

# The Thesis of the PhD dissertation

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HUNGARIAN UNIVERSITY OF  
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HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE  
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PhD THESIS

**MOLECULAR EFFECTS OF  
SILICATE APPLICATION  
ON CUCUMBER SEEDLINGS**

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## **Background and objectives**

Cucumber is a warm seasoned plant that grows rapidly at 24-29°C temperatures. It is native to the tropical regions of South Asia. Its origin dates back to 3000 BC in Indian subcontinents. It is an herbaceous vine that grows, blooms and fructifies normally even on a short-day illumination while requiring high humidity. Greater light intensity and solar radiation however improve its fruit production. It performs reasonably well in a variety of soil with a loose structure. This species is sensitive to a range of environmental stresses, such as water shortage, extreme temperatures and excess salt.

Silicon (Si) fertilizers have been used to improve crop performance for decades. It improves the chemical characteristics of acidic soils, also increases cation exchange capacity. Silicon is absorbed by plants as silicic acid, it is carried by the transpiration stream and deposited in plant tissues as amorphous silica. Numerous positive effects are attributed to silicon on plant production, including reduction of the impact of some diseases, delaying senescence, improving photosynthesis, growth, abiotic stress tolerance and mechanical resistance. Cucumber actively absorbs silicon compared to other eudicots though it is considered as a moderate accumulator. Si transporters involved in Si uptake and distribution have been identified in several plant species, including cucumber. It is directly and/or indirectly involved in enzyme activities, that regulate plants' normal process as well as serve as protection during stress conditions.

Molecular breeding efforts of cucumber are conducted intensively worldwide. Availability of the full genomic sequence of this species by Huang et al., (2009) led to the discovery and characterization of various genes. Due to increasing demand for high quality products from the consumer's side, new hybrids of cucumber are released frequently. Therefore, the most current approaches of breeding are directed to improving relevant traits by properly understanding the molecular basis of the most important physiological processes.

### **Our investigations were aimed to:**

Shed light on cucumber's normal growth and physiology as well as to study how Si is involved in molecular mechanisms regulating certain genes which play key roles in protection and defense mechanisms. Cucumber plant cultivars will be grown in a semi-hydroponic cultivation system to keep control over the experiments, specifically to maintain a steady silicon supply and to avoid interactions with other elements, which might come from the soil. In order to achieve the proposed results, the following objectives are set:

- A. Physiological studies:
  - 1. Determine the fresh weight of the cultivars
- B. Biochemical analysis:
  - 1. Photosynthetic pigment contents will be analysed
  - 2. Abiotic stress inducers such as lipid peroxide, hydrogen peroxide and Thiobarbituric Acid Substances amounts will be measured
- C. Molecular studies:
  - 1. Putative silicon transporter genes will be analysed by RT-qPCR
  - 2. Lipoxygenase genes previously expressed in cucumber will be investigated in response to silicon supply and will be analysed by RT-qPCR

## Materials and methods

### 1. Plant material, growth conditions and Si treatment

Cucumber (*Cucumis sativus* L.) F1 hybrid 'Dirigent' cultivar was chosen as plant material. Seeds were germinated overnight at 24°C in 100 ml distilled water. Seeds were then transferred to pots (7.5 × 7.5 × 6.5 cm) filled with perlite medium (4 seeds in each pot) and grown in a controlled environment chamber. Growth conditions of 14/10 hours' day/night illumination cycle (140 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density) with a relative humidity of 55-60% and temperature of 26°C were applied throughout the experiments. Four pots were placed in a reservoir, with approximately 400 ml culture medium covering the bottom of the pots up to ~2 cm high. The plants were irrigated with distilled water for a week (until cotyledons appear) and then treated with half strength Hoagland solution (pH 5.8) (Millner and Kitt, 1992).

Cucumber plants were treated with and without silicate supplementation (1.67 mM Si in the form of Na silicate, pH 5.8) (Flam-Shephard, 2016).

The fertigation solution in the reservoir was discarded every other day, and pots were flushed with new solution from above. In the days between changing the media, the pH of the reservoir was adjusted to 5.8 with 1M citric acid. Samples were taken between 1-2 pm at day 31 after germination, then frozen immediately in liquid nitrogen and stored at -80°C for further use.

### 2. Element analysis

Leaves were cut individually and cleaned by soaking in tap water for several minutes. The leaf dry weight (DW, g) was measured after the leaves were dried in the 40°C oven for 48 hours.

For nitrogen content determination, 3 ± 0,001 g of dried plant samples were placed in a crucible inside an incandescent oven. The annealing furnace temperature was then raised to 500°C and the samples were incinerated. After cooling, 5 cm<sup>3</sup> of 1 mol/l HCl solution is added to the crucible. The contents of the crucible were transferred to a filtered funnel and washed with distilled water. The filtrates are collected in a 50 cm<sup>3</sup> volumetric flask. Quantification of the nitrogen content is performed according to the standard MSZ-08 1783/6.

Sodium and silicon content were determined according to the standard MSZ-08 1783/5 where 0,5 ± 0,01 g of plant samples were weighed and incinerated. Then hydrochloric acid is added to the ash and the whole is washed with the distilled water into a 100cm<sup>3</sup> volumetric flask. The elements measurements were performed using an ICP-OES Ultima 2 (Jobin Yvon Horiba) instrument.

### 3. Chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were estimated according to the method of Arnon, (1949). Leaf tissue (0.1g fresh weight, FW) was ground in 3ml 80% acetone with a pinch of Sodium carbonate using pre-cooled mortar and pestle. Homogenized samples were transferred to polyethylene conical tubes and the final volume was made up to 10ml with the addition of 80% acetone. The slurry was centrifuged at 1000 rpm for 5min at 4°C. The cleared solution was transferred to cuvettes and the optical density was measured at wavelengths 663 nm, 644 nm and 480 nm against a blank (80% acetone) in a spectrophotometer.

### 4. Thiobarbituric acid reactive substances

To assay lipid peroxidation, the method of Heath and Packer (1968) was adapted with slight modification. The concentration of thiobarbituric acid reactive substances (TBARS) was measured. Leaf samples (0.1g FW each) were taken and ground in 1mL 0,1% trichloroacetic acid (TCA) for extraction. 20μl of 20% butylated hydroxytoluene (BHT) was added to the solution and mixed by vortexing. Homogenized samples were centrifuged at 13000 rpm for 10 min at 4°C to remove insoluble material. 0.25mL supernatant was transferred into a 2mL Eppendorf tube containing 1mL of 0.5% TBA in 20% TCA solution and gently mixed. The samples were kept at 96°C in a heating block for half an hour. Following incubation, tubes were placed on ice for 10 min and centrifuged again at 13000 rpm for 10 min at 4°C. Finally, absorption was measured at

532 nm and 600 nm, data were analyzed with Gen5 software (Powerwave XS2, Biotek, USA). TBARS concentration was calculated using an extinction coefficient  $\epsilon=155\text{mM}^{-1}\text{cm}^{-1}$ .

## 5. Hydrogen peroxide and lipid peroxide assay

For hydrogen peroxide and lipid peroxide assay, 0.2g leaf samples were homogenized in 1mL 10%  $\text{H}_3\text{PO}_4$ . The supernatant was used for the determination of  $\text{H}_2\text{O}_2$  and lipid peroxide according to Wolff's (1994) assay. The reaction mixture for determination of  $\text{H}_2\text{O}_2$  assay contained 100  $\mu\text{M}$  xylenol orange, 250  $\mu\text{M}$  ammonium ferrous sulfate, 100 mM sorbitol, 25 mM  $\text{H}_2\text{SO}_4$  plus 50 $\mu\text{l}$  of sample extract in a total volume of 1mL. Lipid peroxide assay solution was: 100  $\mu\text{M}$  xylenol orange, 250  $\mu\text{M}$  ammonium ferrous sulfate, 4 mM butylated hydroxytoluene, 25 mM  $\text{H}_2\text{SO}_4$  dissolved in 90% methanol and 50  $\mu\text{l}$  of sample extract in a total volume of 1mL. Both assays had the same incubation time and absorbance was read at wavelength 560 nm.  $\text{H}_2\text{O}_2$  was used for calibration.

## 6. RNA isolation

Tissue samples (100mg each) were homogenized in 1mL TRIZOL reagent using liquid nitrogen in a precooled mortar and pestle. Then samples are vortexed vigorously and kept at RT for 5 minutes. Then centrifuged at 13,000 rpm for 15min at 4°C. The supernatant is then transferred to a new 1.5mL Eppendorf tube. Phase separation is carried out using 0.2mL chloroform with centrifugation speed of 12,000rpm for another 15 min at 4°C. After the separation of two phases, the upper aqueous phase (60% of the total reagent used) is transferred carefully to a new tube. RNA is precipitated by mixing it with 0.5mL isopropyl alcohol and incubated at RT for 10 min until centrifugation. After the precipitation RNA is washed with 75% cold ethanol twice and centrifuged for 5 min in between. Finally, the RNA pellet is air dried for 5-10 minutes and dissolved in 50 $\mu\text{l}$  MQ water. RNA concentration was measured with an ND-1000 instrument from NanoDrop®.

## 7. mRNA analysis

### cDNA synthesis

cDNA synthesis was performed on quantitatively normalized total RNA samples with Thermo Scientific First strand cDNA Synthesis kit, used with oligo(dT)<sub>18</sub> primers. All the ingredients in Table 3 were gently mixed and briefly centrifuged then incubated at 37° for an hour. Then the gDNA contamination of the synthesized cDNA was removed by using DNase I, RNase free kit by Thermo Fisher Scientific according to manufacturer's instruction. Each 1  $\mu\text{g}$  of RNA was mixed with 1  $\mu\text{l}$  of 10 × reaction buffer containing  $\text{MgCl}_2$  and another 1  $\mu\text{l}$  of DNase I reagent (RNase-free/1U) and finally, Nuclease free water was added up to 10  $\mu\text{l}$  in volume. Hence, the prepared mixture was incubated at 37°C for half an hour. Then 1 $\mu\text{l}$  50 mM EDTA was added to each tube and further incubated at 65°C for 10 min.

### Primers

Actin7 gene was chosen as the endogenous control (reference gene) in order to normalize the signal value of each sample. A housekeeping gene is required to accomplish the determination of the actual biological difference between control and treated samples. Efforts were also made to minimize mechanical errors such as inconsistent loading. Actin gene and respective primers were selected according to Wan et al., (2010), which showed stable expression on all the tested cucumber samples compared to other housekeeping genes. Other genes and specific primers were selected based on literature data and showed distinctive expression patterns in cucumber cultivars. Silicon influx and efflux transporter genes are chosen according to Sun et al., 2018; and Wang et al., 2014). Moreover, 12 lipoxygenases were selected based on their detectable expression described by Yang et al., (2012).

## RT-PCR

PCR was performed using Dream Taq polymerase (Thermo Scientific®, USA) with silicon transporter genes and primers specific to LOX genes. The thermal cycling parameters were as follows: 30 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1min, with the pre-denaturation cycle at 95°C for 2 min and 30 sec, and a final extension of 72°C for 5 min in Mastercycler (Eppendorf®, Germany). The amplified PCR products were analyzed by electrophoresis on 1.5% agarose gels. Control Actin7 gene (CsGy2G015500) sequences were amplified with the gene specific primers.

## Agarose gel electrophoresis

DNA fragments were separated by 1.5% agarose electrophoresis gel. The gels were prepared by dissolving the LE Agarose (SeaKem®) in the 1 × TBE buffer. To visualize the DNA, Eco Safe nucleic acid staining solution (Pacific image Co) is used. To load the samples, 12 µl PCR product was mixed with 2 µl 6 × DNA loading dye by Thermo Fisher Scientific (In case of Green 10 × Dream Taq buffer, no loading dye is required). Electrophoresis was performed at 80 – 100 V for 30 min to an hour. Amplifications for gene specific primers were visualized under a BIO-RAD gel documentation system and the size of the fragments was determined using GeneRuler 1kb and 100 bp (Thermo Fisher Scientific).

## RT-qPCR

Silicon transporter (Holz et al., 2019) and a control actin gene (XM\_004147305) sequences were amplified with the gene specific primers listed in Table 4. RT-qPCR was done by using HOT FIREPol EvaGreen qPCR supermix (Solis Biodyne, Estonia) in StepOnePlus Real-Time PCR system (Applied Biosystems, USA).

The qPCR cycles were set according to the manufacturer's instruction by Solis BioDyne Super mix dye. PCR amplification was initiated with DNA denaturation at 95 °C for 12 min in order to activate the polymerase. As the amplicon product of the reaction used was assumed to have a length shorter than 150 bp, 20 seconds of annealing and elongation was adjusted. The annealing temperature of the reference gene was different than the other gene specific primers. A melting curve analysis was performed (65–95 °C) at the end of the run and the PCR product specificity was confirmed.

## 8. Statistical analysis

All data were presented as mean ± standard deviation (SD). The comparison of means and the correlation analysis were performed using R (project for statistical computing) software. Significant differences ( $p < 0.05$ ) of means were determined by one-way ANOVA with Duncan's post-hoc test. Fold changes of induction were calculated according to the  $2^{-\Delta\Delta Ct}$  method by Bookout and Mangelsdorf (2003).



## Results and Evaluations

### Element analysis

To estimate the efficiency of silicon supplementation in our experiments, relevant elements of control and treated plants were measured. Treated plants had higher Si levels ( $681.68 \pm 190.33 \text{ mg kg}^{-1}$ ) in leaves when compared with control ( $212.561 \pm 96.104 \text{ mg kg}^{-1}$ ) plants, approving uptake of this element under the conditions applied. Si content of treated leaves in our system was lower than some values reported earlier in other studies. Differences may be due to dissimilar treatment conditions, i.e. continuous exposure from an early stage of development vs. sudden exposure in hydroponics.

The sodium content of leaves was also analyzed in treated and control plants. Interestingly, the sodium content of treated leaves was higher ( $1725.098 \pm 243.48 \text{ mg kg}^{-1}$ ) than that of control ( $743.73 \pm 257.18 \text{ mg kg}^{-1}$ ), which is probably due to supplementation of this element in sodium silicate. Regardless of supplementation of Na, most crops including cucumber translocate very little Na to reproductive structures such as seeds, fruits or storage roots, which are the edible portions of many crops, since translocation of such element is highly restricted in phloem. Besides, plant cells have various transport proteins including antiporters for reducing the internal concentration of this toxic ion (Pessaraki, 2001). Although beneficial effects of the treatment could still be observed, this effect should be routinely considered when Si is supplemented in the form of sodium salt. Especially in irrigated lands since irrigation continuously delivers some Na salts, although at low concentrations. Besides, build-up of Na salt in soil is evident due to the plants' restrictive nature against Na uptake and favoring of K instead (Taiz et al., 2015).

Based on the element analysis conducted on major macro elements, no significant differences have been shown between control and treated plants except for nitrogen (Figure 8). Nitrogen content has decreased significantly with Si supply (Control:  $136.133 \pm 3.729$ ; Si:  $120.528 \pm 3.804 \text{ g kg}^{-1}$ ). Interference of silicon application with N metabolism has been known, as Si has been reported to alleviate stress caused by excessive N. In another example, Si-containing nutrient solution alleviated excess of N application in rice leaves. Moreover, K concentration decreased with Si application ( $78.372 \pm 8.384 \text{ g kg}^{-1}$ ) compared with control ( $88.389 \pm 14.32 \text{ g kg}^{-1}$ ), this effect however was non-significant. Similar results were obtained for potassium with foliar and soil Si treatments, where K concentration was decreased in cucumber leaves.

Potassium concentration also decreased with Si application ( $78.372 \pm 8.384 \text{ g kg}^{-1}$ ) compared with control ( $88.389 \pm 14.32 \text{ g kg}^{-1}$ ), this effect however was not significant. Similar results were obtained for potassium with foliar and soil Si treatments, where K concentration was decreased in cucumber leaves (Emad et al., 2017). This might be due to a competition between two cations ( $\text{Na}^+$  and  $\text{K}^+$ ) since the family of transporters called HAK/KT/KUP are specializes in  $\text{K}^+$  transport across plasma membrane and some of which also mediate  $\text{Na}^+$  influx at high external Na concentrations (Taiz et al., 2015). Since, in our case Si is supplemented as a sodium salt whereas study by Emad et al., (2017) supplemented the Si through soil drenching. Hence, when other cations are abundant in the plant tissue, critical K concentrations kept low but when the cations are low, K concentrations increases depending on the plant species (Taiz et al., 2015).

In respect to Ca content, a modest increase was evident though not significant with Si treatment (Control:  $112.759 \pm 10.885$ ; Si:  $117.564 \pm 19.720 \text{ g kg}^{-1}$ ). A similar finding on cucumber seedlings was observed with exogenous silicon (Jafari et al., 2015). However, according to a study conducted by Khoshgoftarmanesh et al., (2014), a significant increase in Ca content was recorded only in cucumber cultivar exposed to salinity stress. In contrast to different cultivars, increased Ca content was reported in cowpea, wheat, aloe and tomato with the application of silicon. Differences in the studies could be due to Si involvement in uptake and transport of different macronutrients also depending on any stresses applied.

No change was shown in case of P and Mg elements in both control and Si treatment, which were similar to the study conducted by Kamenidou et al., (2008) that have found no differences in the leaf macronutrients including phosphorous and magnesium as well as micronutrients between Si treatments and the control in ornamental sunflower (*Helianthus annuus* L.). Though no change has been associated with the additional Si supply, no deficiency was observed neither on the plant leaves nor in the recorded data. Since phosphorous is an important component of sugar-phosphate metabolites and phospholipids in plant cells, deficient plants show symptoms such as stunted growth and malformation. Leaves also become slightly purple due to excess anthocyanins (Taiz et al., 2015). Mg deficiency is characterized by chlorosis between the leaf veins especially on the older leaves due to its high mobility (Taiz et al., 2015). Similarly, no visible deficiency of Mg was observed on the seedlings.

### **Plant material and growth condition**

The appearance of plants was monitored visually throughout the growth period, with no signs of any malformation or deterioration. The appearance was the same in terms of color and shape but in case for silicate treated plants, width and leaf extension seemed slightly higher.

Application of external silicon as a fertilizer supplement was found beneficial for cucumber yield and stress tolerance (Voogt and Soneveld 2001). The impact of silicon in plants is often tested in hydroponic conditions by transferring seedlings into liquid media containing silicate (Etesami and Jeong 2018, Wang et al, 2015). Although aeration is normally used to decrease hypoxia, these conditions are far from physiological and differ substantially from cultivation practice.

Perlite based semi-hydroponic cultivation system was applied for cucumber plants to minimize the gap between experimental and commercial growth conditions. Since cucumber is sensitive to various stresses, even if no stress condition is applied, minor environmental constraints may create basic, low level strain in plants.

Total fresh weight of cucumber shoots is measured. Si treated ( $5.797 \pm 2.145$ ) plants were significantly higher when compared to control ( $4.193 \pm 1.452$ ) on all biological replications. Under non-stressed conditions, Si treated plants may perform similarly or improve growth. In accordance with other studies, Si was effective to increase total dry weight (Zhu et al., 2004) and shoot dry weight (Maksimović et al., 2012) of *Cucumis sativus* cultivars without any stressor. In comparison with other cultivars, a similar trend was evident on rice cultivar where shoot dry weight was higher with silicon supply (Flam-Shepherd et al., 2018). Moreover, improved performance of plant growth with Si in soybean (Hamayun et al., 2010) under non-stress conditions has also been reported.

Pot studies generally measure the dry weight of young plants rather than the fresh weight due to a more precise estimation of biomass. Zhao et al., (2010) conducted a study on biomass estimation on sugarcane and found the same significance correlated on both FW and DW measurements in a single case and concluded that treatment effect on dry weight had the same effect on FW in most cases. Thus, in order to evaluate the difference between treated and control samples we have measured the fresh weight and dry weight ratio. FW/DW ratio is calculated by dividing a constant weight of 1 g of fresh leaves to the final dried weight ( $1g/n_g = \text{dry weight}$ ). As a result, Si supplied plant was higher ( $0.0855 \pm 0.007$ ) than that of control ( $0.0717 \pm 0.009$ ), which was similar in total FW values (Figure 7).

Under non stress conditions, Si treated plants may perform similarly or improve growth. In accordance with other studies, Si was effective to increase total dry weight and shoot dry weight of *Cucumis sativus* cultivars without any stressor. In comparison with other cultivars, a similar trend was evident on rice cultivar where shoot DW was higher with silicon supply. Moreover, improved performance of plant growth with Si in soybean under non-stress conditions has also been reported.

## **Chlorophyll and carotenoid content**

Photosynthetic pigments like chlorophyll and carotenoids are important indicators determining photosynthetic capacity and hence plant growth. Positive effects of Si on photosynthetic machinery have been reported extensively. Thus, we have measured these photosynthetic pigments in correlation with silicon supplemented fertigation.

Based on our findings, chlorophyll contents of leaves of silicate treated plants ( $2.00 \pm 0.4$  mg g<sup>-1</sup>) exceeded that of control plants ( $1.58 \pm 0.44$  mg g<sup>-1</sup>). The same trend has been found in the case of carotenoids content (Control:  $0.31 \pm 0.07$ , Si:  $0.41 \pm 0.07$  mg g<sup>-1</sup>). Carotenoids are major components to absorb light during photosynthesis, which are also responsible to protect the plants from photo-oxidative damage (Pessarakli, 2001). Our findings are in line with a number of studies reporting increased contents of chlorophyll a, chlorophyll b, and carotenoid in leaves e.g. of wheat (Hussain et al., 2015; Rizwan et al., 2012; Tripathi et al., 2015) and cotton (Farooq et al., 2013) under Si supplemented fertigation.

Stress conditions have often been shown to decrease leaf stomatal conductance which can lead to poor gas exchange and low transpiration rate (Pessarakli, 2001). Low CO<sub>2</sub> availability can lead to overloaded electron transport chain and subsequent ROS production. However, supply of Si has reversed these deleterious effects as was approved by photosynthetic measurements. Thus, Si prevented oxidative damage inside the chloroplast, which could be often observed otherwise in plants under stress conditions (Pessarakli, 2001). Nonetheless, our results indicated that Si supply can ameliorate photosynthetic pigments content even when no apparent stress conditions were applied.

## **TBARS**

The tissue concentration of TBARS is frequently correlated with malondialdehyde level, assumed to be indicative to the extent of lipid peroxidation suffered (Zhu and Gong, 2014). Therefore, the level of MDA, produced during the peroxidation of membrane lipids, is often used as an indicator of oxidative damages. Data showed significant changes of TBARS between control:  $41.05 \pm 7.05$  and Si treatment:  $30.80 \pm 5.72$  nmol g<sup>-1</sup>.

Our results correlated with the study conducted by Liu et al., (2009), where cucumber plants treated with exogenous silicon showed a significant decrease in MDA level during both normal temperature and chilling stress. It is also widely accepted that Si application can ameliorate oxidative stress through regulating antioxidant enzyme activities and non-enzymatic antioxidant substance levels in plants (Liang et al., 2003; Moussa, 2006; Soylemezoglu et al., 2009). Khoshgoftarmanesh et al., (2014) reported that Si supply reduced the malondialdehyde level under salt stress by enhancing antioxidant enzyme activities.

Thus, based on our results Si could prevent the plants from oxidation, regardless of the environmental or soil conditions.

## **Lipid peroxide and hydrogen peroxide content**

Even under normal growth conditions several metabolic pathways produce reactive oxygen species (ROS) as by-products of their primary processes. Some ROS, particularly free radicals, may initiate chain reactions in membrane lipids leading to accumulation of lipid peroxides (LPOs) with subsequent structural and functional alterations in the membranes (Pessarakli, 2001).

Therefore, lipid peroxidation products were measured and were found lower upon Si treatment (Control:  $0.628 \pm 0.093$ , Si:  $0.530 \pm 0.084$  μmol g<sup>-1</sup>). The presented result was in line with the findings of Zhu et al., (2004) which showed decreased lipid peroxidation in cucumber as well as Liang et al., (2003) showed decreased permeability of the plasma membrane of leaf cells and

decreased LPO level in barley. Whereas, in our recent study conducted on another hybrid cultivar of cucumber plant showed decreased LPO without any stressors.

Lower hydrogen peroxide concentration was also demonstrated in treated samples (Control  $2.2\pm 0.45$ , Si:  $1.62\pm 0.1$ )  $\mu\text{mol g}^{-1}$ ). Based on the studies regarding Silicon application, Song et al., (2009) reported reduced  $\text{H}_2\text{O}_2$  contents by enhancing antioxidant enzyme activities and non-enzymatic antioxidant contents under Cd stress. Similarly, determination of  $\text{H}_2\text{O}_2$  content was expressed through bio-photon imaging in our recent study, which showed significant decrease in silicon treated cucumber when no stress was applied. It is well documented that Si addition decreases LPO and  $\text{H}_2\text{O}_2$  content through regulating antioxidant activities, especially in stress conditions. According to Soylemezoglu et al., (2009) Si decreased  $\text{H}_2\text{O}_2$  concentration and lipid peroxidation in grapevine rootstocks through increased catalase (CAT) and superoxide-dismutase (SOD) activity. Furthermore, similar findings have been reported in various species like tomato (Shalata and Tal, 1998), wheat (Meneguzzo et al., 1999), cotton (Gossett et al., 1994) and barley (Liang, 1999).

Zhu et al., (2004) suggest that Si decreases the permeability of plasma membranes and the extent of membrane lipid peroxidation. Thereby Si may maintain membrane integrity and functions in salt-stressed cucumber, thus mitigating against salt toxicity and improving the growth of plants. Therefore, the above findings illustrate, in relation with our present study, that Si treatment can prevent the increase of hydrogen peroxide and lipid peroxide contents in diverse species exposed to various stressors. Our findings therefore demonstrate lower oxidative stress levels in Si treated plants.

### **Si transporter genes**

Extensive studies testified on beneficial effects of Si on plant growth and stress tolerance in several species, however Si accumulation was found variable among plant species. Cucumber is a widely used dicot model for silicon accumulation thus, number of transporters have been identified including efflux transporter *CsLsi2* and two influx transporters *CSiT1* and *CSiT2*.

In connection with silicon accumulation, the expression of known silicate transporters was also investigated in both root and shoot tissues. According to Wang et al., (2015), transcript level of both influx transporters was detected by RT-PCR examination in root, leaves, stems, and flowers of cucumber plants with most abundance in roots and mature leaves. Whereas expression of rice *Lsi1* and *Lsi2* (Ma and Yamaji, 2006), and barley *HvLsi1* (Chiba et al., 2009), the Gramineae counterpart genes, was mainly restricted to roots. Based on our RT-PCR examination of cucumber, both known influx transporters were expressed in roots and mature leaves (2nd and 3rd leaves). The efflux transporter *CsLsi2* was expressed in both roots and leaves which is in line with the findings of Sun et al., (2018) where the transcripts were detected in roots, stems, laminae and petioles.

Quantitative RT-PCR results approved upregulation of *CsLsi2*, a silicon efflux transporter (Sun et al., 2018) as well as two further transporters (*CsiT1* and *CsiT2*) (Wang et al., 2014) in response to 1.67 mM of exogenous silicon supply both in leaves and roots. Influx transporters expression was examined with 1.0 mM Si supply (Wang et al., 2014) whereas efflux transporter was examined with 1.7 mM of exogenous Si (Sun et al., 2018). Thus, these data indicate that the appropriate amount of Si taken up by the cucumber plants may be transported by the putative Si transporters. This is in agreement with the approved role of these genes in Si transport.

### **The effect of Si treatment on the expression of LOX genes**

Numerous studies have suggested that LOXs play a vital role in plants' normal growth and development, synthesis of aroma compounds, ripening and senescence as well as defense against biotic and abiotic stresses. Thus, we have conducted RT-PCR to study the expression of LOX

genes in cucumber leaf tissues. We have chosen twelve genes with transcripts previously detected in cucumber tissues. Our results from RT-PCR have shown that all chosen genes were expressed in the *Cucumis sativus* leaf tissues studied.

As for the molecular mechanism behind the protection Si treatment may offer in oxidative stress, upregulation of antioxidant systems has been frequently evoked. Here we tested whether regulation of lipoxygenase genes may represent an additional possibility contributing to the mitigating effect. As we expected, downregulation of all the genes was evident on Si treated leaves. For further evaluation, quantitative PCR was done on two representative genes *LOX4* and *LOX17*, selected based on their expression pattern. According to RT-qPCR results and the presented  $\Delta\Delta C_t$  data both *LOX4* and *LOX17* genes were found significantly downregulated by Si treatment (Figure 14). This validates the value of the semi-quantitative data obtained by RT-PCR.

Among the complex role of LOXs play in plant growth and homeostasis, participation in stress responses has been described (Viswanath et al., 2020). In our experiments, coordinated downregulation of LOX genes paralleled decreased levels of lipid peroxidation and hydrogen peroxide content in silicated plants. This corresponds with results presented by Gunes et al., (2007) who described alleviating effect of Si on abiotic stress induced lipoxygenases and hydrogen peroxide accumulation. Additionally, our recent research conducted on different cucumber F1 hybrid ('Joker') has shown downregulation of several redox related genes with presumed pro-oxidative effects, including LOXs, due to silicon treatment (Szegő et al., 2021). Results further approved the redox protective potential of downregulating pro-oxidant genes and enzyme activities, resulting in reduced hydrogen peroxide contents as marker of decreased oxidative burden.

Lipoxygenases are involved in the synthesis of fatty acid hydroperoxides and reactive oxygen species, especially in the context of ethylene and Jasmonic acid mediated responses (Kreslavskii et al., 2012; Prasad et al., 2017). These studies also highlighted the signaling roles as to aid in plants' adaptive and tolerance mechanisms. Whether the link between redox protection and downregulation of lipoxygenases is direct through pro-oxidant activity or more complex through e.g., oxylipin biosynthesis and hormonal actions remain to be determined.

## **Consequences and suggestions**

The presented experiments focused on the effects of Si supplementation on cucumber physiology and molecular mechanisms. In these studies, a fertigation solution of half strength HG was applied as control with 1.67mM of Si treatment on an F1 hybrid cultivar of cucumber. All the experiments were conducted using semi hydroponic cultivation system with perlite.

Experimental data allow several conclusions to be drawn. In the soilless perlite medium plants can be subject to a long term, steady supply of Si, which is a more realistic approximation of potential field conditions. Results approved growth promoting potential of Si treatment. This was accompanied by higher chlorophyll and carotenoid contents of leaf tissues.

With respect to molecular mechanisms putative silicon transporter genes were upregulated with Si application in both roots and shoot. Therefore, the appropriate expression of these transporters allowed the increased accumulation of Si uptake and transport in cucumber, which further favors the positive effects correlated with it.

Moreover, our results indicated that Si supply can ameliorate oxidative stress by decreasing important indicators such as hydrogen peroxide and lipid peroxidation levels even when no stress conditions were applied. The background of Si mitigating effect on redox balance was further tested. LOX gene expression was investigated, since it has been frequently found induced in response to biotic and abiotic stress. Our findings showed coordinately downregulated expression of LOX genes in response to silicon supplemented fertigation. This observation draws attention to this class of enzymes as potential players in the context of redox protection offered by Si.

## **New scientific results**

The conducted research on cucumber dirigent cultivar revealed significant differences in both growth parameters and stress induction in response to Si treatment in perlite medium. The obtained results are of high importance with potential application in current cucumber production:

- Our data revealed that a perlite based, semi-hydroponic growth system was suitable to conduct silicon supplemented fertigation experiments on young cucumber plants. Results confirmed several known physiological and molecular effects associated with silicon in cucumber, and also revealed new data with more insight into Si action.
- Genes specific to known silicon transporters were expressed at significantly higher level in silicon treated plants in both roots and shoots.
- Increased silicium and sodium tissue concentrations were approved in leaves of sodium silicate treated plants. This effect correlated with several physiological changes: Chlorophyll and carotenoid contents of silicon treated cucumber were increased compared to control. Significantly higher plant fresh weight of silicon supplied plants was found.
- Higher level of silicium in leaves also correlated with less oxidative damage, illustrated by redox markers, such as malondialdehyde, lipid peroxide and hydrogen peroxide contents.
- Downregulation of all expressed lipoxygenase genes was found in response to Si treatment.

## List of publications providing basis of thesis

### Published papers in referred journals

Bat-Erdene, O., Szegő, A., Gyöngyik, M., Mirmazloun, I., Papp, I., 2021. Effects of silicon in plants with particular reference to horticultural crops (Review article). *International Journal of Horticultural Science* (accepted, in press)

Bat-Erdene, O., Szegő, A., Gyöngyik, M., Mirmazloun, I., Papp, I., 2020. Long term silicon exposure coordinately downregulates lipoxygenase genes, decreases reactive oxygen species level and promotes growth of cucumber plants in a semi-hydroponic cultivation system. *Russian Journal of Plant Physiology*, 68(5) DOI: 10.1134/S1021443721050022. (accepted, in press)

Oszlányi, R., Mirmazloun, I., Pónya, Z., Szegő, A., Jamal, S., Bat-Erdene, O. and Papp, I., 2020. Oxidative stress level and dehydrin gene expression pattern differentiate two contrasting cucumber F1 hybrids under high fertigation treatment. *International Journal of Biological Macromolecules*, 161, pp.864-874.

Szegő, A., Badics, E., Gubala, D., Oszlányi, R., Oyuntogtokh, B.E., Kappel, N., Papp, I. and Kiss-Bába, E., 2019. Diverse responsiveness of dehydrin genes to abscisic acid and water stress treatments in cucumber F1 cultivar hybrids. *The Journal of Horticultural Science and Biotechnology*, 94(6), pp.726-734

Szegő, A., Mirmazloun, I., Pónya, Z., Bat-Erdene, O., Omran, M., Kiss-Bába, E., Gyöngyik, M., and Papp, I., 2021. Downregulation of polyamine and diamine oxidases in silicon treated cucumber. *Plants*, 10. (submitted)

### Conference presentations related to thesis

Bat-Erdene, O., Mirmazloun, I., Szegő, A., Papp, I., 2021. Downregulation of lipoxygenase genes coupled with alleviation of oxidative stress parameters in silicate treated cucumber grown in perlite medium. Spring wind conference, pp. Miskolci University. Miskolc. May. 28 – May. 30 (abstract accepted)

Bat-Erdene, O., Mirmazloun, I., Szegő, A., Papp, I., 2020. Growth promotion in perlite-based cultivation upon silicon treatment is paralleled by expression of silicate transporter genes. *4<sup>th</sup> National Conference on Young Biotechnologists*, pp 36. Debrecen University. Debrecen. Nov. 05 – Nov. 27. ISBN 978-963-490-272-0. (poster)

Mirmazloun, I., Pónya, Zsolt., Oszlányi, R., Szegő, A., Jamal, Shahid., Bat-Erdene, O., Papp, I., 2020. Elevated fertigation level induces oxidative stress and the expression of a dehydrin gene in cucumber F1 hybrids. *4<sup>th</sup> National Conference on Young Biotechnologists*, pp. 30. Debrecen University. Debrecen. Nov. 05 – Nov. 27. ISBN 978-963-490-272-0.

Bat-Erdene, O., Mirmazloun, I., Szegő, A., Papp, I., 2019. Silicate treatment promoted cucumber plant growth paralleled by induction of a Si transporter gene. *SZIENTIFIC Meeting for Young Researchers*, pp. 62-64. Szent István University. Budapest. Dec. 09. ISBN 978-963-269-886-1.

Bat-Erdene, O., Ahmed, M.O., Mirmazloun, I., Oszlányi, R., Papp, I., Szegő, A., 2019. Expression levels of an Ascorbate peroxidase gene in response to Silicate (Si) in salt stressed *Cucumis sativus* L. *Spring wind conference*, pp. 8. Debrecen University. Debrecen. May. 03 – May. 05.



Bat-Erdene, O., Ahmed, M.O., Mirmazloun, I., Oszlányi, R., Papp, I., Szegő, A., 2019.  
Expression profiling of an ascorbate peroxidase gene in *Cucumis sativus* in response to salt and silicate treatments. *3<sup>rd</sup> International Conference for Agriculture and Climate Change*, Elsevier. Budapest. Mar. 24 – Mar. 26. (poster)