and phenolics in plants (Benhamou 1996). In the current experiment we have found similar observations, however there

was significant increase in PPO and POD levels initially and slowly the levels of enzyme and total phenolics compounds showed a decrease with regard to their activity (Tables 2&3).

Table 2: Total amount of phenolic compounds in tomato fruits treated with chitosan loaded haprin nanoparticles (CSHNP), chitosan nanoparticles (CSNP), harpin, chitosan, TPP and water. Fruits wounded and treated with water and unwounded served as controls. Different letters in each column indicate statistically significant difference ($p<0.05$) according to Duncan's multiple range test.

S.No	Treatment	Total Phenolic Compounds			
		Day 0	Day 1	Day 2	Day 3
1	**CSHNP**	1.91 ± 0.1^{a}	1.81 ± 0.8^{d}	0.62 ± 0.5^{c}	0.78 ± 0.5^{d}
2	**CSNP**	1.89 ± 0.3^{c}	1.79 ± 0.5^{b}	0.51 ± 0.4^{b}	0.76 ± 0.4^{c}
3	**Harpin**	1.76 ± 0.2^{b}	1.69 ± 0.4^{c}	0.42 ± 0.7^{d}	0.52 ± 0.7^{b}
4	**Chitosan**	1.52 ± 0.6^{a}	1.42 ± 0.1^{b}	0.41 ± 0.3^{c}	0.38 ± 0.3^{b}
5	**TPP**	1.01 ± 0.4^{d}	1.01 ± 0.2^{d}	0.21 ± 0.2^{d}	0.26 ± 0.2^{a}
6	**Water**	1.12 ± 0.4^{a}	1.02 ± 0.6^{a}	0.28 ± 0.1^{a}	0.19 ± 0.1^{a}
7	**Unwound**	1.21 ± 0.8^{c}	0.79 ± 0.1^{c}	0.18 ± 0.6^{c}	0.18 ± 0.6^{d}

Table 3: P**olyphenol oxidase (PPO) and peroxidase (POD) activities in tomato fruits treated with chitosan loaded harpin nanoparticles (CSHNP), chitosan nanoparticles (CSNP), harpin, chitosan, TPP and water.** Fruits wounded and treated with water and unwounded served as controls. Different letters in each column indicate statistically significant difference ($p<0.05$) according to Duncan's multiple range test.

S.No	Treatment	Day 0		Day 1		Day 2		Day 3	
		PPO	POD	PPO	POD	PPO	POD	PPO	POD
1	**CSHNP**	0.03 ± 0.4^{a}	0.4 ± 0.8^{d}	0.03 ± 0.1^{d}	0.16 ± 0.5^{d}	0.003 ± 0.4^{d}	0.05 ± 0.4^{a}	0.004 ± 0.1^{c}	0.07 ± 0.8^{d}
2	**CSNP**	0.03 ± 0.6^{b}	0.4 ± 0.5^{b}	0.024 ± 0.4^{b}	0.14 ± 0.4^{c}	0.003 ± 0.5^{a}	0.05 ± 0.6^{b}	0.003 ± 0.4^{b}	0.06 ± 0.5^{b}
3	**Harpin**	0.017 ± 0.3^{d}	0.39 ± 0.4^{c}	0.02 ± 0.5^{c}	0.12 ± 0.7^{b}	0.003 ± 0.3^{c}	0.04 ± 0.3^{d}	0.003 ± 0.5^{d}	0.05 ± 0.4^{c}
4	**Chitosan**	0.02 ± 0.5^{c}	0.32 ± 0.1^{d}	0.02 ± 0.4^{a}	0.12 ± 0.3^{b}	0.003 ± 0.6^{c}	0.04 ± 0.5^{c}	0.003 ± 0.4^{a}	0.04 ± 0.1^{b}
5	**TPP**	0.005 ± 0.1^{c}	0.25 ± 0.2^{a}	0.02 ± 0.3^{b}	0.003 ± 0.2^{a}	0.002 ± 0.4^{b}	0.03 ± 0.1^{c}	0.002 ± 0.3^{a}	0.01 ± 0.2^{d}
6	**Water**	0.003 ± 0.2^{d}	0.22 ± 0.6^{d}	0.012 ± 0.6^{d}	0.002 ± 0.1^{a}	0.002 ± 0.6^{b}	0.03 ± 0.2^{d}	0.002 ± 0.6^{d}	0.02 ± 0.6^{a}
7	**Unwound**	0.002 ± 0.3^{a}	0.22 ± 0.1^{c}	0.010 ± 0.1^{a}	0.001 ± 0.6^{d}	0.002 ± 0.9^{d}	0.02 ± 0.3^{a}	0.001 ± 0.1^{c}	0.02 ± 0.1^{c}

Conclusions: Fine nanoparticles were prepared using ionotropic gelation method for the formation of crosslinked harpin loaded Chitosan nanoparticles (CSHNPs) and ionic cross linking for individual Chitosan nanoparticles (CSNPs), which involved deprotonation process by adjusting the pH of TPP to 5.2. Chitosan was cross linked ionically with TPP at lower pH 5.2 by deprotonation mechanism. Nanoparticles were characterized to establish the interplay of concentration, pH, stirring condition on particle stability, size, distribution using TEM, AFM, XRD, ATRIR and UV-Visible spectroscopy followed by testing these nanoparticles on tomato fruit with regard to enhancement of defense markers viz., poly-phenol oxidase (PPO), peroxidase (POD) levels and total phenolics compounds of the fruit. It is well established by the present study that harpin/chitosan individually as well as CSHNPs/CSNPs enhanced the PPO and POD levels with CSHNPs/CSNPs showing much higher efficiency than pure harpin/chitosan. Application of CSNPs and CSHNPs can potentially help in controlling post-harvest storage problems in tomato. Experiments are underway to determine the effect of CSNPs and CSHNPs on fruits when challenged with pathogen *Phytophthoroa infestans.*

Table 1: Possible bonding during CSNPs, CSHNPs formation as observed by ATR-IR peaks.

Possible assignments	CS (cm^{-1})	CSNP (cm^{-1})	CSHNP (cm^{-1})
v(N-H, in NH2)			
v(C-H)	2900	2920	2880
v(C-O), Amide I	1650	1665	1630
v(N-H), Amide II	1550	1500	1530
v(C3-O)	1280	1300	1310
v(C6-O)	1070	1100	1050
v(P-O)	-----	1260	1200
v(P=O)	-----	1120	1150

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