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Induction of β-1,3-Glucanase in Callus Cultures *in vitro*

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Abstract—Sodium salicylate (NaSA) increased induction of both intracellular and extracellular β-1,3-glucanases in calluses of campion and duckweed. NaSA concentrations from 30 to 100 mM were optimal for induction of intracellular glucanase in the campion callus, and for induction of extracellular glucanase the optimal concentration varied from 5 to 100 mM. The glucanase activity in the duckweed callus was lower than in the campion callus, and co-cultivation of the campion callus with *Trichoderma harzianum* mycelium increased the production of intracellular and extracellular β-1,3-glucanases and polygalacturonase in the callus. Biosynthesis by *T. harzianum* of glucanases, extracellular polygalacturonase and xylanase, and of intracellular galactosidase was increased. The co-cultivation was accompanied by increased activity of intracellular acidic isoform of glucanase Glu-3 secreted by the callus cells into the medium, whereas NaSA activated in the callus culture the extracellular acidic isoform Glu-1 and extracellular basic isoform Glu-5. These data indicate the induction of these isoforms and the specificity of protective response of plant cells to different factors.

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Key words: *Silene vulgaris* (M.) G., *Lemna minor* L., *Trichoderma harzianum*, callus, β-1,3-glucanase, polygalacturonase, sodium salicylate

Plants respond to infection with phytopathogenic fungi by triggering complex defense mechanisms [1]. The defense mechanisms include modification of cell walls (lignification and formation of callose), production of reactive oxygen species, and biosynthesis of phytoalexins and pathogenesis-related proteins (PR-proteins) [1-3]. PR-proteins are represented by chitinases and β-1,3-glucanases capable of hydrolyzing chitin and β-1,3-glucans, which are structural components of cell walls of phytopathogens and inhibit phytopathogen growth [4, 5]. β-1,3-Glucanases isolated from various plant species are usually represented by multiple molecular forms with different activities, physicochemical properties, and subcellular locations [6, 7]. Based on the structural similarity and differences in the amino acid sequences, the isoforms are subdivided into acidic and basic proteins. The basic proteins are located in vacuoles and are involved in the normal development of healthy plants but can be induced in response to high concentrations of pathogens causing lysis of plant cell walls [8]. The acidic proteins are mainly extracellular. Some of them are considered to be pathogen-induced PR-proteins of plant defense. In response to biotic and abiotic stress factors, including pathogenic ones, a gene or a number of genes encoding some isoforms of β-1,3-glucanase can be overexpressed. Additional isoforms can also be synthesized and modified post-translationally [7].

Along with fungal, bacterial, and viral infections such exogenous chemical reagents as salicylic acid, sodium salicylate, polyacrylic acid, inorganic salts, chitosan (a component of fungal cell walls), and ozone as a source of reactive oxygen species can induce generation of extracellular proteins and resistance to a subsequent infection by phytopathogens [9]. Salicylic acid and its synthetic derivative benzothiadiazole effectively induce both local and systemic resistance to pathogens. Benzothiadiazole induces resistance to some pathogens in arabidopsis, wheat, tobacco, and sugar beet. Salicylic acid is a crucial signaling component that activates PR-genes [10]. The content of salicylic acid increases in many plants in response to infection with fungi, bacteria, and viruses, and also to abiotic stress (ozone, ultraviolet). Addition of salicylic acid allows researchers to model certain aspects of infection with a pathogen, in particular, activation of

Abbreviations: NaSA) sodium salicylate; PG) polygalacturonase; PR-proteins) pathogenesis-related proteins.

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PR-genes and biosynthesis of β-1,3-glucanases [10]. Induction of β-1,3-glucanase synthesis in response to bacterial and fungal infections has been intensively studied in dicotyledonous plants, whereas data for monocotyledonous plants are rather scarce.

Plant PR-proteins are interesting also in connection with food allergy because they are sources of plant food allergens [11]. Production by biotechnological methods of food allergens (β-1,3-glucanases) can be used for studies on food allergy.

In the present work callus cultures of the campion *Silene vulgaris* (M.) G. (*Oberna behen* (L.) Ikonn) and the duckweed *Lemna minor* L. were used as models for induction of β-1,3-glucanase in cells of dicotyledonous and monocotyledonous plants.

The purpose of this work was to study the effect of sodium salicylate on induction of β-1,3-glucanase in calluses of campion and duckweed and also to investigate the production of β-1,3-glucanase on co-cultivation of the campion callus with *Trichoderma harzianum*.

MATERIALS AND METHODS

Callus culture cultivation. Callus cultures of campion and duckweed were grown on a modified Murashige and Skoog medium [12]. The campion and duckweed calluses were sub-cultivated at 27 ± 1°C in the dark at the interval of 21 and 28 days, respectively.

Treatment with inducer. The callus was treated with sodium salicylate (NaSA) (Diaem, Russia) as an inducer in two ways. In the first way, the campion callus was cultured on medium containing 10, 20, 30, 40, and 100 mM NaSA, and the inducer-free medium was used as a control.

In the other way, the campion callus was sprayed with a sterile solution of the inducer at the exponential phase of the cell growth (16th day), and the enzyme activities were determined in the stationary phase (21st day). The concentrations of NaSA were 2.5, 5, 10, 20, 30, 50, and 100 mM. Distilled water was used in the control. The duckweed callus was sprayed with NaSA (50 and 100 mM) in the exponential phase of cell growth (21st day), and the enzyme activities were determined in the stationary phase (28th day).

Co-cultivation of *S. vulgaris* callus and *T. harzianum* mycelium. Ten campion calluses were planted circumferentially onto solid nutrient medium in Petri dishes. The cells were grown for 7 days at 27°C in a thermostat. On the 8th day, 0.1 ml of suspension containing $3.8 \cdot 10^7$ spores of *T. harzianum* was inoculated into the center of the Petri dish. The callus and mycelium were co-cultivated for 4 days until the mycelium came into contact with the callus. The β-1,3-glucanase activity was determined on the 11th day of the callus growth. The results were analyzed using two controls: first, the callus grown alone, and second, the mycelium grown alone.

General analytical methods. The protein content was determined by the Lowry method [13]. Spectrophotometry was performed using an Ultrospec 3000 apparatus (Pharmacia Biotech, Great Britain).

Contents of reducing sugars were determined as described in [14]. Glucose, D-galacturonic acid, and xylose, respectively, were used as standards for calibration curves on determination of activities of β-1,3-glucanase, polygalacturonase (PG), and xylanase. The enzyme quantity which released 1 μmol reducing sugars per min under the experimental conditions was taken as the activity unit of β-1,3-glucanase, PG, and xylanase.

Analysis of carbohydrases. Wet biomass was homogenized in 0.05 M sodium acetate buffer (pH 5.0) at the biomass/buffer ratio of 1 : 10 and centrifuged at 10,000*g* for 20 min; the supernatant was dialyzed for 3 days against 0.05 M sodium acetate buffer (pH 5.0) at 4°C and then centrifuged. The supernatant was used for determination of intracellular enzyme activities. The agarized cultural medium was centrifuged at 10,000*g* for 20 min, then the supernatant was dialyzed for 3 days against 0.05 M sodium acetate buffer (pH 5.0) at 4°C, centrifuged, and extracellular enzyme activities were determined in the supernatant.

The activity of β-1,3-glucanase was determined by accumulation of reducing sugars upon incubation of the enzyme solution with β-1,3-glucan (laminarin from Sigma (USA)). Laminarin (0.1%) in 0.05 M sodium acetate buffer (pH 5.0) was used as the substrate. The reaction mixture consisting of the enzyme extract and laminarin was incubated for 40 min at 37°C [2].

The activity of PG was determined by accumulation of reducing sugars after incubation of the enzyme solution for 10 min at 50°C with 1% polygalacturonic acid (ICN, USA) in 0.05 M sodium acetate buffer (pH 4.6) [15].

Xylanase activity was determined by accumulation of reducing sugars after incubation of the enzyme solution for 10 min at 50°C with 1% xylan (Sigma) in 0.05 M sodium acetate buffer (pH 5.0) [16].

The activity of β-galactosidase was determined spectrophotometrically at 400 nm using 2-nitrophenyl-β-D-galactopyranoside (Sigma) as the substrate. The calibration curve was determined using *p*-nitrophenol. The enzyme quantity that cleaves 1 μmol substrate in 1 min at pH 4.2 and 30°C was taken as the β-galactosidase activity unit [17].

The enzyme activities were expressed as the enzyme activity unit per mg protein (U/mg protein). The experiments were repeated three or four times.

Electrophoresis. Isoforms of β-1,3-glucanase from lyophilized preparations of the campion callus culture were separated by electrophoresis in a vertical plate of 10% non-denaturing polyacrylamide gel using different buffer systems [18]. For separation of acidic and neutral proteins, we used an alkaline buffer system for the con-

centrating gel (0.074 M Tris-HCl, pH 6.7) and for the separating gel (0.45 M Tris-HCl, pH 8.8) and also an electrode buffer (0.005 M Tris, 0.038 M glycine, pH 8.3). The electrophoresis was performed for 4 h at 400 V and 40 mA. For separation of basic proteins, we used an acidic buffer system for the concentrating gel (0.0719 M KOH/0.075 M CH_3COOH, pH 6.7) and for the separating gel (0.0719 M KOH/0.452 M CH_3COOH, pH 4.3) and also an electrode buffer (0.035 M β-alanine/0.014 M CH_3COOH, pH 4.5). The electrophoresis was performed for 4-5 h at 200 V and 50 mA.

Specimens for electrophoresis contained 50-200 μg protein depending on the enzyme activity in the preparations.

Histochemical determination of β-1,3-glucanase was performed as described in [19]. Upon electrophoresis, the gels were incubated at 40°C in 0.05 M sodium acetate buffer (pH 5.0) for 10-12 h with 0.66% laminarin from *Laminaria digitata* (Sigma) as a substrate. Pinkish-red bands of the β-1,3-glucanase activity appeared several minutes after boiling the gel in 0.15% solution of 2,3,5-triphenyltetrazolium chloride dye (Sigma) in 1 M NaOH.

Statistical analysis. Statistical processing of the data included calculation of the arithmetic mean and the mean square deviation. The significance was evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

Induction of β-1,3-glucanase by sodium salicylate. The activity of intracellular glucanase in the campion callus was inhibited upon cultivation for 24 h in the presence of 20, 40, and 100 mM NaSA (Fig. 1). The glucanase activity in the presence of 0-30 mM NaSA was unchanged on the third day of the growth (lag phase) and decreased in the presence of 40 and 100 mM NaSA. On the ninth day of growth (exponential phase), the enzyme activity was pronouncedly increased in the presence of 20 mM NaSA. On the 21st day of cultivation (stationary phase), the glucanase activity was increased at the NaSA concentrations of 20-40 mM. The maximal activity of the enzyme in the campion callus was observed in the presence of 20 mM NaSA, and this was consistent with earlier data indicating that 20 mM NaSA was the optimal concentration for induction of glucanase in sugar beet [9].

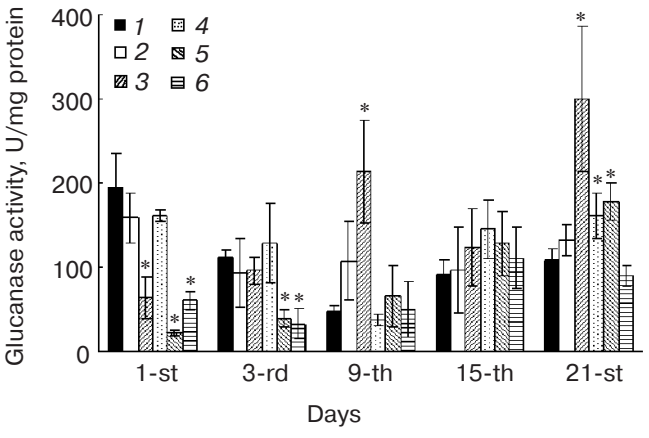

Fig. 1. Effect of NaSA on β-1,3-glucanase activity in callus of *S. vulgaris*. Columns *1-6* show the effects of 0, 10, 20, 30, 40, and 100 mM NaSA, respectively. * Difference is significant at $p < 0.05$. Control, the absence of NaSA.

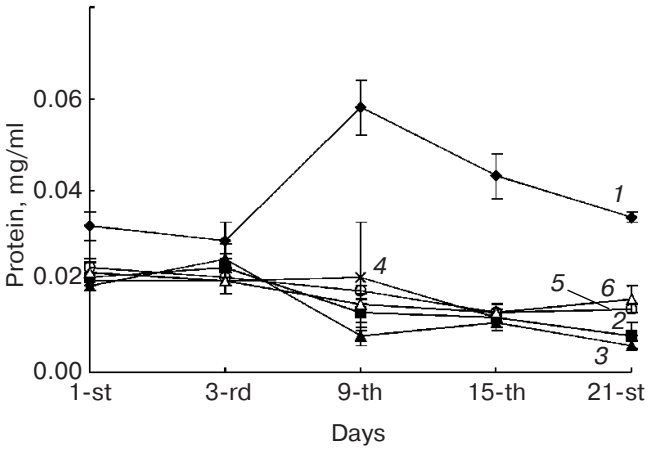

Fig. 2. Effect of NaSA on protein content in callus of *S. vulgaris*: *1-6*) 0, 10, 20, 30, 40, and 100 mM NaSA, respectively.

The activity of extracellular glucanase was not markedly changed on the 1st, 3rd, 15th, and 21st days of cultivation. The maximum induction of the enzyme was recorded on the 9th day, and the enzyme activity was close to that in the control in the presence of 10-20 mM NaSA, was increased in the presence of 30 mM, and decreased at 40 and 100 mM. These data indicated that glucanase was intensively released into the nutritional medium during the exponential phase of cell growth. It seems that during this phase the cells can display the strongest protective response to pathogens or abiotic factors. Salicylic acid is a key regulator of gene expression in response to infection with a pathogen with a resulting biosynthesis of β-1,3-glucanases [10].

Addition into the medium of 10-100 mM NaSA decreased the total protein content in the campion callus and markedly inhibited the callus growth (Fig. 2). Therefore, special conditions were chosen for the treatment of callus with NaSA: the callus was sprayed with a sterile solution of the inducer in the exponential phase of the cell growth.

Activities of intra- and extracellular glucanases in the campion callus were determined in the stationary growth phase upon treatment of the callus with 2.5-100 mM NaSA in the exponential phase of the cell growth (16th

day of cultivation). The intracellular enzyme activity in the experimental calluses was increased two- to threefold in the presence of 30-100 mM NaSA compared to the control specimens (Fig. 3). The extracellular enzyme activity was increased two- to fourfold upon treatment with 5, 20, 30, and 100 mM NaSA. Other intra- and extracellular carbohydrases, such as PG, xylanase, and galactosidase were also found, but their activities in the presence of different concentrations of NaSA were changed insignificantly compared to the control. Thus, the inducer concentration for spraying the callus in the exponential phase may be significantly enhanced without suppression of the cell growth. The NaSA concentrations of 30-100 mM are optimal for inducing intracellular glucanase, whereas to induce the extracellular enzyme the NaSA concentration has to be varied from 5 to 100 mM.

Activities of intra- and extracellular glucanases were determined in the duckweed callus in the stationary growth phase (28th day) upon the treatment of the callus with 50 and 100 mM NaSA in the exponential growth phase (21st day). The intracellular glucanase activity increased upon treatment with 50 and 100 mM NaSA, whereas the extracellular enzyme activity increased at 50 mM of the inducer (Fig. 4). The activities of intra- and extracellular glucanases in the duckweed callus were three- to fourfold and 1.5-3.0-fold, respectively, lower than in the campion callus. The differences in the glucanase activities can be associated with different classes of plants. The presence of intra- and extracellular galactosidases and of intracellular PG was also recorded. Activities of these accompanying enzymes were not markedly changed compared to the control.

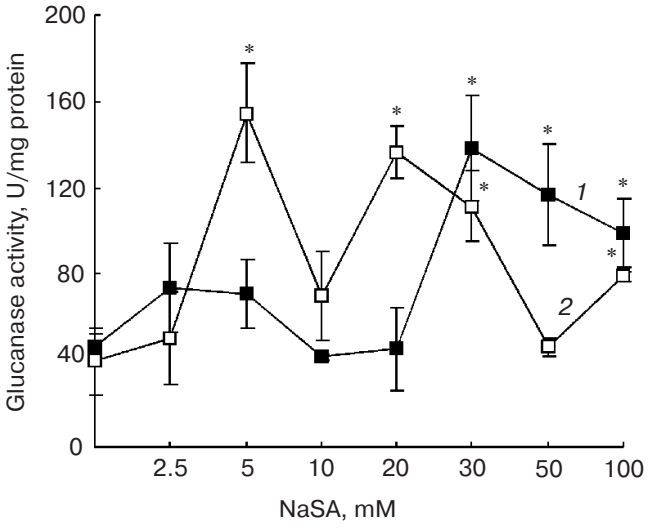

Fig. 3. Effect of NaSA on activity of intracellular (*1*) and extracellular (*2*) β-1,3-glucanase in callus of *S. vulgaris* treated with NaSA in the exponential growth phase (16th day) and analyzed in the stationary growth phase (21st day). * Difference is significant at $p < 0.05$. Control, the absence of NaSA.

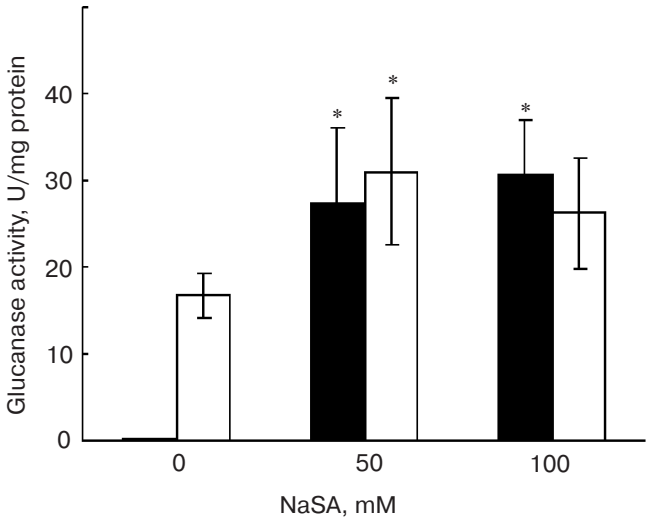

Fig. 4. Effect of NaSA on the activity of intracellular (black columns) and extracellular (white columns) β-1,3-glucanase in callus of *L. minor* treated with NaSA in the exponential growth phase (21st day) and analyzed in the stationary growth phase (28th day). * Difference is significant at $p < 0.05$. Control, the absence of NaSA.

Salicylic acid and its synthetic derivative (benzothiadiazole) were earlier shown to effectively induce both local and systemic resistance to pathogens [9]. NaSA (20 mM) and benzothiadiazole (0.01-0.05%) increased the activity of β-1,3-glucanase in sugar beet [9]. The same was also observed in callus cultures of campion and duckweed, which can be used as model systems for studies of β-1,3-glucanase induction in plant cells.

Production of β-1,3-glucanase on co-cultivation of campion callus and *T. harzianum*. Callus and fungal mycelium co-cultivation was associated with a 5.4-fold increase in the β-1,3-glucanase activity in the campion callus (table). The glucanase activity in the fungal mycelium also increased. On callus and mycelium co-cultivation, the glucanase activity also increased in the culture medium compared with such in the medium from the cultures of either callus or fungus alone. These data suggested that glucanase could be induced in the callus culture by the fungus *T. harzianum*. The enzyme was intensively released by the plant cells into the medium, i.e. the defense mechanism of plants against phytopathogens manifested itself.

Glucanases synthesized by plant cells hydrolyze 1,3- and 1,6-β-glucans of the fungal cell walls that resulted in lysis of hyphae or release of oligosaccharides, which induced additional protective reactions of the plants [8]. The co-cultivation of maize callus with *Aspergillus flavus* was earlier shown to inhibit the growth of the fungus in correlation with an increase in the β-1,3-glucanase activity in the callus and culture medium [2]. A correlation was also observed between β-1,3-glucanase activity and

Activities of carbohydrases* in callus, medium, and mycelium on co-cultivation of the *S. vulgaris* callus and *T. harzianum*

Variant	β-1,3-Glucanase	Polygalacturonase	β-Galactosidase	Xylanase
Callus alone				
callus	105.7 ± 16.1**	0**	133.6 ± 14.3**	0
medium	251.5 ± 37.5**	1.0 ± 0.3**	15.6 ± 2.6	0.15 ± 0.07**
Co-cultivation of callus and mycelium				
callus	572.2 ± 32.7	1.8 ± 1.0	78.7 ± 9.1	0
medium	1471.3 ± 435.5	3.3 ± 0.1	16.4 ± 2.2	0.30 ± 0.04
fungus	3434.4 ± 478.6	3.9 ± 0.3	69.6 ± 1.9	1.5 ± 0.7
Mycelium alone				
fungus	271.6 ± 51.5**	3.4 ± 0.3	20.9 ± 3.8**	1.3 ± 0.6
medium	0**	0.6 ± 0.1**	32.2 ± 5.1	0.3 ± 0.1

* Enzyme activities, U/mg protein. Callus and mycelium cultured separately were used as controls.
** Differences are significant at $p < 0.05$.

resistance of different callus tissues against *A. flavus* infection. Similar data were found for lemon callus co-cultivated with the fungus *Phoma tracheiphila* [20]. Production of glucanases was also studied in leaves and roots of tomatoes infected with *Phytophthora parasitica* [21], in leaves of medic infected with the fungi *Colletotrichum trifolii* [3], and in pea pods treated with *Fusarium solani* [5].

The observed increase in the glucanase activity in the fungus mycelium co-cultivated with the campion callus seems to be associated with degradation of callose (β-1,3-glucan) protecting the callus against the pathogen. Production of β-1,3-glucanase by phytopathogenic fungi was shown earlier [2, 22].

The PG activity in the callus co-cultivated with the mycelium sharply increased (table). The PG activity in the mycelium was high and almost unchanged. Analysis of the medium upon callus and mycelium co-cultivation revealed that the PG activity increased three- to fivefold compared to the control.

The galactosidase activity in the callus co-cultivated with mycelium was 1.7-fold lower than in the control, whereas in the mycelium it was threefold increased. After callus and mycelium co-cultivation, the galactosidase activity in the medium was the same as in the medium after the separate cultivation of the callus. On mycelium cultivation alone, the activity of extracellular galactosidase was higher than on callus and mycelium co-cultivation.

Xylanase activity was not recorded in both the callus grown alone and that co-cultivated with the mycelium. In the mycelium grown alone and co-cultivated with the callus, the xylanase activity was high and changed insignificantly (table). Inoculation of the callus with the fungi was accompanied by a twofold increase in the activity of xylanase compared to that in the culture medium of the untreated callus.

The increased synthesis of extra- and intracellular PG by plant cells and also of extracellular PG and xylanase by *T. harzianum* seems to be favorable for production in the plant cell walls of oligosaccharides, which act as signaling molecules in defense mechanisms of plants. Our findings are consistent with data indicating that an infected plant secrets an enzyme (polygalacturonase). This enzyme induces in the plant's own cell walls production of oligogalacturonide, which stimulates transcription of certain genes resulting in appearance of mRNAs, and the translation of them leads to synthesis of enzymes catalyzing synthesis of phytoalexins [23].

Phytopathogenic fungi are known to secret pectolytic enzymes destroying plant cell walls associated with release of oligosaccharides (oligogalacturonides), which are elicitors of phytoalexins responsible for induced resistance of plants [23]. We have shown that in addition to the increased synthesis of extracellular PG by the fungus *T. harzianum*, activities of extracellular xylanase and intracellular galactosidase also increase, and they hydrolyze the cell wall of the campion callus.

Polyacrylamide gel electrophoresis of β-1,3-glucanase isoforms. Depending on the cultivation time, β-1,3-glucanase from the campion callus is separated by native polyacrylamide gel electrophoresis into four or five bands which, in accordance with decrease in their electrophoretic mobilities, are designated Glu-1 to Glu-5 and correspond to definite isoforms of the enzyme (Figs. 5 and 6). In control specimens of the callus, β-1,3-glucanase is mainly present as isoform Glu-5, which has features of a basic protein and can be revealed in acidic polyacrylamide gel as a single enzymatically active band (Figs. 5b and 6b (lanes *1*)). This major basic form cannot be detected in the culture medium of the control on the 11th day of callus growth, and, thus, seems to be an intracellular enzyme. In alkaline polyacrylamide gel, β-1,3-glu-

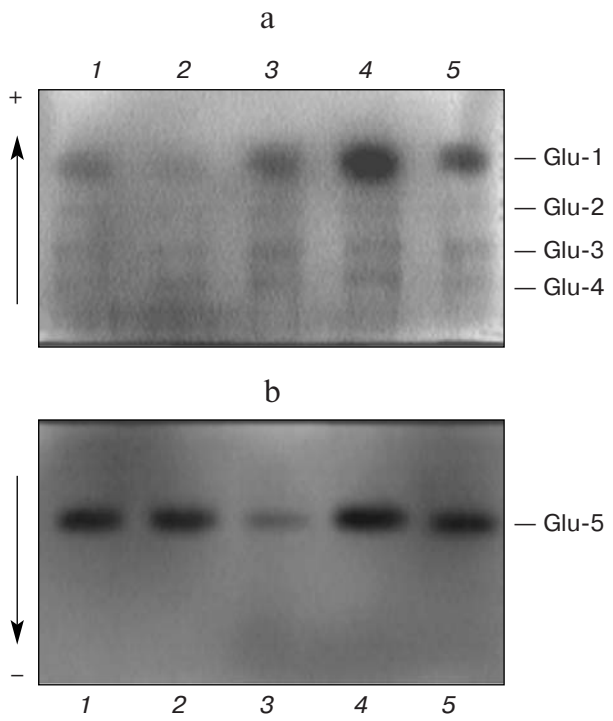

Fig. 5. Effect of NaSA on electrophoretic spectrum of molecular forms of β-1,3-glucanase from campion callus. Electrophoresis in native polyacrylamide gel: a) alkaline gel (pH 8.8); b) acidic gel (pH 4.3). *1*) Callus cells (control); *2*) callus cells upon treatment with 30 mM NaSA; *3*) culture medium (control); *4*) culture medium upon treatment of the callus with 30 mM NaSA; *5*) culture medium upon treatment of the callus with 5 mM NaSA. Glu-1 to Glu-4 are acidic isoforms; Glu-5 is a basic isoform.

canase of the callus cells on the 11th day of growth manifests itself as one weak and two minor bands of acidic proteins corresponding to isoforms Glu-1, Glu-3, and Glu-4 (Fig. 6a, lane *1*), and on the 21st day of growth an additional active band appears corresponding to Glu-2 (Fig. 5a, lane *1*). Acidic isoforms of β-1,3-glucanase are released into the cultural medium by the callus cells in small amounts; therefore, the bands corresponding to these extracellular isoforms are poorly stained on the gel. On the 11th day of growth in the culture medium only acidic isoforms Glu-1 and Glu-3 are displayed (Fig. 6a, lane *4*), and on the 21st day they are supplemented with acidic isoforms Glu-2 and Glu-4, and the alkaline isoform Glu-5 appears in the medium (Fig. 5, lane *3*). Our data on campion callus confirm the previous observations that glucanases are synthesized in small amounts as constitutive proteins also in uninfected plants, and the enzyme concentrations are changing during plant sprouting and development [24].

Treatment of the callus in the exponential growth phase (16th day) with 5 and 30 mM solutions of NaSA resulted in more pronounced extracellular acidic isoform Glu-1 and extracellular basic isoform Glu-5 in the zymogram of the callus cells and culture medium in the stationary phase of development (21st day) as clearly stained intense bands of enzymatic activities (Fig. 5, lanes *4* and *5*).

The systemic acquired resistance of plants which is usually accompanied by synthesis of PR-proteins [25] can be induced not only by inoculation of plants with pathogens, but also by treatment with some chemical compounds. Thus, the treatment of sugar beet with NaSA induced in the intercellular space of the leaves seven acidic and eight basic proteins, and the treatment with benzothiadiazole induced three acidic and one basic isoenzyme of β-1,3-glucanase [9].

An intensively stained band corresponding to the intracellular acidic isoform Glu-3 could be detected in the electrophoregram of callus specimens co-cultivated with fungus *T. harzianum* mycelium on the 11th day of callus growth (Fig. 6a, lane *2*). The acidic isoform Glu-3 became major in cells of the fungus-infected callus and was actively released into the medium (Fig. 6a, lane *3*). An additional activity band appeared that corresponded in mobility to the band of the acidic isoform Glu-2, which could be both intra- and extracellular (Fig. 6a, lanes *2* and

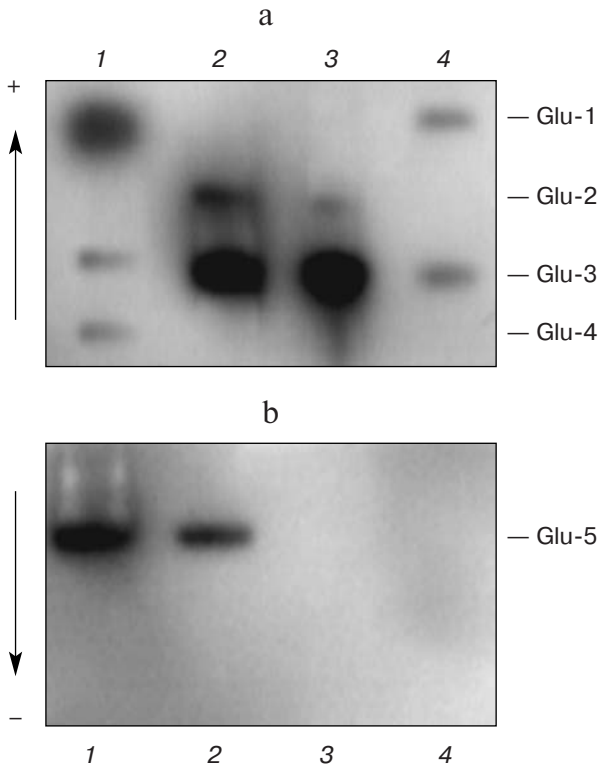

Fig. 6. Influence of co-cultivation of campion callus with fungus *T. harzianum* mycelium on electrophoretic spectrum of β-1,3-glucanase isoforms. Electrophoresis in native polyacrylamide gel: a) alkaline gel (pH 8.8); b) acidic gel (pH 4.3). *1*) Callus cells (control); *2*) callus cells co-cultivated with fungal mycelium; *3*) culture medium of callus and fungus co-cultivation; *4*) culture medium of callus grown alone (control). Glu-1 to Glu-4 are acidic isoforms; Glu-5 is a basic isoform.

3). The alkaline isoform Glu-5 was not revealed in the culture medium on the 11th day of callus growth on the co-cultivation with the fungal mycelium (Fig. 6b, lane *3*).

Induction of additional isoforms was earlier shown in plants infected with fungal pathogens and also under the influence of some compounds on the cells of different tissues [21]. Thus, infection of tomato roots with the fungi *Glomus mosseae*, *G. intraradices*, and *P. parasitica* triggered a defense mechanism represented by induction of additional acidic isoforms [21]. The authors suggest that the acidic proteins are responsible for control of the fungal growth and development inside the tomato roots and that basic isoforms can also be involved in the plant protection against fungal pathogens.

Thus, NaSA increased the induction of both intra- and extracellular β-1,3-glucanase in campion and duckweed calluses. The co-cultivation of the campion callus with *T. harzianum* mycelium caused an increased production in the callus of intra- and extracellular β-1,3-glucanase and PG. The callus and fungal mycelium co-cultivation resulted in more intensive staining of the activity band of the intracellular acidic isoform Glu-3 actively secreted by the callus cells into the medium, whereas the treatment of the callus culture with NaSA strengthened the staining of the bands of extracellular acidic isoform Glu-1 and extracellular basic isoform Glu-5, which indicated the induction of these isoforms. The composition and ratio of the induced isoforms suggested a specificity of the protective response of plant cells to actions of these different factors. Callus cultures can be used as models for studies on plant–pathogen interactions and also are promising for biotechnological production of plant food allergens.

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REFERENCES

1. Dixon, R. A., Harrison, M. J., and Lamb, C. J. (1994) *Ann. Rev. Phytopathol.*, **32**, 479-501.
2. Lozovaya, V. V., Waranyuwat, A., and Widholm, J. M. (1998) *Crop Sci.*, **38**, 1255-1260.
3. Salles, I. I., Blount, J. W., Dixon, R. A., and Schubert, K. (2002) *Physiol. Mol. Plant Pathol.*, **61**, 89-101.
4. Anuratha, C. S., Zen, K.-C., Cole, K. C., and Krishnan, S. M. (1996) *Physiol. Plant.*, **97**, 39-46.
5. Mauch, F., Mauch-Mani, B., and Boller, T. (1988) *Plant Physiol.*, **88**, 936-942.
6. Meins, F., Jr., Neuhaus, J.-M., Sperisen, C., and Ryfal, J. (1992) in *Genes Involved in Plant Defense* (Boller, T., and Meins, F., Jr., eds.) Springer Verlag, Vienna, pp. 245-282.
7. Simmons, C. R. (1994) *Plant Sci.*, **13**, 325-387.
8. Burton, R. A., Qi, Z., Roulin, S., and Fincher, G. B. (1998) *Plant Sci.*, **135**, 39-47.
9. Burketova, L., Sindelarova, M., and Sindelar, L. (1999) *Biol. Plant.*, **42**, 279-287.
10. Mackerness, S. A.-H. (2000) *Plant Growth Regul.*, **32**, 27-39.
11. Breiteneder, H., and Ebner, C. (2001) *Curr. Opin. Allergy Clin. Immunol.*, **1**, 261-267.
12. Murashige, T., and Skoog, S. (1962) *Physiol. Plant.*, **15**, 473-479.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
14. Nelson, N. (1944) *J. Biol. Chem.*, **153**, 375-380.
15. Sapunova, L. I., Mikhailova, R. V., and Lobanok, A. G. (1996) *Russ. J. Appl. Biochem. Microbiol.*, **32**, 506-509.
16. Ghose, T. K., and Bisaria, V. S. (1987) *Pure Appl. Chem.*, **59**, 1739-1752.
17. Polygalina, G. V., Cherednichenko, V. S., and Rimareva, L. V. (2003) *Determination of Enzyme Activity. A Reference Book* [in Russian], DeLi Print, Moscow, pp. 138-140.
18. Maurer, G. (1971) *Disk Electrophoresis* [Russian translation], Mir, Moscow.
19. Pan, S. Q., Ye, X. S., and Kuc, J. (1991) *Phytopathology*, **81**, 970-974.
20. Gentile, A., Tribulato, E., Deng, Z. N., Galun, E., Fluhr, R., and Vardi, A. (1993) *Theor. Appl. Genet.*, **86**, 527-532.
21. Pozo, M. J., Azcon-Aguilar, C., Dumas-Gaudot, E., and Barea, J. M. (1999) *Plant Sci.*, **141**, 149-157.
22. Brockmann, B., Smit, R., and Tudzynski, P. (1992) *Physiol. Mol. Plant Pathol.*, **40**, 191-201.
23. Rodionova, N. A., and Bezborodov, A. M. (1997) *Prikl. Biokhim. Mikrobiol.*, **33**, 467-487.
24. De Carvalho, F., Gheysen, G., Kushnir, S., van Montagu, M., Inze, D., and Gastresana, C. (1992) *EMBO J.*, **11**, 2595-2602.
25. Linthorst, H. J. M. (1991) *Crit. Rev. Plant Sci.*, **10**, 123-150.