The Dominance of Chitosan Hydrochloride over Modern Natural Agents or Basic Substances in Efficacy against Phytophthora infestans, and Its Safety for the Non-Target Model Species Eisenia fetida

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Abstract: Growing pressure to reduce the environmental pesticide burden has the greatest impact on agriculture and crop protection. There is an enormous increase in the demand for research on new, effective, naturally based agents that do not pose an environmental risk. Phytophthora infestans is one of the most destructive phytopathogens, especially in cases where synthetic fungicides are not allowed. This paper describes the high efficacy and safety of the natural polymer chitosan under in vitro and in vivo conditions and its dominance over other natural agents or products. Chitosan demonstrated the highest efficacy against P. infestans. A concentration of 0.2–0.4% was highly effective. The protective effect of chitosan was 99.3% in natural conditions. Direct activity, equivalent to synthetic fungicides (MIC50 0.293 mg/mL), was confirmed. Chitosan was rated non-toxic to useful non-target species. We promote further chitosan expansion within legislation and implementation of chitosan as a safe substance that could reduce the pesticide burden, particularly in eco-friendly plant protection and production of non-harmful foods.

Keywords: chitosan; natural polymer; management; environmental safety; organic farming; basic substances; pre-harvest fungal disease; tomato

1. Introduction

The use of chemical synthetic fungicides in medicine, cosmetics, and other areas has been an integral part of the modern advanced world for many years now. However, the problem of using fungicides does not resonate in any of the aforementioned areas as significantly as in the sphere of agriculture, which increasingly encounters justified or exaggerated environmental obstacles. Research on the anti-fungal properties of many natural substances is promising, and it could replace or reduce the consumption of synthetic pesticides in general. The use of pesticides is currently subject to an increasing number of restrictions due to the rising tendency of environmental protection [1]. The registration process and implementation into the practice of new, less environmentally harmful products are complicated and extremely expensive. One of the ways of ensuring better and faster access to environmentally friendly options of protection was the status of so-called basic substances (BSs) or low-risk active substances (LRASs) implemented in the European Union (EU). The essence of these substances is legislatively defined in (EC) Regulation No. 1107/2009 [2]. One important BS is chitosan, which is only permitted in the EU for the protection of berries, grain, or spices [3]. Chitosan is a natural polymer with significant anti-bacterial and anti-fungal properties. Chitosan also significantly improves the plant’s defense system. In particular, it is mentioned as an elicitor of plant tissue defense mechanisms, including systemic acquired resistance (SAR) to plant pathogens [4–6]. Chitosan also has enormous potential in other areas and is also used in the food industry and for biomedicine and pharmaceutical applications due to its non-harmfulness [7–10]. From a
chemical aspect, this is a polymer of 2-amino-2-deoxy-beta-D-glucopyranose acquired through full or partial deacetylation of the abundant natural substance chitin. This study describes the surprising and very significant dominance of chitosan over other potential eco-friendly substances or products in its efficacy against the very dangerous and destructive plant pathogen *Phytophthora infestans* (Mont.) de Bary, which causes serious symptoms of potato late blight, particularly in potatoes and tomatoes. The importance of this pathogen was historically proven by, for instance, the famine in Ireland in 1845–1847 [11–13]. This pathogen also causes serious problems in other plants. Thanks to the complicated life cycle and biological stages of *P. infestans*, verification of efficacy directly on the plant is crucial. This paper describes the significant efficacy of chitosan against *P. infestans* under not only in vitro but mainly in vivo conditions. This study also compares the effect of a minimum frequency of treatment of plants during vegetation and during simulation of the conditions of constant infectious pressure by a pathogen. Furthermore, our experiments prove the ecological safety and non-harmfulness of chitosan, even in extreme doses on model useful non-target organisms that could be at risk during treatment using synthetic pesticides against *P. infestans*.

2. Materials and Methods

2.1. Substances Used in the Experiment

2.1.1. BSs, LRASs Substance Approved for or Applicable in Organic Farming

Chitosan in the form of water-soluble chitosan hydrochloride (hereinafter chitosan), talc, EDTA, sodium bicarbonate, and lecithin were acquired from the Sigma-Aldrich Company (CR).

2.1.2. Natural Plant Extracts

*Reynoutria japonica* Houtt. leaf water extract and *Humulus lupulus* L. cone water extract were prepared by laboratory maceration and water extraction at a ratio (dried plant/water) of 20/80 w/w for a period of 72 h at a temperature of 21 °C.

2.1.3. Botanical Pesticides

Rock Effect (87% effective agents) and Rock Effect New (56% effective agents) based on oil from the seeds of *Pongamia pinnata* (L.) Pierre were supplied by the manufacturer (Agro CS a.s., Ceska Skalice, Czech Republic). Eco-friendly products applicable in organic farming. Available on the market in the EU.

2.1.4. Reference Fungicide Mixture

The commercial synthetic fungicide Luna Experience containing 40% tebuconazole/fluopyram, 50/50 w/w, was acquired from the Bayer Crop Science Company (CR).

2.2. Efficacy of Chitosan in Comparison with Other Eco-Friendly or BS Compounds

A leaf disk assay was used to determine the in vivo efficacy of chitosan against *P. infestans* on leaf tissues. Other selected natural or eco-friendly substances were included in the experiment as controls and for comparison. A completely randomized design (CRD) was used for the experiment. Disks with a diameter of 1.5 cm were cut out using a cork borer under sterile conditions from fully developed leaves of potato plants (*Solanum tuberosum* L. cv. Agria) grown in a greenhouse. The leaf disks were submerged in a solution of the tested substances, including the tested concentrations of chitosan. After drying, the treated leaf disks were placed in glass Petri dishes (14 cm in diameter), abaxial side up, on moist sterile filter paper. For each tested substance/product, 18 leaf disks in 5 replicates were tested. A 20 µL drop of *P. infestans* inoculum was applied to the surface of each disk. The inoculum was prepared by washing sporangia from 14-day cultures of the pathogen cultivated at a temperature of 18 °C without light on Rye A Agar (RAA). The washed-off sporangia were cultivated for 3 h at a temperature of 4 °C for the purpose of releasing zoospores. The inoculum was adjusted to a concentration of $4 \times 10^4$ zoospores per 1 mL.
using a Bürker chamber. The surface-inoculated leaf disks were placed in closed Petri dishes into a thermostat and cultivated for a period of 12 days at a temperature of 18 °C and a photoperiod of 16/8 h (light/dark). Evaluation of the efficacy of the tested substances against infection by *P. infestans* was performed under a binocular microscope and expressed as a percentage of the affected area from the entire disk surface.

### 2.3. The Efficacy of Chitosan Compared to Other Eco-Friendly or BS Substances on Model Host Plants under Experimental Laboratory Conditions

Verification of the efficacy of chitosan against *P. infestans* was performed under laboratory conditions optimal for the development of the infection. The experiment was conducted in CRD. Young model plants (*Solanum tuberosum* L.) of 20–25 cm were first treated with the tested substances using a hand-held electric sprayer until thoroughly wetted. The inoculum was prepared using the same method as in the previous experiment (see above) and the concentration of zoospores was adjusted to $1 \times 10^4$ per 1 mL. The plants were placed in enclosed cultivation boxes, watered regularly, and cultivated for a period of 14 days at a temperature of 20–21 °C, air humidity of 80%, and a photoperiod of 16/8 h (light/dark). For each tested substance, 4 plants were used. The experiment was repeated 3 times. The experiment was evaluated as a percentage of the entire leaf surface that was affected [14].

### 2.4. Efficacy of Chitosan and the Effect of Repeated Application under Outdoor Conditions

Experimental plants of *S. tuberosum* (cv. Agria) were grown in pots (40 × 40 cm) under outdoor conditions in the experimental plots of the Crop Research Institute, Prague, Czech Republic. The plants were regularly watered and protected against direct sunlight using fine-mesh breathable textile. The experiment was conducted in CRD. The plants were treated using a 0.4% solution of chitosan during the experiment, 1–4x depending on the variant. The simulated rate was 900–1000 L/ha. The control was treated with water. The plants were inoculated using an aerosol of an inoculation solution of zoospores ($1 \times 10^4$), at a dose of 100 mL/plant and 24 h before the first treatment. Applications of chitosan were carried out at intervals of 14–15 days. Each variant contained 10 plants. The plants were grown next to each other in order to simulate dense, susceptible vegetation, with randomly placed pots. Development of the pathogen was promoted by regularly spraying the vegetation with water at least twice a week. The experiment lasted for a period of 2 months, namely May–June. After completion of the experiment, the damage to the plants was evaluated, with a focus on the percentage of damage to the leaf area showing symptoms [14].

### 2.5. Direct In Vitro Inhibitory Efficacy of Chitosan and Minimum Inhibitory Concentration

The agar dilution method was used to test the inhibitory effect of chitosan on the mycelial radial growth of fungi. Chitosan and the fungicide reference standard were properly diluted in RAA at a 2 mg/mL specific concentration. The prepared Petri dishes (9.0 cm diameter) were inoculated aseptically with assay disk (0.4 cm) cuts from the periphery of a culture of the target pathogenic fungus that was 10 days old. The control sets were then prepared using sterile distilled water instead of the tested compounds. All experiments were performed four times. The incubation was performed in the dark for 7 days at 19 °C. The Petri dishes were arranged in a CRD. The percent inhibition of the radial growth of *P. infestans* was calculated using the following formula: Percent inhibition = $(DC - DT)/DC \times 100$, where DC is the colony diameter of the control sets and DT is the colony diameter of the treated sets. The method of graded concentration (0.1–2 mg/mL) in the RAA was used to determine the minimum inhibitory concentration (MIC50) of the tested compounds. The MIC50 was evaluated statistically. The reference standard was the synthetic fungicide Luna Experience. The lowest concentration of compound that inhibits 50% of growth compared to the control sets was identified as the MIC50 [5,15,16].
2.6. Toxicity of Chitosan against Useful Non-Target Organism Eisenia Fetida (Savigny)

Adult earthworms Eisenia fetida with well-developed clitella and weighing between 350–500 mg were obtained from a fixed laboratory colony (more than 20 generations; outcrossed once) following Pavela et al. (2018) at the Crop Research Institute [17], Czech Republic. In each of the 4 replicates, 10 individuals were used. The bioassay was carried out according to the OECD methodology [18]. An artificial soil substrate was prepared by mixing sphagnum peat (10%), kaolinite clay (20%), and quartz sand (70%). The pH level of the mixture was adjusted to 6.0 using calcium carbonate. The substrate was mixed with an experimental quantity of the test substance, the proportion being 1.5, 1, 0.5, 0.25, and 0.1 g/kg of dry weight. Water was used as a solvent. The synthetic commercial fungicide Luna Experience was used as the positive control, the concentrations being 1.4, 1.2, 1, 0.8, and 0.6 g/kg, which is the equivalent of a tebuconazole and fluopyram (50/50) concentration of 0.56, 0.48, 0.4, 0.32, and 0.24 g/kg of dry weight of the substrate—following OECD methodology [18]. Distilled water was a negative control. Treated soil samples (650 g) were placed into 1 L glass jars, covered with gauze [19], and stored in a climate chamber (20 ± 1 °C, 80–85% RH, 16:8 L:D with 600 lux) arranged in a CRD. Mortality was recorded 5 and 10 days after the treatment.

2.7. Statistical Analysis

2.7.1. In Vitro Agar Dilution Experiment and MIC\textsubscript{50} Assessment

BioStat software (version 5) was utilized for the estimation of antifungal efficacy (MIC\textsubscript{50}). MIC\textsubscript{50} values were assessed by an analysis of binomial response variables (Probit analysis) [20]. The obtained MIC\textsubscript{50} values were associated with a 95% confidence interval (CI) and Chi-square values significant at the \( p < 0.05 \) level.

2.7.2. In Vivo Experiments and Toxicity Test Evaluation

Statistica (version 13.3) software was used for statistical evaluation. Percentages were transformed using the arcsine square root (\( \text{arcsine}\sqrt{} \)) transformation before an ANOVA was run. Treatment differences were determined by Tukey’s test (\( p \leq 0.01 \)).

3. Results

3.1. Efficacy of Chitosan in Comparison with Other Eco-Friendly or BS Compounds

The significant effect of chitosan against the development of infection by \( P. \) infestans on the tissue of leaf disks was evident not only at a basic concentration of 1% but also at much lower concentrations. Compared to other eco-friendly substances, the high statistical significance and strong inhibitory effect on the development of the pathogen were proven across the entire gradient of experimental concentrations (1–0.1%). The inhibitory effect of chitosan was 57.8–86.8% depending on the concentration compared to the infected control (Table 1). Other tested eco-friendly substances and products were not effective against the development of \( P. \) infestans and, apart from the moderately effective substance sodium bicarbonate (49.9%), no statistical significance compared to the infected control was proven. On the basis of an assessment of efficacy against \( P. \) infestans in a spectrum of concentrations, a 0.4% concentration was chosen as sufficient, with a high degree of certainty of effective suppression of infection.

3.2. The Efficacy of Chitosan Compared to Other Eco-Friendly or BS Substances on Model Host Plants under Experimental Laboratory Conditions

A similar effect and dominance of chitosan was proven in the following test on entire plants under artificial conditions extremely suitable for the development of the pathogen. A reduced 0.4% concentration of chitosan was sufficient to suppress the development of the pathogen by 84.7% compared to the control. Application of 0.4% chitosan led to only 23.3% leaf area damage compared to the control, on which 95% of the leaf area was devastated. Again, apart from some low efficacy for sodium bicarbonate, no significant effect on the
reduction in infection of the plants was registered during the use of other substances and products (Table 2).

Table 1. Effect of chitosan treatment, concentration, and comparison with other eco-friendly compounds against *Phytophthora infestans* infection in a potato leaf disk assay.

| Treatment and Concentration (%) | Inhibitory Effect (%) ± SD | % of Infected Disk Area ± SD |
|---------------------------------|-----------------------------|-----------------------------|
| Chitosan (1%)                   | 86.84 ± 11.2 ± 8.8 a        | 11.2 ± 8.8 a                |
| Chitosan (0.8%)                 | 84.49 ± 13.2 ± 9.7 a        | 13.2 ± 9.7 a                |
| Chitosan (0.4%)                 | 83.78 ± 13.8 ± 10.6 a       | 13.8 ± 10.6 a               |
| Chitosan (0.2%)                 | 76.26 ± 20.2 ± 11.2 a       | 20.2 ± 11.2 a               |
| Chitosan (0.1%)                 | 57.81 ± 35.9 ± 20.1 b       | 35.9 ± 20.1 b               |
| Sodium Bicarbonate (1%)         | 41.36 ± 49.9 ± 30.1 c       | 49.9 ± 30.1 c               |
| EDTA (1%)                       | 8.11 ± 78.2 ± 20.2 d        | 78.2 ± 20.2 d               |
| Rock Effect (1%)                | 6.35 ± 79.7 ± 17.1 d        | 79.7 ± 17.1 d               |
| Rock Effect New (1%)            | 1.76 ± 83.6 ± 14.3 d        | 83.6 ± 14.3 d               |
| *Humulus lupulus* water extract (1%) | 5.76 ± 80.2 ± 19.3 d   | 80.2 ± 19.3 d               |
| *Reynoutria japonica* water extract (1%) | 0.94 ± 84.3 ± 15.9 d | 84.3 ± 15.9 d               |
| Lecithin                         | −1.18 ± 86.1 ± 15.5 d       | 86.1 ± 15.5 d               |
| Talc (1%)                       | −3.17 ± 87.8 ± 12.1 d       | 87.8 ± 12.1 d               |
| Control                          | 57.81 ± 35.9 ± 20.1 b       | 35.9 ± 20.1 b               |

ANOVA (Df; F; P) 13, 1246; 222.36; p < 0.0001

Means ± SD within a column followed by the same letter do not differ significantly (Tukey’s HSD test, p < 0.05).

% = arcsine square-root transformed data, positive infected control = water, * inhibition % compared to control.

Table 2. Effect of preferred chitosan concentration treatment and comparison with other eco-friendly compounds (agents) against *Phytophthora infestans* infection (14 days) on model plants.

| Treatment and Concentration (%) | Inhibitory Effect (%) ± SD | % of Infected Leaf Area ± SD |
|---------------------------------|-----------------------------|-------------------------------|
| Chitosan (0.4%)                 | 84.72 ± 23.3 ± 7.2 a        | 23.3 ± 7.2 a                 |
| Sodium bicarbonate (1%)         | 15.63 ± 82.1 ± 13.0 b       | 82.1 ± 13.0 b                |
| EDTA (1%)                       | 6.23 ± 90.8 ± 8.2 b,c       | 90.8 ± 8.2 b,c               |
| Rock Effect (1%)                | 3.88 ± 92.1 ± 6.9 b,c       | 92.1 ± 6.9 b,c               |
| Talc (1%)                       | 2.94 ± 92.9 ± 7.8 b,c       | 92.9 ± 7.8 b,c               |
| *Reynoutria japonica* water extract (1%) | 2.47 ± 93.5 ± 7.5 c   | 93.3 ± 7.5 c                 |
| *Humulus lupulus* water extract (1%) | 0.47 ± 95.0 ± 11.5 c  | 95.0 ± 11.5 c                |
| Rock Effect New (1%)            | 0.00 ± 95.4 ± 6.5 c         | 95.4 ± 6.5 c                 |
| Lecithin (1%)                   | −1.06 ± 96.3 ± 4.1 c        | 96.3 ± 4.1 c                 |
| Control                         | 95.4 ± 6.2 c                | 95.4 ± 6.2 c                 |

ANOVA (Df; F; P) 9, 110; 86.819; p < 0.0001

Means ± SD within a column followed by the same letter do not differ significantly (Tukey’s HSD test, p < 0.05).

% = arcsine square-root transformed data, positive infected control = water, * inhibition % compared to control.

3.3. Efficacy of Chitosan and the Effect of Repeated Application under Outdoor Conditions

Practical application of chitosan against *P. infestans* was simulated during an in vivo experiment under outdoor conditions. In this case, the effect of chitosan, within the meaning of its protective effect in correlation with repeated treatment of plants, was also monitored. This experiment clearly demonstrates, with high statistical significance, that even under natural conditions, environmentally safe chitosan is very effective against *P. infestans*. Average damage of over 76% was observed in the control plants. In treated variants with 1–4 applications of chitosan, the final damage to the plants ranged from 48% to 0.5%. Expressed as values of the final inhibitory protective effect, a single application of 0.4% solution of chitosan was shown to provide statistically significant inhibition and an inhibitory effect of 37%. In cases where chitosan was applied four times, an inhibitory effect of up to 99.3% was demonstrated. The effect of chitosan significantly correlated
to the frequency of chitosan application. The protective effect was the most statistically significant in variants with three to four applications (Table 3).

Table 3. Effect of preferred 0.4% chitosan concentration and number of treatments (during two months) on final rate of Phytophthora infestans infection of potato plants.

| Chitosan (0.4%) | Inhibitory Effect (%) * | % of Infected Leaf Area ± SD |
|----------------|--------------------------|-----------------------------|
| 1 treatment    | 37.25 b                  | 48.0 ± 20.8 b               |
| 2 treatments   | 52.94 b                  | 36.0 ± 12.3 b               |
| 3 treatments   | 90.46 a                  | 7.3 ± 14.4 a                |
| 4 treatments   | 99.35 a                  | 0.5 ± 2.8 a                 |
| Control        | 76.5 ± 10.0 c            |                             |

ANOVA (Df; F; P) 4, 45; 84.309; p < 0.0001

Means ± SD within a column followed by the same letter do not differ significantly (Tukey’s HSD test, p < 0.05).

% = arcsine square-root transformed data. Control = water. * inhibition % compared to control.

3.4. Direct In Vitro Inhibitory Efficacy of Chitosan and Minimum Inhibitory Concentration

The direct anti-fungal activity of chitosan against P. infestans was confirmed using an in vitro dilution test at a basic concentration of 2 mg/mL RAA. At this concentration of chitosan, a significant inhibitory effect against radial growth at a level of 99.4% was registered. A mixture of fungicides (tebuconazole/fluopyram 50/50), which was used as a reference standard for comparison, demonstrated an inhibitory effect of 97.3%. Evaluation of the inhibitory concentration gradients led to the determination of similar MIC50 values. In regard to chitosan—0.293 mg/mL; in regard to the commercial fungicide mixture—0.258 mg/mL (Table 4).

Table 4. Inhibitory effect of chitosan on radial growth of Phytophthora infestans at the basic experimental concentration 2 mg/mL and MIC50 (reference standard—tebuconazole/fluopyram 50/50).

| Compound | Inhibition (%) | S.D. | MIC50 a (mg/mL) | CI 95 b | Chi-Square c |
|----------|----------------|------|-----------------|---------|--------------|
| Chitosan | 99.04 ± 0.05   | 0.293| 0.238–0.356     | 5.405   |
| Teb/Flp  | 97.28 ± 0.47   | 0.258| 0.204–0.311     | 6.194   |

a Minimum inhibitory concentration of compound that resulted in a 50% radial growth inhibition. b 95% confidence intervals. c Chi-square value, significant at p < 0.05 level.

3.5. Toxicity of Chitosan against Useful Non-Target Organism

Testing of the toxicity of chitosan against non-target useful organisms, which was carried out on the model organism E. fetida, confirmed the absolute non-toxicity of chitosan, even in extreme doses. The commercial mixture of synthetic fungicides demonstrated toxicity with an average mortality of 13.3–66.7% at a dose of 0.4–0.56 g/kg after just 7 days, and 13.3–100% at a dose of 0.32–0.56 g/kg after 14 days. Compared to this, in the case of chitosan, no mortality was observed even at an extreme dose of 1.5g/kg, not even after 14 days (Table 5).

Table 5. Toxicity of chitosan and tebuconazole/fluopyram (50/50) on Eisenia fetida earthworms.

| Treatment and Concentration (g/kg) | 7th Day * (Mortality % ± SD) | 14th Day * (Mortality % ± SD) |
|-----------------------------------|------------------------------|-------------------------------|
| Chitosan 1.5                      | 0.0 ± 0.0 a                  | 0.0 ± 0.0 a                   |
| Chitosan 1.2                      | 0.0 ± 0.0 a                  | 0.0 ± 0.0 a                   |
| Chitosan 1.0                      | 0.0 ± 0.0 a                  | 0.0 ± 0.0 a                   |
| Chitosan 0.8                      | 0.0 ± 0.0 a                  | 0.0 ± 0.0 a                   |
| Chitosan 0.6                      | 0.0 ± 0.0 a                  | 0.0 ± 0.0 a                   |
Table 5. Cont.

| Treatment and Concentration (g/kg) | 7th Day * (Mortality % ± SD) | 14th Day * (Mortality % ± SD) |
|-----------------------------------|-----------------------------|-----------------------------|
| Teb/Flp 0.56                     | 66.7 ± 23.6<sup>b</sup>     | 100.0 ± 0.0<sup>c</sup>     |
| Teb/Flp 0.48                     | 16.7 ± 12.5<sup>a</sup>     | 73.3 ± 23.6<sup>c</sup>     |
| Teb/Flp 0.40                     | 13.3 ± 12.5<sup>a</sup>     | 40.0 ± 8.2<sup>b</sup>      |
| Teb/Flp 0.32                     | 0.0 ± 0.0<sup>a</sup>       | 13.3 ± 9.4<sup>a,b</sup>    |
| Teb/Flp 0.24                     | 0.0 ± 0.0<sup>a</sup>       | 0.0 ± 0.0<sup>a</sup>       |
| Control                           | 0.0 ± 0.0<sup>a</sup>       | 0.0 ± 0.0<sup>a</sup>       |

ANOVA (Df; F; P) 10, 22; 10.282; p < 0.0001 10, 22; 38.625; p < 0.0001

* Average mortality of E. fetida (±SD) achieved on the 7th and 14th day after application of chitosan and tebuconazole/fluopyram (50/50 w/w) (Teb/Flp). Means ± SD within a column followed by the same letter do not differ significantly (Tukey’s HSD test, p < 0.05). % = arcsine square-root transformed data. Negative control = water.

4. Discussion

The great antifungal potential of chitosan hydrochloride has already been described in our previous studies [5]. In the specified case, excellent inhibitory values were achieved on model filamentous fungi from the pathogenic and toxicogenic groups Aspergillus, Fusarium, and Penicillium [5]. However, we did not expect such excellent results, particularly against the evolutionarily and biologically different pathogen P. infestans (Oomycetes) [21], mainly due to the complicated biology of this pathogen as well as the very different infectious stages in the process of pathogenesis itself [22]. The initial phase of attack and destruction of tissue usually takes place extremely quickly and with released motile flagellated zoospores [23]. This biological fact evidently leads to essentially in vivo inefficacy in other cases of very effective substances [14,24]. Some studies mention the more likely indirect secondary effect of chitosan applied to plants, such as the increased resistance of plants thanks to the elicitation of defensive mechanisms in the tissue, together with the mechanical barrier of the polymer chitosan layer [25,26]. However, our abovementioned previous study also confirmed the significant direct in vivo antifungal effect of chitosan [5], which was also experimentally confirmed in the case of P. infestans in this study. The efficacy of chitosan is most probably caused by many mechanisms, such as the electrostatic interaction of positively charged molecules of chitosan with cellular membranes. This leads to disruption of the osmotic balance of cytosols or to direct destabilization of membranes. Important metal ions are blocked, particularly Ca<sup>2+</sup> [4,27], as a result of the strong chelating properties of chitosan. We believe that chelation and the blocking of Ca<sup>2+</sup> is the crucial mechanism against the initial infectious phase of P. infestans on plants. The Ca<sup>2+</sup> ion is essential for the orientation and motility of flagellated zoospores [28,29]. Neither mode of action precludes the other, but rather they both suitably complement each other from a practical aspect. The direct in vitro effect of chitosan was compared with the efficacy of commercial mixtures of frequently used synthetic fungicides for the reference purposes of subsequent research. The results indeed demonstrate a comparable effect expressed by MIC50 levels. On the basis of a toxicity test against non-target useful organisms, it is, however, clear that compared to the reference commercial mixture of frequently used synthetic fungicides, chitosan did not demonstrate toxicity, not even at extreme doses. We assume that mainly tebuconazole causes the greatest mortality in test individuals of E. fetida [30,31]. Despite this fact, tebuconazole is abundantly used in conventional agriculture and is permitted as an active agent in many commercial products [32]. The high efficacy of chitosan and its proven ecological safety support its expanded use in the sphere of protection against P. infestans. Even in conventional agriculture, where synthetic fungicides can be used, the P. infestans pathogen is considered dangerous and destructive. Our results indicate that a single application of this natural polymer can significantly reduce the impact of infection in host plants. Repeated application of this substance leads to highly significant suppression of the pathogen. The use of synthetic fungicides is not permitted.
in organic farming, and the situation during the occurrence of *P. infestans* is often critical. Research on environmentally safe compounds or plant substances has not yet resulted in many agents that are highly effective in vivo. It is remarkable that, for example, chitosan still has not been recognized within EU legislation as a BS, or as a natural and environmentally safe product directly intended for the protection of a crop as important as the potato against *P. infestans* [3]. In particular, organic farming systems where conventional synthetic pesticides cannot be used remain virtually defenseless in situations with an increased presence of this destructive pathogen. In our opinion, and also on the basis of our results, it seems that chitosan hydrochloride has enormous potential to provide environmentally safe and effective protection against this pathogen. However, it should be noted that even such a safe substance [33,34] can be hazardous if handled improperly. The safety data sheet clearly warns of various risks such as eye or respiratory tract irritation. These risks arise mainly from the powder formulation of chitosan on the market, which can be inhaled or can affect the eyes if handled improperly [35]. The risks of toxicity to selected aquatic organisms are also mentioned. Toxicity is associated mainly with the use of acetic acid as a solvent (acidification) and is also affected by the formulation of the tested chitosan [36,37].

5. Conclusions

Chitosan could help not only in environmentally friendly organic agriculture but also in the growing worldwide effort to reduce the consumption of synthetic pesticides in conventional agriculture, given the often problematic issue of protecting against *P. infestans* while producing safe food without problematic residues that are a risk to the environment and consumer health. Increasing worldwide restrictions in the use of conventional pesticides means increased scientific efforts to seek out environmentally safe and effective natural alternatives and approaches. This paper described and verified chitosan, a natural polymer, as a very effective and environmentally safe substance, with strong practical potential. We believe that our work could also serve to support further chitosan expansion within the legislation for BSs. Obviously, this is one of the best possibilities for effectively suppressing this significant pathogen and would have great benefits for environmentally sound systems in plant protection.

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