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Effect of Immunomodulators on Potato Resistance and Susceptibility to *Phytophthora infestans*

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Abstract—The mechanisms of induced resistance and susceptibility of potato (*Solanum tuberosum* L.) tubers to late blight agent (*Phytophthora infestans* Mont de Bary) were studied using an elicitor chitosan and an immunosuppressor laminarin. It was elucidated that treatment of disks from potato tubers with chitosan resulted in salicylic acid (SA) accumulation due to activation of benzoate-2-hydroxylase and hydrolysis of SA conjugates. Such SA accumulation in potato tissues inhibited one of the antioxidant enzymes, catalase, inducing an oxidative burst and resistance development. The mechanisms of induced susceptibility to the late blight causal agent were studied using an unspecific immunosuppressor, laminarin, an analogue of natural specific suppressor of potato immune responses, β-1,3,β-1,6-glucan. It was established that the development of immunosuppression in tissues treated with laminarin did not affect the SA level in tissues. However, catalase sensitivity to SA reduced in laminarin-treated tissues, and the enzyme activity increased. In its turn, this might result in the reduced level of hydrogen peroxide in the cells and, as a sequence, in the increased potato susceptibility to late blight.

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INTRODUCTION

One of the hot problems of current agriculture is plant defense against pathogens and pests, which cause an annual loss of 15 to 30% of yield. Until now, plant treatment with pesticides is a basic way of plant defense. However, pesticides not only kill their targets, pathogenic organisms, but also damage the surrounding beneficial microflora. Some pesticides are known to display site-specificity, and modification of a sensitive site might result in the accumulation of resistant pathogen forms in their population [1].

Currently, researchers attempt to reduce pesticide application, replacing it by using other defense methods based on the increase in plant resistance [2]. The most well-known among them is the application of elicitors. Elicitors are biogenic or abiogenic compounds; their action is directed not to parasite elimination but to induction of organism defense properties. After treatment with elicitors, plants acquire resistance to a wide range of pathogens, i.e., unspecific resistance [1].

However, even the usage of efficient elicitor and its competent application do not ensure successful elicitation. This might be related to the presence in the pathogens of suppressors, which action is opposite to that of elicitors, i.e., compounds inducing an increased susceptibility of plant tissues to diseases [3].

The mechanisms of elicitor action are currently under intense investigation [2], whereas suppressor action is almost unstudied. Since these compound actions are opposite to this of elicitors, a comparison of their action mechanisms is of a great interest.

One of the contributors to the signal system formation responsible for resistance development is salicylic acid (SA), which is synthesized in plants and accumulates in the loci of resistance formation [4, 5]. In plant tissues, SA can be in free or bound form with a predominance of *O*-β-D-glucoside among the latter. Free SA is capable of resistance induction. Bound SA does not possess such capability, but it is a store of SA in tissues [6, 7]. The role of SA in immunosuppression is unclear.

The objective of this work was a comparison of elicitor and suppressor actions on resistance and susceptibility of potato plants to the causal agent of late blight. Such investigation seemed rather interesting because it permitted a comparison of the signaling pathways of elicitation and immunosuppression of potato tubers infected with *Phytophthora infestans.*

Abbreviations: AT—3-amino-1,2,4-triazole; BA—benzoic acid; BA-2-H—benzoate-2-hydroxylase; SA—salicylic acid; DMTU—1,3-dimethylthiourea; DPI—diphenyleneiodoniumchloride; ROS—reactive oxygen species; TEA—Tris-ethanol-amine.

MATERIALS AND METHODS

Experiments were performed with tubers of potato (*Solanum tuberosum* L., cv. Istrinskii) plants harboring the R_1 gene of resistance to late blight. Tubers were harvested in fields of the experimental farm Bol'shevik, stored at 4°C, and used during a single season of storage.

Late blight causal agent (*Phytophthora infestans* Mont de Bary) was grown in tubes on oat–agar medium for 11 days. To extract zoospores from zoosporangia, fungus mycelium was poured over with distilled water and kept in a refrigerator for 45 min and then at room temperature for 45 min.

A water-soluble chitosan with an average mol wt of 2–6 kD determined by viscosimetry and a deacetylation degree of 85% was used as an elicitor. It was produced at the Center of Bioengineering of Russian Academy of Sciences by the enzymatic hydrolysis of crab chitin and gifted us by Drs. V.P. Varlamov and A.V. Il'ina [8]. The elicitor was most efficient at the concentration of 100 μg/ml.

Laminarin (β-1,3/β-1,6-glucan, Sigma, United States) was used as an immunosuppressor. Laminarin is a homologue of a race-specific immunosuppressor, which was earlier isolated from the mycelium of the late blight agent and identified by the authors of publications [9, 10]. Glucans close to laminarin in their structure (pachiman (β-1,3-glucan), postulan (β-1,6-glucan), and β-1,3/β-1,6-glucan from yeast cell walls) did not suppress potato immune responses [11]. Only laminarin from brown algae (β-1,3/β-1,6-glucan) containing 25–30 glucose residues exhibited an immunosuppressor effect; its most efficient concentration was 300 μg/ml [11].

The effects of immunomodulators and inhibitors of NADPH-oxidase system on potato resistance to late blight were assesses using disks (7 mm in height and 16 mm in diameter) excised from tubers. 50 μl of the solutions tested were dropped on the surface of disks. After 72 h, this surface was infected with zoospores of the late blight causal agent at the load of 10^4 spore/ml. Water was applied to the surface of control disks. Control and experimental disks were kept on the moistened support in petri dishes.

When disk surface was infected with incompatible pathogen race, necrotic lesions appeared and the pathogen hyphae penetrated through dead cells. In 72 h, the number of necrotic cells was counted [12].

Development of tolerance or susceptibility to the pathogen was expressed in percents of immunomodulating effect. Control values were taken as 100%. In this case the values were below 100% for immunizing effect and above 100% for immunosuppressing effect.

In definite time intervals, the 3-mm upper layer was removed from each disk. These cut tissues were used for preparation of the acetone powder. To this end, 3 g of tissue were ground in liquid nitrogen, washed twice with acetone cooled to –70°C and then with diethyl ester (10 ml of solvent per 1 g tissue).

Soluble enzymes, catalase and benzoate-2-hydroxylase, were extracted from acetone powder with respectively 67 mM K,Na-phosphate buffer, pH 7.0, and 20 mM TEA buffer, pH 7.4, containing 1 mM EDTA and 5 mM sodium ascorbate [13]. The homogenates were filtered through a double layer of cheesecloth and centrifuged at 17000 *g* for 10 min. The supernatants were used for enzyme assays. All procedures were performed at 4°C.

Catalase (EC 1.11.1.6) was assayed from a decrease in the hydrogen peroxide concentration [14]. The reaction medium (a final volume of 0.5 ml) contained 67 mM K,Na-phosphate buffer, 20 mM hydrogen peroxide, and 30 μl of enzyme extract. Measurements were performed with a Backman Coulter-2000 spectrophotometer at 240 nm during 5 min after the reaction start. Thereafter, repeated measurements were performed every 10 min during 1 h to control the dynamics of enzyme activity. Catalase specific activity was expressed in μmoles of hydrogen peroxide per 1 mg of protein per 1 min.

The reaction mixture for BA-2-H assay (0.5 ml) contained 10 mM TEA buffer, pH 7.4, 1 μM BA, 1 μM NADPH, and 200 μl of the enzyme preparation [15]. The reaction mixture was incubated at 30°C for 30 min in the water thermostat. The reaction was stopped by the addition of 2.5 ml of 15% TCA. The precipitate was sedimented by centrifugation at 5000 *g*. SA was extracted from the supernatant twice with the mixture of ethyl acetate and cyclohexane (1 : 1, v/v). Then the mixture was separated in a separatory funnel, and SA was isolated from the organic phase as described below. BA-2-H activity was expressed in ng SA/(mg protein min).

Protein content was determined by the method of Bradford [16].

SA was extracted in two steps [17]. During the first step, free SA was extracted. 5 g of tissue from potato tuber disks was frozen in liquid nitrogen, ground, and extracted with 15 ml of 70% ethanol. After centrifugation at 15 000 *g*, the pellet was resuspended in 15 ml of 90% ethanol and centrifuged repeatedly. The supernatants were combined and centrifuged for the third time at 4500 *g* for 10 min. Ethanol was evaporated under vacuum, and 1 ml of 5% TCA was added to the water phase. The mixture was centrifuged at 3000 *g* for 10 min; the supernatant was extracted twice with the mixture of ethyl acetate and cyclohexane (1 : 1, v/v). The upper organic layer containing phenolic compounds was separated in a separatory funnel; the solvent was evaporated under vacuum; dry residue was dissolved in a mobile phase used for HPLC.

The second step included acidic hydrolysis of SA conjugates with 8 N HCl at 80°C for 1 h with subsequent centrifugation of the hydrolyzed mixture at 3000 *g* for 10 min. Phenolic fraction containing SA was obtained from the supernatant, as described above.

Table 1. The effect of immunomodulators on the activity of benzoate-2-hydroxylase in potato tuber disks

Treatment of the disk surface	BA-2-H activity, ng SA/(mg protein min)
Water (control)	64.0 ± 1.3
Chitosan, 100 μg/ml	76.0 ± 2.4
Laminarin, 300 μg/ml	46.0 ± 1.5

Table 2. Inducing activity of hydrogen peroxide

Treatment of the disk surface	No. necrotic cells, $M \pm \Delta$*	Immunomodulating effect, % of control
Water (control)	13.6 ± 0.2	100
H_2O_2, 5 μM	8.9 ± 0.3	66
H_2O_2, 25 μM	8.4 ± 0.2	62
H_2O_2, 100 μM	11.0 ± 0.4	81

* Percent of total number of cells in the four surface layers. See Materials and Methods section for explanation.

SA was quantified by HPLC using a fluorescence detector (extinction at 305 nm, emission at 407 nm) of the Staier installation (Russia). Luna C-18(2) column (250 mm × 4.6 mm) (Phenomenex, United States) was used. Isocratic elution was performed with 0.43% H_3PO_4 and acetonitrile (55 : 45) at the flow rate of 1 ml/min. *o*-Anisic acid (100 μg/ml) was used as an internal standard.

Computer program STRAZ was used for statistical processing of data on enzyme activity and SA content [18].

The number of necrotic cells was counted under microscope, and the results were processed statistically. Tables 2 and 3 present mean values and absolute maximum error Δ at $P = 0.95$.

RESULTS AND DISCUSSION

Induced resistance and susceptibility was modulated by potato tuber disk treatment with chitosan (elicitor) and laminarin (suppressor), respectively. Control disks were treated with water.

After 24 h, SA was isolated from potato disk tissues and quantified. Chitosan treatment resulted in a substantial increase in the content of free SA, whereas the amount of its conjugates decreased. Laminarin did not essentially affect the content of either free or bound SA (Fig. 1).

Free SA can accumulate due to its liberation from conjugates and to its synthesis under the effect of chitosan. In fact, earlier we established that elicitation acti-

Table 3. The effect of inhibitors of potato late blight development

Treatment	Inhibitor concentration	No. necrotic cells, $M \pm \Delta$*	Immunomodulating effect, % of control
Water (control)	–	10.4 ± 0.4	100
Apocynin, mM	100	13.0 ± 0.3	125
	50	12.8 ± 0.4	123
	25	17.0 ± 0.2	164
	2.5	17.2 ± 0.4	165
	1	17.3 ± 0.4	166
DPI, mM	100	10.7 ± 0.3	103
	50	11.1 ± 0.5	107
	25	10.6 ± 0.2	102
	2.5	10.9 ± 0.3	105
	1	12.4 ± 0.2	119
AT, M	100	9.9 ± 0.3	95
	10	7.9 ± 0.1	76
	1	8.4 ± 0.2	81
	0.1	8.7 ± 0.2	84
DMTU, M	300	11.4 ± 0.4	110
	30	11.6 ± 0.3	112
	3	11.2 ± 0.4	108
	0.3	11.2 ± 0.4	108

* Percent of total number of cells in the four surface layers. See Materials and Methods section for explanation.

vated phenylalanine ammonia-lyase, a key enzyme of SA and phenylpropanoid syntheses [19].

In order to detect a possible de novo synthesis of SA, we assayed the activity of benzoate-2-hydroxylase (BA-2-H), the enzyme responsible for SA synthesis. Table 1 shows that chitosan treatment of potato tuber disks enhanced somewhat this enzyme activity, whereas treatment with laminarin suppressed it. Therefore, we can conclude that elicitation released SA from its conjugates and simultaneously enhanced its synthesis, whereas during immunosuppression SA synthesis was inhibited.

Chen et al. [20] elucidated that SA was capable of catalase binding, a critical enzyme for hydrogen peroxide breakdown in plant tissues. Such binding with SA inactivated the enzyme. This discovery permitted a supposition that catalase inhibition is one of the SA action mechanisms. In its turn, this resulted in the accumulation of hydrogen peroxide, one of reactive oxygen species involved in the development of plant defense responses [21, 22]. However, it was found later that SA could inhibit far from all plant catalases [2].

In addition, it is now known that SA can bind not only active centers of some catalases but also of other Fe-containing enzymes (xanthine oxidases, peroxidases, etc.) [20, 23]. It is of interest that all these enzymes are required for ROS detoxification, in particular, hydrogen peroxide degradation. Therefore, their activity suppression by binding with SA might result in the oxidative burst, which, in its turn, induces expression of defense genes.

The role of catalase in the development of induced susceptibility is essentially unstudied. We may only suppose that the activity of this enzyme does not change or increases to prevent oxidative burst in response to penetration of compatible pathogen race into host tissues.

In this connection, we determined catalase activity in tissues of potato tubers treated with chitosan (resistance inducer) or laminarin (susceptibility inducer). It was found that, as early as after 5 h, chitosan reduced catalase activity markedly, whereas laminarin increased it (Fig. 2). The effects were most pronounced 24 h after treatments.

It is worth mentioning that we met some contradiction in experiments with laminarin. In fact, laminarin did not essentially affect the content of free SA in treated disks, whereas it increased substantially catalase activity. In order to explain such a contradiction, we tested a capability of SA to inhibit activities of catalase preparations isolated from tissues treated with elicitor or immunosuppressor. It turned out that catalase isolated from tubers immunized with chitosan was much more sensitive to SA than calatase isolated from laminarin-treated tubers (Fig. 3).

As a result of catalase inhibition by SA, hydrogen peroxide should accumulate in potato tissues, which is most stable ROS. Since hydrogen peroxide is believed

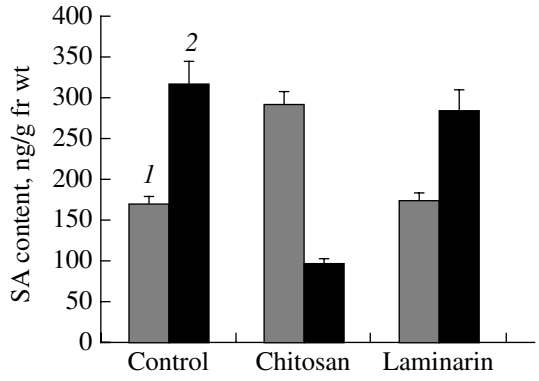

Fig. 1. Content of (*1*) free and (*2*) conjugated salicylic acid in disks from potato tubers treated with immunomodulators. (Exposure for 24 h).

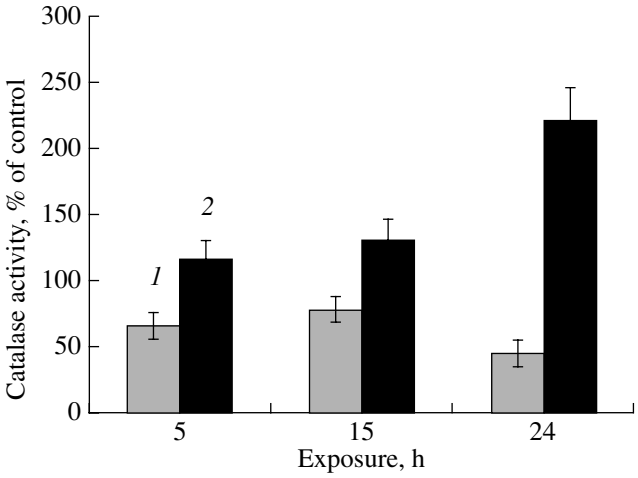

Fig. 2. Catalase specific activity in disks from potato tubers treated with (*1*) chitosan and (*2*) laminarin.

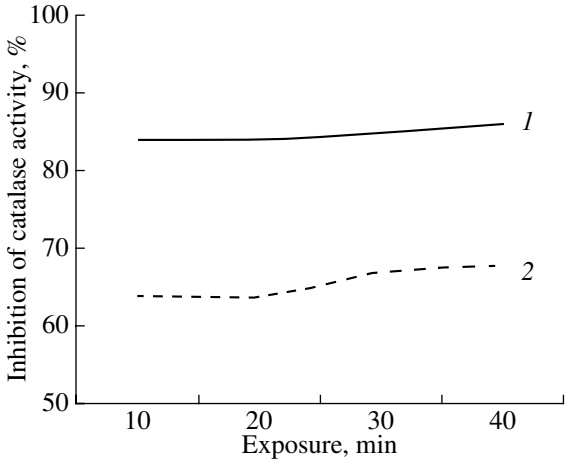

Fig. 3. Inhibition by salicylic acid of the activity of catalase isolated from potato tuber disks treated with (*1*) chitosan and (*2*) laminarin.

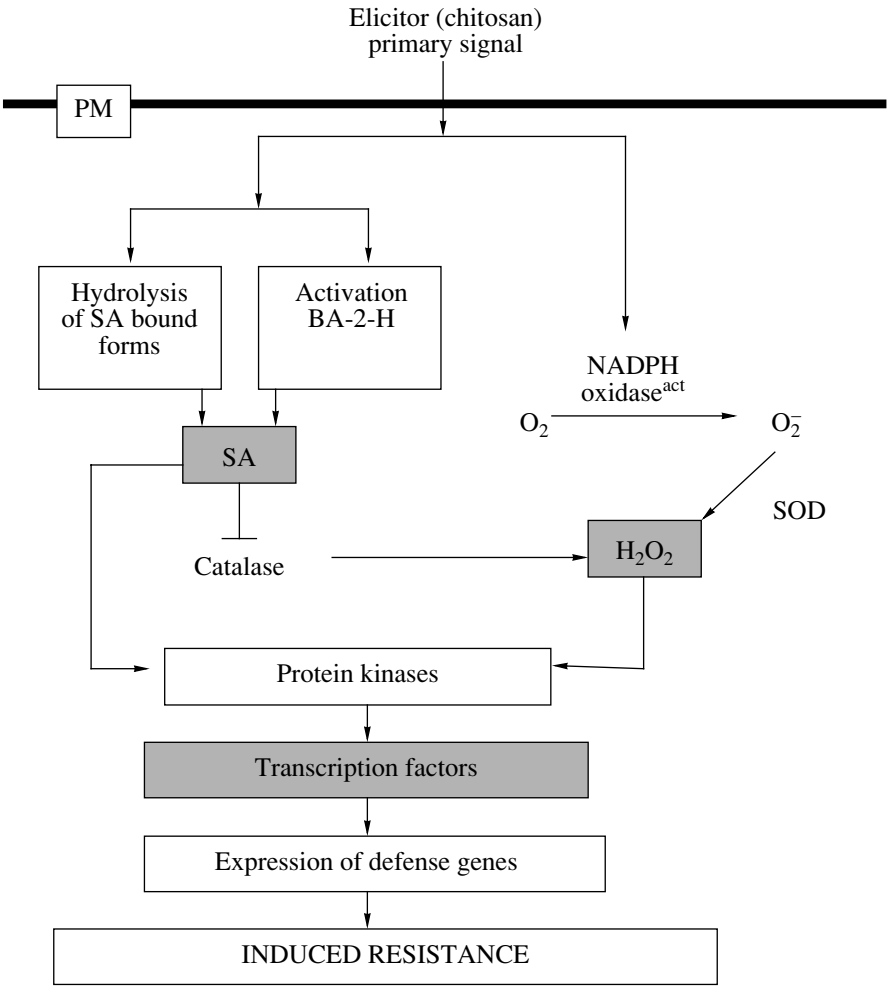

Fig. 4. Hypothetic model of the induction of potato late blight resistance by chitosan.
PM—plasma membrane. Along with NADPH oxidase, other enzymes (superoxide dismutase (SOD), pH-dependent peroxidase of the cell wall, xanthine oxidase, glycolate oxidase, and diaminoxidase) are involved in hydrogen peroxide generation. Along with catalase, ascorbate peroxidase, glutathione reductase, guaiacol peroxidase, and other peroxidases degrade hydrogen peroxide.

to serve a second messenger triggering the cascade of plant defense responses [24], it is reasonable to elucidate whether it can display an independent inducing activity.

Hydrogen peroxide action on plant cells depends on its concentration. Thus, at the concentration of 2.5 mM, hydrogen peroxide damages plant cells irreversibly, whereas at a concentration of 250 μM, the damage was reversible. At still lower concentrations (0.1–50 μM), cells appear to be undamaged, but they experience some metabolic changes, possibly leading to resistance induction [24].

Table 2 shows that tuber disk treatment with hydrogen peroxide induced a defense response with the highest effect at the concentrations of 5–25 μM. The effect was somewhat lower at a higher hydrogen peroxide concentration. These results support the idea that hydrogen peroxide accumulation is required for plant tissue immunization.

The level of hydrogen peroxide in plant tissues is known to be under the control of several enzymes. Its degradation is mainly determined by catalase, whereas NADPH oxidase is responsible for its accumulation.

In order to elucidate whether NADPH oxidase, catalase, and hydrogen peroxide are the components of the signaling pathway resulting in the development of resistance or susceptibility in potato tuber tissues, we used an inhibitor analysis. Potato tuber disks were treated with the following inhibitors of these enzymes (50 μl/disk): a specific inhibitor of NADPH oxidase apocynin [25]; a less specific inhibitor of NADPH oxidase diphenyleneiodoniumchloride (DPI); the inhibitor of catalase 3-amino-1,2,4-triazole (AT) [26]; and 1,3-dimethyl-2-thiourea (DMTU), a compound binding hydrogen peroxide, which can be conventionally called as hydrogen peroxide scavenger.

It turned out that, after treatment with the inhibitors of NADPH oxidase, potato tuber tissue developed a

susceptibility to pathogen. The more specific inhibitor (apocynin) provoked a stronger suppression of immune responses, as compared to less specific DPI. It seems likely that NADPH inactivation reduced the ROS level in tissues and, as a sequence, induced their susceptibility (Table 3).

AT-induced inhibition of catalase induced potato tuber resistance to late blight, which indicates the involvement of hydrogen peroxide in the process of immunization. This is supported by experiments with tissue treatment with DMTU, a hydrogen peroxide scavenger, which resulted in increased susceptibility.

Based on data obtained, we supposed a hypothetic model of chitosan-induced potato resistance to late blight (Fig. 4). According to this model, the following successive regulatory events function in resistance induction: elicitation results in the accumulation of free SA, which inhibits catalase activity; as a result, hydrogen peroxide accumulates (oxidative burst) and induced resistance is developed.

At induction of susceptibility with laminarin, SA is not accumulated, the sensitivity of catalase declines, and its activity increases. As a result, a development of induced susceptibility is not accompanied by the accumulation of hydrogen peroxide; moreover, due to catalase activation, its level decreases. A decrease in the ROS concentration in the cells does not permit a resistance development, and tubers acquire a susceptibility to pathogens.

In conclusion, it is necessary to investigate the complex and diverse mechanisms of induced resistance and susceptibility for production of novel ecologically safe and economically efficient defense preparations.

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