Research article

Tobacco plants (*Nicotiana benthamiana*) were influenced by silicon and were not infected by dodder (*Cuscuta europaea*)

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ABSTRACT

The effect of silicon (Si) on tobacco (*Nicotiana benthamiana*) development and dodder (*Cuscuta europaea*) – tobacco interaction were studied. Three Si application approaches were tested: tobacco seed priming (2.5 mM Si and 5 mM Si; 2.5S, 5S), watering tobacco plants with Si solution (2.5 mM Si and 5 mM Si; 2.5W, 5W) and foliar application (1 mM Si and 2.5 mM Si; 1F, 2.5F). Dodder was not able to infect the host plant in almost all Si treatments. Only in the control and 2.5W treatments was dodder able to infect its host. A significant increase in all observed antioxidant enzymes activities (POX, CAT and SOD) occurred in the plants of 2.5W treatment after infection in comparison with the uninfected 2.5W treatment and control plants, which indicated the importance of antioxidant enzymes activities in the plant parasite – host interaction. Resistance of Si treated plants to dodder could have been due to the changes in the cell wall properties of the epidermis and cortex where activity of POX was confirmed histochemically.

The growth and development of tobacco shoots were evaluated after four and eight weeks of cultivation in the individual Si treatments. The development of shoots was enhanced after eight weeks of cultivation in the 2.5S, 5S, 2.5W and 5W treatments in comparison with the control treatment. However, a negative effect of Si was observed in 1F and 2.5F treatments. In the majority of cases, the plants treated with Si had decreased chlorophyll content when compared to control, except for chl *a* in 5W plants after 8 weeks of cultivation. Contrary to this, carotenoids increased in all Si treated plants after eight weeks cultivation in comparison with the control. The secondary xylem formation in tobacco was enhanced after 4 and 8 weeks cultivation in shoots of plants receiving the 2.5S, 5S, 2.5W and 5W treartments. The cambium was the most active in producing secondary xylem in the 2.5S treatment. Protein profile and antioxidant enzymes activities (POX, CAT and SOD) were altered by Si treatment. After 8 weeks of cultivation, activities of POX were significantly decreased in 2.5S, 5S, 2.5W and 5W in comparison with control. Catalase was decreased in 2.5S, 5S and 5W in comparison with the control, however, 1F and 2.5F treatments had significantly increased CAT and SOD activities. The specific activity of POX was confirmed histochemically in Si treated plants in the cell walls of several stem tissues like the epidermis, cortex and pith. A small amount of H_2O_2 was detected in leaves in the control and Si treated plants. The amount of O_2^- decreased in all treatments with time. The highest Si concentration in the plants (almost 800 mg · kg^{-1} d. w.) was detected in the 2.5W, 5W treatments.

1. Introduction

Parasitic plants which obtain nutrients and water from host plants, have an extraordinary lifestyle. They possess root-like structures called haustoria which penetrate the host and enter its xylem or phloem. One of the most important genera of parasitic plants are dodders (*Cuscuta* spp.) which can cause serious economic losses to host crops. Their seeds germinate in the soil and the young plants can detect organic compounds that are released into the air by nearby host plants and grow towards them. These obligate stem holoparasites can parasitize multiple host plant species and once they infect a host, their roots die. The whole mechanism of attaching and infecting host plants is described in several studies (e. g. Vaughn, 2002; Hong et al., 2011; Svubova et al., 2013, 2017). Many *Cuscuta* species are not able to parasitize monocotyledonous plants despite having a broad host spectrum. Dawson et al. (1994) suggested this could be due to the arrangement of vascular bundles or incompatibility of signals that are important for forming interspecies connections of vascular strands. In some cases of *Cuscuta* –

host plant interactions, a defence program in the host plant was recognised. A secretion of soluble phenylpropanoids starts during an interaction of dodder with host plants, and increasing accumulation and activity of peroxidases (POX) occurs. These enzymes are important for linking phenylpropanoids with other components of the cell wall such as proteins, pectins, or cellulose fibers (Löffler et al., 1995, 1997; Sahm et al., 1995). This modified cell wall is thought to prevent penetration of the host plant by dodder. Several authors studied the role of POX in the processes of invasion and destruction of tissues of the host by dodder (López-Curto et al., 2006; Svubova et al., 2017). The activity of POX generating reactive oxygen species (ROS), polymerising cell wall compounds, and regulating H_2O_2 levels was shown to be associated with the processes of invasion and destruction of host tissues and the morphogenesis of the adherence structures in *Cuscuta jalapensis* (López-Curto et al., 2006). In a previous study, these antioxidant enzymes were described (Svubova et al., 2017) in *Cuscuta europaea* as important components involved in an effective mechanism making the host's surface cell walls more flexible.

Nowadays, there is a massive effort worldwide to alleviate the impact of various biotic or abiotic stresses which devastate plants. One of the most studied elements in this aspect is silicon (Si). Silicon is the second most abundant element in soils present as silicic acid at 0.1–0.6 mM concentrations. Plants differ in their ability to absorb it and, therefore, Si concentration in the plant ranges from less than 1% of the dry matter to 10% or higher in some plant species (Epstein, 1994, 1999; Hodson et al., 2005). Even though it is not considered as "essential" for higher plants, some authors consider it as quasi-essential (Epstein and Bloom, 2005) or a beneficial element (Ma et al., 2001; Lu et al., 2017). A major role in moving Si throughout the plant is transpiration (Kumar et al., 2017). There are several recent studies presenting Si as an important element for plant growth, mechanical strength and several other aspects of plant life. When supplied to the growth medium, plant vigour and resistance to biotic and abiotic stresses increases (e.g. Hattori et al., 2005; Vaculik et al., 2009; Lukacova et al., 2013; Azeem et al., 2015; Coskun et al., 2016; Johnson et al., 2018). The protective role of Si was initially attributed to creating a physical barrier fortifying the cell wall or modifying properties of cytoplasmic membranes (Chérif et al., 1992; Liang et al., 1999; Kim et al., 2002; Lux et al., 2002; Guerriero et al., 2016). Silicon can also influence water relations in plants, by inducing the formation of a silica cuticle double layer which reduces water losses from the leaf (Gong et al., 2003). However, several studies have shown that the action of this element on plants is more complex and Si can be involved in plant metabolism, transcription and cell signalling at various levels. For example, Van Bockhaven et al. (2015) showed it can induce the upregulation and down regulation of 35 and 121 transcription factors, respectively, and Fauteux et al. (2005) reported that by binding to hydroxyl groups of proteins, Si is involved in signal transduction leading to resistance induction. Rodrigues et al. (2004, 2005) reported Si upregulates transcription level of POX, responsible for the lignin biosynthesis and growth regulation in tomato challenged by *Ralstonia solanacearum*. Silicon also upregulates mitogen-activated protein kinase (MAPK19), WRKY transcription factors and linker histones (H1 and H5) (Ghareeb et al., 2011) and the expression of a leucine rich repeat receptor-like kinase (LRR-RLK) in rice (Fleck et al., 2011), which is a protein involved in intracellular signal transduction. As a result, Si improves defence against biotic stresses caused by fungi, bacteria, viruses and by animals (Bélanger et al., 1995; Hunt et al., 2008; Zellner et al., 2011; Bathoova et al., 2018). There is a wide range of specific mechanisms of Si alleviating biotic stresses such as increasing the abrasiveness or physical strength of plant tissues and thus reducing digestibility for herbivores (Massey and Hartley, 2009; Schurt et al., 2014; Frew et al., 2016), induction of defensive compounds such as phenolics, phytoalexins and momilactones (Remus-Borel et al., 2005), and activation of defensive enzymes such as POX, polyphenol oxidase (PO), lipoxygenase (LOX) and phenylalanine ammonia lyase (PAL)

(Rahman et al., 2015).

Most authors conclude that the beneficial effects of Si are more apparent under stress conditions (Ma, 2004; Hattori et al., 2005; Vaculik et al. 2009, 2015; Liang et al., 1999; Lukacova Kulikova and Lux, 2010; Lukacova et al., 2013; Vaculikova et al., 2014, 2016). There are many papers reporting that Si plays a crucial role in alleviating stresses especially in so-called Si accumulating plants, such as grasses like rice facing sheath blight and blast infection (Rodrigues et al., 2001; Seebold et al., 2001), or in dicots such as cucumber or vines against powdery mildews (Renolds et al., 1996; Fauteux et al., 2005). In Si accumulating monocots, the effect of Si and its accumulation is relatively well documented. However, much less is known about this phenomenon in dicots especially in species not known to accumulate Si. It was shown, that Si primes defence responses in Si non-accumulator tomato facing *Ralstonia solanacearum* (Kiirika et al., 2013). After Ghareeb et al. (2011), tomato was protected against *R. solanacearum* by Si treatment by an upregulation of genes involved in ethylene and jasmonic acid signalling. There is also very limited information about the role of Si in alleviating (a)biotic stresses in tobacco plants. Application of exogenous Si enhanced the growth and photosynthetic pigments content of tobacco plants after treatment with Cd (Lu et al., 2017). Liang et al. (2015) studied the crosstalk between Si and ethylene signalling in tobacco BY-2 cell cultures and summarized that ethylene played an important role in Si function.

The objective of this study was to determine if Si could alter the growth and development of tobacco plants (*Nicotiana benthamiana*) and alleviate the biotic stress of tobacco facing an attack of dodder (*Cuscuta europaea*).

2. Materials and methods

2.1. Plant material cultivation and growth conditions

In vivo cultivated tobacco (*Nicotiana benthamiana* Domin.) as the host plant and parasitic dodder (*Cuscuta europaea* L.) were used in this study. Seeds of *N. benthamiana* were obtained from Gene Bank in Gatersleben, Germany and seeds of *C. europaea* originated from the locality Ivanka pri Dunaji (2015, Slovak Republic, latitude 48°19′, longitude 17°22′). The plant material (tobacco plants alone or with dodder) was cultivated in soil in the growth chamber with a 16/8 h photoperiod at 23 ± 2 °C and 100 µmol m^{-2}s^{-1} PAR. The soil used in our experiment (N max 1.9%, P_2O_5 max 0.5%, K_2O max 0.9%, pH 5–7) contained 42 mg · kg^{-1} Si.

Tobacco plants were subjected to one of seven treatments;

C – control.

2.5S – seeds of tobacco were treated with 2.5 mM Si solution before sowing.

5S – seeds of tobacco were treated with 5 mM Si solution before sowing.

1F – foliar application of 1 mM Si solution 7, 14 and 21 days after germination.

2.5F – foliar application of 2.5 mM Si solution 7, 14 and 21 days after germination.

2.5W – plants were watered with 2.5 mM Si solution 7, 14 and 21 days after germination.

5W – plants were watered with 5 mM Si solution 7, 14 and 21 days after germination.

There were 15–20 plants per treatment, and the experiment was repeated twice. The tobacco seeds primed with Si (2.5S and 5S treatments) were soaked in the 2.5 mM Si or 5 mM Si solution for 2 h before seeding. Plants treated by foliar application (1F and 2.5F) were sprayed with 2.5 mM or 5 mM Si solution with an appropriate solution volume to wet the whole aboveground plant, while the soil was covered to avoid the contact of the solution with the soil. Plants watered with Si solution (2.5W and 5W treatments) were normally watered, and on the 7th, 14th and 21st days after germination, they were treated with

100 ml of 2.5 mM or 5 mM Si solution. Silicon was added in a form of water glass ($Na_2SiO_3 \cdot xH_2O$) and pH of every solution was adjusted to 5.8. No additional fertilisation of the plants occurred.

All tobacco plants were cultivated like the control and various Si treatments for ten weeks. Four plants were analysed after the first four weeks of cultivation and then, other four plants, after eight weeks of cultivation. Remaining plants were left in the pots for other two weeks and cultivated with the dodder seeds.

Dodder seeds were scarified by soaking in concentrated H_2SO_4 for 15 min, washed with distilled water and sown in soil around the base of 8 weeks-old control tobacco plants and all Si-treated plants that were not removed for the analysis. Approximately twenty seeds were used per one pot.

2.2. Lignin deposition in tobacco stems

The influence of various Si treatments on the lignin deposition in the tobacco plants was observed using free hand sections. We analysed the 4th youngest stem internodium of four plants from the 1st and the 2nd experimental runs for each treatment. This approach allows analysis and comparison of the same developmental stage of the stems. The sections were stained with fluoroglucinol – HCl and the lignin deposits were also evaluated by autofluorescence by an Axioskop 2 plus microscope (Carl Zeiss, Germany), equipped with excitation filter TBP 400 + 495 + 570, chromatic beam splitter TFT 410 + 505 + 585 and emission filter TBP 460 + 175 530 + 610 (wavelengths are in nm), and photographed using an Olympus DP 72 camera system.

2.3. Anatomy of leaves and stems

Pieces of leaves and the 4th youngest stem internodium of four plants per treatment from each experimental run were fixed in formalin–acetic acid–alcohol (FAA) for 24–48 h. Fixed material was dehydrated through a graded ethanol series and embedded in paraffin wax. Transverse sections (10 µm thickness) were cut using a Microm HM 325 (Thermo Scientific) rotary microtome. Samples were deparaffinized with xylene, and then gradually hydrated through a decreasing alcoholic series (ethanol 100%, 90%, 70%, 50%, distilled water). Histochemical staining of sections was performed using safranin O and alcian blue. After staining, slides were dehydrated using an increasing ethanol series (50%, 70%, 90%, 100%, xylene) and mounted with glycerol – albumin. The sections were observed using an Axioskop 2 plus microscope (Carl Zeiss, Germany) and photographed using an Olympus DP 72 camera system.

2.4. Determination of chlorophylls and carotenoids concentration

Chlorophyll *a, b* (Chl *a* and Chl *b*) and carotenoids were extracted from the leaves (the youngest and the oldest leaves were not taken into consideration) with 80% acetone and thereafter their amounts determined spectrophotometrically (Jeanway 6400, London, UK). The pigment concentrations were calculated according to Lichtenthaler (1987). Three samples from each treatment were taken in each experimental run.

2.5. Protein separation

Samples (100 mg of the leaves from the central tobacco stem area) were ground in liquid nitrogen and suspended in protein extraction buffer [28 mM dithiotreitol, 175 mM sucrose, 28 mM Na_2CO_3, 10 mM EDTA, 5% (w/v) SDS (Sigma-Aldrich, USA)] with antiprotease pill (Roche). After 30 min incubation at 70 °C and 15 min centrifugation ($12.100 \times g$), the supernatant was used for determination of protein concentration using a Bichinonic Acid Kit for Protein Determination (Sigma-Aldrich, St. Louis, MO, USA). Protein samples (25 µg) were separated on a 12% SDS-polyacrylamide gel and stained using Coomasie

Brilliant Blue. The leaves of three plants per treatment were assessed for each experimental run.

2.6. Assays of antioxidant enzyme activities

The effect of Si on the activity of antioxidant enzymes – guaiacol peroxidase (POX, E.C.1.11.1.7), superoxide dismutase (SOD, E.C.1.15.1.1) and catalase (CAT, E.C. 1.11.1.6) in the 3rd tobacco leaf of three plants of each treatment for each experimental run was compared. The total protein content was established according to Bradford (1976). The isoenzymes of POX were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970) with some modifications. Isoenzymes were separated at 4 °C and 200 mV using a BioRad Electrophoresis System (BioRad, USA). The gels were incubated in acetate buffer. For isoenzyme visualisation, 50 mM guaiacol and 50 mM H_2O_2 were used as a substrate. The activity of guaiacol peroxidase was established according to Frič and Fuchs (1970), the activity of superoxide dismutase according to Beauchamp and Fridovich (1971) and activity of catalase according to Hodges et al. (1997). The specific activity of CAT was calculated according to Claiborne (1985). The leaves were analysed after the first four weeks of cultivation, and at the end of the cultivation, after eight weeks of cultivation. After another two weeks, when dodder infected the 2.5W treated plants, another analysis was done. The activities of POX, SOD and CAT of tobacco stems with developed haustoria of the 2.5W treatment were compared with the activities of the stems of uninfected control plants and uninfected 2.5W treated plants.

2.7. H_2O_2 and O_2^-, visualisation

The presence of H_2O_2 and O_2^- was detected in the 3rd leaves of one plant of each treatment for each experimental run after Kumar et al. (2014). The plant samples were placed in the test tubes and immersed in DAB (3,3′-diaminobenzidine) for H_2O_2 and in NBT (nitrotetrazolium blue chloride) solution for O_2^- visualisation. The test tubes were incubated overnight in the dark at room temperature. Chlorophylls were then removed from the leaves in absolute ethanol heated in a boiling water bath for 10 min. H_2O_2 was visualised on the leaf as reddish-brown stain formed by the reaction of DAB with the endogenous H_2O_2. The O_2^- content was detected as dark blue stain of formazan compound formed as the result of NBT reacting with the endogenous O_2^-.

2.8. In situ POX activity

The *in situ* POX activity was detected after incubation of hand-made cross sections (0.5 mm thick) of tobacco stems in 100 mM Na-acetate buffer (pH 5.2) with 5 mM 4-methoxy-1-naphthol (4-MN) in ethanol for 15 min at 30 °C (after Ferrer et al., 1990; Ďurčeková et al., 2007). Similar to lignin deposition analysis, the 4th internodium was analysed in control and the Si treatments (4 plants per treatment were analysed in each experimental run).

2.9. Determination of Si concentration

Dry plant samples of two plants per each treatment for each experimental run were dissolved in concentrated HNO_3 and heated at 150 °C for 2 h. Subsequently, H_2O_2 and HF were added and after heating at 150 °C for 2 h, H_3BO_3 was added. The concentration of Si in tobacco shoots were determined by atomic absorption spectrometry (AAS) (PerkinElmer, #1100). The concentration of bioavailable Si from the soil was analysed according to Rodrigues et al. (2003) with modification after Bokor et al. (2017). After extraction by 0.5 M acetic acid, Si was measured by ICP-MS in place of colorimetric determination using the blue silicomolybdous acid procedure.

Fig. 1. Tobacco plants infected by dodder. Treatments: **control**, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination.

2.10. The statistical analysis

One-way and two-way ANOVA was performed using Statgraphics Centurion XV.I software for statistical significance at P < 0.05. Analysing the tobacco plants treated with Si, two factors were taken into consideration in two–way ANOVA – factor time (four or eight weeks of cultivation), factor treatment and also the interaction between these two factors. All the results were expressed as mean ± SD (standard deviation) in the three independent replications. Means were separated using the LSD (least significant difference) test at a 5% level of significance.

3. Results

Most of the Si treated tobacco plants were not infected by the parasitic dodder, although tobacco is a compatible host and the control plants were parasitized. Germinated dodder plants were able to infect after a few days, but only in the control and 2.5W treatments (Fig. 1). In the case of 2.5S and 5S treatments, dodder plants grew toward and wrapped around tobacco stems but they did not create any prehaustoria or haustoria and died after a few days. Dodders around the tobacco plants of the 5W, 1F and 2.5F treatments germinated but the parasite died one week after germination without initiating infection. Measurements of AOE were done in the stems of the control, the 2.5W treatment without infection and the 2.5W treatment after dodder infection. Activity of POX, SOD and CAT after dodder infection increased dramatically in comparison with noninfected plants in the control and 2.5W treatment (Fig. 2).

Shoots of plants of the 2.5S and 5S treatments had significantly shorter shoots in comparison to the control plants after four weeks of cultivation but after eight weeks of cultivation, shoots of the 2.5S, 5S, 2.5W and 5W treatment were significantly longer than the control shoots (Fig. 3). The 5W treatment achieved the best growth. The worst cultivation effect of Si treatment was achieved in the 1F and 2.5F treated plants after 8 weeks. The plants were completely wilted and the shoot biomass was less in comparison with the control plants. The leaves of 2.5S, 2.5F and 2.5W treatments had very prominent veins in comparison with the control and other Si treated tobaccos. The midveins and secondary veins especially became very large. All changes were compared anatomically (Fig. 4). The midvein in the Si treated leaves had different organisation with higher number of xylem vessels

in comparison with the control. Also an increased number of mesophyll cell layers on the abaxial side of the leaf midvein was observed in Si treatments.

Silicon treatment influenced the content of assimilation pigments; they mostly decreased (Fig. 5). Content of chl *a* after four weeks of cultivation decreased in 2.5S and 5W significantly compared with the controls and, similarly, chlorophyll *b* decreased in 2.5S, 5S and 5W treatments. The most notable decrease in chlorophyll content occurred in the 2.5S and 5S treatments. The content of carotenoids also decreased in all Si treatments except for the 2.5W treatment. At eight weeks, the chl *a* and chl *b* concentrations increased in the control and in some of the Si treated plants. Only in the treatments with foliar Si application did the amount of chlorophylls decrease at eight weeks in comparison with the four weeks old shoots. On the contrary, the concentration of carotenoids dramatically increased in 1F and 2.5F treatment at eight weeks of cultivation. Two – way ANOVA analysis revealed that factors of time and treatment played different significant role in the pigment composition. The amount of chl *a* was significantly influenced by the "treatment" and also the interaction of "time" and "treatment" while chl *b* was influenced by "time", "treatment" and their interaction. The carotenoids were influenced by "time" and the interaction of "time" and "treatment" (data not shown).

Stem development was also changed by Si treatment. The majority of Si treated stems accelerated secondary xylem formation after the first four weeks of cultivation compared to the controls (Fig. 6). The highest rate of stem lignification and secondary growth was observed in the 2.5S and 5S treatments and the 2.5W and 5W treatments. The acceleration in the stem development under treatment with Si was also observed after eight week cultivation (Fig. 7). The most visible enhancement in the secondary xylem production with the most active cambium was seen in the 2.5S treatment.

Silicon treatment resulted in a changed protein profile of the tobacco shoots. Individual treatments differed in protein composition as well as in the amount of proteins (Fig. 8).

Specific activities of peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) from the tobacco leaves were found in four and eight weeks old tobacco shoots (Figs. 9 and 10). The highest activity of POX in the four week cultivation period was achieved in 2.5S, 5W and 2.5F treatments, where the increases were approximately 100%, 50% and 40%, respectively, compared to the control. The only significant decrease was in the 5S treatment. Measurements after eight weeks cultivation also revealed changes in antioxidant enzymes activities. A negative trend in POX activity was noticed in leaves of the 2.5S, 5S, 2.5W and 5W treatments, most noticeably in the 2.5W and 5W treatments. On the other hand, there was no difference in POX activity in 1F and 2.5F treatment when compared with the control. A massive increase in CAT activity after four weeks of cultivation was observed only in the 5S treatment. A significant increase in CAT activity after eight weeks of cultivation was observed in the 2.5W, 1F and 2.5F treatment. Catalase activity increased in control shoots after eight week cultivation by more than 100% compared with the control after four weeks. In 2.5S, 5S and 5W treatment, the activity of CAT after eight weeks decreased significantly compared to the control and were also lower in comparison with corresponding treatments after four weeks cultivation. The most visible increase in CAT activity at eight weeks of cultivation
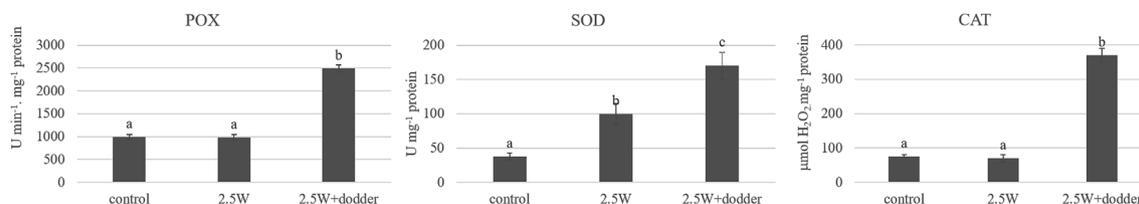
Fig. 2. The activity of POX, SOD and CAT in the stems of the uninfected control and 2.5W treatment and in the 2.5W treatment infected by dodder. Different letters indicate significant difference at P < 0.05, bars are means of 3 experimental runs ± SD.
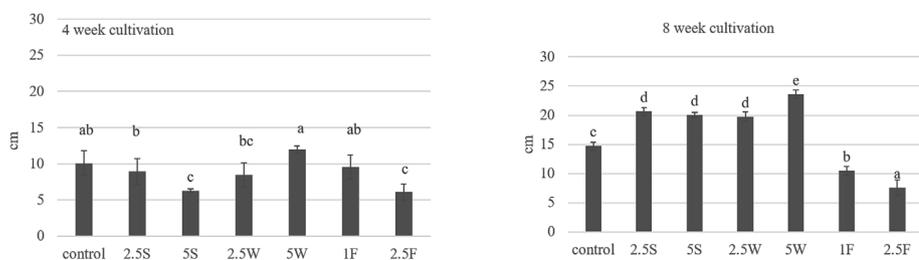
Fig. 3. The length of the tobacco plant shoots treated as a control or with Si for 4 or 8 weeks. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Different letters indicate significant difference at P < 0.05, bars are means of 3 experimental runs ± SD.

was noticed in the 1F and 2.5F treatments (Fig. 9). Similarly to CAT, SOD activity increased the most dramatically in 5S treatment at the end of the first four weeks of cultivation (Fig. 10). In general, the activity of SOD was significantly higher in the Si treated plants (except for 5W) than in the controls. Dramatical changes in SOD activity were also noticed after eight weeks cultivation. A decrease in the SOD activity occurred in the 5S treatment, but, in all other treatments, there was a dramatical increase in SOD activity compared to the four weeks old plants. The highest SOD activity was in the 1F and 2.5F treatments in comparison with the control. The two-way ANOVA revealed "time" and "treatment" to be significant factors.

Superoxide radicals' visualisation analysis after four and eight weeks of cultivation is shown in Fig. 11. In general, very little H_2O_2 was visualised except for the leaves of 1F and 2.5F treatments at eight weeks of cultivation. We detected more superoxide radicals on Si treated leaves after four weeks of cultivation compared to eight weeks of cultivation. On the contrary, the four week old control leaves were almost without any visualised superoxide, but with visible blue spots indicating its presence after eight weeks cultivation.

In control conditions as well as in Si treatments with lower Si concentrations (2.5S, 1F and 2.5W), the positive results (blue colour) of peroxidase histochemical analysis were achieved just in the areas of secondary wall formation (forming secondary xylem) (Fig. 12). However, in 5S and especially in 2.5F treatment, it was observed in the pith and primary cortex cell walls. In 5S treatment, the activity was also noticeable in the outer epidermal cell walls.

Tobacco shoots under Si treatments accumulated unexpectedly high amounts of Si (Fig. 13). In all Si treated plants, the amount of Si was significantly higher than in the controls. The highest Si concentration was detected in the 2.5W and 5W (almost 800 mg $\cdot$kg^{-1} d. w.).

4. Discussion

Silicon, considered as plant nutrient "anomaly", is presumably not essential for plant growth and development (Epstein, 1991; Coskun et al., 2018). However, soluble Si has enhanced the growth, development and yield of several plant species including rice, sugarcane, wheat, maize, horse-tail and some dicotyledonous species (Jones and Handreck, 1967; Elawad and Green, 1979; Datnoff et al., 2001). Behind this, Si provides many benefits to plants facing stresses including protection against attack from fungal or bacterial pathogens (Bélanger et al., 1995). The interaction between plant parasite (*Cuscuta europaea*) and its host (*Nicotiana benthamiana*) that was primed or treated by Si was investigated for the first time in this study.

Dicots in general are known as poor Si accumulators (Datnoff and Rodrigues, 2005; Hodson et al., 2005). However, the range of Si uptake in dicot plants can vary substantially from more than 12,000 mg Si per kg of dry tissue for plants such as *Zinnia elegans* (a high Si accumulator) (Frantz et al., 2011), to less than 300 mg Si per kg of dry tissue in plants such as *N. tabacum* (a low Si accumulator) (Zellner et al., 2011), which corresponds with our control treatment (Fig. 13). However, tobacco plants watered with Si (2.5W and 5W), were able to accumulate almost 800 mg Si per kg of dry weight, and a significant increase in the shoot Si concentration was observed also in the tobacco plants with primed seeds (2.5S and 5S). Interestingly, in addition to the control plants, only tobacco plants from the 2.5W treatment were attacked by *Cuscuta* with successful haustoria development (Fig. 1). The protective effect of Si in the other Si treatments was therefore not related to the amount of Si accumulating in the shoots. On the other hand, plants of the 2.5W treatment had the significantly lowest POX activity in comparison with all other treatments after eight week cultivation. No presence of POX activity was detected histochemically in outer stem tissues in the 2.5W
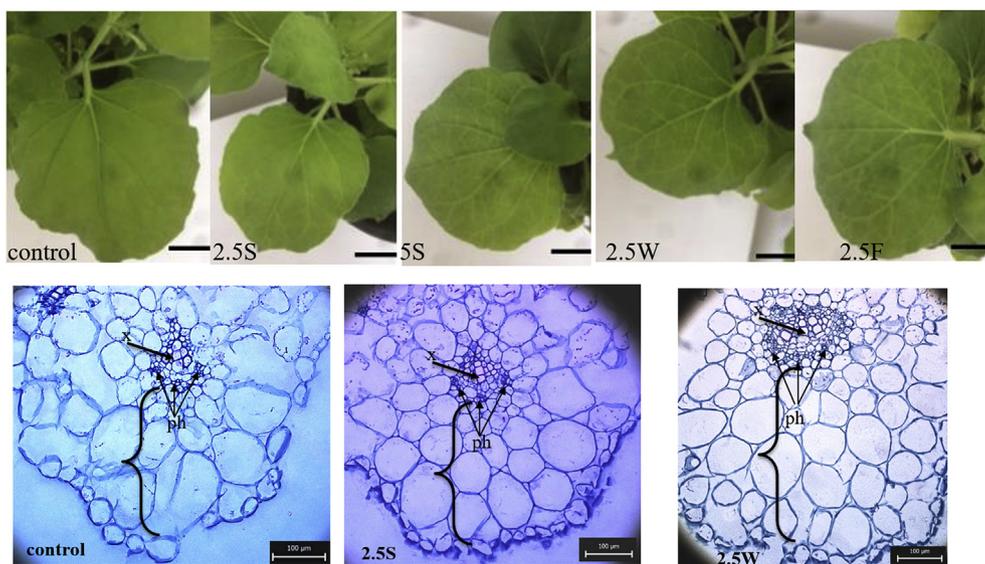
Fig. 4. Leaves veins (macro and anatomy) in control and Si treatments in tobacco leaves after 4 week cultivation. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Arrows pointing at the xylem vessels (x) and phloem (ph), parenthesis mark the abaxial side of the leaf mesophyll. Scale bar = 1 cm (macros) and 200 or 500 mm (anatomical pictures).
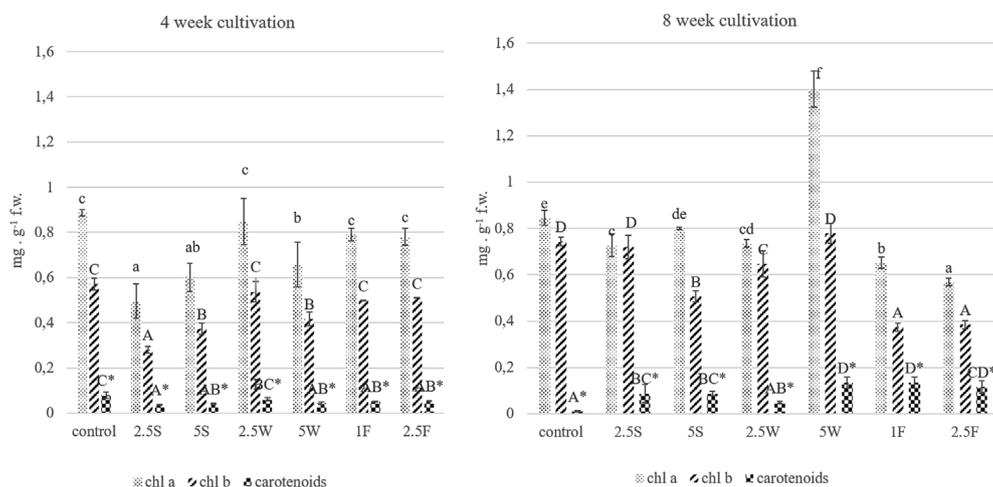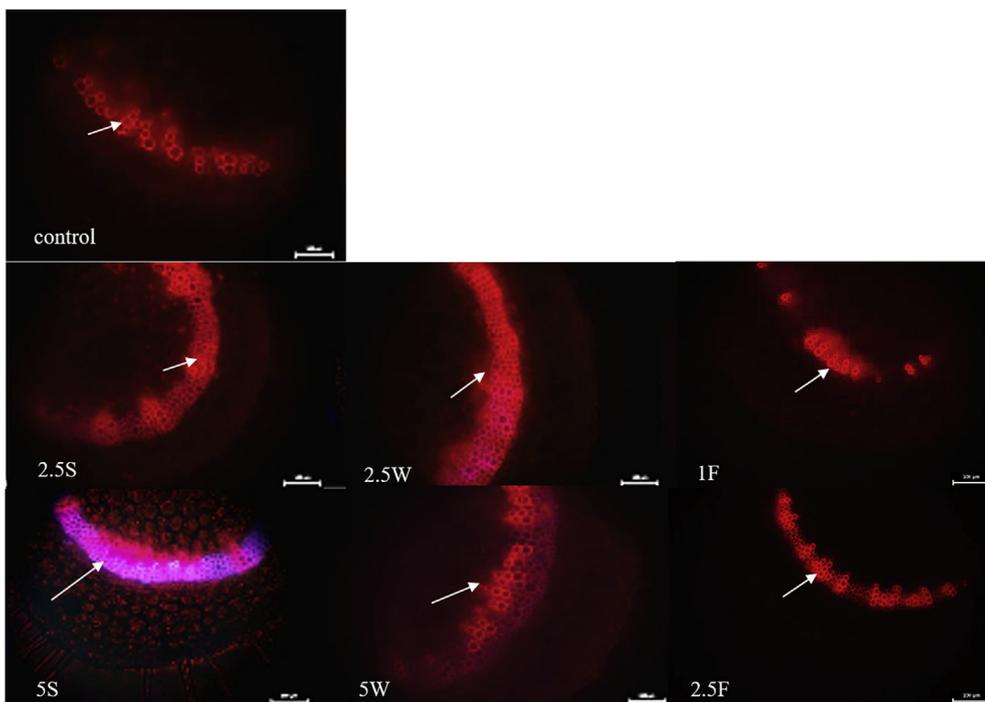
Fig. 5. Content of chlorophyll *a, b* and carotenoids in tobacco leaves after 4 and 8 weeks of cultivation. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Different letters indicate significant difference at P < 0.05, bars are means of 3 experimental runs ± SD.

treatment, while in other Si treatments it was found in the outer cortex or epidermal cell walls. Peroxidases are activated in response to pathogen attacks and various roles have been attributed to plant peroxidases in host – pathogen interaction (Passardi et al., 2005). They can have a cell wall cross-linking activity (formation of lignin and suberin) (Bernards et al., 1999; Blee et al., 2003) and create a highly toxic environment by massively producing ROS (oxidative burst). Plants exposed to stress had up-regulated their overall POX activity in a number of experiments (e.g. Lukacova et al., 2013; Howladar et al., 2018; Pereira et al., 2018). When *Cuscuta* infected the tobacco plants in the 2.5W treatment, the activities of all measured antioxidant enzymes were several times higher than in the control plants and also significantly higher than in the uninfected 2.5W treated plants (Fig. 2). This points to the importance of the system of antioxidant enzymes activities in the defence reactions against a plant parasite in the tobacco plants. We detected the higher activity of POX in outer stem tissue cell walls in the 2.5S and 5S treatment which probably corresponded with building up a stronger and more rigid cell walls blocking the dodder penetration and haustorium development (Fig. 12). Also, in previous work (Svubova et al., 2017), we focused on the role of POX, its content,

activities and changes in isoenzymes composition in tobacco stems attacked by dodder and concluded that the highest POX activities were observed on both sides of the functional connection, the haustorium, confirming the importance of these antioxidant enzymes in the infection process.

There are several studies confirming accelerated root development in terms of xylem lignification and endodermal cell wall formation in the plants facing stress caused by heavy metals, especially cadmium (Cd) (Ďurčeková et al., 2007; Vaculik et al., 2012; Vatehova et al., 2012; Lukacova et al., 2013). It is one of the plant defensive reactions which retains the toxic element in the roots, preventing the damage of the aboveground plant part. When the negative effect of the toxic element was ameliorated by addition of Si, in the most cases the development of the root endodermis and xylem lignification was enhanced when compared to Cd treatments (Vaculik et al., 2012; Vatehova et al., 2012; Lukacova et al., 2013). In the present study it was observed that Si accelerated the development of the tobacco stems (Figs. 6 and 7). A massive secondary xylem production by cambium as a result of Si treatment was seen in comparison with control stems, especially in the 2.5S, 5S, 2.5W and 5W treatments. We have concluded that the reaction

Fig. 6. Stem lignification of tobacco plants in different Si treatments after 4 week cultivation. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Arrows pointing at the secondary xylem in the 4th youngest internodium. Scale bar = 100 mm.
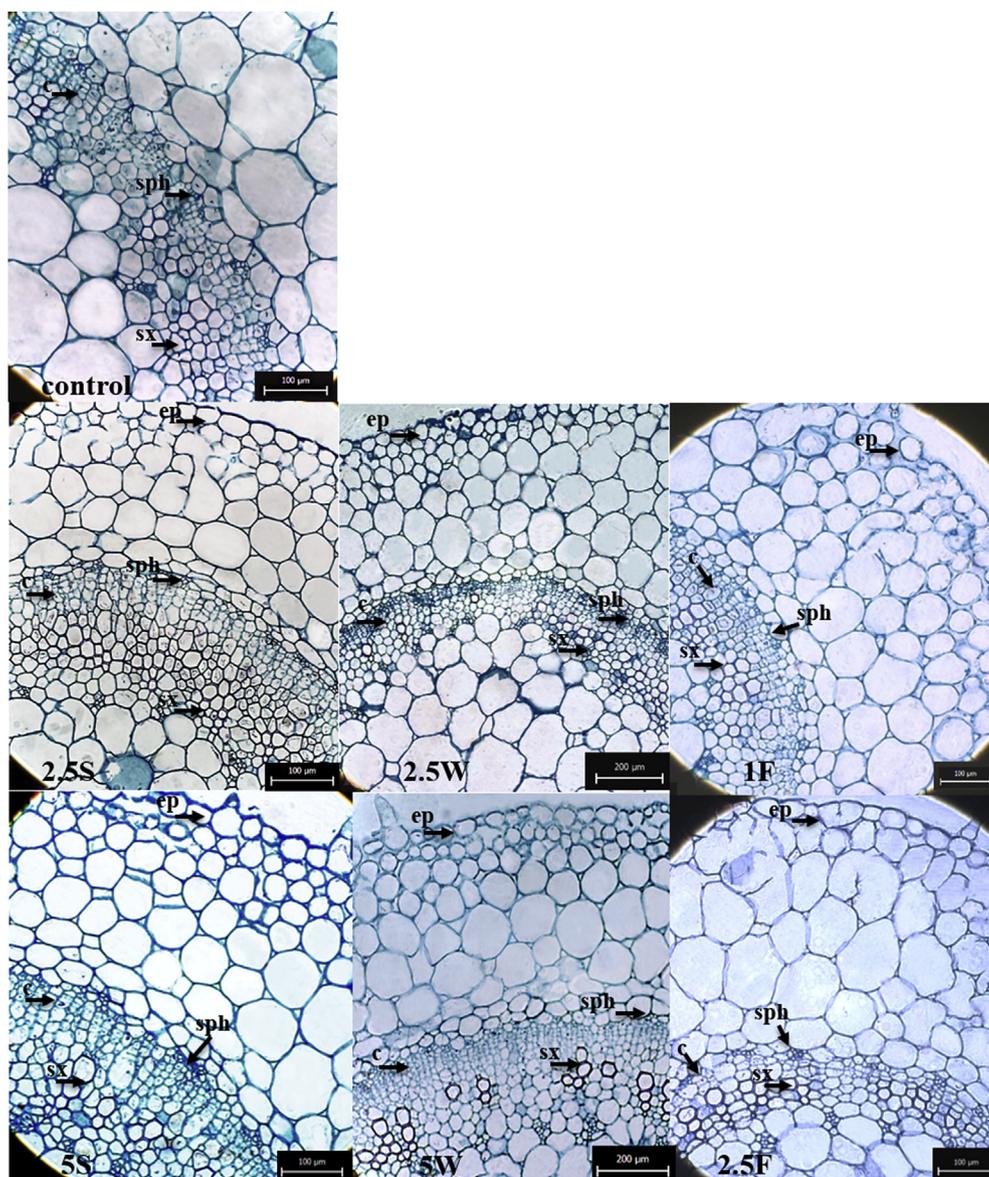
Fig. 7. Stem anatomy of tobacco plants in different Si treatments after 8 weeks of cultivation. The picture shows secondary xylem formation in the 3rd internodium in control and Si treated plants after eight weeks of cultivation. Treatments: control, 2.5S – seeds pre-treated with 2.5 mM Si, 5S – seeds pre-treated with 5 mM Si, 2.5F – foliar treatment with 2.5 mM Si on the 7th, 14th and 21st day after germination, 2.5W – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination. Arrows pointing at the secondary xylem (sc) and (sph) phloem as well as secondary meristem cambium (c) and epidermis (ep). Scale bar = 100 (control, 2.5S, 1F, 5S, 2.5F) or 200 (2.5W, 5W) mm.

of the tobacco plants with the Si treatment as not being a result of stress: more lignified tissues in the tobacco stems would make them mechanically stronger and more xylem elements would increase the tissue water capacity thus increasing overall plant fitness. Few brownish or blue spots, indicating the presence of free H_2O_2 and O_2^- in leaves, were noticed after four and eight week cultivation in 2.5S, 5S, 2.5W and 5W treatment (Fig. 11), indicating that plants were not stressed, and the increased POX activity was therefore probably associated with accelerated secondary tissues development (Figs. 6 and 7).

Changes in the xylem vessels number and organisation were observed in most Si treated leaves in the present study (Fig. 4). There were markedly more cell layers on the midvein abaxial side in the Si treated leaves when compared to the control. The main leaf veins in most Si treatments became macroscopically more prominent. There is very limited information on how Si can modulate the leaf anatomy and especially vascular bundles formation. Doncheva et al. (2009) and Vaculik et al. (2015) observed an increase in maize leaf thickness in Si treatments.

The effect of Si on tobacco plant growth and biotic stress caused by the parasitic plant *Cuscuta europaea* is influenced by relevant factors. The first is time. In the first four weeks the effect of applied Si was not positive in most treatments, however, significant improvement in shoot growth was observed after eight week cultivation (Fig. 3). The second factor was the method and dose of Si application. When Si was applied to the foliage, plants grew markedly worse than control and other Si treatment plants. There was a massive presence of H_2O_2 in the leaves after eight week cultivation in foliar treated plants (1F, 2.5F), and CAT activity was the highest. At the same time, in the 1F and 2.5F treatments, we observed the highest POX activity at the end of cultivation. However, the plants were obviously stressed. They were completely wilted with the lowest amounts of chlorophylls and the highest carotenoids concentrations. Therefore, we consider foliar Si treatment as not suitable for tobacco. In all other Si treatments, the growth and the development of tobacco shoots was enhanced. According to Habibi (2015) and Karmollachaab and Gharineh (2015), the improvement of growth by Si could be due to its deposition as silicate crystals on epidermal tissues, which composes a barrier to water transpiration through the cuticles and stomata. Latef and Tran (2016), who primed maize seeds with Si, concluded priming plays a pivotal role in alleviating the negative effects of alkaline stress on maize growth by improving water status, enhancing photosynthetic pigments, accumulating osmoprotectants rather than proline, activating the antioxidant machinery, and maintaining the balance of K^+/Na^+ ratio.

The content of photosynthetic pigments during the first four weeks
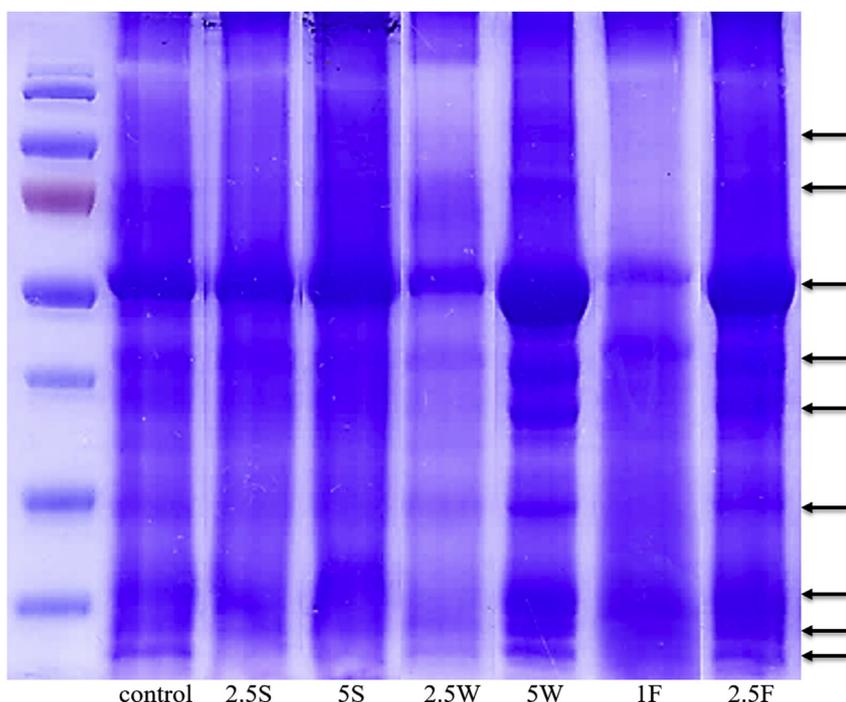
Fig. 8. Protein profile of tobacco plants treated as a control or Si treatments. **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination.

control   2.5S   5S   2.5W   5W   1F   2.5F

of our study decreased in Si treated plants in comparison with the control (Fig. 5). However, after eight weeks of cultivation, chl *a* content in the 5W treatment increased dramatically. Also, the content of carotenoids increased in the majority of Si treated plants at the end of cultivation. Carotenoids act as effective scavengers of free radicals provoked by reactive oxygen species (Gururani et al., 2015). In general, many authors conclude that Si alleviates the stress response in plants by increasing the antioxidant enzymes activities (e. g. Neuman and Zur Neiden, 2001; Zhu et al., 2004; Lukacova et al., 2013).

Plants can be infected by various pathogens including plant parasites. The fact that plants are still surviving on the Earth despite their sessile way of life supposes that they must be able to defend themselves. The plant immune system produces mechanical (cell walls) (Martinka et al., 2014) or biochemical barriers (activation of signalling pathways that induce defence reactions) (Jones and Dangl, 2006). Our research is pioneering work dealing with the interaction of the plant parasite *Cuscuta* and its host *N. benthamiana* treated with Si. It opens a new area of Si research which has been focused in the past few years on other

stress factors like heavy metals, toxic elements or fungal, bacterial and viral infections. In continuing our work, we believe that in the future, we will be able to clarify the mechanism of how Si is connected to the plant immune system, allowing tobacco plants to defend themselves against dodder infection.

5. Conclusion

Silicon altered the growth and development of tobacco (*Nicotiana benthamiana*) shoots and the method and time of application were important; treatments with primed seeds or watered with Si solution seems to be more suitable.

Tobacco plants with primed seeds (2.5 mM and 5 mM Si) had accelerated development, longer shoots and more intensive secondary xylem formation. After eight week cultivation, they had decreased POX, SOD and CAT activities in comparison with the controls and less superoxide in the leaves. Histochemical staining of POX in the stems revealed some atypical locations of its activity, such as in the cell walls of
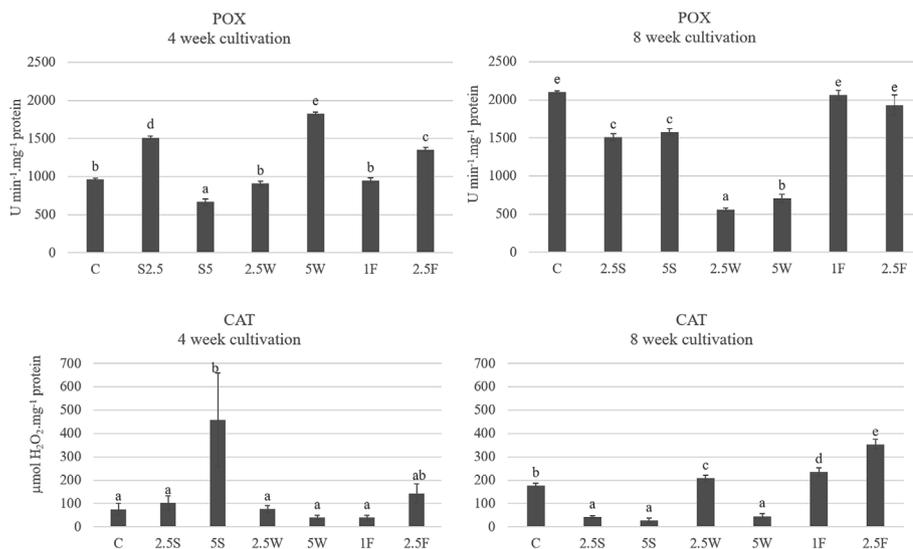
Fig. 9. Peroxidase and catalase activity of tobacco plants treated as a control or Si treatments after 4 or 8 weeks of cultivation. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Different letters indicate significant difference at P < 0.05, bars are means of 3 experimental runs ± SD.
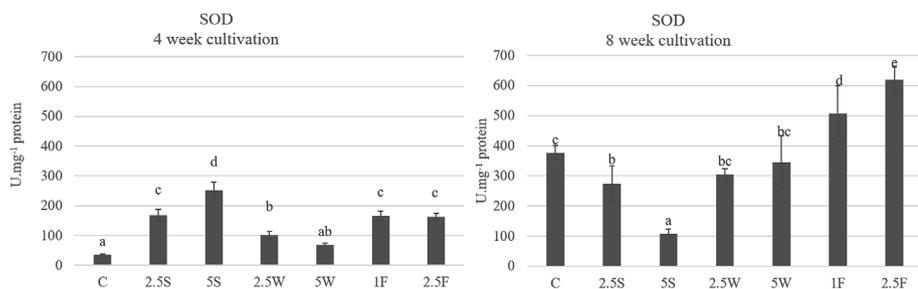
Fig. 10. Superoxide dismutase activity of tobacco plants treated as a control or Si treatments after 4 or 8 week cultivation. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Different letters indicate significant difference at P < 0.05, bars are means of 3 experimental runs ± SD.

outer tissues: epidermis or cortex. Tobacco plants were able to accumulate up to 500 mg · kg^{-1} Si d. w. Dodder was able to wrap around the host, but it did not penetrate the stems and died after a few days.

Tobacco plants watered with Si (2.5 mM Si and 5 mM Si) grew significantly better than the control. We detected more chl *a* in 5W treatment when compared to the control, and secondary tissue formation in the stems was accelerated. Shoots achieved less POX than controls and also less CAT activity in 5W treatment than controls. A significant increase in CAT activity was detected in the 2.5W treatment. There were no differences among treatments in SOD activities. The shoots in the 2.5W and 5W treatments accumulated the highest Si; almost 800 mg · kg^{-1} Si d. w. The dodder was able to wrap around the stems and penetrate the host tissues only in 2.5W treatment, where no POX histochemical activity was observed in outer tissues, just in the places of secondary cell wall formation. Activities of POX, SOD and CAT in the stems increased dramatically after dodder's attack in the 2.5W treatment in comparison with the uninfected control plants and uninfected plants of 2.5W treatment at the same age.

Tobacco plants treated with Si by foliar application (1 mM Si and 2.5 mM Si) grew worse than the control. They had significantly less chlorophylls but more carotenoids than controls. Secondary tissues formation was not accelerated in comparison with the control. Activities of SOD and CAT were increased in comparison with the control, and in the leaves, there were high amounts of ROS. The histochemical POX activity was high and it was present in the cell walls of outer tissues. Shoots accumulated up to 650 mg · kg^{-1} Si d. w. Dodder was not able to wrap around and infect these stems.

Contributions

Zuzana Lukacova: designed the experiment, worked on the anatomy of tobacco leaves and stems, visualised the ROS in the tobacco leaves, statistically analysed the reached results, wrote the text of manuscript.

Renata Svubova: designed the experiment, cultivated the plant
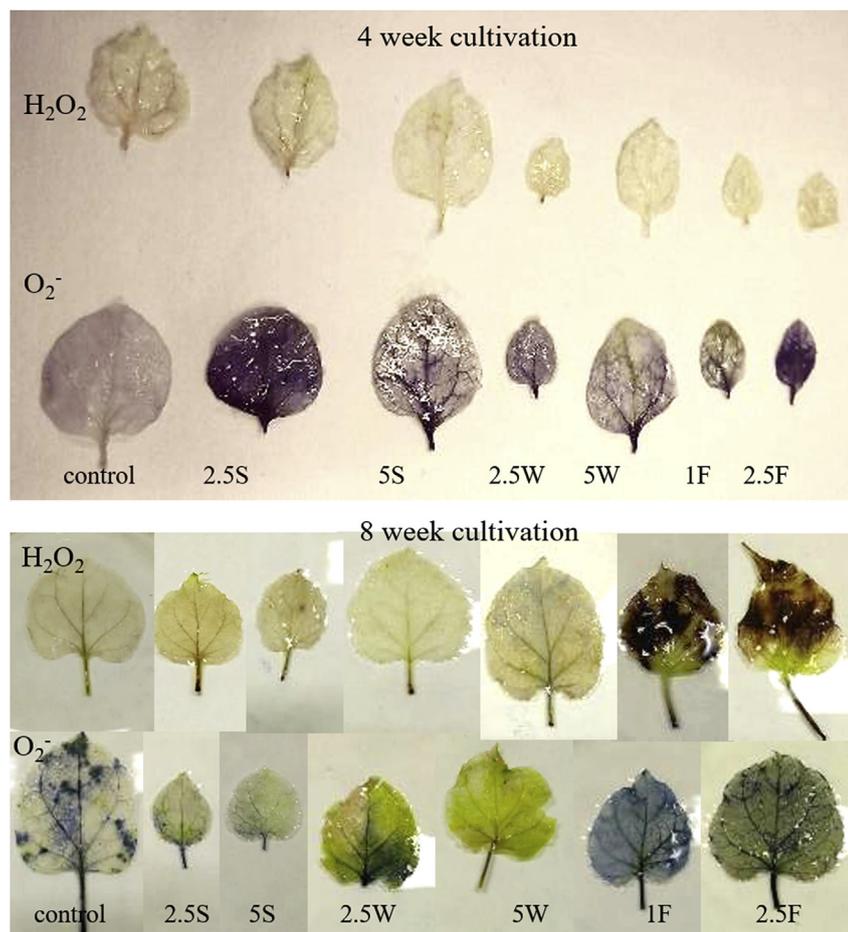
Fig. 11. Peroxide and superoxide visualisation by using DAB and NBT after 4 or 8 week cultivation in tobacco leaves of control and Si treatments. The presence of H_2O_2 and O_2^- is confirmed by brownish and blue spots, respectively. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
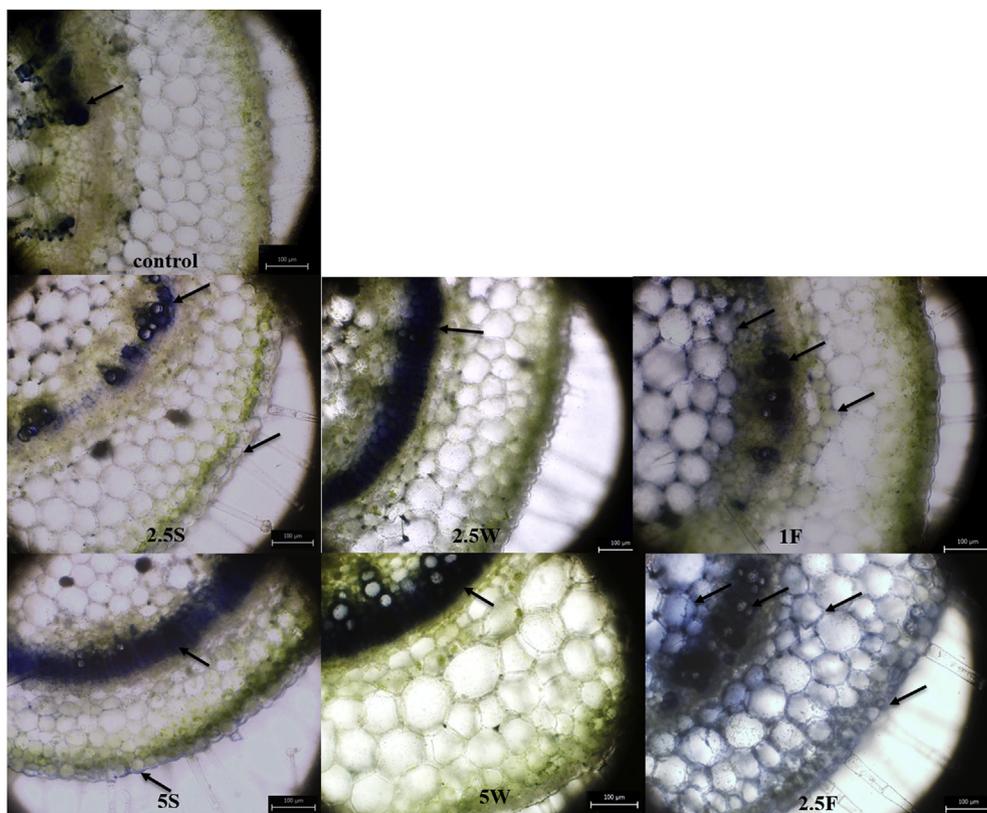
Fig. 12. Histochemical analysis of POD in the tobacco stems (the 4th internodium) of control and Si treatments. The presence of O_2^- is confirmed by dark blue stain (**arrows**). Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Scale bar = 100 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
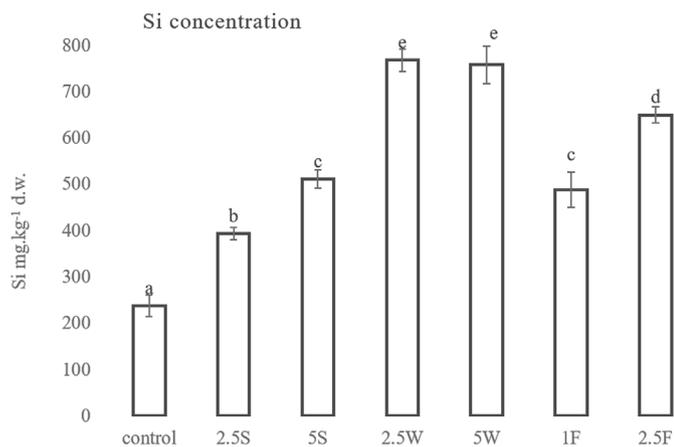
Fig. 13. Silicon concentration in tobacco shoots after 4 weeks of cultivation. Treatments: **control**, **2.5S** – seeds pre-treated with 2.5 mM Si, **5S** – seeds pre-treated with 5 mM Si, **2.5W** – watering with 2.5 mM Si on the 7th, 14th and 21st day after germination, **5W** – watering with 5 mM Si on the 7th, 14th and 21st day after germination, **1F** – foliar application with 1 mM Si on the 7th, 14th and 21st day after germination, **2.5F** – foliar application with 2.5 mM Si on the 7th, 14th and 21st day after germination. Different letters indicate significant difference at P < 0.05, bars are means of 3 experimental runs ± SD.

material, did the protein separation and antioxidant enzymes activities, worked on *in situ* POX activities.

Simona Janikovicova: observed the lignin deposition in tobacco stems.

Zuzana Volajova: determined the concentrations of chlorophylls and carotenoids.

Alexander Lux: co-worked on the anatomy of leaves, critically read the manuscript.

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