

Effect of Externally Applied Fungal Polysaccharides on Fusariosis in Tomato Plants

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The chitin—glucan complex isolated from the waste mycelia of filamentous fungi *Aspergillus niger* that are left behind upon the industrial production of citric acid, β -D-glucan from baker's yeast (*Saccharomyces cerevisiae*), and mannan from *Candida albicans* has been investigated. Five different water-soluble polysaccharide derivatives were obtained and used in the assay of the antifungal activity against plant pathogen *Fusarium oxysporum* f. sp. *lycopersici* in tomato (*Lycopersicon esculenta* L.). In the experiments, application of the polysaccharides led to the diminished infestation as well as to slightly increased productivity of fresh mass of the plants. The results demonstrated that the external application of the polysaccharides led to changes in production of cell-wall, as well as of some outer- and integral-membrane-bound proteins. Although the nature of the observed proteins has not been yet established, it can be speculated that they represent certain enzymes involved in the infective or anti-infective mechanisms in plants.

It has been documented that polysaccharides isolated from the fungal cell walls induce a specific response in plants that leads to the enhanced resistance of the polysaccharide-treated plants against subsequent infestation with plant pathogens [1–3]. In all fungi, polysaccharides represent a major component of the cell wall accounting for up to 75 % of its dry mass [4]. The major common types of the polysaccharides present in the cell walls of many different fungi are α -D-mannans, β -D-glucans, and chitin, that is covalently linked to (1→3)- β -D-glucan forming a chitin—glucan complex [4, 5]. It has been demonstrated that application of fungal polysaccharides to plants leads to increased production of proteins similar to those involved in the physiological response of the plant to an attack of infectious agents. Thus, chitosan induced accumulation of phytoalexins in tomato [6], fungal (1→3)- β -D-glucans activated production of plant (1→3)- β -glucan hydrolases and phytoalexins [7–9], while application of yeast mannan and glucomannan led to increased levels of peroxidase which resulted in significant inhibition of the number of viral lesions in cucumber and bean [10]. Recently it was shown that in maize application of water-soluble derivative of fungal chitin—glucan resulted in pronounced changes in content of some hydrolytic enzymes, including chitinase and nonspecific esterase, isolated from various parts of the plant [11]. In this paper the results of investigation of an effect of several fungal polysaccharides

on anti-infective resistance, changes in composition of certain intra- and extracellular proteins, and productivity parameters of tomato are presented.

EXPERIMENTAL

All reagents and solvents used were commercial products of analytical purity grade.

The microorganisms used were: *Saccharomyces cerevisiae* (baker's yeast, Slovlik, Trenčín, Slovak Republic), *Candida albicans* (strain 29-3-109, serotype A, Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences), *Aspergillus niger* used for commercial production of citric acid (Biopo, Leopoldov, Slovak Republic), *Fusarium oxysporum* f. sp. *lycopersici* from infected tomato fruits of Slovak provenance isolated by A. Šrobárová.

Relative molecular masses of the polysaccharides were established using high-performance gel permeation chromatography (HPGPC) on Separon HEMA-BIO 1000 columns (Tessek, Prague, Czech Republic) calibrated by a set of pullulan standards (Shodex Standard P-82, Macherey—Nagel, Düren, Germany) [12].

Carboxymethylglucan

Water-insoluble (1→3)- β -D-glucan was obtained from the cell walls of *Saccharomyces cerevisiae* using

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subsequent alkaline and acid extractions and the product was carboxymethylated with monochloroacetic acid in isopropyl alcohol according to a previously described procedure [12]. The degree of carboxymethylation was established by means of potentiometric titration [13] to be 0.56 and relative molecular mass was estimated to be 350000.

Carboxymethyl(chitin—glucan) and Sulfoethyl(chitin—glucan)

The crude chitin—glucan complex was isolated from the mycelium of industrial strain of the filamentous fungus *Aspergillus niger* according to a previously described procedure [11]. Carboxymethylation of chitin—glucan and ultrasonic treatment of the obtained carboxymethylated chitin—glucan were carried out according to *Machová et al.* [14]. Degree of carboxymethylation determined by potentiometric titration was 0.43. Two fractions obtained upon 60-min ultrasonication were separated by size-exclusion chromatography on Sepharose CL-6B (Pharmacia, Uppsala, Sweden) and their relative molecular masses were established by HPGPC [15] to be about 600000 for high-molecular-mass and about 20000 for low-molecular-mass fractions.

Sulfoethylation of chitin—glucan was performed using sodium β -ethyl sulfonate in isopropyl alcohol as previously described [16]. The content of sulfur in the prepared sulfoethyl(chitin—glucan) was 2.30 %, which corresponded to a degree of substitution 0.26. Its relative molecular mass was determined by HPGPC to be about 600000.

Cell-Wall Mannan

Cell-wall mannan was isolated from the collection strain of *Candida albicans* using precipitation with Fehling reagent as previously described [17]. The relative molecular mass of the isolated mannan was 87000.

Tomato Seedlings Infection and Evaluation

Tomato (*Lycopersicon esculenta* L.) seeds (susceptible cultivar Lucy obtained from Novosev, Nové Zámky) were pretreated (5 min, 100 seeds in 100 cm³ of solution) with polysaccharide solution (0.01 % concentration) and infected with *Fusarium oxysporum* f. sp. *lycopersici* after 5 weeks of growing. The fungus was cultivated in a thermostat on potato-dextrose agar (PDA, Sigma) with added streptomycin (0.02 %) and penicillin (0.01 %) at 25°C. After 14 d of cultivation, conidia were removed from aerial mycelium with a scalpel and adjusted using a Burkner chamber and light microscopy.

Pots (20 × 20 cm) containing five plants planted

in commercial garden soil were cultivated in a greenhouse in March. The temperature of 21/15°C and 16/8 day/night light regimen provided by white fluorescent light were maintained. The inoculation with the infective agent and the assay of the results were carried out according to *Kroon and Elgersma* [18]. 0.02 cm³ of a conidial suspension (10⁵ cm⁻³) of the pathogen were injected 1 cm above the cotyledons, one for each of the main vascular bundles of the plants, and covered by a plastic foil. The equal amount of distilled water has been injected into plants of control variant. The above soil part of each plant was sprayed with polysaccharide solution (0.01 %, 5 cm³) starting from the day after the inoculation with the infective agent, twice a day for the whole 10 d of duration of the experiment. The growth and productivity of the above soil part of the plants of each variant was compared with the control plants (noninfected with and without polysaccharide spraying, and infected without polysaccharide spraying but sprayed with distilled water). The mean value from the two measurements is presented. The fresh mass of the above soil parts was estimated by weighing.

Occurrence of disease symptoms was evaluated in 15 plants (two repetitions) of each group according to the index 0—5, where 0 indicated a healthy plant; 1 – implied epinasty of leaves; 2 – wilting of leaves; 3 – yellowing and necrosis of some leaves, wilting of all leaves; 4 – yellowing and necrosis of most of leaves, some leaves fallen, 5 – plant death. The final degree of infestation was calculated as a sum of individual degrees obtained for each plant divided by 15. The spread of the fungus in the plant was determined by placing slices of the stem on PDA with added streptomycin (0.02 %) and penicillin (0.01 %).

Protein Assay

Evaluation of the proteins was performed in tomato leaves (*Lycopersicon esculenta* L.) infected with *Fusarium oxysporum* f. sp. *lycopersici*. Collected leaves were frozen in liquid nitrogen and ground to a fine powder in a cooled mortar. The process of proteins extraction and evaluation was performed as previously described [19]. The powder was homogenized using a homogenizer in an extraction buffer (in they ratio 1:2) (50 mM-Tris (pH 8.0) containing 1 mmol dm⁻³ ethylenediaminetetraacetic acid (EDTA), 1 mmol dm⁻³ dithiothreitol (DTT), and 3 % poly(vinylpyrrolidone) (PVP)). After filtration, the homogenate was fractionated using differential centrifugation, first at 1500 × *g* for 5 min as a cell-wall fraction and supernatant was subsequently re-centrifuged at 14000 × *g* for 10 min followed by ultracentrifugation at 150 000 × *g* for 30 min (Beckman L8-M ultracentrifuge) to obtain the microsomal membrane fraction, while the supernatant represented the cytoplasmic membrane fraction.

Cell-wall proteins

The cell-wall fraction was washed with 40 cm³ of distilled water and re-centrifuged at 500 × *g* for 10 min (three times). Proteins from purified cell-walls were eluted and re-centrifuged with three different solutions to obtain three fractions of the cell-wall proteins: fraction I in 10 mmol dm⁻³ Tris—maleate buffer (pH 7.3) that contained 0.15 mol dm⁻³ NaCl, 1 mmol dm⁻³ EDTA or 10 μg cm⁻³ butylhydroxytoluene (BHT); fraction II in 10 mmol dm⁻³ Tris—maleate buffer (pH 7.3) that contained 1 mol dm⁻³ NaCl, 1 mmol dm⁻³ EDTA and 10 μg cm⁻³ BHT; and fraction III in 10 mmol dm⁻³ Tris—maleate buffer (pH 7.3) containing 1 % SDS. Proteins were obtained from each fraction by precipitation with ice-cold acetone.

Peripheral and microsomal membrane fractions

The pellet was washed with 10 mmol dm⁻³ Tris—maleate buffer (pH 7.3) that contained 0.15 mol dm⁻³ NaCl, 1 mmol dm⁻³ EDTA, and either 10 μg cm⁻³ BHT for fraction I, or the same buffer containing 1 mol dm⁻³ NaCl, 1 mmol dm⁻³ EDTA, and 10 μg cm⁻³ BHT for fraction II, or 0.1 mol dm⁻³ Na₂CO₃ for fraction III. The pellet after the third washing represents an integral-membrane proteins fraction, which was subsequently fractionated into three subfractions according to *Pryde and Philips* [20].

Cytoplasmic proteins

After passing through Sephadex G-25 column and concentration, proteins were fractionated on an anion-exchange column Bio-Scale Q2 in 25 mM-Tris buffer (pH 8.00). Fractions were eluted with a linear 0—1.0 M-NaCl gradient in the same buffer.

Gel electrophoresis

The proteins were precipitated overnight at -20°C with twofold volume of ice-cold acetone. Proteins were solubilized and separated under denaturing conditions on 15—20 % gradient polyacrylamide gel using discontinuous buffer system as described by *Lämmli* [21]. Protein concentration was determined according to *Lowry et al.* [22] using bovine serum albumin (BSA) as the standard.

RESULTS AND DISCUSSION

One of the symptoms observed in the plants affected by biotic or abiotic stress factors is the change in the accumulation of various proteins differing in their localization within the plant cells and their function in the plant organism. Pathogen attack or subjection to harsh stress conditions (such as increased tem-

perature, draught, salinity, *etc.*) also cause evident decrease in plant growth and, as a result, the decreased productivity. Since changes in production of proteins reflect alterations in the expression of certain genes in plants subjected to different types of stresses, investigation of the changes occurring in the protein composition and relative amounts can lead to elucidation of genetic mechanisms of the plant response to stress, while search for the substances that enhance production of certain important proteins may result in the development of novel plant-protective and productivity-boosting agents.

In our early work we have demonstrated occurrence of pronounced changes in the activity of several hydrolytic enzymes during pathogenesis [23, 24]. Later we have shown that treatment of plants with the fungal polysaccharide (chitin—glucan complex) that is ubiquitously present in the cell walls of fungal pathogens elicits in plants a very similar reaction resulting in significant changes of many hydrolytic enzymes (*e.g.* chitinase, β-glucosidase, leucine aminopeptidase, *etc.*) [11]. Thus, it was obvious that prophylactic application of fungal polysaccharides somehow mimicked fungal attack on a plant and evoked reaction somewhat resembling vaccination in mammals, triggering the protective response in the plant organism. Now we have tried to evaluate how fungal polysaccharides may affect productivity parameters of tomato infected with *Fusarium oxysporum* and how does polysaccharide application affect composition of proteins isolated from leave cell walls, cell membranes, and cytoplasm of infected tomato plants.

Five different fungal polysaccharide preparations have been used throughout our study and will be thereafter referred to according to the designation provided in the following description: P1 – carboxymethylglucan prepared from baker's yeast *Saccharomyces cerevisiae* (relative molecular mass 350000); P2 – carboxymethyl(chitin—glucan) prepared from the filamentous fungus *Aspergillus niger* (low-molecular-mass fraction obtained upon ultrasonic treatment, relative molecular mass 20000); P3 – carboxymethyl(chitin—glucan) prepared from the filamentous fungus *Aspergillus niger* (high-molecular-mass fraction obtained upon ultrasonic treatment, relative molecular mass 600000); P4 – sulfoethyl(chitin—glucan) prepared from the filamentous fungus *Aspergillus niger* (relative molecular mass 600000); P5 – mannan from the pathogenic yeast *Candida albicans* serotype A (relative molecular mass 87000). Some of these or similar polysaccharides have been previously shown to efficiently inhibit viral infection caused by tobacco necrosis virus and tobacco mosaic virus in beans and cucumber [25, 26].

The results of evaluation of the action of prophylactic application of the fungal polysaccharides to tomato plants are presented in Table 1.

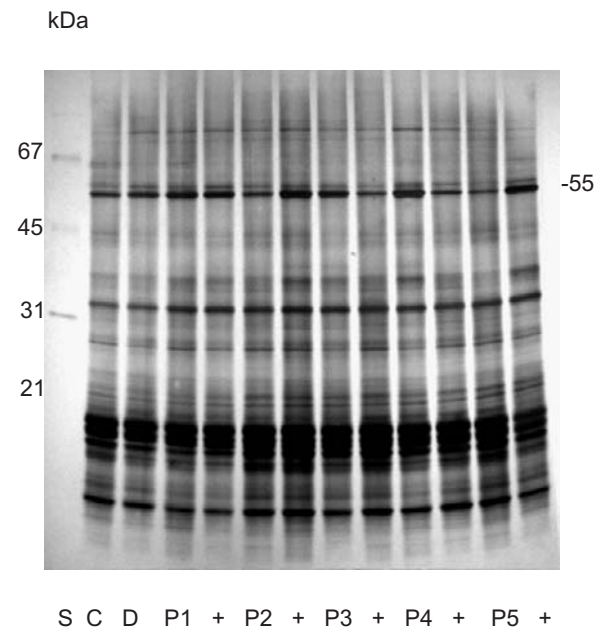
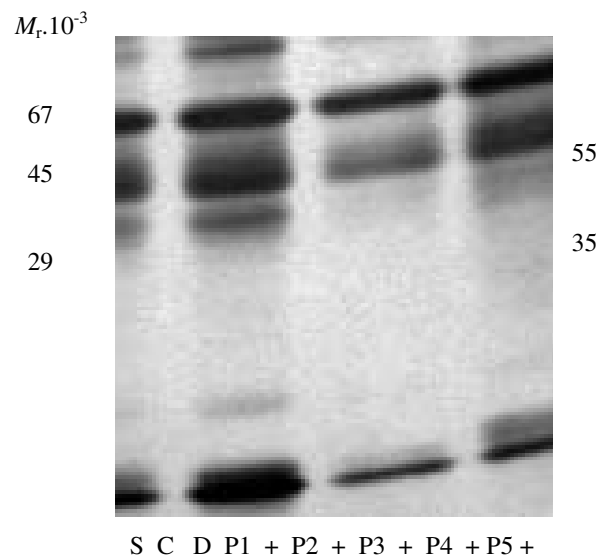
Table 1. Effect of Application of Fungal Polysaccharides to Tomato on the Extent of Infestation with *Fusarium oxysporum* and on the Fresh Mass of the Above Soil Parts of Plants

Experiment model	Infestation		Fresh mass/g	
	Degree	%	Infected	Noninfected
Sprayed with P1	2.0	90	32.90	33.90
Sprayed with P2	1.4	40	36.20	34.23
Sprayed with P3	2.5	100	33.60	33.68
Sprayed with P4	1.0	80	29.22	35.95
Sprayed with P5	3.5	100	26.73	36.15
Noninfected control	0	0		25.24
Infected control	1.6	80	22.99	

As can be seen, upon 10 d of experimental infection, the spread of disease was between 40 % and 100 % of the plants and spraying with certain polysaccharide solution has in fact led to increased incidence and degree of infestation (P1, P3, P5). Application of low-molecular carboxymethyl(chitin—glucan) (P2) resulted in the strong inhibition of infection spread, while sulfoethyl(chitin—glucan) (P4) caused most marked decrease in the degree of infestation in the infected plants. Interestingly, despite equivocal effect of the applied polysaccharides on the effect of infection, all polysaccharides demonstrated clear and pronounced productivity-enhancing effect, which resulted in increased fresh plant mass in both infected and noninfected plants. To our knowledge, this is for the first time that application of fungal polysaccharides is demonstrated to elicit productivity-enhancing effect on the agricultural plant.

Effect of the tested polysaccharides on composition of the tomato leaves cell-wall proteins is shown in Fig. 1. The electrophoregram shows that in the polysaccharide-treated plants the fractions of cell-wall proteins with relative molecular mass (M_r) between 45000 and 67000 were accumulated and their quantity was obviously higher than in the control nonsprayed plants (both noninfected and infected – lanes C and D, respectively). It is known that proteins between 60000—80000 may be involved in the export and assembly of semitrial subunits across the outer membrane [27] or may serve as organic solvent tolerance protein precursors involved in outer membrane tolerance to toxins [28]. It has been described that by increasing the content of cell-wall proteins, polysaccharides can contribute to enhancement of plant cell-wall resistance to pathogens [29, 30].

Analysis of the composition of cytoplasmic proteins revealed (Fig. 2) that application of polysaccharides P4 and P5 resulted in decreased amount of produced cytoplasmic proteins with M_r 45000 and 60000, while a larger amount of the protein with M_r 55000 was produced in the infected plants sprayed with these

**Fig. 1.** SDS-PAGE analysis of cell-wall proteins of tomato infected with *F. oxysporum*. S – standards; C – control plants; D – infected plants nontreated with polysaccharides; P1—P5 – plants sprayed with individual polysaccharide solutions; + – infected plants sprayed with the polysaccharide solution indicated in the column on the left.**Fig. 2.** SDS-PAGE analysis of cytoplasmic proteins of tomato infected with *F. oxysporum*. Designation of the symbols as in the legend to Fig. 1.

polysaccharides. This protein was not detected in the control variant. On the other hand, both used samples of carboxymethyl(chitin—glucan) (P2 and P3) caused increased induction of the proteins with M_r around 35000, not only in the polysaccharide-treated variants, but also in the infected variants.

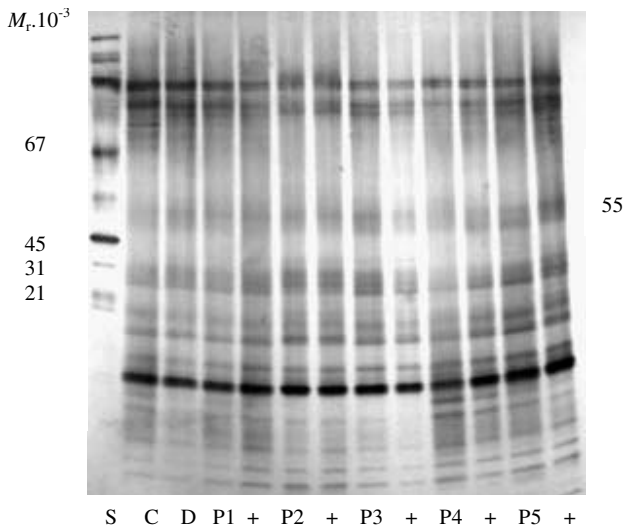


Fig. 3. SDS-PAGE analysis of integral-membrane proteins of tomato infected with *F. oxysporum*. Designation of the symbols as in the legend to Fig. 1.

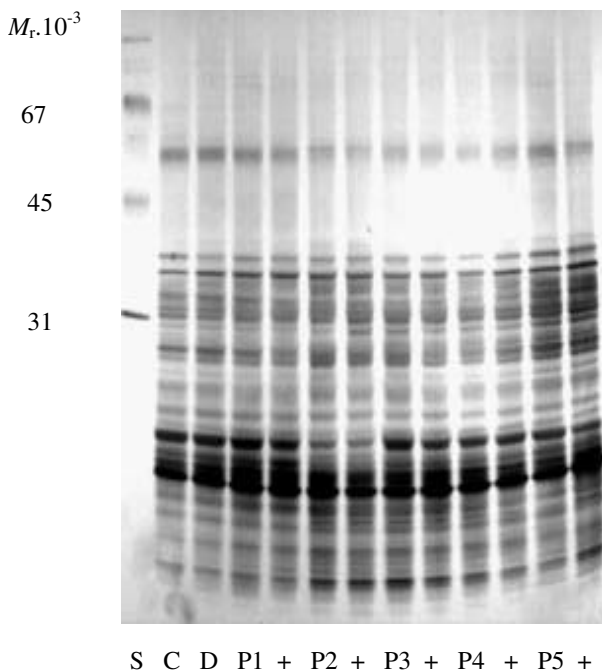


Fig. 4. SDS-PAGE analysis of peripheral-membrane proteins of tomato infected with *F. oxysporum*. Designation of the symbols as in the legend to Fig. 1.

Two integral-membrane proteins (IMP) have been accumulated that were associated with polysaccharide application (Fig. 3). The one with the M_r between 50000 and 60000, namely around 55000, detected in all variants may probably represent a hypothetical fusaric acid resistance protein Fus A [31] or may be associated with the efflux pumps that can protect cells from a range of toxic compounds [32], or may be simply associated with the disease process. The used strains

of *F. oxysporum* produced fusaric acid both *in vitro* and *in vivo* [33]. The only other significant difference in protein accumulation was observed in the quantitative content of a protein with M_r around 21000 between the samples treated and nontreated with the polysaccharides.

Higher content of the two peripheral-membrane proteins (PMP) with the M_r around 29000 and 45000 was detected upon the treatment with the polysaccharides, especially with P3 and P4 (Fig. 4). However, no correlation between these proteins and protective action was observed, since while P3 increased the degree of infestation, P4 has caused its decrease. In the case of PMP, less visible differences were observed among the plant samples treated with different polysaccharides.

CONCLUSION

The results obtained in our study indicate that in general, application of fungal polysaccharides induces accumulation of some proteins which can be involved in strengthening, stabilizing, or accelerating the recovery of the plant cell membranes damaged as a result of the action of metabolic products of pathogenic fungi. Some of these as well as of the previously described proteins may possess enzymatic activity related to resistance of the plants or may be involved in a plant response to infection by the agents of fungal and viral origin. Thus, the observed activity of the fungal polysaccharides may serve as a foundation for future study of the plant—pathogen relationships, their mechanisms, and potential implementation in design of plant growth-stimulating substances on the basis of microbial polysaccharides.

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