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Review Article

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 inductor 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).

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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).

Table 1. Main sources of chitin

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 camembertii*	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 [H_2SO_4], acetic acid [CH_3COOH] and formic acid [CH_2O_2]) to eliminate mineral compounds (calcium carbonate [$CaCO_3$] and calcium phosphate [$Ca_3[PO_4]_2$]); 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 RNAm 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, ascetic, 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).

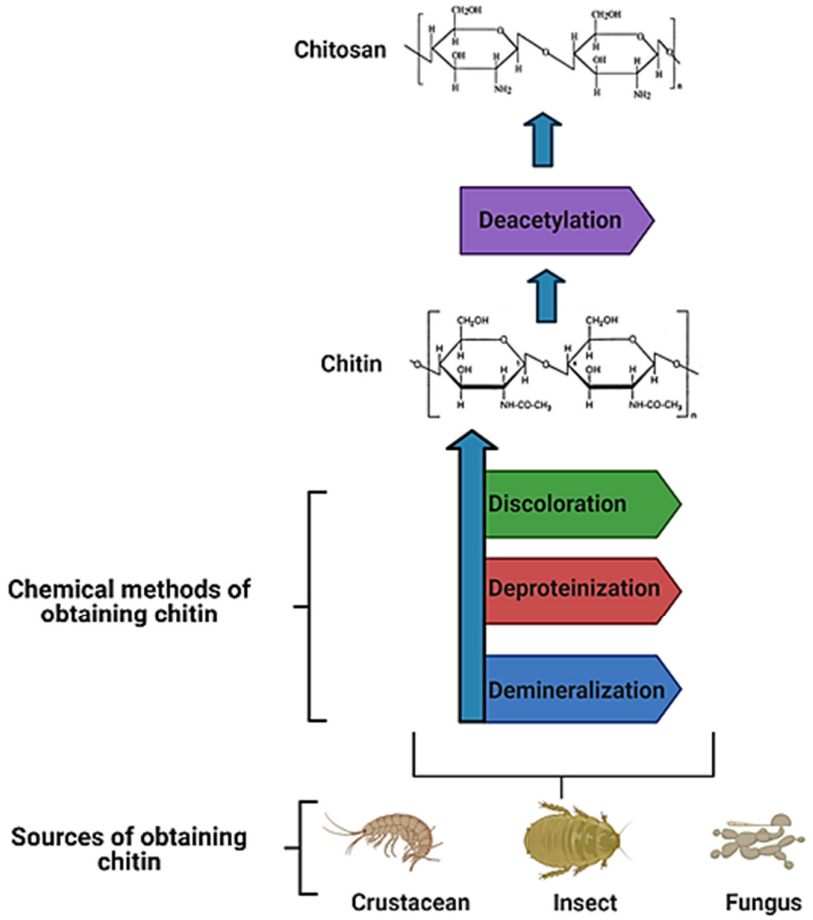

Figure 1. Chemical method of obtaining chitin
Source: Authors

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).

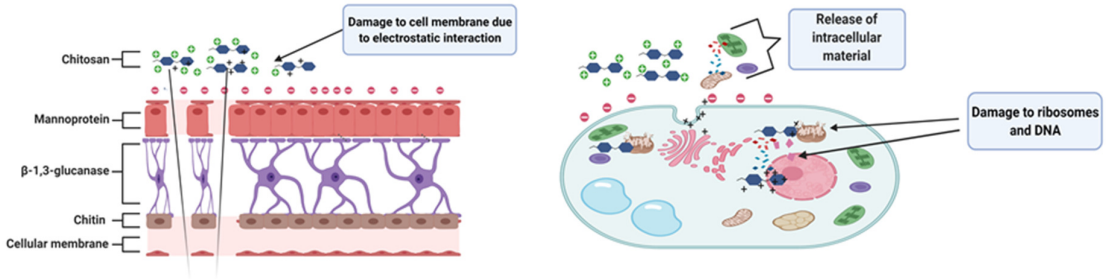

Figure 2. Chitosan antimicrobial activity in cells
Source: Authors

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[§*]	Mw (kDa)[¥]	DD (%) [£]	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 inductor

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).

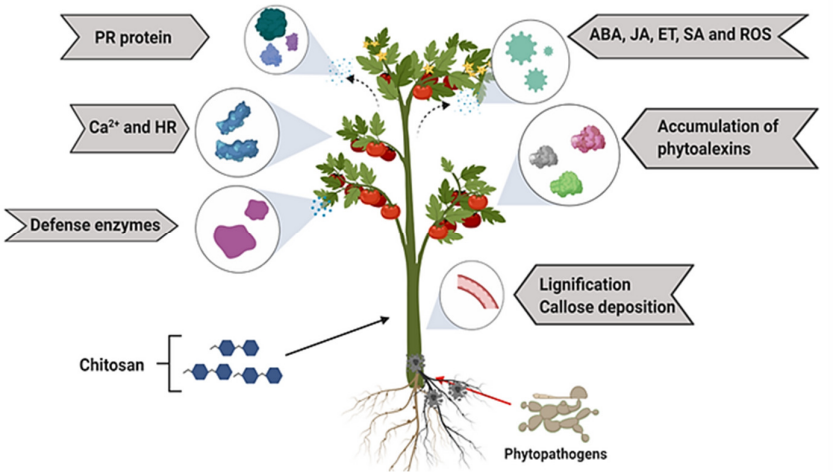

Figure 3. Defense responses induced by chitosan
Source: Authors

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 (H_2O_2) in microorganisms, implied in H_2O_2 in H_2O and O_2 degradation (Yang and Poovaiah, 2002). Peroxidase is an enzyme that catalyzes ROS oxidation, such as H_2O_2 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_2O_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 W5I0R4_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).

Table 3. Chitosan effects on fruits

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	Jongsri *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	Jongsri *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 inhited 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 ascetic acid when compared with non-treated seedlings (Muthukrishnan *et al.*,

2019). 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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