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Defence responses against TNV infection induced by galactoglucomannan-derived oligosaccharides in cucumber cells

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Abstract

Galactoglucomannan-derived oligosaccharides (GGMOs) showing biological activity in growth, morphogenesis and cell viability were tested in a host pathogen interaction. As a model system, cucumber (*Cucumis sativus* L. cv. Laura) reacting hypersensitively to tobacco necrosis virus (TNV) was used. The defence reactions were dependent on the degree of polymerisation and concentration of oligosaccharides, as well as on the time of application of virus to plant cotyledons. Disease symptoms were inhibited by 60–75%. The average number of lesions per cotyledon was significantly decreased when oligosaccharides were used simultaneously or 24 h prior to virus inoculation. Significant changes in peroxidase, β-glucanase and chitinase activities accompanied the defence reaction. It can be concluded that oligosaccharides derived from spruce galactoglucomannan induce non-specific resistance to local viral infection in plants. GGMOs probably act as inhibitors of the virus infection, rather than inhibitors of direct virus multiplication.

Introduction

Plants and animals have evolved signalling mechanisms to regulate the expression of genes that are essential for their growth, development and defence against pests. In some of these signalling mechanisms oligosaccharides, released from cell wall polymers, are involved (Albersheim et al., 1992).

The first oligosaccharides identified as elicitors of defence responses in plant cells were of fungal origin – glucans, chitosans and chitin oligosaccharides (Coté and Hahn, 1994). Their size, polyanionic character, and molecular shape appear to be important features for the biological activity of oligosaccharides acting in defence responses of plant cells, which sometimes occur at exceedingly low concentrations (Aldington et al., 1991). Oligogalacturonides, released from pectins of the plant cell wall can be involved in plant defences, as well as processes of plant growth and development under the control of phytohormones (Marfá et al., 1991; Bellincampi et al., 1993). A cell-wall-derived oligosaccharide with antiauxin activity is xyloglucan nonasaccharide (XXFG), which is formed *in vivo* by the partial cleavage of cell wall xyloglucan, catalysed by hydrolases and/or endotransglycosylases (Fry, 1996). However, xyloglucan-derived oligosaccharides are also reported to influence defence reactions and related enzymes (Šubíková et al., 1994; Slováková et al., 1994).

Thus, some of defence processes, such as hypersensitive response (HR), can be induced by the above mentioned substances (Conrath et al., 1989; Lesney, 1990; Slováková et al., 1994). The HR is a frequent reaction of plants that prevents the spreading of the invader,

and is accompanied by a rapid and localised necrosis of the infected tissue. Hypersensitivity is a complex biochemical process, which includes the synthesis and appearance of new host-coded proteins and other substances (Masuta et al., 1991). Further changes are connected with the increased synthesis and secretion of specific enzymes including chitinase (Boller et al., 1983), 1,3-β-glucanase (Moore and Stone, 1972), and peroxidase (Hammerschmidt et al., 1982). Increased activity of peroxidase is associated also with induced systemic resistance in cucumber to a variety of pathogens and in tobacco to tobacco mosaic virus (TMV) (Ye et al., 1990). Both chitinase and β-1,3-glucanase are coordinately synthesised as a defence response since they split complementary linkages of pathogen derived polysaccharides (Hughes and Dickerson, 1991). The supposed role of acidic isoforms of the β-1,3-glucanases and chitinases secreted into extracellular spaces of plants is to degrade the pathogen cell wall leading to the destabilisation of the organism (Van den Blucke et al., 1990; Ji and Kuc, 1996). The presence of these enzymes was largely correlated with pathogen distribution (Benhamou et al., 1990). Another role of these isoforms is associated with the release of elicitors which probably serve as information to the plant about the presence of pathogens resulting in distribution of plant metabolic energy towards the resistance mechanism (Van den Blucke et al., 1990).

Oligosaccharides derived from poplar galactoglucomannan are biologically active in plant cell elongation, differentiation and cell viability (Auxtová et al., 1995; Lišková et al., 1995; Auxtová-Šamajová et al., 1996). Similar data were obtained with spruce galactoglucomannan-derived oligosaccharides (GGMOs) (unpublished). From this point of view it was of interest to examine the activity of these oligosaccharides in the induction of defence responses in the model system cucumber–tobacco necrosis virus (cucumber–TNV).

Materials and methods

Plant and virus material

Cucumber (*Cucumis sativus* cv. Laura) plants were grown from seeds in a greenhouse under controlled conditions (23±1 °C, 16 h illumination, 80 μmol m^{-2} s^{-1} irradiance, and relative air humidity 60%; the dark period – 8 h, 18 ± 1 °C, relative humidity 65%).

A spindle tree (*Euonymus europaeus* L.) isolate of TNV was purified by differential and density gradient centrifugation (Šubíková, 1994). Cucumber cotyledons were inoculated with virus (500 μg ml^{-1}) by the use of a leaf rub method with carborundum as an abrasive.

The number of local lesions, 3–4 days after TNV inoculation was statistically determined by Student's *t*-test and ANOVA. The percentage of disease symptom inhibition was calculated according to Kopp et al. (1989).

Isolation of the galactoglucomannan

A modified procedure of Mills and Timell (1963) for the isolation of galactoglucomannan from spruce secondary cell walls was used (Karácsonyi et al., 1996). The delipidized (benzene–methanol) and delignified (sodium chlorite) sawdust (25 g) prepared from the trunk of spruce (*Picea abies* L. Karst) was successively extracted, twice (in all following steps) with boiling water (250 ml) for 3 h, 0.5% aqueous ammonium oxalate (250 ml) for 3 h at 60 °C, dimethyl sulphoxide (250 ml) for 48 h at laboratory temperature, aqueous 15% potassium hydroxide (250 ml) containing 10 mM solution of $NaBH_4$ for 2 h at laboratory temperature, and aqueous 17.5% sodium hydroxide containing 4% boric acid (250 ml) and 10 mM solution of $NaBH_4$ for 2 h at laboratory temperature. The final alkaline extracts were combined, neutralised with acetic acid, dialysed, and freeze-dried (Capek et al., 1998).

Partial acid hydrolysis of the galactoglucomannan – source of oligosaccharides

Galactoglucomannan was partially depolymerised with 0.4 M trifluoroacetic acid for 70 min at 100 °C. Trifluoroacetic acid was evaporated and a mixture of mono- and oligosaccharides was separated on a column (200 × 2.5 cm) of Bio-Gel P-2 by water elution. Fractions (4 ml) were collected and analysed for the carbohydrate content by phenol–sulphuric acid assay (Dubois et al., 1959). The elution profile of GGMOs is shown in Figure 1. Their degree of polymerisation (DP) was identified by comparison with the elution volumes of malto-oligosaccharides (Serva, Germany) used as a reference standard.

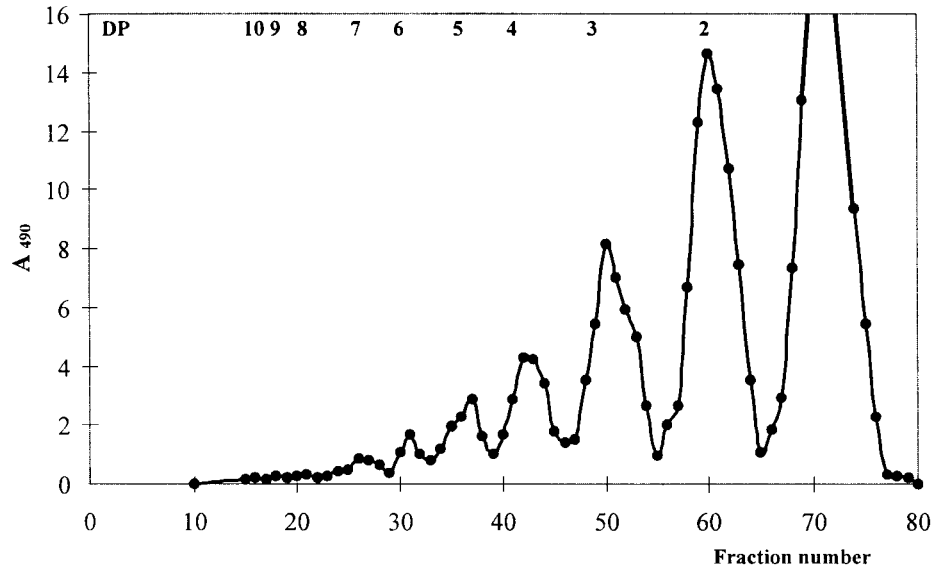

Figure 1. Gel filtration pattern (Bio-Gel P2) of GGMOs.

Monosaccharide analysis

The monosaccharides and oligomers derived from galactoglucomannan were identified after total hydrolysis with 2 M trifluoroacetic acid for 1 h at 120 °C and reduction, in the form of their trifluoroacetates (Shapira, 1969) by gas chromatography on a Hewlett-Packard Model 5890 Series II instrument equipped with a PAS 1701 column (0.32 mm × 25 m) at the temperature program of 110–125 °C (2 °C/min) to 165 °C (20 °C/min) and flow rate of nitrogen 20 ml/min.

Application of GGMOs

Oligosaccharide fractions DP 3, 4, 5, 6–7 (not separated), and MIX (mixed oligomers DP 3–7) were applied with glass pad to the surface of cucumber cotyledons in water solution 24 h before, simultaneously, or 2 and 4 h after virus inoculation at 5, 50, 500 and 5000 mg l^{-1}. In control treatments only distilled water was applied.

Determination of enzyme activities

Water extracts from intercellular spaces were prepared by modified Klement's (1965) method (Slováková et al., 1994). The enzyme activity was determined after 0, 24, 48, 72, 96 and 120 h of GGMO treatment.

For total proteins the method of Bradford (1976) was used. Peroxidase activity was determined by the method of Frič and Fuchs (1970), β-1,3-glucanase activity by that of Tuzun et al. (1989), and chitinase by that of Wirth and Wolf (1990).

All experiments were repeated at least three times, with a total of at least 25 replicates.

Results

The tentative structure of the galactoglucomannan of spruce secondary cell walls (Figure 2) shows an irregular main chain with side chains arranged at random. This indicates great structural variability which was confirmed by their monosaccharide composition (Table l).

Timing of GGMOs application

The GGMOs MIX were most effective when applied simultaneously with TNV inoculum (0 h) (Figure 3). The number of local lesions significantly decreased compared with the control, and the inhibition of disease symptoms varied from 60% to 75%. Application of these oligosaccharides 24 h before inoculation of TNV also resulted in a significant decrease of lesion number, but the inhibition of disease symptoms was

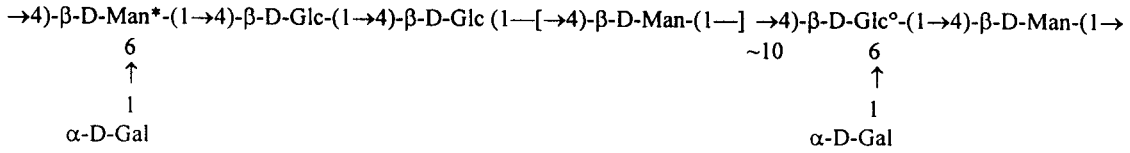

Figure 2. Tentative structure of the spruce galactoglucomannan. About each 33rd D-mannose (Man*) and 66th D-glucose (Glc°) unit is substituted with monomeric D-galactose residue.

Table 1. DP and sugar composition of GGMOs

Oligomer DP	Mole ratio		
	Gal	Glc	Man
3	1.0	9.2	66.1
4	1.0	8.6	40.4
5	1.0	7.1	35.4
6–7	1.0	4.9	31.1
4–8	1.0	5.0	14.4

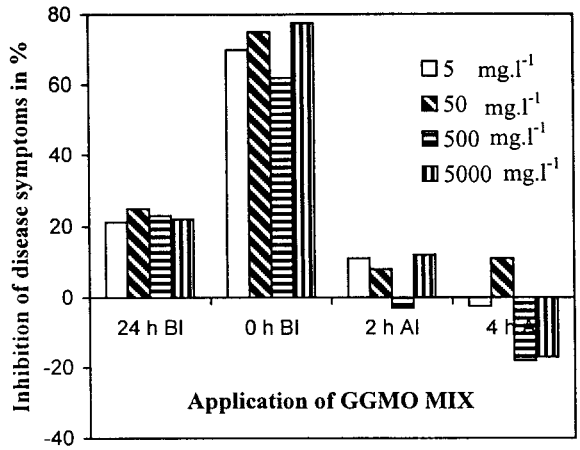

Figure 3. Effect of GGMOs MIX (applied to cucumber cotyledons) on TNV disease symptoms. GGMOs were applied 24 h before TNV inoculation (BI), together with TNV (0 h BI), and 2 h and 4 h after TNV inoculation (AI) at different concentrations. Inhibition of disease symptoms in % was calculated as described in Materials and methods.

less than that achieved with simultaneous application of GGMOs and TNV (0 h). The treatment of cotyledons with GGMOs 2 and 4 h after the inoculation with TNV did not affect disease symptom development. The effect of oligosaccharides decreased with their prolonged application time after TNV inoculation. No significant differences between concentrations used were observed (Figure 3).

Biological activity of separated GGMO fragments

Application of GGMOs DP 3 (trisaccharides) to the surface of cucumber cotyledons 24 h prior to TNV inoculation resulted in significant decrease of average lesion number per cotyledon compared with control except at a concentration 500 mg l^{-1} (Figure 4). The inhibition of disease symptoms varied between 20% and 45% (Figure 5). The effect of various concentrations of oligosaccharides DP 3 is shown in Table 2. The biological activity of GGMOs DP 4 (tetrasaccharides) was not significant (Figures 4 and 5). Treatment of cucumber cotyledons with GGMOs DP 5 (pentasaccharides) at 50 mg l^{-1} and 500 mg l^{-1} inhibited the disease symptoms from 27% and 37%, respectively (Figure 5). The highest inhibition of disease symptoms induced by GGMOs with the DP 6–7 (hexa- and heptasaccharides) was observed at the lowest concentration (Figure 5). Similar effects to DP 3 oligosaccharides were obtained with GGMOs MIX (Figure 4). On the basis of the results obtained it is evident that except for DP 5, the most effective concentration applied was 5 mg l^{-1}.

A comparison of the GGMOs fractions and their effect on defence reactions of cucumber cotyledons to TNV is summarised in Table 2.

Influence of GGMO fragments on PR-proteins

Based on foregoing results, in this study only the most effective GGMO fragments and concentrations were used. Application of 5 mg l^{-1} GGMOs DP 3, 6–7, or MIX 24 h prior to TNV inoculation induced changes in studied peroxidase, glucanase and chitinase activity involved in HRs of cucumber cotyledons.

Peroxidase activity, measured in the extracellular space of GGMOs-treated cotyledons, oscillated between 75% and 130% compared with the healthy control. Differences in the activity of peroxidase in GGMOs-treated and control plants were higher after TNV inoculation (Figure 6A). In comparison to the TNV-inoculated cotyledons, the activity of peroxidase

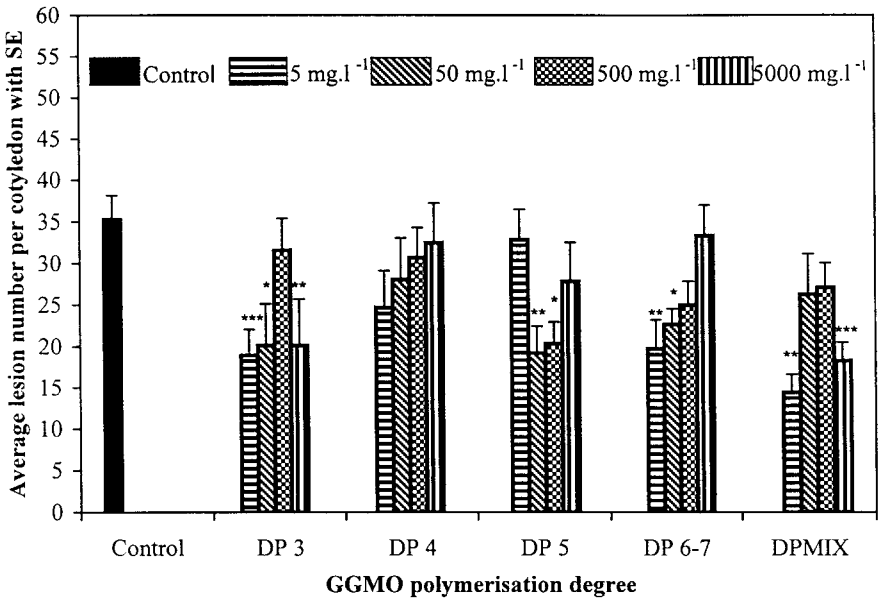

Figure 4. Effect of GGMOs DP 3, 4, 5, 6–7, and MIX 4–8 (applied to cucumber cotyledons 24 h before TNV inoculation) on average lesion number in comparison with control (untreated TNV inoculated cotyledons). Significance: $*** - p \leq 0.001$, $** - p \leq 0.01$, $* - p \leq 0.05$. The graphic expression of average lesion number is one characteristic experiment from six independent experiments.

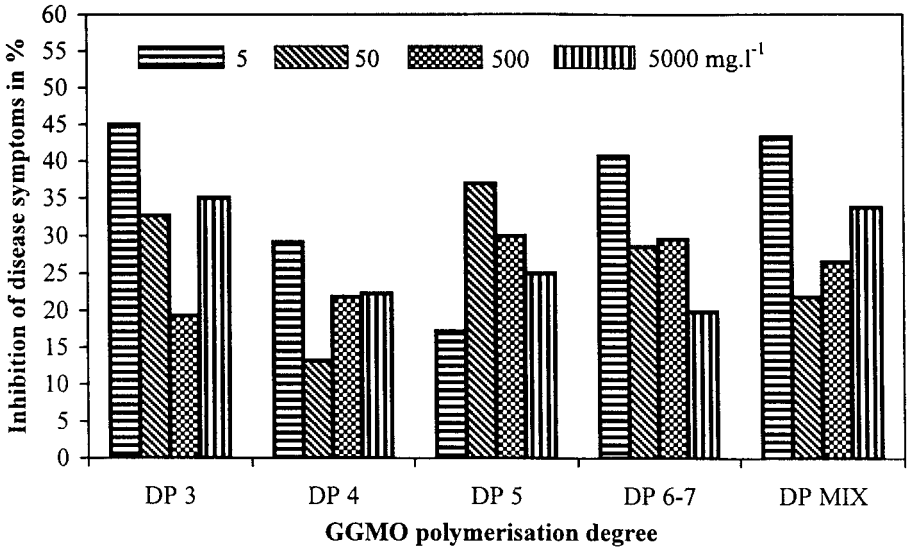

Figure 5. Effect of GGMOs DP 3, 4, 5, 6–7, and MIX 4–8 (applied to cucumber cotyledons 24 h before TNV inoculation) on inhibition of disease symptoms. Inhibition of disease symptoms in % was calculated as described in Materials and methods (average of six independent experiments.)

in GGMOs-treated cucumber cotyledons decreased. Ninety-six hours after inoculation this activity rapidly decreased to 10% and 40% of the activity in the infected control (Figure 6B).

Application of all three GGMOs to cucumber cotyledons resulted in an increase of extracellular β-1,3-glucanase activity compared with the healthy plants 24 h after application. Oligosaccharides of DP 3

Table 2. Effect of different DP of GGMO applied to cucumber cotyledons 24 h prior to TNV inoculation on necrotic local lesion number

DP of GGMO	Concentration (mg l^{-1})	Multifactorial ANOVA multiple range analysis 95% LSD intervals											
		Experiment no. 1 (October 10 1997)[c]				Experiment no. 2 (October 21 1997)				Experiment no. 3 (March 15 1998)			
		Average[a]	Standard error	Variance	Homo-geneous group[b]	Average	Standard error	Variance	Homo-geneous group	Average	Standard error	Variance	Homo-geneous group
3	5	18.9	3.15	109.69	***	31.30	2.87	82.45	*****	12.50	3.13	98.55	***
	50	20.1	5.0	250.1	******	31.12	6.55	342.55	*****	14.20	2.45	60.40	***
	500	31.60	3.82	146.48	***	39.22	7.25	473.94	****	11.50	36.27	2.00	***
	5000	20.12	5.56	247.55	***	20.12	5.56	247.55	**	8.70	1.15	13.34	***
4	5	24.66	4.44	236.96	***	51.12	4.30	148.12	*	13.22	1.96	34.96	***
	50	28.00	5.08	258.44	*******	50.75	4.97	249.64	*	14.60	5.44	296.48	**
	500	30.70	3.62	131.56	****	35.88	4.97	247.95	****	14.50	2.02	40.94	*
	5000	32.50	4.72	267.90	**	35.50	7.13	508.72	****	11.80	1.79	32.17	***
5	5	32.83	3.66	161.60	******	40.00	4.84	234.88	****	12.20	3.33	111.06	***
	50	19.16	3.22	124.51	***	50.20	6.08	370.17	*	12.10	2.65	70.54	***
	500	20.30	2.60	68.01	***	36.60	4.50	202.93	*****	8.00	2.00	40.00	*
	5000	27.81	4.72	245.36	******	29.90	3.32	110.54	****	11.20	2.10	44.84	***
6–7	5	19.70	3.51	123.34	***	24.00	3.02	91.55	**	7.80	0.87	7.73	*
	50	22.66	1.88	42.42	******	27.18	3.60	142.56	***	9.60	1.62	26.48	***
	500	23.00	2.85	81.33	*******	33.60	5.95	355.15	*****	8.00	1.02	10.44	*
	5000	33.37	3.64	106.26	**	43.40	5.86	344.26	***	12.00	2.21	49.11	***
MIX	5	14.41	2.23	60.08	*	21.20	3.95	156.40	*	8.10	1.03	11.25	**
	50	26.3	4.92	242.67	******	40.30	6.25	392.45	****	9.75	1.25	15.51	***
	500	27.10	3.64	146.09	******	32.90	3.64	146.09	*****	9.50	1.40	19.87	***
	5000	18.25	2.28	62.56	**	34.70	3.87	150.45	*****	11.25	1.78	31.45	***

[a] Average lesions number per cotyledon; [b] asterisks present evaluation by ANOVA multiple range analysis 95% LSD intervals; [c] the date indicates the beginning of the experiment which took 10 days.

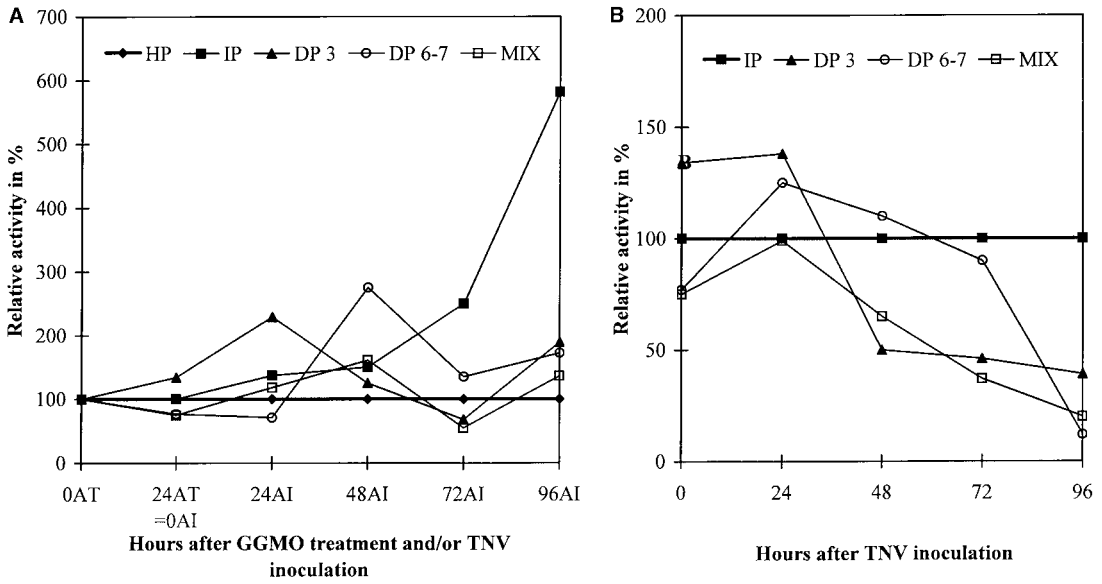

Figure 6. Changes in peroxidase activity in the fluid from intercellular space of GGMOs-treated cucumber cotyledons (24 h prior to TNV inoculation) compared with A – untreated healthy plants (100%) and B – TNV-inoculated plants (100%). HP – healthy plants, IP – untreated plants inoculated with TNV, DP – degree of GGMOs polymerisation 3, 6–7, and MIX (mixed oligomers of DP 3–7), AT – hours after GGMOs treatment, AI – hours after TNV inoculation, cotyledons were inoculated with virus 24 h after treatment with GGMOs.

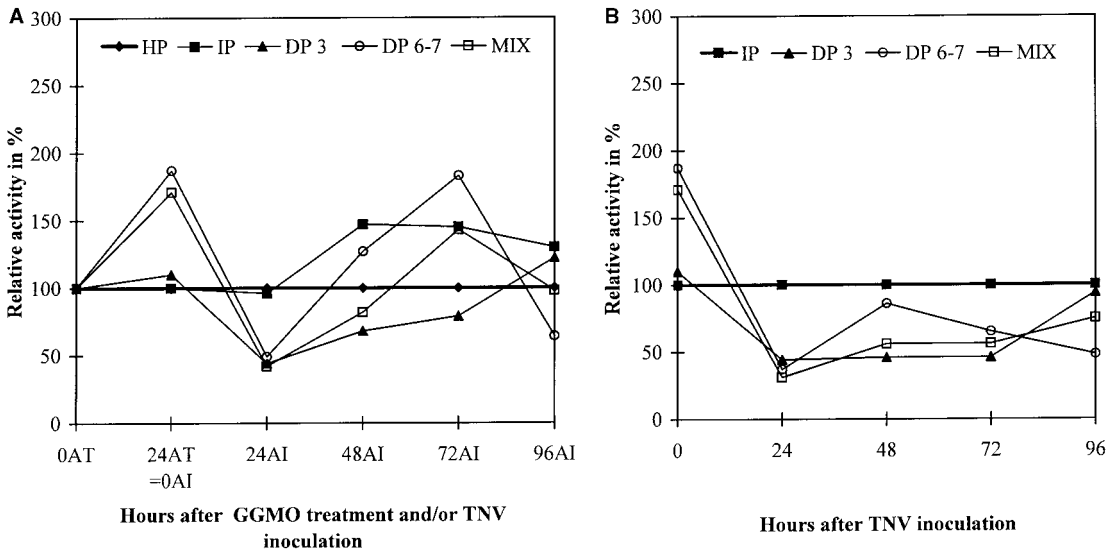

Figure 7. Changes in β-1,3-glucanase activity in the fluid from intercellular space of GGMOs-treated cucumber cotyledons (24 h prior to TNV inoculation) compared with A – healthy plants (100%) and B – TNV-inoculated plants (100%). For expansion of abbreviations see caption of Figure 6.

increased the enzyme activity to 7%. In the other two applications (DP 6–7 and MIX), the activity varied between 160% and 180% of the healthy plants (Figure 7A). Rapid changes in enzyme activity were observed 24 h after TNV inoculation. The activity decreased after the use of all three GGMOs similarly to 50% of healthy plants. Seventy-two hours of TNV inoculation β-1,3-glucanase activity increased in samples

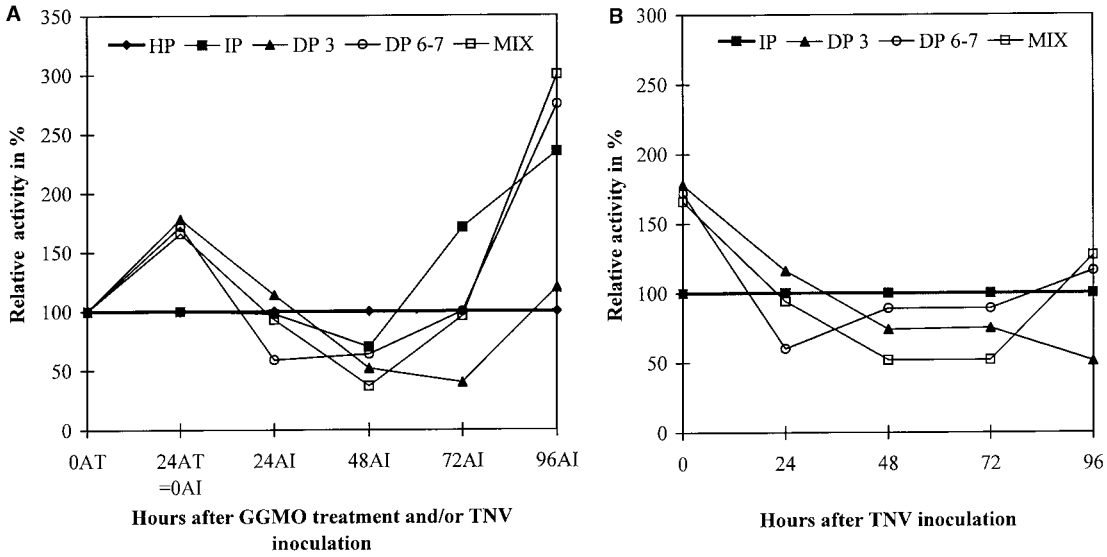

Figure 8. Changes in chitinase activity in the fluid from intercellular space of GGMOs-treated cucumber cotyledons (24 h prior to TNV inoculation) compared with A – healthy plants (100%) and B – TNV-inoculated plants (100%). For expansion of abbreviations see caption of Figure 6.

treated with oligosaccharides DP 3 and reached 115% of the healthy plants, while in the other two samples (DP 6–7, and MIX) an increase of the activity from 120% to 160% was observed (Figure 7A). Compared with the TNV inoculated, but untreated cotyledons, the glucanase activity rapidly decreased by 40% within 24 h after TNV inoculation, and the values were less than in the diseased plants by between 40% and 85% (Figure 7B).

Chitinase accumulation in the extracellular space of cucumber cotyledons increased by about 60% after treatment with all three GGMO derivatives compared with untreated uninoculated cotyledons. After TNV inoculation, a decrease of this activity (after 48 h of TNV inoculation) to about 50% was observed. Application of oligosaccharides DP 6–7 and MIX, 96 h after TNV inoculation, caused rapid changes in the chitinase activity which reached 250% of the healthy control (Figure 8A). Enzyme activity in GGMOs-treated cotyledons compared with TNV inoculated control decreased during the whole experiment (Figure 8B).

Discussion

The biological activity of GGMOs on the hypersensitive reaction of cucumber cotyledons to TNV infection was studied. Results presented show that application of GGMOs derived from spruce cell walls induced a decrease in local lesion number on cucumber cotyledons induced by TNV infection. Plant virus inhibitors can be divided into two groups: inhibitors of virus multiplication which prevent virus replication and spreading when applied to leaves already infected, and inhibitors of virus infection which prevent virus infection when applied before or simultaneously with the virus (Bawden, 1954; Hirai, 1977). Since GGMOs significantly inhibited the disease symptoms when applied to cucumber cotyledons before or together with virus inoculum, it is probable that the virus infection was influenced during the early stages of infection. We therefore suggest that these oligosaccharides belong to the second group of virus inhibitors.

For plant virus inhibiting activities of poly- and oligosaccharides DP is one of the very important attributes. A water-soluble β-(1→3)-linked D-glucan, with an average DP of 23 glucose units with a single branching point, derived from *Phytophthora infestans* mycelium, completely inhibited local lesion development of potato virus Y on *Nicotiana tabacum* Samsun without inhibition of virus multiplication (Singh et al., 1970; Wood et al., 1971). Generally, oligosaccharides able to induce a biological response have a DP higher than 4 (Darvill et al., 1992). Oligogalacturonides released from plant cell walls

by pathogen endopolygalacturonases e.g., elicited defense-related responses in various plants only, if the number of galacturonic acid residues was not lower than 10 (Cervone et al., 1989; Mathieu et al., 1991).

In contrast to these observations, in our experiments, one of the most effective substances besides the oligosaccharides of DP 6–7 and the MIX, was the fragment DP 3. GGMOs from the secondary cell walls of spruce (*Picea abies* Karst) wood consist of a backbone of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues distributed at random, having single stubs of (1→6)-linked α-D-galactopyranosyl residues attached to both mannosyl and glucosyl residues, but with slightly preferable substitution of mannosyl residues. It seems that the biological activity of GGMOs tested depends not only on the mutual proportions of the individual monosaccharide components building up their backbone or degree of side branching at the position O-6 of D-glucose and D-mannose units, but also on the conformation shape of oligosaccharide molecules.

The extracellular fluid of cucumber after TNV inoculation contains three acidic isozymes of peroxidase (Repka and Slováková, 1994). They could play a role in at least four defence-related events that occur in the extracellular matrix: generation of H_2O_2 which can be the source of antifungal activity, suberin synthesis (Mohan and Kolattukudy, 1990; Peng and Kuc, 1992), lignin synthesis (Lagrimini and Rothstein, 1987), and formation of intermolecular linkages (Fry, 1986). Rapid accumulation of new anionic peroxidase isoenzymes in the first 24 h was detected in soyabean cotyledons after treatment with cell wall glucan derived from *Phytophthora megasperma* (Graham and Graham, 1991). In contrast to these results, no significant changes in extracellular peroxidase activity were detected in our experiments with all GGMOs fractions 24 h after treatment.

The rapid increase of peroxidase activity after inoculation of TNV to GGMOs-treated cotyledons reached a maximum after 24–48 h. In this time, local lesions as a consequence of hypersensitive reaction were not visible. In comparison to this, the peroxidase activity in healthy and untreated plants, was lower and the increase in activity started after this time. Our observations support the conclusions of Fritig et al. (1987) that in early stages of infection the activity of phenylpropanoid pathway, in which peroxidase is also involved, is increased to supply precursors of lignin just before appearance of lesions. Application of GGMOs probably affected the early stages of hypersensitive reaction. In late stages of infection, when local lesions became visible, the activity of peroxidase in treated cotyledons decreased compared with the infected control.

The increase of glucanase and chitinase activity in our experiments 24 h after treatment was not dependent on the size of oligosaccharides. Successive decrease of their activity after TNV inoculation compared with the control is probably a consequence of lower lesion number on treated cotyledons. Elicitation of chitinase and glucanase activity was probably an expression of non-specific defence reaction. In many cases, these two enzymes are coordinately synthesised as a defence response since they hydrolyse complementary linkages in pathogen cell wall polysaccharides (Hughes and Dickerson, 1991).

The stimulation of release and accumulation of peroxidase, glucanase and chitinase into the extracellular fluid was similar to that found in our previous work with xyloglucan oligosaccharides (Slováková et al., 1994).

From the results presented, it can be concluded that GGMOs derived from spruce cell walls can induce a non-specific resistance in plants to local viral infection on leaf surfaces. This induction is probably accompanied by changes in plant metabolism resulting in accumulation of PR-proteins in early stages of infection which can prevent infection. On the contrary to this, Kopp et al. (1989) pointed out that glucan-induced resistance of tobacco against viruses does not depend on the induction of PR-proteins. The induced resistance by GGMOs is manifested by the decrease of local lesion number. It can be suggested that there is also inhibition of virus entry by modifying infectible sites on the leaf surface as described by Singh et al. (1970) and Wood et al. (1971). GGMOs probably act as inhibitors of virus infection rather than inhibitors of direct virus multiplication.

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