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Antifungal Activity of Chitosan Oligomers–Amino Acid Conjugate Complexes against *Fusarium culmorum* in Spelt (*Triticum spelta* L.)

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Abstract: Fusarium head blight (FHB) is a complex disease of cereals caused by *Fusarium* species, which causes severe damages in terms of yield quality and quantity worldwide, and which produces mycotoxin contamination, posing a serious threat to public health. In the study presented herein, the antifungal activity against *Fusarium culmorum* of chitosan oligomers (COS)–amino acid conjugate complexes was investigated both in vitro and in vivo. The amino acids assayed were cysteine, glycine, proline and tyrosine. In vitro tests showed an enhancement of mycelial growth inhibition, with EC_{50} and EC_{90} effective concentration values ranging from 320 to 948 $\mu g \cdot mL^{-1}$ and from 1107 to 1407 $\mu g \cdot mL^{-1}$ respectively, for the conjugate complexes, as a result of the synergistic behavior between COS and the amino acids, tentatively ascribed to enhanced cell membrane damage originating from lipid peroxidation. Tests on colonies showed a maximum percentage reduction in the number of colonies at 1500 $\mu g \cdot mL^{-1}$ concentration, while grain tests were found to inhibit fungal growth, reducing deoxynivalenol content by 89%. The formulation that showed the best performance, i.e., the conjugate complex based on COS and tyrosine, was further investigated in a small-scale field trial with artificially inoculated spelt (*Triticum spelta* L.), and as a seed treatment to inhibit fungal growth in spelt seedlings. The field experiment showed that the chosen formulation induced a decrease in disease severity, with a control efficacy of 83.5%, while the seed tests showed that the treatment did not affect the percentage of germination and resulted in a lower incidence of root rot caused by the pathogen, albeit with a lower control efficacy (50%). Consequently, the reported conjugate complexes hold enough promise for crop protection applications to deserve further examination in larger field trials, with other *Fusarium* spp. pathogens and/or *Triticum* species.

Keywords: amino acids; cereal; chitosan oligomers; FHB; fungicide; synergism; wheat

1. Introduction

Fusarium head blight (FHB) is a devastating fungal disease that affects wheat and other small-grain cereals worldwide, caused by several species belonging to the genus *Fusarium*. Besides causing significant yield losses and reducing grain quality [1], these species are also able to biosynthesize mycotoxins harmful to both humans and animals [2,3]. *F. culmorum* (W. G. Smith) Sacc. and *F. graminearum* Schwabe are generally considered the two most important FHB causal agents worldwide [4], but *F. poae*, *F. asiaticum*, *F. boothii*, *F. vorosii* and *F. cortaderiae* also pose a serious threat [5].

Depending on their chemotype, infection by *F. culmorum* and *F. graminearum* can result in cereal grain contamination with different Type B trichothecenes: nivalenol (NIV) and its acetylated derivatives, deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON), or DON and 15-acetyldeoxynivalenol (15-ADON) [6,7]. Nonetheless, some recently identified *F. graminearum* strains produce Type A trichothecenes (NX-2 and NX-3), similar in structure to DON and 3-ADON, but differing in the presence of a ketone at C_8 [8].

At present, no complete FHB-resistant wheat varieties are commercially available, so, in order to control the disease and limit mycotoxin contamination, the use of less susceptible genotypes in combination with the application of fungicides is used worldwide. However, the extensive use of tebuconazole, metconazole, prothioconazole, prochloraz and other commonly used fungicides of wheat is exerting a selective pressure and is influencing population dynamics of *Fusarium* species [5]. Moreover, the European legal framework (Article 14 in European Directive 2009/128/EC) enforces their use in a sustainable way, but low concentrations of the aforementioned fungicides may cause an incomplete reduction of fungal development [9]. Hence, alternative control strategies have become the subject of intense research, including biocontrol agents [10], the selection of resistant cultivars [11,12], or agronomic practices (e.g., influence of tillage and cover crop [13]).

Another approach to address this challenge involves the replacement of conventional antifungals with novel preparations from natural products. This latter option would be favored by the recent regulatory change at a European level, given that new preparations based on natural products are contemplated in Product Function Category (PFC) 6 within the Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019, which requalifies 'biostimulants' as fertilizers (and not as phytosanitary products). It should also be taken into consideration that, according to Regulation 834/2007 on organic production and labeling, as well as Regulation 889/2008, which establishes provisions for the application of Regulation 834/2007, and its subsequent modifications, products and by-products of plant origin are considered as substances suitable for application in Organic Farming. For example, chitosan, common horsetail (*Equisetum arvense* L.) and common nettle (*Urtica dioica* L.) vegetable extracts, rich in polyphenolic compounds, are widely used as agricultural biostimulant products.

As a response to this regulatory change, investigations on the efficacy against FHB of aromatic carboxylic acids present in barley-root exudates [14], commercial essential oils (garlic, grapefruit, lemon grass, tea tree, thyme, verbena, cajeput and *Litsea cubeba* Pers.) [15], essential oils from lemon-scented gum (*Corymbia citriodora* (Hook.) K.D. Hill and L.A.S. Johnson) [16], essential oils of Moroccan wormseed (*Dysphania ambrosioides* (L.) Mosyakin and Clemants) [17], walnut (*Juglans regia* L.) green husk extracts [18], extracts from buckwheat grain and hulls [19], phenolic-rich bee products (propolis, bread and pollen) [19], or polyphenol inclusion compounds and conjugate complexes [20,21], among others, have been recently reported.

In the work presented herein, novel formulations based on chitosan (which has been previously assayed against *Fusarium* spp. [22–25], and which has been put forward as a sustainable alternative in crop protection [26]) in combination with amino acids have been tested against *F. culmorum*, both in vitro and in vivo, with a view to assessing if an enhanced behavior resulting from synergies between these natural products can be attained.

2. Materials and Methods

2.1. Reagents and Fungal Isolates

High molecular weight chitosan (CAS No. 9012-76-4; 310,000–375,000 Da) was purchased from Hangzhou Simit Chemical Technology Co., Ltd. (Hangzhou, China). Citric acid (CAS 77-92-9; 99.5%) and Tween® 20 (CAS 9005-64-5) were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Neutrase® 0.8 L enzyme was supplied by Novozymes (Bagsvaerd, Denmark). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Becton, Dickinson and Company

(Franklin Lakes, NJ, USA). Cysteine (Cys, CAS No. 52-90-4), glycine (Gly, CAS No. 56-40-6), proline (Pro, CAS No. 147-75-3) and tyrosine (Tyr, CAS No. 60-8-4), all with 99% purity, were purchased from Panreac S.L.U (Barcelona, Spain).

The *Fusarium culmorum* strain used in the present study (CECT 20493) was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain). The chemotype was 3-ADON [7].

2.2. Preparation of Chitosan Oligomers and Bioactive Solutions

Chitosan oligomers (COS) were prepared following the procedure described by Buzón-Durán et al. [27]. The amino acid-only bioactive solutions were prepared by dissolving the amino acids in distilled water, without further purification, at an initial concentration of 3000 $\mu g \cdot mL^{-1}$. The COS–amino acid conjugate complexes (viz., COS–cysteine, COS–glycine, COS–proline and COS–tyrosine) were prepared by mixing in a 1:1 (v/v) ratio of the respective solutions (COS solution at 1500 $\mu g \cdot mL^{-1}$ + amino acid solution at 1500 $\mu g \cdot mL^{-1}$), followed by sonication with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) for 15 min in five 3-min periods, controlling the temperature so as to keep it below 60 °C.

2.3. In Vitro Tests of Mycelial Growth Inhibition

The biological activity of the treatments was determined using the agar dilution method (or "poisoned food method") [28], incorporating aliquots of stock solutions into the PDA medium to provide final concentrations of 62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000 and 1500 $\mu g \cdot mL^{-1}$, i.e., the usual ones defined in the Clinical & Laboratory Standards Institute (CLSI) or in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard antifungal susceptibility testing procedures. Mycelial disks of *F. culmorum* (5 mm in diameter) from the edges of a 7-day-old culture were transferred to plates filled with these media (in triplicate), using plates containing only the PDA medium as the control.

Radial mycelial growth was determined by calculation of the average of two perpendicular colony diameters for each replicate. Mycelial growth inhibition for each treatment and concentration after 7 days of incubation, at 25 °C in the dark, was calculated according to the formula:

$$((d_c - d_t)/d_c) \times 100 \quad (1)$$

where d_c is the average diameter of fungal colony in the control and d_t is the average diameter of the fungal colony treated with the tested composite.

Results were also expressed as half maximal and 90% maximal effective concentrations (EC_{50} and EC_{90}, respectively), estimated by regressing the radial growth inhibition values (%) against the $\log_{10}$ values of the treatment concentrations.

2.4. Preparation of Inoculum

Conidial suspensions were obtained following the procedure described by Khan et al. [29], with minor modifications. *F. culmorum* conidia were harvested from 7-day-old PDB cultures (200 mL cultures incubated in the dark at 25 °C, 140 rpm in an ECOLAN 60 (Labolan; Esparza de Galar, Navarra, Spain) orbital stirrer incubator). The suspension obtained was then filtered through two layers of sterile muslin to remove hyphal fragments. Spore concentration was determined using a hemocytometer (Weber Scientific International Ltd., Teddington, Middlesex, UK), and was adjusted to a final concentration of 5×10^4, 1×10^5 or 1×10^6 spores (conidia) mL^{-1}, with 0.2% Tween® 20, depending on the experiment to be conducted afterwards.

2.5. Inhibition of Colonies Formation

In vitro tests aimed at determining the inhibition rate percentage of the number of colonies formed were carried out according to the procedure reported by Kheiri et al. [30]. 0.5 mL of the

conidial suspension (5×10^4 conidia·mL^{-1}) was mixed with different concentrations (500, 1000 and 1500 μg·mL^{-1}) of the solution of COS–tyrosine conjugate complex (for which the lowest EC_{90} value was obtained, as discussed below) to a final volume of 2 mL. Conidial suspension was also prepared with distilled water and 0.5% v/v acetic acid aqueous solution as the control. The resulting solutions were incubated in the dark at 25 °C for 24 h. Aliquots of 50 μL of each dilution were spread on PDA and incubated at 25 °C, counting the number of colonies formed after 5 days. Each treatment was replicated 3 times. The percent inhibition rate was estimated as: % Inhibition rate = (number of colonies formed in control plate − number of colonies formed in treated plates)/number of colonies formed in control plate × 100.

2.6. Effect of Conjugate Complexes on Mycotoxin Production and Mycotoxin Chemical Analysis

The effect of the conjugate complex with the best performance on the growth of *Fusarium* fungi on wheat grain was investigated using the method described by Perczak et al. [31]. 5 mL of the COS–tyrosine solution at a concentration of 1500 μg·mL^{-1} was mixed with 25 g of sterile spelt grain in an Erlenmeyer flask. The mixture was vigorously stirred, and three rings (6 mm) of solid culture of *F. culmorum* were then added to each Erlenmeyer flask and mixed. Solutions of Tween® 20 and deionized water were added to the control sample, without the addition of bioactive compound. Next, the prepared mixtures were stored in the dark at 25 °C for 28 days. After incubation, samples were dried, milled, homogenized and prepared for chromatographic analysis.

The organic extracts were obtained by soaking of the samples in a mixture of water, methanol and acetonitrile in a 10:10:30 v/v ratio, followed by sonication for 15 min in five 3-min periods. The supernatant solution was filtered with Whatman nº 4 paper and stored at 4 °C until the analytical determinations were carried out. The determination of mycotoxins was carried out according to the procedure recommended by Jeyakumar et al. [32], using a X500R (Ab Sciex Spain S.L., Madrid, Spain) quadrupole time-of-flight mass spectrometer (QTOF-MS) coupled to an ExionLC series two-dimensional ultra-high performance liquid chromatography (2D-UHPLC) system, at the Laboratorio de Técnicas Instrumentales facilities (Universidad de Valladolid, Valladolid, Spain).

2.7. In Vitro Seedling Tests

The potential ability of the conjugate complex treatment to induce resistance in seedlings of spelt against *F. culmorum* was evaluated following the methodology reported by Orzali et al. [33], with minor modifications. Spelt grains were first surface-sterilized for 3 min by immersion in 2% NaOCl and then rinsed with water three times. The seed treatments (100 seeds per treatment) were performed by immersion in 100 mL of conjugate complex solution (at a concentration of 1500 μg·mL^{-1}, with 0.2% Tween® 20) at room temperature, under stirring for 1 h. Distilled water with 0.2% Tween® 20 was used in the positive and negative controls. The seeds were then air-dried for 30 min, and inoculated by immersion in 100 mL of the 1×10^6 conidia·mL^{-1} suspension, with 0.2% Tween® 20, for 30 min. The seeds were finally air-dried again for 30 min.

The physiological quality of the seeds for each treatment (negative control, positive control, phytotoxicity test and treatment with the bioactive formulation) was evaluated by germination, as described in the International Rules for Seed Testing [34]. For each treatment, 3 replicates of 100 seeds were placed in glass plates, using the between paper method, and kept under constant humid conditions. Germination was evaluated after 4 days, in such a way that a seed was considered germinated if it produced a well-developed seedling with three roots and a shoot present.

The efficacy of the antifungal treatment was then assessed by planting the seeds in pots filled with autoclaved peat-based substrate, with a procedure similar to those described by Lozano-Ramírez et al. [35] and Koch et al. [36]. Seedlings were grown in greenhouse conditions at 25 °C. After 2 weeks, they were removed from the substrate and, based on their appearance, classified as healthy or diseased. Symptoms in the roots and the internode were visually evaluated, using a 0 to 5 scale [37].

2.8. Field Trials

Small-scale field trials with spelt plants were carried out in micro-plots (8 × 1 m) located in the ETSIIAA facilities (University of Valladolid, Palencia, Spain). The soil is classified as Eutric Fluvisol, commonly known as 'meadow' soil, easy to till, with good permeability, with a balanced and uniform texture along the profile, good depth and pH above 7. Sowing was conducted in mid-November 2019 with a trial sowing machine, at a dose of 250 kg/ha. The rest of the agricultural practices were the usual ones in the area. At the beginning of spring, an herbicide treatment was applied, with a mixture of Bromoxinil, Typhensulfuron and Diflufenican. A NPK(S) 20:10:10(7.5) fertilizer with 3,4-dimethylpyrazole phosphate (DMPP) nitrification inhibitor was used for fertilization in a single application, at a rate of 350 kg/ha, in mid-February 2020.

Climatologically, the agricultural year was marked by frequent rainfall in the first half of spring. Until then, the rainfall was that of the average year, with normal nascence. Since April 2020, the rainfall was higher than in the average year, with mild temperatures and almost no frost, resulting in good ear formation and—in absence of artificial inoculation—good grain filling.

Artificial inoculation tests were conducted in agreement with the procedure described by Brennan et al. [38], with minor modifications. At growth stage (GS) 65 (mid-anthesis; 21 May), 100 heads (1 head per plant) were sprayed to run-off (ca. 4 mL/ear)—using a hand-held atomizer—with the COS–tyrosine solution (at a 1500 $\mu g \cdot mL^{-1}$ concentration) containing 0.2% Tween® 20. After 24 h, the same 100 heads were artificially inoculated with *F. culmorum* by spraying the prepared conidial suspension (1×10^5 conidia$\cdot mL^{-1}$) onto ears at a rate of ca. 4 mL of spore suspension per ear. 100 heads were sprayed with distilled water containing 0.2% Tween® 20 (negative control), 100 heads with the conidial suspension and 0.2% Tween® 20 (positive control) and another 100 heads with the COS–tyrosine solution (phytotoxicity test). Heads subjected to treatments were secondary heads. All plants were enclosed for 24 h using clear polythene bags to increase humidity and promote disease development.

The FHB disease symptoms (% infected spikelets per head) were visually assessed at GS73, 77, 83 and 87 (soft dough). Severity was assessed using a scale similar to that of Parry et al. [39], and by applying the following formula: number of heads in each class × each evaluation class/total number of heads. Efficacy percentage was determined by applying Abbott's formula: (% infection control − % treated infection)/(% infection control) × 100.

2.9. Statistical Analyses

Data were subjected to analysis of variance (ANOVA), followed by post hoc comparison of means through Tukey's HSD (honest significant difference) test at $p < 0.05$. SPSS Statistics v.25 software (IBM; Armonk, NY, USA) was used.

3. Results

3.1. In Vitro Tests of Mycelial Growth Inhibition

The results of the sensitivity tests are summarized in Figure 1. The treatments based solely on amino acids showed a lower performance than the treatments including COS, either alone or in combination with the amino acids (Figure 2). Upon increase of the concentration, higher inhibition was attained for all treatments, except for the glycine only treatment, in which fungal inhibition remained below 30%.

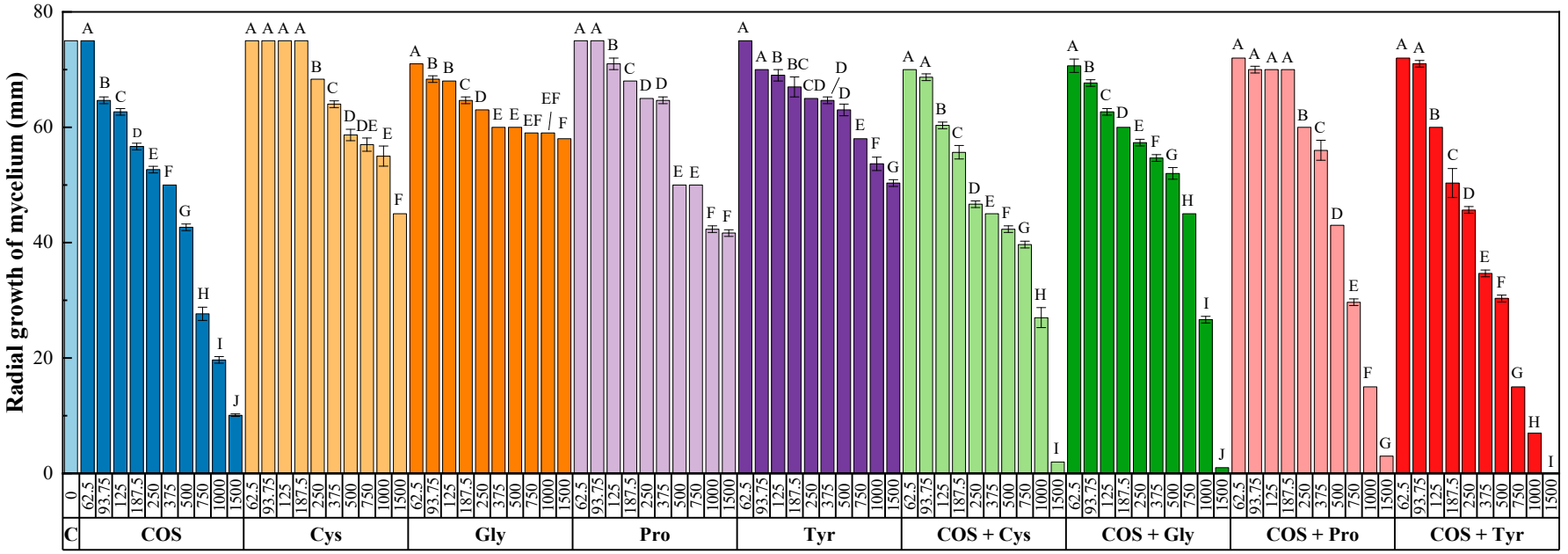

Figure 1. Radial growth values of *F. culmorum* in the presence of the different treatments, which consisted of chitosan oligomers (COS), amino acids (cysteine, Cys; glycine, Gly; proline, Pro; tyrosine, Tyr), and the conjugate complexes consisting of COS–amino acids (1:1 v/v). A 75 mm radial growth was obtained for the potato dextrose agar (PDA) control (C). Concentrations labelled with the same letters are not significantly different at $p < 0.05$ by Tukey's test. All values are presented as the average of three repetitions. Error bars represent the standard deviation across three replicates.

Figure 2. Sensitivity test. Radial growth of mycelium for: (**a**) control (PDA), (**b**) COS only, (**c**) COS–Cys, (**d**) COS–Gly, (**e**) COS–Pro, (**f**) COS–Tyr. From top to bottom: 62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000 and 1500 $\mu g \cdot mL^{-1}$. Only one replicate is shown.

The effective concentrations are presented in Table 1 for comparison purposes. In view of the EC_{90} values, a synergistic behavior was observed for all the conjugate complexes, which was particularly evident for tyrosine. In fact, the lowest EC_{50} and EC_{90} values were obtained for this amino acid, which was then used in the rest of the experiments.

Table 1. Effective concentrations that inhibited mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively).

Concentration ($\mu g \cdot mL^{-1}$)	COS	Cys	Gly	Pro	Tyr	COS–Cys	COS–Gly	COS–Pro	COS–Tyr
EC_{50}	680.27	1516.7	-	726.7	3524.8	820.45	948.39	675.69	320.46
EC_{90}	2230.26	8150.2	-	3460.3	79,197.5	1406.87	1359.08	1372.58	1106.87

COS = chitosan oligomers; cysteine = Cys; glycine = Gly; proline = Pro; tyrosine = Tyr.

3.2. Inhibition of Colonies Formation

The number of colonies were counted five days after incubation on the COS–tyrosine supplemented medium, which showed various levels of inhibition of the number of colonies as a function of the bioactive product dose (Figure 3). The number of colonies was decreased with all concentrations in comparison with the control, reaching full inhibition at a concentration of 1500 μg·mL^{-1}. The percentage of reduction of the number of colonies for 500 and 1000 μg·mL^{-1} concentrations was 55.6% and 66.7%, respectively.

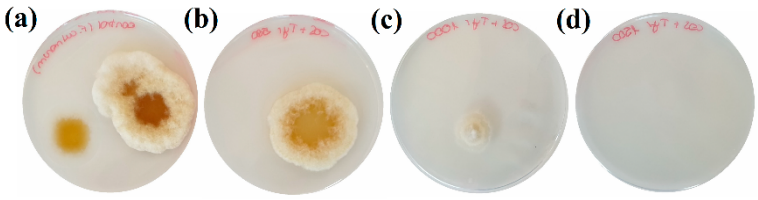

Figure 3. Effect of the COS–tyrosine conjugate complex treatment on the number of colonies formation of *F. culmorum* after 5 days. Suspension of fungal spores soaked for 24 h at different concentration of conjugate complex, and 50 μl of treated spores were spread on PDA medium. (**a**) control, (**b**) 500 μg·mL^{-1}, (**c**) 1000 μg·mL^{-1} and (**d**) 1500 μg·mL^{-1}. Only one replicate is shown.

3.3. Effect on Mycotoxin Production

After incubation of the grain samples inoculated with *F. culmorum* for 28 days, differences in the growth of the mycelium were observed for the grains treated with COS–Tyr, as compared to control trials (Figure 4). The treatment, at a concentration of 1500 μg·mL^{-1}, clearly had an inhibitory effect. From the analyses, DON content was reduced by ca. 91% vs. The control sample (in which DON content reached 8.12 ± 1.53 μg·mL^{-1}).

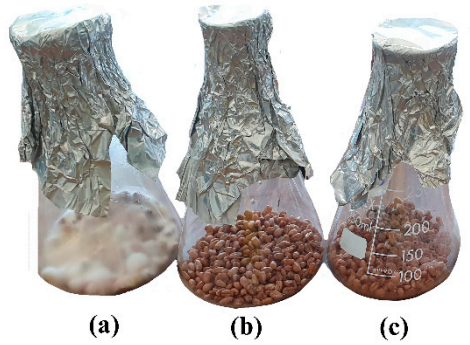

Figure 4. Effect of the application of COS–tyrosine conjugate complex on the growth of *F. culmorum* on spelt grain: (**a**) positive control, (**b**) negative control, (**c**) treated grain at a dose of 1500 μg·mL^{-1}.

3.4. Seedling Tests

Concerning the germination tests (Figure 5), no significant differences were observed between the negative control and the seeds treated with the conjugate complex and not inoculated with *F. culmorum* (100% and 99% germination percentages, respectively), thus suggesting that the bioactive product based on chitosan would not be phytotoxic. The germination rate for the positive control (inoculated and not treated seeds) was noticeably lower (48%), but it was clearly improved for the inoculated and treated seeds (89.5%).

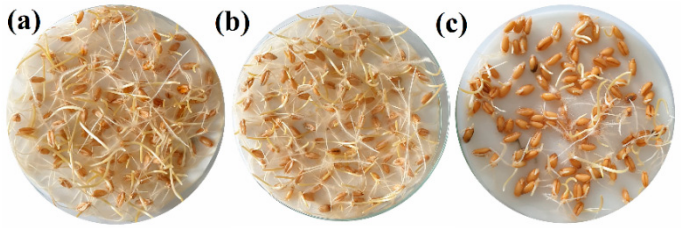

Figure 5. Germination tests: (**a**) negative control, (**b**) treated seeds, (**c**) positive control. Only one replicate is shown.

With regard to seedling symptoms after two weeks, in the negative control, no root rot symptoms were observed; in the positive control, the average disease severity was 28% (with some seedlings reaching a 3 in the 0 to 5 scale, showing clear wilting and browning of coleoptiles and roots, Figure 6), and in the artificially inoculated seedlings treated with the COS–Tyr conjugate complex, the average disease severity was close to 14%. Hence, the control efficacy of the treatment was moderate (50%).

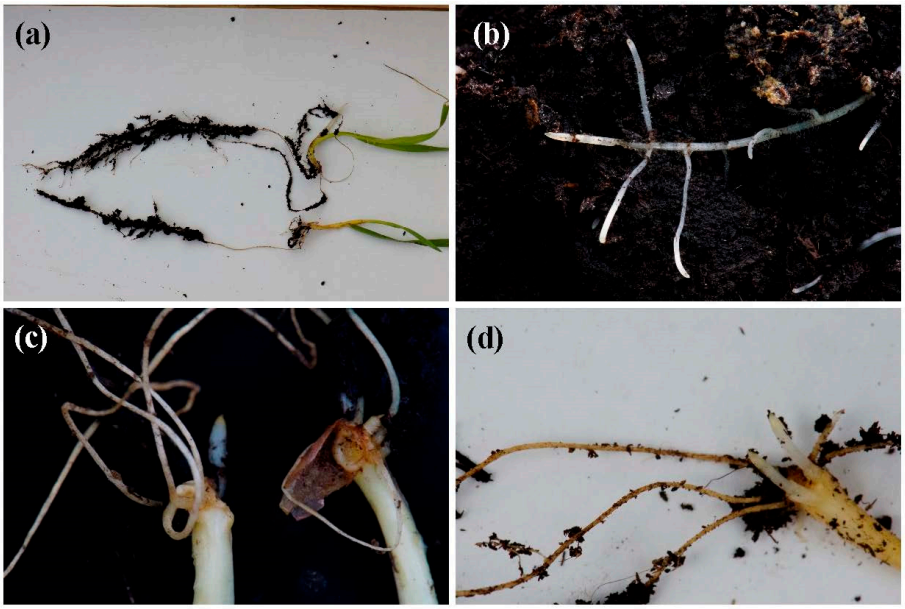

Figure 6. (**a**) Healthy (top) vs. infected (bottom) seedlings, (**b**) healthy seedling roots, (**c**) root rot symptoms, (**d**) root shortening.

3.5. Field Trials

No disease symptoms were observed in the negative control heads, and no signs of phytotoxicity were observed in the heads that had been treated with the COS–Tyr solution. In GS 87, the average disease severity reached 45.2% for the positive control plants (Figure 7), while in the treated plants, it was noticeably lower (only 7.45%), thus indicating a good control efficacy (83.5%) of the chosen treatment.

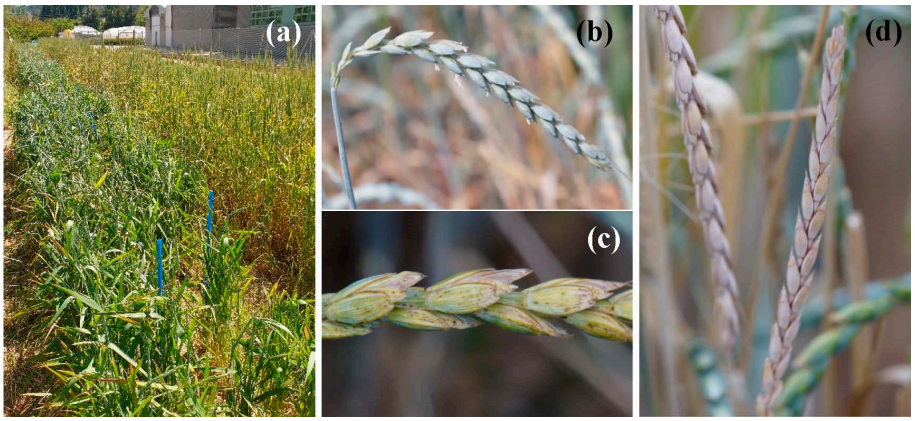

Figure 7. (**a**) Microplots used in the field trials, with spelt plants in growth stage (GS) 65, (**b**) healthy treated ear, (**c**) ear with attack of intermediate severity, (**d**) positive control, entirely affected.

4. Discussion

4.1. Comparison of Treatment Efficacy

Fusarium spp. do not have a normal minimum inhibitory concentration (MIC) and minimum effective concentration (MEC) distribution, so prediction of the antifungal susceptibility of a single strain is difficult ([40] and references therein). Moreover, as the susceptibility profile is isolate-dependent, comparisons of the effective concentrations below should be taken with caution.

A thorough bibliographical survey yielded no studies on the effects of amino acids on *Fusarium* spp., but the results presented herein may be compared with those of other treatments based on chitosan. In relation to the in vitro mycelial growth inhibition tests, contrasting results have been reported: while Xing et al. [25] found no mycelial growth inhibition against *F. culmorum* at concentrations of up to 2 mg·mL^{-1}, Al-Hetar et al. [24] reported an EC_{50} of 1.4 mg·mL^{-1} and a maximum inhibition of 76.36% at 8 mg·mL^{-1} for *F. oxysporum* f. sp. *cubense*. Park et al. [23] obtained EC_{50} values in the 1.5–4.0 and 1.8–3.2 mg·mL^{-1} range for *F. graminearum* and *F. oxysporum* respectively, and Kheiri et al. [30] reported that ≥85% inhibition of *F. graminearum* on PDA was attained with 5 mg·mL^{-1} of chitosan nanoparticles. The EC_{50} value reported herein for COS (0.68 mg·mL^{-1}) was approximately half of those reported by Al-Hetar et al. and Park et al., and the EC_{90} value was half of that reported by Kheiri et al., but it should be stressed that the pathogen was different.

Concerning the inhibition of colonies of *F. graminearum*, Kheiri et al. [30] reported 43.95% and 72.8% inhibition of colonies treated with 5 mg·mL^{-1} of chitosan nanoparticles and chitosan, respectively. In our study, for *F. culmorum*, full inhibition was reached at a lower dose (1.5 mg·mL^{-1}).

Regarding in vivo tests, Khan et al. [41] evaluated the ability of chitosan to inhibit *Fusarium* seedling blight disease of wheat (cv. GK-Othalom) caused by *F. culmorum* (strain FCF 200). Regarding its effect on the germination of wheat seedlings, they found that wheat seeds inoculated with *F. culmorum* failed to germinate, and that treatment with chitosan did not ameliorate the effect of *F. culmorum* inoculation on wheat seedling germination (in contrast with our results, in which almost half of the positive control spelt seeds germinated and the treatment had a marked positive effect on the germination rate). Nonetheless, it is worth noting that the authors claimed that, when used as a stem base treatment, chitosan (at 1 mg·mL^{-1}) was among the most effective treatments to reduce *Fusarium* seedling blight disease symptoms (by 89% vs. The positive control).

Orzali et al. [33] evaluated the effect of chitosan seed treatment as an elicitor of resistance to *F. graminearum* in wheat (cv. Simeto and cv. Creso) in greenhouse. They found that wheat seed treatments with different concentrations of chitosan led to no significant changes in germination and vigor index values compared with the untreated control, confirming that chitosan is not phytotoxic for the seeds (in good agreement with the results obtained in this study). The treatment with chitosan at

a concentration of 5 mg·mL^{-1} resulted in a disease severity in the seedling roots of 21% and 22.3% vs. 38.4% and 39.5% for the positive controls (for cv. Simeto and cv. Creso, respectively). The disease severity observed in our study for spelt (28% and 14% for the positive control and the treated seeds) was lower, but differences may be ascribed to differences in the pathogen and in the sensitivity of the cultivar (*T. spelta* has been reported to have a weaker response to *F. culmorum* infections than other *Triticum* spp. [42]). If efficacies are compared, those attained by Orzali et al. were 45.3% and 43.5%, lower than the one obtained herein (50%) at a significantly lower bioactive product dose (1.5 vs. 5 mg·mL^{-1}).

With reference to the field trials, Khan et al. [29] also studied the efficacy of chitosan for the control of FHB and associated mycotoxin contamination of grain. Spraying of winter wheat (cv. GK-Othalom) heads with chitosan (at 1 mg·mL^{-1}) resulted in an 81% and 76% reduction in FHB disease symptom development in greenhouse trials and in small-scale field trials respectively, slightly lower than the reduction reported herein (83.5%). Chitosan also significantly reduced the concentration of DON under both glasshouse and field conditions (≥74% reduction). The reduction in our in vitro experiments was higher (89%), albeit lower than that reported by Perczak et al. [31], who obtained trichothecenes concentration reductions in the 94.51–100% range by using essential oils.

In greenhouse experiments, Kheiri et al. [30] reported that spraying with chitosan nanoparticles and chitosan (at 1 mg·mL^{-1}) resulted in disease severity percentages of 26.87% and 28.74% respectively, three weeks after fungus inoculation. These results apparently are much higher than those reported herein (only 7.45%), but the severity in the positive controls was also higher (94.25% vs. 45.2%). Upon comparison of efficacies calculated with Abbott's formula (71.5% and 69.5% for chitosan nanoparticles and chitosan in the study by Kheiri et al. [30] respectively, and 83.5% in this study), the difference is reduced, but the complex conjugate still seems to have a better performance than the chitosan-only treatment.

4.2. Mechanism of Action

The inhibition mode of chitosan is based on three main mechanisms, according to Ing et al. [43]: (i) the interaction of its positive charge with the negatively charged phospholipid components of the fungal membrane, which results in an increase in its permeability and in the leakage of cellular contents, (ii) its behavior as a chelating agent, given that its binding to trace elements causes the unavailability of the essential nutrients needed for normal growth of fungi and (iii) its ability to penetrate the fungal cell wall and to bind to its DNA, which inhibits mRNA synthesis and affects essential proteins and enzymes production.

The existence of reactive groups in COS has been explored for the conjugation with other small chemical groups that primarily act by modulating the physicochemical properties of the molecule. Conjugation with amino acids entails the formation of hydrogen bonds (Figure 8), already observed in the interaction of chitosan and chitosan oligomers with the hydrophobic amino acid DL-Tyrosine [44].

The conjugation of COS and Tyr (mainly, L-Tyr) reduces the crystallinity of COS and (in a spontaneous and endothermic process) generates a rearrangement of their chains, a decrease of the intercatenary spacing and, mainly, an increase of their cationic surface charge. We believe that the occurrence of this latter feature enhances the linkage to the negatively charged site-specific binding receptors on the fungal membrane through electrostatic interactions.

Whereas chitosan-resistant fungi such as *Pochonia chlamydosporia* or *Beauveria bassiana* have low-fluidity membranes (enriched on saturated free fatty acids, FFA), the membranes of chitosan-sensitive fungi such as *F. oxysporum* or *Neurospora crassa* are highly fluid (rich in polyunsaturated FFA, such as linolenic acid) [45] and more susceptible to peroxidation. The lipid peroxidation in the fungal membrane is enhanced with the concomitant presence of amino acids, which have been shown to possess potential pro-oxidant capacity in linoleic acid. This pro-oxidative activity could be attributed to the presence of the α-amino group in the form H3-N-R, and the difference in this activity would mainly be due to the functional groups attached to β-carbon in the amino acid molecules.

In fact, a linear relation between concentration of hydroperoxides and time during the early stages of oxidation has been reported for cysteine [46], and a similar behavior is expected for tyrosine. Thus, the enhancement of mycelial growth inhibition of *F. culmorum* observed, which evidences a synergistic behavior between COS and amino acids, can be referred to as enhanced damage (permeabilization) of the fungal membrane via lipid peroxidation induction. Future studies should investigate the generation of oxylipins, metabolites derived from lipid peroxidation [47].

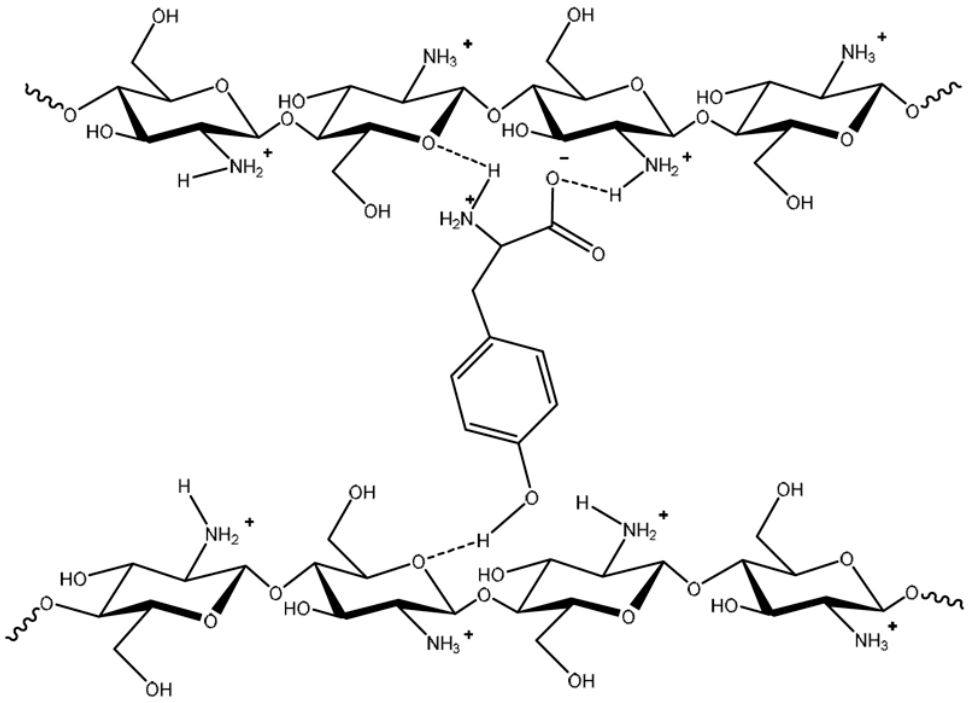

Figure 8. Hydrogen bonding in the COS–tyrosine conjugate complex.

4.3. Significance of the Reported Findings

Although follow-up studies will be necessary to draw firm conclusions on the effectiveness of the application of the proposed treatments (see Section 4.4 below), the fact that the conjugate complexes reached higher mycelial growth inhibition than other chitosan-based treatments makes them promising candidates for the effective control of FHB.

It is also worth noting that *F. culmorum* is not only a pathogen of wheat, but also of other commercially important cereal crops, such as barley, corn, sorghum, oats or rye. In addition, it has been isolated from sugar beet, flax, carnation, bean, pea, asparagus, red clover, hop, leeks, Norway spruce, strawberry and potato tuber [48]. Consequently, the results of this study may also find application in other pathosystems, resulting in a higher ecological and economic impact.

4.4. Limitations of the Study and Further Research

Although the preliminary results may be suggestive of a noticeable antifungal activity of the proposed conjugate complexes against *F. culmorum*, further research is needed before a conclusion can be made on their real applicability for wheat crop protection applications. Apart from the caveats noted above about the differences in resistance among different *Fusarium* species and strains, field tests on other *Triticum* spp. genotypes with different ploidy levels would also need to be carried out, provided that their responses to FHB infection have been reported to vary in a significant manner [42]. Moreover, the impact of the treatments on other wheat diseases (caused by, for instance, *Puccinia* spp., *Tilletia* spp., etc. [49]) should also be taken into consideration if traditional fungicides are to be replaced with

this natural product-based alternative, although it is worth noting that in our field tests, the chosen formulation appeared to control *Alternaria* spp. infection too.

In addition, a detailed assessment the effect of timing of fungicide application and dose rate on the development of FHB and the accumulation of DON (similar to that reported in References [50,51]) would be required before other key practical aspects (e.g., cost, degradation tolerance and efficacy of protection over time, etc.) can be factored in.

5. Conclusions

From in vitro mycelial growth inhibition tests, conjugate complexes of chitosan oligomers and amino acids were found to feature an enhanced antifungal behavior, tentatively ascribed to enhanced damage of the fungal membrane via lipid peroxidation (due to the pro-oxidative activity of the amino acids). The best treatment, based on the COS–tyrosine, with EC_{50} and EC_{90} values of 320 and 1107 $\mu g \cdot mL^{-1}$, was further tested for the inhibition of colonies formation and to avoid mycotoxin production in grain, with positive results: full inhibition of colonies was attained at a concentration of 1500 $\mu g \cdot mL^{-1}$, and the same dose reduced DON content by 89%. In planta assays, conducted both in greenhouse trials with *T. spelta* seedlings and in small-scale field experiments, showed that the chosen formulation had no phytotoxic effects and significantly reduced the severity of FHB symptom development (by 50% in seedling roots after two weeks, and by 83.5% in artificially inoculated heads after three weeks). These promising results call for further studies with other fungal pathogens and *Triticum* species to elucidate the potential of these conjugate complexes as an alternative to conventional fungicides.

6. Patents

The work reported in this manuscript is related to the following Spanish patents: Universidad de Valladolid. Solución acuosa que comprende un complejo de inclusión, método de obtención y su uso para aplicación en cultivos y la mejora de su rendimiento [Aqueous solution comprising an inclusion complex, its method of obtention and its use for crop application and yield improvement]. Application number P201931118, 17 December 2019; Universidad de Valladolid. Complejo conjugado para el tratamiento de agentes patógenos presentes en la agricultura y la naturaleza [Conjugate complex for the treatment of pathogens present in agriculture and nature]. Application number P201831106, 15 November 2018.

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