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CROSSTOLERANT EFFECT OF SALT PRIMING AND VIRAL INFECTION ON *NICOTIANA BENTHAMIANA*

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ABSTRACT

Currently, the salinization of soil one of the factors, which determine the productivity of crop plants. Under severe conditions, plants reveal the retardation in growth of shoots and roots,the decrease of productivity, because of biochemical and physiologicaleffects of salt high concentrations. To avoid any severe effect of biotic and abiotic stress is applied to the method of seed priming. Primed seed preserves the resistance signaling pathway for upcoming stress conditions. During biotic and biotic stress firstly accumulates reactive oxygen species which are the secondary messenger molecules in many signaling pathway of plant organisms. Excessive amounts of reactive oxygen species lead to macromolecules damage, such as lipids of cell walls, proteins, nucleic acids. To overcome the severe effect, plants evolved enzymatic and non-enzymatic systems of protection. The crucial enzymes are catalase, aldehyde oxidase, superoxide dismutase. The main components of the non-enzymatic antioxidant system are vitamins, low molecular mass substances such as glutathione. The phenomenon "cross-tolerance" suggests the resistance of plant organism to one stress by the exposure to another one. Early exposure salt stress to *Nicotiana benthamiana* leads to resistance to viral infection, change the activity of antioxidant enzymes (catalase, aldehyde oxidase). The analysis of viral particles revealed the decrease of viral particles content in salt primed plants.

Keywords: salinity, priming, viral infection, cross-tolerance, reactive oxygen species.

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INTRODUCTION

Salinity is one of the crucial environmental factors, which leads to crop productivity restrictions because the majority of crops are sensitive to salinization caused by high levels of salts in the soil. The area affected by high salt concentration grows every day. The loss of yield among cultural plants ranges from 20% to 50%, and the causes of such losses are mainly drought and severe salinization of the soil [1].

Elucidation of the adaptation mechanisms of plant organisms that allow them to survive under saline conditions is an important direction in the physiology of plant resistance [2].

In consequence of review the effect of medium salinity, the following factors are identified: the difficulties of water supply in the whole plant and, therefore, negative changes in the functioning of osmoregulation mechanisms; imbalance in the mineral

composition of the medium, resulted in disturbances of the plants' mineral nutrition; stress on severe salinization; toxicity.

Plants respond to severe environmental conditions, such as abiotic and biotic stress bythe generation of active oxygen species. For the majority of organisms the oxygen presence plays a crucial role in cell energetics, because of nutrients oxidation capability.

Molecular oxygen is not toxic for cells, but products of oxygen incomplete oxidation suggest the danger represents incomplete oxidation of oxygen products: peroxide compounds, superoxide radicals, singlet oxygen, and others. Due to the high biological activity they are named reactive oxygen species (ROS). The appearance of ROS is caused by the fact that molecular oxygen (O_2) can intercept electrons from certain electron transport chain carriers [3]. As a result of a single-electron reduction of the oxygen molecule, superoxide radical or anion radical is formed. Superoxide radical is a charged particle surrounded by water molecules. Therefore, O_2 cannot pass the membrane, it is "locked" in the cell and becomes the source of other ROS forms, for example, hydrogen peroxide. Hydrogen peroxide, in turn, is restored and gives a hydroxyl radical. Hydroxyl radicals oxidize almost any substance of the cell, including DNA.

Reactive oxygen species take part in defensive reactions, for example, under the action of pathogens, and also serve as secondary intermediaries in signaling. Cell protection is ensured by the operation of the antioxidant system, which can be accomplished by enzymatic and non-enzymatic pathways. The main method of protection against ROS is inactivation. This is achieved by the work of special enzymes: superoxide dismutases, catalases, peroxidases [4].

Superoxide dismutase (SOD) is an enzyme that is widely distributed in nature. The active center of SOD contains metal ions (copper, iron, manganese, zinc). Thus, Mn-SOD is contained in mitochondria, Fe-SOD in chloroplasts, and Cu-Zn SOD in cytoplasm and peroxysomes. SOD is present in all aerobic organisms and serves to effectively remove superoxide radicals. SOD catalyzes the reaction of the conversion of two anion radicals to hydrogen peroxide (H_2O_2) and molecular oxygen. Catalase breaks down hydrogen peroxide with the formation of water and molecular oxygen, and peroxidases restore hydrogen peroxide with special substrates, for example, a glutathione[5]. Catalases are a tetrameric heme-containing enzyme found in all aerobic organisms. It is one of the most active natural catalysts that break down hydrogen peroxide (H_2O_2). Catalase is a tetramer of four polypeptide chains, each of which consists of more than 500 amino acids [6]. It contains four groups of heme porphyrin, which contribute to the interaction of the enzyme with hydrogen peroxide. As a result of the interaction, catalase converts (H_2O_2) into the water and molecular oxygen [7].

Catalase activity depends on many factors, one of which is salicylic acid. Thus, SA can inhibit the activity of catalase under the influence of a pathogen on plants, as a result of which the accumulation of H_2O_2in the lesion increases to induce the expression of resistance genes associated with systemic acquired resistance [8].

The antioxidant system also includes low molecular weight antioxidant substances capable of reacting with ROS without the participation of enzymes. Such substances include carotenes, vitamins (A, E, C), others. Thus, ascorbic acid (vitamin C) can react with superoxide and hydroxyl radicals and thereby reduce their concentration in the cell. The degree of damage from ROS depends on the effective operation of the antioxidant system and is determined by the resistance of plants. In resistant plants, the activity of antioxidant enzymes and the content of vitamins E and C are higher. It has been shown that the introduction of SOD into tissues suppresses the formation of excess ROS and reduces the death of cells due to the action of pathogens[9].Transgenic plants with

increased activity of SOD turns out to be more resistant to many stress factors, including water deficiency.

Seed priming induces various protective mechanisms under stress[10].High salinity results ina production loss of 1.5 million hectares of land each year [11].Seed germination and seedling growth are two critical steps for creating crops. These stages are most sensitive to abiotic stress [12]. The seed priming method is designed to alter the processes associated with germination. [13].The seed priming process includes, firstly, exposure to a factor for resistance to future stress exposure [14].

Priming is a procedure that moisturizes seeds in a specific environment, after which the seeds dried so that germination processes begin [15].This process improves the protective mechanisms of germination, which enable seeds to overcome environmental stresses[16].

TBSV is a virus that is a species of the *Tombusvirus* family, the genus *Tombusviridae*. TBSV and the suppressor protein P19 are effective tools for studying the molecular interactions of plants and viruses [17].Currently, the Tombusviridae virus family includes thirteen viral genera that contain single chain (+) ssRNA and isometric virions [18]. According to the latest data from the International Committee on Taxonomy of Viruses (ICTV), 71 representatives of the *Tombusviridae* family are registered, which infect both monocotyledonous and dicotyledonous plant representatives[19]. In plants, one of the common mechanisms of plant resistance is RNA-interference. In plants, RNA interference acts as an antiviral system, so successful viral infection requires inhibition of RNAi. Many viral suppressors have been identified but the molecular basis for RNAi suppression is still poorly understood. P19 also inhibits RNAi in the heterologous *Drosophila in vitro* system, preventing the inclusion of siRNAs in the RISC complex. During infection, p19 markedly reduces the number of free siRNAs in cells, forming P19-siRNA complexes, thereby making siRNAs inaccessible to effector complexes of the RNAi mechanism. Besides, the results suggest that P19-mediated siRNA binding in virus-infected cells blocks the spread of mobile, systemic RNAi [20].

Materials and methods

Seed priming. Previously, the seeds of *N. benthamiana* were sterilized with a solution of 25 ml of sodium hypochlorite (NaClO) and 25 ml of distilled water for 10 minutes, then they were washed three times with distilled water and dipped in a solution of 70% alcohol for 10 seconds, then rinsed again with distilled water. After drying, the seeds were primed, soaked in 25 mM, 50 mM, 100 mM NaCl solutions at room temperature for 4, 6, 8 hours, and the seeds of the control group were soaked in distilled water. In the end, the seeds were washed with distilled water, dried for 10 hours. Then, 12 seeds of each treatment and control variant were sown in pots with sterilized soil.

Plant and virus materials. Seeds of plants *Nicotiana benthamiana*were grown in the soil containing biohumus and basic nutrients such as nitrogen (NH_4+NO_3) 150 mg/l, phosphorus (P_2O_5) 270 mg/l, potassium (K_2O) 300 mg/l, with a pH of 6.0-6.5 in the greenhouse. The greenhouse is equipped with white-fluorescent lamps of 230 V, a timer with a 16-hour operation, and a temperature regime of 25/20°C (day and night) with a humidity of 80%. Plants were watered 3 times a week with 50 ml of water. Plants of 25-35 days of the same size were selected to provide equal experimental conditions. 20 μl of an inoculation buffer (10 mM sodium phosphate buffer, pH 6.9) containing virions and carborundum (d= 0,037мм) was applied on the surface of the middle leaves of each plant.

Plants inoculation by TBSV RNA transcripts. For plants inoculation *in vitro* generated transcripts of full-length TBSV cDNAs were used [21,22]. For this, plasmids containing the inserts were linearized at the 3′ -end of the viral cDNA sequence by restriction SmaI enzyme digest. Transcripts were synthesized using T7 RNA polymerase, and the resulting transcripts were used for the inoculation of plants as previously described [23]. Control plants were mock-inoculated by using phosphate buffer without viral RNA. Healthy and infected plants were grown separately in the same conditions.

Western blot analysis. Protein samples extracted from mock-inoculated and TBSV infected plants were separated by 12% SDS - polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Osmonics, Westborough, MA). After transfer the membranes were stained with Ponceau S (Sigma, St. Louis, MO) for verification of protein transfer efficiency. The resulting membrane was incubated with diluted primary antibodies (1:5000) raised against P19 TBSV protein. Alkaline phosphatase-conjugated to goat anti-rabbit antiserum (Sigma) was used as a secondary antibody at a dilution of 1:3000, and the immune complexes were visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as the substrate.

Detection of TBSV virions in inoculated plants. Antibodies against TBSV virions were received from BALB/c mice by long period immunization. The immunization was carried out by Freund's complete and incomplete adjuvants [24]. For immunization 200 ml of virions and 200 ml of adjuvant were added. Upper non-inoculated leaves were analyzed for the presence of TBSV virions. For each experiment samples were combined from three different plants. Samples from healthy and inoculated by wild type TBSV plants were separately homogenized in TRIS/EDTA (TE) buffer in ratio 1/2 (sample/buffer) on ice. After homogenization, all samples were centrifuged at 12 000 rpm (4 C) for 20 min and the resulting supernatant was transferred in the new tubes. Then 30 ml of each sample were mixed with 6X Loading buffer and separated in 1% agarose gel with ethidium bromide for 40 min with Tris/Borate/ EDTA buffer. Viral particles were detected in agarose gel using UV light. To detect TBSV virions, a capillary transfer onto the nitrocellulose membrane was performed followed by immunostaining with TBSV virion-specific polyclonal antibodies. Membrane blocking and visualization of phosphatase activity after incubation with antibodies, were performed like it was described for Western blotting.

Samples preparation for non-denaturing gel electrophoresis and *in vitro* assays. Freshly collected upper non-inoculated leaves of N. benthamiana were detached from plants, weighted and then frozen in liquid nitrogen, grounded in an ice-cold mortar with cold extraction buffer in 1:2 ratio (w/v). For each experiment samples were combined from three different plants. The buffer contained 250 mM Tris-HCl (pH 7.5), 250 mM Sucrose, 0.05 mM Sodium molybdate dihydrate (Na2MoO4$2H2O), 1 mM ethylenediaminetetraacetic acid (EDTA), 4 mM 1,4-Dithiothreitol (DTT), 5 mM L-cysteine, 0.001 mM aprotinin, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.001 mM pepstatin. After homogenization all samples were centrifuged at 12 000 rpm (4 °C) for 20 min. The resulting supernatant was transferred to the new tubes and kept on ice until the non-denaturing PAGE, which was performed as previously described [25].

Analysis of AO *in gel* activity. After non-denaturing electrophoresis resulting gels were rinsed with distilled water and stained in a substrate mixture, containing 0.1 M TRIS-HCl, pH 7.4, 1 mM indole-3-carboxaldehyde, 0.5 mM thiazolyl blue

tetrazolium bromide (MTT) as an indicator and 0.5 mM phenazine methosulfate (PMS) as electron carrier at 37 C for 45 min [26]. To determine the ability of AO to produce superoxide anions using aldehydes, PMS was excluded from the substrate solution. AO production of O_2^- using NADH as a substrate was tested as it was described before with 0.25 mM NADH instead of indole-3-carboxaldehyde [27].

Analysis of CAT *in gel* activity. After non-denaturing electrophoresis resulting gels with leaves samples of mock- and TBSV inoculated *N. benthamiana* plants were rinsed with distilled water 3 times and then soaked in 0.003% H_2O_2 solution for 15 min. Gels were then stained in a solution of 2% potassium ferricyanide and 2% ferric chloride until the appearance of the achromatic bands [28].

RESULTS

The effect of salt priming on the growth of *N. benthamiana*. After priming, *N. benthamiana* seeds were grown on sterilized soil with 12 seeds each for further direct assessment of the effect of various concentrations of sodium chloride on physiological parameters (% seed germination, growth rate, germination index) and morphometric parameters (root and stem lengths). On the 10th day after planting, ascended plants from 12 planted seeds were counted (Figure 1). The results of 4 hours of seed priming in 25 mM, 50 mM, 100 mM on day 10 of the experiment are presented below in figures 1 and 2.
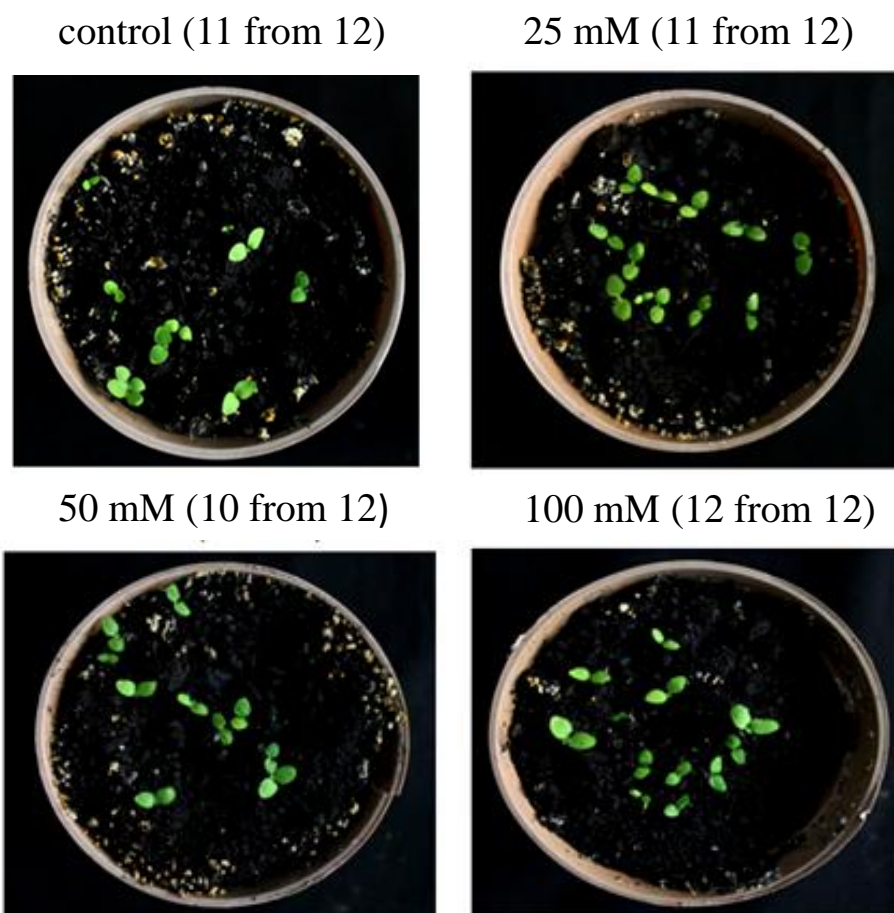

Fig.1. The growth of *N. benthamiana* after salt priming with various concentrations of NaCl on day 10 of cultivation
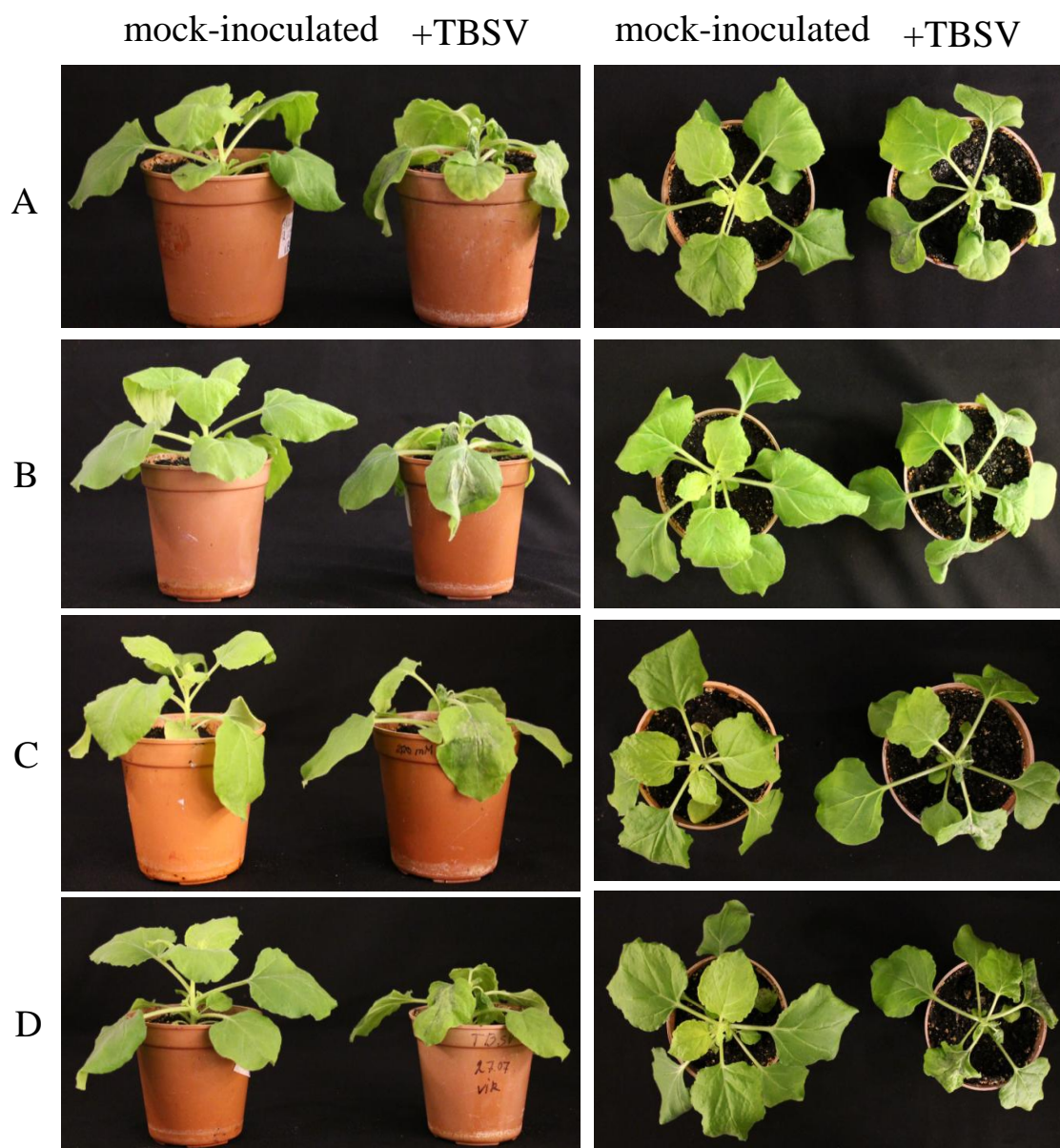
A - distilled water priming (control plants); B - 25 mm salt priming; C - 50 mM salt priming; D - 100 mm salt priming.

Fig. 2. The effect of 4 hour NaClpriming on the growth of *N. benthamiana* on day 30 of cultivation

As a result of the priming of *N. benthamiana* seeds at 4 and 6-hour primings, no significant difference in plant growth was observed. Slight growth retardation was observed when priming with 50 and 100 mM salt as compared to control and primed with 25 mM salt plants. And at 8 hours of priming with various concentrations of salts, *N.benthamiana* seeds did not germinate.

The effect of salt priming on plants antiviral resistance. Plants primed with various concentrations of salts of *N. benthamiana* at the age of 30 days were inoculated in vitro with wild-type TBSV transcripts (Figure 3).

A - distilled water priming (control plants); B - 25 mm salt priming; C - 50 mM salt priming; D - 100 mm salt priming.

Fig. 3. The manifestation of symptoms of TBSV infection with preliminary salt priming of *N. benthamiana* by 7 dpi.

To detect viral infection, an express method for analyzing the presence of virions was performed (Figure 4).
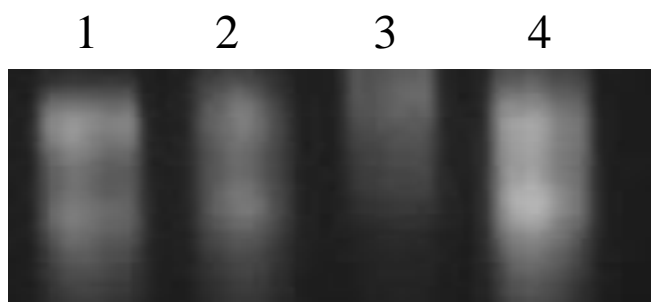
1– priming with distilled water, TBSV inoculated; 2 - 25 mM salt priming, TBSV inoculated; 3 - 50 mM salt priming, TBSV inoculated; 4 - 100 mM salt priming, TBSV inoculated.

**Fig.4.** Express detection of TBSV virus particles in infected plants

According to the result of analysis of the presence of viral particles in infected plants in plants previously primed with 50 mM NaCl, less than other plants. Corresponding results were also shown by visual symptoms of a viral infection (Figure 4).

The effect of salt priming of seeds on oxidative stress enzymes. Isoforms of the catalase enzyme and the aldehyde oxidase molybdoenzyme in the activation of an oxidative explosion during plant priming are studied. In this experiment, seeds pre-primed using 25 mM, 50 mM, 100 mM *NaCl* were used. On the 35th day of their development, the plants were analyzed using native gel electrophoresis to determine the activity of the aldehyde oxidase and catalase enzymes (Figure 5, Figure 6).With an increase in the treated concentration of *NaCl*, the activity of the aldehyde oxidase in the gel increases. The higher the concentration of *NaCl*, the higher the activity of aldehyde oxidase compared with the control sample.With an increase in the treated concentration of *NaCl*, the activity of catalase decreases compared with the control sample.
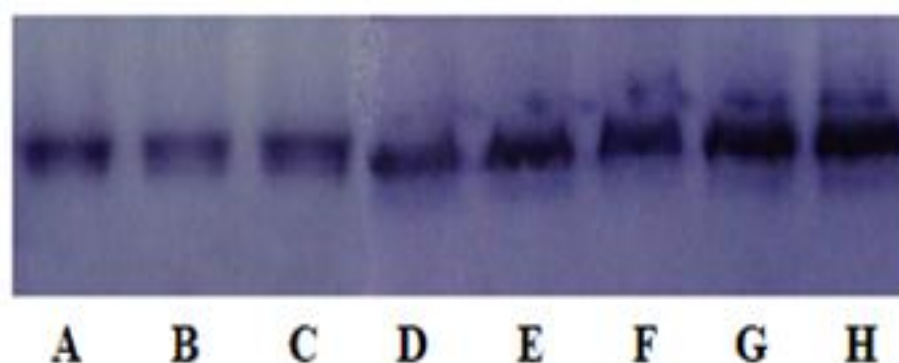
A - control plant distilled water primed; B - unprimed virus inoculated plant; C - 25 mM NaCl primed; D – virus inoculated, 25 mM NaCl primed; E - 50 mM NaCl primed; F -50 mM NaCl primed, virus inoculated; G - 100 mM NaCl primed; H - 100 mM NaCl primed, virus inoculated.
Fig.5. The effect of salt priming of seeds on aldehyde oxidase activity determination *in gel*
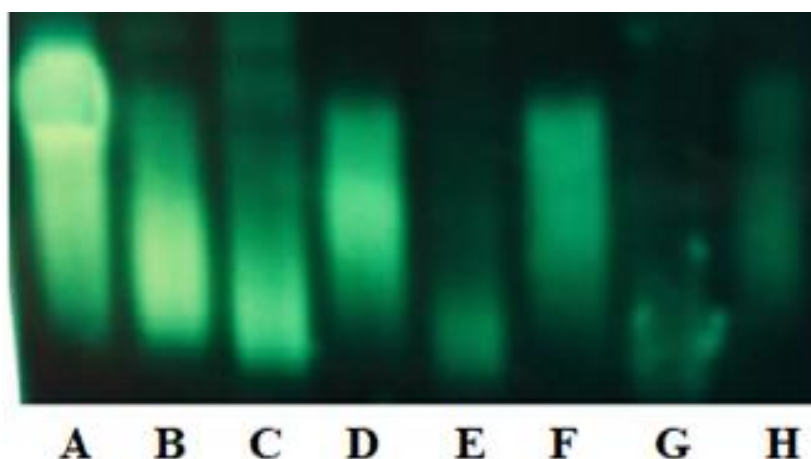
A - control plant distilled water primed; B - unprimed virus inoculated plant; C - 25 mM NaCl primed; D – virus inoculated, 25 mM NaCl primed; E - 50 mM NaCl primed; F -50 mM NaCl primed, virus inoculated; G - 100 mM NaCl primed; H - 100 mM NaCl primed, virus inoculated.
Fig. 5. The effect of salt priming of seeds on catalase activity *in gel*

DISCUSSION

As mentioned before, seed priming prepares plants to overcome upcoming environmental stress. Proposed priming mechanisms include the occurrence of epigenetic changes, as well as the accumulation of transcription factors and inactive

forms of signaling proteins. These mechanisms are modulated under the influence of stress, which leads to a more effective protective mechanism. This leads to faster and more uniform germination and the emergence of seedlings [29], improve crops under stressful conditions [30]. There are various methods of priming, namely hydropriming, halopriming, osmopriming, priming using the matrix.

The positive effects of priming under salinization conditions were noted in many cultures, such as tomato*(SolanumlycopersicumL.)*, hot pepper (*Capsicum annuum var. Acuminatum L.*), lettuce (*Lactuca sativa L.*), corn (*Zea mays L.*) [31], okra (*Abelmoschus esculentusL.)*, pea*(Pisum sativum L.)*, pepper *(Capsicum annuum L.)*, soy*(Glycine max L.)* [32].

Chemical treatment of plants using priming elicitors leads to the primary transmission of signals leading the plants to the priming stage, in which the signal network remains inactive. Exposure to environmental stress stimulates a genetically programmed defensive response along with activation of the primary signaling network associated with priming. This structure provides Spatio-temporal patterns of expression of genes and proteins associated with stress and ends with the reprogramming of a specific proteome, which ultimately leads to stress acclimatization. Despite the agronomic and environmental importance of priming, little is known about molecular mechanisms [33, 34].

It was confirmed that in the case of pretreatment of NaCl on *Glycine max* seedlings for acclimatization to subsequent salt stress, *Deschampsia antarctica* to cold stress. Interestingly, the primed state can also be induced in plants after the initial exposure to the agent, such natural or synthetic compounds as nitric oxide (NO).

 [35], hydrogen peroxide (H_2O_2)[36], hydrogen sulfide (H_2S), β-aminobutyric acid (BABA), polyamines. Also, by analogy with the priming of vegetative parts of plants, water-based seed priming is widely used to revitalize and accelerate their germination [37].

Cross-tolerance is a phenomenon that explains how plant resistance to stress leads to resistance to a completely different kind of stress [38, 39], [40]. For example, it was shown that plants of the *Tabacum* family exposed to UV radiation showed resistance to the virus [41, 42].

In most stressful situations, signals are recognized by receptors, then the signals are transmitted to the generation of secondary messengers, also known as reactive oxygen species [43]. Secondary messengers can modulate extracellular calcium levels, usually initiating cascading protein phosphorylation reactions that lead to the activation of proteins directly involved in cellular defense.

In Arabidopsis, the expression of various transcripts has been studied using the microarray method and is evidence of the intersection of signals and the interaction of various biotic and abiotic stresses. Based on these results, the stress response genes were divided into 2 groups, the early stress response genes, which are mainly constitutive in most plants, and the late stress response genes, which mainly encode "effector proteins", like metabolic enzymes participate in physiological mechanisms adaptation [44].

The phenomenon "salinization and viral infection" is still poorly understoodbut interesting literature has already appeared based on the resistance of the plant *N. benthamiana* to the EMCV virus (*Eggplant mottled crinkle virus*) during plant development under salt stress [45], the resistance of plants *N. Glutinosa* to the TBSV virus under conditions like salt stress (200 mM), darkness, and in a combination of salinization and lack of light (incubation period 48 hours) [46].

CONCLUSIONS

Studying the cross-tolerance phenomenon "salinization and viral infection" allowed us to draw the following conclusions.

1. The neutral effect of salt priming of *N. benthamiana* seeds on plant growth parameters was established;

2. It was determined that salt priming of *N. benthamiana* seeds inhibits viral infection. It was determined that at 50 mM NaCl priming of *N. benthamiana*, the accumulation of viral proteins, respectively, of viral infection is inhibited;

3. It was found that the higher the concentration of NaCl for priming with *N. benthamiana* viral infection, the higher the activity of aldehyde oxidase compared with the control sample;

4. It was found that, with an increase in the treated concentration of NaCl without viral infection, the activity of catalase decreases compared with the control sample, and with an increase in concentration during viral infection, the activity of catalase in the gel increases in comparison with control samples treated with NaCl.

Acknowledgemets

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ЭФФЕКТ ПЕРЕКРЕСТНОЙ ТОЛЕРАНТНОСТИ У *NICOTIANA BENTHAMIANA* ПРАЙМИНГА СЕМЯН И ВИРУСНОЙ ИНФЕКЦИИ

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АБСТРАКТ

В настоящее время засоление почв является одним из факторов, определяющих продуктивность сельскохозяйственных культур. Во избежание какого-либо серьезного воздействия биотического и абиотического стрессов применяется метод прайминга семян. Праймированное семя сохраняет сигнальный путь сопротивления для наступающих стрессовых условий. Во время биотического и абиотического стресса сначала накапливаются активные формы кислорода, которые являются вторичными молекулами-мессенджерами во многих сигнальных путях растительных организмов. Чрезмерное количество активных форм кислорода приводит к повреждению макромолекул, таких как липиды клеточных стенок, белки, нуклеиновые кислоты. Чтобы преодолеть серьезный эффект, у растений развивались ферментативные и неферментативные системы защиты. Ключевыми ферментами являются каталаза, альдегидоксидаза, супероксиддисмутаза. Основными компонентами неферментативной антиоксидантной системы являются витамины, низкомолекулярные вещества, такие как глутатион. Феномен «перекрестной толерантности» предполагает устойчивость растительного организма к одному стрессу при воздействии другого. Раннее воздействие солевого стресса на *Nicotiana benthamiana* приводит к его устойчивости к вирусной инфекции, изменению активности антиоксидантных ферментов (каталазы, альдегидоксидазы). Анализ вирусных частиц выявил снижение содержания вирусных частиц в праймированных солью растениях.

Ключеыве слова: соленость, праймирование, вирусная инфекция, перекрестная толерантность, активные формы кислорода.

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ТҮЙІН

Қазіргі уақытта топырақтың тұздануы ауыл шаруашылығы дақылдарының өнімділігін анықтайтын факторлардың бірі болып табылады. Ауыр жағдайларда өсімдіктерде сабақтары мен тамырлардың өсуінің баяулауы, жоғары концентрациялардағы тұздардың биохимиялық және физиологиялық әсерінен өнімділіктің төмендеуі анықталады. Биотикалық және абиотикалық стресстердің қандай да бір елеулі әсерін болдырмау үшін тұқым праймингі әдісі қолданылады. Праймингтен өткен тұқым келе жатқан стресстік жағдайлар үшін кедергінің сигналдық жолын сақтайды. Биотикалық және абиотикалық стресс кезінде алдымен өсімдіктің көптеген сигналдық жолдарында екінші молекулалар-мессенджерлер болып табылатын оттегінің белсенді түрлері жинақталады. Оттегінің белсенді формаларының шамадан тыс мөлшері жасушалық қабырғалардың липидтері, ақуыздары, нуклеин қышқылдары сияқты макромолекулалардың зақымдануына әкеледі. Елеулі әсерді жеңу үшін өсімдіктерде ферментативті және ферментативті емес қорғау жүйелері дамыды. Негізгі ферменттер-каталаза, альдегидоксидоксид, супероксидисмутаза. Ферментативті емес антиоксиданттық жүйенің негізгі компоненттері-витаминдер, глутатион сияқты төмен молекулалық заттар. "Тоғыспалы төзімділік" феномені өсімдік ағзасының бір стреске төзімділігін басқаның әсерінен болжайды. *Nicotiana benthamiana*-ға тұзды стресстің ерте әсері оның вирустық инфекцияға төзімділігіне, антиоксиданттық ферменттердің (каталазалар, альдегидоксидазалар) белсенділігінің өзгеруіне әкеледі. Вирусты бөлшектерді талдау праймирленген тұз өсімдіктердегі вирусты бөлшектер құрамының төмендегенін анықтады.