Thiosulfate stimulates growth and alleviates silver and copper toxicity in tomato root cultures

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Abstract

The influence of supplemented thiosulfate ($S_2O_3^{2-}$) as well as a complex of either Ag^+ or Cu^{2+} with $S_2O_3^{2-}$ in the culture medium on proliferating root cultures of tomato (*Solanum lycopersicum*) was investigated. The presence of 10–300 µM sodium thiosulfate ($Na_2S_2O_3$) in half-strength Murashige and Skoog (MS) basal salt medium promoted root elongation and proliferation of lateral roots. Growth was enhanced by 1–2 µM $AgNO_3$, but was completely arrested at 5 µM $AgNO_3$; moreover, growth inhibition was elicited by dissolved silver (Ag^+) and by silver in silver precipitate particles. Root elongation was also inhibited by 50 µM $CuSO_4$ supplemented to the basal medium. Roots subjected to either $AgNO_3$ or $CuSO_4$ growth inhibiting treatments were unable to recover following transfer to medium lacking either Ag^+ or Cu^{2+}. When the basal medium was supplemented with either silver or copper in the form of silver thiosulfate complex or copper thiosulfate complex, root cultures continued to elongate and proliferate, thus either completely alleviating or diminishing the inhibitory effects of Ag^+ and Cu^{2+}, respectively. It was concluded that tomato roots sensed and responded to $S_2O_3^{2-}$, hence root proliferation could be promoted by adding $Na_2S_2O_3$ to the medium. Moreover, a complex of Ag^+ with $S_2O_3^{2-}$ detoxified dissolved Ag^+ and prevented the generation of toxic silver particle precipitates. Consequently, silver thiosulfate was superior to $AgNO_3$ in enhancing root culture. Finally, a complex of Cu^{2+} with $S_2O_3^{2-}$ ligand reduced toxicity of Cu^{2+} to root cultures of tomato.

Keywords Copper – Root development – Silver – *Solanum lycopersicum* – Thiosulfate sensing – Toxicity

Abbreviations *BM*
Basal medium
- *CuTS*

Copper thiosulfate
- *LR*
Lateral root
- *MES*
2-(N-morpholino) ethanesulphonic acid
- *MS*
Murashige and Skoog medium
- *PRS*
Primary root segment
- *STS*
Silver thiosulfate

Introduction

Sulfur is an essential element for both prokaryotic and eukaryotic cells. Plant cells take up sulfur from the external environment mostly as sulfate ion. Bacteria take it up as sulfate or thiosulfate compounds, and thiosulfate assimilation in bacteria has been extensively studied (Ghosh and Dam 2009). Although fragmented information can be found on thiosulfate in plants, reviews and research articles on the biochemistry and molecular biology of plant sulfur metabolism scarcely refer to it, reflecting a lack of knowledge on its importance to cell metabolism and to regulation of plant growth (Hell et al. 2010; Yi et al. 2010; Mugford et al. 2011).

Plant tissue cultures are nourished with sulfate salts, and they are provided with thiosulfate only exceptionally, when silver thiosulfate (STS) complex is added to the medium. Recently, Steinitz et al. (2010) found that supplementation of sodium thiosulfate ($Na_2S_2O_3$) to the medium markedly stimulates development in *Corymbia maculata* cultures, indicating that the thiosulfate ($S_2O_3^{2-}$) component of STS contributes to culture growth. However, whether $S_2O_3^{2-}$-dependent stimulation of organogenesis is a phenomenon unique to *C. maculata* or is valid for other plant species as well remains an open question.

Silver (Ag^+, dissolved $AgNO_3$) reacts with various compounds such as lipopolysaccharides, amino acids, proteins, RNA, and DNA to form silver nanoparticles. Dissolved Ag^+ and silver nanoparticles bond with multiple critical biotic receptors and therefore are generally highly cyto- and genotoxic to unicellular and multicellular organisms, including plants (Ratte 1999; Ma et al. 2010; Park et al. 2010; Yin et al. 2011). Plant cells are also sensitive to low, non-toxic concentrations of Ag^+ at which the metal ion interferes with ethylene and auxin signaling and thereby alters, for instance, root development (Strader et al. 2009; Zhao and Hasenstein 2009). Accordingly, silver has been successfully utilized over the last four decades to regulate development in tissue culture, including root induction and growth (Kumar et al. 2009; Kanwar et al. 2010; Liu

and Pijut 2010; Sridevi et al. 2010; Steinitz et al. 2010; Parimalan et al. 2011). Despite the relative abundance of silver toxicology literature, reports on silver's detrimental impact on plant tissue culture are lacking. De Klerk et al. (1999) and Steinitz et al. (2010) suggested that $S_2O_3^{2-}$ could decrease silver toxicity, but this proposal was advanced with no supporting data because, as far as we know, there are no reports documenting STS being less toxic than $AgNO_3$ to *in-vitro* plant cultures.

Copper (Cu^{2+}), an essential micronutrient, may become phytotoxic when present in excess in the growth medium (Gori et al. 1998; Kartosentono et al. 2002). Exposure of plants to excess Cu^{2+} generates oxidative stress, leading to cellular damage generated by reactive oxygen species (ROS), elevations in H_2O_2, and significant DNA impairment (Lequeux et al. 2010; Cuypers et al. 2011; İşeri et al. 2011). Given that copper undergoes complexation with organic compounds that could modify its toxicity (Jung et al. 2003), and since copper can form a copper thiosulfate (CuTS) complex (Fischmann et al. 2008), we examined the potential of $S_2O_3^{2-}$ for decreasing the detrimental properties of copper in root cells.

A root culture system, allowing growth and propagation through repeated segmentation of roots and subculture in medium without exogenous growth regulators (White 1934), was used. We determined the ability of roots detached from the shoot to respond to $S_2O_3^{2-}$ by modified growth and assessed the capacity of $S_2O_3^{2-}$ to decrease deleterious effects of Ag^+ or Cu^{2+} on root growth.

Materials and Methods

Plant material and conditions for root growth in culture

Seeds of *Solanum lycopersicum* (L.) Miller line MP1 were surface-disinfected in 1% sodium hypochlorite solution containing a few drops of Tween-20 for 20 min, rinsed with sterile distilled water, and germinated in the dark for 4 days at 29 ± 1°C on filter paper wetted with sterile distilled water in Petri plates (9 cm x 2 cm). Apical root tips from 20 seedlings, 1.5 cm long, were excised and plated for culture initiation in a root propagation medium (RPM). Four days after culture initiation, a single 1.5-cm long apical tip was selected and excised from the fastest growing root. The selected tip was used to establish a clone of primary roots (Fig. 1a). Lateral roots appearing on long primary roots were harvested and used as new primary root tips in subculture. Repeated harvest of lateral root tips and subculture in fresh RPM was performed at 5- to 7-day intervals. Two types of explants were prepared from primary roots for the experiments: (i) 2.5-cm long apical root segment from which the apical meristem was excised, used for root organogenesis (Fig. 1b-d, Table 1); (ii) 1.5-

cm long apical primary root segment with apical root meristem, used for root elongation assays (Figs. 2-6).

The RPM consisted of 0.5X Murashige and Skoog (1962) (MS) basal salt mixture (Duchefa, Haarlem, The Netherlands), 1.0 mM $MgCl_2$, 1.5 mM $CaCl_2$, Gamborg B5 vitamins (Gamborg et al. 1968) (Duchefa, Haarlem, The Netherlands), 0.4 µM calcium pantothenate, 0.4 µM biotin, 60 mM sucrose, 1.17 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), and 60 mM sucrose. The pH (5.8) was adjusted after addition of 5 g l^{-1} Phytagel, before autoclaving at 120 kPa for 20 min at 121°C. Filter-sterilized vitamins were added to the cooling autoclaved medium. Sucrose, MES, Phytagel, and calcium pantothenate were obtained from Sigma Co. (St. Louis, MO, USA). The propagation culture was grown in Petri plates (14 cm x 2 cm) in a vertical position, with gravity-oriented root tips (Fig. 1a), in the dark at 29 ± 1°C.

Experiments on organogenesis (Table 1) and elongation (Figs. 2-6) were conducted in liquid media. Root organogenesis medium was the same as RPM but without the added 1.0 mM $MgCl_2$ and 1.5 mM $CaCl_2$ and without Phytagel. Root elongation medium had the same composition as root organogenesis medium but with a reduced sugar level of 30 mM sucrose. The pH (5.8) was adjusted before sterilization. Liquid media were filter-sterilized with a 250-ml polysterene vacuum filter bottle system having a cellulose acetate membrane with 0.22-µm pores (Corning Inc., Corning, NY, USA). The following supplements were examined for their influence on root growth: 1-20 µM $AgNO_3$, 1-20 µM STS, 10-300 µM $Na_2S_2O_3$, 10-300 µM Na_2SO_4 and 1-50 µM $CuSO_4$; the concentrations applied are specified for each experiment. Small volumes of stock solutions of the supplements were filter-sterilized in a 0.22-µm pore syringe-driven filter unit (Millex-GV, Millipore Corporation, Bedford, MA, USA). Unless otherwise specified, $AgNO_3$, STS, $Na_2S_2O_3$, Na_2SO_4 and $CuSO_4$ stock solutions were filter-sterilized before they were added to the filter-sterilized root organogenesis medium or root elongation medium.

Liquid medium (10 ml) was poured into a 125-ml Erlenmeyer flask, and a single explant was cultured per flask. Flasks closed with an air-permeable paper fiber Steristopper (model 22K, Heinz Herenz, Hamburg, Germany) were placed on a rotary shaker at 100 rpm (G10 Gyrotory Shaker, New Brunswick Scientific, Edison, NJ, USA). Liquid cultures were exposed to 10 µmol m^{-2} s^{-1} continuous fluorescent white light (Osram 36W10), at 25 ± 1°C.

Preparation of STS and CuTS

Ready-to-use stock solutions of 0.1 M $AgNO_3$, 0.1 M $Na_2S_2O_3$ and 0.1 M $CuSO_4$ were stored in the dark until STS or CuTS preparation. STS was prepared before use by pouring $AgNO_3$ stock solution into stirred $Na_2S_2O_3$ stock solution at a 1:4 molar ratio of silver to thiosulfate. Unless otherwise indicated, CuTS was

prepared before use by pouring $CuSO_4$ stock solution into stirred $Na_2S_2O_3$ stock solution at a 1:5 molar ratio of copper to thiosulfate.

Experimental design and data analysis

Elongation (mm) of control roots incubated in a basal medium was considered 100% elongation. The ratio "elongation (mm) in treated roots:elongation (mm) in control roots" was used to calculate the relative elongation of treated roots. Elongation of treated roots was expressed in Table 1 and in Figs. 2-6 in percent relative to control roots.

Experiments consisted of five roots (five Erlenmeyer flasks) per treatment per experiment. Independent experiments were repeated at least four times on different dates. The mean value of a treatment in an independent replicate was calculated, and data representing the mean value of these replicates were subjected to ANOVA. The data shown represent the mean ± SE. Differences between mean values were assessed by Duncan's multiple comparison test or, as specified in the Results, means were compared by *t*-test (least significant difference, LSD) and were considered significant at $P \leq 0.05$.

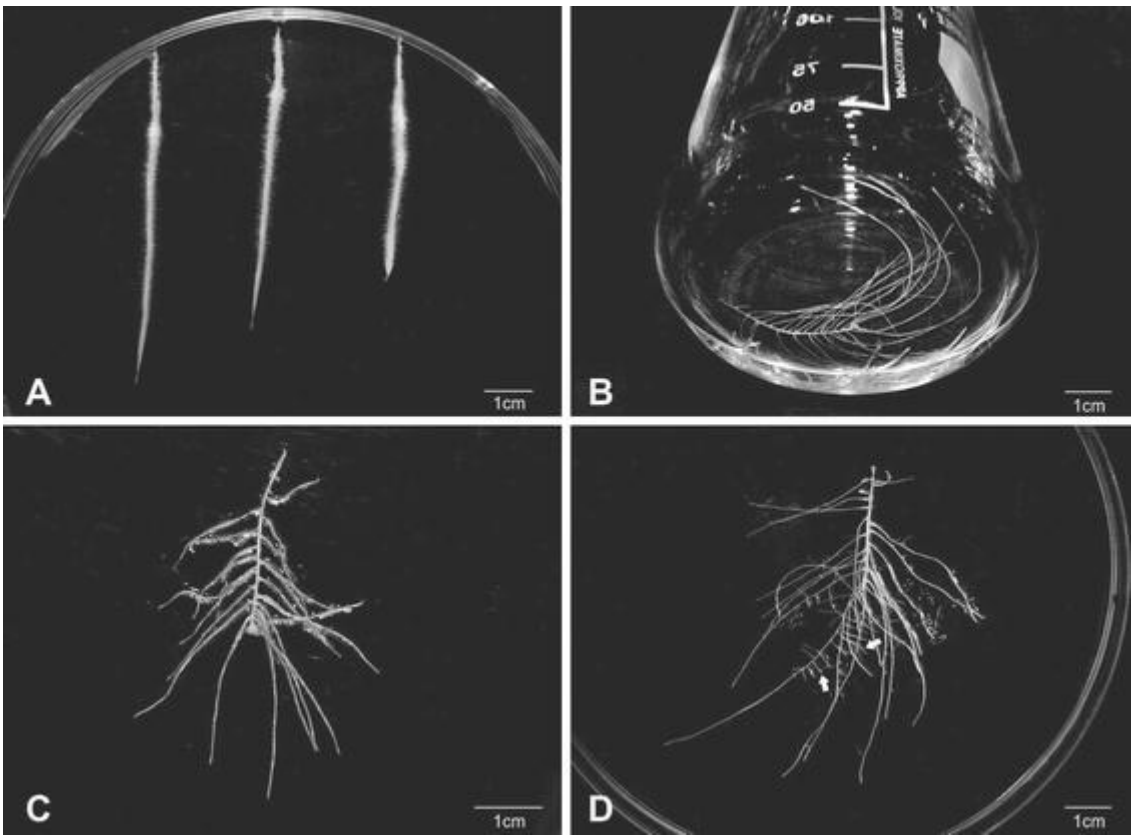

Fig. 1 *Solanum lycopersicum* root culture. **a** Elongating primary roots grown vertically in root propagation medium gelled with Phytagel. **b** Decapitated primary root with lateral roots, incubated in liquid root organogenesis medium in an Erlenmeyer flask. **c** Primary root with first-order lateral roots grown in liquid root organogenesis medium (control). **d** Primary root with first- and second-order lateral roots (*arrows*) grown in liquid root organogenesis medium supplemented

with 10 µM $Na_2S_2O_3$. (**c**–**d**) Roots are shown 7 days after decapitation of the apical meristem from the bottom pole of the primary root. *Bar* = 1 cm

Results and discussion

Root organogenesis

Root morphogenesis was evaluated in root cultures grown on one of the following three media: a Murashige and Skoog (MS) basal medium (BM) used as a control, BM supplemented with either $Na_2S_2O_3$ or Na_2SO_4, each provided at equimolar concentrations. Three morphological indices of root organogenesis were recorded: length of first-order lateral roots (LRs) generated on the primary root segment (PRS), number of first-order LRs with second-order LRs per PRS, and total number of second-order LRs found on a PRS (Table 1).

Table 1 Development of *Solanum lycopersicum* root culture modulated by $Na_2S_2O_3$ or Na_2SO_4 medium supplement

Concentration (µM)	**0**	**10**		**100**		**300**	
Supplement	**Control**	**$Na_2S_2O_3$**	**Na_2SO_4**	**$Na_2S_2O_3$**	**Na_2SO_4**	**$Na_2S_2O_3$**	**Na_2SO_4**
Elongation of 1st order LR (%)	100 ± 6 c	123 ± 6a	112 ± 4b	133 ± 9a	101 ± 1c	124 ± 7a	88 ± 6c
No. of 1st order LR with 2nd order LR per PR	6 ± 1.3c	16 ± 0.6 a	10 ± 0.8 b	15 ± 2.6 a	10 ± 1.3b c	14 ± 1.9 a	8 ± 1.9b c
Total No. of 2nd order LR per PR	19 ± 4c	80 ± 11a	39 ± 5b	86 ± 19a	40 ± 14b	86 ± 18a	23 ± 3c

Following apical meristem excision, primary root (*PR*) segments were incubated in a liquid medium composed of 0.5X MS salts, Gamborg B5 vitamins, 0.4 µM calcium pantothenate, 0.4 µM biotin, 60 mM sucrose, 1.17 mM 2-(*N*-morpholino) ethanesulphonic acid (*MES*), and the indicated $Na_2S_2O_3$ or Na_2SO_4 concentrations. Development of 1st and 2nd order lateral roots (*LRs*) was determined 7 days after excision of the PR apical meristem. Elongation of roots incubated in a control basal medium without supplement was defined as 100%, and elongation of roots treated with $Na_2S_2O_3$ or Na_2SO_4 is expressed in percent of the control reference. Values represent the means ± SE of four independent experiments. Mean values followed by the same letter within a row are not significantly different at 0.05 probability level

Sodium thiosulfate enhanced elongation of first-order LRs by 23 to 33% over the control at all concentrations employed, whereas only 10 µM Na_2SO_4 led to a minor length increment (12%) (Table 1). The number of first-order LRs bearing second-order LRs increased significantly at all $Na_2S_2O_3$ concentrations, but to a lesser extent at 10 and 100 µM Na_2SO_4. The total number of second-order LRs generated per PR explant was quadrupled by $Na_2S_2O_3$ treatment but

only doubled by 10 and 100 µM Na_2SO_4, and remained unchanged in cultures exposed to 300 µM Na_2SO_4, relative to controls. Overall, Table 1 displays two main observations: (1) roots sensed and responded to $S_2O_3^{2-}$ in the medium; (2) at a given supplement concentration and for a given examined developmental trait, the extent of the response was always highest in medium with $Na_2S_2O_3$, indicating that the cells discerned $S_2O_3^{2-}$ from SO_4^{2-} while the added Na^+ played no detectable role.

Two differences between tomato and *C. maculata* roots in their response to $S_2O_3^{2-}$ should be noted: first, the reaction of tomato roots with regard to root elongation and number of LRs formed was nearly saturated at 10 µM $Na_2S_2O_3$ (Table 1) whereas in *C. maculata*, saturation was reached at one order of magnitude higher concentrations (150 µM $Na_2S_2O_3$ for root elongation and 200 µM $Na_2S_2O_3$ for LR generation, see Table 3 in Steinitz et al. 2010). Second, the number of second-order LRs in tomato was fourfold at most that of LRs in the control (Table 1), whereas in *C. maculata*, the increase was almost 13 times that of control (see Table 3 in Steinitz et al. 2010). Genotypic differences between species could underlie quantitative differences in sensitivity and extent of response to $S_2O_3^{2-}$. In addition, the present and former studies were conducted in very different systems: in *C. maculata*, stimulation of root elongation and generation of LRs occurred in adventitious roots attached to a shoot, i.e. roots grew in a whole-plant context and benefited from exchange of signals and growth factors with the shoot. Similar circumstances supporting root development were absent from the tomato root culture used here.

It is proposed that facilitation of root elongation and lateral root formation in a medium supplemented with 10–100 µM $S_2O_3^{2-}$, on background of 820 µM SO_4^{2-} of the basal medium (Table 1), is not due to amendment of a sulfur or sulfate nutrient deficiency of the basal medium. Rather, roots sensed $S_2O_3^{2-}$ and responded to it by alterations in growth rate and development. Due to a lack of information on uptake, and on molecular and metabolic assimilation of $S_2O_3^{2-}$ in plant cells, the pathways ultimately leading to the observed augmentation of growth by $S_2O_3^{2-}$ remain obscure. However, the clear stimulation of LR generation by $S_2O_3^{2-}$ implies that multiplication of roots in culture can be significantly enhanced by adding $Na_2S_2O_3$ to the root propagation medium. This finding could be of benefit for root or cell cultures systems utilized for production of high-value metabolites (e.g. Weathers et al. 2010). The option to facilitate and improve culture performance with a $S_2O_3^{2-}$ medium supplement certainly warrants further investigation with other species and different culture systems.

Effects of $AgNO_3$ and STS on root growth

Results regarding the impact of the chemical formulation of silver ($AgNO_3$ vs. STS) on elongation of primary roots are presented in Fig. 2. Root elongation was accelerated by 1 and 2 μM silver administered as either $AgNO_3$ or STS. However, increasing $AgNO_3$ concentration to 5 μM or higher caused severe suppression of elongation. Moreover, roots browned or blackened within a day of culture initiation in media with a 5 μM or higher concentration of $AgNO_3$. Roots incubated in media with 5 to 20 μM silver as STS elongated to the same extent as roots incubated in media with 1 to 2 μM STS or $AgNO_3$, and their color remained white, similar to control roots grown in medium without silver supplement.

Roots that were first incubated for 1 week in a medium with 5 to 20 μM $AgNO_3$ and then transferred for 7 days to a BM without supplemented silver remained brown-black and did not grow at all (data not shown). Complete inability to recover and regain elongation indicated irreversible poisoning and functional impairment of the roots during incubation in media with 5 to 20 μM $AgNO_3$. Results (Fig. 2) demonstrated unequivocally that complexation of silver with thiosulfate ligand very effectively prevents acute silver toxicity, and root-elongation ability was fully preserved. To the best of our knowledge, this is the first study presenting evidence for STS being less toxic than $AgNO_3$ to in vitro plant tissue cultures. This finding is in line with previous studies showing that Ag^+ can lead to cell death (De Jong et al. 2002), and that Ag^+ toxicity decreases with formation of the STS complex (Hiriart-Baer et al. 2006; Ratte 1999; Rose-Janes and Playle 2000).

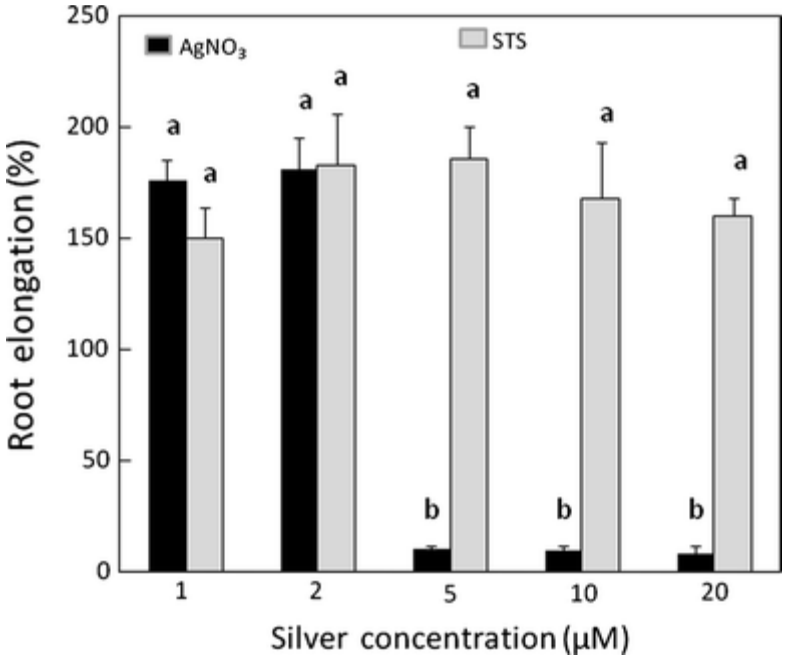

Fig. 2 Inhibition or stimulation of root elongation elicited by $AgNO_3$ and silver thiosulfate complex (STS) medium supplements. Elongation of control roots,

incubated in basal medium (BM) without silver supplement, was defined as 100%. Elongation of silver-treated roots was expressed relative to elongation of control roots. Root length was measured 7 days into the assay. *Columns* and *bars* represent mean ± standard error (SE) of four independent experiments. Pairs of means were compared for a given silver concentration. Same letters in a pair are not significantly different at α = 0.05 using the LSD test

Since basal salts and vitamin mixtures for tissue culture media contain chloride (Gamborg et al. 1968; Murashige and Skoog 1962), AgCl precipitates are formed as a result of the addition of $AgNO_3$ to the medium (Steinitz et al. 2010). Sterilization of a liquid medium with $AgNO_3$ by filtration removes silver precipitate particles larger than the pore size of the filter and consequently, reduces the total amount of silver (i.e. dissolved Ag^+ plus silver precipitate) remaining in the medium. If, however, filter-sterilized $AgNO_3$ stock solution is added to a medium that was filter sterilized separately, precipitation will occur and the initial total amount of silver will be retained. It is therefore argued that a culture's response to silver could depend on the procedure of filter sterilization during medium preparation. Furthermore, given that precipitates do not form when silver is provided as STS (Steinitz et al. 2010), the extent of a culture's response to STS should remain unchanged by filter sterilization procedure. In Fig. 3, results of an experiment examining these premises are shown. Roots were treated with 5 μM silver as either $AgNO_3$ or STS (toxic and non-toxic silver treatment, respectively). For each silver formulation, two media were prepared: (1) silver stock solutions (of $AgNO_3$ or STS) and BM were filter-sterilized separately before the supplement was added to the medium; (2) filter sterilization was performed after addition of the supplement to the medium.

Growth was only drastically inhibited when roots were incubated in the medium made up of separately filter-sterilized $AgNO_3$ stock solution and BM (Fig. 3). In contrast, in medium filter-sterilized after addition of $AgNO_3$, root elongation was permitted and even promoted, suggesting that the total amount of silver remaining after filtration and partial removal of silver precipitate particles was below the critical level required to decrease or block growth. In STS media, root elongation was unaffected by sterilization procedure, because in the absence of silver particles, silver concentration in the medium remained unchanged.

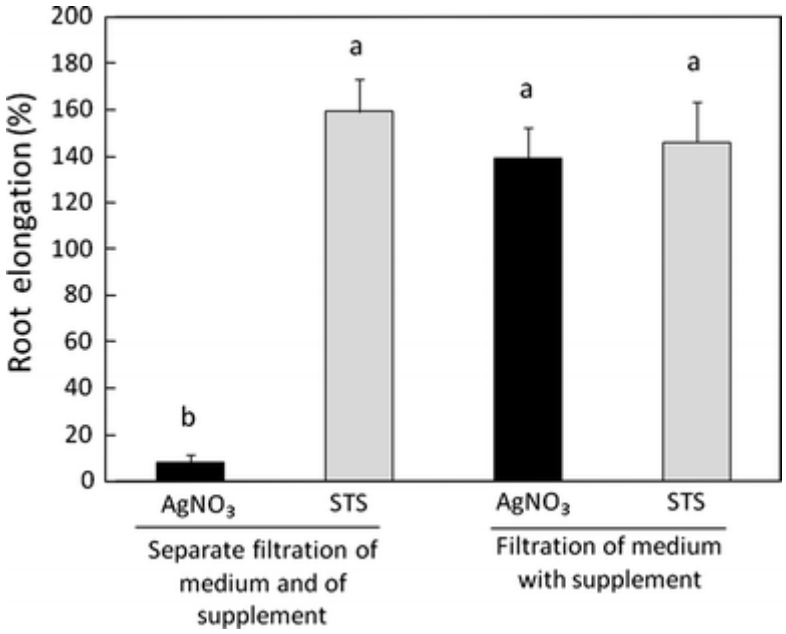

Fig. 3 Root elongation in media with 5 μM $AgNO_3$ or 5 μM silver thiosulfate (STS), as influenced by medium filter-sterilization protocol. (1) Silver stock solutions and basal medium (BM) filter-sterilized separately before addition of the supplement to the medium. (2) Filter sterilization of the medium including silver supplement. Root length was measured 7 days into the assay. Elongation of control roots in BM without supplement was defined 100%. Elongation of silver-treated roots was expressed relative to elongation of control roots. *Columns* and *bars* represent mean ± SE of four independent experiments. Columns with the same letter were not significantly different by Duncan's multiple comparison test ($P \leq 0.05$)

Two inferences emerge from Fig. 3: (1) silver precipitate particles are toxic to roots, in agreement with recent reports on biotoxicity of silver particles (Navarro et al. 2008; Johnston et al. 2010; Kumari et al. 2010; Ma et al. 2010). According to chemical studies, silver in particle form can be slowly released over time as Ag^+ to the aqueous solution (Kittler et al. 2010; Liu et al. 2010), a phenomenon that presumably underlies the particle toxicity observed in the present work; (2) a chemical change during complexation with $S_2O_3^{2-}$ leads to loss of the toxic potency of dissolved silver (Fig. 3), and concomitantly avoids generation of toxic silver precipitates (see Steinitz et al. 2010). This would explain the efficient facilitation of root elongation observed in STS-treated roots (Fig. 2). The present findings unequivocally show that the detrimental potential of Ag^+ and silver precipitates can be conveniently avoided by using STS instead of $AgNO_3$ as the growth-regulating agent in root culture.

Detoxification of silver by the $S_2O_3^{2-}$ ligand is a property of the chemical system, stemming from the chemical configuration of silver in the complex. Thus, the non-deleterious character of STS is probably not biological system-specific: it is likely to be valid for tissue culture systems other than tomato root culture. A search of the ISI Web of knowledge database set up by Thomson Reuters for the period 1980–2010, consisting of several hundreds of articles reporting on the

use of silver in in vitro plant studies, revealed that in more than 80% of those studies, $AgNO_3$ had been chosen as the silver agent to regulate culture growth. Taken together with the observations made by Steinitz et al. (2010), the present findings on the non-toxicity of STS (Figs. 2 and 3) and the probability of the more general validity of these observations, it is suggested that a preliminary assessment be conducted to clarify which silver formulation better suits a particular culture system before making a final choice for routine use. Use of STS could prevent toxicity and the disappointing results obtained with $AgNO_3$ as a growth regulator.

Impact of copper on root growth

In a preliminary investigation, it was found that 50 µM $CuSO_4$ supplemented to BM impedes root elongation. Roots incubated for 1 week in this medium were subsequently unable to recover growth when transferred to a BM without supplement (data not shown). This indicated that 50 µM Cu^{2+} is highly toxic to root cells, but unlike roots exposed to toxic levels of $AgNO_3$ which turned brown-black, roots treated with toxic levels of Cu^{2+} remained white.

Different structures are established during the generation of CuTS complex (Fischmann et al. 2008). In the absence of any previous information on the consequences of CuTS complex configuration with regard to biological activity in plants, the bioactivity of CuTS generated by mixing 50 µM $CuSO_4$ with $Na_2S_2O_3$ at different molar ratios was assessed (Fig. 4). While growth was virtually blocked by 50 µM $CuSO_4$, elongation suppression decreased with increasing copper-to-thiosulfate ratio, and leveled off when complexes were generated by mixing 50 µM $CuSO_4$ with 150 to 300 µM $S_2O_3^{2-}$. Copper-to-thiosulfate ratios of 1:3 to 1:6 permitted elongation by 70 to 80% over control roots (Fig. 4). A copper-to-thiosulfate molar ratio of 1:5 was used in subsequent experiments.

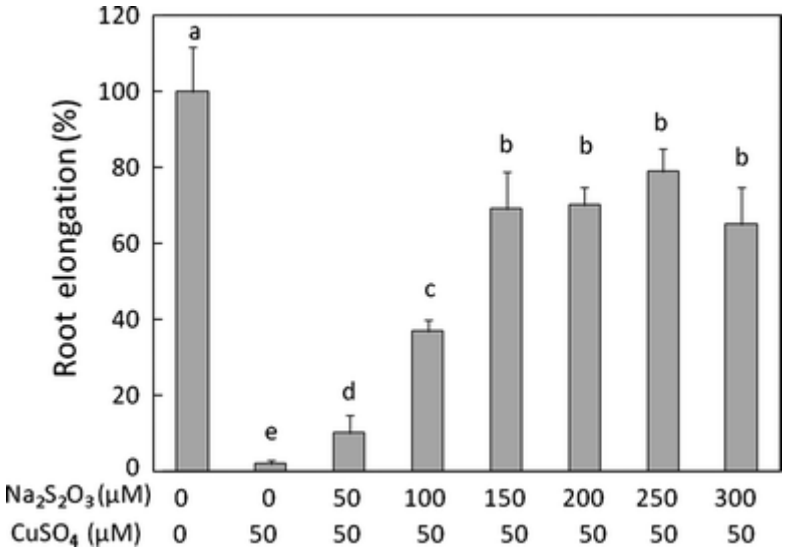

Fig. 4 Root elongation response to supplement of $CuSO_4$ or copper thiosulfate complex (CuTS). Complexes were prepared by mixing $CuSO_4$ with $Na_2S_2O_3$ at the indicated ratios. Elongation of control roots in BM without supplement was defined 100%. Elongation of copper-treated roots was expressed relative to elongation of control roots. Root length was measured 7 days into the assay. *Columns* and *bars* represent mean ± SE of four independent experiments. Columns with the same letter were not significantly different by Duncan's multiple comparison test ($P \leq 0.05$)

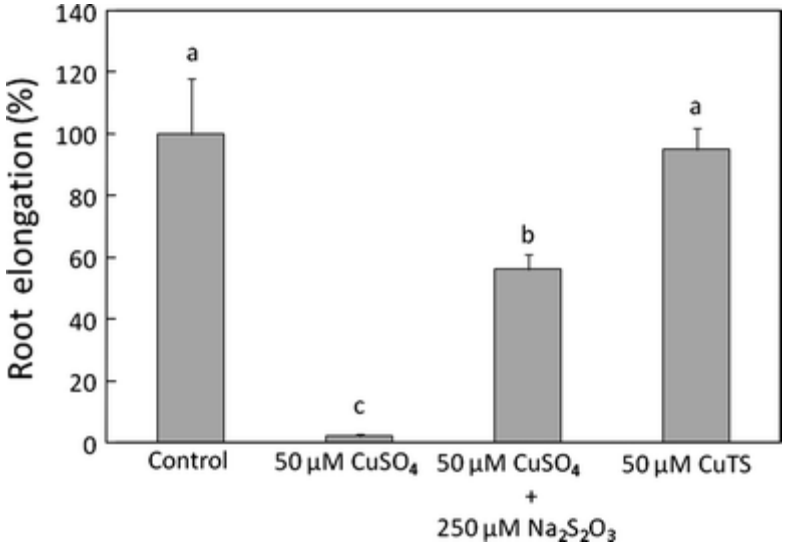

Fig. 5 Root elongation dependency on preparation protocol of media supplemented with $CuSO_4$ or copper thiosulfate complex (CuTS). Stock solutions of $CuSO_4$, $Na_2S_2O_3$ and CuTS were filter-sterilized before adding to filter-sterilized basal medium (BM). Supplements were added to the medium separately ($CuSO_4$, $Na_2S_2O_3$) or were mixed to form the complex before adding to the medium (CuTS). Elongation of control roots in BM without supplement was defined as 100%. Elongation of copper-treated roots was expressed relative to elongation of control roots. Root length was measured 7 days into the assay. *Columns* and *bars* represent mean ± SE of four independent experiments. Columns with the same letter were not significantly different by Duncan's multiple comparison test ($P \leq 0.05$)

We next explored whether it is important to generate the complex prior to its addition to the medium, or if the complexation can occur in the medium following addition of the separate components ($CuSO_4$ and $Na_2S_2O_3$), using the following approach: (1) 50 µM $CuSO_4$ and 250 µM $Na_2S_2O_3$ were added separately, and the medium with supplements was filter-sterilized (Fig. 5, BM + $CuSO_4$ + $Na_2S_2O_3$); (2) $CuSO_4$ and $Na_2S_2O_3$ stock solutions were mixed at a 1:5 molar ratio to generate the CuTS complex; this CuTS stock solution and the BM were then filter-sterilized separately, prior to adding CuTS to the medium (Fig. 5, BM + CuTS); (3) the $CuSO_4$ stock solution was first filter-sterilized, then added to filter-sterilized BM (Fig. 5, $CuSO_4$). Root elongation in media with $CuSO_4$ or CuTS was compared to elongation of roots incubated in control BM. The results revealed that the elongation-blocking effect of 50 µM $CuSO_4$ is largely prevented when using CuTS, provided the complex is generated and filter-sterilized prior to addition to filter-sterilized BM. Suppression of elongation was only partially avoided by a medium sterilized after addition of $CuSO_4$ and $Na_2S_2O_3$ (Fig. 5). Evidently, the extent of copper toxicity alleviation depends on the medium preparation procedure. Perhaps some BM constituent(s) negatively interferes with, and reduces the efficiency of the CuTS complexation reaction, resulting in partial retention of copper's toxicity (Fig. 5, BM + $CuSO_4$ + $Na_2S_2O_3$). In the former (Fig. 4) and subsequent (Fig. 6) experiments, $CuSO_4$ and CuTS stock solutions were first filter-sterilized and only then added to filter-sterilized BM.

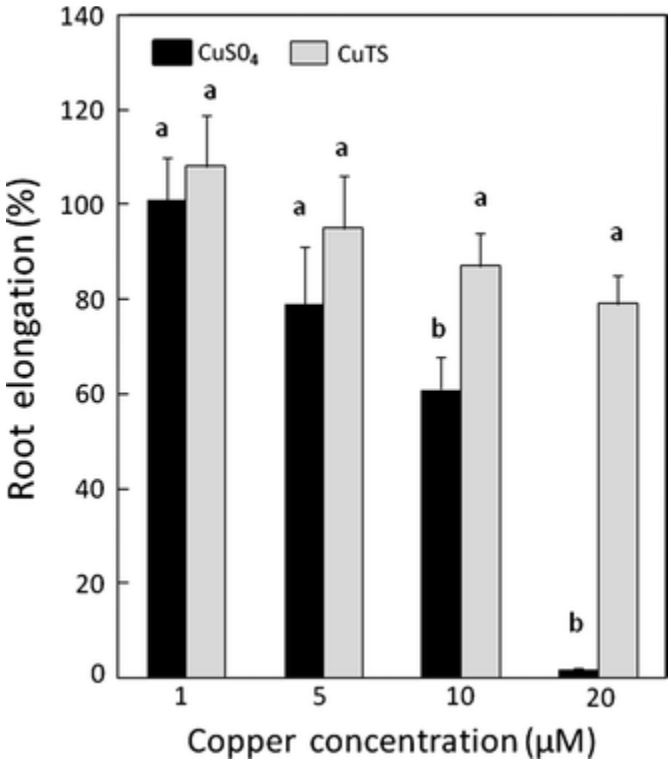

Fig. 6 Root elongation response to copper supplement concentration. Supplements were $CuSO_4$ or copper thiosulfate complex (CuTS). Complexes were prepared by mixing $CuSO_4$:$Na_2S_2O_3$ at a 1:5 molar ratio. Elongation of control roots in basal medium (BM) without supplement was defined 100%. Elongation of copper-treated roots was expressed relative to elongation of control roots. Root length was measured 7 days into the experiment. *Columns* and *bars* represent

mean ± SE of four independent experiments. Pairs of means were compared for a given copper concentration. Same letters in a pair are not significantly different at α = 0.05 using the LSD test

The concentration of $CuSO_4$ used in our experiments (50 μM) is generally higher than the environmentally relevant toxic levels of Cu (~10 μM). The implications of complexation were also assessed with Cu^{2+} at concentrations that cause non-acute toxicity (Fig. 6). Intermediate suppression of root elongation was elicited by 5 or 10 μM $CuSO_4$, and nearly full or partial alleviation of suppression, respectively, was achieved by complexation of Cu with $S_2O_3^{2-}$ (Fig. 6).

Reduction of copper phytotoxicity by extracellular complexation of the metal with different chemical ligands has been reported by Jung et al. (2003), Yruela (2009) and Cui et al. (2010). At least partial detoxification of copper and other metals can also occur by binding the metal ions with specific groups of proteins, such as chelation by phytochelatins, metallothioneins, heat-shock proteins, organic acids, and by sequestration and storage in specific cell compartments (Cobbett and Goldsbrough 2002; Yruela 2009). Attenuation of Cu^{2+} toxicity by $S_2O_3^{2-}$ has been found in *Daphnia magna* neonate cultures (Oh et al. 2008); to the best of our knowledge, our work is the first to show a similar phenomenon in plant cells.

Complexation of metal ions by $S_2O_3^{2-}$ modifies metal uptake rate, increases its bioavailability to cells, and facilitates its translocation through the plant's vascular tissue (Fortin and Campbell 2001). It would be interesting to investigate whether a better remedy for copper deficiency in plants can be achieved by providing CuTS instead of $CuSO_4$ as the copper nutrient. In addition, since $S_2O_3^{2-}$ can be metabolized, it is envisioned that, following uptake, STS or CuTS complexes might decompose. Should STS or CuTS not accumulate but be degraded biochemically, the detoxified state of the metals would have to be retained by substituting thiosulfate with chelation, neutralization, translocation and/or discharge into a subcellular compartment. Such putative processes, and the biochemical fate of $S_2O_3^{2-}$ in cells as part of sulfur metabolism and metal homeostasis, await further investigation.

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