A biopolymer with antimicrobial properties and plant resistance inducer against phytopathogens: Chitosan

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Abstract

Some synthetic fungicides have been currently prohibited due to their adverse effects; thus, searching for alternatives to decrease their application is a priority worldwide. An alternative to the application of synthetic fungicides is chitosan -a natural biopolymer- because of its biocompatibility, biodegradability, and bioactivity. Chitosan has been used in different industries, such as cosmetology, pharmaceutics, food, among others. In agriculture, it has been used as a resistance inducer and bio-fungicide because of its antimicrobial activity and for plant development as growth promoter. Although many works have been published on chitosan for its characteristics and mode of action, the direct effects on agriculture -both in plant and fruit phytopathogens- have not been reported. Therefore, the objective of this review is to summarize recent advances and achievements of chitosan application in agriculture with special attention to its antimicrobial properties and plant defence induction mechanisms.

Keywords: antimicrobial activity; induced systemic resistance; main sources of chitin; fruit protection; chitosan nanoparticles

Introduction

Synthetic fungicides have an important role in phytopathogen control (Massi et al., 2021). Nonetheless, their application generates resistance to phytopathogens and affects the environment, human and animal health negatively (Meena et al., 2020b). Currently, interest exists in friendly agriculture that produces healthy food and minimizes the use of agrochemicals (Baker et al., 2020). Using biopolymers, such as cellulose, starch, galactomannan, among others, has gained importance for controlling diverse plant diseases (Malerba and Cerana, 2018) where chitosan stands out as the most used biopolymer in agriculture (Yang et al., 2021).
Chitosan is extracted from chitin found in the exoskeleton of crustaceans (Fournier et al., 2020), fungal cell wall (Chang et al., 2019), insect cuticle (Luo et al., 2019), among others. Chitosan has been used in waste water treatment (Uragami et al., 2015), cosmetology (Aranaz et al., 2018), medicine (Ahsan et al., 2018), pharmaceutics (Khan et al., 2019), food and beverage (Rocha et al., 2017), and paper industries (Song et al., 2018), among others.

In agriculture, chitosan has been used to induce plant resistance (Coutinho et al., 2020), increasing the antagonistic capacity of beneficial microorganisms (El Amerany et al., 2020) and crop productivity (Rahman et al., 2018). In phytopathogen control, chitosan induces morphological changes and structural alterations in fungal cells that cause cell death (Berger et al., 2016). When chitosan is used to improve plant defense, it induces reactive oxygen species (ROS) production (Silva et al., 2018), hydrolytic enzymes (Obianom et al., 2019), pathogenesis-related (PR) proteins (Liu et al., 2019), phytoalexins (Gai et al., 2019), callose formation (de Lamo et al., 2020), and promotes lignification (Jiang et al., 2018). Chitosan structure, synthesis and antimicrobial properties in vitro have been discussed widely (Verlee et al., 2017), but few studies have focused on its effects on agriculture.

**Chitosan**

*Chemical characteristics and main sources*

Chitosan is the most important chitin by-product, which is obtained by thermo-alkaline deacetylation. It is a lineal polymer formed by N-D glucosamine (2-amino-2-deoxy-β-D glucopyranose) monomers, bound by β-1-4 (Katiyar et al., 2015). Chitin is a natural and abundant biopolymer found in many organism (Fournier et al., 2020; López-Corona et al., 2020) (Table 1) and is compound of 2-acetylamine-2-deoxy-β-D-glucopyranose units (Peter et al., 2020).

| Source       | Specie                        | Reference               |
|--------------|-------------------------------|-------------------------|
| Cockroach    | *Periplaneta americana*       | Kaya et al., 2015a      |
| Spider       | *Caribena versicolor*         | Machałowski et al., 2019 |
| Scarab       | *Goliathus orientalis*        | Fournier et al., 2020   |
| Crab         | *Portunus segnis*             | Hamdi et al., 2017      |
| Bryozoa      | *Plumatella repens*           | Kaya et al., 2015b      |
| Mollusk      | *Chiton sp.*                  | Rasti et al., 2017      |
| Norway lobster | *Nephrops norvegicus*          | Sayari et al., 2016     |
| Shrimp       | *Penaeus monodon*             | Srinivasan et al., 2018 |
| Yeast        | *Saccharomyces cerevisiae*    | Sun et al., 2018        |
| Fungus       | *Penicillium camemberti*      | Aili et al., 2019       |

The main forms of application of chitosan are: seed coating, soil enrichment, foliar spraying, fruit coating, nanoparticles, among others (Morin-Crini et al., 2019). Chitin is white or yellowish in its pure state and highly hydrophobic, thus, insoluble in water and on organic solvents (Cheba, 2011). To obtain chitin, chemical and biological methods are used. The first one has a disadvantage (environmental pollution due to generated waste), but its short processing time turns it into the most commercially used (Hamed et al., 2016). This method implies three steps; demineralization, deproteinization and discoloration (Figure 1). The first step consists of processing raw matter in dust with strong acids (hydrochloric acid [HCl], sulfuric acid [H₂SO₄], acetic acid [CH₃COOH] and formic acid [CH₂O₂]) to eliminate mineral compounds (calcium carbonate [CaCO₃] and calcium phosphate [Ca₃[PO₄]₂]); for deproteinization, an alkaline treatment is used where proteins are eliminated with (sodium hydroxide [NaOH]); in discoloration, if a colorless product is
expected, organic or inorganic solvents (acetone, sodium hypochlorite and hydrogen peroxide) are used to eliminate pigments (astaxanthin and β-carotene) (Santos et al., 2020).

Chitosan is described according to the degree of deacetylation and molecular weight. The degree of deacetylation establishes the content of free amino groups and allows differentiating chitin from chitosan (Taşkın et al., 2014). In general, the greater deacetylation degree, the greater solubility in acid conditions, positive charge, thus, antimicrobial activity (Tolaimate et al., 2003). Chitosan is classified according to its molecular weight in high (> 300 kDa)-medium (> 190 kDa up to 300 kDa)-or low (> 16 kDa up to 190 kDa)-molecular-weight and oligochitosan (≤ 16 kDa) (Verlee et al., 2017). High and medium-molecular-weight chitosan coats the cell surface, blocking nutrient transport to the microbial cellular membrane and causing cell lysis (Li et al., 2010). Low-molecular-weight chitosan and oligochitosan go through cellular membranes of microorganisms, bind to DNA, and inhibit RNA synthesis (Chien et al., 2016). Moreover, oligochitosan produces changes in internal cell structure causing cell lysis and releasing intercellular components (Kulikov et al., 2014). The main difference between chitin and chitosan is the content in amino groups and their physical-chemical properties related with flocculation, chelation and biological functions (Xia et al., 2011). Chitosan in addition to organic acids, such as formic, ascorbic acids forms chitosonium acids salts and turns soluble in water, which confers greater versatility when compared to chitin (Vinsova and Vavrikova, 2008; Philibert et al., 2017).

![Chemical method of obtaining chitin](image)

*Figure 1.* Chemical method of obtaining chitin

*Source: Authors*
Chitosan antimicrobial properties

Chitosan antimicrobial activity depends on the type of microorganism, molecular weight, deacetylation degree, besides inoculant concentration, temperature, culture medium, pH, among others (Wang et al., 2020b). The types of microorganisms sensitive to chitosan are grouped into Gram-positive and Gram-negative bacteria, sensitive and resistant fungi (Palma-Guerrero et al., 2010).

Chitosan interacts with the cell surface of microorganisms, which leads to affectations in cell membrane permeabilization (Wang et al., 2015). This interaction is mainly electrostatic because of the presence of amino (NH$_3^+$) glucosamine groups and their capacity to interact with surface components with a negative charge and many microorganisms (lipopolysaccharides in Gram-negative bacteria, teichoic acid in Gram-positive bacteria and cell membrane phospholipids in fungi). These interactions cause extensive alterations in cell surface, which leads to integral cell wall and membrane loss, release of intracellular material and cell death (Ma et al., 2017) (Figure 2).

Chitosan also has antimicrobial activity by chelation of essential nutrients and metals (zinc [Zn], copper [Cu], cobalt [Co], manganese [Mn], nickel [Ni] and cadmium [Cd]) (Divya et al., 2017). Wang et al. (2004), demonstrated that when Zn ions were chelated, the positive charge strengthened in the chitosan amino group, increasing its capacity to interact with cell surface components of the microorganisms. Furthermore, chitosan (especially low-molecular-weight) causes damage in ribosomes and DNA; it penetrate the cell wall of microorganisms and bind to DNA, inhibiting DNA/RNA synthesis and protein translation (Schelegueda et al., 2016). Moreover, chitosan forms a layer in cell surface that avoids nutrient entrance (Liu et al., 2004).

Chitosan antimicrobial activity in cells

Source: Authors

Chitosan effect on phytopathogenic fungi

Chitosan is efficient in inhibiting spore germination, germinal tube and mycelium elongation of phytopathogenic fungi. Chitosan antifungal mechanism implies alteration and rupture of the cell wall that interferes directly on phytopathogen growth (Chun and Chandrasekaran, 2019). Permeabilization of the phytopathogen plasmatic membranes by chitosan depends on fluidity; the membranes of sensitive fungi to chitosan are rich in polyunsaturated fatty acids (PUFA) (linoleic acid), so they are very fluid (Palma-Guerrero et al., 2010) (Table 2).

Resistant fungi have low-fluidity membranes enriched with saturated fatty acids (SFA) (palmitic or stearic) (Palma-Guerrero et al., 2010). Lopez-Moya et al. (2015), demonstrated that chitosan permeabilized Neurospora crassa cell membrane, which triggered intracellular production of ROS and cell death; intracellular ROS production oxidizes PUFA of cell membranes, permeabilizing the plasmatic membrane and causing cell lysis.
### Table 2: Chitosan effects on phytopathogenic fungi

| Chitosan $^v$ | Mw (kDa) $^v$ | DD (%) $^v$ | Phytopathogenic fungi | Biological effect | Reference |
|---------------|---------------|-------------|------------------------|-------------------|-----------|
| 5 mg mL$^{-1}$ | 350           | 90          | *Penicillium expansum* | Alteration of plasma membrane; pleomorphic and anamorphic spores | Wang *et al.*, 2014 |
| 0.1%          | 100           | 93          | *Aspergillus ochraceus* | Wilting, abnormal branching, and vacuolation in hyphae | Meng *et al.*, 2020 |
| 5 mg mL$^{-1}$ | not specified | 85          | *Fusarium andiyazi*    | Membrane rupture and leakage of cellular components | Chun and Chandrasekaran, 2019 |
| 1.25 g L$^{-1}$ | 350           | 90          | *Alternaria tenuissima* | Damage to plasma membrane | Liu *et al.*, 2019 |
| 2.5 mg mL$^{-1}$ | 50            | 90          | *Botryosphaeria sp.*   | Mycelial growth inhibition | Wang *et al.*, 2017 |
| 0.32%         | not specified | 90          | *Colletotrichum capsici* | Mycelial growth inhibition and germination of conidia | Long *et al.*, 2018 |
| 8 mg mL$^{-1}$ | not specified | 88          | *Fusarium oxysporum f. sp. cubense* | Agglomeration of hyphae, abnormal forms, vesicles, or empty cells devoid of cytoplasm in the mycelium | Al-Hetar *et al.*, 2011 |

### Chitosan effect in plants

**Resistance inducer**

Plant resistance is activated by a great number of inductors biotic (fungi, bacteria, virus, phytoplasma and insects) and abiotic (chemical and physical) known as induced resistance (*Meena* *et al.*, 2020a). Two types of induced resistance are known -systemic acquired resistance (SAR) and induced systemic resistance (ISR)-mediated by phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (*Malerba* and *Cerana*, 2016). SAR depends on SA signal molecule and ISR depends on JA and ET (*Vlot* *et al.*, 2020).

The first line of recognition in plants toward phytopathogens is through pattern recognition receptors (PRR), which recognize microbial compounds, such as bacterial flagellum or fungal chitin - called by pathogen-associated molecular pattern (PAMP), microbe-associated molecular pattern (MAMP), or damage-associated molecular patterns (DAMPs) (*Mauch-Mani* *et al.*, 2017). Pattern recognition translates in first line of defense called activated immunity by PAMP or PAMP-triggered immunity (PTI) (*Bigeard* *et al.*, 2015). Chitosan behaves as a general elicitor, inducing resistance by a mediated PRR recognition (*Lopez-Moya* *et al.*, 2019). The defense responses caused by chitosan include an increase of cytosolic Ca$^{2+}$, callose deposition, oxidative explosion, hypersensitive response (HR), abscisic acid (ABA), ET, JA, SA, enzymes related with defense, phytoalexins, and PR protein (*Gai* *et al.*, 2019; *de Lamo* *et al.*, 2020; *Dubin* *et al.*, 2021) (Figure 3).

Chitin recognition by plants is associated to proteins (CEBiP/CERK), but chitosan lacks specific receptors (*Yin* *et al.*, 2016). Thus, chitosan is a molecular pattern associated to less efficient phytopathogens than chitin (*Lopez-Moya* *et al.*, 2019).
Proteins related with pathogenicity

Chitosan in plants induces protein production related with pathogenesis with antimicrobial activity to protect itself from phytopathogens (Liu et al., 2019). An example of these proteins related to pathogenesis are chitinase and β-1,3-glucanase (Hadwiger, 2013). The cell wall is responsible for cell physical integrity, and in the case of fungi, it is formed by chitin layers and β-1,3-glucan (Spadaro and Droby, 2016); β-1,3-glucan hydrolyzes β-D-glycoside bonds of β-1,3-glucan; chitinases hydrolyze β-1,4 bonds of N-acetyl-β-D-glucosamine obtained from chitin, breaking phytopathogen cell walls (Kaur et al., 2005).

Chitosan activates a subset of genes (PR-genes), which are called genes related to pathogenicity that cause disease resistance (Dubin et al., 2021). Chitosan increases transcription of these genes by activating cell or membrane receptor surface by the plant DNA interaction, which in turn influences on genetic transcription (Hadwiger, 2013). In general, chitosan direct interactions with DNA influence gene transcription related to pathogenicity and PR protein synthesis (Loschke et al., 1983).

Enzymes related with defense

The actions of enzymes related to plant defense, such as polyphenol, phenylalanine ammonia lyase, catalase, peroxidase and superoxide dismutase, increase with chitosan application, inducing plant resistance against phytopathogens (Gutiérrez-Martínez et al., 2017).

Polyphenol oxidase catalyzes phenolic substances to synthesize lignin, strengthen cell wall structure and avoid entrance and colonization of phytopathogens toward plants (Avdiushko et al., 1993). Moreover, catalyzes oxidation of phenolic compounds to quinone (antimicrobial compounds), which are toxic for phytopathogens (Soliva et al., 2000). Phenylalanine ammonia-lyase is important in phenylpropanoid pathway and catalyzes L-phenylalanine conversion into trans-cinnamic acid (Bhattacharyya and Ward, 1988).

Phenylalanine ammonia-lyase products are modified through phenylpropanoid metabolism to secondary metabolites (lignin, flavonoids, and phytoalexins), which are important in plant resistance against phytopathogens (Morrison and Buxton, 1993). Catalase is the main enzyme for eliminating hydrogen peroxide (H₂O₂) in microorganisms, implied in H₂O₂ in H₂O and O₂ degradation (Yang and Poovaiah, 2002). Peroxidase is an enzyme that catalyzes ROS oxidation, such as H₂O₂ that has antifungal activity facing diverse phytopathogens and participates in various physiological processes, such as lignification, suberization and auxin catabolism (Hiraga et al., 2001). Superoxide dismutase is responsible for eliminating ROS species to protect plants from oxidative stress during phytopathogen invasion (Lamb and Dixon, 1997).
**Phytoalexins**

Phytoalexins are low-molecular-weight compounds with antimicrobial properties, which are synthesized in low concentrations when plants are healthy but accumulate in great concentrations in response to a phytopathogen or an exogenous inductor (Keen and Bruegger, 1977). Phytoalexins are toxic and inhibit germ tube elongation and growth, decrease mycelial growth, and limit glucose absorption in fungi (Hammerschmidt, 1999). They are specific plants for each family with different chemical structures and synthesized by different enzymes, which make their mechanisms of action complex to study (Shamshina et al., 2020). Some phytoalexins synthesized by treated plants with chitosan are type formononetin, calicosine, phenylpropanoid and triterpenoid (Lucini et al., 2018; Gai et al., 2019).

**Lignin**

Lignin—jointly with cellulose and hemicellulose—contributes to hardening plant cell wall (Rajan et al., 2005). The lignification of cell walls is a mechanism of plant resistance to phytopathogens. Moreover, chitosan application forms a physical barrier in plants, which avoids phytopathogen entrance and colonization (Liu et al., 2019). Furthermore, PR protein accumulation and ROS stimulation as H$_2$O$_2$ by the effect of adding chitosan, induce the formation of phenolic compounds, such as phytoalexins that promote lignification (Chun and Chandrasekaran, 2019).

**Callose**

Callose deposition is a plant reaction to biotic and abiotic stress, such as lesions and infection caused by phytopathogens; it also isolates stress impact in the tissue locally by depositing a physical barrier (Farrokhi et al., 2006). Chitosan application promotes an increase of Ca$^{2+}$ concentrations (Zuppini et al., 2004) and induces callose deposition in plants (Luna et al., 2011). Callose synthesis is correlated with an increase in Ca$^{2+}$ net absorption by cells; Ca$^{2+}$ has access to the cytoplasm and act as a second messenger capable of directly activating β-1-3-glucan synthase located in the plasmatic membrane and dependent on Ca$^{2+}$, making callose deposition locally (Waldmann et al., 1988).

**Chitosan molecular recognition**

In Arabidopsis sp. kinase 1 (CERK1) chitin receptor has demonstrated to be fundamental in molecular pattern recognition associated to phytopathogens (Miya et al., 2007). The affinity of a specific protein (lectin) for glucosamine oligomers has been demonstrated by chitosan affinity chromatography, starting from Rubus fruticosus L. cultured cells. Lectine is a receptor of oligomers derived from chitosan with defense response inductor activity against phytopathogens (Liénart et al., 1991).

Chitosan binding proteins are determined in tobacco crop and Arabidopsis sp. plasmatic membrane (Yin et al., 2009; Yin et al., 2016). Tobacco protein is 75 kDa, similar to CERK1 chitin receptor. Nevertheless, the studies that have been performed are not sufficient to determine whether it is a receptor or not. Furthermore, Arabidopsis sp. protein is small (12 kDa), which suggests it is not a receptor (Yin et al., 2016).

Additionally, chitosan interaction has been demonstrated marked with fluorescence with wheat leaves and chitosan interaction with plasmatic membrane proteins, such as W5G2U8_WHEAT (a potential kinase receptor protein associated to the wall), W5HY42_WHEAT, and W510R4_WHEAT (serine/threonine-protein kinase similar to lectin receptor S type G) as potential chitosan receptors (Liu et al., 2018). Another point of view proposed that chitosan does not have plant specific receptors. Chitosan cationic properties have been established to allow plant plasma membrane binding.

However, another cation-oligomer material (poly-L-lysine) does not inhibit chitosan binding to colza membrane; thus, the bond does not depend only on cationic property (Yin et al., 2013). Chitosan structural complexity makes its understanding difficult, which is why chitosan receptor in plants has not been clarified yet (Li et al., 2020).
Chitosan effect on fruit protection against phytopathogens

Postharvest diseases are the main cause of fruit loss, which tend to have short shelf life (Ye et al., 2021). The adhesive nature of chitosan, its biodegradability, and antifungal activity make chitosan coating application an option to extend fruit shelf life (Wang et al., 2020a). Chitosan coating form a semipermeable film in fruit surface, minimizing respiration rate, decreasing water loss and weight, and extending fruit quality attributes effectively (Table 3) (Silva et al., 2018). Furthermore, chitosan coating helps to avoid phytopathogen colonization (Gutiérrez-Martínez et al., 2017).

| Chitosan\% | Mw (kDa)\$ | DD (%)£ | Fruit | Biological effect | Reference |
|-----------|------------|---------|-------|-------------------|-----------|
| 3%        | not specified | 98 | Guava | Breathing, fresh weight loss, firmness, color and antioxidant activity | Silva et al., 2018 |
| 1%        | 360 | 85 | Mango | Fruit ripening and weight loss | Jongsi et al., 2016 |
| 1%        | 17.4 | 75-85 | Soursop, mango, banana | Firmness and weight loss | Gutiérrez-Martínez et al., 2017 |
| 1.5%      | not specified | not specified | Strawberry | Fruit softening | Wang et al., 2020a |
| 1.9 mg mL$^{-1}$ | 50 | 90 | Pear | Fruit decomposition, defense enzymes, nutritional value, and weight loss | Wang et al., 2017 |
| 1%        | 360 | 85 | Mango | Fruit softening, accumulation of phenolic compounds and defense enzymes | Jongsi et al., 2017 |
| 1%        | not specified | not specified | Jujube | Fruit senescence, nutrient and antioxidant content | Kou et al., 2017 |

* = \$ Chitosan concentration, \$ Molecular weight, £ Degree of deacetylation

Chitosan nanoparticles in agriculture

Chitosan nanoparticles (CNP) are being used in agriculture to promote plant growth (Chun and Chandrasekaran, 2019). Their main effects against phytopathogens are related with antimicrobial activity (Sathiyabama and Parthasarathy, 2016; Varamin et al., 2020). Moreover, CNP treatment improves plant immune response, increasing the activity of enzymes related with PR protein defense, as well as raising total phenol levels (Chandra et al., 2015). Furthermore, CNP are used as nano-porters; nano-encapsulation increases bioavailability, solubility, and retention time of bioactive compounds (Muthukrishnan et al., 2019).

A recent study demonstrated that CNP inhibited Pyricularia grisea, A. solani, F. oxysporum growth in vitro, and chickpea seeds treated with CNP have a greater germination percentage, seed vigor index, and vegetative seedling biomass (Sathiyabama and Parthasarathy, 2016). Abdel-Aliem et al. (2019), demonstrated CNP antifungal effects against A. tenuis, Beauveria bassiana, F. graminearum, F. oxysporum, A. niger, A. flavus, Penicillium sp. and Sclerotium rolfsii. CNP also inhibited mycelial growth in C. gloeosporioides, Phytophthora capsici, S. sclerotiorum, F. oxysporum and Gibberella fujikuroi (Oh et al., 2019). Additionally, the use of copper nanoparticles coupled to chitosan inhibited Rhizoctonia solani and Pythium aphanidermatum causal agent of damping-off disease (Vanti et al., 2020).

CNP application charged with thiamine in chickpea seedlings improved germination index, growth and improved production of indole asctetic acid when compared with non-treated seedlings (Muthukrishnan et al.,
Salicylic acid nanoparticles and chitosan increased the production of defense antioxidant enzymes, improving ROS equilibrium, increasing lignin deposition in cell wall, improving growth and disease control in maize (Kumaraswamy et al., 2019). CNP in tomato crop induced PR protein expression (PR-1, PR-2, PR-8, and PR-10) and controlled wilting disease produced by *F. andiyazi* (Chun and Chandrasekaran, 2019).

**Conclusions**

From the ecological point of view, chitosan constitutes an option for agriculture since it does not pollute the environment and is not harmful for human health. Chitosan is important to control phytopathogens that colonize plants, generating a struggle between plant immunity and fungus virulence. Chitosan activates plant defense increasing callose deposition, production of enzymes related to defense, phytoalexins and proteins. Furthermore, chitosan application improves growth, development, and yield parameters in numerous crops. A great number of recently published articles testify the interest of scientists for the use of this biopolymer in agriculture, which lead to an extension of its use and benefits for the environment.

**Authors’ Contributions**

Conceptualization: LGHM, JJRP, JATR; Methodology: LGHM, JJRP, JATR; Project administration: LGHM; Validation: CA, TRC, EQA; Writing: LGHM, JJRP, JATR; Review and editing: LGHM, JJRP, CA, TRC. All authors read and approved the final manuscript.

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**Conflict of Interests**

The authors declare that there are no conflicts of interest related to this article.

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