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THE COMBINED EFFECT OF TBSV P19 MUTANTS AND HEAVY METALS ON ANTIOXIDANT ENZYME ACTIVITY

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ABSTRACT

Food-value crops are frequently exposed to viral infections. Therefore, crops might experience a noticeable decline or even perish. For instance, the *Tomato Brown Wrinkle Virus*, whose primary hosts are tomatoes and peppers, was first identified in Kazakhstan in 2021. In farms where the virus was found, yield loss varied from 30 to 70% at the same time. To increase plant stress tolerance to viral infection, it is crucial to develop techniques. This manuscript's scientific uniqueness comes from the fact that no previous research has been done on the combined impact of viral infections and heavy metals on plants. Reactive oxygen species content in plant cells is tightly controlled by antioxidant enzymes. Superoxide radicals are neutralised by the enzyme catalase, which initiates the breakdown of hydrogen peroxide into water and molecular oxygen. Therefore, antioxidant enzymes stop tissue deterioration and necrosis. Reactive oxygen species can be produced by molybdenum enzymes under unfavourable circumstances like pathogen infection or dehydration. In molybdoenzymes, molybdenum is a crucial component of the Moco cofactor, but tungsten has the capacity to replace molybdenum, leading to a reversible loss of enzyme activity. Consequently, tungsten acts as a stressor for plants. Inoculation of plants with *Tomato Bushy Stunt Virus* of the wild type leads to their death. At the same time, when infected with *TBSV 157, RMJ1* and *RMJ2* mutants, the plants recovered after some time. Plants are expected to be more viable when the subject is exposed to heavy metal solutions and inoculated with viruses. As a result of this study, it was found that a viral infection increased the activity of catalase. The combined effect of TBSV mutants and heavy metals on enzyme activity was directly proportional to the effects of metals alone.

Keywords: *Tomato bushy stunt virus*, molybdoenzymes, heavy metals, molybdenum, tungsten, catalase.

INTRODUCTION

Biological significance of molybdenum and the role of molybdoenzymes in plants

Molybdenum binds to the organic part of molybdopterin to form the molybdenum cofactor (Moco) and acquires redox properties. Moco is in the active centre of molybdoenzymes, which are used to function as small electron transfer chains and are involved in nitrogen and sulfur metabolism, hormone biosynthesis, transformation of toxic compounds and other important processes not only in plants, but also in many other living things [1].

Mo was found as a cofactor in the active centers of more than fifty enzymes. Most molybdenum-containing enzymes are found in prokaryotes, while only five of them have been identified in plants: nitrate reductase, sulfitoxidase, aldehydoxidase, xanthine dehydrogenase, and an amidoxime reducing component [2].

The process of Moco synthesis in plants is very conservative and includes 4 stages. During the first stage, the conversion of 5'-GTP to cyclic pyranopterin monophosphate (cPMP) takes place in mitochondria [3]. This reaction is catalyzed by two proteins: CNX2 and CNX3 (Figure 1) [4, 5].

The formed cPMP must pass through the mitochondrial membranes into the cytosol, where

The formation of Moco will go on. This is made possible by the ABC family member transport protein ATM3, which is located on the inner mitochondrial membrane [6].

The second step involves the synthesis of molybdopterin (MPT) by introducing the dithiolene group by transferring two sulfur atoms to cPMP using MPT synthase. MPT synthase is a heterotetrameric complex consisting of two small CNX7 subunits and two large CNX6 subunits. Two steps are required for

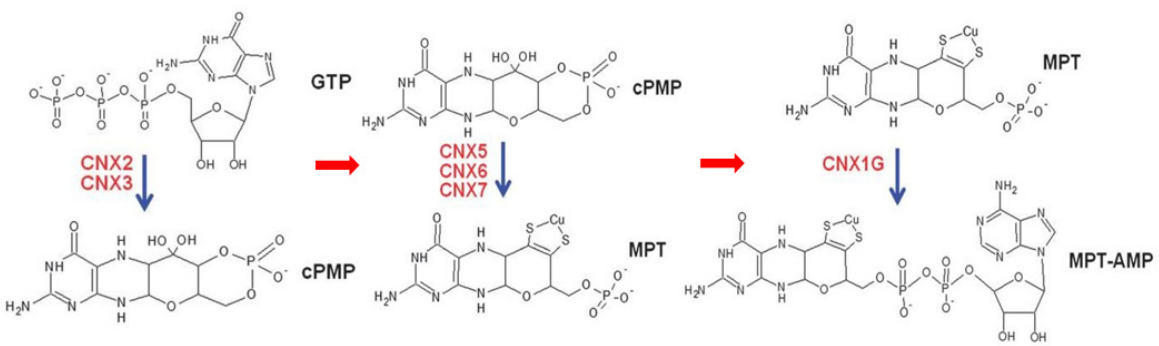

Figure 1 - Stages of Moco synthesis

molybdopterin formation because each small subunit carries one sulfur atom and the MPT-synthase must be resulfured by CNX5 sulfurase each time [7, 8].

MPT is activated by adenylation in the third stage, which results in the production of the complex MPT-AMP [9]. The reaction is catalyzed by the CNX1 protein, which consists of two domains: the large N-terminal domain of CNX1E and the small C-terminal domain of CNX1G. Binding of MPT to the CNX1G domain results in adenylation activation [9]. Then, in the fourth step, MPT-AMP is transferred to the CNX1E domain, where it is de-adenylated, and molybdenum derived from molybdate is attached [10]. This reaction is Zn2+/Mg2+-dependent [11].

Xanthine dehydrogenase is a homodimer with a molecular weight of 300 kDa [12], each of whose subunits has catalytic activity when separated [13]. The monomers are subdivided into three distinct domains: the N-terminal domain required for binding the two clusters [2Fe-2S], the FAD binding site, and the C-terminal domain to which Moco and the second monomer attach (Figure 2).

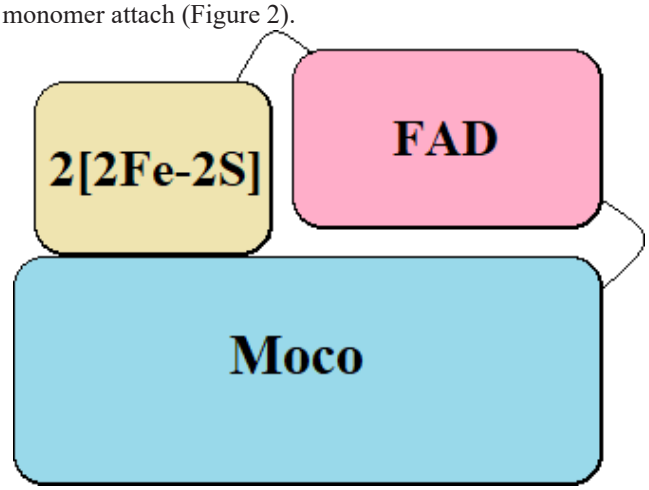

Figure 2 – Domain structure of xanthine dehydrogenase [14]

XDH catalyses the two-step oxidation of hypoxanthine and xanthine to uric acid, but the enzyme is also capable of oxidizing about thirty aliphatic and aromatic aldehydes with lower affinity to the substrate [15]. Xanthine dehydrogenase is involved in the catabolism of purines formed during nitrogen fixation by legumes [16] and has NADH-oxidase activity. Electrons formed after the interaction of the enzyme with the substrate are transferred from Mo via [2Fe-2S] clusters to the FAD cofactor. When NADH is formed, the electrons are transferred to either NAD+ or molecular oxygen, forming superoxide anions. The production of reactive oxygen species by xanthine dehydrogenase is of physiological significance, since it has been shown that FAD activity and ROS production increase during plant-pathogen interactions, drought, natural aging, hypersensitivity and viral infection [13].

Aldehydoxidase has a similar structure and amino acid sequence to xanthine dehydrogenase. Due to the high degree of homology, it is assumed that aldehyde oxidase evolved from CDH by gene duplication or they have a common ancestor [17]. Also, both enzymes can catalyze the oxidation of various non-aromatic and aromatic aldehydes and heterocycles, converting them into the corresponding tricarboxylic acids. AR is involved in the biosynthesis of phytohormones: abscisic and indolylacetic acids. The most preferred substrate for the enzyme is abscisic aldehyde, after oxidation of which abscisic acid is formed, while catalysis of the reaction of indolylacetic acid formation from indole-3-acetaldehyde occurs at a slower rate. Aldehydoxidase uses molecular oxygen as an electron acceptor, forming superoxide anions and hydrogen peroxide [13], and does not use NAD+ at all. Unlike CDH, the FAD domain in AR does not contain the FFLGYR motif, which is presumably responsible for NADH/NAD+ binding. However, it has been shown that stimulation of aldehyde oxidase by the addition of NAD+ increases its activity [18].

Nitrate reductase is a homodimer with a molecular weight of about 200 kDa. Each of the subunits includes three prosthetic groups: flavinadenindinucleotide (FAD), cytochrome b557, and the molybdenum cofactor. These domains are connected by two protease-sensitive hinge regions [19]. The enzyme plays an important role in nitrogen metabolism and is localized in the cytosol of the plant cell. Nitrate reductase catalyzes the reduction reaction of nitrate (NO-) to nitrite (NO-) [28].

$$NO_3^- + NADH \rightarrow NO_2^- + NAD^+ + OH^- \quad (1)$$

The reaction uses NADH electrons, which distinguishes it from the processes catalyzed by xanthine dehydrogenases, aldehydoxidases, and sulfitoxidases, where electrons are released on the contrary. Nitrite is further reduced to ammonium in the plastids by nitrite reductase. Also plant nitrate reductases are able to use nitrite as a substrate and produce nitrogen monoxide (NO) [20]. Nitrate reductase-derived nitric oxide is involved in antioxidant metabolism, stomatal opening, respiration regulation, root development, responses to osmotic stress and cold stress. External input of nitrate activates the expression of nitrate reductase genes, while ammonium suppresses them. Under conditions of molybdenum deficiency, the enzyme activity also decreases, but when the metal is added to optimal concentrations, activity is restored.

Accordingly, tungstate inhibits the activity of this molybdoenzyme. In the dark, the nitrate reductase protein is phosphorylated, which allows stoichiometric binding to the inhibitor protein. When leaves are illuminated, dephosphorylation and dissociation of the inhibitor occur, causing the enzyme to reactivate [21].

Sulfitoxidase plays an important role in the detoxification of sulfite, that is, the oxidation of sulfite to sulfate. As a homodimer, the enzyme has a molecular mass of 90 kDa. This protein is highly conserved among plants and is considered to be one of the simplest molybdenum enzymes found among eukaryotes. The redox center of sulfitoxidase is represented only by the molybdenum cofactor. Sulfitoxidase is localized in peroxisomes and uses molecular oxygen as an electron acceptor and forms hydrogen peroxide. This in turn explains the location of sulfitoxidase, since the excess hydrogen peroxide is neutralized here by catalase [22].

Reactive oxygen species

Plants everywhere face adverse conditions caused by abiotic and biotic factors such as drought, soil salinity, exposure to pathogens and heavy metals. As a consequence of such stress, plant cells can reproduce reactive oxygen species.

AOS, in turn, can both initiate oxidative stress, accompanied by cell damage or death, and act as signaling molecules, inducing an increase in the stress tolerance of the organism [23].

ROS include a set of reactive oxygen species. As a rule, their lifespan is short. Reactive oxygen forms include free radical particles - superoxide radical anion (- O-), hydroxyl radical (- OH), peroxyl radicals (- RO-), alkoxyl radicals (- RO) and nonradical molecular forms such as hydrogen peroxide (H2O2) and singlet oxygen (1O2). However, the most common ones are superoxide, hydrogen peroxide and hydroxyl radical [24]. The reduction of molecular oxygen leads to the formation of superoxide, which then dismutase into hydrogen peroxide. H2O2 can be partially reduced to a hydroxyl radical or completely reduced to water [25].

Antioxidant enzymes

Catalase is an iron-containing enzyme consisting of four subunits with a total molecular weight of about 240 kDa. The tetramer catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen [26]. The mechanism of catalase action includes four stages. In the first stages, hydrogen peroxide is reduced by splitting the O-O bond to form the first water molecule and intermediate compounds, the oxyferryl and the porphyrin cation radical. As the oxyferryl is reduced, the second hydrogen peroxide molecule is oxidized to molecular oxygen and this results in the release of bound oxygen as a second water molecule (Figure 3).

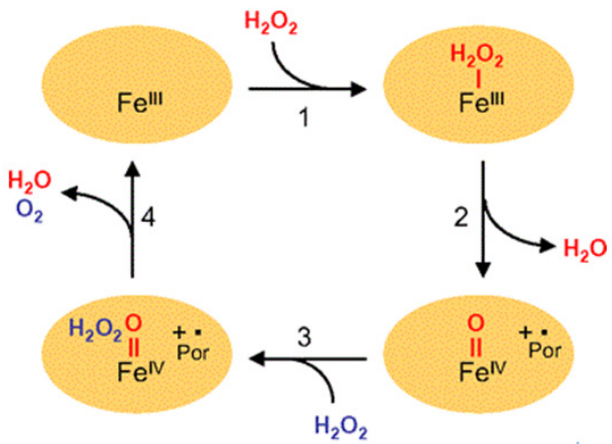

Figure 3 – Stages of the reaction catalyzed by catalase [26]

MATERIALS AND METHODS

Study objects: *Nicotiana benthamiana,* TBSV and its mutants 157, RMJ1 and RMJ2.

Tomato bushy stunt virus (TBSV) is a typical member of the genus *Tombusvirus* of the family *Tombuviridae*. Virions are shell-free icosahedral particles assembled from 180 protein subunits of P41, the arrangement of which gives the surface a granular appearance. The particles are about 33 nanometers in diameter and consist of 17% ribonucleic acid and 83% protein (Figure 4).

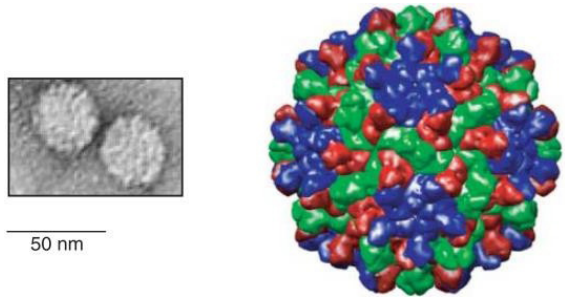

Figure 4 — (a) viral particles of TBSV in a transmission electron microscope and (b) atomic model of the crystallographic structure of the TBSV virion [27]

The TBSV virus genome is a single-stranded positive-sense RNA consisting of 4,776 nucleotides. The genomic RNA (gRNA) lacks both a 5'-cap and a 3'-polyadenine tail and encodes five open reading frames (ORF). The genome contains five genes encoding two viral replicase proteins (P33 and P92), a capsid protein (P41), an RNA interference suppressor (P19), and a transport protein (P22) [27].

P33 and P92 proteins are translated directly from the genomic RNA, with P92 being expressed via ribosomal reading of the P33 stop codon, resulting in the accumulation of more P33 relative to P92. The P33 protein is responsible for binding to RNA and forming a replicase complex together with the RNA-dependent RNA polymerase P92 [28]. Both proteins localize in membranes. The replicase complex uses genomic RNA as a matrix for the synthesis of complementary minus-sense RNA, which will serve as a matrix for positive-sense RNA. When the first unit of P33 binds to the RNA, the ribonucleic acid is quickly coated with additional P33 molecules, which can be useful for the replication process. Also, P33-coated viral RNAs may be less susceptible to nucleases and gene silencing. However, without the P19 suppressor, the viral RNA remains unstable to RNA interference [27]. The above characteristics belong to the wild-type *tomato bushy stunt virus* (TBSV-WT). However, the mutants differ in the expression of some proteins: the TBSV 157 mutant (ΔP19) does not express the interfering RNA suppressor P19 [29]; the TBSV P19/75-78 mutant contains a substitution of two amino acids of arginine for glycine at positions 75 and 78. Thus, a P19 protein is formed that cannot specifically bind to short interfering RNAs [30]; in the TBSV RMJ1 mutant genome, the gene encoding the capsid protein P41 has been completely deleted and replaced with a gene for green fluorescent protein (GFP), whose product exhibits bright green fluorescence when exposed to light ranging from blue to ultraviolet. The suppressor RNAi is identical to the wild type [31]; TBSV RMJ2 also has GFP instead of capsid protein and a replacement of the P19 start codon ATG by CTG, resulting in a shorter amino acid sequence of the protein than wild-type P19. P19 expression is reduced but can still be detected; TBSV RMJ3 RNA is similar to TBSV RMJ2, but additionally has a premature stop codon in the P19 gene, which leads to destabilization and rapid degradation of the suppressor protein (Figure 5) [32].

The P19 protein of *tomato bushy stunt virus* is a suppressor of RNA interference, resulting in the spread of systemic infection. P19 dimers correspond to 21 nucleotide duplexes

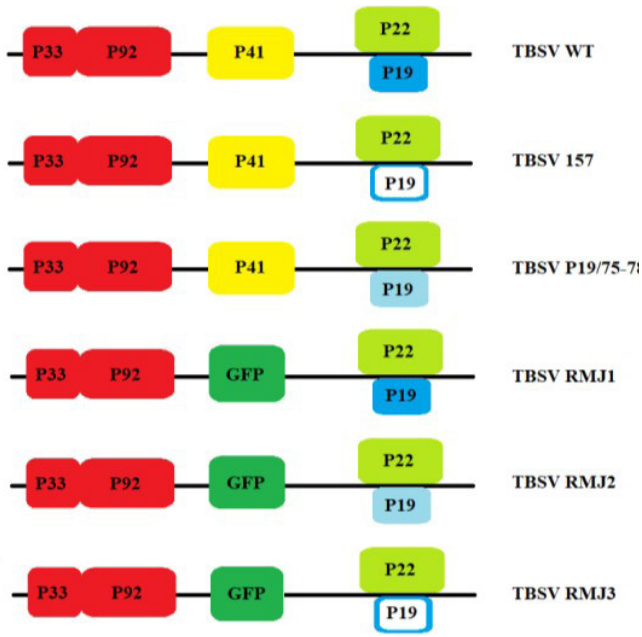

Figure 5 — Comparative characterization of the TBSV wild-type and its mutants [33]

of short interfering RNAs formed by DCL. During TBSV replication, high levels of genomic single-stranded and double-stranded RNA accumulate in plants and can be used as a substrate for cleavage into short interfering RNAs. The single-stranded siRNAs then bind to Ago and form the RISC complex, forming a catalytic structure that targets viral RNA degradation. To prevent this, the suppressor homodimeric protein TBSV binds to the siRNAs and thus makes them inaccessible for RISC formation [34]. Thus, infection with mutants defective in the P19 protein does not lead to necrosis and the plant can recover.

Grows of *N. benthamiana*

Prepared universal soil was used for planting, which was mixed with vermiculite in the ratio of 3 parts soil and 1 part vermiculite. The soil was moistened with 40 ml of water and the seeds were planted at a frequency of 1-2 per square centimeter. Watering was carried out regularly by 10-20 ml. On day 7-10 after planting, each seedling was transplanted separately into a 250 ml pot. Watering was carried out by 30 ml once every two days.

After reaching thirty days, the plants were watered daily for one week with 20 ml of heavy metal solutions (Mo, W, MoW) of two concentrations, 2.5 mM and 5 mM. On the thirty-seventh day, plants were inoculated with TBSV WT virus and its mutants, 157, RMJ1, and RMJ2.

Heat-shock transformation of *E.coli*

The transformation was performed by heat-shock. Competent cells are kept on ice throughout the procedure since they must be stored at -80°C. pUC19 is a genetically engineered construction which contains built-in viral sequences of TBSV WT, 157, RMJ1 and RMJ2. To 50 µl of competent cell culture (*Escherichia coli*, strain XL10) were added 5 µl of pUC19 plasmids containing DNA fragments of *tomato bushy stunt virus*.

The pUC19 plasmid is a high-copy cloning vector consisting of 2,686 base pairs. The plasmid includes an ampicillin resistance gene used for selection and a polylinker site (multiple cloning site, MCS) embedded in the lacZ gene encoding ß-galactosidase and containing 13 different sites for the restrictases Acc I, BamH I, EcoR I, Hinc II, Hind III, Kpn I, Pst I, Sac I, Sal I, Sma I, Sph I, Xba I and Xma I.

The cells were left on ice for half an hour, then placed in a water bath heated to 42°C for 2.5 minutes and transferred back to ice for 30 minutes. Next, 1 ml of LB liquid medium was added and incubated for one hour in a heat shaker at 37°C and 150 rpm. After incubation, cells were centrifuged for three minutes at 3000 rpm. The supernatant was removed to 100-150 microliters, and the remaining precipitate was transferred by the Koch method to Petri dishes with solid LB agarized medium containing ampicillin. At 37°C overnight, colonies consisting only of cells containing pUC19 plasmids grew because they contain the ampicillin resistance gene.

Plasmids isolation

Isolation of plasmids performed by using thermo scientific GeneJET Plasmid Miniprep Kit.

Restriction of plasmids

Restriction was performed using the restrictase SmaI. The restriction site of SmaI is CCC↓GGG. One microliter of SmaI restrictase, one microliter of plasmids, five µl of buffer, and 50 milliliters of sterile injection water were added to an Eppendorf tube. Incubated for 15 minutes at room temperature.

Purification of DNA

To 50 µl of DNA, 450 µl of distilled H_2O and 500 µl of phenol chloroform were added. Stirred by inverting the vial for 30 seconds. It was centrifuged at 10,000 rpm and 4°C for fifteen minutes. 400 µl of supernatant was carefully taken from the upper phase and 40 µl of 3 molar sodium acetate and 560 µl of chilled 96% ethyl alcohol were added. Incubated for 40 minutes at -20°C in an upright position. Centrifuged for ten minutes, after which the contents were poured off. The precipitate was washed with 500 µl of 70% ethyl alcohol, centrifuged for five minutes, and drained again. This step was repeated twice. The precipitate was dried at 37°C and resuspended in 10 µl of H_2O.

***In vitro* transcription**

6 µl of Tango buffer were added to 4 sterile Eppendorf tubes, 1 µl adenosine triphosphate, 6 µl uridine triphosphate, 6 µl cytidine triphosphate, 6 µl guanosine triphosphate, 6 µl purified restrictants containing TBSV coding DNA WT, 157, RMJ1 and RMJ2, 6 µl T7 DNA dependent RNA polymerase, and 21 µl sterile water. Incubated for

for three hours in a water bath at 37°C. The presence of transcripts was checked using an agarose gel.

Inoculation of plants

The solution containing the virus RNA was brought to a volume of 350 µL with 10 mM phosphate buffer. After a week of metal treatment, two leaves on each plant were infected with the addition of selite and 25 µl of virus RNA and then the leaf was gently rubbed to allow the RNA to penetrate the cells. One week after infection, specimen isolation was performed. 0.2 g of the leaves were homogenized with 400 µl of single-TE buffer by pestle grinding in a chilled mortar, then transferred to tubs. Centrifuged at 4°C and 10,000 rpm for 30 min, the supernatant was collected in clean tubes and stored in the freezer at -20°C.

Western blotting

Western blotting is required to determine the presence of contamination. Gels after vertical SDS-PAGE electrophoresis were used to transfer proteins to a nitrocellulose membrane. Transfer buffer and the following parameters were used: current voltage 180 V, current strength 300 mA, and transfer duration 120 minutes. Protein transfer efficiency was checked using Ponceau S dye. To avoid nonspecific binding, the membrane was placed for one hour in a blocking solution prepared from 3.5 g of skim milk powder and 50 ml of single TBS/TWEEN. Immunoblotting was then performed for one hour with specific antibodies induced against the TBSV P19 protein. The membrane was washed three times with a single TBS/TWEEN solution. Next, the membrane was primed with secondary anti-rabbit antibodies labeled with alkaline phosphatase and washed three times after one hour as well. Substrate NBT/5-bromo-4-chloro-3-indolyl phosphate was used to visualize the results.

Express method for determination of infection

For plants infected with the TBSV 157 mutant, the above described method of determining infection using the P19 protein is not applicable, because this protein is absent. Therefore, the express method was used for it. Proteins separated in 1% agarose gel were transferred to a nitrocellulose membrane by capillary forces using a stack of filter paper. Blocking was performed similarly, but primary antibodies were used to the anti-mouse capsid protein. Secondary antibodies were also anti- mouse, labelled with alkaline phosphatase, and NBT/BCIP substrate.

Native protein electrophoresis in polyacrylamide

For the bottom gel, 2.82 mL of 40% acrylamide solution, 3.75 mL of 1 M TRIS solution (pH 8.8), 8.44 mL of distilled water, 100 µl of 10% ammonium peroxodisulfate, and 15 µl of TEMED were used. The top gel was prepared by adding 2.5 ml of 6.25% acrylamide solution, 1.25 ml of 3 molar TRIS solution (pH 6.8), 1.25 ml of distilled water, 100 µl of 10% ammonium peroxodisulfate, and 5 µl of TEMED. The buffer used was UPPER, current voltage 110 V, current strength 50 mA, and time three hours.

Determination of catalase activity

After electrophoresis of native proteins, the gels were washed three times with distilled water and left for ten minutes in 0.03% hydrogen peroxide solution. Then, they were washed again with distilled water and simultaneously filled with 2% iron (III) chloride solution and 2% potassium hexacyanoferrate solution.

RESULTS AND DISCUSSION

Preparation of TBSV RNAs for plant inoculation *N. benthamiana*

After transforming competent *E.coli* cells with pUC19 plasmids, the cells were multiplied and then plasmids were isolated from them. The presence of plasmids in solution was checked by electrophoresis of DNA in a 1% agarose gel (Figure 6). Then, the restrictions were purified from possible impurities and contaminants. The TBSV genome is a single-stranded RNA molecule; therefore, transcripts must be used for infection. For this purpose, in vitro transcription was performed, the results of which were verified by electrophoresis.

Determination of infestation of *N. benthamiana* plants

Nicotiana benthamiana plants were treated with Mo, W, and MoW solutions at concentrations of 2.5 mM and 5 mM on day 30 after planting. Seven days later, plants were infected with TBSV transcripts. On the 7th day after infection

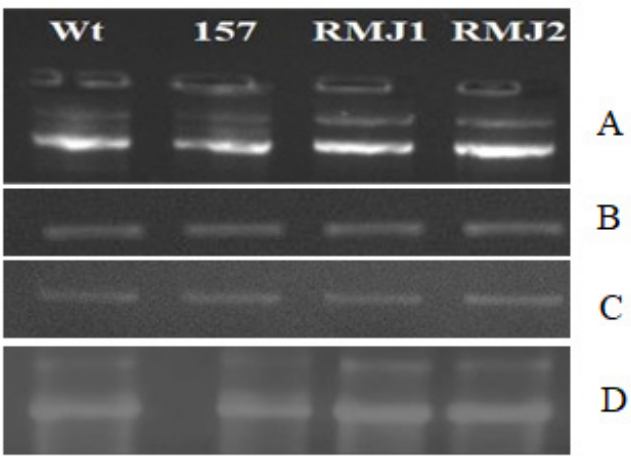

Figure 6 - Preparation of TBSV RNAs

A – Results of electrophoresis of isolated plasmids; B - Plasmid DNA was linearized using the SmaI endonuclease; C - Restrictions in an agarose gel; D - Purified TBSV RNAs

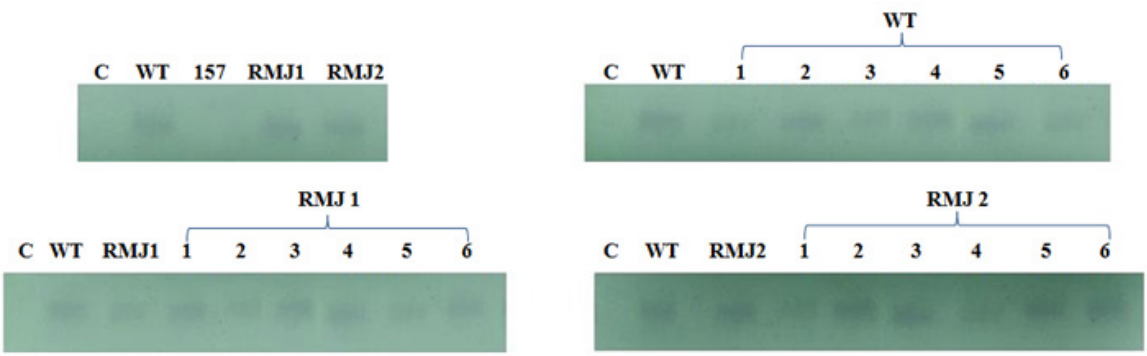

Figure 7 – P19 proteins on the membrane

C – negative control (H_2O); WT – wild type, positive control; RMJ1 – viral mutant; RMJ2 – viral mutant; 1 - 6 indicate the concentration of Mo, W and MoW solutions with viral mutants, where 1 - Mo 2,5 mM; 2 - W 2,5 mM; 3 - MoW 2,5 mM; 4 - Mo 5mM; 5 - W 5mM; 6 - MoW 5mM. P19 protein encoded by TBSV is presented in a dimeric form on the membrane.

of the plant with virions, the viral infection did not spread due to the absence of the capsid protein and the plant did not die, but growth slowed down.

On day 4-5 after infection, GFP proteins could be observed under UV light on plants inoculated with TBSV RMJ1 and RMJ2, indicating the presence of viral particles.

Another week later, proteins were isolated from plant leaves. Using electrophoresis of proteins in polyacrylamide gel and its further staining with Coomassie Brilliant Blue, the presence of proteins was determined.

After checking protein transfer, the dye was washed with a single TBS/TWEEN solution and Western blotting was performed to detect the P19 protein using anti-rabbit antibodies (Figure 7). p19 protein encoded by TBSV is presented in a dimeric form on the membrane.

To determine infestation in plants inoculated with TBSV 157 mutant, a rapid method of capsid protein determination was used (Figure 8).

Determination of catalase activity

To determine catalase activity, native protein samples were separated on a 7.5% polyacrylamide gel and stained with the substrate. The degree of activity was analyzed using ImageJ software. The data were converted into percentage values.

When exposed to viruses expressing P19 protein, catalase activity was decreased. The TBSV 157 mutant does not contain P19; therefore, the activity remains close to the control sample (Figure 9).

The level of catalase decreased when treated with metals with 5 mM concentration, but remained quite high after exposure to 2.5 mM tungsten solution (Figure 10).

In plants infected with TBSV WT, the enzyme activity decreased, but at the same time, the combined effect of this pathogen and metal solutions led to its increase, especially in combination with 2.5 mM tungsten (Figure 11).

The effect of TBSV 157 mutant and metals is similar to the effect of metal solutions on uninfected plants, from which we can conclude that infection had no effect on enzyme activity (Figure 12).

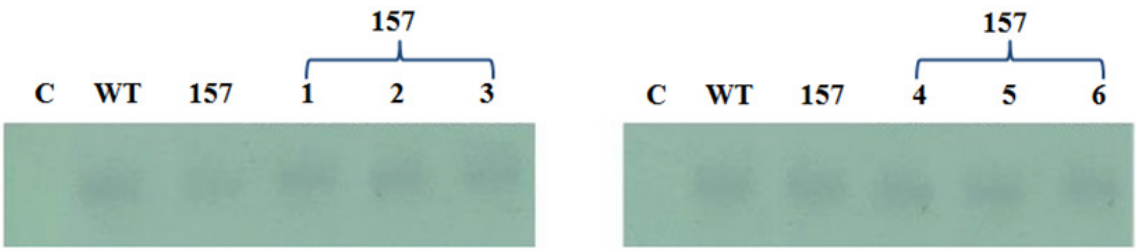

Figure 8 – P41 proteins on the membrane

C – negative control (H_2O); WT – wild type, positive control; 157– viral mutant; 1 - 6 indicate the concentration of Mo, W and MoW solutions with viral mutants, where 1 - Mo 2,5 mM; 2 - W 2,5 mM; 3 - MoW 2,5 mM; 4 - Mo 5mM; 5 - W 5mM; 6 - MoW 5mM.

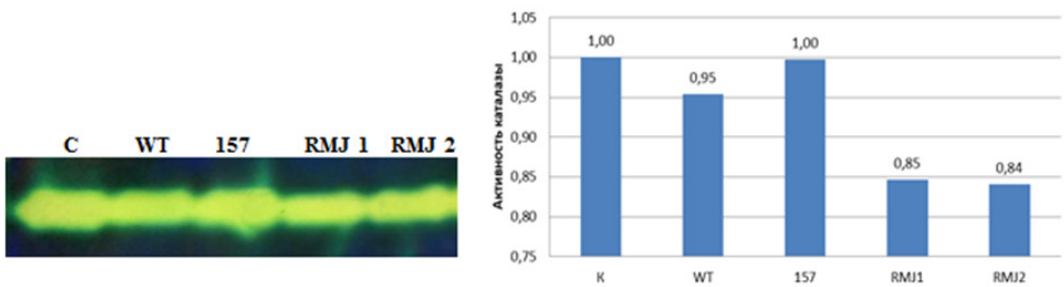

Figure 9 – Catalase activity when exposed to TBSV. On the left is *in gel* activity of catalase, and on the right is a diagram showing the percentage values of activity

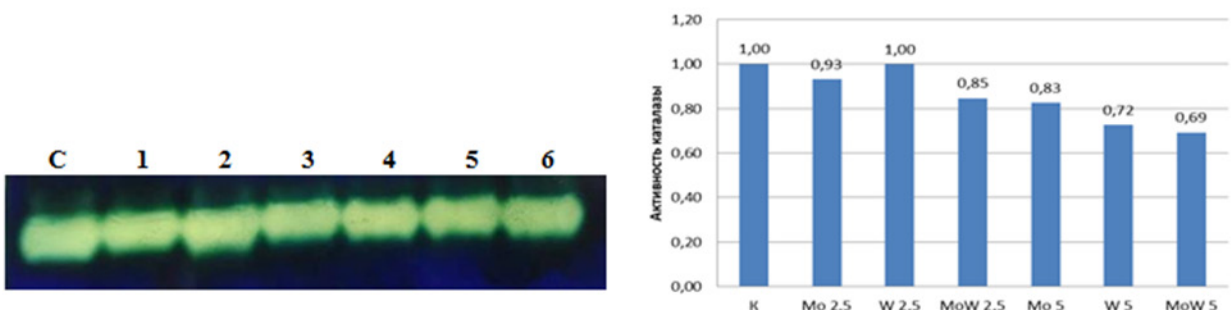

Figure 10 – Catalase activity after metal treatment

C – negative control (H_2O); 1 - 6 indicate the concentration of Mo, W and MoW solutions with viral mutants, where 1 - Mo 2,5 mM; 2 - W 2,5 mM; 3 - MoW 2,5 mM; 4 - Mo 5mM; 5 - W 5mM; 6 - MoW 5mM.

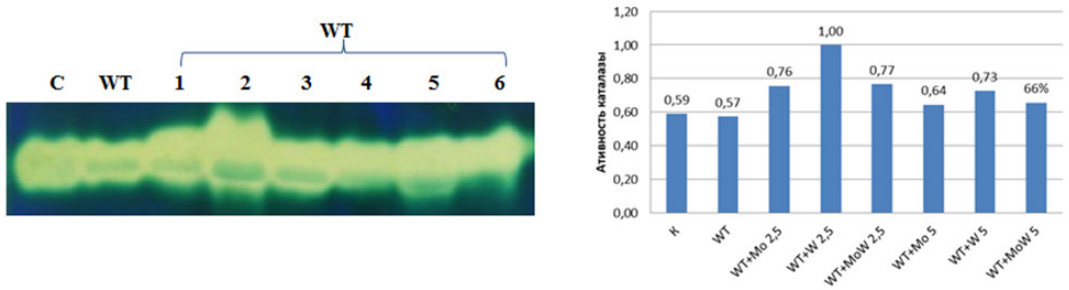

Figure 11 – Catalase activity when co-treated with heavy metals and TBSV WT

C – negative control (H_2O); WT – wild type, positive control; 1 - 6 indicate the concentration of Mo, W and MoW solutions with viral mutants, where 1 - Mo 2,5 mM; 2 - W 2,5 mM; 3 - MoW 2,5 mM; 4 - Mo 5mM; 5 - W 5mM; 6 - MoW 5mM.

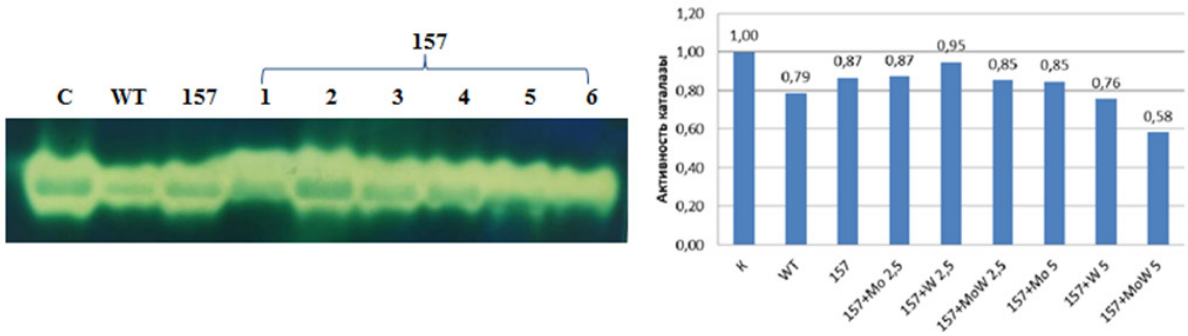

Figure 12 – Catalase activity when exposed to heavy metals and TBSV 157 mutant

C – negative control (H_2O); WT – wild type, positive control; 157– viral mutant; 1 - 6 indicate the concentration of Mo, W and MoW solutions with viral mutants, where 1 - Mo 2,5 mM; 2 - W 2,5 mM; 3 - MoW 2,5 mM; 4 - Mo 5mM; 5 - W 5mM; 6 - MoW 5mM.

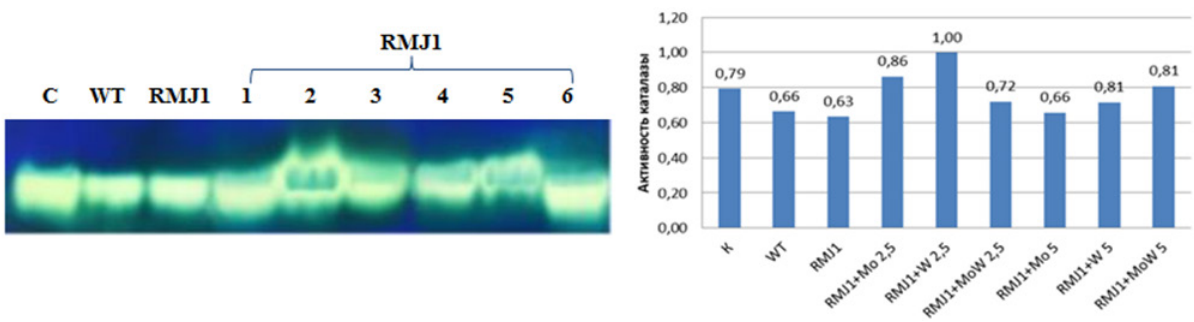

Figure 13 – Catalase activity in plants treated with metal solutions and the TBSV RMJ1 mutant

C – negative control (H_2O); WT – wild type, positive control; RMJ1 – viral mutant; 1 - 6 indicate the concentration of Mo, W and MoW solutions with viral mutants, where 1 - Mo 2,5 mM; 2 - W 2,5 mM; 3 - MoW 2,5 mM; 4 - Mo 5mM; 5 - W 5mM; 6 - MoW 5mM.

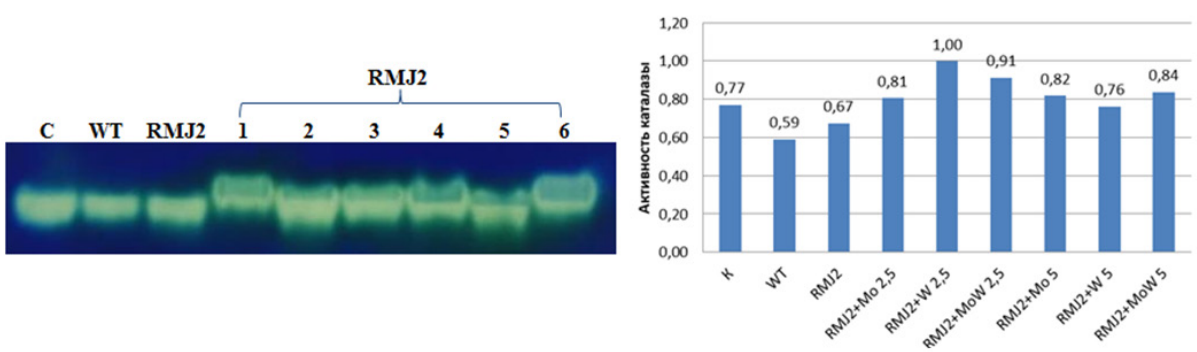

Figure 14 – Catalase enzyme activity in heavy metals and the TBSV RMJ2 mutant

C – negative control (H_2O); WT – wild type, positive control; RMJ2 – viral mutant; 1 - 6 indicate the concentration of Mo, W and MoW solutions with viral mutants, where 1 - Mo 2,5 mM; 2 - W 2,5 mM; 3 - MoW 2,5 mM; 4 - Mo 5mM; 5 - W 5mM; 6 - MoW 5mM.

The RMJ1 mutant together with metal solutions led to an increase in catalase activity, the maximum of which was recorded at 2.5 mM concentration of tungsten (Figure 13).

Exposure to the TBSV mutant RMJ2 and heavy metals led to an increase in activity, and just as in the results with RMJ1, the maximum value was reached under the action of a concentration of 2.5 mM tungsten (Figure 14).

Identification of differences in plant morphology and symptoms

Plants of *N.benthamiana* treated with 2.5 mM molybdenum solutions showed no distinguishing features from control plants. At a concentration of 5 mM, growth retardation was observed.

Plants treated with 5 mM tungsten solution showed stunted growth, yellowing and wilting of leaves. When treated with the metal within 10 days, 50% of plants died, indicating the destructive effect of tungsten. At a tungsten concentration of 2.5 mM, a decrease in leaf plates was also observed compared to the control plant, which was watered only with water.

The effect of MoW solutions at a concentration of 2.5 mM affected the wilting of lower leaves. The concentration of 5 mM had a more aggressive effect on the plant, similar to the same concentration of tungsten. When watered with the solution for 10 days, 40% of the plants died (Figure 15).

Infection with TBSV WT showed the presence of chlorotic spots on the leaf surface, curling and wrinkling of the upper leaves. When infected with mutant TBSV 157, the symptoms were expressed only as chlorotic spots. No symptoms were observed in plants inoculated with RMJ1 and RMJ2 mutants other than leaf wilting through which infection occurred (Figure 16).

Infected plants pre-treated with heavy metal solutions showed a combination of traits from the two stressors. However, the plants survived and returned to normal growth after 10-14 days.

CONCLUSION

The presence of capsid protein, P19 protein, and GFP expression in leaves infected with RMJ1 and RMJ2 mutants served as a marker for viral infection in *N. benthamiana* plants. Plants were inoculated with transcripts of viral mutants that contain a modified viral genome according to the constructs specified in the materials and methods.

Molybdenum exposure showed almost no effect on the plants, whereas tungsten exposure caused stunted growth, leaf yellowing, and decreased viability. Molybdenum and tungsten treatment in combination caused leaf wilting. The largest negative impact occurred at a dose of 5 mM. Plants with TBSV WT infections have chlorotic patches and wrinkled leaves. The higher leaves also started to curl. Only chlorotic patches were produced by the TBSV 157 mutant. The plants' outward characteristics were unaffected by the RMJ1 and RMJ2 mutations. Together, heavy metals and viruses had a combined effect, yet viability was boosted. Of all the TBSV mutants and metal solutions tested, it was discovered that the 5 mM tung-

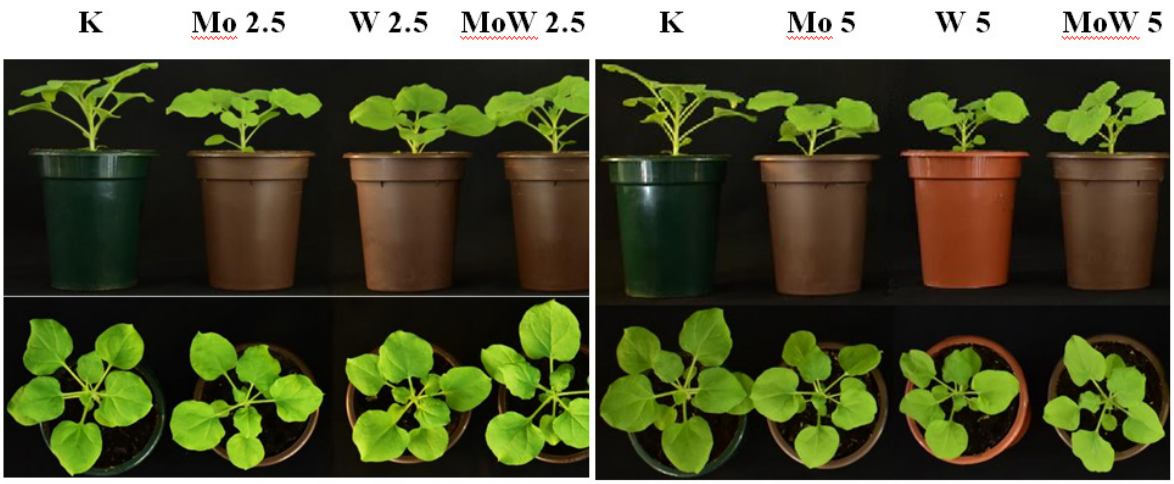

Figure 15 – Effect of heavy metal solutions on plants

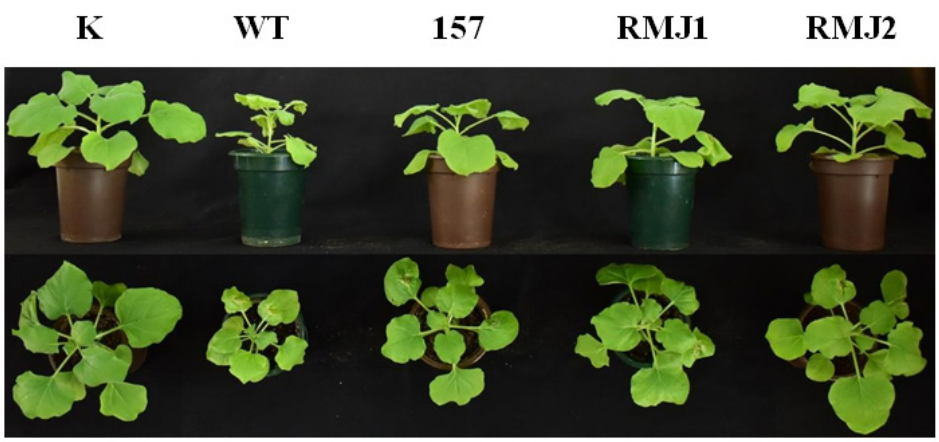

Figure 16 – Plants infected with TBSV and its mutants

sten and TBSV WT solutions produced the most stress.

Catalase activity was highest when exposed to TBSV 157, which does not contain the P19 protein responsible for suppressing RNA interference. Plants treated with 2.5 mM tungsten solution also had high catalase activity. All solutions with a concentration of 5 mM resulted in relatively low activity, both in uninfected plants and in combination with viruses. Thus, catalase activity increased upon exposure to 2.5 mM solutions of tungsten and viruses, indicating a high concentration of hydrogen peroxide in the cells, hence, the oxidative stress from the combination of these factors was the greatest. The low catalase activity in plants treated with metal concentrations of 5 mM was probably related to the accumulation of other forms of AOS, which in turn led to impaired plant growth. The effect of the combined effect of TBSV mutants and heavy metals on enzyme activity was directly proportional to exposure to metals alone, and in addition, viral infection increased catalase activity. It was determined that catalase activity increases with exposure to 2.5 mM tungsten and viral solutions, and low catalase activity was found in metal-treated concentrations of 5 mM.

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СОВМЕСТНОЕ ВЛИЯНИЕ МУТАНТОВ TBSV P19 И ТЯЖЕЛЫХ МЕТАЛЛОВ НА АКТИВНОСТЬ АНТИОКСИДАНТНЫХ ФЕРМЕНТОВ

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АБСТРАКТ

Культуры, имеющие пищевую ценность, часто подвергаются воздействию вирусных патогенов. Следовательно, посевы могут заметно уменьшиться или полностью погибнуть. Например, в 2021 году в Казахстане был обнаружен вирус коричневой морщинистости томатов (*Tomato Brown Wrinkle Virus*), основными хозяевами которого являются томаты и перец. В то же время в хозяйствах, где был обнаружен возбудитель, потери урожая составляли от 30 до 70%. Поэтому важно разработать методы, направленные на повышение стрессоустойчивости растений к вирусной инфекции. Научная новизна этой статьи заключается в том, что ранее одновременное воздействие вирусных патогенов и тяжелых металлов на растения не изучалось. Антиоксидантные ферменты играют важную роль в регулировании концентрации активных форм кислорода в клетках растений. Фермент каталаза катализирует превращение перекиси водорода в воду и молекулярный кислород, тем самым нейтрализуя супероксидные радикалы. Таким образом, антиоксидантные ферменты предотвращают повреждение тканей и некроз. Молибдоферменты могут вырабатывать активные формы кислорода при воздействии неблагоприятных условий, таких как заражение патогенами или засуха. Молибден является неотъемлемой частью кофактора Moco в молибденовых ферментах, но вольфрам обладает способностью заменять молибден, что приводит к обратимой потере функции фермента. Следовательно, вольфрам действует как стрессор для растений. Инокуляция растений диким типом вируса кустистой карликовости томатов (Tomato Bushy Stunt Virus) приводит к их гибели. В то же время, при заражении мутантами TBSV 157, RMJ1 и RMJ2 растения через некоторое время выздоравливали. Ожидается, что растения будут более жизнеспособными, если субъект подвергнется воздействию растворов тяжелых металлов и прививке вирусами.

Ключевые слова: TBSV, молибдоферменты, тяжелые металлы, молибден, вольфрам, каталаза.

TBSV P19 МУТАНТТАРЫ МЕН АУЫР МЕТАЛДАРДЫҢ АНТИОКСИДАНТТЫ ФЕРМЕНТТЕРДІҢ БЕЛСЕНДІЛІГІНЕ БІРЛЕСКЕН ӘСЕРІ

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АБСТРАКТ

Тағамдық құндылығы бар дақылдар көбінесе вирустық қоздырғыштардың шабуылына ұшырайды. Сондықтан дақылдар айтарлықтай азаюы немесе толығымен өлуі мүмкін. Мысалы, 2021 жылы Қазақстанда қызанақтың қоңыр бүрісу вирусы (*Tomato Brown Wrinkle Virus*) табылды, оның негізгі иелері қызанақ пен бұрыш болып табылады. Сонымен қатар, қоздырғыш табылған шаруашылықтарда егіннің шығыны 30-дан 70% - ға дейін болды. Сондықтан өсімдіктердің вирустық инфекцияға стресске төзімділігін арттыруға бағытталған әдістерді әзірлеу маңызды. Бұл мақаланың ғылыми жаңалығы - бұрын вирустық қоздырғыштар мен ауыр металдардың өсімдіктерге бірлескен әсері зерттелмеген. Антиоксидантты ферменттер өсімдік жасушаларында оттегінің белсенді түрлерінің концентрациясын реттеуде маңызды рөл атқарады. Каталаза ферменті сутегі асқын тотығының суға және молекулалық оттегіге айналуын катализдейді, осылайша супероксид радикалдарын бейтараптандырады. Осылайша, антиоксидантты ферменттер тіндердің зақымдануы мен некроздың алдын алады. Молибдоферменттер қоздырғыш инфекциясы немесе құрғақшылық сияқты қолайсыз жағдайларға ұшыраған кезде оттегінің белсенді түрлерін шығара алады. Молибден Молибден ферменттеріндегі Moco кофакторының ажырамас бөлігі болып табылады, бірақ вольфрам молибденді алмастыра алады, бұл фермент функциясының қайтымды жоғалуына әкеледі. Сондықтан вольфрам өсімдіктер үшін стресс ретінде әрекет етеді. Қызанақтың бұталы ергежейлі вирусының (*Tomato Bushy stunt Virus*) жабайы түрімен өсімдіктерді жұқтыру олардың өліміне әкеледі. Сонымен қатар, TBSV 157, RMJ1 және RMJ2 мутанттарымен жұқтырған кезде өсімдіктер біраз уақыттан кейін қалпына келді. Егер субъект ауыр металл ерітінділерімен өңделсе және вирустармен егілсе, өсімдіктер өміршең болады деп күтілуде.

Кілтті сөздер: TBSV, молибдоферменттер, ауыр металдар, молибден, вольфрам, каталаза.