Use of chitosan nanoparticles loaded with biologically active substances for pre-harvest plant protection from pathogens (a review)

S B Popletaeva and L R Arslanova

State Scientific Institution "All-Russian Research Institute of Phytopathology", Russian Agricultural Academy, own. 5, Institute st., Bolshie Vyazemy, Odintsovo distr., Moscow reg., 143050, Russia

E-mail: unavil@yandex.ru

Abstract. Chitosan is a biopolymer that readily forms nanoparticles, with or without additional biologically active substances loaded into them. Chitosan nanoparticles can include hydrophobic or hydrophilic compounds, metal ions, compounds poorly soluble in water, etc. Chitosan is biodegradable, biocompatible, non-toxic to plants, humans and animals. It also stimulates plant immunity and enhances crop yields. Because of that properties, there are some works about chitosan nanoparticles loaded with biologically active substances for plant protection. The major advantages of these nanoparticles are: good solubility in water, large surface for interaction with the pathogen, possibility of gradual release of active substances, protection of active substances from damage, easy entrance of the nanoparticles into plant cells.

In this review current research works about use of chitosan nanoparticles that are loaded with active substances for pre-harvest plant protection are summarized.

1. Chitosan

Chitosan is a biopolymer that can be obtained by deacetylation of chitin, and also is a natural component of the crustacean shells, insect exoskeletons, and the cell wall of fungi of the Zygomycota division. Chitosan consists of D-glucosamine and N-acetyl-D-glucosamine units connected by β-(1-4) bonds and arranged in a random order.

This compound is biocompatible, biodegradable, non-toxic, not harmful to human and animal health, relatively easy to obtain. It is also possible to obtain chitosan nanoparticles, including those loaded with some substances. These properties of chitosan have led to the fact that chitosan nanoparticles are now widely used in medicine for drug delivery, also because chitosan nanoparticles are better soluble in water than usual chitosan [1, 2].

Also, because of growing demand for biopesticides as an alternative to synthetic pesticides, there exist works on the use of chitosan for plant protection. It negatively affects phytopathogens (fungi, bacteria, viruses) in a direct way, and also activates the immune mechanisms of living plants, i.e. it is an elicitor (inducer of plant disease resistance) [3, 4]. It is non-toxic to plants [5]. A number of studies have noted its positive impact on plant productivity and crop quality (see, for example, a review [6] by...
Mukhtar Ahmed et al.). Another advantage of using chitosan as a material is that it can easily enter plant cells due to the presence on the cell membrane a receptors that recognize chitosan [7, 8].

It is possible to include both hydrophilic and hydrophobic compounds in chitosan nanoparticles. Also chitosan (usual or in the form of nanoparticles) readily forms complexes with metal ions. Chitosan nanoparticles can include both well-and poorly water-soluble compounds [9] It is also possible to synthesize chitosan nanoparticles containing chitosan together with other biopolymers, for example, poly-γ-glutamic acid [10], alginate—a polysaccharide derived from algae [11, 12], or pectin— polysaccharide from vegetables [13]. It should also be noted that microparticles based on chitosan and dextran and loaded with an elicitor protein were also obtained [14].

In view of the above, there already exist some research works about use of chitosan nanoparticles in pre-harvest plant protection, in particular those loaded with active substances.

Their advantages for plant protection compared to usual chitosan are: 1) better solubility of chitosan nanoparticles in water compared to usual (bulk) chitosan; 2) larger surface area available for interaction with the pathogen; 3) the possibility of their use as a "nanocontainers" for the gradual release of active substances; 4) the possibility of protecting active substances from the damaging effects of the environment (for example, protection of proteins and peptides from solar radiation); 5) readily entrance into plant cells because of small particle size [15, 16, 17].

However, there is still a small amount of works on the use of chitosan nanoparticles for pre-harvest plant protection. The consideration of such works seems appropriate, since in comparison with experiments with pathogens in vitro, experiments on living plants (or their leaves) additionally involve the elicitory effect of chitosan, i.e., the activation of the plant's own immunity by this biopolymer.

All works on the use of chitosan nanoparticles for pre-harvest plant protection are very recent, no earlier than 2015, and such use is shown, as a rule, on the example of protection against phytopathogenic fungi and oomycetes, there are no works on bacteria and viruses yet. This may be due to the fact that fungi are the most important group of phytopathogens [18, 19]. All these works will be discussed in more detail in section 2. Active substances and host plants are summarized in Table 1.

2. Active substances
This subsection contains some information about recently used in chitosan nanoparticles active substances.

Copper compounds are traditionally used in plant protection [20]. Zinc in plants is involved in a variety of vital processes, such as gene expression, electron transport, and protein metabolism [21, 22]. Zinc deficiency leads to a decrease in yield due to delayed growth and development of plants [23].

Salicylic acid is an important plant hormone involved in plant response to pathogen attack [24, 25]. Harpins are a group of proteins present in various phytopathogenic bacteria and are elicitors (inducers of disease resistance) in plants that are non-host to these bacteria [25, 26].

PPIase from Pseudomonas fluorescens also is an elicitor protein [27].

Hexaconazole and dazomet, as it seems, were chosen as an example demonstrating the possibility of producing nanoparticles of chitosan with pre-loaded synthetic fungicides.

Table 1. Use of chitosan nanoparticles loaded with biologically active substances for pre-harvest plant treatment for protection from fungal pathogens.

| Host plant       | Pathogen             | Active substance          | Reference |
|------------------|----------------------|---------------------------|-----------|
| Maize            | Curvularia lunata    | zinc (II) sulfate         | [15]      |
| Maize            | Curvularia lunata    | copper (II) sulfate       | [28]      |
| Tomato (the leaves were detached after treatment) | Rhizoctonia solani | Pss harpin protein       | [29]      |
| Maize            | Fusarium verticillioides | salicylic acid       | [30]      |
| Tomato           | Alternaria solani    | and copper (II) sulfate  | [16]      |
3. Examples of the use of chitosan nanoparticles loaded with biologically active substances for plant protection from pathogens

Choudhary et al. [15] synthesized zinc-containing chitosan nanoparticles with a size 387.7±4.0 nm. Zinc was present in the nanoparticles in the form of ions captured by chitosan as a chelating agent. Maize seeds and leaves were treated in the experiment with potted plants and in the field experiment with various preparations, the effects of which was tested against the fungus *Curvularia lunata*, that causes Curvularia leaf spot disease.

For the experiments with plants in pots, maize seeds were soaked before sowing, for 4 hours, and then on the 35th day after sowing the plants were sprayed with various types of treatments. The following treatments were used: chitosan nanoparticles in concentration 0.01%, 0.04%, 0.08%, 0.12% and 0.16%, chitosan solution (0.01%), ZnSO$_4$ (0.01%), commercial fungicide (Bavistin, 0.1%) and water (control). The plants were grown in the net house. At 10 days after spraying with the preparations, the plants were infected with *C. lunata*. At 15 days after inoculation, data was recorded to calculate disease severity. In the field experiment, the conditions of treatment with preparations and pathogen were the same as in the experiment with potted plants, but the data for calculating disease severity was recorded at 20 days after inoculation.

In the experiment with potted plants, control plants showed disease symptoms on the 3rd–4th day after inoculation, and as a result, the affected leaves dried up. In the plants treated with nanoparticles, disease symptoms appeared as small lesions, without necrosis of the leaves, on the 7th–8th day after inoculation. On the 15th day after inoculation, disease severity was 43.3% (a) in the control, 30.0% (bc) for usual chitosan, 32.0% (b) for ZnSO$_4$, 30.7% (bc) for commercial fungicide Bavistin. For nanoparticle concentrations 0.01%, 0.04%, 0.08%, 0.12% and 0.16%, it was 29.3% (bcd), 23.3% (de), 22.0% (e), 21.3% (e), and 24.7% (cde), respectively.

Different Latin letters here and further in the text are marking values, the differences between which are statistically significant.

Thus, the disease protection decreased for the highest concentration of zinc-chitosan nanoparticles. The authors [15] explain this by statement that zinc in high doses is toxic to plants, that is consistent, for example, with the review [33] on zinc in plants.

In the field, there was trend similar to that in the net house. The disease severity was 61.2% (a) in the control, 47.3% (b) for conventional chitosan, 47.8% (b) for ZnSO$_4$, and 44.6% (bc) for Bavistin. For nanoparticle concentrations 0.01%, 0.04%, 0.08%, 0.12% and 0.16%, it was 29.3% (bcd), 23.3% (de), 22.0% (e), 21.3% (e), and 24.7% (cde), respectively.

The difference between the indoor and field experiments was the stronger effect of Bavistin in the field, but among the different concentrations of nanoparticles in both cases, one of the best was the concentration of 0.12%.

It should be noted that in field experiments, treatment of maize seeds and leaves with zinc-chitosan nanoparticles increased the grain yield compared to the control. The grain yield (in kg per plot) was 1.86 (de) in the control, 2.38 (bcd) for usual chitosan, 2.16 (bcd) for ZnSO$_4$, and 1.29 (e) for Bavistin. For nanoparticle concentrations 0.01%, 0.04%, 0.08%, 0.12% and 0.16% it was 3.09 (bcd), 3.06 (a), 2.81 (ab), 2.58 (abc) and 2.34 (bcd), respectively. The decrease in grain yield with an increase in the

| Plant       | Pathogen                    | Chemicals                              | [Reference] |
|-------------|------------------------------|----------------------------------------|-------------|
| Finger millet (Eleusine coracana) | *Fusarium oxysporum* separately | Copper (II) sulfate                     | [31]        |
| Oil palm    | *Pyricularia grisea*         | Hexaconazole; dazomet; hexaconazole and dazomet simultaneously | [32]        |
| Tobacco leaves (detached) | *Alternaria longipes* | Peptidyl-prolyl cis/trans isomerase (PPIase) from *Pseudomonas fluorescens* | [14]        |
concentration of zinc-chitosan nanoparticles the authors [15] also explain by the toxicity of high zinc doses to plants. This explanation is the same as in case of disease protection and is consistent with the review [33].

Choudhary et al. [28] also used copper-containing chitosan nanoparticles with a size of 361.3±2.1 nm, also on maize against the fungus *Curvularia lunata*. Copper, like zinc in [15], was present in the nanoparticles in the form of ions captured by chitosan as a chelating agent.

The treated seeds were grown in pots in the net house. The leaves of the plants were sprayed on the 35th day after sowing with the same preparations that were used for seed treatment. 10 days after treatment with the preparations, the plants were inoculated with *C. lunata*. Data for calculating disease severity was recorded on the 15th day after infection.

Additionally, a field experiment (July-December 2016) was conducted. Seed treatment, leaf treatment, inoculation by the pathogen, and disease severity calculation were performed in the same way as in the case of experiments with potted plants. Data for calculating disease severity was recorded on the 20th day after inoculation.

In the experiments with potted plants, the symptoms of the disease in control plants began to appear after 3–4 days of fungal inoculation. A small chlorotic spot appeared on leaves, gradually turning into a large eye-shaped lesion, leading to the formation of necrosis. At the same time, in plants treated with nanoparticles, symptoms of the disease in the form of small lesions without chlorosis appeared after 7-8 days of fungal inoculation, and in the following days spread and severity of the disease were also small.

Disease severity for the potted plants was as follows: control — 44.0% (a), usual chitosan — 32.7% (b), CuSO₄ — 32.0% (bc), Bavistin — 29.3% (bc). For copper-chitosan nanoparticles in concentrations 0.01%, 0.04%, 0.08%, 0.12% and 0.16% disease severity was 28.7% (c), 24.7% (d), 24.7% (d), 23.3% (d), and 22.7% (d), respectively.

In the field experiment, the disease severity was: control—64.3% (a), usual chitosan—48.2% (b), CuSO₄ — 47.5% (bc), Bavistin — 47.0% (bc). For copper-chitosan nanoparticles in concentrations 0.01%, 0.04%, 0.08%, 0.12% and 0.16% disease severity was 46.4% (bc), 46.3% (bc), 45.9% (bc), 44.7% (cd), and 42.5% (d), respectively.

Thus, the lowest values of disease severity both in the field and in the net house were observed in the case of nanoparticles treatment, and they decreased with the increasing of nanoparticles concentration.

It should be noted that in the field experiment, weight of ears per plot, grain yield per plot and 100 grain weight were higher than in the control, in the case of treatment with nanoparticles at a concentration of 0.12% - 0.16%. The weight of ears per plot, grain yield per plot and 100 grain weight in the control were 2.83 kg (ab), 2.08 kg (abcd), and 25.55 g (b), respectively. The weight of the cobs, the grain yield, and the weight of 100 grains when treated with nanoparticles at a concentration of 0.16% were the highest among all tested variants and were 3.10 kg (a), 2.69 kg (a), and 29.80 g (a), respectively. As one can see from the data provided, disease protection increased with the increase in the concentration of copper-chitosan nanoparticles, and the highest weight of ears per plot, grain yield per plot and 100 grain weight were also observed at the highest concentration of nanoparticles. Thus, the improvement in these parameters could be associated with better disease protection provided by the highest concentration of nanoparticles.

Nadendla et al. [29] obtained chitosan nanoparticles (H-CSNPs), 133.7 ± 3.6 nm in size, loaded with the Pss harpin protein from *Pseudomonas syringae* pv. *syringae*. This protein is an elicitor (inductor of plant disease resistance). The authors of this study point to poor uptake of this protein itself by plants, which is an obstacle to its use in plant protection. Therefore, the authors [29] synthesized chitosan nanoparticles containing harpin Pss to improve the delivery of this protein into plant cells and so improve its bioavailability. Also they synthesized chitosan nanoparticles without harpin (CSNPs).

In this work, experiments were carried out with *Rhizoctonia solani* on leaves separated from tomato plants after treatment with preparations. The plants were sprayed with harpin Pss,
H-CSNPs, CSNPs and buffer (control). After 24 hours, the leaves were separated and inoculated with *R. solani*. After 5 days of inoculation, disease severity was determined by the diameter of the lesions caused by the pathogen on the leaves. The lesion diameter in the control was approx. 3 cm, with harpin Pss — approx. 1.5 cm, with CSNPs — approx. 1.3 cm, with H-CSNPs — approx. 0.3 cm. There was statistically significant difference between these values, except for difference between harpin Pss and CSNPs. Thus, the use of H-CSNPs reduced disease severity by about 10 times compared to the control.

Kumaraswamy et al. [30] synthesized chitosan nanoparticles, 368.7±0.05 nm in size, loaded with salicylic acid, which was covalently bound to chitosan (SA-CS NPs) and conducted experiments with them to protect maize from the fungus *Fusarium verticillioides*, which causes post-flowering stalk rot.

Maize seeds were treated for 4 hours using the following variants: 1) water (control), 2) usual chitosan at concentration of 0.01%, 3) salicylic acid at concentration of 0.01%, 4) SA-CS NPs at concentration of 0.01%, 0.04%, 0.08%, 0.012% or 0.16%. Then they were planted in pots in the net house. On the 55th day after sowing, the leaves were sprayed using the same variants, from 1) to 4). At the beginning of the flowering stage (60 days after sowing), the soil near the plant roots was inoculated with *F. verticillioides*. On the 95th day after sowing (time of maturity) disease severity was evaluated. In addition, a field experiment was conducted, in which the same methods of treatment and disease severity evaluation were used as in the indoor experiment.

In the experiment with potted plants, disease severity for various types of treatment was as follows: control — 99.7% (a), usual chitosan — 91.2% (a), salicylic acid — 91.2% (a). For SA-CS NPs used in the concentration of 0.01%, 0.04%, 0.08%, 0.012% and 0.16%, disease severity was 62.3% (b), 50.3% (c), 51.3% (c), 50.3% (c), and 50.3% (c), respectively.

The disease severity for various types of treatment was: control — 55.6% (a), ordinary chitosan — 48.6% (ab), salicylic acid — 49.2% (ab). For SA-CS NPs, used in the concentration 0.01%, 0.04%, 0.08%, 0.012% and 0.16%, disease severity was 25.3% (c), 33.1% (abc), 29.7% (bc), 22.5% (cd) and 28.9% (bc), respectively.

Thus, both indoors and in the field the best variant of treatment was chitosan nanoparticles loaded with salicylic acid.

Saharan et al. [16] used for tomatoes treatment chitosan nanoparticles, 374.3 ± 8.2 nm in size, loaded with copper sulfate. Experiments were conducted with potted tomato plants in the net house with *Alternaria solani* and *Fusarium oxysporum*, separately.

For experiments in pots, 20-day-old plants were inoculated with *A. solani*. After 3–4 days of inoculation, the plants were sprayed with aqueous suspension of nanoparticles with concentrations of 0.08%, 0.10%, and 0.12%. In parallel, *F. oxysporum* was added into the soil in the pots near the stem-root junction of the tomato plants. Three days after inoculation, the soil at the root zone was treated with aqueous suspension of nanoparticles with concentrations of 0.08 %, 0.10% and 0.12%, 10 ml per plant. The commercial fungicide Mankozeb (Dithane M 45) at a concentration of 0.2% served as a positive control in experiments with both pathogens.

Disease severity values for early blight were as follows: control — 55.0% (a), Mancozeb — 18.0% (e), nanoparticles at concentrations of 0.08%, 0.10% and 0.12% — 15.3% (b), 8.6% (b) and 6.6% (b), respectively. For Fusarium wilt values of disease severity were: control — 66.6% (c), Mancozeb — 18.0% (a), nanoparticles at concentrations of 0.08%, 0.10% and 0.12% — 50.0% (b), 41.6% (b) and 33.3% (ab), respectively.

Thus, for the control of early blight, the most effective treatment was chitosan nanoparticles with copper sulfate, and for the control of Fusarium wilt they were on the second place after the commercial fungicide.

Sathiayabama and Manikandan [31] synthesized chitosan nanoparticles with copper sulfate (CuChNp), 88.2 nm in size. The obtained nanoparticles were used to treat finger millet (*Eleusine coracana*) plants growing in a greenhouse. This plant is an important grain crop in some regions of India and North Africa.
Preliminary experiments were conducted on the effect of these nanoparticles on plant growth, using different concentrations of CuChNp (0.01%, 0.05%, 0.1%, and 0.15%). Based on that, the 0.1% concentration of CuChNp was chosen for further experiments. Leaf spraying or seed soaking for 12 hours in combination with leaf spraying was used. 20-day-old plants were sprayed with 0.1% CuChNp. Then spraying was repeated 2 times with 10-day intervals, up to the 40th day. Water sprayed plants served as control.

30-day-old treated plants (leaf spraying or seed soaking+leaf spraying) were infected with *Pyricularia grisea*, which causes blast disease. During the next 50 days, the appearance of the first visible symptoms of the disease and the further development of the plants was observed and the disease incidence was calculated.

After 15 days of inoculation, the leaves of the control plants developed typical symptoms of the disease in the form of dark brown lesions. The symptoms progressed rapidly, and on the 50th day, 100% disease incidence was observed in the control. Treatment with CuChNp delayed the onset of symptoms. The appearance of the first visible symptoms occurred on the 25th day in the case of only leaf spraying and on the 30th day in the case of the combination of seed treatment and leaf spraying. On the 50th day after inoculation, the treated plants had a disease incidence of 25% (seed coat+foliar spray) or 28% (foliar spray).

Maluin et al. [32] synthesized chitosan nanoparticles (CEN, 2 nm in size), as well as chitosan nanoparticles loaded with commercial fungicides: hexaconazole (CHEN, 18 and 168 nm), dazomet (CDEN, 7 and 32 nm) and hexaconazole and dazomet simultaneously (CHDEN, 5 and 58 nm).

Commercial 3-month-old oil palm plants were purchased and then grown in the nursery. The following treatments were used: T1—control (untreated plants); T2—CEN, 2 nm; T3—CHEN, 18 nm; T4—CHEN, 168 nm; T5—CDEN, 7 nm; T6—CDEN, 32 nm; T7—CHDEN, 5 nm; T8—CHDEN, 58 nm. The following concentrations were used: for T2—3; 9 and 15 g/l, for T3—5 g/l, for T3-T8—1; 3 and 5 g/l. For each treatment, 10 ml was injected into the soil. Then, after 2 weeks, the plants were inoculated with the fungus *Ganoderma boninense*, that causes basal stem rot. The treatment was carried out 2 more times: 3 months and 5 months after inoculation, moistening the soil near the plants with 20 ml of the appropriate type of treatment.

The external manifestations of the disease were evaluated 2, 4, 6 and 8 months after inoculation. The disease incidence for the treated plants was less than in the control.

The best results among each of the treatments T2-T8 were observed for the highest concentration of each treatment. There was no statistically significant differences between the control and the treatments at 4 months after inoculation, except T3, T5 and T7 at 5 g/l, where disease incidence was approx. 10%, 12% and 5%, respectively, compared to approx. 30% in the control. At 6 months the best results were also for T3, T5 and T7 at 5 g/l each: approx. 30%, 30% and 20% respectively, and approx. 90% in the control. At 8 months best results were for T7 and T8 at 5 g/l each: approx. 50%, and 60% respectively, and approx. 95% in the control.

Thus the best treatment was chitosan nanoparticles containing both hexaconazole and dazomet and having the smallest size from two size variants (T7).

It should be noted that in biotechnology and medicine the term “nanoparticles” can mean particles up to 1000 nm in size [33]. Therefore, the work [14] with chitosan/dextran-based particles of 0.5—2 microns in size, should also be noted as adjacent to the topic of chitosan nanoparticles use. The microparticles [14] consisted of dextran (70%), chitosan (20%), and an active component (10%), an elicitor protein from *Pseudomonas fluorescens*, which is a peptidyl-prolyl cis/trans isomerase (PPIase). Dextran was used to form the microparticles themselves, and chitosan was used to gradually release the active component. The protective activity of the microparticles was tested on detached tobacco leaves against *Alternaria longipes*, which causes tobacco brown spot. The controls were as follows: 1)particles consisting of 75% dextran and 25% chitosan without PPIase and 2)purified PPIase preparation.

One half of the tobacco leaves was treated with a solution of the preparation with a concentration of 1 mg/ml of PPIase (the same amounts of PPIase for the chitosan/dextran/PPIase particles and for
the purified PPIase). The second (control) half was treated with a buffer, on the basis of which the preparation solution was made. A day later, the leaves were inoculated with *A. longipes*, with evenly distribution of the pathogen spores suspension over the surface of the entire leaf. 5 days after inoculation, the number of necroses on each of the leaf halves was counted. The results of this experiment are given in Table 2. As one can see from Table 2, PPIase encapsulated into chitosan/dextran microparticles was able to prevent the disease development on tobacco leaves and demonstrated the same activity as the initial preparation of the intact protein. Dextran and chitosan used as protectors also demonstrated some protective activity, but it was significantly lower than that of the intact PPIase and the microparticles with PPIase.

**Table 2.** Protection of tobacco leaves from tobacco brown spot (*A. longipes*) using chitosan/dextran-based microparticles with an elicitor protein from *Pseudomonas fluorescens*, which is a peptidyl-prolyl cys/trans isomerase (PPIase) [14].

| Treatment                          | Number of necroses on leaf halves |
|------------------------------------|-----------------------------------|
|                                    | Treated with preparation | Control                  |
| PPIase/chitosan/dextran microparticles | 313 ± 37.8                   | 469.3 ± 60.4              |
| Controls                           | PPIase                        | 237.8 ± 38.2              |
|                                    | chitosan/dextran microparticles | 447.7 ± 72.5              |
|                                    |                                 | 553.3 ± 78.1              |

4. Conclusions
As one can see from works summarized in section 2, chitosan nanoparticles, loaded with biologically active substances, are an effective tool for plant protection and in some cases for increasing crop yield.

Acknowledgments
This work (the literature review) was conducted as a part of state assignment № 0598-2019-0002.

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