Toxicity of Copper oxide nanoparticle on physiology and biochemistry of *Solanum lycopersicum* as evident by growth, photosynthetic pigment content and PS II photochemistry: toxicity alleviation by calcium

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Abstract

Copper oxide nanoparticles (CuONPs) toxicity has greatly affected the environment and particularly plants. It causes rapid changes in plant morphology and physiology; however, the changes can be restored *via* exogenous supplementation of Calcium (Ca). Thus, the present work has been undertaken to elucidate the impacts of Ca supplementation on reducing the CuONPs toxicity in *S. lycopersicum*. CuONPs at both the doses (500 and 1000 µM) showed significant reduction in growth, protein content, photosynthetic pigments, PS II photochemistry, contrastingly the reactive oxygen species (SOR and H_2O_2) were significantly raised as confirmed by *in- vitro* and *in-vivo* visualization of SOR and H_2O_2 that eventually leads to cell death as evidenced. Now days the nutrient management techniques have been widely used for the release of toxic effects caused by the pollutants in plants. In this study, the toxicity alleviation is achieved by exogenous application of calcium (Ca). Ca is a macronutrient that acts as a signalling molecule and produces a remarkable enhancement in growth attributes, protein content, and photochemistry of PS II. Besides physiological responses Ca also reduced the ROS generation. Therefore, the study concludes that, Ca not only shields the growth and development of plants from CuONPs toxicity but also reduce the generation of ROS.

Keywords: Growth, photosynthetic pigment, protein, *in- vitro* and *in-vivo* ROS generation.

1.0 INTRODUCTION

The advancement in nano-technology has contributed to the indiscriminate usage of nanoparticles (NPs) in numerous commercial sectors that have resulted in contamination of the environment (Yang et al., 2017). In agricultural process several nano-technological practices are used, like delivery of pesticides, fertilizers, growth

promoting substances, plant pathogen identification as well as monitoring soil conditions (Duhan et al., 2017). Recent studies have illuminated tha,t nanoparticles particularly copper oxide nanoparticles releases from industrial and medical products including cosmetics, fabrics, paints and other surfaces (Woodrow Wilson International Center for Scholars. 2007; Ravindran et al., 2011). The NPs differ from the bulk particles of same materials due to their unique physiochemical and toxicological nature; they also possess the ability of penetrating membrane (Wang et al., 2016). The enhanced level of CuONPs in environment have caused an adverse impact on plants i.e., it causes reduction in plant growth, photosynthetic pigment content, and chlorophyll florescence. Studies have also suggested that NPs can penetrate the cell membrane and induce the generation of reactive free radicals that ultimately causes oxidative stress (Lv et al., 2019).

Plants are known as an important link of an ecosystem that drives the whole food chain. Any damage to the environment ultimately causes diminution in plant growth and productivity that leads to crop loses and food scarcity. These metal-based pollutants primarily CuONPs easily enters the plant membrane and transported *via* the vascular system to the entire plant cellular system (Shi et al., 2014). Thus, on entering the plant system it oddly affects the growth and development process, primary and accessory photosynthetic pigments i.e. chlorophyll (Chl *a* and b) and carotenoid (Car) content, the disturbance in the chlorophyll content ultimately decreases the photosynthetic rate and also interrupts the PS II photochemistry. The reduced efficiency of PS II photochemistry and in photosynthesis leads to disturbance in the electron transport chain and this disturbance in the leakage of electron from the electron transport chain induces oxidative stress in plants by generating reactive oxygen species (Jalilian et al., 2020). Generation of ROS such as; superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) interferes with cell metabolic processes and eventually leads to cell death and protein disruption (Thwala et al., 2016; Mylona et al., 2020).

Essential macronutrient primarily calcium (Ca) regulates the growth and development processes in plants, it also stabilizes the cell membrane, balances pH of the cell, and checks solute leakage from the cytoplasm (Hirschi, 2004; Hepler, 2005). Several studies have illuminated that Ca is involved in signalling and gene expression under changing environmental conditions (He et al., 2005). Calcium effectively alleviates abiotic stresses such as salt, drought, light and heavy metals (Bonilla et al., 2004; Österås and Greger, 2003). Heightened

level of Ca alleviates the negative impact of heavy metal *via* precipitation and complex formation and thereby regulating the plant metabolism (Le et al., 2012).

Calcium is also found to regulate the cell division, extension and synthesis, (McLaughlin and Wimmer, 1999). Therefore, the present study has been undertaken to evaluate the toxic effects induced by CuONPs and alleviating effect of Ca through regulation of CuONPs toxicity in *Solanum lycopersicum.*

2.0 Materials and Methods

2.1. Plant and growth conditions

Seeds of *Solanum lycopersicum* (tomato) were purchased from certified seed agency of Prayagraj, Uttar Pradesh. The seeds were surface sterilized with 2 % sodium hypochlorite and washed thoroughly with DDW followed by soaking for 12 hr in dark. The germinated seeds were selected and kept in growth chamber with photosynthetically active radiation (PAR) of 300 µmol photons $m^{-2}s^{-1}$ and light-dark regime of 16:8 h with relative humidity of 60–70 % at 26 ±1 °C during growth.

2.2. Treatments design and dose selection

Following 7 days of acclimatization, uniform sized seedlings were selected and treated with CuONPs and Ca alone and in combination. The, experimental set-up comprises of (i) Control (half strength Hoagland solution) (ii) 500 µM CuONPs, (iii) 1000 µM CuONPs (iv) Ca (2Mm) (v) 500 µM CuONPs + Ca (vi) 1000 µM CuONPs + Ca. Treatments were changed at three days of interval and aerated regularly to avoid root anoxia. All the experiments were performed after 7 days of seedlings growth.

After following a series of screening experiment, the two doses of CuONPs were selected i.e. 500 and 1000 µM corresponding to LC 15 and LC 30. Similarly, screening experiment was also performed for the dose selection of Ca and 2mM (18% alleviation) was selected for the further study.

2.3. Growth behaviour

Growth in control and treated seedlings was analysed in terms of shoot length and root length.

2.4. Protein content

Protein content in treated and untreated samples was measured by following the method of Lowery et al. (1951).

2.5. Photosynthetic Pigments content

The Photosynthetic pigment i.e. total chlorophyll *a* **(**Chl *a* + Chl b) and carotenoids (Car) were analysed by following the method of Lichtenthaler (1987). The absorbance was read out at 663.2, 646.5 and 470 nm respectively for Chl *a* + Chl b and Car.

2.6. PS II photochemistry

The PS II photochemistry was analyzed by chlorophyll *a* florescence by using (FluorPen FP 100, Photon System Instruments, Czech Republic) in dark adapted samples for 30 min following the method of Strasser et al. (2000).

2.7. *In-vitro* and *in-vivo* assessment (superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)

The *in-vitro* SOR ($O_2^{\bullet-}$) and H_2O_2 content in treated and untreated samples were analysed in leaf by following the method of Elstner and Heupel (1976) and Velikova et al. (2000) respectively. Further to verify biochemical experiments, the *in-vivo* localization of $O_2^{\bullet-}$ and H_2O_2 were also done in leaf by following the method of Frahry and Schopfer (2001), Thordal-Christensen et al. (1997) After staining and proper washing stained leaves were photographed with Nikon coolpixW150.

2.8. Statistical analysis

The data was statistically analyzed by analysis of variance (ANOVA). Duncan multiple range test (DMRT) was performed for the significant differences among treatments at $P<0.05$ levels.

3.0 Results

3.1. Growth attributes

The result related to growth attributes that has been analyzed in terms of root length and shoot length has been presented in Fig 1. The result clearly depicts that both the doses of CuONPs caused a significant reduction of 15 and 31% in shoot and 17 and 30% in root length respectively. The exogenous supplementation of Ca alone raised the growth by 15 and 17% in shoot and root respectively. However, combining with the doses of CuONPs the extent of damage is reduced to only by 5 and 12% in shoot and 6 and 11% in root respectively attesting towards the beneficial role of Ca in alleviating CuONPs toxicity.

3.2. Protein content

The protein content in tested seedling has been presented in Fig 2. The results clearly reveal that the protein content was significantly declined by 17 and 32 % under CuONPs in concentration dependent manner. However, under exogenic Ca the protein content was raised by 15% and along with CuONPs the protein content was found to be only reduced by 6 and 14% (200 and 500 µM CuONPs) pointing towards beneficial role of Ca in alleviating stress condition.

3.3. Photosynthetic pigment

The result related to pigment content i.e. total chlorophyll and carotenoids is presented in Fig 3. The results clearly depict that the total Chl content was significantly declined by 14 and 29% under both the doses of CuONPs similarly Car content was declined by 16 and 30% with respect to control. Further exogenous supplementation of Ca alone and in combination alleviated the toxicity caused by CuONPs as alone it improved the pigment content by 15 and 17% respectively and under similar condition with CuONPs. It reduced the extent of damage as it only caused 3 and 12% reduction in total Chl and 5 and 17% reduction in Car respectively.

3.4. PS II photochemistry

The reduction in photosynthetic pigment content leads to significant impact on the efficiency of PS II photochemistry that is shown in Fig 4. The CuONPs at both the test doses significantly declined the chlorophyll *a* fluorescence kinetics parameter like quantum yield of primary photochemistry (Phi_P_0), yield of electron transport per trapped excitation (Psi_$_0$), quantum yield of electron transport (Phi_E_0), and performance index of PS II (PI_{ABS}). Contrary to this loss in kinetics parameters the energy flux parameters like ABS/RC, TR_0/RC, DI_0/RC and ET_0/RC was remarkably elevated in comparison to control seedlings. Whereas addition of Ca in growth medium alone showed contrary result to CuONPs treated seedlings, in which kinetics parameter increased and energy flux per reaction centre were decreased.

3.5. *In-vitro* and *in-vivo* assessment of oxidative stress biomarkers (SOR and H_2O_2) content

The result related to biochemical experiment of SOR and H_2O_2 content has been depicted in Fig 5. The result clearly reveals that under tested doses of CuONPs the SOR and H_2O_2 content raised by 27 and 52% and 29 and

56% respectively. However exogenous supplementation of Ca lowers the generation of oxidative stress biomarker i.e. by 20 and 23% alone while along with CuONPs the SOR and H_2O_2 generation was reduced as it showed only 16 and 27% enhancement in SOR content and 18 and 27 % in H_2O_2 content respectively.

Further to confirm the biochemical results the *in-vivo* localization for SOR, H_2O_2 was also performed, the SOR and H_2O_2 content in leaves react with the NBT and DAB and stains blue and brown colour respectively and the intensity of colour was found to be more intense on increasing the doses of CuONPs while exogenic Ca reduces the extent of damage by showing less intense colour.

4.0. Discussion

The present study focuses on the ameliorating impact of Ca in removing CuONPs toxicity in crop plant primarily *Solanum lycopersicum*. The growth of plants was significantly affected under both the doses of CuONPs and the pronounced reduction in growth (Fig. 1) is allied with increased Cu accumulation thus causing prominent reduction in photosynthetic pigment (Tab. 1) and protein contents (Fig. 2) and thereby alterating the PS II photochemistry (Fig 3) and disturbing the electron leakage that ultimately leads to oxidative stress (Fig. 4 and 5) similar to our findings studies have also been reported by Kolbert *et al*. (2012) and Nair and Chung (2014). The photosynthetic pigments are primarily responsible for the process of photosynthesis, the Chl *a* and Chl b are essential while Car act as an accessory pigment and also involved in photo protection. The tested doses of CuONPs significantly declined the photosynthetic pigment content either due to replacement of co-factors required for the Chl biosynthesis or degradation of enzyme involved in chlorophyll synthesis (Mishra et al., 2016), addition to this alteration of the photosynthetic machinery that damage the photosynthetic pigments is might be a probable reason (Stoeva et al., 2005) concurrent to our study Kolbert *et al*. (2012) also showed the similar result.

The reduced status of the photosynthetic pigment content under CuONPs toxicity hints towards the alteration in the PS II photochemistry, the values related to the energy flux parameters i.e. (Phi_P_o), ($Psi\__o$), (Phi_E_o) and ($Pi\__{ABS}$), were found to be decrease with increasing stress condition whereas the corresponding ratios the energy flux ratios like ETo/RC and TRo/RC, DIo/RC were found to increase. The reduction in (Phi_Po) is might be due to reduced primary charge separation or by cessation of some minor antenna from PS II. (Singh et al., 2019). Concurrent to our study, Wang et al., (2018) also published similar result in tomato plant under ZnONPs.

The major reduction in growth parameters ultimately reduces the photosynthetic efficiency by altering pigment status and PS II photochemistry, which eventually leads to electron leakage and generation of reactive oxygen species (ROS). Raised generation of ROS in due course causes oxidative stress thereby leading to cell death (Baxter et al., 2014). In our findings CuONPs stress tempted the oxidative burst in tested seedlings, as evident by sharp enhancement of SOR and H_2O_2, which probably be due to either impairment in the photosynthetic process and leakage of electron or amplified Cu level that accelerated free radical formation (Singh et al., 2018; Mylona et al., 2020). The enhanced oxidative stress (Fig. 4 and 5) possibly related with increased membrane damage, which ultimately indicates that the Cu peroxidises the plasma membrane lipids which is confirmed by *in-vivo* staining of SOR and H_2O_2. Similar result was also reported by Lequeux *et al*. (2010). Lee *et al*. (2013) have also reported genotoxicity in *Fagopyrum esculentum* as a result of oxidative stress due to CuONPs exposure.

However, exogenous supplementation of Ca significantly improves the growth attributes and reduces the generation of reactive oxygen species i.e. $O_2^{\bullet-}$ and H_2O_2, as shown in *in-vitro* and *in-vivo* results, these findings also suggest that Ca has capability of protecting plants under CuONPs toxicity by reducing oxidative stress. So, the supplementation of Ca against the CuONPs toxicity balances the Ca content in plant that might be the reason of increased chlorophyll content, carotenoids as well as repair in photochemistry of PS II. Consequently, leading to increased plant growth. On similar note, addition of Cu enhanced the photosynthetic pigment content, which recovers the efficiency of PS II photochemistry thus improved growth.

Exogenously applied calcium Ca reduces the generation of ROS as it activates the antioxidant defense system in plant cell, which maintains redox homeostasis of cell and also maintains balance between oxidants and antioxidants (Jiang et al., 2014; Yan and Chen, 2019).

Conclusion

The current study concludes that CuONPs remarkably reduced the growth of *S. lycopersicum* due to increased Cu accumulation as well as reduction in pigment content and increased oxidative stress that leads to cell death. While, addition of Ca protected the test seedling against CuONPs toxicity by increasing pigment content, protein, as well as reducing ROS generation through activating antioxidant defense system. Hence, the study recommends

the mitigation of CuONPs toxicity by exogenous supplementation of minerals (Ca) to increase the plant growth and productivity.

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Conflicts of interest

Authors declare no conflict of interest

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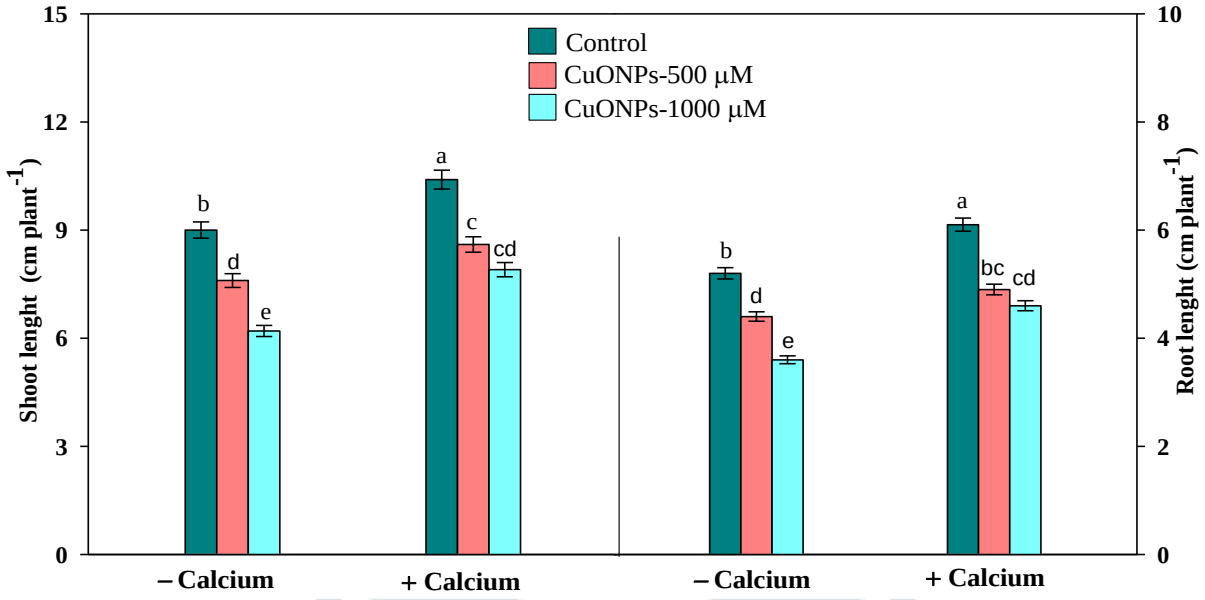

Figure 1: Growth parameters (Length of Shoot and Root) of *S. lycopersicum* seedlings grown in the presence of CuONPs alone or in combination of Ca. Data are means ± standard error of three replicates. Bars with different letters show significant differences at $p<0.05$ according to the Duncan's multiple range test.

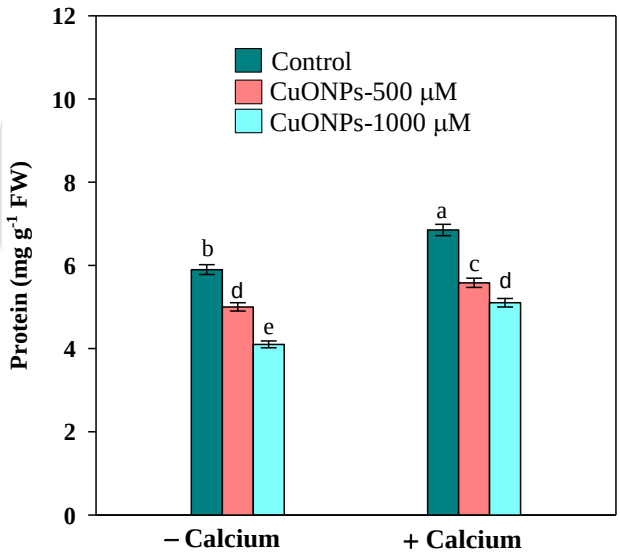

Figure 2: Protein content of *S. lycopersicum* seedlings grown in the presence of CuONPs alone or in combination of Ca. Data are means ± standard error of three replicates. Bars with different letters show significant differences at $p<0.05$ according to the Duncan's multiple range test.

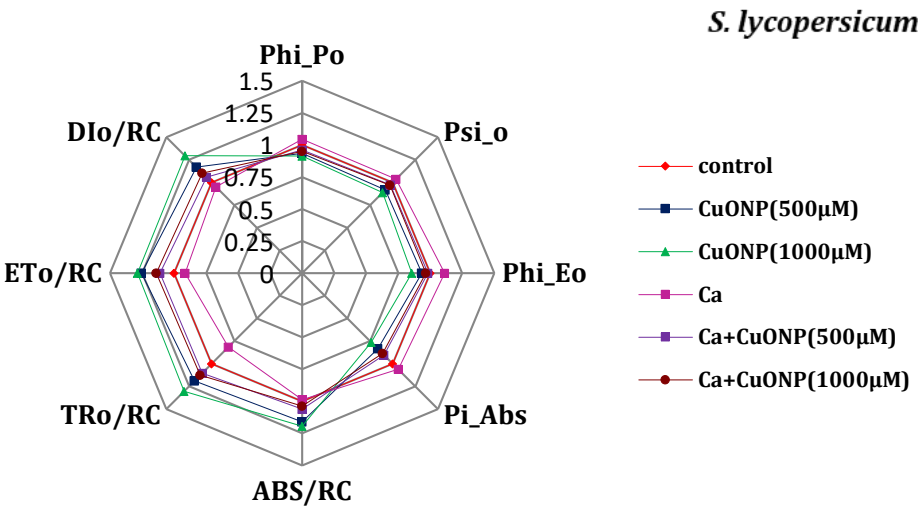

Figure 3: Radar graph showing the altered kinetics of PS II in *S. lycopersicum* seedlings in the presence of CuONPs alone or in combination with Ca.

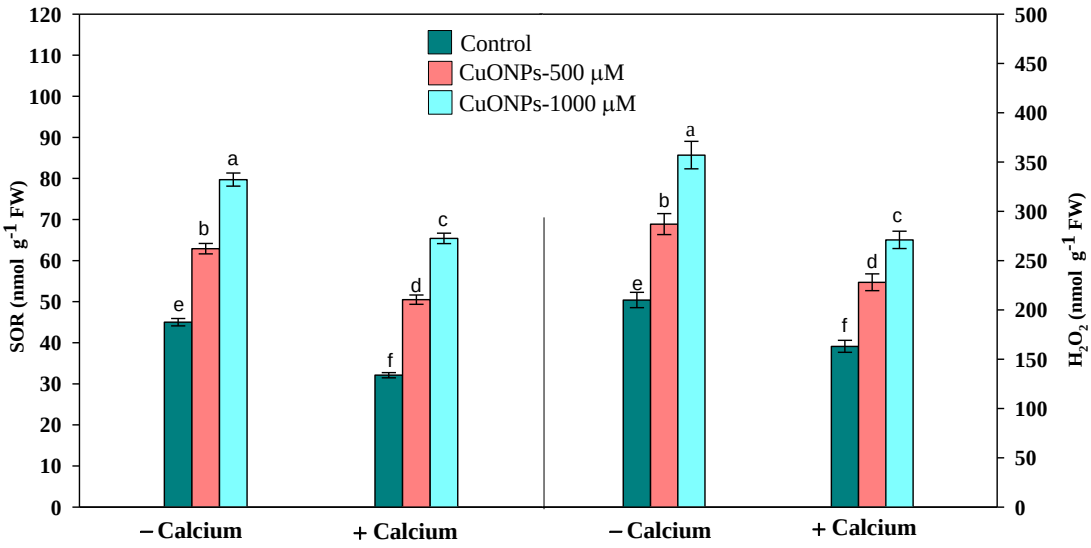

Figure 4: SOR and H_2O_2 accumulation in *S. lycopersicum* seedlings grown in the presence of CuONPs alone or in combination of Ca. Data are means ± standard error of three replicates. Bars with different letters show significant differences at $p<0.05$ according to the Duncan's multiple range test.

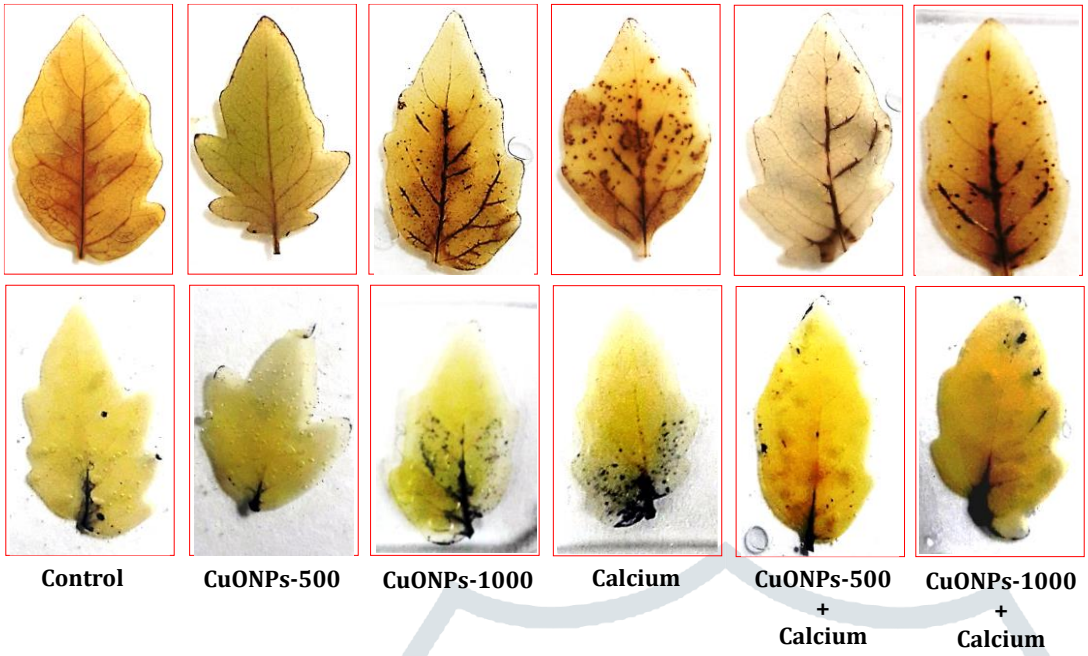

Figure 5: *In-vivo* production of H_2O_2 and SOR in *S. lycopersicum* seedlings grown in the presence of CuONPs alone or in combination of Ca

Table 1; Effect of CuONPs and Ca alone and in combinations on Total Chl and Carotenoid content in *S. lycopersicum*. The data are means ± standard error of three replicates. Different letters on values within same column show significant difference at $P < 0.05$.

Treatments	Total chl (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)
Control	1.813 ± 0.027^{b}	0.389 ± 0.007^{b}
CuONPs-500µM	1.518 ± 0.022^{d}	0.329 ± 0.006^{e}
CuONPs-1000µM	1.286 ± 0.019^{e}	0.279 ± 0.005^{f}
Calcium	2.086 ± 0.031^{a}	0.451 ± 0.009^{a}
CuONPs-500µM+ Calcium	1.746 ± 0.026^{c}	0.376 ± 0.007^{c}
CuONPs-1000µM + Calcium	1.580 ± 0.023^{d}	0.339 ± 0.006^{d}