Final report of the PD 131589 project

The two face of nanoparticles: metal-oxide nanoparticle-induced nitro-oxidative stress in crop seedlings and its possible alleviation by nano-silicon seed priming

The project was carried out over three years (01. 12. 2019 – 30. 11. 2022), in three equal annual periods.

1. Theoretical background

The early development of the root system is crucial in terms of the life of a plant; besides ensuring physical stability for the whole plant, it is responsible for water and nutrient uptake and also might be practically (phytoremediation) relevant. The development of the root system is regulated by a complex and a diverse signalling network (Jung and McCouch, 2013). Besides hormonal factors, reactive oxygen (ROS) - and nitrogen species (RNS) play an important role in the regulation of root development. The gaseous signal molecule nitric oxide (NO) is considered to possess a principle role in the complex signal transduction network behind the whole process (Yu *et al*., 2014).

Formation of ROS, such as superoxide anion, hydroxyl radical, hydroperoxide radical, hydrogen-peroxide and singlet oxygen (Gill et al., 2010) can be linked to a wide range of stress responses (Apel and Hirt, 2004). This suggests an important role as intermediates in metal stress responses, as well as their connections to the signalisation of RNS. RNS as a group of molecules consists of several reaction products of NO, such as the radical nitrogen-dioxide, or the non-radical peroxynitrite and S-nitrosoglutathione (Molassiotis and Fotopoulos, 2011). The metabolism of ROS and RNS are connected at various points. The concept of nitro-oxidative stress has only recently become the subject of research in the field of plant biology (Corpas and Barrosso, 2013). RNS express their bioactivity through different covalent modifications on specific parts of the proteins. In the process of protein tyrosine nitration induced by RNS, peroxynitrite (product of the reaction of NO and superoxide radical) reacts with tyrosine amino acids in a two-step process, modifying the structure and activity of proteins (Corpas *et al*., 2013). Before now, numerous proteins with diverse functions have been identified as being involved in this reactions (Astier and Lindermayr, 2012; Lozano-Juste *et al*., 2011), however in the case of heavy metal excess our workgroup was the first to investigate the presence of protein tyrosine nitration (Feigl *et al*., 2015). The presence of this PTM has been proved in control, healthy plants as well (Corpas *et al*., 2013; Feigl *et al*., 2015; Lehotai *et al*., 2016).

Besides NO, hydrogen-sulphide (H_2S) has emerged in the last decade as a new gaseous signalling molecule plant cells and is as important as NO, carbon monoxide, or hydrogen peroxide (Aroca *et al.* 2018). H_2S is a powerful inducer of osmolytes and enzymatic or non-enzymatic antioxidants, moreover it interacts with the Ca^{2+} and NO signalling pathways as well. H_2S also protects stress-sensitive proteins by cysteine persulphidation, resulting highly effective ROS scavenger persulphides (Banerjee *et al.* 2018), thus its detection has a promising importance.

The delicate balance of the endogenous signal system can be affected by various environmental stimuli, such as the excess of metal oxide nanoparticles (NPs) in the environment. The rapidly growing application of nanomaterials inevitably lead to their accumulation in the ecosystems, and according to recent studies, through sludge application and irrigation approximately 30% of the total amount of nanomaterials released to the environment reaches the soil in Europe (Sun *et al.* 2014). Many studies conclude that metal-oxide NPs interfere with the redox homeostasis, reducing or most likely increasing oxidative stress (Tripathi *et al.* 2017). While oxidative burst has been regularly reported in plants exposed to toxic levels on NPs, still very little is known about the effect of NPs on RNS. Exposure to Ag, ZnO and aluminium oxide NPs induced NO formation besides ROS in duckweed (Thwala *et al.* 2013), corn (Zhao *et al.* 2012) and Tobacco BY2 cells (Poborilova *et al.* 2013), but the examination of the effect of metal-oxide nanoparticles on the nitro-oxidative homeostasis of crop seedlings is yet to be performed.

It is not yet clear whether copper oxide (CuO) nanoparticles are more toxic (i.e., they inhibit plant growth at the same concentration) compared with larger-sized copper oxide particles (Kumar *et al*., 2021; Roy *et al*., 2022). Copper ions released from the particles are able to bind to the thiol groups of proteins, causing the proteins to undergo conformational changes (Nekrasova and Maleva, 2007). Another mechanism of toxicity occurs through the Fenton reaction, in which copper ions convert hydrogen peroxide into hydroxyl radicals, which in turn, damage the surrounding macromolecules (Chung *et al*., 2019).

The effects of copper oxide nanoparticles have been investigated in a relatively few number of plant species. In most cases, monocots did not respond or responded negatively to copper oxide nanoparticle treatment, while other species that were positively influenced by copper oxide nanoparticles were predominantly dicotyledonous.

Silicon (Si) is not an essential plant nutrient, however many studies have shown its beneficial effects in numerous species under several environmental circumstances. Si has been reported to alleviate a wide range of abiotic stresses, including metal toxicity, such as cadmium (Shao *et al.* 2017), manganese (Che *et al.* 2016), aluminium (Wang *et al.* 2004) or copper (Mateos-Naranjo *et al.* 2015). Si seems to alleviate oxidative stress (a hallmark feature of different stress processes), however this does not mean that Si is directly involved in antioxidant activity, since no such evidence exists. In a recent review of Coskun *et al.* (2019), the authors seek the ultimate causes of Si-provided alleviation of abiotic stress and propose a working model called the apoplastic obstruction hypothesis, which means that Si deposits around and fortifies apoplastic barriers surrounding the vascular bundles, inhibiting the transport and accumulation of harmful materials (like metals or metal NPs) to the shoot, mitigating downstream stress events. Si might also co-precipitate with toxic materials in the extracellular matrix, further protecting tissues against stress (Coskun *et al.* 2019). Despite the growing (and sometimes confusing or contradictory) evidence of the stress alleviating effect of Si, the interaction of Si NPs and metal or nano-metal stress is not yet well known.

2. Introduction – setting up the experimental system and optimization

2.1. Setting up the *in vitro* plant growing system

In vitro plant growing system was adapted and established. Glass petri dishes with the diameter of 100 mm was used. Petri dishes were lined with 2 layers of Grade 1602/N type qualitative filter paper and then autoclaved to avoid bacterial and fungal infections of the growing seedlings.

The filter paper in the petri dishes was only moistened before placing seeds onto them. For moistening, besides distilled water as control, a wide range of zinc oxide (ZnO) and CuO nanoparticle concentrations were used (10; 25; 50; 75; 100; 125; 150; 200; 300 and 400 mg/l).

Seeds were surface sterilized; in every petri dish 10 seeds were placed on the soaked filter paper (or in some cases between the two layers) at equal distances from each other. The dishes were sealed with plastic foil and placed in a greenhouse (12/12 h day/night period).

For most of the examined species in the pilot experiments, 5 days growing period was sufficient for the seedlings to develop roots which were still possible to separate without damage and could be measured.

After 5 days, dishes were opened and seedlings were scanned (Czur Shine 800 Pro, Czur Tech Co. Ltd., Dalian, China), placed next to a scale. Digital photos were examined with Fiji (imageJ) software.

2.2. Testing a wide range of nanoparticle (copper- and zinc-oxide) concentrations, to determine their effect *in vitro*, involving numerous crops

Nanoparticles were manufactured by the Applied Chemistry Department of the University of Szeged.

Seeds were obtained from the Cereal Research Non-Profit Ltd. Szeged and in some cases from commercial sources. Among monocotyledons wheat, durum wheat, spelt, triticale, sorghum, millet, rye and setaria; among dicotyledons sunflower, flax, pea, tomato, rapeseed, soybean and buckwheat were tested.

After 5 days, digital images were taken and the seedlings' roots were characterised with Fiji (imageJ) software by measuring root length and counting lateral roots (in case of dicots) roots in the developing fibrous root system (in case of monocots).

It was found that **monocots did not response even to the highest applied ZnO nanoparticle treatment**, while they caused serious growth inhibition of dicot seedlings. Interestingly, **dicots did not response even to the highest applied CuO nanoparticle treatment**, while they caused serious growth inhibition of monocot seedlings, which means that the two nanoparticle have the exact opposite effect on the investigated plant species.

Due to the promising results, and the relatively large amount of available data on the effect of ZnO nanoparticles on plants in the literature compared to CuO nanoparticles, I decided to prioritise experiments and **focused on the effect of CuO nanoparticles on monocotyledonous species**. Among these, sorghum (*Sorghum bicolor* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.) and triticale (*x Triticosecase*) were investigated using 50% growth inhibition-causing CuO NP concentrations in an *in vitro* setup.

2.3. Silicon dioxide nanoparticles, as seed priming agent

After reviewing the methods in the literature, seeds were first surface sterilized and then incubated in a SiO_2 suspension (100-800 mg/L concentration) for six hours. After the incubation period, seeds were drained and dried; once reached their initial weight, seeds were stored in sealed centrifuge tubes on 4°C, similar to unprimed seeds.

2.4. Rhizotron plant growing system

Custom-made plexi panels were ordered and assembled into 15 cm wide, 30 cm tall and 1,6 cm thick rhizotrons, using polifoam sheets and screws with wing nuts. The front panel is made of 3 mm thick, anti-glare, 100% transparent plastic, while the back panel is a 3 mm thick non-transparent black sheet; the thickness of the soil layer inside the rhizotron is 1 cm.

The rhizotrons are filled with 250 grams of “Klasmann Potgrond P” blocking substrate (100% frozen through black peat with a fine structure of maximum 8 mm size, pH 6,0; 210 mg N/l; 240 mg P_2O_5/l) mixed with 20% sand; the initial water content is set to 70%.

3. Applied methods

During the course of research copper and zinc nanoparticles were used as stressors in several concentrations, to determine their 50% growth-inhibition causing concentrations. In the priming experiments, silicon nanoparticles were used in several concentrations to determine their optimal concentration as seed pre-treatment, alleviate the later metal-oxide nanoparticle-induced stress processes. The applied nanoparticles were synthetized and characterized in collaboration with the Applied Chemistry Department of the University of Szeged.

Numerous, widely cultivated crops were used in in vitro experimental system. In the in vitro setup seeds were germinated in filter paper wetted with metal-oxide nanoparticle suspension in different concentrations for 5 days.

During screening experiments morphological and biomass-production measurements (seedling root length, fresh and dry weight), viability tests (fluorescent microscopic analysis of the root apical meristem) and detection of protein tyrosine nitration, an RNS dependent posttranslational modification (western blot) were conducted.

Detailed experiments were conducted with the metal-oxide nanoparticle concentration resulting in 50% root growth inhibition in the in vitro system. These include light- and fluorescent microscopic analysis of the root tips, e.g. root tip macromorphology, meristem viability, cell cycle labelling, and root cell wall modifications. The ROS and RNS content and the antioxidant activity of the root tissues were determined by microscopy and spectrophotometry. Subsequent changes, such as protein nitration, GSNO, GSNOR and lipid peroxidation were detected by immunfluorescent- and light microscopy. Hydrogen sulphide and flavonol levels were measured by fluorescent microscopy.

4. Difficulties encountered in the research and their impact on the effectiveness of the work

Due to the escalated COVID-19 situation and disruptions caused by the recent energy crisis, realization of the project faced some unforeseeable but serious obstacles. As a result, it has become difficult to schedule and perform experiments, so the implementation of the measurements was often behind schedule.

These constraints have led to delays in the preparation of the manuscripts, compounded by the fact that the publication process has slowed down considerably worldwide, which means that the results of the work are still under publication. However, the commitment made in the work plan has been fulfilled, i.e. one manuscript has been submitted and another is in preparation. Furthermore, due to the complications of the international travel ban, I have been attending domestic and online meetings instead of international conferences.

Nevertheless, I believe that the main hypotheses of the project were successfully tested, and the experiments performed have provided useful new information to better understand the relationship between nanoparticles and plants.

5. Key results

5.1 Exploration the homeostasis of signaling molecules in monocotyledonous crops with different CuO nanoparticle tolerance

The results of this part of the project is submitted for publication at the time the report is written in *Plant Direct* (Online ISSN:2475-4455).

Summary

In this study, the concentration of CuO NP that inhibits 50% of root growth was determined using sorghum, wheat, rye, and triticale as model plant species and the NP-induced stress response and the balance of reactive molecules were assessed. Based on the effective concentration of CuO NP, wheat, rye, and triticale were more tolerant compared with sorghum and entirely different response mechanisms in the homeostasis of reactive signal molecules were observed. For the sensitive sorghum roots, the amount of reactive molecules was not significantly altered, whereas a significant increase in protein tyrosine nitration indicated a severely stressful state caused by CuO NPs. In contrast, the amount of reactive molecules increased significantly in the roots of the relatively tolerant species, and while the appearance of lipid peroxidation indicated oxidative stress, different changes in protein tyrosine nitration was associated with tolerance. The significant CuO NP-induced rise of endogenous H_2S content in the root tips may be partly responsible for the relative tolerance of wheat, rye, and triticale compared with sorghum. Overall, the results demonstrate that while monocotyledonous species with different CuO NP sensitivities may exhibit similar growth responses, the underlying changes in the dynamics of reactive molecules influence their resilience.

5.1.1. CuO NP-induced root morphological changes

The primary aim of this study was to determine the CuO NP concertation that induces 50% root growth inhibition, in terms of root length. Sorghum was more sensitive compared with wheat, rye, and triticale, as 50 mg/L CuO NP treatment was able to induce the anticipated root growth response, whereas in the others, 150 mg/L of CuO NP was required for the same effect. Although longitudinal root growth was significantly inhibited, fibrous root numbers of wheat, rye, and triticale seedlings decreased only slightly and the lateral root formation of sorghum was not affected by CuO NP stress. Because of the inhibition of root elongation, the fresh weight of the root system was also significantly decreased by CuO NP stress in all the plant species examined (Table 1).

Species	Treatment	Root length		(Lateral) root number		Root fresh weight		Viability - FDA fluorescence	
		(mm)		(pieces)		(mg)		(pixel intensity)	
Sorghum	Control	5.67 ± 0.19	a	3.47 ± 0.28	f	23.60 ± 1.68	c	7659.60 ± 693.72	a
	50 mg/L CuO	2.49 ± 0.09	de	3.71 ± 0.21	f	9.96 ± 0.62	e	3433.03 ± 293.79	b
Wheat	Control	3.74 ± 0.18	bc	4.83 ± 0.09	abcd	48.20 ± 3.40	b	4508.75 ± 607.57	b
	150 mg/L CuO	1.96 ± 0.06	e	4.38 ± 0.10	cde	16.46 ± 1.11	de	1154.15 ± 162.11	c
Rye	Control	4.08 ± 0.18	b	4.84 ± 0.21	abc	50.24 ± 3.93	b	907.45 ± 120.85	c
	150 mg/L CuO	2.42 ± 0.08	d	4.69 ± 0.16	abcde	19.57 ± 1.68	cd	690.75 ± 142.14	c
Triticale	Control	4.07 ± 0.23	bc	5.37 ± 0.20	a	67.85 ± 4.51	a	1330.01 ± 134.53	c
	150 mg/L CuO	2.01 ± 0.05	e	5.00 ± 0.15	ab	27.16 ± 1.99	c	1171.43 ± 178.72	c

Table 1. CuO nanoparticle-induced changes in root length, root number, and lateral root number, and the viability of the apical meristematic zone of sorghum, wheat, rye, and triticale. The results are expressed as the mean ± s.e. Different letters indicate significant differences according to Duncan’s test ($P \leq 0.05$).

CuO NP stress significantly decreased apical meristem viability of the relatively sensitive sorghum. Among the relative tolerant species, growth-inhibiting CuO NP treatment significantly decreased the viability of wheat root tips, whereas in rye and triticale, only some minor inhibition was detected (Table 1). While the root growth results and the root tip viability data may appear contradictory in the last two species, root elongation is regulated by a complex network of factors (e.g., metabolic, hormonal, etc.), not just the metabolic activity of the apical meristem cells.

5.1.2. Changes in the dynamics of reactive signaling molecules in the root tips

CuO NP-induced changes in reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulphur species (RSS) homeostasis was detected in the meristematic zone of the root tips. In general, whereas the trend of the alterations was mostly similar in all monocot species, a significant difference was observed in the extent of changes between the relatively tolerant wheat, rye, and triticale species and compared with the relatively sensitive sorghum.

The superoxide anion content of the root tips of sorghum was not affected by 50 mg/L CuO NP stress. In contrast, 150 mg/L (50% root growth-inhibiting) CuO NP treatment significantly increased superoxide anion levels in the meristematic zones of the relative tolerant species (Fig. 1A). Similarly, CuO NP stress significantly induced the accumulation of hydrogen peroxide in the root tips of wheat, rye, and triticale. In contrast to superoxide, the amount of hydrogen peroxide was slightly, but visibly increased, in the root tips of sorghum (Fig. 1B). A comparison of the amount of GSH and hydrogen peroxide in the root tips indicated that the relatively sensitive sorghum contained the most GSH under control circumstances, but was decreased significantly with increasing H_2O_2 during CuO NP stress (Fig. 1B and C). In the relative tolerant species, there was no significant difference in GSH content in the CuO NP-stressed root tips; however, both GSH content in the control roots and the degree of reduction were different in the three species (Fig. 1C). The appearance of pink coloration associated with lipid peroxidation correlated with high ROS levels in the root tips, because it was observed in the relatively tolerant species (Fig. 1D).

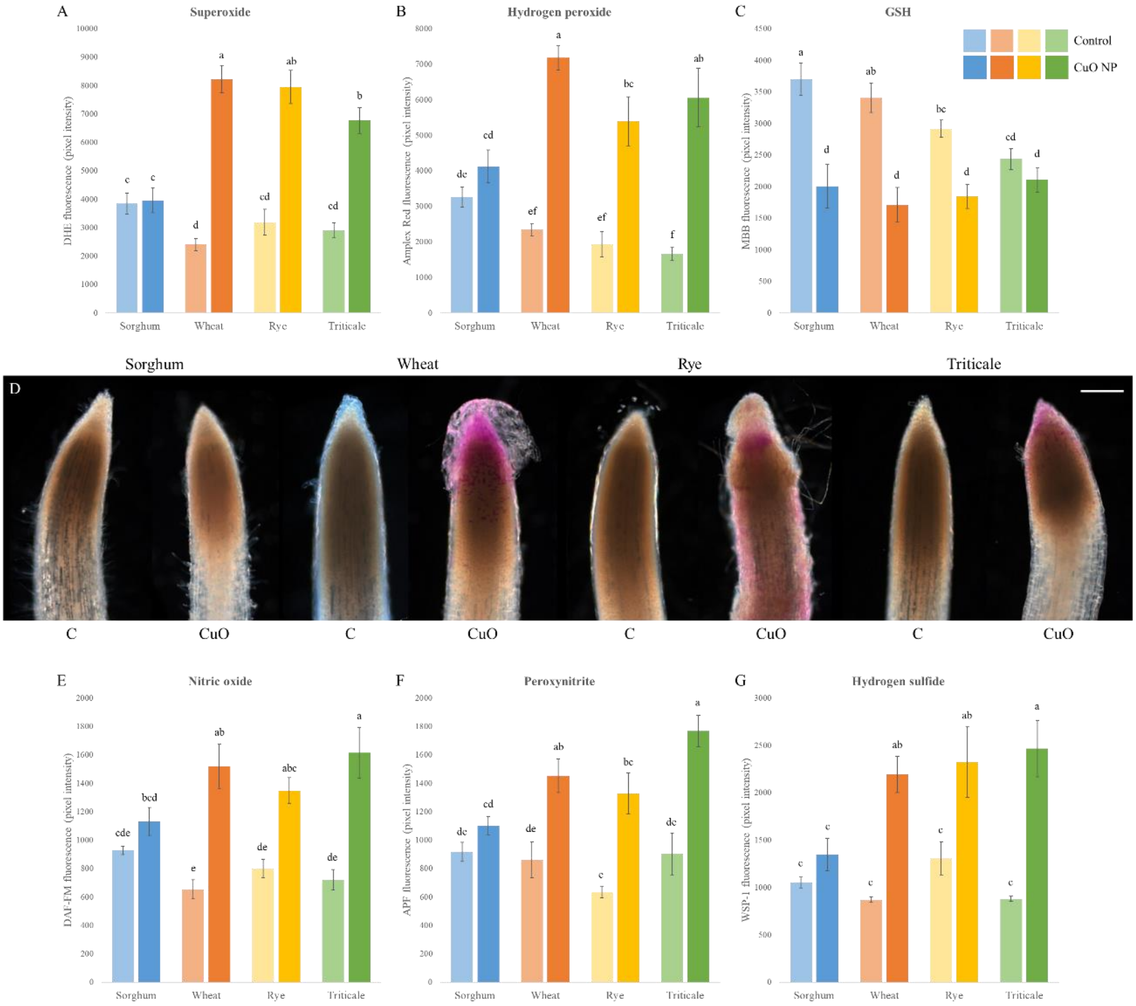


Figure 1. CuO nanoparticle-induced changes in the levels of reactive molecules and lipid peroxidation in sorghum, wheat, rye, and triticale root tips. Superoxide (A), hydrogen peroxide (B), glutathione (C), malondialdehyde (D), nitric oxide (E), peroxynitrite (F) and hydrogen sulfide (G) content. The results are expressed as the mean ± s.e. Different letters indicate significant differences according to Duncan's test ($P \leq 0.05$).

Compared to the relationship between CuO NPs and ROS, less is known regarding the effect of CuO NPs on the homeostasis of RNS. The tendency of CuO NP-induced changes in RNS was similar to that for ROS. In the relatively sensitive sorghum root tips, CuO NP stress induced only slight NO accumulation; however, among the control roots, NO content was the highest of all species examined. On the other hand, in the relatively tolerant wheat, rye, and triticale, growth-inhibiting CuO NP stress induced significant NO accumulation (Fig. 1E). In the more tolerant species and in correlation with the NO levels discussed above, $ONOO-$ content was significantly increased by CuO NP stress. Moreover, a moderate $ONOO^-$ accumulation could also be detected in sorghum root tips (Fig. 1F), which is probably due to a slight increase in the amount of NO. The observed changes in H_2S content of plant root meristematic zones were similar to other reactive molecules. In relatively sensitive sorghum, the levels did not change significantly, whereas in the relatively tolerant species, CuO NP stress induced significant H_2S accumulation (Fig. 1G).

5.1.3. CuO NP-induced changes in protein tyrosine nitration in the roots

Despite the fact that CuO NP did not cause a significant change in the amount of reactive molecules in sorghum, the level of protein tyrosine nitration (PTN), a marker of nitro-oxidative stress, increased significantly in CuO NP-treated roots. Regardless of the uniform increase in reactive molecules in the relatively tolerant species, CuO NP-induced changes in the nitroproteome exhibited a species-specific response (Fig. 2). In the roots of wheat, nitration levels were unchanged compared with the control and the overall amount of nitrated proteins was low. In the roots of rye, CuO NP increased the nitration of proteins in the size range of 70 and 25 kDa; moreover, several new immunopositive bands appeared in the proteome of the stressed root (50, 35, and 23 kDa). In triticale, several nitrated protein bands were visible in the control samples (70, 35, 25, and 23 kDa), but with the exception of the 70 kDa band, the nitration signal decreased under CuO NP stress. Although showing similar growth response accompanied by analogous changes in the homeostasis of the reactive molecules in their roots, the relative tolerant species exhibited three different nitration responses. It is also important to note that the species-specific nitration response was significantly less pronounced compared with the large increase in nitration resulting from CuO NP stress in the relatively sensitive sorghum root.

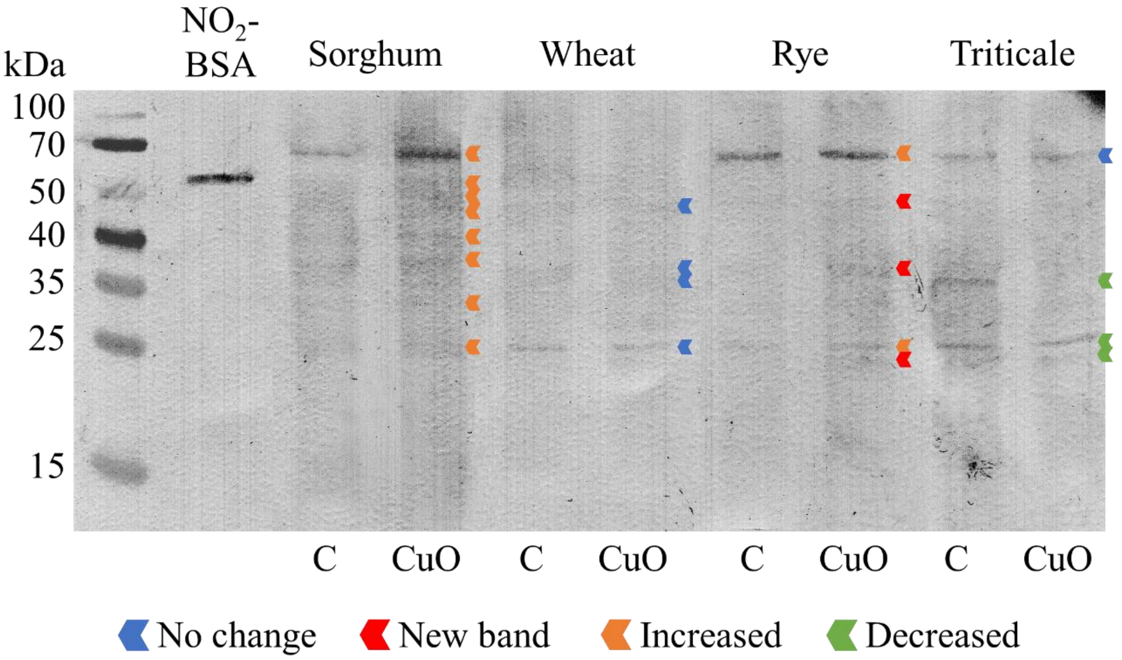
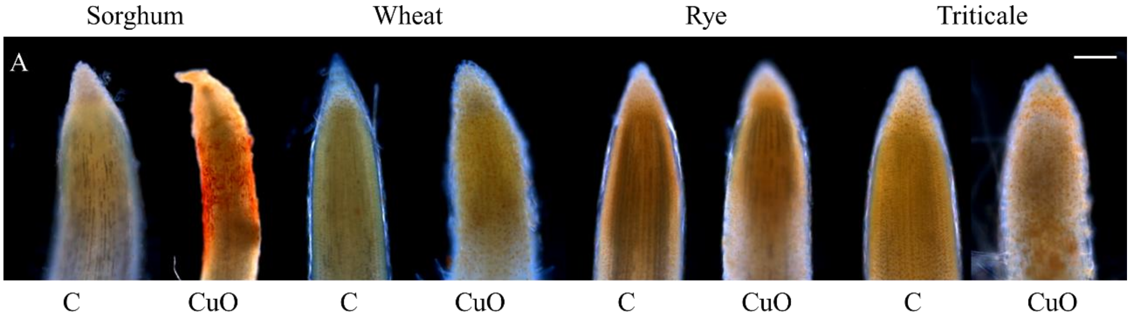


Figure 2. Representative immunoblot indicating protein tyrosine nitration in the roots of sorghum, wheat, rye, and triticale under control conditions and subjected to CuO NP stress. Blue arrows show nitrated protein bands with no change compared with the control, red arrows indicate new nitrated bands, orange arrows represent protein bands with increased nitration compared with the control, green arrows indicate protein bands with decreased nitration compared with the control. (NO2-BSA: nitrated bovine serum albumin, used as positive control).

5.1.4. CuO NP-induced changes in the cell wall-mediated defense system

CuO NP stress induced various modifications in the root tip cell wall of the relatively sensitive sorghum plant compared with that in other tolerant species. Of the four species, CuO NP caused lignin encrustation only in the root tips of the sorghum plant (Fig. 3A). In contrast, callose accumulation was not observed, only in the more tolerant species (Fig. 3B). The amount of quercetin, a flavonoid component of the cell wall-related defense machinery, was only increased during CuO NP-induced stress in the root tip of wheat (Fig. 3C). Compared with the more CuO NP-tolerant species, the quercetin content of sorghum root tips was significantly lower and not increased by CuO NP stress. The amount of quercetin detectable in the root apex was inversely proportional to the degree of nitration, suggesting a possible connection between the two. In sorghum, in which the least amount of quercetin was detected in the roots, nitration increased significantly following CuO NP treatment, whereas in wheat, a significant increase in quercetin content was observed and the level of nitration did not change during CuO NP stress. In rye and triticale, CuO NP stress did not induce quercetin accumulation; however, in rye, the quercetin content was significantly lower compared with triticale (or wheat), protein nitration was increased, and a higher amount of quercetin was accompanied by decreased PTN levels.



Species	Treatment	**B** **Callose** Aniline blue fluorescence (pixel intensity)		**C** **Quercetin** DPBA fluorescence (pixel intensity)	
Sorghum	Control	6607.53 ± 344.68	b	398.53 ± 18.64	e
	50 mg/L CuO	4213.13 ± 242.32	ef	792.89 ± 104.28	e
Wheat	Control	3907.85 ± 140.19	f	5295.47 ± 406.56	c
	150 mg/L CuO	5985.35 ± 580.69	bc	12556.28 ± 846.42	a
Rye	Control	5194.16 ± 407.19	cde	2495.42 ± 476.73	d
	150 mg/L CuO	9372.28 ± 521.79	a	3236.91 ± 349.20	d
Triticale	Control	4181.83 ± 156.09	ef	7176.23 ± 573.17	b
	150 mg/L CuO	5486.91 ± 444.58	bcd	6898.73 ± 670.88	bc

Figure 3. CuO nanoparticle stress-induced cell wall-mediated defenses. Lignin (A), callose (B), and quercetin (C) content of the root tips of sorghum, wheat, rye, and triticale. The results are expressed as the mean ± s.e. Different letters indicate significant differences according to Duncan’s test ($P \leq 0.05$).

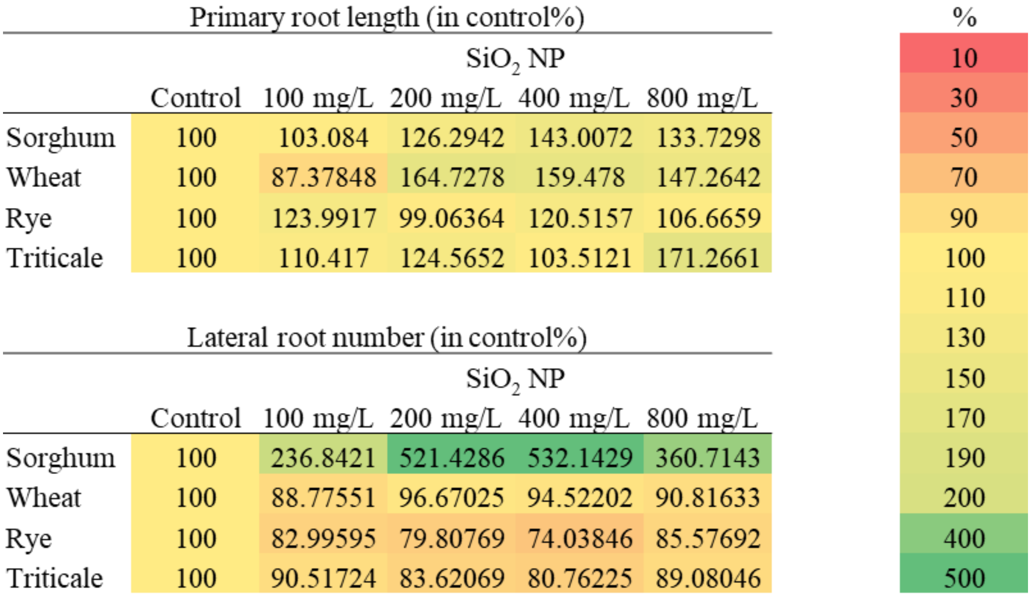
5.2. Counteracting effect of silicon nanoparticle priming on subsequent CuO nanoparticle-induced early stress response of monocotyledonous crops

The results of this part of the project are partly presented at “FIBOK 2022” 5th National Conference of Young Biotechnologists (Szierer *et al.*) and being prepared for publication at the time the report is written.

In this study, as a continuation of the work presented above, the effect of silicon dioxide nanoparticle seed priming was determined using sorghum, wheat, rye, and triticale as model plant species under control circumstances and also under the 50% growth-inhibiting effect of CuO NPs. Besides root growth parameters, the NP-induced changes in the balance of reactive molecules are being assessed.

5.2.1. Effect of SiO_2 nanoparticle seed priming on root growth parameters

Figure 4 shows that, compared to the control, silicon nanoparticle pretreatment positively affected the root length growth of the four studied species. For sorghum, higher concentrations, especially 400 mg/L, had a positive effect. For wheat, the 100 mg/L concentration had a slightly inhibitory effect, while the 200, 400 and 800 mg/L concentrations all had a stimulating effect. For rye, the 100 and 400 mg/L concentrations were stimulatory, while for the others there was no statistically significant effect. In contrast, for triticale, the 200 and 800 mg/L concentrations had a stimulatory effect, while the others were neutral.



Primary root length (in control%)

		SiO_2 NP			
	Control	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	100	103.084	126.2942	143.0072	133.7298
Wheat	100	87.37848	164.7278	159.478	147.2642
Rye	100	123.9917	99.06364	120.5157	106.6659
Triticale	100	110.417	124.5652	103.5121	171.2661

Lateral root number (in control%)

		SiO_2 NP			
	Control	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	100	236.8421	521.4286	532.1429	360.7143
Wheat	100	88.77551	96.67025	94.52202	90.81633
Rye	100	82.99595	79.80769	74.03846	85.57692
Triticale	100	90.51724	83.62069	80.76225	89.08046

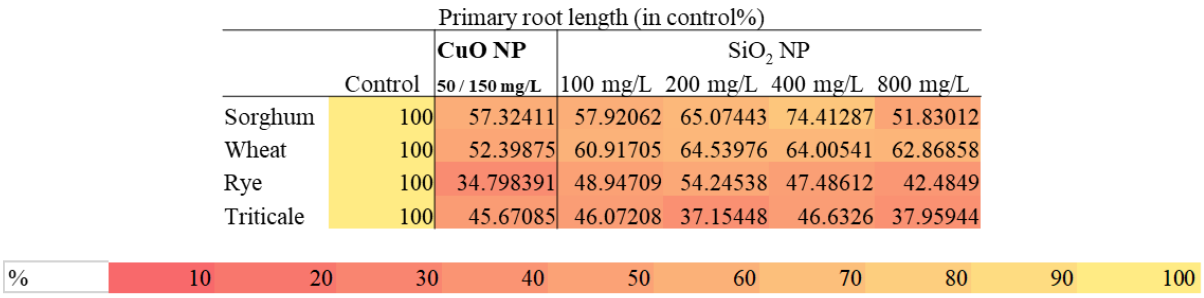
%
10
30
50
70
90
100
110
130
150
170
190
200
400
500

Figure 4. Effect of SiO_2 nanoparticles on the primary root length and lateral root number. Data are presented in control %, shades of red represents growth inhibition, shades of green represents positive growth response.

The effect of the treatment on the root and lateral root numbers was also investigated, the results are also shown on Figure 4. From the data obtained, it can be said that the silica nanoparticle pre-treatment had a positive effect on the lateral root number of sorghum, while it reduced the root number of wheat, rye and triticale. For sorghum, the treatment with concentrations of 200 and 400 mg/L had the greatest effect, while the effect of 800 mg/L was less significant. For wheat, the correlation between root number and the concentration of the silica nanoparticle mixture used for pretreatment did not show a strong correlation, with a slight decrease observed at the 100 mg/L concentration. For rye, the inhibitory effect was more significant, with pre-treatments at concentrations of 100, 200 and 400 mg/L having an increasingly significant inhibitory effect, while the effect was weaker at 800 mg/L pre-treatment. For triticale, a similar trend to rye was observed, but here the effect of the 100, 200 and 400 mg/L pretreatments were highly significant, the inhibitory effect increasing spectacularly with increasing concentration, and at 800 mg/L the inhibition was also weaker and less significant.

5.2.2. Combined effects of copper oxide and silicon nanoparticles on root development

In the next part of the series of experiments, it was investigated whether the silicon nanoparticle pretreatment could mitigate the effects of the inhibitory copper oxide treatment and whether the combined effect of the two was species dependent. As shown in Figure 5, silica nanoparticle pretreatment of seeds was able to counteract the root growth inhibition caused by copper oxide nanoparticles in sorghum, wheat and rye, but not in triticale.



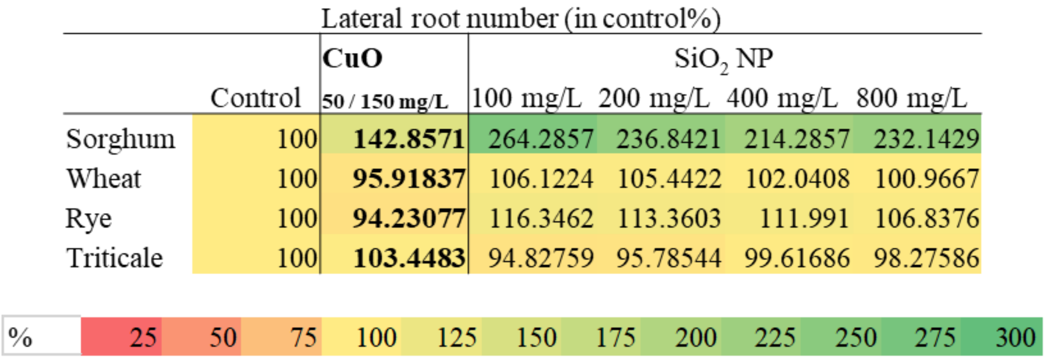
Primary root length (in control%)

	Control	**CuO NP**	SiO_2 NP			
		50 / 150 mg/L	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	100	57.32411	57.92062	65.07443	74.41287	51.83012
Wheat	100	52.39875	60.91705	64.53976	64.00541	62.86858
Rye	100	34.798391	48.94709	54.24538	47.48612	42.4849
Triticale	100	45.67085	46.07208	37.15448	46.6326	37.95944

%	10	20	30	40	50	60	70	80	90	100

Figure 5. Effect of SiO_2 NP priming on the primary root length of CuO NP stressed plants. Data are presented in control %, shades of red represents the degree of growth inhibition.

The results shown in Figure 6 indicate that copper oxide nanoparticle stress was able to counteract the root number reducing effect of silica nanoparticles in wheat and triticale, but the effect was most pronounced in rye. Also, silicon dioxide nanoparticle pre-treatment had a positive effect on the number of lateral roots in sorghum, while it reduced the number of roots in wheat, rye and triticale under CuO NP stress.



Lateral root number (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	100	**142.8571**	264.2857	236.8421	214.2857	232.1429
Wheat	100	**95.91837**	106.1224	105.4422	102.0408	100.9667
Rye	100	**94.23077**	116.3462	113.3603	111.991	106.8376
Triticale	100	**103.4483**	94.82759	95.78544	99.61686	98.27586

%	25	50	75	100	125	150	175	200	225	250	275	300

Figure 6. Effect of SiO_2 NP priming on the lateral root number of CuO NP stressed plants. Data are presented in control %, shades of red represents growth inhibition, shades of green represents positive growth response.

5.2.3. Changes in the dynamics of reactive signaling molecules in the root tips

As mentioned above, CuO NP stress significantly disrupted the balance of reactive molecules (ROS, RNS, RSS) in the root tips of the relative tolerant wheat, rye and triticale, while in the relative sensitive sorghum their level did not increase significantly. SiO_2 NP priming was able the counteract the CuO NP-induced growth inhibition of sorghum, wheat and rye, but not in case of triticale.

In the background of the growth inhibition reducing effect of SiO_2 NP priming, a complex change in the balance of reactive molecules can be detected (Figure 7). While some experiments are still being carried out or repeated, SiO_2 priming seems to have different effects on the levels of different reactive molecules in the root tips of the species studied. For example, silicon was able to decrease superoxide content of the root tips under CuO NP stress in sorghum, wheat and rye, and the significant root growth recovery of sorghum induced by 400 mg/L SiO_2 NP is accompanied with a control-like NO level in the root tip.

$O_2^{\cdot -}$ (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	78.73801	100	84.69527	72.04022	76.69575	61.0168
Wheat	51.04927	100	70.63533	86.70817	96.3299	116.1708
Rye	37.75537	100	59.48698	83.22091	101.4545	102.7754
Triticale	55.59811	100	121.0251	91.70822	101.987	71.64833

H_2O_2 (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	96.13847	100	143.8485	136.7369	133.017	110.5222
Wheat	32.24757	100	122.8144	108.7015	135.7859	144.3239
Rye	35.95522	100	95.60542	172.3491	127.1577	82.08097
Triticale	25.01993	100	106.3988	101.7991	119.5819	130.0447

H_2S (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	79.34503	100	99.12894	108.7727	131.176	94.85568
Wheat	39.58761	100	113.5085	82.59112	104.4506	100.5842
Rye	93.26162	100	124.6478	141.8355	104.3251	179.427
Triticale	39.0217	100	127.4579	79.88098	102.2585	82.4497

Callose (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	103.1067	100	101.827	84.51215	79.09079	83.60406
Wheat	57.70769	100	98.98505	89.51455	95.02767	116.2989
Rye	70.04876	100	98.16117	92.82817	107.5422	93.27421
Triticale	52.4996	100	113.2054	106.0178	117.649	103.9807

NO (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	83.5291	100	147.0885	168.6009	82.48696	127.1186
Wheat	38.0968	100	130.4953	104.6039	87.1509	104.6223
Rye	35.29902	100	113.6135	128.2184	154.2631	134.3604
Triticale	26.94283	100	95.54056	125.3725	154.7421	93.16258

$ONOO^-$ (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	86.94291	100	90.25851	120.0058	92.2747	89.27179
Wheat	45.10799	100	106.7319	98.66267	86.75531	98.79406
Rye	34.48482	100	139.8768	132.6244	165.7401	107.7514
Triticale	43.41671	100	118.4097	89.87842	104.7675	67.52014

Quercetin (in control%)

		CuO	SiO_2 NP			
	Control	**50 / 150 mg/L**	100 mg/L	200 mg/L	400 mg/L	800 mg/L
Sorghum	51.658	100	46.78714	62.72117	66.29016	93.88542
Wheat	36.51294	100	160.1142	138.4164	325.4744	96.89289
Rye	68.3242	100	61.26294	62.15095	91.66841	109.913
Triticale	104.8724	100	34.34162	77.72706	73.39222	77.87239

%
10
20
30
40
50
60
70
80
90
100
125
150
175
200
225
250
275
300
325
350

Figure 7. CuO and SiO_2 nanoparticle-induced changes in the levels of reactive molecules and quercetin in sorghum, wheat, rye, and triticale root tips. Data are presented in control % (where results obtained from CuO NP-stressed roots were selected as 100%), shades of red represents decreased levels of molecules, shades of green represents increased reactive molecule and quercetin content.

While it would be too early to draw far-reaching conclusions from the data, it seems clear that priming the seeds with SiO_2 nanoparticles has an effect on the signaling processes underlying the growth responses.

5.2.4. Effect of SiO_2 NP priming on the CuO NP-induced changes in protein tyrosine nitration in the roots

As mentioned above, CuO NP stress caused different changes in the nitration level and pattern in the roots of the different species. Although final experiments are still underway when this report is written, it seems clear that the changes in the balance of reactive molecules are accompanied by an alteration in the level of nitration detectable in the roots of species.

The results obtained suggest that the treatment with copper oxide nanoparticles induces stress in plants, which can be reduced in some cases by silicon nanoparticle pretreatment based on our morphological and proteomic results. In sorghum, pretreatment with silicon nanoparticle solution at a concentration of 400 mg/L was the most effective. For wheat, there was no remarkable difference on Western blot but the nitration levels are slightly lower in the treated bands. Rye gave variable results but there was a reduction in nitration at concentrations of 100 and 400 mg/L SiO_2 NP. The case of triticale is of particular interest because the morphological test results showed no improvement in plant stress status with silicon nanoparticle pretreatment over any of the silicon concentration ranges tested, but Western blot showed a reduction in nitration rates at, for example, 100 mg/L, so the results of the two test methods appear to contradict each other, which remains to be resolved in the future.

5.3. Examination of the seedlings root architecture subjected to silicon priming and grown on 50% growth inhibiting metal-oxide nanoparticle concentrations in rhizotron system

In case of the rhizotron setup, investigation of the effect of CuO nanoparticles and silicon priming on root system architecture was attempted.

Due to the promising results of the *in vitro* experiments, pilot rhizotron trials were assembled in order to shed light the effect of CuO NPs on root development in soil. During these pilot experiments, a wide range of CuO NP concentrations were tested, but interestingly, these failed to induce growth responses similar to those seen *in vitro*, even at higher concentrations. Based on the pilots, after evaluating the results of the experiments, I did not continue with the rhizotron experiments as they did not seem scientifically justified.

6. Conclusions

The project has revealed a number of new information about the relationship between plants and metal oxide nanoparticles.

Firstly, monocotyledons and dicotyledons responded very differently to ZnO and CuO nanoparticles, with monocotyledons tolerating the presence of ZnO well, while CuO was able to significantly inhibit their growth. The situation was reversed for dicotyledons, whose growth was inhibited by ZnO, while they proved tolerant to CuO stress.

Among the monocotyledons, there is a significant difference between the responses of relatively sensitive sorghum and relatively tolerant wheat, rye and triticale to CuO nanoparticle treatments that cause 50% growth inhibition. The results of this part of the project indicate that although different monocotyledonous species with different CuO NP sensitivities exhibit similar growth responses, diverse changes may occur in the balance of reactive molecules in their roots, which can indicate and influence their resistance to CuO NPs as a stressor.

SiO_2 nanoparticle priming of the seeds was able to counteract the root growth inhibition caused by copper oxide nanoparticles in sorghum, wheat and rye, but not in triticale. It seems clear that priming the seeds with SiO_2 nanoparticles has an effect on the signaling processes underlying the growth responses.

7. Dissemination

Gábor Feigl, Árpád Molnár, Dóra Oláh, Kolbert Zsuzsanna
Role of Nitric Oxide in Plant Abiotic Stress Tolerance
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XIII. Hungarian Congress of Plant Biology

Horváth Edit, Bela Krisztina, Hajnal Ádám, Feigl Gábor, Kulman Kitti, Gaál Marcell, Csiszár Jolán (2021)
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XIII. Hungarian Congress of Plant Biology

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Different nitro-oxidative response of tomato cultivars to salt- and osmotic stress
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Barbara Kacziba, Ádám Szierer, Enikő Mészáros, Andrea Rónavári, Zoltán Kónya, Gábor Feigl
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Submitted

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