biologia plantarum
an international journal for experimental botany

BIOLOGIA PLANTARUM (2023) 67: 159-165 DOI: 10.32615/bp.2023.014

Special Issue "Plant-Microbe Interactions"

Thiosulphonate-rhamnolipid-glycanic complexes as inducers of virus resistance in hypersensitive plants

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Abstract

Involving the natural host-resistance mechanisms to pathogens are essential and one of the most promising approaches in development of first-line defenses against viral plant diseases. Polysaccharides isolated from natural sources are considered the most active resistance inducers. The biological activity of polysaccharides depends on the nature and chemical structure of the constituent components of complex preparations. In this view, the objective of our study was to evaluate the biological activity of complex preparations composed of glycans, rhamnolipids, and thiosulfonates as inducers of natural plant resistance and inhibitors of tobacco mosaic virus (TMV). Complex preparations were obtained using the following components: biogenic glycolipids - rhamnolipids of the *Pseudomonas* sp. strain PS-17, glycans - *Ganoderma adspersum* glucan and *Candida maltosa* mannan, as well as synthetic biocides - thiosulfonates (methylthiosulfanilate). The biological activity of the preparations was investigated in the host-virus model system *Nicotiana tabacum* L. and TMV. It was shown that preparations at concentrations of 10 and 100 μg mL^{-1} were active plant resistance inducers in *N. tabacum* cv. Immune 580, hypersensitive to TMV. At the same concentrations, complex preparations also reduced infectivity of TMV on *Datura metel* L. acting as viral infection inhibitors. The inducing activity of the complex preparations is sensitive to well-known transcription inhibitor actinomycin D (10 μg mL^{-1}). This fact may indicate the important role of RNA synthesis in the activation of plant virus resistance by the studied preparations.

Keywords: *Datura metel*, glycans, glycolipids, liposomes, *Nicotiana tabacum*, thiosulfonates.

Introduction

Viruses as disease-causing entities of plants are of considerable economic importance as result of their worldwide distributions and wide host range among crops, ornamentals, and wild plants. Plant viruses are responsible for severe diseases leading to significant crop losses. At present, one of the disease control strategy and plant virus management relies on preventing viruses from entering plants using insecticides against natural vectors, quarantine, removal of virus reservoirs (infected plants and weeds). Use of pesticides for controlling viral diseases have certain disadvantages, above all, there is environmental pollution and negative impact on human

Received 14 September 2022, *last revision* 26 April 2023, *accepted* 26 April 2023.
Abbreviations: AMD - actinomycin D; Glu - glucan; MTS - methylthiosulfanilate; RL - rhamnololipids; TMV - tobacco mosaic virus; TRc - liposomal thiosulfonate-rhamnolipid complex; TRGc - liposomal thiosulfonate-rhamnolipid-glycan complex.
Acknowledgements: This work was carried out with funds supplied by the National Academy of Sciences of Ukraine (governmental registration numbers of works are #0116U007265; #0119U002996) and funded by Ministry of Education and Sciences of Ukraine #0118U005306.
Conflict of interest: The authors declare no conflict of interest.

health. The most effective, economically safe, and environmentally sustainable approach of managing plant viruses is to use virus-resistant and high yielding crop cultivars. However, plant breeding is a long-term and time-consuming process with significant financial costs and besides of this genetically controlled plant resistance is often quickly overcomed by viruses. The most appropriate and quite justified approach, in our opinion, is the development of environmentally friendly antivirals capable of stimulating the plant's own defense mechanisms by inducing the resistance responses. A one of the ways to enhance the plant's own innate immunity is the use of biogenic poly- and oligosaccharide preparations, which can be involved in a plant signaling pathways and trigger defense responses in plants enhancing protection against pathogens (Chong *et al*. 2002, Vera *et al*. 2011, 2012). The use of biological resistance inducers as well as natural plant defense mechanisms is an alternative to chemical control and resistance breeding (Laporte *et al.* 2007, Dodds and Rathjen 2010, Mejía-Teniente *et al.* 2010, Burketova *et al.* 2015, Tyuterev *et al.* 2015). A strategy based on the activation of natural plant defense mechanisms provides a long-term systemic effect and contributes to the fight of plants against a wide and diverse range of pathogens. In solving tactical tasks according to this concept, an important place belongs to saccharides and sugar-containing polymers. The role of carbohydrates acting as plant defense elicitors or signaling molecules in plant immunity has been proven for various crops (Smeekens 2000, Trouvelot *et al*. 2014). Plant cellular components responsible for the induction and manifestation of plant defense mechanisms can be targets for such substances (Whitham and Wang 2004).

However, there is a problem of penetration efficiency of biopolymers in plants. The use of biosurfactants, in particular glycolipids, allows for increasing the permeability of cell membranes and the bioavailability of drugs and thereby increase their effectiveness (Whitham and Wang 2004). The findings of our previous studies have showed an increase in the antiviral activity of fungal glycans in case of using in complex preparations rhamnolipids of *Pseudomonas* sp. PS-17, which also possess antiviral and antimicrobial properties (Kovalenko *et al*. 2008). A successful attempt was made to prepare liposomal preparations composed of rhamnolipids and glycans obtained from basidiomycetes and yeasts (Kovalenko *et al*. 2013). We showed that the obtained complex drugs had a prophylactic antiviral effect and at the same time had a positive effect on the symbiotic properties of the rhizobial microflora of leguminous plants in field conditions, and were also effective as means of healing bean callus tissues from viral diseases *in vitro* (Kovalenko *et al.* 2017, 2019). Recently, we also showed that thiosulfonates (synthetic analogues of natural biocides) are appropriate for the liposome formation as well (Kovalenko *et al*. 2016). However, the biological activity of the latest generation of liposomal antiviral drugs has not been investigated.

Thus, the purpose of this study was to evaluate the activity of complex preparations composed of glycans, rhamnolipids and thiosulfonates as resistance inducers in plants hypersensitive to tobacco mosaic virus (TMV) on *Nicotiana tabacum* and *Datura metel* models.

Materials and methods

Virus and plants: The common strain of the tobacco mosaic virus (TMV-U1) was used as the research object in all experiments. The virus was purified from the harvested leaves of systemically inoculated *N*-gene deficient mutant *Nicotiana tabacum* L. cv. Immune 580 by differential centrifugation (Gooding and Hebert 1967). Obtained TMV particles were diluted to the required concentration (4 - 10 μg mL^{-1}) with 0.01 M phosphate-buffered saline (PBS) before use. Antiviral and phytotoxic activity of preparations was evaluated using seeds of hypersensitive tobacco cv. Immune 580, which carry resistance *N* gene derived from *Nicotiana glutinosa* as a result of interspecies hybridization (Kovalenko and Kyrychenko 2004). The effect of preparations on the development of infection caused by TMV has been studied in leaves of semi-hypersensitive *Datura metel* L. plants, reacting by local necrosis and systemic mosaic on TMV infection (Kovalenko and Kyrychenko 2004). The plants were cultivated in an insect-free greenhouse in the spring and summer period at an average daily temperature of 20°C for 6 - 8 weeks. In each variant of the experiment, at least 10 experimental and control plants of a similar age and size were used.

Thiosulphonate-rhamnolipid and thiosulphonate-rhamnolipid-glycanic complexes: Complex preparations (liposomes) composed of: chemically pure glucan (Glu) – aqueous mycelium extracts of *Ganoderma adspersum* (Schulzer) Donk (Kovalenko and Wasser 2016); mannan oligosaccharides (Man), obtained from *Candida maltosa* cells by extraction with 6% CH_3COOH (Podgorsky *et al.* 2010); rhamnolipids (RL) – product of the *Pseudomonas* sp. PS-17 strain isolated from the cell-free culture liquid in the acidic medium and pH 3 (10% HCl) followed by extraction with mixture of chloroform/methanol (2:1, v/v) and vacuum evaporation (Pokynbroda *et al*. 2019, Semeniuk *et al*. 2020); methylthiosulfanilate (MTS) – synthetic structural analogue of organic sulfur compounds of the garlic and onions biocides with a wide range of biological activity (Lubenets *et al*. 2013, 2019). In this study, liposomal preparations were prepared according to the method mentioned in the previous work (Kovalenko *et al.* 2022). To prepare the liposomal delivery system, the ingredients (MTS and RL) were mixed in a 1:3 ratio and dissolved in a chloroform-methanol mixture (2:1). Therefore, the mixture was poured into three flasks equally and the solvents were evaporated on a rotary evaporator under vacuum pump at a temperature of 50°C. After the removal of the organic solvent, a homogeneous, a methylthiosulfanilate-rhamnolipid film was formed on the inner surface of the flasks. The hydration of the film in every flask was performed using a distilled water (preparation 1), aqueous solutions of *G. adspersum* glucan (preparation 2), or *C. maltosa* mannan (preparation 3) in

appropriate concentration. The initial concentrations of liposome-forming ingredients, as a rule, were: 10 mg mL^{-1} for glycans, 9 mg mL^{-1} for rhamnolipids, and 3 mg mL^{-1} for MTS. The flasks were placed in a water bath (15 min, 50°C), periodically shaken to wash off the film, hydrate and emulsify substances. As a result, three liposomal thiosulfonate-rhamnolipid-glycan complexes (TRc and TRGc) were obtained: TRc-1 – MTS + RL (empty, unloaded liposomes, in biological experiments studied primarily as control); TRGc-2 – MTS + RL + Glu (liposomes loaded with glucan); TRGc-3 – MTS + RL + Man (liposomes loaded with mannan). To avoid spontaneous aggregation of liposomes, all TRGs were sonicated (10 min, 22 kHz) in an ultrasonic bath at 25°C and stored in a refrigerator (6 - 8°C) until analysis (Bang *et al.* 2009).

Microscopic analysis of TRG complex: For the imaging of TRGc, freshly prepared liposomal complexes were examined by phase-contrast microscopy (*Meiji Techno 3000*, Japan).

The effect of TRG complex on plant germination was studied using seeds of *N. tobacco* cv. Immune 580, which served in our experiments as a model object for antiviral evaluation. The experiments were carried out in a luminestat at a controlled temperature (25 ± 2°C) and a 16-photoperiod (138 µmol m^{-2} s^{-1}). Germination analyses were performed according to Leubner-Metzger *et al*. (1995). In brief, 25 tobacco seeds were placed in Petri dishes (Ø 12 mm) on filter paper soaked with each of TRG complex solutions (10 and 100 µg mL^{-1}), in three replicates. In control, the seeds were placed on top of filter paper containing distilled water. During the experiment (9 d), the young tobacco seedlings were moistened with the same solutions or distilled water. The germination of seeds was monitored every day. The effect of TRG complex on the tested seeds was determined by recording the number of germinated seeds in every variant. The seed was considered germinated when the radicle came out of the pericarp.

Analysis of the protective activity of TRG complex: The preparation's protective activity was primarily evaluated as a decrease in lesion number generated on the leaves of *D. metel*, semi-sensitive to TMV (Kovalenko and Kyrychenko 2004). For this, we used the half-leaf method. Water solutions of the tested TRG complex (10 and 100 µg mL^{-1}) were mixed with viral suspension (4 µg mL^{-1}) at a 1:1 ratio and incubated 30 min under the laboratory conditions. The resulting mixtures (50 - 100 µL) were then inoculated on the left side of *D. metel* leaves along the main vein, whereas TMV suspension at the same concentration without TRG complex was inoculated on the right side of the same leaf as a negative control. Inoculation was made with a glass spatula by rubbing leaves pre-dusted with abrasive material (fine-grained carborundum). In each version, at least 10 leaves and three test plants were used. The local lesion numbers in the halved leaves were recorded 7 d after inoculation and inactivation efficacies of TRG complex against TMV was evaluated as inhibition rates of local lesions on *D. metel* leaves. The TMV inhibition rates (I [%]) of the TRG complex were calculated according to the formula: I [%] = [(1 - E)/C] × 100%, where E is the average number of local lesions for the treatment; C is the average number for the negative control. At least ten leaves were used per treatment. Each experiment was repeated 3 times.

Studies of TRG complex as inducers of plant resistance: Effect of TRG complex as a plant inducer against TMV infection was assessed on tobacco plants cv. Immune 580, hypersensitive to TMV. In the first set of experiments, TRG complex solutions (100 µg mL^{-1}) were injected into the intercellular spaces of the leaves left halves; right (control) halves were treated in with distilled water. 1, 3, 5, and 7 d after the injections, the whole leaf was inoculated with a suspension of TMV (10 µg mL^{-1}) as described above. In each version, at least 10 leaves and three test plants were used. 7 d after inoculation, the number of viral local lesions in the experiment and in the control was calculated and the induced resistance was estimated according to the formula given above.

In a second series of experiments, the effect of the inhibitor of DNA-dependent RNA-polymerase actinomycin D (AMD) on viral plant resistance induced by TRG complex was studied. First, the left halves of plant leaves were injected with a solution of TRG complex as a resistance inducer (10 µg mL^{-1}), and the right halves were treated in the same way with water (control). After 1 d, the leaves of the first group of plants (experiment) were completely treated in the same way with an aqueous solution of AMD (10 µg mL^{-1}), in the second group of plants (control) with distilled water. Experimental and control groups of plants were inoculated with TMV (10 µg mL^{-1}) 2 d after treatment with the studied substances, and 7 d after inoculation, the number of local lesions was counted. Note that in these experiments, we did not evaluate the degree of induced resistance, but the degree of susceptibility of plants to viral infection as a more convenient indicator of differences with a small difference in the number of lesions between the experimental and control variants (Kovalenko and Wasser 2016).

Statistical analysis: The experimental results are presented as the means ± standard deviations. Experimental data were statistically processed using the *Microsoft Excel-2010* software package. In addition, the differences between the experimental data were statistically analyzed using the *Statistica* software package *version 12.0* (*StatSoft*, Tulsa, OK, USA). Differences were considered as statistically significant at $P<0.05$ or $P<0.01$.

Results

The liposomes obtained in the work by our preparation technique represent an opalescent, slightly brownish-colored homogeneous solution in concentrated form, are stable at room or low temperatures (+2°C), and

autoclaving (50 kPa, at a temperature of 121°C for 1 h) and freeze-drying. The morphological and surface evaluation performed in a light microscope show that liposomes presented as spherical structures of different sizes ranging from 0.1 to 0.5 μm (Fig. 1). Moreover, empty liposomes have, as a rule, more uniform size than those glycans-loaded. Multilamellar structures sized of 2 - 3 μm and were more observed in loaded liposomes and were not detected in empty ones. The formation of these structures can be prevented by treating the emulsion with ultrasound (22 kHz, 10 min).

An important first step in the evaluation of new antiviral drugs is the determination of their non-toxic concentrations and the exclusion of possible toxic effects on the plant. Experiments were conducted on seeds and seedlings of *N. tabacum* (cv. Immune 580), which in our work was the main model plant in the virus-host system. The possible toxicity of liposomal complexes was evaluated by the ability of seeds to germinate and by taking into account the percentage of germinated seeds treated with TRG complex solutions (10 and 100 μg mL^{-1}). The results of this study showed that seed treatment with TRc-1 (empty liposomes) at a concentration of 100 μg mL^{-1} significantly reduced seed germination and inhibited the germination process compared to the control (treated with H_2O), which may indicate its phytotoxicity (Fig. 2*A*) in contrast to TRGc-2/TRGc-3. This toxicity of TRc-1 is probably due to the relatively higher content of MTS and RL compared to TRGc-2/TRGc-3, which contain non-toxic *G. adspersum* glucan and *C. maltosa* mannan along with MTS and RL. Some delay in the process of germination of tobacco seeds under the influence of TRGc-2/TRGc was detected only at the early stage of seed germination (Fig. 2*B*,*C*). But in general, it did not affect the output of germinated plants on the 9th day of the experiment.

The results of inactivation efficacies of TRG complex (antiviral activity) at concentration 10 and 100 μg mL^{-1} against TMV on *D. metel* indicator plants are given in Table 1.

The highest inhibition of local TMV infection in *D. metel* was observed when using both concentrations of TRGc-2: 43.9% (100 μg mL^{-1}) and 34.2% (10 μg mL^{-1}). The activity of TRGc-3 in relation to local infection in this plant did not exceed that of the TRGc-3 preparation

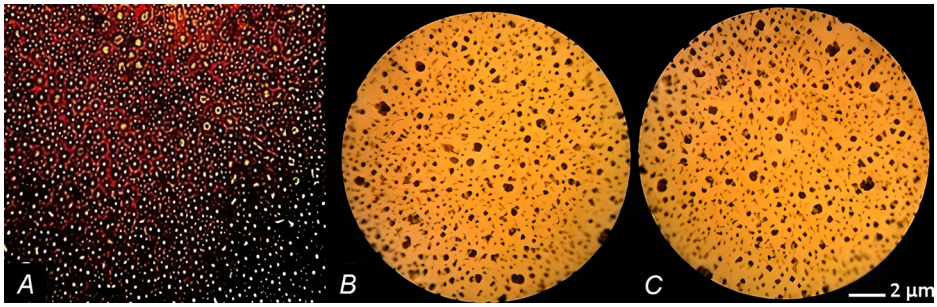

Fig. 1. Optical (phase-contrast) microscope images of liposome formulations (×400): *A* - empty liposomes (TRc-1); *B*, *C* - liposomes loaded with *G. adspersum* glucan (TRGc-2) and *C. maltosa* mannan (TRGc-3), respectively.

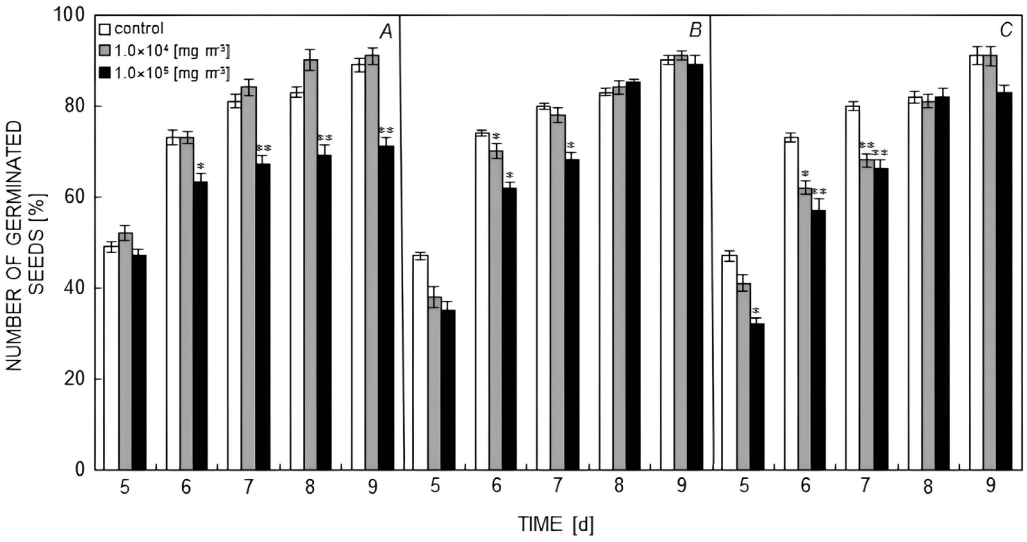

Fig. 2. Effect of *N. tobacco* cv. Immune 580 seed treatment with TRGc-1 (*A*), TRGc-2 (*B*), and TRGc-3 (*C*) on the germination rate and dynamics of seed germination. Means ± SDs, $n = 10$; * - $P<0.05$ and ** - $P<0.01$ significant differences between the treated and control plants, respectively.

Table 1. Effectiveness of tobacco mosaic virus inactivation by TRG complexes. TRGc-1: empty liposomes; TRGc-2: glucan-loaded liposomes; TRGc-3: mannan-loaded liposomes; significant difference (* - $P<0.05$) between the treated and control plants.

Liposomal complex	Concentration [μg mL^{-1}]	Average inhibition rate [%]
TRGc-1	100	32.5 ± 1.1
	10	22.0 ± 0.8
TRGc-2	100	43.9 ± 0.7*
	10	34.2 ± 1.0*
TRGc-3	100	26.6 ± 0.9*
	10	14.3 ± 1.2*

taken by us as a control. Moreover, the latter in high concentration (100 μg mL^{-1}) caused leaf tissue damage. Probably, the moderate antiviral activity of TRG complex revealed in our experiments is related to some phytotoxicity of surfactants, in particular the control drug TRGc-3, which contained the highest relative concentration of these substances. However, some "additive" antiviral effect of the glucan component in TRGc-2 most likely has a different nature and is related to deep processes underlying the interaction of viral and cellular genomes.

The effect of TRGc on the mechanism of natural resistance was studied on tobacco plants hypersensitive to TMV. It was established that both TRGc-2 and TRGc-3, introduced into the intercellular space of leaf parenchyma, are capable of inducing plant resistance to viral infection (Fig. 3). The process of induction of virus resistance is dynamic, as it depends on the duration of contact of the inducer with the cells of the host plant. Thus, when they were in contact with plant cells from 1 to 3 d, there was an increase in the resistance of plants to viral infection by 55 - 63% compared to the control. And after the extension of the "incubation" period of the inducers in plant tissues from 5 - 7 d, the resistance of plants increased to 70 - 72% in relation to the control plants treated with water during the corresponding periods. Probably, for the development and implementation of plant resistance, a certain period is necessary, sufficient for the realization of the inductive potential of the investigated supramolecular complexes (3 - 4 d in our experiments). Drug TRc-1 (empty liposomes) was not tested as a control in these experiments due to its high phytotoxicity (Fig. 2*A*).

The mechanisms of resistance caused by TRG complexes are not yet known. In order to reveal the role of the cellular genome in the protective effect of the studied complexes, we tested the known inhibitor of DNA-dependent RNA polymerase actinomycin D. An aqueous solution of the antibiotic (10 μg mL^{-1}) was injected into a part of the plants pretreated with TRGc-2 or TRGc-3, and the second part of the plants treated with the same inducers were treated with water accordingly. The experimental results showed that the susceptibility of tobacco tissues to TMV increased by TRGc-2 and TRGc-3

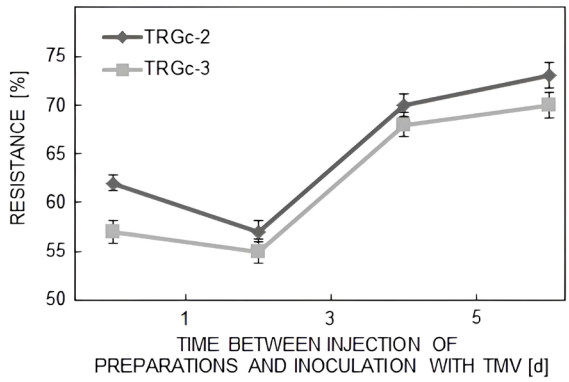

Fig. 3. Development of resistance to TMV infection induced in tobacco cv. Immune 580 by TRGc-2 and TRGc-3 preparations at a concentration of 100 μg mL^{-1}.

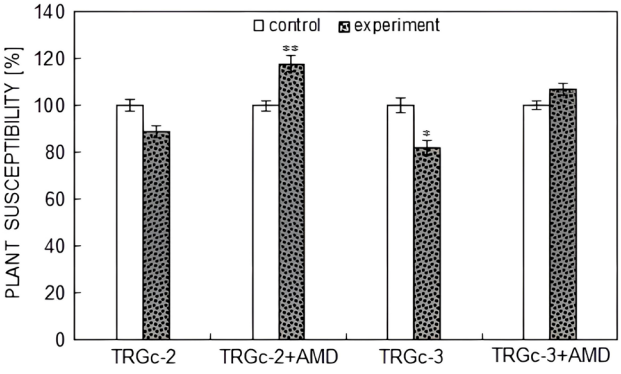

Fig. 4. The effect of antimetabolite AMD (10 μg mL^{-1}) on the susceptibility of tobacco plants cv. Immune 580 to TMV infection, reduced by the use of TRGc-2 and TRGc-3 (10 μg mL^{-1}); significant difference at * - $P<0.05$ and ** - $P<0.01$ between the treated and control plants.

(10 μg mL^{-1}) was sensitive to the action of actinomycin D (Fig. 4). These data proved the participation of the *de novo* RNA transcription process on the cellular DNA matrix in the development of resistance induced by liposomal drugs.

Discussion

The work is devoted to the development of an important direction in the protection of plants from viral diseases through the use of their own protective mechanisms, which higher plants have acquired as a result of coupled evolution with their viral pathogens. As is known, the protein-carbohydrate interaction and carbohydrate polymers as signaling molecules play a significant role in the functioning of such protective mechanisms (Kovalenko *et al.* 2000, Smeekens 2000, Kyrychenko and Kovalenko 2011, Trouvelot *et al.* 2014, Tyuterev 2015). Moreover, it is the protein-carbohydrate interaction that is obviously the molecular mechanism of pathogen recognition by the host and, accordingly, the basis of the affinity of the plant and animal defense system against

viral pathogens (De Schutter and Van Damme 2015). Since glycopolymers have a limited permeability into plant tissues and cells (Kovalenko and Kluge 1988), we started the search for substances that contribute to the delivery of active components of carbohydrate nature to their targets. Such substances were found among diphilic substances of the glycolipid type, which are biosurfactants (Banya *et al.* 2015) and have biological, including anti-phytoviral (Kovalenko *et al.* 2008) properties. As it turned out later, these polymers have the ability to form supramolecular structures – liposomes (Kovalenko *et al.* 2013). In this work, in addition to rhamnolipids, substances from the class of thiosulfonates, in particular methylthiosulfanilate, are involved in the formation of liposomal structures, which also have biological activity in the pathogen-host system due to their antioxidant properties (Banya *et al.* 2015, Liubas *et al.* 2022). These substances, as donors of amine groups, served in our studies to bind the carboxyl groups of fatty acids of rhamnolipids, which in the process of hydration led to the formation of liposomes. The latter in the water environment were able to absorb dissolved components: *C. maltosa* mannan and *G. adspersum* glucan. As a result of this procedure, we obtained complex preparations that have appropriate biological properties, namely: they show the ability to induce resistance to TMV infection in hypersensitive *N. tabacum* plants. Inhibition of local infection of the model virus (TMV) in quasi-hypersensitive *D. metel* is not a significant feature of liposomal preparations of glycans, since in this respect one of them exceeded (TRGc-3), the other did not exceed (TRGc-2) the activity of the control preparation (TRc-1, empty liposomes). It should be noted that due to the pronounced phytotoxicity of the preparation of "empty" liposomes, we could not test their ability to induce plant resistance to viral infection. However, it is possible that the latter may contribute to the metabolic processes of plants leading to the development of induced viral resistance. Our further research will show this. It seems obvious that liposome-based glycan preparations are less active in semi-hypersensitive *D. metel* plants than in hypersensitive *N. tabacum* (Table 1, Fig. 3). We noted this in relation to other liposomal glycoconjugates. The difference in the activity of this class of antiviral agents is probably due to the fact that the first plants, unlike the second, lack protective mechanisms of acquired, induced resistance controlled by the plant genome (Kovalenko and Kyrychenko 2004). Therefore, it is consistent with the data that the liposomal forms of glycans we studied, as well as the glycans themselves (Kovalenko *et al.* 2009, Kovalenko and Wasser 2016), can act as *de novo* inducers of viral resistance in plants. In the process of developing induced resistance, mRNA transcription processes on the DNA matrix of the cell are involved. This is evidenced by our data on the sensitivity of their induced resistance to the specific inhibitor of the synthesis of DNA-dependent RNA polymerase actinomycin D (Fig. 4). In addition, as we recently showed, such drugs can be useful as antiviral heathy agents in plant tissue culture *in vitro* (Kovalenko *et al.* 2019).

The authors express the hope that the research they started on new nanomaterials and artificial molecular structures formed on their basis have a perspective for their further study in biotechnology, plant breeding, and medicine.

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