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EFFECT OF CHITOSAN FORMULATIONS OF DIFFERENT BIOLOGICAL ORIGIN ON TOBACCO (*NICOTIANA TABACUM L.*) PR-GENES EXPRESSION

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ABSTRACT

During the life cycle, plants sustain a significant impact of external abiotic and biotic stress factors. Their reactions to the factors are complex and multi-level; they include changes in the transcriptomic, cellular, and physiological levels. The effect of chitosan formulations of different biological origin and molecular weight on the relative expression pattern of five pathogenesis-related (PR-)genes of tobacco (PR1a, PR2a, PR2b, PR3a and PR3b) was studied. Based on the analysis of nucleotide sequences deposited in the GenBank, specific primers for the amplification of exon regions of the five PR-genes were designed and the optimal conditions for real-time PCR, using SYBR Green dye determined. The relative expression pattern of the genes under study was influenced by the molecular weight of the chitosan used, the time passed after treatment and the very gene; The expression was generally higher when low-molecular chitosan fractions were used. Regardless of the biological origin of the elicitor, the formulations had the smallest effect on the expression of gene PR1a and the highest effect on the expression of the main chitinase gene (PR3b). *Nicotiana tabacum* L. leaves possess sensory systems triggering response reactions on the transcriptional level. The nature of the interaction between chitosan and cell sensor may differ, however, depending on the biological origin of chitosan, its molecular weight and the degree of deacetylation. The obtained results prove high sensitivity of defensive plant systems to the tested chitosan formulations and the ability of these formulations to penetrate tissues and interact with the relevant cellular sensors.

Keywords: response reactions, real-time PCR, reverse transcription, defence genes

INTRODUCTION

Biological formulations on the basis of chitosan and its modifications are becoming a topic of increased interest in plant pest and disease control (**Bykova, 2002**). Widespread use of chitosan is determined by its physical and chemical properties and the ability to mobilize plant defensive system against phytopathogens. Therefore, introducing a biological plant protection method based on chitosan is a promising alternative to the use of pesticides (**Khor E., Lim, 2003**).

Chitosan is a natural safe biopolymer (poly-1,4-2-deoxy-2-amino-D-glycopolysaccharide) obtained through deacetylation of the natural polysaccharide chitin of crustaceans, insects and certain types of fungi by enzymatic (chitin deacetylase or alkali (40–50 % NaOH) treatment (**Brine et al., 1992; Chi Fai Cheung et al., 2015**). The biological action of chitosan is associated with an increase/stimulation in the processes of seed germination, photosynthesis, absorption of nutrients and the development of resistance to abiotic stress. It can also act as anti-transpiration agent associated with the induction of synthesis of jasmonate and abscisic acids (**Iriti M., Faoro,2008**; **Sharp, 2013**)

Chitosan can act as potent agent to elicit stronger plant defense reactions and inhibit the growth of several phytopathogenic fungi and bacteria (**Shibuya et.al., 2001**). It was found that chitosan effectively reduces the amount of polygalacturonases produced by *Botrytis cinerea*, which causes severe cytological damage to the hyphae invading bell pepper fruit (**El Ghaouth et.al., 1997**). Chitosan induced the synthesis of phytoalexin, a potent suppressor of fungal growth, in rice leaves (**Agrawal et.al., 2002**). The chitinase and peroxidase activity increased and growth of *Botrytis cinerea* was successfully inhibited when cucumber plants were sprayed with chitosan or chitin before *Botrytis cinerea* inoculation (**Ben-Shalom et.al., 2002**).

Treating plants with chitosan may induce defensive mechanisms, leading to an increase in the intensity of the cell ligation process, changes in ion flux, cytoplasmic oxidation, membrane depolarization, PR-proteins (pathogenesis-related proteins) activation, etc. (**Coqueiro et al., 2015**). The effect of chitosan is also associated with the synthesis of phytoalexins, generation of active oxygen, biosynthesis of jasmonic acid and the expression of unique 'early' defensive genes in response to stress both in monocotyledons and dicotyledons (**El-Mohamedy et al.,2014; Katiyar et al., 2014**).

At present, there is no clear understanding of the effect of chitosan on reducing the frequency and intensity of plant diseases. However, in the literature, there are experimental data on the direct influence of chitosan on pathogens through a cytotoxic effect and through chelation of minerals and nutrients necessary for the life of pathogens. Due to its physical and chemical properties, this biopolymer exhibits the ability to form physical barriers around certain pathogens, preventing their spread to healthy tissues and to trigger a series of signal reactions (**El Hadrami et al., 2010**).

The eliciting effect of chitosan on pathogenic organisms occurs as a result of interaction with the pathogen through chelation of metals or binding to negatively charged components of the cell wall of microorganisms (**Raafat et al., 2008**), or as an inducer of immunity due to a high affinity with transmembrane receptors of plants (**Povero et al.,2011**). Chitosan formulations, due to its biological origin of different animal sources, are structurally heterogeneous, which affects their immunomodulating properties. The key parameters of the biological activity of chitosan include its molecular structure and weight, as well as the degree of deacetylation (**Kulikov et.al., 2008**).

The aim of this study was to examine the effect of chitosan formulations of two different biological origins (fungus and crustaceans) and molecular weight on the relative expression pattern of the tobacco PR-genes.

MATERIAL AND METHODS

As a model, we used plants of *Nicotiana tabacum* L., cultured *in vitro* and adapted to the conditions of covered soil. After adaptation, plants were grown on soil substrate (peat:perlite, 3:1) at a temperature of 25 °C, humidity 70 % and a 16-hour photoperiod in growth chamber.

Chitosan treatment of tobacco plants

Plants were treated with 0.4 % solutions of chitosan of. low-molecular weight (LMC) and high-molecular weight (HMC) The first fraction of chitosan was obtained from champignon (*Agaricus bisporus*) fruiting bodies by the method of enzymatic hydrolysis (low molecular weight chitosan, LMC) and the second fraction was a commercial chitosan formulation made from crustaceans (high molecular weight chitosan, HMC, Sigma Aldrich) (**Subin et al., 2018**). The chitosan powder was dissolved in 0.05 N HCl and adjusted to pH 5.6 with 0.05 N NaOH. Distilled water served as a control. Sampling of treated leaves was performed before and 1, 12, 24, 48 and 72 h after treatment.

Total RNA isolation and primer design

Total RNA isolation was carried out with 100 mg of tobacco leaves using the RIBO-sorb Nucleic Acid Extraction Kit (InterLabService, Russia). Quantitative analysis of the selected samples was performed UV-spectrophotometrically on a NanoDrop 1000 (Thermo Fisher Scientific, USA). The integrity of the RNA was evaluated by electrophoresis in a denaturing urea polyacryle amide gel: 6 % polyacrylamide 6 M urea and 0.5 M TBE buffer. Site-specific primers were designed in our study using GeneRunner (Hastings Software) and the PRIMER3 software program (http://primer3.sourceforge.net) (Table 1).

Reverse transcription

Before carrying out reverse transcription, RNA was treated with the enzyme DNase I (Thermo Fisher Scientific, USA). The reaction was carried out in a 20-µL solution containing 10 µL of RNA, 1 U of DNase I, 2 µL of 10x reaction buffer (NB: from which company?) and 7 µL of DEPC deionized water and incubated at 37 °C for 1 h. The enzyme was inactivated by adding 2 µL of 0.5 M EDTA and heating the mixture at 65 °C for 15 min.
The reverse transcription reaction was performed using reagents RevertAid and First Strand cDNA Synthesis Kit Random from Hexamer Primer (Thermo Fisher Scientific, USA) according to the instruction. Synthesis of cDNA was carried out in a 20µL sample at 42 °C for 60 min (in accordance with the manufacturer's instructions). The obtained cDNA samples were stored at -20 °C for no more than three days until conducting PCR.

Analysis of the tobacco PR-genes expression

Analysis of the tobacco PR-genes expression was carried out by real-time PCR on a CFX96 (BioRad, USA) amplifier using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA). The PCR protocol was 95 °C for 10 min, 42 cycles: 95 °C for 20 s, 60 °C for 20 s (signal removal) and 72 °C for 30 s. Each 20-µL sample of reaction mixture contained 10 µL of 2x Master-Mix, 200 ng of cDNA, 0.3–0.5 µM forward and backward primers. The relative expression pattern of the investigated genes (RQ) was calculated by the $2^{-\Delta\Delta Ct}$ method (**Livak et al., 2001**). Specificity of amplification in the experiments was by determining if there was a single peak in the melting curve. Control over the absence of nonspecific PCR products was carried out in 2 % agarose gel. The molecular size of the amplification products was determined by GeneRuler 100 bp marker (Fermentas, USA) using software Quantity One, version 4.6.3 (BioRad, USA).

RESULTS AND DISCUSSION

We studied the effects of low and high molecular weight chitosan solutions of two different sources on the expression of five tobacco PR-genes: *PR1a, PR2a, PR2b, PR3a* and *PR3b*. Based on the analysis of nucleotide sequences of these genes deposited in GenBank, we developed specific primers for amplifying exon sites of the genes under study (**Table 1**). As a reference, we used the tobacco actin, which constitutive expression was shown in other models described in literature (**Coqueiro et al., 2015**). Examination of the effectiveness of site-specific amplification of selected genes, matching molecular size, and optimizing PCR conditions was performed by analysing genomic DNA of tobacco.

Table 1 Nucleotide sequences of the primers designed by us for studying the expression of tobacco *PR*-genes

№	Gene	Primer sequence 5′→ 3′ (forward/reverse)	GenBank accession number	PCR efficiency (%)	Amplicon size (bp)
1	Actin	GCCGTGGTGGTGAAAGAG TGGACTCTGGTGATGGTGTC	U60489.1	98,84	154
2	PR1a	ACCTGGAGGATCATAGTTGC GATGTGGGTCGATGAGAA	X12485.1	99,42	191
3	PR2a	TGGCTAAGAGTGGAAGGT GCACCATTTGTTGCTCCT	DQ206348.1	97,93	211
4	PR2b	TGTTGATGCCATTGTTGGCTTC CCCTACAGATGCCCCTCCTG	M59442.1	102,41	241
5	PR3b	TGGTACCAGTGGCGATACCAC AGTCGCCGGGGCTACCTT	S44869	98,32	421
6	PR3a	GATGACACCACAGGACAACAAG TCCACTGCGTCATTCCGTC	M29868.1	99,64	175

Our study allowed to determine the factors that most influenced the amplification efficiency. To this end the optimal conditions for PCR for all the primer pairs used were determined. This allowed obtaining clear amplicons of a definite molecular size without additional nonspecific amplification products (**Figure 1**). It shows that for these regions of PR-genes and This greatly enhances the analysis of the expression of the 5 genes.

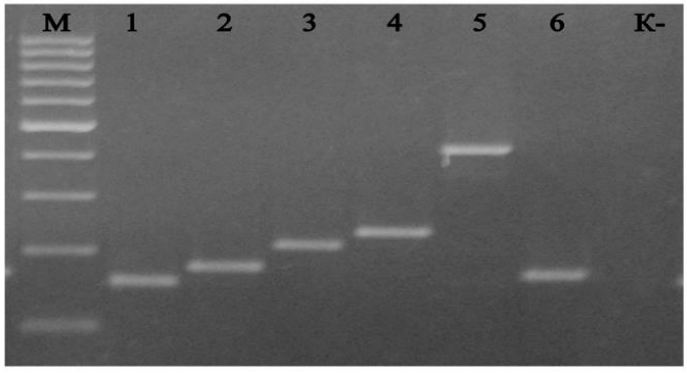

Figure 1 Examination of the specificity of the designed primers: *A* agarose electrophoresis (*M* molecular marker, *1–6* PCR product of the investigated genes (lane numbers correspond with the primer numbering in Table 1), *K* negative control of PCR, (using RNA as matrix for PCR);

To confirm the efficiency of the reverse transcription reaction and purification from genomic DNA, the RNA preparations were treated with the enzyme DNase I. A specific amplification product was formed solely on the cDNA matrix. At the same time, the concentration of total RNA of all samples examined, was adjusted to the same level, which allowed using the spectrum of different Ct values corresponding to the concentration of a certain mRNA in the sample as one of the criteria for the stability of gene expression.
Chitosan treatment of tobacco plants lead to expression of all five PR-genes. The level of expression, however, of these genes considerably differed between LMC and HMC chitosans different physical and chemical properties, time passed after the treatment and the very genes (**Figure 2** and **Table 2**). In general, the relative expression pattern of PR genes in plants treated with low molecular weight chitosan was higher (NB: how much higher in %, please be specific) as compared to high molecular weight chitosan. This indicates the different nature of the response reactions of the plant organism to stimulation with chitosan. That is confirmed by our previous results (**Subin et.al.2018**). Given that the concentration and conditions of treatment of the leaf surface with the active substances were the same, there is a reason to reckon that the degree of chitosan polymerization, i.e. is the length of the polyamine chain, and its biological origin is decisive for plants. Regardless of the chitosan origin, the two formulations had the slightest effect on the expression of the *PR1a* gene, and the largest effect on the expression of the main chitinase gene (*PR3b*).

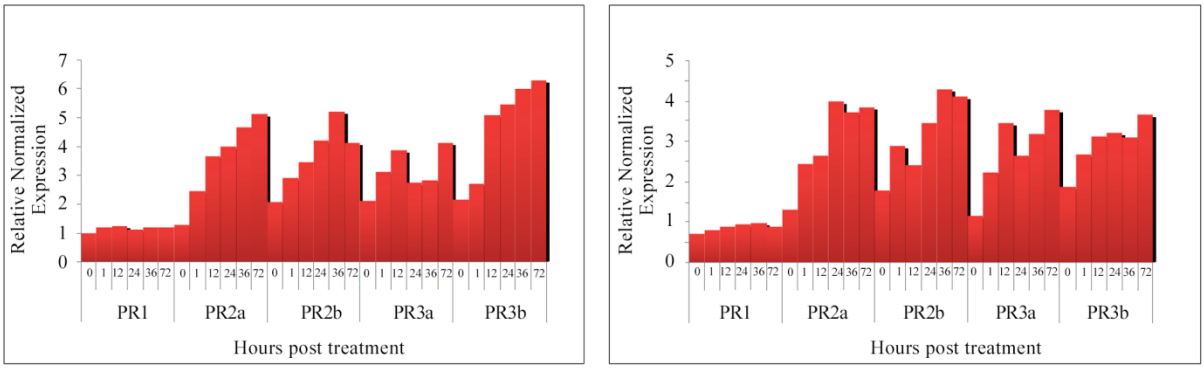

Figure 2 Relative expression (log2 ratio) of the examined PR-genes of tobacco in relation to the reference gene of actin: *A* treatment with low molecular chitosan (LMC), *B* treatment with high molecular chitosan (HMC)

Table 2 The results of amplification of the genes of PR proteins of tobacco

Treatment	1*		2		3		4		5		6	
(hour)	ΔRn**	Ct	ΔRn	Ct	ΔRn	Ct	ΔRn	Ct	ΔRn	Ct	ΔRn	Ct
0	770,28	30,27	660,26	33,29	456,73	36,88	465,24	38,56	544,27	29,46	690,22	35,26
1	680,34	34,22	620,34	36,42	497,22	37,44	477,22	36,77	533,47	34,26	528,36	37,72
12	736,42	29,64	590,88	35,76	589,34	36,22	469,46	39,21	514,75	33,56	511,75	35,98
24	790,67	33,18	670,55	36,33	672,33	38,73	478,94	38,48	628,57	36,18	640,59	37,43
36	800,06	33,43	720,93	34,67	867,84	34,57	486,74	35,44	727,44	38,11	746,88	38,55
72	740,37	31,37	680,44	38,87	672,88	37,46	488,49	37,59	770,36	34,67	850,36	37,98

Note: * – 1 – Actin; 2 – Pathogenesis-related protein PR1a; 3 – Acidic beta 1,3 glucanase gene; 4 – Basic-1,3 glucanse gene; 5 – Basic chitinase; 6 – Pathogenesis-related protein Q (acidic chitinase); ** - ΔRn - normalized signal SYBR Green, Ct - threshold cycle value

Except for the *PR1a* gene, of which the expression practically did not change during the experiment, for all four other genes expression was detected already in the first hours for both chitosan formulations. It is known that PR1a-proteins are resistant to proteases and occur only in plant tissues affected by viruses, bacteria and fungi (**Sharipova et al., 2015; Hernandez et al., 2005**). Since pathogenic organisms were not involved in our model experiment, the lack of reliable differences and stable trends with respect to the total mRNA content under the effect of chitosans indicates that there may be other triggers for regulation of PR-proteins activity. Earlier it was shown that the expression of the *PR1a* gene is associated with the activation of Ca-dependent protein kinases (**Sharipova et al., 2015**). Under stress conditions, this activation occurs due to an increase in nitric oxide concentration in cells (**Durner et al., 2015**). In plant cells, nitric oxide takes part in a wide range of defensive reactions, including in the process of pathogenesis (**Delledonne, 2005**).

Under normal circumstances it has been observed that sensory systems are activated, the transcriptional response of the tobacco plants to treatments with chitosan solutions occurs within 24 h. For example, increasing nitric oxide synthesis in tomato leaves was observed within 12 h after inoculation with tobacco mosaic virus (**Fu et al., 2010**). In our experiment, when the HMC or LMC elicitor directly targeted plant cell, the physiological reaction occurred quite quickly.

In the plant leaves treated with LMC, already one hour later, the relative number of mRNA copies gradually increased and reached its maximum on the third day for acidic β-1,3-glucanase (*PR2a*) and sharply (5.1–6.3 times) increased within 12 h for the main chitinase (*PR3b*). In the case of the major β-1,3-glucanase (*PR2b*), the expression had a wave-like character, with maximum expression at 36 h. A similar trend in the expression (with minor differences) was observed after the plant treatment with a high molecular weight chitosan polymer, indicating a high biological activity of both formulations under study.

The bulk amount of β-1,3-glucanases, as expressed by PR2a genes are endoglucanases catalyzing the hydrolysis of internal bonds of β-glucans that are the part of the cell walls of fungi (**Okinaka**, **1995**). Expression of *PR2a* genes is apparently due to the ability of LMC to interact with sensor systems of cells. In plants, fungal pathogenesis usually occurs against the background of cytoplasm acidification. An increase in the concentration of protons leads to the development of oxidative stress reactions and the accumulation of proteins (**Roos et al., 2006**). Along with the move of low molecular weight glucosamines (elicitors) resulting from enzymatic hydrolysis in the apoplast, defensive reactions spread through plant tissues. Since under the stress conditions, the extracellular medium becomes more alkaline, the conditions optimal for *PR3b* are being gradually formed. This class of PR-proteins represents the most effective defensive enzyme system destructing phytopathogenic fungi.

CONCLUSION

The obtained results confirm the sensitivity of the tobacco plant defensive systems to chitosan, its ability to penetrate into the tissues and interact with corresponding cellular sensors and stimulate the expression of at least 4 of 5 PR genes tested. Importantly, the nature of such interaction can significantly vary depending on the target gene, and possibly on the origin and molecular weight of the chitosan.

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