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Zinc alleviates cadmium induced heavy metal stress by stimulating antioxidative defense in soybean [*Glycine max* (L.) Merr.] crop

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Abstract
The interaction between cadmium- a toxic metal and zinc- an essential micronutrient was investigated in influencing the activity of various antioxidant enzymes and related metabolites in soybean [*Glycine max* (L.) Merr.]. Higher levels of cadmium (Cd) stimulate the activity of potential enzymes like ascorbate peroxidase (APX), superoxide dismutase (SOD) accompanied by the buildup of non-enzymatic metabolites, hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and proline due to rise in oxidative stress of plants. Also, the reduced activity of catalase (CAT), glutathione reductase (GR) and ascorbic acid (AsA) content was based upon Cd treatment levels. Application of zinc (Zn) combination enhances the activity of enzymes like APX, GR, CAT and SOD in Cd treatments, also confirmed with the depleted levels of H_2O_2. Zn alone treatment had no significant effect on the activity of such enzymes indicating the toxicity owing to Cd treatments only. The accumulation behavior of other non-enzymatic metabolites like MDA, proline and ascorbic acid also get reversed with metal combination treatment. Moreover, the efficacy of Zn was more when applied in higher concentrations with low Cd. Thus, Zn plays a key role in plants to counter heavy metal stress by elevating antioxidative defense with higher activity of enzymes and reduced levels of non-enzymatic metabolites, and efficacy of Zn in combination is dose dependent.

Keywords: Abiotic stress, Antagonistic interactions, Grains, Legumes, Pulses

INTRODUCTION

Cadmium (Cd) is a toxic heavy metal that occurs naturally in soil with no known biological function in the plants and animals. Various anthropogenic activities are mainly responsible for a range of issues such as over exploitation of resources, salinity, acidification and contamination by metal pollutants. Such activities have dangerously added to the chances of entry of heavy metals into our ecological food chains and risking human health (Lantzy and Mackenzie, 1979; Galloway *et al*., 1982; Angelone and Bini, 1992). Many such metal elements with no metabolic function greatly reduce the crop productivity in their supra-optimal range (Rascio and Navari-Izzo, 2011; Pierart *et al*., 2015). And more seriously, such heavy metals exhibit very high stability rate in soil due to lack of biodegradability (Smolders *et al*., 1999; Singh and Prasad, 2015). Various negative effects of such metals have been reported affecting the development of roots and shoots (Lux *et al*., 2011; Gallego *et al*., 2012). Cadmium accumulation interferes with the enzymes of Calvin cycle, carbohydrate metabolism, photosynthesis (Shi *et al*., 2010) and alters the antioxidant metabolism (Khan *et al*., 2009). Cd triggers the oxidation of NADPH resulting in extracellular production of toxic superoxide ($O_2^{\cdot-}$) and accumulation of H_2O_2 (Kawano *et al*., 2001; Brahim *et al*., 2010). Destabilization of cell membrane enhances due to generation of ROS causing lipid peroxidation (Smeets *et al*., 2005). Such oxidation effects can be controlled with the stimulation of antioxidant enzymes and non-enzymatic metabolites via ascorbate-glutathione cycle (Foyer and Noctor, 2003). Depending upon the severity of metal toxicity, response of antioxidant machinery varies among species and different tissues (Hassan *et al*., 2005a). The defense response is essentially related to metal ion acquisition and ion homeostasis for the survival of plants, pathogens and herbivores (Morkunas *et al*., 2018).

Zn, an essential micronutrient actively participates in various biological functions such as cell membrane integrity, chlorophyll biosynthesis, photosynthesis, enzyme activation, carbohydrate metabolism, fertility, protein synthesis, gene expres-

sion and regulation (Nishizawa, 2005; Broadley *et al*., 2007; Chasapis *et al*., 2012; Marschner and Marschner, 2012). Structurally, it is an integral component of more than 300 enzymes including RNA polymerase, alcohol dehydrogenase, alkaline phosphatase and carbonic anhydrase (Guerinot and Eide, 1999; Auld, 2001). Zn protects the vital components of cell such as chlorophyll, membrane lipids and -SH group of proteins against ROS (Cakmak, 2000). Zn fertilization is thus, necessary to protect the plant cell from oxidative damage being a cofactor of antioxidative enzyme SOD (Alscher *et al*., 2002; Alloway, 2004). Processing and the subsequent release of zinc to environment is normally accompanied by cadmium as pollutant (Ullrich *et al*., 1999) because, generally zinc ores (ZnS) contain upto 5% or even more of cadmium (Adriano, 1986). Due to their chemical similarity both Cd and Zn are taken up by plants as divalent cation and compete at the plasma membrane (Hart *et al*., 2002). In yeast cells, the intake of Cd occurs through Zn carrier proteins at the plasma membrane (Gomes *et al*., 2002). Looking at the present information, it was thought worthwhile to study the role of Zn in ameliorating heavy metal Cd induced stress by assessing activity of both enzymatic and non-enzymatic antioxidants in soybean crop.

MATERIALS AND METHODS

Soybean (*Glycine max* (L.) Merr. Palam soya) seeds were procured from Himachal Pradesh Agriculture University, Palampur, Himachal Pradesh, India. Healthy seeds were surface sterilized with 0.01% $HgCl_2$ followed by thorough washing with distilled water and overnight soaking in thick slurry of rhizobium culture mixed with activated charcoal and acacia gum. The plants were raised in earthenware pots filled with approximately 5kg of washed river sand and were lined with perforated polythene bags. Only three healthy plants were selected after thinning in each pot. The plants were grown and maintained in natural daylight conditions in dome shaped out-house. Cd ($Cd_{0.3}$ and $Cd_{0.6}$ mM as $CdSO_4.7H_2O$) and Zn ($Zn_{0.3}$ and $Zn_{0.8}$ mM as $ZnSO_4.7H_2O$) treatments were given 8 DAS (days after sowing) alone and in combination along with the nutrient medium (Minchin and Pate, 1975). Plants irrigated with distilled water served as control. The observations were recorded at the reproductive stage using fresh leaves.

Standardized procedure was followed for the measurements of MDA content (Heath and Packer, 1968) using extinction coefficient 155 $mM^{-1}cm^{-1}$, H_2O_2 (Velikova *et al*., 2000), Ascorbic acid (Mukherji and Chaudhari, 1983) and Proline (Bates *et al.,* 1973). The activity of enzymes was assayed by method of Nakano and Asada, 1981 (APX activity) using molar extinction coefficient 2.8 $mM^{-1}cm^{-1}$, Teranishi *et al.,* 1974 (CAT) using molar extinction coefficient 36 $mM^{-1}cm^{-1}$, Mavis and Stellwagen, 1968 (GR), Dhindsa *et al.,* 1981 (SOD) and one unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT.

Statistical analysis: All the values were in triplicates and represented as mean ± SE (standard error). Data was statistically analysed using one-way ANOVA in SPSS-16 by taking the probability level of 5%. Least significant difference (LSD) post hoc test was used to compare the multiple comparisons of mean.

RESULTS

Malondialdehyde (MDA) content: MDA content increased to 26.81% and 41.16% in $Cd_{0.3}$ and $Cd_{0.6}$ mM treatments to that of control. Zn alone and in all combination treatments with Cd reduced the enhanced MDA content. In $Zn_{0.3}$ and $Zn_{0.8}$ mM alone treatment, MDA content was lowered by 7.25% and 23.50% in comparison to control, respectively. In combined treatments Zn supplementation decreased MDA content to 0.78% and 17.82% in $Cd_{0.3}$+$Zn_{0.3}$ mM and $Cd_{0.3}$+$Zn_{0.8}$ mM; to 9.30% in $Cd_{0.6}$+$Zn_{0.8}$ mM in comparison to control. Similarly in $Cd_{0.6}$+$Zn_{0.3}$ mM treatment, Zn was able to reduce the MDA content to some extent in which MDA content was 15.14% more than that of control. Thus, Zn supplementation was effective in lowering MDA content in Cd treated plants (Fig. 1a).

Proline content: The content of proline, known osmoprotectant was enhanced upto 20.33% and 35.16% in $Cd_{0.3}$ and $Cd_{0.6}$ mM treatments in comparison to control. Zn combination was able to check proline accumulations with a rise of only 13.55% and 1.69% in $Cd_{0.3}$+$Zn_{0.3}$ mM and $Cd_{0.3}$+$Zn_{0.8}$ mM; 27.54% and 6.77% in $Cd_{0.6}$+$Zn_{0.3}$ mM and $Cd_{0.6}$+$Zn_{0.8}$ mM treatments, respectively. In $Zn_{0.3}$ and $Zn_{0.8}$ mM alone treatments, a drop in proline content upto 2.96% and 10.59% was observed. It was noticed that higher concentration of Zn (0.8 mM) in combination was more effective in restoring proline levels (Fig. 1b).

Hydrogen peroxide (H_2O_2): The content of hydrogen peroxide, another parameter related to oxidative stress increased by 25.02% and 38.56% in $Cd_{0.3}$ and $Cd_{0.6}$ mM in comparison to control. Zn supplementation in combination treatments checked such accumulation with a rise of only 13.81%, 4.57% in $Cd_{0.3}$+$Zn_{0.3}$ mM and $Cd_{0.3}$+$Zn_{0.8}$ mM; and 31.66%, 19.19% in $Cd_{0.6}$+$Zn_{0.3}$ mM and $Cd_{0.6}$+$Zn_{0.8}$ mM treatments, respectively. In $Zn_{0.3}$ and $Zn_{0.8}$ mM alone treatments, reduction in H_2O_2 level was noticed upto 3.41% and 18.74%, respectively. Efficacy of Zn was more when used in higher concentrations (Fig. 1c).

Ascorbic acid (AsA): There was a drop of 43.57% and 52.79% in level of Ascorbic acid with $Cd_{0.3}$ and $Cd_{0.6}$ mM treatments in comparison to

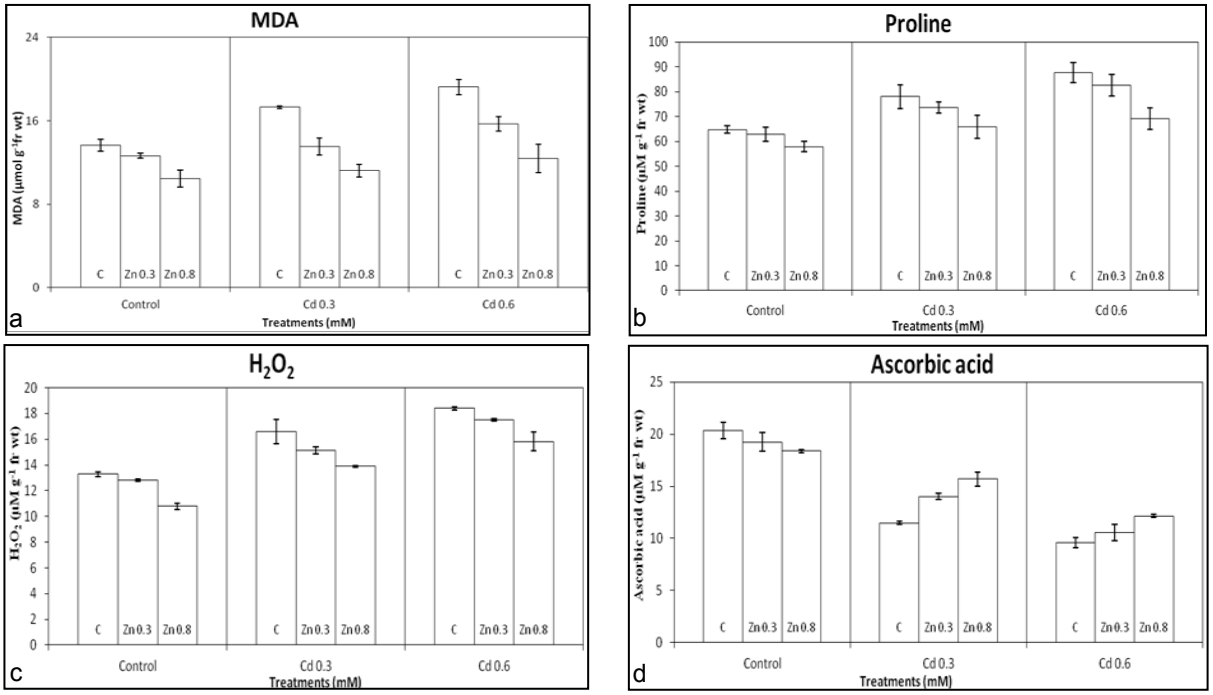

Fig. 1. *Effect of Cd and Zn alone and in combination on (a) MDA content ($LSD_{0.05}$=2.18), (b) Proline content ($LSD_{0.05}$=10.71), (c) Hydrogen peroxide content ($LSD_{0.05}$=1.25), (d) Ascorbic acid content ($LSD_{0.05}$=1.68) in soybean plants. Each value represents mean ± SE of three replicates.*

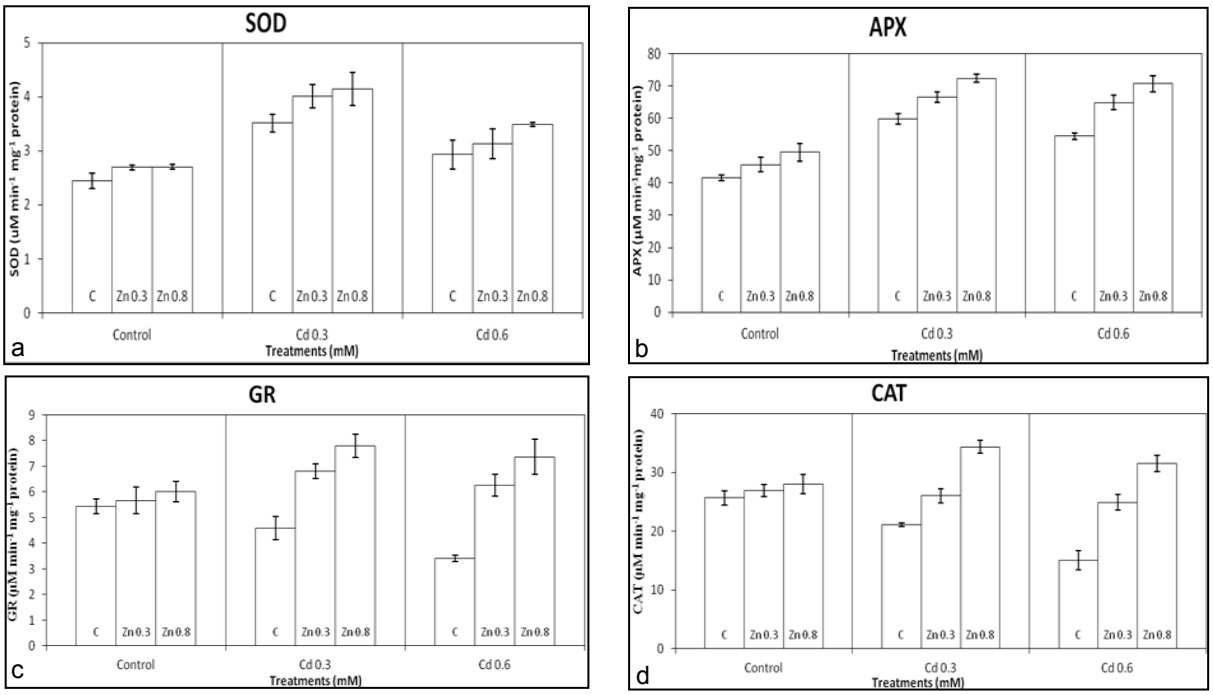

Fig. 2. *Effect of Cd and Zn alone and in combination on (a) Sodium dismutase activity ($LSD_{0.05}$=0.58), (b) Ascorbate peroxidase activity ($LSD_{0.05}$=5.57), (c) Glutathione reductase activity ($LSD_{0.05}$=1.27), (d) Catalase activity ($LSD_{0.05}$=3.74) in soybean plants. Each value represents mean ± SE of three replicates.*

control. Even, $Zn_{0.3}$ and $Zn_{0.8}$ mM alone treatments had decreased AsA contents upto 5.41 and 9.71%. Zn combination checked such losses to 31.07%, 22.96% in $Cd_{0.3}$+$Zn_{0.3}$ mM, $Cd_{0.3}$+$Zn_{0.8}$ mM; and to 48.12%, 40.29% in $Cd_{0.6}$+$Zn_{0.3}$ mM and $Cd_{0.6}$+$Zn_{0.8}$ mM treatments, respectively. Efficacy of Zn treatment was more with lower concentrations of heavy metal Cd (Fig. 1d).

Superoxide dismutase (SOD) activity: The activity of enzyme SOD was enhanced by 10.10% and 10.61% in $Zn_{0.3}$ and $Zn_{0.8}$ mM, alone treatments, respectively in comparison to control. Cd treatment had also raised its activity upto 43.66% ($Cd_{0.3}$ mM) and 19.88% ($Cd_{0.6}$ mM) of the control values. In combination treatment, the activity was further raised upto 64.05% and 69.05% in $Cd_{0.3}$+$Zn_{0.3}$ mM and $Cd_{0.3}$+$Zn_{0.8}$ mM; and upto 28.03% and 42.8% in $Cd_{0.6}$+$Zn_{0.3}$ and $Cd_{0.6}$+$Zn_{0.8}$ mM, respectively (Fig. 2a).

Ascorbate peroxidase (APX) activity: APX enzyme activity enhanced upto 43.9% ($Cd_{0.3}$ mM) and 31.05% in ($Cd_{0.6}$ mM) in comparison to control. Even, Zn alone treatment resulted in an increase of 9.89 ($Zn_{0.3}$ mM) and 19.17% ($Zn_{0.8}$ mM). Zn in combination treatments with Cd further raised its activity upto 59.97% and 74.18% in $Cd_{0.3}$+$Zn_{0.3}$ mM and $Cd_{0.3}$+$Zn_{0.8}$ mM; and upto 56.13 and 70.16% in $Cd_{0.6}$+$Zn_{0.3}$ mM and $Cd_{0.6}$+$Zn_{0.8}$ mM treatments, respectively (Fig. 2b).

Glutathione reductase (GR) activity: Activity of GR was reduced with an increase in Cd treatment. The decrease was upto 15.46% ($Cd_{0.3}$ mM) and 37.45% ($Cd_{0.6}$ mM) to that of control. Zn supplementation had a positive effect in raising enzyme activity levels. Zn supplementation in combination treatments raised activity levels upto 25.09% and 43.26% in $Cd_{0.3}$+$Zn_{0.3}$ mM and $Cd_{0.3}$+$Zn_{0.8}$ mM; and 14.94% and 35.55% in $Cd_{0.6}$+$Zn_{0.3}$ mM and $Cd_{0.6}$+$Zn_{0.8}$ mM treatments, respectively (Fig. 2c).

Catalase (CAT) activity: Cadmium treatment caused sharp drop in activity of enzyme CAT was upto 17.67% ($Cd_{0.3}$ mM) and 41.24% ($Cd_{0.6}$ mM) in comparison to control. Zn alone application was promotory in raising activity up by 4.97% ($Zn_{0.3}$ mM) and 9.17% ($Zn_{0.8}$ mM). Zn in combination treatment checked the drop in enzyme activity to 1.46% in $Cd_{0.3}$+$Zn_{0.3}$ mM and 2.89% in $Cd_{0.6}$+$Zn_{0.3}$ mM. In $Cd_{0.3}$+$Zn_{0.8}$ mM and $Cd_{0.6}$+$Zn_{0.8}$ mM the activity was enhanced upto 33.82% and 22.99% in comparison to control (Fig. 2d).

DISCUSSION

Our findings clearly revealed that soybean plants countered heavy metal Cd induced oxidative stress through enzymatic and non-enzymatic activity of metabolites. Higher levels of Cd stimulates the activity of potential enzymes like APX, SOD accompanied by accumulated contents of non-enzymatic metabolites, H_2O_2, MDA and proline as a marker of rise in oxidative stress of plants. Such multiple responses were due to production of the reactive oxygen species (ROS), and were indicative of plant being under oxidative stress (Ahmed *et al*., 2008, 2010). Cd induced oxidative stress negatively affect the defense system of wheat with an overproduction of ROS (Qayyum *et al*., 2017; Hussain *et al*., 2018; Rehman *et al*., 2018). Proline accumulation is considered as one of the most sensitive response to abiotic stress including heavy metal Cd (Ashraf and Harris, 2004; Chen *et al*., 2004; Mishra and Dubey, 2006; Kalai *et al*., 2014). Lipid peroxidation occurs as malondialdehyde content (MDA) enhances with Cd treatment (Singh *et al*., 2006, Tkalec *et al*., 2014; Kapoor *et al*., 2016). Also, the reduced activity of CAT, GR and ascorbic acid content was based upon level of Cd treatment. SOD can convert $O_2^{\cdot-}$ to H_2O_2 while CAT decomposes H_2O_2 to H_2O and oxygen molecules, and similarly other antioxidant enzymes also play role in ROS scavenging in plants (Mittler, 2002). Higher concentration of $O_2^{\cdot-}$ inactivates the enzyme CAT (Cakmak, 2000) while GR is highly sensitive to inhibition by heavy metals ions (Smith *et al*., 1989). Various genes were differentially regulated in response to abiotic stress to induce a similar kind of defense response resulting in the enhanced levels of several metabolites and proteins (Ozturk *et al*., 2002).

Zn plays a vital role in production and activity of enzymes to detoxify the reactive oxygen species (Tavallali *et al*., 2010; Weisany *et al*., 2012). In the present studies enhanced activity of such enzymes like APX, GR, CAT and SOD was noticed in Cd-Zn combination. Higher activity of SOD, CAT, APX and GR in Cd and Zn combinations was due to Zn against heavy metal induced oxidative stress in *Ceratophyllum demersum* (Aravind and Prasad, 2003, 2005).

Similar findings have also reported that the reduced activity of SOD, CAT, APX and GR during oxidative stress was up-regulated by lower concentrations of Zn (Cherif *et al*., 2011). Foliar spray of ZnO nanoparticles reduce electrolyte leakage, MDA and H_2O_2 content; raising the content of chlorophyll and activities of SOD, CAT, APX and POD in maize crop grown in Cd contaminated soil (Rizwan *et al*., 2019). Zn supplementation inhibited NADPH oxidation and formation of $O_2^{\cdot-}$ radical to prevent the formation of ROS in Cd treated plants (Aravind *et al*., 2009, Cherif *et al*., 2011). Zn supplemented Cd plants promotes the APX activity to control H_2O_2 levels thus, preventing cell damage more efficiently (Asada, 1992; Shigeoka *et al*., 2002; Dikkaya and Ergün, 2014). As reported earlier, our results have also indicated the depletion in H_2O_2 levels with Zn combination treatment (Cho and Seo, 2005; Mobin and Khan, 2006; Markovaska *et al*., 2009; Gill *et al*., 2012). As, Zn alone treatment in plants did not show any significant change in the activities of SOD, CAT, APX and GR indicating toxicity effects due to Cd treatments only (Arvind and Prasad, 2003; Cherif *et al*., 2011). Other non-enzymatic metabolites like MDA, proline and ascorbic acid also reversed their accumulation behavior in Zn supplemented Zn-Cd combination treatment compared to Cd only treatments. Addition of Zn to Cd stressed plants helps

in lowering MDA content and proline accumulations (Khalid and Hendawy, 2005; Subba *et al*., 2014; Qiao *et al*. 2015). Zn supplementation lowers the abiotic stress including that of heavy metal Cd by raising ascorbic acid levels (Ozturk *et al*., 2003, Ma *et al*., 2017; Seminario *et al*., 2017). Zn stabilizes and protects the bio-membrane proteins and phospholipids from the oxidative damage (Powell, 2000). An antagonistic interaction between Cd and Zn lowers the heavy metal Cd induced oxidative stress and its accumulation in plants (Wu and Zhang, 2002; Hassan *et al*., 2005b; Akay and Koleli, 2007; Sarwar *et al*., 2010; Balen *et al*., 2011; Trakal *et al*., 2012). Moreover, it was also noticed that efficacy of the Zn in combination treatment was more when Cd was in lower concentrations.

Conclusion

Heavy metal cadmium exposure induces an oxidative stress in soybean plants. Zn treatment effectively protects the plant from heavy metal stress by lowering the accumulation of stress related metabolites, inhibiting membrane lipid peroxidation and enhancing activity of enzymes that play vital role in ROS scavenging. Efficacy of Zn supplementation in combination treatment is dose dependent and more with lower Cd concentrations.

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