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Effect of Chitosan on Disease Control and Growth of Scots Pine (*Pinus sylvestris* L.) in a Forest Nursery

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Abstract: Chitosan has become a promising biological agent for disease control and plant growth promotion. The objective of this study was to assess the effects of chitosan, applied as an active ingredient of Beta-chikol (Poli-Farm, Łowicz, Poland), to control damping-off and *Lophodermium* needle cast on Scots pine seedlings growing in field conditions. Beta-chikol was used for seed treatment and as a foliar spray at recommended rates and concentrations. For each experimental variant (chitosan, fungicides, unprotected), inventories of seedlings were performed, after germination and again after six weeks. In the aboveground parts of seedlings, the concentration of endogenous salicylic acid was determined by HPLC. At the end of the growing season, seedling growth parameters were determined. Beta-chikol used as foliar spray limited infection by the damping-off fungi but was ineffective when used as a seed treatment. *Lophodermium* needle cast was not observed during the study period. After the application of Beta-chikol, the concentration of salicylic acid did not increase. The application of Beta-chikol enhanced all growth parameters under investigation. Our results indicate the possibility of using chitosan in the form of Beta-chikol to stimulate plant growth and protect pine seedlings against parasitic damping-off in forest nurseries.

1. Introduction

Polymers have been used worldwide for the last few decades. They have a number of applications, ranging from daily needs to biomedical and defence fields [1–5]. Among various polymers, biorenewable polymers such as cellulose, hemicellulose, lignin, and chitosan have attracted the attention of researchers and manufactures as low cost materials for several industrial applications [2–6]. Among these biorenewable polymers, polysaccharides are the most abundant [3–6]. Their unique properties include flexibility, biodegradability, environmental friendliness, easy processing, recyclability, impressive physico-machanical properties, and easy availability [5]. Chitosan is one of the most important natural polymers [6].

The intensive production of seedlings creates favorable conditions for the development of fungal pathogens, so it is necessary to use plant protection products in forest nurseries. Whenever possible, the use of chemicals should be minimized, while alternative substances and biological methods should be preferred. One such substance is chitosan [7,8].

Chitosan is an organic polysaccharide also known as deacetylchitin, as it is a deacetylated derivative of chitin. On a large scale, it is produced from the skeletons of shrimps and crabs. Its advantages include non-toxicity, biodegradability, and high bioactivity, as well as sorption and chelating properties [9].

Chitosan is an exogenous elicitor of plant defense responses. It induces local and systemic acquired resistance, reflected in the synthesis of salicylic acid, phytoalexins, and pathogenesis-related proteins (chitinase and β-1, 3-glucanase), the lignification of cell walls, and callose synthesis [8,10–12]. In seeds treated with chitosan, increasing concentrations of phenolic compounds—derivatives of hydroxycinnamic acid (*p*-cumaric, caffeic and feluric) and benzoic acid (benzoic, pirotocatechulic and gallic)—increase seed resistance to infection due to lignin synthesis and accumulation [13,14]. Chitosan affects plant defences in two ways, as it not only activates the genes responsible for the initiation of resistance mechanisms in plants, but it also has antiviral [15], antibacterial [9,16], and antifungal properties [17,18].

Moreover, chitosan stimulates plant growth and development. Seed treatment with chitosan promotes germination, increases seedling emergence, and accelerates further plant growth [19,20]. Many studies have shown that plants treated with chitosan are taller and have larger and more numerous leaves. Chitosan application also shortens the vegetative phase of plants and accelerates flowering; the treated plants produce more numerous and larger flowers. In addition, crop yields are increased [21].

Salicylic acid is a phenolic compound that is a derivative of benzoic acid commonly found in plants in small concentrations, below 1 mg/kg fresh weight [22]. However, in infected plants its concentration can increase 20-fold, activating the genes responsible for the synthesis of defense-related proteins [23]. Both endogenous and exogenous salicylic acid induces local resistance and is a signal molecule for the

development of systemic acquired resistance [24]. Salicylic acid increases plant tolerance to abiotic stressors: low temperature, soil salinity, and herbicide treatment [25–27]. Moreover, salicylic acid is an endogenous regulator of plant growth and development [28]. There are also studies that show a positive effect of exogenous salicylic acid on aboveground and root dry mass, the regeneration of flower buds, flowering, and tuber formation [26,29].

Research on chitosan as a plant protection product and growth stimulant has so far concerned various herbaceous crop plants, while only few studies have focused on woody plants, including forest trees [8,21,30]. This study aimed to assess the effectiveness of chitosan applied in the form of Beta-chikol as a seed treatment agent and foliar spray to protect Scots pine (*Pinus sylvestris* L.) against parasitic damping-off and *Lophodermium* needle cast in a forest nursery (in the ground). In the experiment, we also assessed the effect of this bioproduct on plant growth. Our null hypotheses were that (1) Beta-chikol limits seedling infection from both diseases; and (2) it has a favorable effect on pine growth. We also assumed that the major compound that determines the effectiveness of chitosan as a stimulant of pine resistance and growth is endogenous salicylic acid, whose concentration would increase as a result of the Beta-chikol treatment.

2. Materials and Methods

2.1. Study Site

Field research was conducted in the forest nursery of the Spała Forest District (51.54121 N; 20.14754 E), located approximately 120 km SW of Warsaw in Poland. The nursery started to produce tree seedlings in 1958. The annual mean temperature in the study area is 7.2 °C. The warmest month is July (17.3 °C), and the coldest month is January (−3.2 °C). The growing season (mean daily air temperature above +5 °C) begins in early April and lasts on average 210 days. The long-term mean annual precipitation is 596 mm, of which approximately 420 mm fall during the growing season [31].

2.2. Filed Experiment, Application of Chitosan and Fungicide

The experiment compared 3 variants: pine seedlings protected with either chitosan or fungicides against parasitic damping-off and *Lophodermium* needle cast and unprotected seedlings. Seeds were sown on 8 May, 2013, because of excessive precipitation earlier in the year. Chitosan was applied in the form of Beta-chikol (Poli-Farm, Łowicz, Poland) as an organic plant growth stimulant. This chitosan formulation is in liquid form for dilution with water. The concentration of chitosan in the product is 2%. We used it for seed priming (soaking before sowing) and as a foliar spray at a concentration of 2%. The seeds were soaked in the solution for 5 hours before sowing. For spraying, 1000 L of the solution were used per hectare. The fungicide treatment involved a dry seed treatment with Sadoplon 75 WP and spraying with Previcur Energy 840 SL, Gwarant 500 SC, Amistar 250 SC, and Falcon 460 EC. The fungicides were applied according to the standard procedures in Poland, defined by the Forest Research Institute [32].

Chitosan was applied 3 times after seed germination: on 7 June, 12 July, and 26 August. Fungicides were applied on the same dates and an additional 8 times (11 in total: for protection against damping-off

and needle cast). These included 6 treatments every 5–7 days against parasitic damping-off (alternating Previcur Energy and Gwarant) and 5 treatments every 3–4 weeks against needle-cast (alternating Amistar and Falcon).

2.3. Seedling Inventory and Growth Measurement

Seedling emergence was estimated twice, on the days of the 1st and 2nd chitosan treatments. Seedlings were counted in 1-metre segments of individual rows of the seedbed, spaced 5 m apart. For each variant, 32 such 1-metre segments were taken into account. In late October, 60 seedlings of each variant were collected to measure biometric characters. For each seedling, we measured the shoot length, root collar diameter, dry mass of aboveground parts and roots, and we counted lateral shoots. Using the WinRHIZO software (Regent Instruments Inc., Québec, Canada), the root length and number of root tips were estimated.

2.4. Salicylic Acid Content

To measure the salicylic acid concentration, seedlings were sampled twice: 5 and 12 days after the 2nd chitosan treatment. A single sample consisted of 10 seedlings. In total, 18 samples were taken, 3 for each variant on the 2 dates.

The salicylic acid content was determined using reversed-phase–high-performance liquid chromatography (RP-HPLC). For this purpose, the aboveground parts of the pine seedlings were homogenized by grinding in liquid nitrogen. One milliliter of 5% trichloroacetic acid (TCA) was added to 200 mg of the homogenized tissues. After vigorous mixing, the samples were incubated for 15 min at 75 °C. Samples were then centrifuged at $16,000 \times g$ for 10 min. Supernatants were retained, and the pellets were re-extracted in the same manner with 0.5 mL of 5% TCA. After centrifugation, the pellets were discarded and the supernatants were pooled with those obtained after the first extraction step. Subsequently, the TCA extracts were extracted with portions of ethyl acetate in volumes of 500 µL (first portion) and 300 µL (second and third portion). All three portions of ethyl acetate were pooled and then subjected to drying in a vacuum centrifuge at 60 °C. The residues were dissolved in 200 µL of methanol, and the resultant solutions were analyzed using RP-HPLC. Chromatographic separations were carried out at room temperature using a HPLC system composed of a pump (model 1525, Waters), an autosampler (model 717plus, Waters), a fluorescence detector (model 474, Waters, detection was carried out at $\lambda_{ex} = 310$ nm/$\lambda_{em} = 410$ nm), C8 column (Symmetry 4.6 × 150 mm, 5 µm, Waters) connected to a C8 guard column (Symmetry 3.9 × 20 mm, 5 µm, Waters), and a personal computer with Breeze software (Waters). We used a 60 mM phosphate buffer at a pH 6.8 and acetonitrile with H_2O (4:1) as eluents. Chromatographic separations were carried out at a flow rate of 1 mL/min using a gradient elution. The sample injection volume was 25 µL. The concentration of salicylic acid in the samples was determined from the area of the corresponding peak on the basis of the prepared calibration curve.

2.5. Statistical Analyses

Before significance tests, the normality of the data distribution was verified by the Shapiro-Wilk test and the equality of variances was assessed by the Levene test. For many parameters (shoot length, root collar diameter, dry mass of aboveground parts and roots, and number of seedlings/m of row), the data distribution did not differ significantly from the normal distribution, and the variances of the compared variants were homogeneous, so the significance of differences was assessed by a one-way analysis of variance (ANOVA) and a *post hoc* Fisher's least significance difference (LSD) test. For the salicylic acid concentration, a two-way ANOVA was used (seedling treatment and date of assessment). For the remaining traits (number of lateral shoots, root length, number of root tips), nonparametric tests were used (non-parametric ANOVA, *i.e.*, Kruskal-Wallis test).

3. Results

During the first inventory, an average of 103 seedlings/m of row were recorded in the variant treated with fungicides. In the other variants, seedling numbers were significantly smaller: control 78, chitosan 77 ($p = 0.0015$, Figure 1a). Relative to the number of sown seeds, seedling emergence reached 57% in the variant treated with fungicides and 48% in the remaining variants.

During the second inventory, an average 72 seedlings/m of row survived after fungicide applications, which corresponds to 40% of the sown seeds. In the variant with chitosan, 52 seedlings (29%) were recorded, compared to 37 seedlings (20%) in the control (Figure 1b). Differences between the variants were significant ($p < 0.0001$). Seedling emergence was affected most strongly by parasitic damping-off, and its symptoms were observed in all variants of the experiment. Symptoms of *Lophodermium* needle cast were not noticeable in any of the experimental variants.
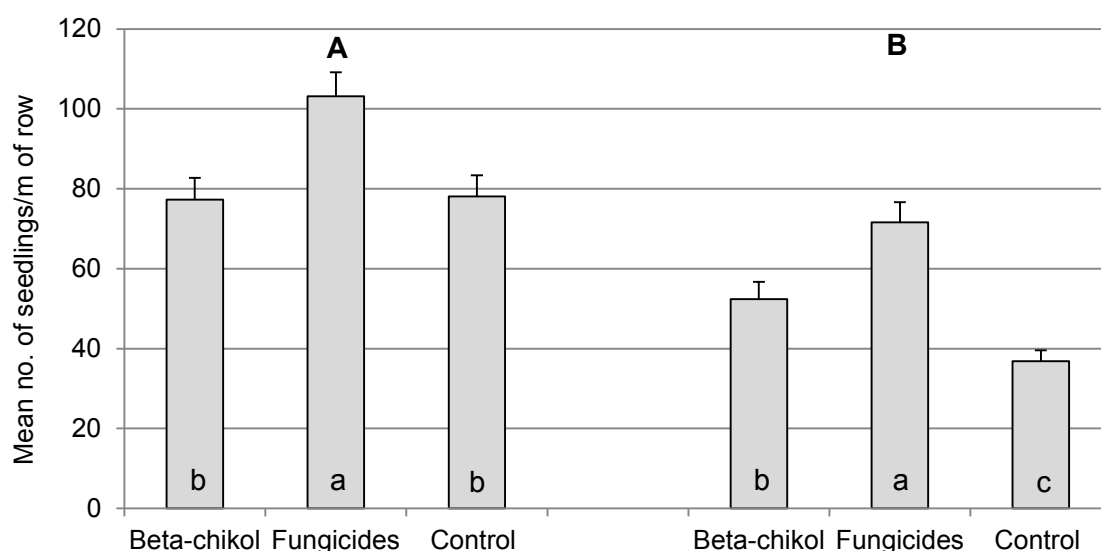
Figure 1. Mean number of Scots pine seedlings in 1-meter segments of the seed row (**A**) after seed germination and (**B**) approximately 7 weeks after germination in variants with Beta-chikol, fungicides, and no protection (control). Means marked with different letters are significantly different ($p < 0.05$, Fisher's LSD test). Error bars denote standard deviation.

All the analyzed biometric parameters of pine were affected similarly. The highest significant values of individual growth parameters were in the group treated with chitosan: shoot length 9.3 cm ($p < 0.0001$), root collar diameter 2.23 mm ($p < 0.0001$), number of lateral shoots 3.5 ($p < 0.0001$), aboveground dry weight 0.737 g ($p < 0.0001$), root dry weight 0.319 g ($p < 0.0001$), root length 332.7 cm ($p < 0.0001$), and number of root tips 796 ($p < 0.0001$). Pine seedlings protected with fungicides and unprotected seedlings (control) had lower values of these parameters, which did not differ significantly (Table 1).

Table 1. Biometric parameters (mean and standard deviation in brackets) of Scots pine (*Pinus sylvestris*) protected with Beta-chikol, fungicides, and of the unprotected control.

Treatment	Shoot Length (cm)	Root Collar Diameter (mm)	Aboveground Dry Mass (g)	Root Dry Mass (g)	No. of Lateral Shoots	Root Length (cm)	No. of Root Tips
Beta-chikol	9.3 a (1.9)	2.2 a (0.4)	0.737 a (0.287)	0.318 a (0.138)	3.9 a (1.7)	332.7 a (107.5)	795 a (222)
Fungicides	8.1 b (1.8)	1.8 b (0.4)	0.515 b (0.261)	0.183 b (0.091)	3.2 b (2.5)	178.4 b (85.1)	424 b (182)
Control	7.6 b (1.5)	1.6 b (0.4)	0.455 b (0.208)	0.169 b (0.078)	2.3 b (2.3)	192.0 b (89.7)	486 b (175)

Means marked with different letters within columns are significantly different ($p < 0.05$).

The salicylic acid content in the seedlings five days after treatment ranged from 81.03 ng/g fresh weight (in one sample from the group treated with fungicides) to 113.56 ng/g fresh weight (in one of the control samples). On the date of the first assessment, the salicylic acid content of seedlings did not differ significantly. However, 12 days after treatment, the salicylic acid content was the highest in control pines: on average 188.26 ng/g fresh weight (treatment type $p < 0.0001$, date of assessment $p < 0.0001$, treatment type × date of assessment $p < 0.0001$) (Figure 2).
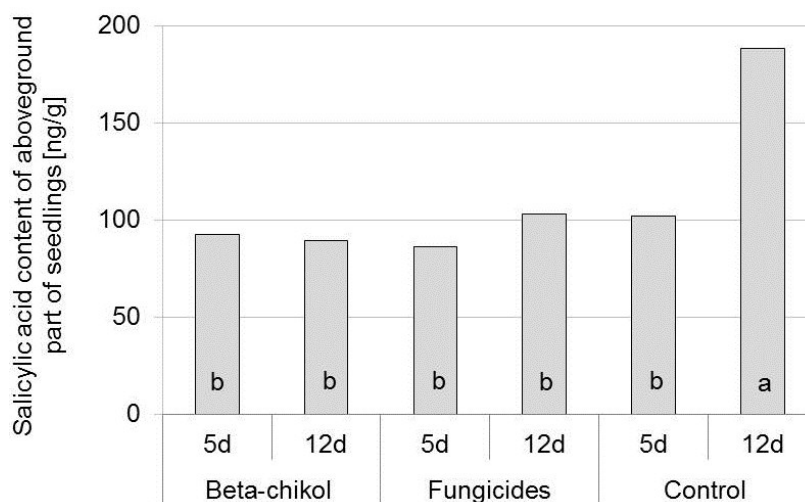
Figure 2. Salicylic acid content of Scots pine seedlings 5 and 12 days after chitosan treatment in variants with Beta-chikol, fungicides, and no protection (control). Explanations are as in Figure 1.

4. Discussion

The forest nursery of the Spała Forest District has produced seedlings of forest trees for 50 years. The risk of parasitic damping-off (caused by fungi) is high in the nursery, which is confirmed by the results of our study. In the variant treated with fungicides, healthy seedlings developed from only 40% of sown seeds, while in the other variants, the corresponding values were even lower.

Chitosan applied in the form of Beta-chikol limited the rate of infection of pine seedlings with parasitic damping-off but was less effective than fungicides. The major reason for the low effectiveness was the lack of effect of seed treatment. Seedling emergence after seed priming with Beta-chikol was very similar to that of the control variant (no seed treatment).

Many studies indicate that the treatment of seeds of various herbaceous plants with chitosan increases the number of germinated seeds and decreases the number of infected seedlings [20,33], but the effectiveness of chitosan as a seed treatment depends on its molecular weight and concentration, the pH of the solution and the duration of seed soaking or number of layers of seed treatment powder [19,33]. Our results indicating a lack of protective effect of the Beta-chikol used for seed priming could be due to its application according to the manufacturer's instructions without taking into account the factors mentioned above. Unfortunately, published data do not allow us to determine unambiguously which values of individual factors are optimal. The varied effect of chitosan on seed germination may also be associated with local environmental conditions, e.g., the temperature and moisture of the soil surrounding the treated seeds [30,34].

Our results may be because the effectiveness of chitosan (applied as Biochikol 020 PC) as a forest seed treatment depends on the tree species, the fungal species causing the damping-off, and the concentration of the working solution. In a pot experiment, a seed treatment with 1% and 2% Biochikol proved to be ineffective at protecting against *Rhizoctonia solani* [35].

The effectiveness of pine protection against parasitic damping-off by foliar spraying with Beta-chikol in this study was comparable with that of chemical protection. Similar results of experiments with woody and herbaceous plants have been reported by many authors, e.g., [30,35]. The lack of symptoms of *Lophodermium* needle cast made it impossible to check the effectiveness of the tested product in protection against this disease.

Sathiyabama and Balasubramanian [12] showed that foliar application of chitosan on *Arachis hypogaea* caused a nearly four-fold increase in endogenous salicylic acid 8 days after treatment and more than a seven-fold increase after 12 days. Salicylic acid is a signal molecule that induces a cascade of signals that trigger plant defense reactions [23]. In our experiment, a significant increase in salicylic acid content was observed only in unprotected seedlings during the second assessment (12 July). Such a result indicates that the plant initiated natural defense responses induced by infection with the pathogen [23].

Chitosan application, in addition to causing a rise in the salicylic acid concentration, may trigger many other plant defense responses [8], which could also be the case in our experiment. The effective protection of seedlings against parasitic damping-off by foliar application of Beta-chikol, without any simultaneous increase in the salicylic acid content of the plants, suggests that some other defense mechanism(s) could be initiated, unrelated to the plant reaction. Patkowska *et al.* [36] reported that legume seed treatment and spraying with a solution containing chitosan (Biochikol 020 PC) resulted in

microbiological changes in the rhizosphere. The treatment resulted in a 30% lower number of isolates of pathogenic fungi, including those causing parasitic damping-off, and higher numbers of isolates of bacteria of the genera *Bacillus* and *Pseudomonas*, as well as fungi of the genera *Aspergillus*, *Gliocladium*, and *Trichoderma*. The stimulation of growth of these groups of soil microorganisms results from their ability to synthesize chitinases, *i.e.*, the enzymes that determine their resistance to the influence of chitosan [8,21]. Simultaneously, the high chitinolytic activity makes it possible to use the above-mentioned genera of bacteria and fungi for plant production, as plant-growth-promoting rhizobacteria (PGPR) and plant-growth-promoting fungi (PGPF). These bacteria and fungi induce plant resistance to diseases, abiotic stresses, stimulate plant growth, and indirectly affect pathogenic fungi [37,38].

The effectiveness of chitosan in plant protection in this study could also result from a direct effect of the Beta-chikol on the fungi and oomycetes causing the parasitic damping-off, reflected in the inhibition of hyphal growth and spore germination [17,39]. Another mechanism could be the ability of chitosan molecules to bind mycotoxins secreted by facultative pathogens, such as those causing damping-off, to enable them to colonize plant tissues [40].

Chitosan applied as Beta-chikol resulted in the intensive stimulation of pine growth. Chitosan applies to all the analyzed growth parameters of both the roots and aboveground parts. A similar stimulation of growth was observed in many other studies [19,41–43]. The mechanism of this phenomenon has not been fully explained so far. The stimulation can result from a direct effect of chitosan uptake by roots and use in metabolic processes by the plant as an additional source of nutrients [42]. Chitosan is a rich source of nitrogen and calcium and contains also micronutrients, including copper, zinc, and iron [40,44]. Better availability of nutrients to the plants may also result from the chelating properties of chitosan [45]. Moreover, the plant growth stimulation may be an effect of the microbial changes in the soil described above. The increased abundance of PGPR and PGPF leads to better growth of plants [37,38].

There probably exists a still unknown mechanism of plant growth stimulation as a result of chitosan application because this phenomenon was also observed in experiments with very low concentrations of chitosan, making it impossible to use them as a source of nitrogen and carbohydrates. Moreover, plant growth was also stimulated by chitosan in experiments performed in aseptic conditions, where the effects of PGPR and PGPF were impossible [21].

5. Conclusions

Chitosan is an organic, environmentally friendly, biodegradable product that is non-toxic to higher organisms. Its effectiveness in the protection of Scots pine seedlings against parasitic damping-off by foliar spraying is comparable with that of a fungicide treatment. It can be used in forest nurseries as an alternative to chemical methods and as a product stimulating seedling growth. Modern forest management in the European Union seeks environmentally friendly technological solutions; thus, chitosan should find its place in the Integrated Pest Management (IPM) systems of forest nurseries.

Acknowledgments

Author Contributions

Marta Aleksandrowicz-Trzcińska: idea, laboratory work, data interpretation and writing; Anna Bogusiewicz: filed and laboratory work; Michał Szkop: measured the salicylic acid concentration; Stanisław Drozdowski: performed the statistical analysis.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Thakur, V.K.; Kesster, M.R. Self-healing polymer nanocomposite materials: A review. *Polymer* **2015**, *69*, 369–383.
2. Thakur, V.K.; Thakur, M.K. Recent advances in green hydrogels from lignin: A review. *Int. J. Biol. Macromol.* **2015**, *72*, 834–847.
3. Pappu, A.; Patil, V.; Jain, S.; Mahindrakar, A.; Haque, R.; Thakur, V.K. Advances in industrial prospective of cellulosic macromolecules enriched banana biofibre resources: A review. *Int. J. Biol. Macromol.* **2015**, *79*, 449–458.
4. Thakur, V.K.; Thakur, M.K.; Gupta, R.K. Review: Raw natural fiber-based polymer composites. *Int. J. Polym. Anal. Charact.* **2014**, *19*, 256–271.
5. Thakur, V.K.; Thakur, M.K. Processing and characterization of natural cellulose fibers/thermoset polymer composites. *Carbohydr. Polym.* **2014**, *109*, 102–117.
6. Thakur, V.K.; Thakur, M.K. Recent advances in graft copolymerization and applications of chitosan: A review. *ACS Sustain. Chem. Eng.* **2014**, *2*, 2637–2652.
7. Chandler, D.; Bailey, A.S.; Tatchell, G.M.; Davidson, G.; Greaves, J.; Grant, W.P. The development, regulation and use of biopesticides for integrated pest management. *Philos. Trans. R. Soc. B* **2011**, *366*, 1987–1998.
8. El Hadrami, A.; Adam, L.R.; El Hadrami, I.; Daayf, F. Chitosan in plant protection. *Mar. Drugs* **2010**, *8*, 968–987.
9. Raafat, D.; Sahl, H.-G. Chitosan and its antimicrobial potential—A critical literature survey. *Microb. Biotechnol.* **2009**, *2*, 186–201.
10. Manjunatha, G.; Niranjan Raj, S.; Prashanth, G.N.; Deepak, S.; Amruthesh, K.N.; Shetty, H.S. Nitric oxide is involved in chitosan-induced systemic resistance in pearl millet against downy mildew disease. *Pest Manag. Sci.* **2009**, *65*, 737–743.
11. Zuppini, A.; Baldan, B.; Millioni, R.; Favaron, F.; Navazio, L.; Mariani, P. Chitosan induces Ca^{2+}-mediated programmed cell death in soybean cells. *New Phytol.* **2003**, *161*, 557–568.

12. Sathiyabama, M.; Balasubramanian, R. Chitosan induce resistance components in *Arachis hypogaea* against leaf rust caused by *Puccinia arachidis* Speg. *Crop Prot.* **1998**, *17*, 307–313.

13. Reddy, M.V.B.; Arul, J.; Angers, P.; Couture, L. Chitosan treatment of wheat seeds induces resistance to *Fusarium graminearun* and improves seed quality. *J. Agric. Food Chem.* **1999**, *47*, 1208–1216.

14. Fajardo, J.E.; Waniska, R.D.; Cuero, R.G.; Pettit, R.E. Phenolic compounds in peanut seeds: Enhanced elicitation by chitosan and effects on growth and aflatoxin B_1 production by *Aspergillus flavus*. *Food Biotechnol.* **1995**, *9*, 59–78.

15. Pospieszny, H.; Chirkov, S.; Atabekov, J. Induction of antiviral resistance in plants by chitosan. *Plant Sci.* **1991**, *79*, 63–68.

16. Li, B.; Liu, B.; Shan, C.; Ibrahim, M.; Lou, Y.; Wang, Y.; Xie, G.; Li, H.; Sun, G. Antibacterial activity of two chitosan solutions and their effect on rice bacterial leaf blight and leaf streak. *Pest Manag. Sci.* **2013**, *69*, 312–320.

17. Laflamme, P.; Benhamou, N.; Bussières, G.; Dessureault, M. Differential effects of chitosan on root rot fungal pathogens in forest nurseries. *Can. J. Bot.* **2000**, *77*, 1460–1468.

18. Hernández-Lauzardo, A.N.; Vega-Pérez, J.; Velázquez-Del Valle, M.G.; Sánchez, N.S.; Peña, A.; Guerra-Sánchez, G. Changes in the functionality of plasma membrane of *Rhizopus stolonifer* by addition of chitosan. *J. Phytopathol.* **2011**, *159*, 563–568.

19. Cho, M.H.; No, H.K.; Prinyawiwatkul, W. Chitosan treatments affect growth and selected quality of sunflower sprouts. *J. Food Sci.* **2008**, *73*, 70–77.

20. Manjunatha, G.; Roopa, K.S.; Prashanth G.N.; Shetty H.S. Chitosan enhances disease resistance in pearl millet against downy mildew caused by *Sclerospora graminicola* and defence-related enzyme activation. *Pest Manag. Sci.* **2008**, *64*, 1250–1257.

21. Sharp, R.G. A review of the applications of chitin and its derivatives in agriculture to modify plant-microbial interactions and improve crop yields. *Agronomy* **2013**, *3*, 757–793.

22. Raskin, I.; Skubatz, H.; Tang, W.; Meeuse, B.J.D. Salicylic acid levels in thermogenic and non-thermogenic plants. *Ann. Bot.* **1990**, *66*, 369–373.

23. Malamy, J.; Carr, J.P.; Klessig, D.F.; Raskin, I. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **1990**, *250*, 1002–1004.

24. Raskin, I. Role of salicylic acid in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 439–463.

25. Janda, T.; Szalai, G.; Tari, I.; Páldi, E. Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. *Planta* **1999**, *208*, 175–180.

26. Kaydan, D.; Yagmur, M.; Okut, N. Effects of salicylic acid on the growth and some physiological characters in salt stressed wheat (*Triticum aestivum* L.). *Tarim Bilim. Derg.* **2007**, *13*, 114–119.

27. Żurek, A.; Grzyś, E. Wpływ kwasu salicylowego na parametry fizjologiczne siewek ogórka traktowanych herbicydem Reglone. *Post. Ochr. Roślin* **2007**, *47*, 325–329. (In Polish with English summary).

28. Matysiak, K.; Adamczewski, K. Regulatory wzrostu i rozwoju roślin—Kierunki badań w Polsce i na świecie. *Post. Ochr. Roślin* **2009**, *49*, 1810–1816. (In Polish with English summary).

29. Handro, W.; Mello, C.M.; Manzano, M.A.; Floh, E.I.S. Enhancement of stem elongation and flower bud regeneration by salicylic acid. *Rev. Bras. Fisiol. Veg.* **1997**, *9*, 139–142.

30. Hadwiger, L.A. Multiple effects of chitosan on plant system: Solid science or hype. *Plant Sci.* **2013**, *208*, 42–49.

31. *Forest Management Plan 2012–2021*; Spała Forest District: Spała, Poland. Unpublished work, 2012. (In Polish)

32. Głowacka, B. *Środki Ochrony Roślin Oraz Produkty do Rozkładu Pni Drzew Leśnych Zalecane do Stosowania w Leśnictwie w Roku 2013*; IBL: Sękocin Stary, Poland, 2012; pp. 1–72.

33. Ziani, K.; Ursúa, B.; Maté, J.I. Application of bioactive coatings based on chitosan for artichoke seed protection. *Crop Prot.* **2010**, *29*, 853–859.

34. Guan, Y.J.; Hu, J.; Wang, X.J.; Shao, C.X. Seed priming with chitosan improves miaze germination and seedling growth in relation to physiological changes under low temperature stress. *J. Zhejiang Univ.—Sci. B* **2009**, *10*, 427–433.

35. Duda, B.; Oszako, T.; Piwnicki, J. Possibilities of chitosan use in forestry. *Bull. Pol. Acad. Sci. Biol. Sci.* **2003**, *51*, 213–220.

36. Patkowska, E.; Pięta, D.; Pastucha, A. *The Effect of Biochikol 020 PC on Microorganism Communities in the Rhizosphere of Fabaceae Plants*; Polish Chitin Society: Łódź, Poland, 2006; pp. 171–178.

37. Harman, G.E. Multifunctional fungal plant symbionts: New tools to enhance plant growth and productivity. *New Phytol.* **2011**, *189*, 647–649.

38. Ramamoorthy, V.; Viswanathan, R.; Raguchander, T.; Prakasam, V.; Samiyappan, R. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. *Crop Prot.* **2001**, *20*, 1–11.

39. Palma-Guerrero, J.; Jansson, H.-B.; Salinas, J.; Lopez-Llorca, L.V. Effect of chitosan on hyphal growth and spore germination of plant pathogenic and biocontrol fungi. *J. Appl. Microbiol.* **2008**, *104*, 541–553.

40. Bornet, A.; Teissedre, P.L. Chitosan, chitin-glucan and chitin effects on minerals (iron, lead, cadmium) and organic (ochratoxin A) contaminants in wines. *Eur. Food Res. Technol.* **2008**, *226*, 681–689.

41. Aleksandrowicz-Trzcińska, M.; Hamera-Dzierżanowska, A.; Żybura, H.; Drozdowski, S. Wpływ mykoryzacji i chitozanu na wzrost sosny zwyczajnej (*Pinus sylvestris* L.) w szkółce i na uprawie. *Sylwan* **2013**, *157*, 899–908. (In Polish with English summary).

42. Ohta, K.; Morishita, S.; Suda, K.; Kobayashi, N.; Hosoki, T. Effects of chitosan soil mixture treatment in the seedling stage on the growth and flowering of several ornamental plants. *J. Jap. Soc. Hortic. Sci.* **2004**, *73*, 66–68.

43. Ohta, K.; Taniguchi, A.; Konishi, N.; Hosoki, T. Chitosan treatment affects plant growth and flower quality in *Eustoma grandiflorum*. *HortScience* **1999**, *34*, 233–234.

44. Ramírez, M.Á.; Rodriguez, A.T.; Alfonso, L.; Peniche, C. Chitin and its derivatives as biopolymers with potential agricultural applications. *Biotecnol. Apl.* **2010**, *27*, 270–276.

45. Ravi Kumar, M.N.V. A review of chitin and chitosan applications. *React. Funct. Polym*. **2000**, *46*, 1–27.