A Review on the Physicochemical and Biological Aspects of the Chitosan Antifungal Activity in Agricultural Applications

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Abstract: The antifungal activity of the chitosan biopolymer has been extensively studied for several decades. However, the mechanisms of action associated with this process have not been fully clarified yet. To a large extent, this situation is due to the lack of systematization with which, in general terms, the subject has been approached. However, it seems to have begun to change in recent years with the appearance of several papers reviewing the accumulated knowledge on the beneficial effects shown by chitosan in agricultural applications and putting forward it in a more systematic mode. In this work, the most relevant mechanisms of action proposed for chitosan regarding its antifungal activity will be briefly presented, i.e., disruption and changes in the fungal plasma membrane, alteration of gene expression, inhibition of RNA and protein synthesis, Ca^{2+} channel blocker, to then address the main factors that influence this antifungal activity, observed mainly in studies focused on phytopathogenic species, which have been grouped into three main blocks: those related exclusively to the chitosan molecules, those associated to the fungal itself and those having to do with the environment where the processes take place. Additionally, a brief section addressing some possibilities on which future studies on this topic should focus is also included.

Keywords: Action mode, Radial growth inhibition, Membrane disruption, Fungal chitosan, Development stage.

1. INTRODUCION

Research on natural products to obtain novel biomaterials for the control of pathogens is a very active area due to the benefits that its developments can bring in topics such as crop improvements, environmental protection, food preservation, human health, etc. Chitosan, a polysaccharide consisting of randomly linked glucosamine and N-acetyl-glucosamine monomeric units, whose average fractions in the polymers chains are f_A and $(1\text{-}f_A)$, respectively, with $f_A > 0,5$, is one of the most studied biomaterials for these purposes due to all the enormous benefits that have been attributed to it [1]. Despite the numerous studies that have been developed on the general chitosan and chitin antimicrobial activity, and on its antifungal activity in particular, the knowledge about the different mechanisms proposed to explain its action has been little systematized. However, recently there seem to be more interest in trying to arrange the accumulated knowledge on the beneficial effects shown by this biopolymer in agricultural applications [2-5], both in plant growth and in the control of diseases associated with different pathogens.

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This paper presents a panoramic view of the main factors influencing the antifungal activity of chitosan, and its interrelation, which in many cases are ignored and usually prevent comparative analysis of experiments carried out in different laboratories.

2. PROPOSED CHITOSAN ACTION MECHANISMS

The effectiveness of chitosan against many phytopathogenic fungi has been known for a long time [6-8], having been notorious from the initial studies that its effect was lower on fungi containing it in their plasmatic membrane [9]. However, the mechanisms of its fungicidal action have not been fully clarified to date. In this section, the most relevant mechanisms of action proposed for chitosan concerning its antifungal activity will be briefly discussed, to the address in the next section the main factors that influence this antifungal activity, observed mainly in studies focused on phytopathogenic species.

2.1. Disruption and Changes in the Plasma Membrane of Fungi

Morphological changes, alteration of the cell membrane and loss of cellular material in various phytopathogenic fungi, such as *Penicillium expansum* [10], *Fusarium oxysporum* [11], *Ustilago maydis* [12], when treated with chitosan, have been well documented. Additionally, inhibition on spore germination and mycelial growth are some of the

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effects of chitosan against certain phytopathogenic fungi [11].

Similarly to that observed in filamentous fungi used as study models, such as *Neurospora crassa*, it has been found that chitosan permeabilizes the plasma membrane in chitosan-sensitive fungi [13], a fact that does not seem to occur in not-sensitive fungi to chitosan. Thus, the characterization of the components of the plasma membrane, both resistant and sensitive fungi to chitosan, has allowed infer that sensitivity in the latter is conferred by the fluidity of the plasma membrane, which depends on the composition of the lipid fraction, specifically of a relatively higher content of polyunsaturated fatty acids, i.e., linolenic acid. In contrast, the plasma membrane in resistant-chitosan fungi, which has a higher content of saturated fatty acids such as palmitic and stearic acid, seems to act as barrier to chitosan molecules [14]. The interplay of the plasmatic membrane with chitosan, trough electrostatic interactions between the negative phospholipid head groups in the membranes and the positively charged protonated amine groups on the chitosan molecules, leads to its disruption, later affecting the cooperativity and anisotropy of their gel and liquid-crystalline phases.

2.2. Alteration of Gene Expression in Fungi

The induction of genes related to plasmatic membranes, response to stress and cell wall integrity has been reported when the model organism *Saccharomyces cerevisiae* was exposed to chitosan [15]. This chitosan treatment also resulted in increased resistance of cells to β-1,3-glucanase, which is characteristic of cell wall-stressed cells and indicative of activation of the cell wall integrity pathway. Additionally, a very interesting study has shown that activation of specific genes in the fungus is an even more complex process in the presence of the host, the latter also experiencing changes in its gene expression due to the chitosan/pathogen/host interaction [16]. This situation should lead to rethink the usual correlations made on the protection that chitosan provides to a crop against a specific pathogen without considering these interactions.

2.3. Inhibition of RNA and Protein Synthesis

From the earliest studies it has been proposed that chitosan can penetrate fungal cells and then inhibit or slow down the synthesis of messenger RNA and proteins, additionally to activate defensive genes in plants [7]. More recently, proteomic changes in *Penicillium expansum* after chitosan treatment were observed for 26 proteins, which were identified and categorized according to their putative biological function; proteins related to DNA or protein biosynthesis, carbohydrate metabolism and energy production were decreased in relative protein abundance, while proteins involved in antibiotics resistance, and antioxidant defense response increased in relative protein abundance [10].

2.4. Chitosan as Ca^{2+} Channel Blocker

A recent study has found that antifungal activity of chitosan against *Penicillium italicum*, one of the pathogens responsible for the blue mold infection on citrus, is dependent on molecular weight and concentration of chitosan; furthermore, a specific fraction of chitosan oligomers was found to be more effective in inhibiting fungal growth than starting chitosan of high molecular weight [17]. The antifungal effect of the identified fraction become reduced in response to exogenously added Ca^{2+}, suggesting that it acts via disruption of a Ca^{2+} gradient and further may serve as Ca^{2+} channel blocker.

In the other hand, it has been recently proposed that both pH and Ca^{+2} ions are critically important to the viability of *Penicilium italicum* and it may probably develop calcium ion channels to protect itself from the pH decreasing [18].

3. FACTORS INFLUENCING THE CHITOSAN ANTI-FUNGAL ACTIVITY

In general, factors affecting the fungicidal activity of chitosan can be grouped into three main blocks: those related exclusively to the chitosan molecules, those associated with the fungal species and those having to do with the environment where the processes take place. Figure **1** put forward a more detailed view of these factors.

3.1. Origin

The origin of the chitin used in the production of chitosan is an important factor to consider because the production treatments will depend on it, which in turn will affect the properties of the chitosan to be obtained. Thus, β-chitin –which is obtained from squid pens and whose fibrils are formed by chains oriented in parallel is easier to hydrolyze than α-chitin (fibrils with chains in anti-parallel orientation) from others sources, i.e., crustacean shells and fungi. In this sense, it is

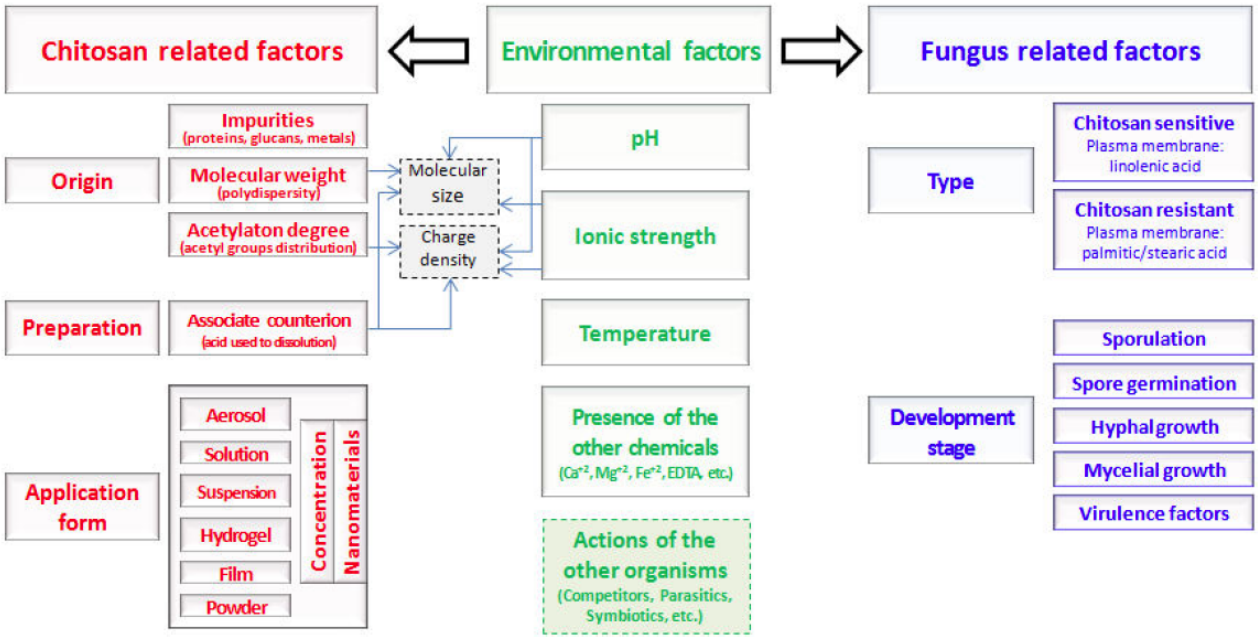

Figure 1: A panoramic view of the main factors influencing the chitosan antifungal activity.

important to know, at least approximately, the mineral and protein content of the starting material to apply a specific treatment. An illustrative work of the substantial differences that can arise when are used different marine sources of chitin (shrimp residues, crab shells and cuttlefish bones) shows that the molecular weights and acetylation degrees obtained are quite different, leading to noticeable differences in their antifungal activity [19].

The main production source of chitosan nowadays is the chitin extracted from crustaceans. However, recently chitin and chitosan obtained from fungal resources have attracted the attention of scientific researchers and industrial producers due to the best benefits assumed for these materials in the care of the environment and human health [20]. Thus, fungal chitosan could be advised in the drug delivery field as a carrier with similar benefits to those of crustacean chitosan but less allergenic; however, it has been pointed out that when the purification processes of crustacean chitosan have been correctly conducted, the differences between these materials should only be those derived from their physicochemical properties [21].

The fundamental characteristics differentiating the crustacean chitin from the fungal one surge of the type of chemical structures which they are associated in their natural starting materials. Crustacean chitin normally posses minimal residual protein and binds with sclerotized proteins and minerals, whereas fungal chitin is associated with other polysaccharides, such as glucan, which can even occur in quantities exceeding the chitin content [22]. Additionally, fungal chitin should not contain any heavy metals like nickel and copper; furthermore, high molecular weight chitosan is usually obtained from crustaceans, i.e., $1.5x10^6$ Da, while medium molecular weight values ($1–12x10^4$ Da) are obtained from fungal mycelia [23].

Although there are still not many works comparing the antimicrobial properties of both types of chitosan, there is already evidence that material obtained from fungi seems to be slightly superior to inhibit the growth of fungi and bacteria, showing be more effective when it was applied as nanoparticles [23]; however, this work is not conclusive in this respect because the molecular weights of tested materials were not reported.

In this context, it is also convenient to indicate that there are two ways of synthesizing chitosan: (a) by deacetylation of chitin, a process that usually takes place in heterogeneous conditions, which can be conducted by chemical [24] and biological [25] methods, including ultrasound-assisted chemical treatments of fungal [26] and crustacean chitin [27], and (b) by reacetylation in a homogeneous phase of highly deacetylated chitosan samples, which gives rise to chitosans with properties different from chitosans with similar degrees of acetylation obtained by deacetylation of chitin, presumably due to a random distribution of the acetylated groups introduced under homogeneous conditions [28]. The chitin deacetylation

process lead to irregular distributions of the N-acetyl-D-glucosamine and D-glucosamine residues, with possibility of formation of some acetyl blockwise groups along the polymeric chains, whereby its solubility and degree of aggregation could have a variable behavior, like as its biological activities, even for samples having the same average DA. The physicochemical properties of these chitosans appear different from those obtained under homogeneous conditions (randomly acetylated) but, until now, comparative studies on their anti-microbial activities have not been reported.

3.2. Effects of the Molecular Weight

Diverse studies have proven that antifungal activity of chitosan is dependent on its molecular weight for some phytopathogens but not others, as it has been observed during studies with *Fusarium oxisporum* and *Aspergillus niger*, respectively [29]. Usually, the chitosan and derivatives of chitosan oligomers show a higher antifungal activity [30]. Studies carried out on the phytopathogen *Botrytis cinerea* Pers demonstrated a greater inhibitory effect on the growth of this fungus in the presence of samples of high molecular weight of quaternized chitosan, compared to those with a low molecular weight [31].

Furthermore, an interesting and methodic study carried out by Oliveira *et al*. [32] demonstrated that antifungal activity of chitosan samples with a low acetylation degree increases when its molecular weight decreases; however, the authors also report that for other degrees of acetylation values the trend is ambiguous and even reverses for the higher values studied. Thus, these authors have proposed the possibility that the molecular weight dependency of the chitosan inhibitory activity changes with the acetylation degree; thereby, while slightly acetylated chitosans exhibit their inhibitory activity best as small to medium-sized polymers, the inhibitory activity of highly acetylated chitosans may be highest at very large polymer sizes.

On the other hand, it is important also consider that usually chitosan samples have different molecular size distributions, whereby the effectiveness of a determined chitosan sample will be defined by its greater content of either smaller species with high acetylation degree or larger species with low acetylation degree. In such respect, more research using samples with narrower molecular weight distributions is required; however, these are not always easy to prepare because, additionally, the samples should have similar degrees of acetylation for an effective comparison.

3.3. The Acetylation Degree Effects

As it has been previously mentioned, the molecular weight and the acetylation degree of chitosan appear to be closely related concerning the observed effects on its antimicrobial activity. This relationship has not securely been fully established due to the methodical lacks in the work that had been carried out, a difficult situation to resolve due to the difficulties in obtaining chitosan samples with different molecular weights and acetylation degrees, under the same preparation scheme and from the same origin. A phytopathogen for which the influence of the chitosan acetylation degree on its *in vitro* antifungal effect has been demonstrated is *Alternaria solani*, with the chitosan activity decreasing when its acetylation degree increases and its molecular weight is maintained around 70-85 kDa [23].

3.4. Associated Counter Ions in the Salt Form

In aqueous environments the chitosan-associated counter ions can shield its positive charges to a variable extent, among other reasons due to steric effects [33], which in turn would alter the strength of the chitosan electrostatic interaction with the negatively charged phospholipids components in the fungal membranes. A study on the activity of chitosan dissolved in different acids on *Escherichia coli* confirmed that chitosan solutions prepared with small organic acids, i.e., formic acid and acetic acid, had better bactericidal properties than those resulting from solutions with propionic acid. [34]. These results seem to indicate that chitosan associated with anions causing a minor steric shielding, which makes its positive charges more expose, has greater antibacterial effectiveness; however, others factors such as the chitosan solubility (1.36, 1.12 and 0.96 g/l, respectively) and the pK_a values (3,75, 4,76 and 4,86, respectively) have been also be considered. Although no similar reports have been found about the antifungal activity of chitosan, similar behavior cannot be ruled out.

3.5. Chitosan Concentration

The inhibition of fungal growth by chitosan has also been reported as chitosan concentration-dependent for a wide variety of phytopathogens such as *Fusarium oxysporum* [35], *Bipolaris oryzae* [36], *Alternaria alternate and Colletotrichum gloeosporioides* [37].

Moreover, in some cases a linear relationship is observed between the chitosan-logarithmic concentration and the antifungal activity [35]. Notwithstanding, it is important to be alert because some systems have shown a maximum antifungal activity followed by a gradual fungal growth as the polymer concentration increases [38]. Some authors have proposed that at higher chitosan concentrations, fungal enzymes can degrade it to glucosamine units [39], which could be used as nutrients by the fungus, accelerating its growth. Thus, as it can be appreciated, the chitosan antifungal performance is tightly related to the application of an appropriate rate.

3.6. Application Form

One of the greatest advantages of chitosan as an antifungal agent is its versatility in terms of application. The most common forms of use are solutions and films for wrapping agricultural products and prepared foods. However, other routes have also been tried successfully. Thus, it has been used in aerosol form to treat the purple blotch pathogen (*Alternaria porri*) of onion; *in vitro* results showed that as the concentration of chitosan increased from 0.2 to 2.4%, the inhibition of spore germination was increased from 51.69 to 79.18% while during the *in vivo* assays with chitosan 0.4% (sprayed 30, 45 and 60 days after transplanting) the disease intensity was minimum (7.81 percent disease index) as compared to untreated control treatment (water spray) as 37.25 percent disease index [40].

On the other hand, when chitosan was applied in hydrogel form, as a protective coating on freshly cut cherry tomatoes, it appeared to be very effective in forming stable films and preventing fungal infestation, showing an antifungal activity surprisingly greater than its derivative *N,N,N*-trimethylchitosan [41]; probably chitosan has better filmogenic ability because it can form a greater number of hydrogen bonds during the hydrogel-drying process.

The versatility of chitosan to be used as antifungal by different application routes has been substantially increased by the ease that it also offers for the preparation of nanomaterials, such as films loaded with nanoparticles, nanoparticle suspensions that can be sprayed, nanoparticles-containing hydrogels, etc. It is also possible to prepare nanoparticles loaded with metals such as silver [42,43], or nanocomposites with metal oxides [44], which have shown superior antimicrobial properties. Thus, the immobilization of nano ZnO on a chitosan matrix through a simple and facile *in situ* method allowed obtaining nanocomposites with excellent antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* [44], which should encourage testing these materials as antifungal.

3.7. Aspects Related to Fungal Species

3.7.1 Type of Fungal

Comparing the chitosan antifungal effects on different types of fungi is difficult to carry out, especially when the work has been carried out in different laboratories because the multiple factors that must be considered, i.e., the molecular weights (including polydispersity and the associated counter ion) and the degree of acetylation (including the distribution of the acetylated groups along the chain, a factor rarely considered in most studies) of the chitosans used, the sequence with which the samples are applied during *in vitro* studies, etc. A study that allows an adequate comparison of the effect of chitosan on three different phytopathogenic fungi is the work published by Ziani *et al*. [45], in which the same chitosan samples were applied, under identical conditions, to study its inhibitory effect on radial growth; thus, when a 3% solution of low molecular weight chitosan was used, the phytopathogens *Alternaria alternata*, *Rhizopus oryzae* and *Aspergillus niger* showed different sensitivities to the same treatment, i.e., radial growth inhibition of 100%, ~62% and ~45%, respectively. These results will surely be associated with the composition of the cell wall of each fungal species, and especially with the organization of the different layers forming these in each particular species.

3.7.2. Fungal Development Stages

The development of most fungi includes several stages that generate dramatic changes in the cell shape, which are related to the formation, reformation and repairing of their cell walls. Thus, the molecular architecture of the cell wall is not fixed. The cell can make considerable adjustments to the composition and structure of its wall, for example, during the cell cycle or in response to environmental conditions (such as nutrient and oxygen availability), temperature, and pH. When the cell wall is defective, dramatic changes can occur in its molecular architecture, pointing to the existence of cell wall repair mechanisms that compensate for cell damage [46]. In connection with the above, various studies have reported that chitosan sensitivity of fungal pathogens varies with its development stage. In this regard, Liu *et al*. found that chitosan had a better inhibitory effect on the

germinability of *Penicillium expansum* spores than those of *Botrytis cinerea*, while the opposite effect was observed for its mycelial growth [47]. These responses turned out similar to those shown by other phytopathogens treated with the chemical fungicide fluazinam, for which the germinating spores of *Botryosphaeria parva* results more sensitive as compared to that of *Colletotrichum gloeosporioides*, while a contrary effect was observed for the mycelial growth inhibition [48].

3.8. Environmental Aspects

3.8.1. pH Effect

The pK_a value of chitosan is dependent on both, its molecular weight and its acetylation degree, as it has been demonstrated by Wang *et al*., whose found that pK_a value changed of 6.39 to 6.51 when Mw changed from 1370 to 60 kDa and it increased from 6.17 to 6.51 for a variation from 5.4 to 26.7% in the acetylation degree [49]. Thus, it can be considered that for lower pH values than 6.17, the majority of the glucosamine residues in chitosan molecules will carry a cationic charge due to protonation of its amino groups, which enables them to interact with anionic components of the cell surface such as proteins, anionic polysaccharides, fatty acids, bile acids and phospholipids. Furthermore, it is also important to consider that positively charged groups will have greater accessibility because the repulsion between them generates polymer chains more extended. On the other hand, chitosan begins to lose its charges from pH values > 6.51, which induce its precipitation from its aqueous solutions due to deprotonation of the amino groups. Because of these particularities, it would be expected that aqueous solutions of chitosan show a greater antifungal activity as the pH becomes more acidic, which has been observed during *in vitro* studies of *Mucor racemosus* using chitosan glutamate (2 g/l), where the growth rate underwent a reduction of around 75% and 53% at pH values of 4.5 and 5.2, respectively [50].

3.8.2. Ionic Strength

The shielding of the positive charges in the chitosan polymer chains can also occur due to the presence of external ions (sometimes added to the medium to control the ionic strength), which modify the magnitude of the interactions between them and affecting, among others, the expansion of the polymer chains [51]. Nonetheless, it should also be considered that chitosan molecules have a greater solubility at higher ionic strength values, compensating for the greater flexibility they acquire under these conditions; a higher chitosan bactericidal activity observed against *Escherichia coli* and *Staphylococcus aureus* for high ionic strength values seems to demonstrate it [34]. Probably, similar behavior can be observed if chitosan is applied to fungal control in an aqueous medium with high ionic strength values.

3.8.3. Temperature

Like as for whatever biochemical process, temperature is an important parameter to consider respect the antifungal activity of chitosan. Temperature can have important effects both on the physicochemical stability of aqueous chitosan solutions and on the susceptibility of a determined type of fungus. Thus, it is well known that chitosan solutions maintained at higher temperatures show greater alterations in their properties, i.e., the diminution of its viscosity over time as a consequence of acid hydrolysis reactions which cut the polymer chains diminishing its molecular weight [52]. Also, simultaneously with chain scission other chemical reactions can occur such as cleavage and/or destruction of its functional groups and formation of free radicals which induce oxidation processes [53]. Therefore, the temperatures and sterilization times of chitosan solutions during *in vitro* inhibition studies must be carefully considered. On the other hand, greater stability has been reported for aqueous chitosan solutions when these are maintained at 5 °C [54].

Regarding the temperature effects on the fungal susceptibility, it could be expected a behavior similar to that observed in bacteria, i.e., the susceptibility of *Escherichia coli* to chitosan increased upon increasing temperature from 4 to 37 °C, suggesting the low-temperature stress was capable of changing the cell surface structure in a way that decreased the number of surface binding sites for chitosan [55]. Surprisingly, one of the scarce related studies reported that affectation of the growth rate of *Mucor racemosus* by chitosan glutamate was greater at 18 °C (25%) than at 25 °C (20%) [50].

3.8.4. Others Factors

The presence of others chemical substances such as metal salts can selectively to affect the antifungal activity of the chitosan as Kim *et al*. have demonstrated by evidencing that the antifungal activity of chitosan oligomers was significantly decreased after addition of Ca^{+2} ions while it was little affected by Mg^{+2}, Mn^{+2} and

Zn^{+2} ones (all of them as chloride salts) [56]. Furthermore, a notorious synergistic effect against *Penicillium italicum* has been observed when chitosan oligomers were combined with a Zinc-EDTA chelate, an unexpected result because the chetate alone did not show any antifungal activity [18].

On the other hand, the presence of other live organisms (competitor, symbiotic, parasitic, etc.) could also influence the chitosan antifungal activity [57], although to date there is little documentation in this regard.

According to what is glimpsed in the previous part, the presence others chemical substances and/or other live organisms are very important factors to take into account regarding to the antifungal chitosan activity, especially during *in vivo* and greenhouse studies.

4. FUTURE TRENDS

The need to reduce the negative impact of conventional antifungal treatments based on synthetic chemicals on the environment and human health will continue to be the driving force of the new developments to come, which could to be accelerated by a greater awareness of society and by the hardening of the laws regulating these issues. In this context, chitosan and its derivatives will continue to be materials offering multiple opportunities for the control of pathogenic fungi, either due to the obtaining of new methods to refine their physicochemical properties bearing a greater specificity of its biological activities or through the preparation of new materials with enhanced antifungal activities based on them.

Because marked effects of different chitosan samples have been observed in many cases, such as the higher antifungal activity shown by chitooligomers with higher degrees of acetylation [32], news methods for the meticulous preparation of this type of materials could yield megascopic results. In this sense, the picture is as broad as can be inferred from the approach to the chitooligomers release from chitosan nanosystems [4]. Antifungal treatments with a combination of different well-defined fractions are also a promising possibility, i.e., high acetylation degree chitooligomers in combination with high molecular weight chitosan of low acetylation degree.

On the other hand, research into new derivatives of chitosan resulting in materials more soluble in aqueous neutral or alkaline media (pH > 6.2) and with a higher density of positive charges, can provide materials with better antifungal properties [58,59].

The enormous development of nanotechnology has opened new frontiers for chitosan and derivatives based systems, introducing them into the fields of already successful nanobiotechnology and emerging agro-nanotechnology [60], with excellent perspectives for the development of agro-nanosensors [61,62], nano-fungicides [57], detection and control of the plant diseases [63], among others. Nevertheless, as for other related areas, it is necessary to streamline studies of the long-term effects of nanosystems on human health and environment. Education of user communities, creation of national regulations and its communication to citizens, among other aspects, is a practically virgin field to work.

5. CONCLUDING REMARKS

Despite more than 40 years have passed since its first steps as an antifungal agent, chitosan has not lost its relevance in this field. On the contrary, this material has become increasingly current because the development of its multiple forms of application seems to run parallel to the development of nano- and biotechnologies, such as the development of nanosensors, nanogels, films based on nanohybrid materials, etc., potentially useful in agriculture. Therefore, due to all their peculiarities, the list of challenges having recently been undertaken with these materials, or can be undertaken quickly, seems to grow more and more every day. In this sense, it could be affirmed, without fear of error, that researching on antimicrobial applications of chitosan will continue to be a highly active sector for the next years, especially concerning its antifungal activity, even beyond its applications in agriculture. Thus, the production of new chitosan-based materials with improved antifungal activities should consider, in addition to the type of fungus and its development stage, the main combinations of its physicochemical properties that favour these, i.e., oligomers with narrow distributions and high degrees of deacetylation, samples with high molecular weights and low degrees of deacetylation, chitosan derivatives with higher positive charge densities, use of counterions with a low shielding effect on their cationic charges, etc.

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