

Summer School of Science, Visnjan

# Impact of stress on the plant's ability to fight against a pathogen

P.S. PLANTS ARE UNDER STRESS TOO

Students: Nea Baus, Mia Radovic and Liisa Veerus

Supervisors: Kristina Majsec, B.Sc. and Petra Pusic, B.Sc

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## INTRODUCTION

Stress is reaction of an organism to a change in the environment. A stressor is anything in the outside world that knocks an organism out of balance. The stress response enables the individual to meet danger and be prepared for the worst to overcome the obstacles. Plants also react to stress but in a different manner than humans, because their reactions are usually internal, and not external. Plants cannot run away from stress, therefore they use more complex ways to fight with stressors. Stressors for plants are: drought, flood, salinity, pollution, climate change, too much or not enough sunlight, mechanical damage, viruses, bacteria and insects.

In our project we researched how do plants (*Nicotiana glutinosa* L.) react to an infection caused by a virus (Tomato bushy stunt virus, TBSV) after we put them under stress for 48 hours. *Nicotiana glutinosa* L. is a plant that belongs to genus *Nicotiana* widely known as tobaccos which are, except their commercial use in industry, very important experimental plants in biology. TBSV is an RNA virus that infects wide range of plants. Our goal was to prove that plants use cross-resistance to fight against virus. Cross-resistance is a phenomenon when a resistance to one stress induces acclimatization (increasing the level of resistance by exposing the plants to minor stress) to another stress. It is only possible when stressors share many similar features.

## MATERIALS AND METHODS

To perform the experiment, plants were divided into 4 groups: control (no treatment), darkness (complete lack of sunlight), salinity (200mM) and combination of darkness and salinity; with 7 plants in each group. After 48 hours of stress, they had 24 hours recovery time under normal conditions and then were infected with TBSV by mechanical inoculation of the homogenate of infected tissue in potassium phosphate (KP)-buffer. Two leaves per plant were first mechanically damaged and then inoculated, so the virus could easily invade the plant. The results of infection should be visible macroscopically after 48 hours as necrotic (dark, tiny) spots. To check if TBSV is really present in plant leaf tissue of infected *N. glutinosa* plants microscopical screening for virus inclusions in leaf hair cuticle cells was done.

Different kinds of methods were used for measuring plant biomarkers of stress, among them thin layer chromatography (TLC) of plant pigments, free proline concentration and phenolic compounds concentration measurement by spectrophotometer and guaiacol peroxidase enzyme assay.

Thin layer chromatography is a method of separating mixture of substances into their pure components by passing the mixture over an insoluble material to which the substances stick to varying degrees. Substances that stick tightly move very slowly, while those that stick loosely or do not stick at all move rapidly. To separate the mixture of plant pigments silica-gel was used as the stationary phase which is very polar and mixture of the non-polar solvents acetone and petroleum ether (3:7). Pigments that are non-polar travel faster on the silica-gel and the ones that are more polar travel slower. In this manner it was checked if there are some changes in composition of pigments during stress (especially darkness).

Concentration (c) of free proline, an important plant osmolyte, was measured to obtain difference between stressed plants. It was done indirectly by measuring the absorbance (A) of formed free proline-nynhidrin complex at wavelength of 515nm by spectrophotometer. First, a calibration curve *A versus c* of known proline concentrations in complex with nynhidrine was made. Then from obtained absorbance of treated samples, the concentration was calculated according to Beer-Lambert law (Bates *et al.*, 1973).

Concentration of phenolic compounds, versatile secondary metabolites, was also determined spectrophotometrically in a reaction with Folin-Ciocalteu (FC) reagent from calibration curve by sample absorbance at 765nm (Singleton *et Rossi*, 1965).

Plant proteins were isolated and their concentration was determined in reaction with Bradford reagent in acid conditions from calibration curve by sample absorbance at 595 nm (Bradford, 1976).

Activity of non-specific (guaiacol) peroxidases (POX), important stress enzymes, was determined in reaction with guaiacol by measurement of sample absorbance at 470nm. POX activity was expressed as concentration of tetraguaiacol (tG) produced by 1mg of total proteins in the sample during 1minute period (Chance *et* Maehly, 1955).

## RESULTS

Thin layer chromatography is basically a qualitative method that we made semi-quantitative by applying same amount (65µL) of sample leaves extract in same condition to the silica-gel plate. In this way it was possible for us to correlate the intensity of developed spots with concentration of pigments in the leaf sample. Results showed that leaves of *N. glutinosa* plants that were exposed to darkness for 48 hours had more chlorophyll; they had more intensive chlorophyll bands compared to the control and other treated plants, while leaf extract from salinity stressed plants showed paler carotenoids (xanthophylls and carotenes) bands. Leaf extracts of plants exposed to combination of stressors (salinity and darkness) showed paler carotenoids bands and also higher Rf values for all pigments, except for carotenes, in comparison to other groups which had constant Rf values for each pigment among them.

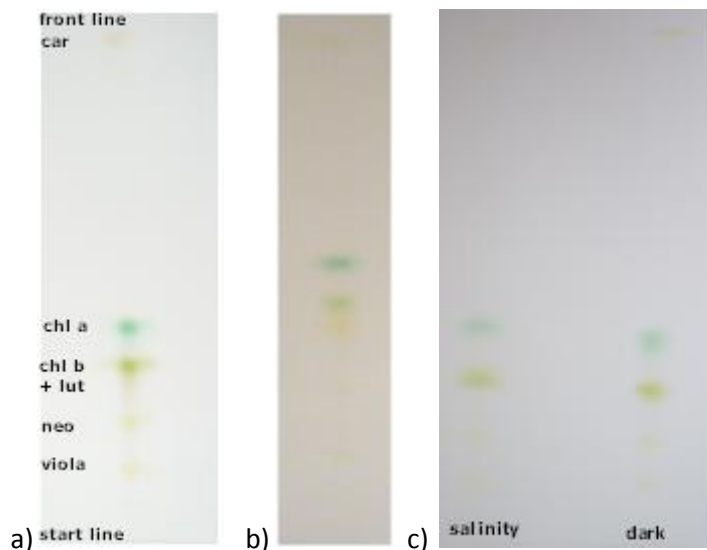


Figure1. Chromatograms of plant pigments from leaves of four experimental groups of plants before infection: a) control b) combination of salt (200mM) and darkness after 48h c) salinity (200mM), darkness (viola-violaxanthin, neo-neoxanthin, lut-lutein, chl b-chlorophyll b, chl a-chlorophyll a, car-carotene)

Free proline concentration in comparison to control showed more than 2.5 fold greater amount of free proline in salinity -stressed plant leaves before infection in comparison to darkness- and combination-stressed plant leaves before infection. The combination- and darkness-stressed plant leaves before infection showed similar concentration of free proline in comparison to control. Free proline concentration in comparison to control after infection showed a significant increase in salinity- and combination-stressed plant leaves, while darkness-stressed plant leaves did not change almost at all (Figure 2).

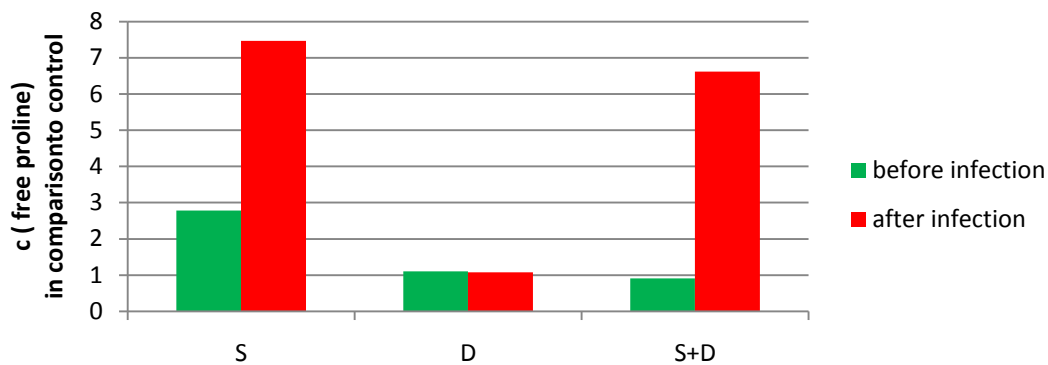


Figure2. Free proline concentration in comparison to control in leaves of treated plants before and after infection (S-salinity-stressed plant leaves, D-darkness-stressed plant leaves, S+D- combination- stressed plant leaves).

Phenolic compounds concentration in comparison to control showed an evident increase of phenolic compounds concentration in comparison to control before infection and a decrease in it after infection in leaves of all three groups of stressed plants (Figure 3.).

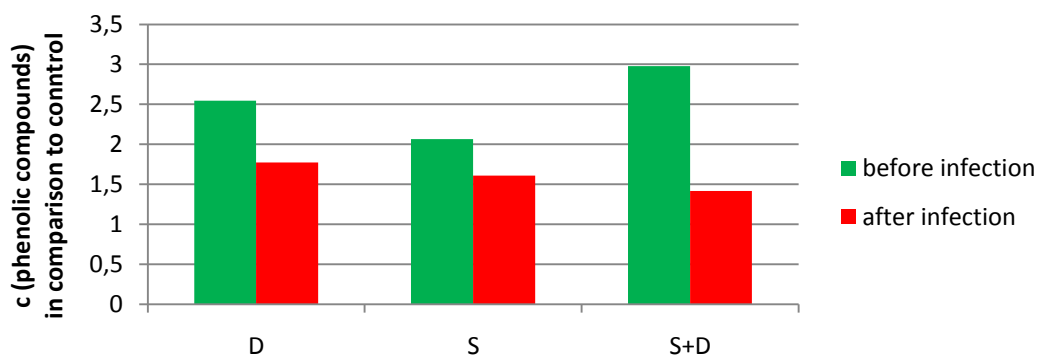


Figure3. Phenolic compounds concentration in comparison to control in leaves of treated plants before and after infection (S-salinity-stressed plant leaves, D-darkness-stressed plant leaves, S+D- combination- stressed plant leaves).

There is an evident increase in POX activity in comparison to control before infection and a decrease in it after infection in all groups of stressed plant leaves, except in darkness-stressed plant leaves where almost no change occurred (Figure 4.).

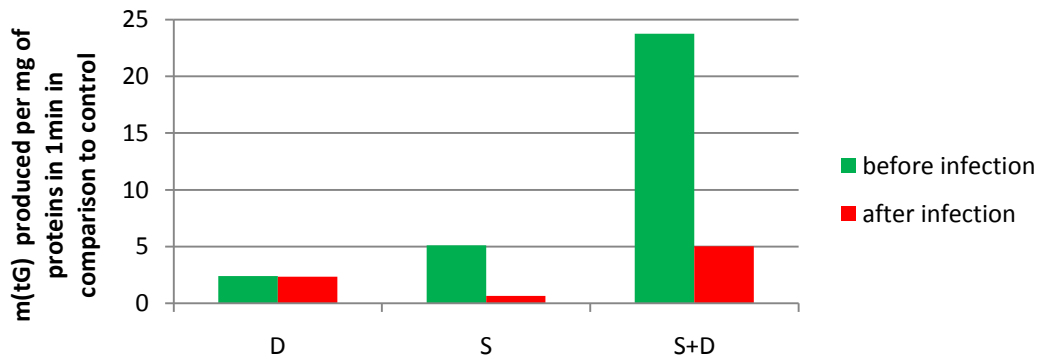


Figure4. POX activity in comparison to control in leaves of treated plants before and after infection (S-salinity-stressed plant leaves, D-darkness-stressed plant leaves, S+D- combination- stressed plant leaves).

After 48 hours after infection of *N. glutinosa* plants with Tomato bushy stunt virus we did not observe any macroscopical symptoms of successful infection. Microscopical screening of leaf hair cuticle revealed formation of non-specific virus inclusions (mainly crystallic formations of virus inside of cell) that are probably result of infection with Tomato bushy stunt virus.

## DISCUSSION

Obtained results from TLC of plant pigments from leaves of stressed plants and control are consistent with our expectations. Pigments are chemical compounds that reflect and absorb certain wavelengths. Chlorophylls (especially chlorophyll a) are the most important pigments for the plant because they are key players in absorbance of sunlight and transferring the obtained energy to photo-systems involved in photosynthesis. Plants have also other pigments, such as carotenoids, a group of accessory pigments that can be divided to carotenes (orange) and xanthophylls (yellow) (Grotewold, 2006). Plants that we exposed to darkness were only for a short period of time in dark. In this short period of time plants try to cope with lack of light, which is vital for the process of photosynthesis to occur, by increasing the amount of chlorophylls. Considering the salinity stressed plants, it is known that salinity stress causes increase in oxygen radicals (reactive oxygen species, ROS) levels in plants (Mittler, 2002). To cope with that plants use several different mechanisms. One of them is linked with carotenoids potential role in oxidation and detoxification of ROS (Salguero *et al.*, 2003,). Excessive amount of ROS causes direct conversion of zeaxanthin to violaxanthin (types of xanthophylls), also carotenoids serve as precursor for abscisic acid (ABA), important stress hormone (Grotewold, 2006), which all together causes paler carotenoids bands. Leaf extracts from plants that were stressed by a combination of stressors (200Mm NaCl and lack of sunlight) showed also paler carotenoids bands due to same reasons as salinity-stressed plants, as well as increase in Rf values of the pigments. Rf value represents a ratio of the distance travelled by the pigment relative to the distance travelled by the solvent. These values should be the same for particular pigment, regardless of the extent of travel of the solvent, and in theory are independent of a single experimental run. They do depend on the solvent used, and the type of TLC plate. We can explain the difference in Rf in leaf extracts of plants treated with combination of stressors as a result of change in structure of pigments due to process going on in the plants, which affected the change in polarity properties of the pigments. Chlorophylls probably became more unpolar, so they travelled further than usually, while carotenes more polar so they travelled less.

Free proline concentration is a common molecular marker in research of osmotic stress in plants caused by salinity. In this conditions water tends to move from a place of its higher concentration and higher potential (in plants), to a place where it has lower concentration and lower potential (out), so the uptake of water from soil is very difficult and plants tend to dry. To prevent this plants produce compatible osmolytes, one of them is free amino acid proline. It can balance the water circulation and ease the water stress in plants by acting as a substitute for water were possible (at the surface of proteins) or binding water in order to reduce apparent quantity of water within a cell

(Roger, 2001). The results showed that in leaves of salinity-stressed plants a greater amount of proline was synthesized in comparison to other stress-treated group in both cases, before and after infection. The results before infection are consistent with osmotic stress theory. Increase after infection can be explained through greater sensitivity of salinity stressed plants to additional changes in water potential. They already have their osmotic balance system alerted, so they fight against the water loss due to mechanical damage of leaves during the inoculation of TBSV into *N. glutinosa* plants more vigorously. Interestingly, leaves from plants exposed to dark, did not show any significant change before and after infection and stayed all the time low in their free proline content. We can conclude that they developed some kind of resistance in this manner. Leaves of the plants that were treated with combination of stressor showed before the infection the lowest amount of free proline content, that was a bit lower than in darkness stressed group before infection which is probably due to delayed reaction caused by too much stress. After infection they showed a great increase in free proline content, but it was a bit lower than in salinity-stressed group after infection.

Phenols are versatile secondary metabolites that have among other functions, important role in oxidative stress as antioxidants, as well in strengthening the cell walls during infection (Roger, 2001). Phenolic compounds concentration in comparison to control showed increase in leaves of all three groups of stressed plants before infection and decrease after infection. From two of stressors darkness had a bit surprisingly higher amount of phenolic compounds than salinity before infection which indicates that darkness may also cause induction of synthesis of phenolic compounds. Decrease after infection is possible due to an exhaustion of the whole system after infection of plants by TBSV. Leaves of the plants that were treated with combination of stressor showed before the infection the highest amount and after infection the lowest amount of phenolic compounds content. This is in accordance to our theory of exhaustion.

POX is not really just one enzyme, rather a group of similar enzymes that can catalyze same reactions but are localized in different compartments of the cell playing important role in anti-oxidative reactions under normal and stress-caused conditions. Basic reaction of POX enzymes is reduction of hydrogen peroxide into water, and simultaneously oxidation of a substrate (Gaspar *et al.*, 1991). Obtained POX activities in comparison to control indicate synergic effect in leaves of plants that were stressed with combination of stressors in comparison to single stressor treated plant leaves in both cases before and after infection. Increase in POX activity in comparison to control before infection and a decrease after infection in salinity and combination stressed plant leaves is consistent with our theory of exhaustion. In darkness-stressed plant leaves almost no change occurred in POX activity in comparison to control. These results are consistent with other measured parameters and together they all imply that plant response to lack of sunlight has improved resistance against virus infection.

Main reason for not observing any macroscopical symptoms of infection of *N. glutinosa* plants with Tomato bushy stunt virus are probably unfavourable weather conditions (rainy and cold weather) after incubation. This prolonged the time of successful replication of Tomato bushy stunt virus to level of concentration (virus titre) high enough to cause macroscopically visible symptoms. This is supported by our microscopically obtained results of possible TBSV presence in form of virus inclusions.

## CONCLUSION

In summary, we can conclude that we surely have stressed our plants. From our results we can assume that pre-treatment with darkness could be a potentially good tool for development of resistance against a virus infection because there is no change in measured molecular markers, except for the change of chlorophyll and phenolic compounds content, but additional research on other plant species is needed. Salinity proved to be a bit too strong stressor, as well as combination of salinity in darkness which might have caused a collapse in defence system as indicate the results from phenolic compounds and POX measurements. Stress in plants is an interesting research field which has many economically important applications. One of them is the application of cross-resistance to develop plant tolerance to other stressors. In the light of obtained results, we propose that a S3 workshop for next year should look into some other aspects of this interesting phenomenon.

## LIST OF REFERENCES

Bates L.S., Waldren R.P., Teare I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant Soil* 39, 205–207.

Bradford M.M. (1976) A rapid and sensitive assay for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Chance B., Maehly A. C. (1955) Assay of catalases and peroxidases. In: *Methods in Enzymology*, ed. Colowick S.P., Kaplan N.O., New York: Academic Press, Vol. 2, 764-775.

Gaspar Th., Penel C., Hagege D., Greppin H. (1991) Peroxidases in plant growth, differentiation and developmental processes. In: *Biochemical, Molecular and Physiological Aspects of Plant Peroxidases*, ed. Lobarzewski J., Greppin H., Penel C., Gaspar Th., University M. Curie-Sklodowska, Lublin, Poland and University in Geneva, Geneva, 249-280.

Grotewold E. (2006) The genetics and biochemistry of floral pigments. *Annual Review in Plant Biology* 57, 761–780.

Mittler R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* 7, 405-410.

Roger M.J.R. (2001) Handbook of Plant Ecophysiology techniques. *Kluwers Academic Publishers*.

Salguero A., de la Morena B., Vigarra J., Vega J. M., Vilchez C., Leon R. (2003) Carotenoids as protective response against oxidative damage in *Dunaliella bardawil* *Biomolecular Engineering* 20, 249-253.

Singleton V.L., Rossi J.A. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16, 144-158.