Full title: Copper sensitivity of *nia1nia2noa1-2* mutant is associated with its low nitric oxide (NO) level

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Abstract

Copper (Cu) in excess can disturb the cell redox status maintained by reactive oxygen- (ROS) and nitrogen species (RNS). With the help of the nitric oxide (NO)-deficient *nia1nia2noa1-2* mutant, the role of NO in copper stress tolerance and its relationship with ROS was examined. Under control conditions and also during Cu exposure, the NO level in the cotyledon and root tip of the mutant was significantly lower compared to the wild-type (WT) suggesting the contribution of the nitrate reductase (NR)- and nitric oxide associated 1 (NOA1)-dependent pathways to NO synthesis. The cell viability decrease was more pronounced in the triple mutant and the originally low growth rate was maintained under Cu stress. The endogenous NO level of the mutant was increased by NO donor addition and its cell viability significantly improved suggesting that the Cu sensitivity of the *nia1nia2noa1-2* mutant is directly associated with its low NO content. As the effect of Cu increased ROS formation occurred in WT roots, while the originally high ROS levels of the triple mutant slightly decreased, still remaining significantly higher than those in the WT. In the cotyledons of the triple mutant 5 µM Cu induced ROS production but NO formation failed, while in the WT cotyledons NO but no ROS accumulation was observed. The promoting effect of NO deficiency on ROS production assumes an antagonism between these molecules during Cu stress. Based on the results, it can be concluded that NO contributes to copper tolerance and its deficiency favours for ROS production.

Key words: copper stress, *nia1nia2noa1-2*, nitric oxide, reactive oxygen species

Introduction

Despite its essentiality, copper (Cu) in excess can have several toxic effects in plants. Being a transition metal, it directly catalyzes the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) or hydroxyl radical ($OH^{\cdot}$) leading to oxidative damage of macromolecules and membranes. Moreover, copper can possibly replace other essential metal ions in proteins and it is highly reactive to thiols, therefore Cu homeostasis of plant cells must be precisely controlled (Burkhead et al. 2009). The main reason for Cu toxicity is the disturbance of the cells' redox status, which is maintained by redox active compounds such as reactive oxygen- and nitrogen species and their interactions (Potters et al. 2010). Reactive nitrogen species (RNS) are nitric oxide (NO)-derived radical or non-radical molecules (e.g. peroxynitrite, $ONOO^-$ or S-nitrosoglutathione, GSNO) possessing multiple roles in plant development and stress tolerance (Wang et al. 2013). The central molecule, nitric oxide was found to act in developmental processes such as germination (Liu et al. 2011), in acclimation to abiotic stresses such as chilling (Esim and Atici 2014) or salt (Liu et al. 2014) and during plant-pathogen interactions (Jian et al. 2015). Plant cells respond to heavy metal stress by modifying their NO status, which is strictly regulated by its synthesis, removal and transport (Xiong et al. 2010). The biosynthesis of NO in plants is quite complex since multiple enzymatic and non-enzymatic pathways were evidenced. One of the major enzymes playing a role in NO synthesis is nitrate reductase (NR) and the NR-deficient *nia1nia2* mutant contains lower NO level compared to the wild-type. Its contribution to NO synthesis was observed during e.g. stomatal movements, pathogen interactions, floral development, osmotic stress, auxin-induced lateral root fomation (Desikan et al. 2002, Jian et al. 2015, Kolbert et al. 2008, 2010). The another enzyme playing direct or indirect role in NO production of plant cells is the Nitric Oxide-Associated1/Resistant to Inhibition by Fosfidomycin1 (AtNOA1/RIF1) protein (Gupta et al. 2011). Recently, the *rif1* mutant was isolated, carrying a null mutation in the AtNOA1 locus (At3g47450), and the function of AtNOA1/RIF1 in the expression of chloroplast-encoded proteins was revealed (Flores-Pérez et al. 2008). However, since then the involvement of AtNOA1 in NO synthesis was questioned and the protein was identified as cGTPase (Moreau et al. 2008). In 2010, a plant NOS showing ~45% homology to mammalian one was described in the *Ostreococcus tauri* algae (Foresi et al. 2010), but the existence of a NOS or a NOS-like enzyme in higher plants remained questionable. In order to get more accurate view about the functions of NO biosynthetic pathways and their contribution to NO synthesis in higher plants, Lozano-Juste and León (2010) generated the triple *nia1nia2noa1-2* mutant that is impaired in nitrate

reductase (NIA/NR)- and Nitric Oxide-Associated1 (AtNOA1)-mediated NO biosynthetic pathways and it contains extremly low NO level in their roots.

The main goal of our work was to characterize the NO, ROS production and copper sensitivity of *nia1nia2noa1-2* mutant and to draw conclusions about the involvement of NO in Cu stress responses and its interactions with ROS.

Materials and methods

Plant material and growth conditions

Seven-days-old wild-type (*Col-0,* WT) and *nia1nia2noa1-2* mutant *Arabidopsis thaliana L.* seedlings were used for the measurements. The *nia1nia2noa1-2* triple mutant was created and described by crossing the nitrate reductase (NR)-deficient *nia1nia2* with *noa1-2* mutant by Lozano-Juste and León (2010). The seeds were surface sterilized with 5% (v/v) sodium hypochlorite and transferred to half-strength Murashige and Skoog medium (1% (w/v) sucrose and 0.8 % (w/v) agar) supplemented with 0, 5 or 25 µM $CuSO_4$. The Petri dishes were kept in a greenhouse at a photo flux density of 150 µmol m^{-2} s^{-1} (12/12 day/night period) at a relative humidity of 55-60% and 25 ± 2°C. As an NO scavenger, 50 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid potassium salt (cPTIO) was used. Also, sodium nitroprusside (SNP) as an NO donor was applied at a concentration of 10 µM. These chemicals were added to the nutrient media before the seeds were planted.

Morphological observations

Fresh weights (FW, mg) of 10 whole seedlings were measured using a balance and were expressed as average weight (mg/seedling). Seedling morphology of the WT and the *nia1nia2noa1-2* mutant was observed under Zeiss Axioskope 200-C stereomicroscope (Carl Zeiss, Jena, Germany).

Fluorescence microscopy

Nitric oxide levels in *Arabidopsis* root tips and cotyledons were analyzed by 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). This fluorophore does not react with hydrogen peroxide or peroxynitrite, but it responds to NO donors and/or scavengers, therefore it can be considered as a NO-specific fluorescent probe (Kolbert et al. 2012a). Whole seedlings were incubated in 10 µM dye solution (in 10 mM Tris-HCl, pH 7.4) for 30 min and were washed twice with Tris-HCl (Feigl et al. 2013). Fluorescein diacetate (FDA), a cell-permeable esterase substrate was used for the determination of cell viability in the root tip and in the cotyledons (Harvey et al. 2008). Whole seedlings were incubated in 10 µM dye solution (prepared in MES/KCl buffer, pH 6.15) for 30 min in darkness (Feigl et al. 2013). For the visualization of intracellular reactive oxygen species (mainly H_2O_2, hydroxyl radical, superoxide anion, peroxynitrite) as a general oxidative stress indicator, 10 µM (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester) (CM-H_2DCFDA) was used at 37°C for 15 min, then the samples were washed in 20 min with 2-N-morpholine-ethansulphonic acid/potassium chloride MES/KCl (pH 6.15) buffer. Highly reactive ROS, such as hydroxyl radical or peroxynitrite was detected by incubating the

samples in 10 µM aminophenyl fluorescein solution (APF, prepared in 10 mM Tris-HCl buffer, pH 7.4) for 60 min (Feigl et al. 2013). The specificity of DAF-FM, APF and H_2DCFDA was tested both *in vivo* and *in vitro* (Kolbert et al. 2012a). Investigations were carried out using a Zeiss Axiovert 200M-type inverted-fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with filter set 10 (excitation: 450-490 nm, emission: 515-565 nm). Fluorescent intensities (pixel intensity) were measured on digital images using Axiovision Rel. 4.8 software (Carl Zeiss, Jena, Germany). In case of the meristematic and elongation root zones, the measurements were done within area of circles with 60 µm radii; in cotyledons circles with 120 µm radii were applied. The radii of circles were not modified during the experiments. The selected fluorescent images are representatives of similar results from the 2 repetitions.

Statistical analysis

All experiments were carried out at least two times. In each treatment at least 10-15 samples were measured. Results are expressed as mean ± SE. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P<0.05$) and Duncan's test. In some cases, Microsoft Excel 2010 and Student's t-test was used (*$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$).

Results and discussion

Copper in excess disturbes the NO homeostasis of WT and *nia1nia2noa1-2* plants

Under control conditions, the cotyledon and primary root of *nia1nia2noa1-2* showed significantly reduced NO content compared to the wild-type (Fig 1), suggesting that the largest proportion of NO in *Arabidopsis* seedlings is produced by the NR- and the NOA1-dependent enzymatic pathways. It is known from early works that cotyledons show relatively high NR activity, NR protein and mRNS levels (Beevers et al. 1965, Rajasekhar et al. 1988). Moreover, the participation of NR in NO generation in aerial plant parts was reported, *inter alia*, in *Betula pendula* and *Arabidopsis* (Zhang et al. 2011, Zhao et al. 2009). In the roots, nitrate reductase can considered to be the major enzymatic source of NO (Xu and Zhao 2003). Similarly to our results, Lozano-Juste and León (2010) published that the triple mutant has an extremly reduced NO level in their roots, which proved to be lower than that of the NR-deficient *nia1nia2*. Since the triple mutant possessed a basal NO content, we can not exclude the exsistence of other (even non-enzymatic) mechanisms of NO generation as well. In case of the triple mutant, NO accumulation induced by the low Cu concentration (5 µM) in the cotyledons could not be observed, suggesting the involvement of both enzymatic pathways (NR- and NOA1-dependent) in this process. In roots of WT and triple mutant plants, 25 µM Cu caused the heavy reduction of NO levels; and those levels were comparable in the plant lines. The copper-triggered changes in NO homeostasis showed organ-specificity and concentration-dependence in *Arabidopsis* seedlings, since 5 µM Cu was able to induce NO generation only in the cotyledons, while more serious Cu excess caused significant NO level decrease only in the roots. Indeed, the effects of heavy metals (like copper) on NO levels can be dependent on several factors such as the duration and concentration of the metal treatment applied, the plant species, age etc. (Kolbert et al. 2012b). Earlier we published, that WT *Arabidopsis* shows significant and concentration-dependent copper accumulation in both organs (Pető et al. 2013), which can explain the relevant effects of copper treatments in the root- and shoot system as well.

Nia1nia2noa1-2 shows more pronounced copper sensitivity than the WT

In order to reveal the Cu endurance of the seedlings, fresh weights and cell viability of WT and mutant plants were determined and compared. The control mutant possessing low NO levels showed overall growth reduction (Fig 2A a and c) and remarkably decreased fresh weights (Fig 2B) compared to the WT, which suggest the fundamental role of NO in the

regulation of seedling development (Lozano-Juste and León 2010). Moreover, the low NO containing *nia1nia2* showed smaller stem and root size compared to the WT, which further supports the pivotal regulatory role of NO in plant development (Pető et al. 2011, 2013). Whilst copper exposure decreased the fresh weight of WT seedlings in a non-significant but concentration-dependent manner, the seriously reduced fresh weight of *nia1nia2noa1-2* did not decrease further as the effect of Cu exposure, which means that it maintains its growth even under Cu stress (Fig 2B). The growth maintenance of the triple mutant under Cu stress supposes the lack of its ability to rearrange its means from development to defence, which can make the mutant more susceptible for Cu stress. Indeed, the viability of cotyledon cells decreased in both plant lines as the effect of Cu exposure (Fig 2C); although the viability loss was more pronounced in the triple mutant, since it occurred already in the case of 5 µM Cu. Interestingly, the applied Cu concentrations did not have remarkable reducing effects on cell viability in the primary root tissues, even it enhanced in the elongation zone of the 5 µM Cu-treated WT roots. Possibly, the low Cu concentration could have a positive effect on esterase activity thus fluorescence increased reflecting viability enhancement. Contrary, in the *nia1nia2noa1-2* mutant, the cells of both root zones suffered Cu-induced viability loss (Fig 2D). The intensified loss of viability further supports the Cu sensitivity of *nia1nia2noa1-2* compared to the WT.

Copper sensitivity of the *nia1nia2noa1-2* is associated with its low NO level

Although, our results pointed out the enhanced Cu sensitivity of the triple mutant, the involvement of NO in this phenomenon was needed to be elucidated as well. We applied NO donor (SNP) and scavenger (cPTIO) treatments in order to biochemically modify the endogenous NO content of the WT and the *nia1nia2noa1-2* plants; seedling fresh weight and cell viability in their cotyledons and roots was detected. The cPTIO treatment slightly reduced, while SNP increased the DAF-FM fluorescence indicating NO contents in both organs of both plant lines (Fig 3). In case of the wild type, NO donor prevented Cu-induced FW loss, while cPTIO resulted in a more pronounced decrease of the seedling weight in case of 5 µM Cu. However, cPTIO+25 µM Cu –treated plants showed increased FW (by 40%) compared to plants treated with 25 µM Cu alone (Fig 4A). In the *nia1nia2noa1-2* mutant, NO addition was able to cause ~30% and ~20% increase in FW, respectively, while FW remained ~100% in plants treated with copper alone (Fig 4B). In WT cotyledons, exogenous NO unequivocally intensified the Cu-induced viability loss, while cPTIO had no effect in case of low Cu concentrations and reduced the viability in 25 µM Cu-exposed *Arabidopsis* compared

to plants treated with copper alone (Fig 5A). SNP had no significant effect on viability of the root meristem, but NO elimination by cPTIO resulted in the aggravation of viability loss (Fig 5B). The exogenous NO treatment of Cu-exposed *nia1nia2noa1-2* caused viability improvement in both organs (Fig 5CD), suggesting the direct involvement and promoting role of NO in Cu tolerance. Also, the stress mitigating effect of NO under copper stress was evidenced in *Panax ginseng* where NO treatment reduced cell death and membrane damages (Tewari et al. 2008). In another paper, exogenous NO mitigated Cu stress of tomato by improving plant growth, alleviating oxidative stress and reducing lipid peroxidation (Cui et al. 2010). In general, NO exerts its protecting role against Cu stress not by preventing Cu uptake, rather principally by reducing oxidative damage through the regulation of antioxidant contents and activities (Zhang et al. 2009).

The *nia1nia2noa1-2* mutant shows higher ROS levels under control conditions and also during Cu stress

Being a transition metal, Cu has a great ability to directly induce the formation of different ROS. In order to reveal the effect of the *nia1nia2noa1-2* mutation on ROS levels, we applied two staining procedure to detect the level of highly reactive oxygen radicals (e.g. $ONOO^-$, $OH^\cdot$ and OCl^-) and intracellular ROS (e.g. H_2O_2, $O_2^{\cdot-}$, $OH^\cdot$). The *nia1nia2noa1-2* triple mutant possessed notably higher hROS content compared to the WT in its cotyledons and roots during control circumstances and even under Cu stress (Fig 6AB and E). Also, it has to be mentioned that as the effect of Cu, WT roots showed ROS formation, while in the triple mutant the originally high ROS levels slightly decreased, even so those remained significantly higher than that of the WT. Interestingly, in cotyledons of the triple mutant 5 µM Cu was able to cause ROS accumulation (Fig 6C) but NO formation failed (see Fig 1), while in the WT cotyledons opposing phenomenon was observed: 5 µM Cu triggered NO accumulation (see Fig 1), but it did not cause ROS generation. All these results reflect the promoting effect of NO deficiency on ROS production both in non-stressed and Cu-exposed plants. Antagonism between ROS (H_2O_2) and NO was supposed also in the roots of selenite-treated *Arabidopsis* (Lehotai et al. 2012). The antagonism can originate from direct chemical interactions between ROS and NO and enzymatic or non-enzymatic background mechanisms. Indeed, NO is capable of regulate ROS levels by modifying the activities of antioxidant enzymes such as glutathione transferase, glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase (Polverari et al. 2003) or by inducing the expression of the biosynthetic genes of (Innocenti et al. 2007) or increasing the concentration of antioxidants such as glutathione (Xu

et al. 2010). Question arises, whether the notably elevated ROS content contributes to the developmental defects of the *nia1nia2noa1-2* mutant. Plants with lower ascorbate content and consequently elevated ROS level (*vtc2-1* and *vtc2-3*) showed WT-like root and shoot size, suggesting that ROS levels do not significantly influence the seedling development of *Arabidopsis* (Pető et al. 2013).

Conclusions

The significantly lower NO content of the *nia1nia2noa1-2* compared to the WT suggests that in the cotyledons and roots of *Arabidopsis* seedlings NO is produced mainly by the NR- and the NOA1-dependent enzymatic pathways. The lack of the copper (5 µM)- induced NO accumulation in the cotyledons of *nia1nia2noa1-2* implies the involvement of both enzymatic pathways (NR and NOA1-dependent) in the NO formation as the effect of Cu excess. Presumably, copper-exposed wild-type plants reduce their growth in order to develop defence strategies, while the triple mutant did not show remarkable growth inhibition, which means that it lacks the ability to rearrange its means from development to defence. Indeed, *nia1nia2noa1-2* mutant suffered more intense viability loss under Cu stress, which further supports the increased sensitivity of it. The exogenous NO treatment of Cu-exposed *nia1nia2noa1-2* improved cell viability in both organs suggesting the direct involvement and promoting role of NO in Cu tolerance. The *nia1nia2noa1-2* mutant possessing low NO levels shows high ROS content, which assumes the antagonistic relationship between these molecules under control conditions and even during Cu stress.

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Figure legends

Fig 1 Nitric oxide levels (pixel intensity) in the cotyledons (A) and primary root tips (B) of WT and *nia1nia2noa1-2* mutant treated with 0, 5 or 25 µM Cu for 7 days. Different letters indicate significant difference according to Duncan's test (n=10-15, P≤0.001). (C) Representative fluorescent microscopic images of cotyledons of control and 5 µM Cu-treated WT and mutant *Arabidopsis* stained with DAF-FM DA. Bar= 1 mm.

Fig 2 (A) Representative stereomicroscopic images of 7-days-old WT and *nia1nia2noa1-2* seedlings treated with 0 or 25 µM Cu. a= WT, control; b= WT, Cu-treated; c= *nia1nia2noa1-2*, control; d= *nia1nia2noa1-2*, Cu-treated. Bar= 3 mm. (B) Average fresh weights (mg) WT and mutant *Arabidopsis* seedlings. The lack of significancy was indicated by n.s.= non-significant. Cell viability (pixel intensity, in control%) of cotyledons (C) and primary root tips (D) of WT and mutant *Arabidopsis* treated with 0, 5 or 25 µM Cu. Asterisks indicate significant differences according to Student's t-test (n=10-15, **P≤0.001, ***P≤0.0001). n.s.=non-significant, MZ=meristematic zone, EZ=elongation zone.

Fig 3 Nitric oxide levels (pixel intensity) in cotyledons and root tips of wild-type (WT, A) and *nia1nia2noa1-2* (B) mutant *Arabidopsis* grown on agar plates without (-SNP/-cPTIO) or with 10 µM SNP or 50 µM cPTIO. Different letters indicate significant differences according to Duncan's test (n=10-15, P≤0.001).

Fig 4 Fresh weight (mg/seedling, in control%) of the wild-type (WT, A) and *nia1nia2noa1-2* mutant (B) *Arabidopsis* grown on agar plates without (-SNP/-cPTIO) or with 10 µM SNP (+ SNP) or 50 µM cPTIO (+ cPTIO). The significant differences according to Student's t-test (n=10-15, **P≤0.001, ***P≤0.0001) are indicated.

Fig 5 Cell viability in the cotyledon (A, pixel intensity, in control%) and in the root tip (B) of the WT treated with different copper concentrations without (-SNP/-cPTIO) or with 10 µM SNP (+SNP) or 50 µM cPTIO (+cPTIO). Cell viability in the cotyledon (C) and in the root tip (D) of *nia1nia2noa1-2* treated with different copper concentrations without (-SNP) or with 10 µM SNP (+SNP). The lack of significancy (n.s.) or significant differences according to Student's t-test (n=10-15, **P≤0.001, ***P≤0.0001) are indicated.

Fig 6 The level of highly reactive oxygen species (hROS, pixel intensity, A and B) and intracellular ROS (pixel intensity, C and D) in the cotyledon (A, C) and in the root tip (B, D) of WT and mutant *Arabidopsis* treated with 0, 5 or 25 μM Cu. Different letters indicate significant difference according to Duncan's test (n=10-15, $P \leq 0.001$). (E) Representative fluorescent microscopic images of control and 25 μM Cu-treated WT and *nia1nia2noa1-2* root tips stained with H_2DCF-DA or APF. Root apical meristem (the site of fluorescence measurement) was indicated by an arrow. Bar= 1 mm.

Figure1
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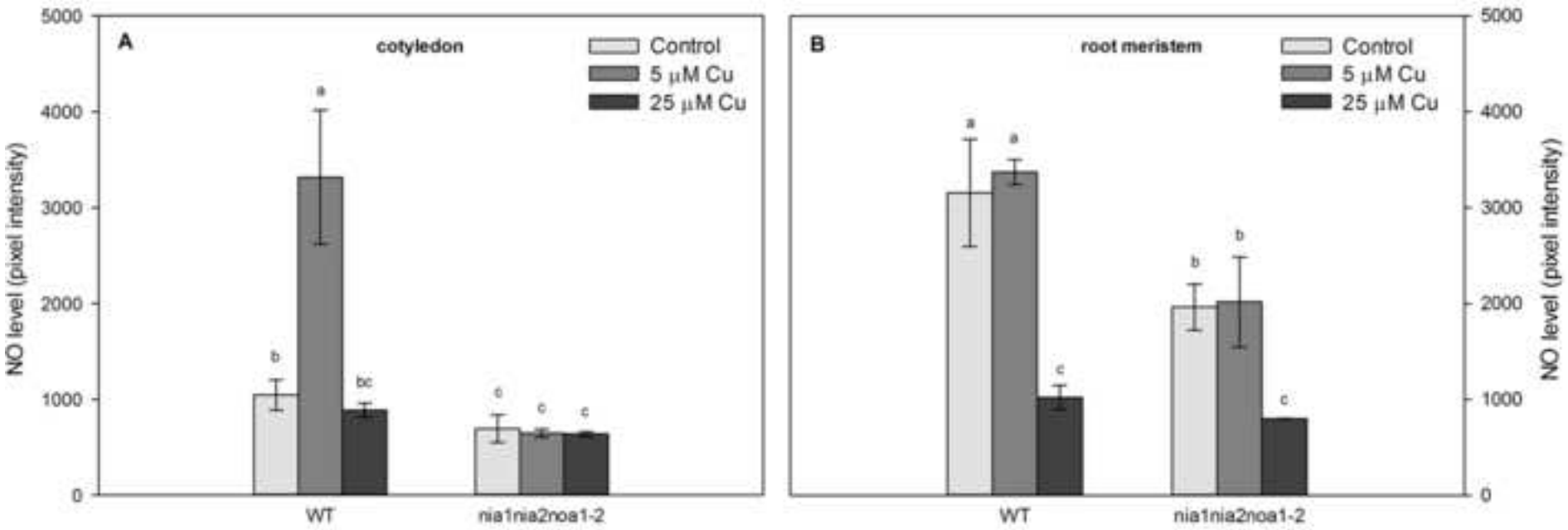

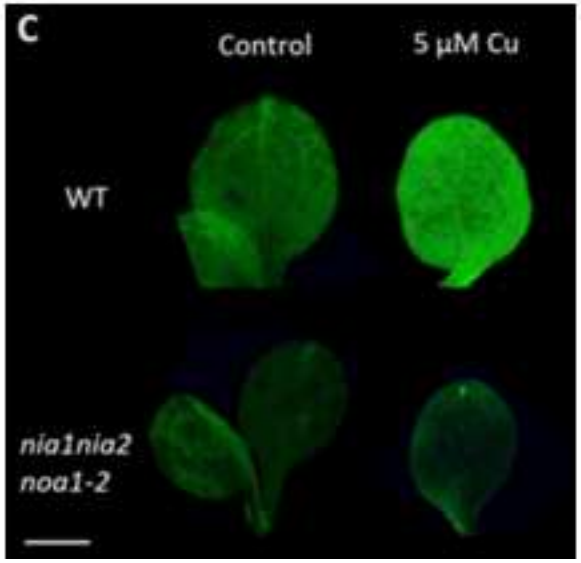

Figure2
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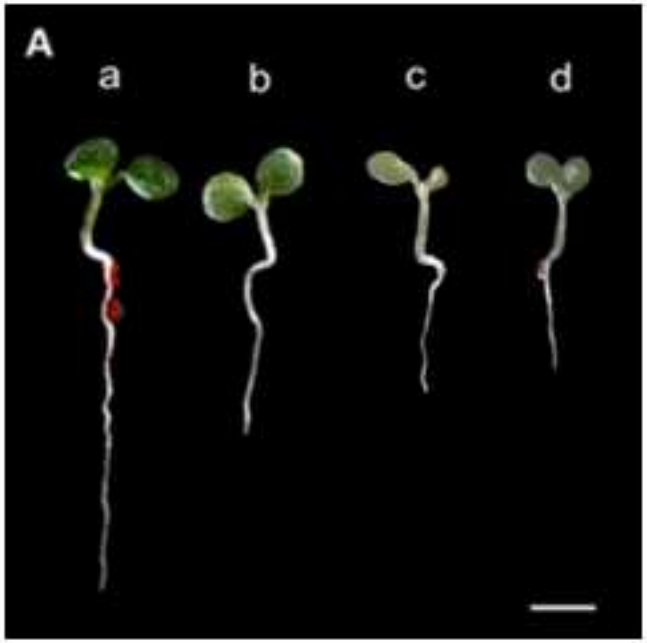

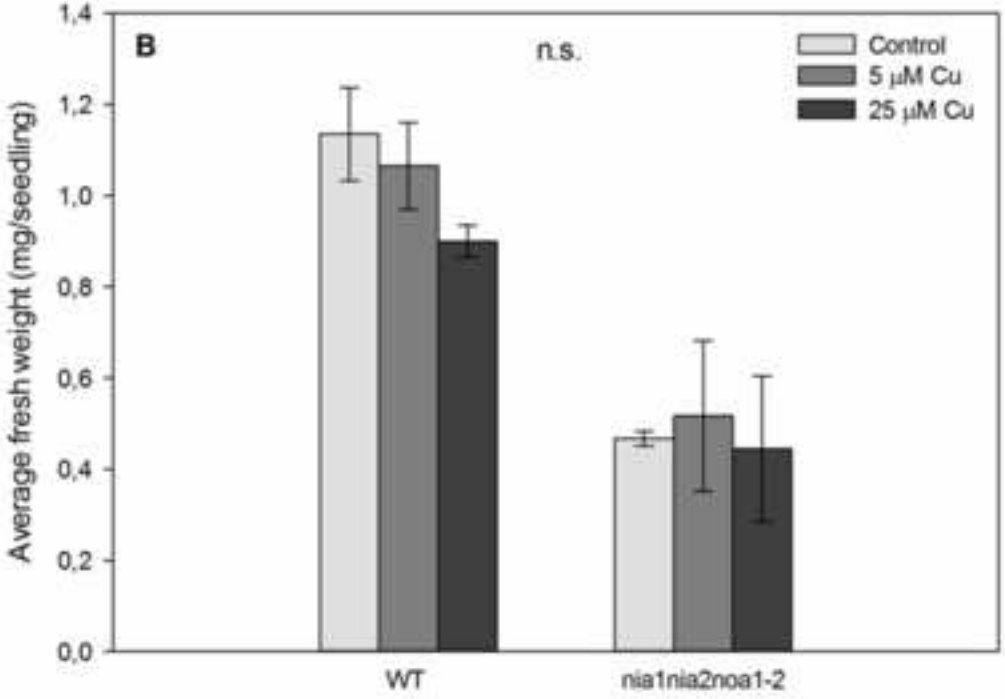

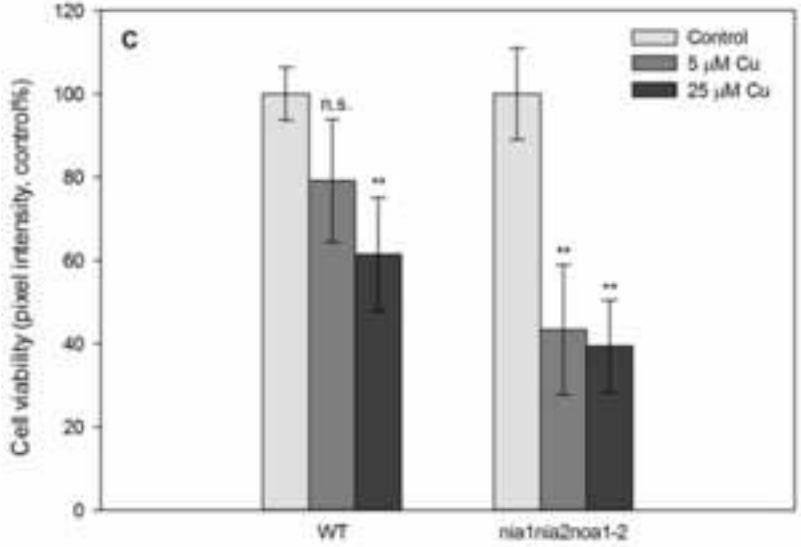

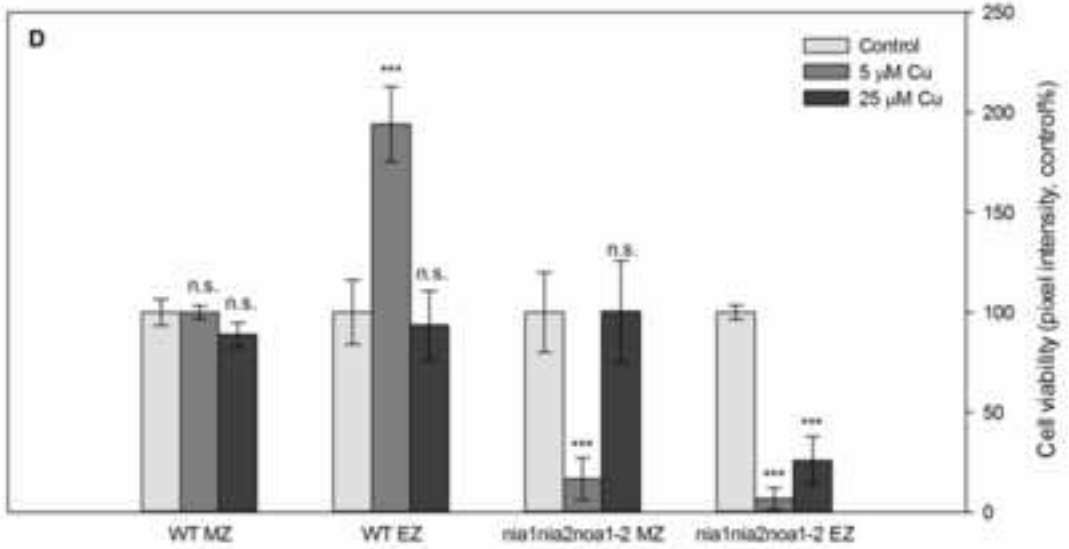

Figure 3
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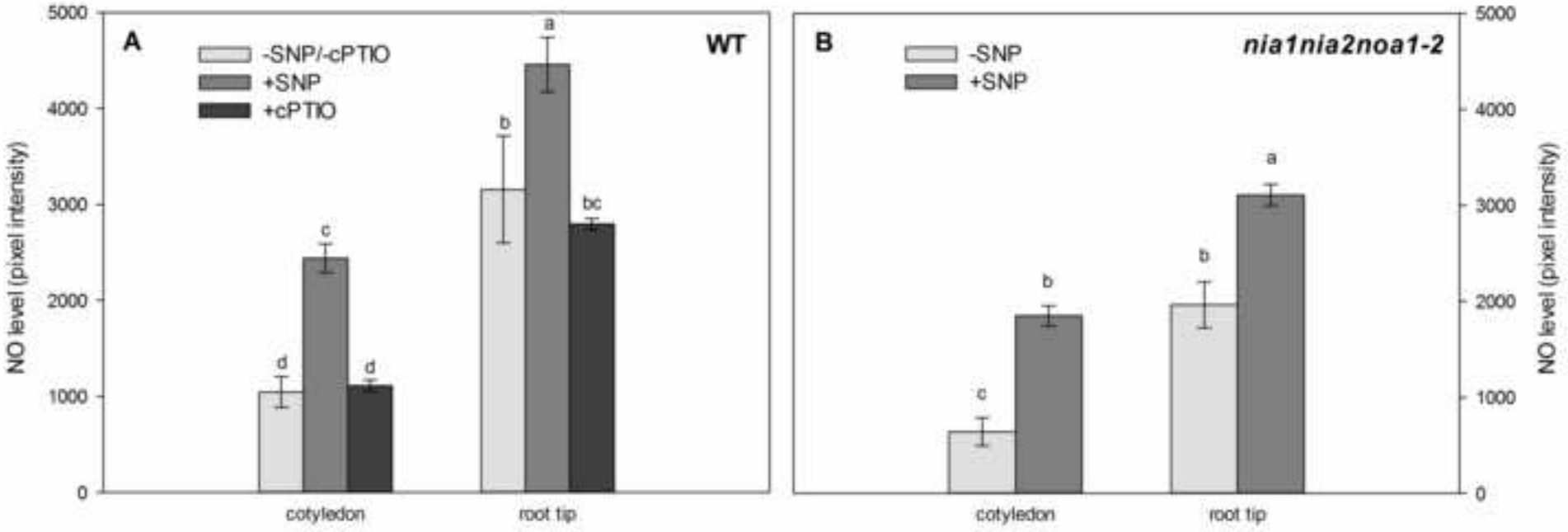

Figure 4

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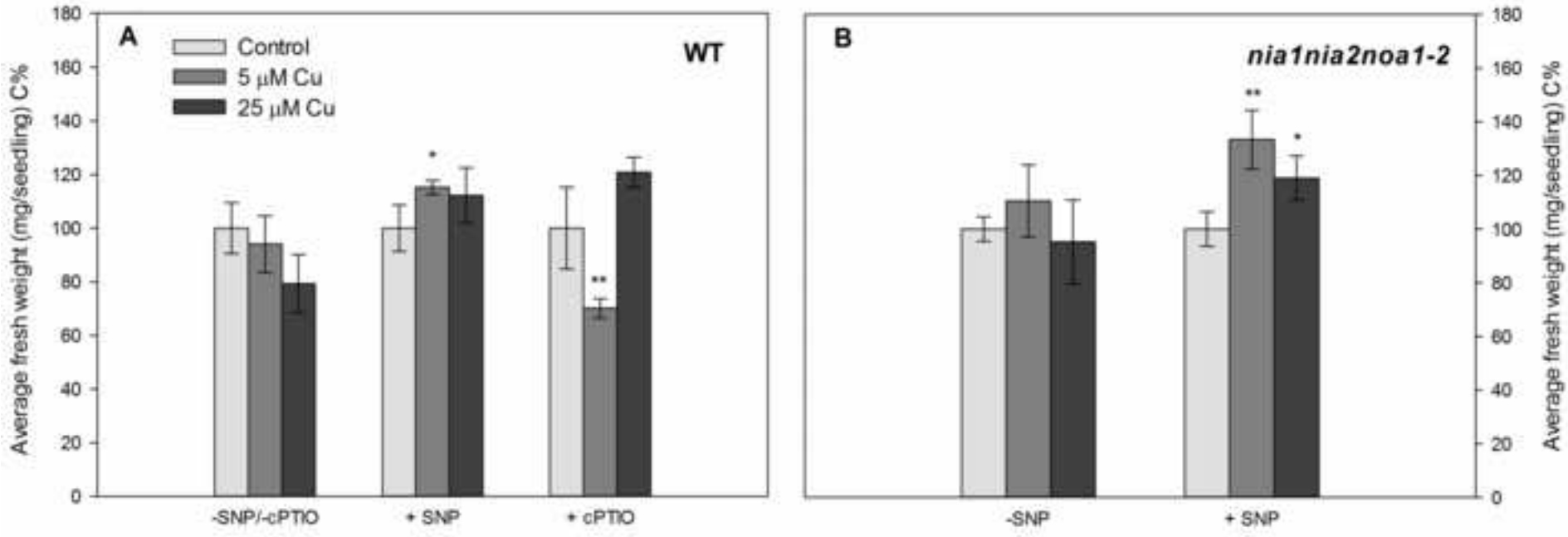

Figure 5
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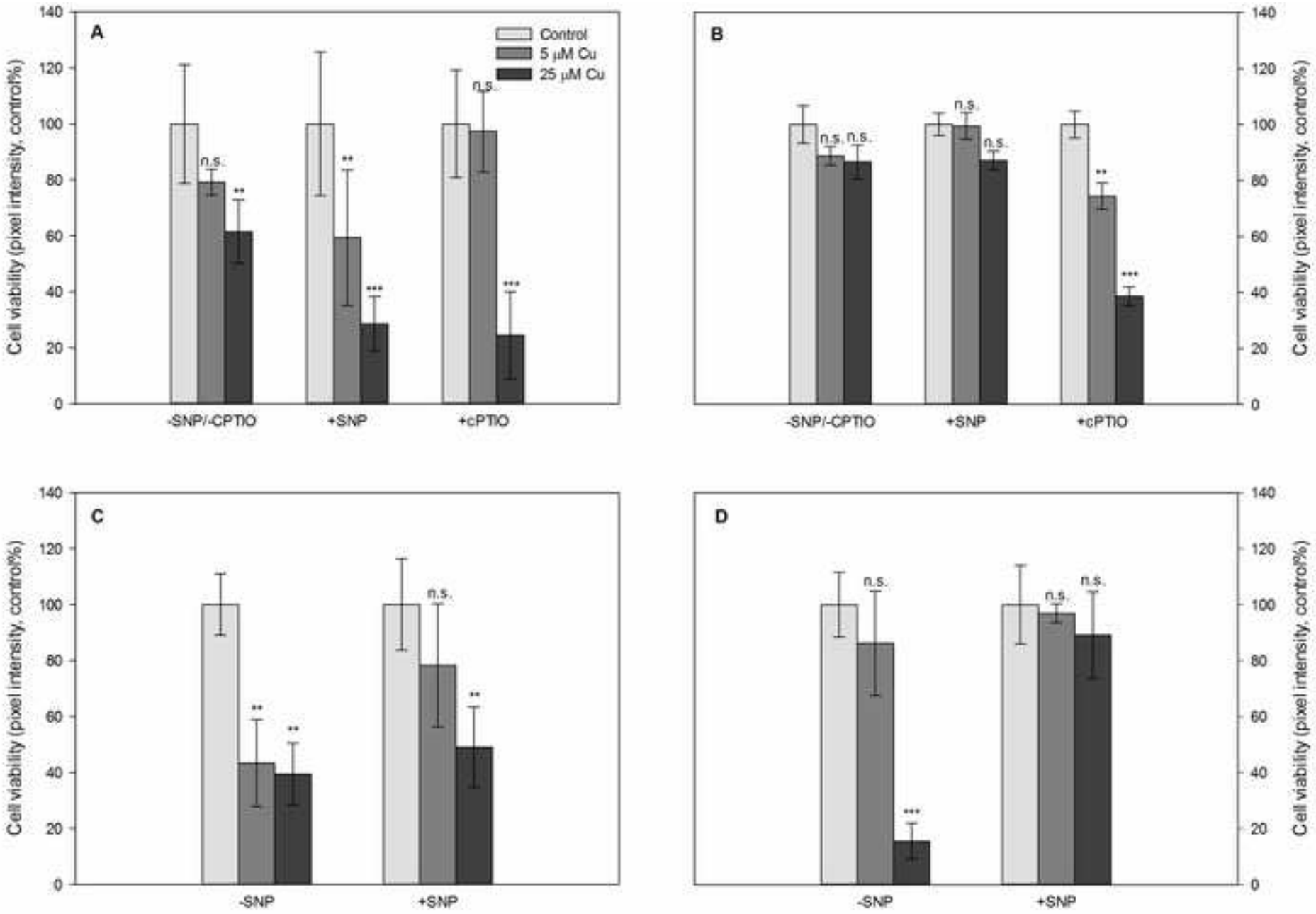

Figure 6

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