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SEED GERMINATION AND SEEDLING GROWTH OF *DIOSCOREA DELTOIDEA* WALL. EX GRISEB. FROM KINNAUR (HIMACHAL PRADESH) AS AFFECTED BY ZINC AND COPPER

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We examined the effects of zinc (100-1000 μM) and copper (50-500 μM) on seed germination and seedling growth of a population of *D. deltoidea*, an endangered medicinal plant species, from Kinnaur (Himachal Pradesh). Zn and Cu treatment of seeds led to enhanced seed germination and seedling growth. In general, Zn was more effective than Cu. Organ-specific differences in response to Zn and Cu were evident as the root growth promotion was found to be stronger than that of shoot. Due to Zn and Cu treatment, TTC reduction ability, a measure of the activity of dehydrogenases, and α-amylase activity in the seeds were enhanced. Concomitantly, the lipid peroxidation in seeds was marginally enhanced by certain concentrations of Zn and Cu that was accompanied by unaltered catalase (CAT) and enhanced ascorbate peroxidase (APX) activity. The promotory influence of Zn and Cu on seed germination and seedling growth could be ascribed to the observed metabolic changes representing diverse processes.

Key words: Copper, *Dioscorea deltoidea*, Seed germination, Seedling growth, Zinc

In recent years, there has been a tremendous increase in demand for medicinal plants. This could be ascribed largely to the resurgence of interest in herbal medicine that has fewer side effects and hence greater acceptability. Most of the demand for medicinal plants is met from wild populations causing a great loss to medicinal plant wealth (Canter *et al.* 2005). Like elsewhere, Himachal Pradesh, a rich repository of medicinal plant species, has also registered losses to medicinal plants (Goraya *et al.* 2013). A practical solution to ensure their sustainability would be to develop the concerned medicinal plants as small scale farming crops (Van Staden 1999). However, the growth conditions including the soil properties are likely to deviate from those prevailing in the natural habitats of the medicinal plants. Plants are likely to encounter two major kinds of possible deviations. On the one hand, they might experience abiotic stresses such as water-deficit, salinity etc. while on the other, soils might be deficient in different aspects including the micronutrients such as Cu, Zn etc. An understanding of the responses of medicinal plants to the stated deviations is of immense significance in a meaningful realization of their ex situ cultivation. The non-essential heavy metals (HMs) such as Cd, Pb, Hg etc. are invariably phytotoxic even at low concentrations. In contrast, the essential HMs such as Zn, Cu, Ni are required for normal plant growth and development as they serve as enzyme cofactors and constituents of diverse bio-molecules. Zn is involved in carbohydrate and nucleic acid metabolism. Besides, it is important for auxin formation (Cakmak *et al.* 1989). Likewise, Cu plays an essential role in signalling of transcription, oxidative phosphorylation and Fe mobilization (Yruela 2005). Both Zn and Cu are a component of superoxide dismutase (SOD). However, the essential HMs turn toxic at higher concentrations (Marschner 2012).

Dioscorea deltoidea Wall. Ex Griseb. (Family Dioscoreaceae), locally known as Shingli-Mingli, is an important medicinal plant. Extracts of *D. deltoidea* rhizomes have anti-rheumatic properties and are used to treat roundworm infection. Dried rhizomes contain diosgenin which is widely used as a precursor in the synthesis of steroid hormones such as progesterone, corticosteroids and anabolic steroids. In a CAMP (Conservation Assessment and Management Planning) workshop, .*D. deltoidea* has been assessed as endangered in Himachal Pradesh (Goraya *et al.* 2013). In the present study, we have determined the effects

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of zinc and copper on seed germination and seedling growth of a *D. deltoidea* population from, Kinnaur (Himachal Pradesh).

MATERIALS AND METHODS

Seed source

Seeds of *D. Deltoidea* were collected from Nichar (2110 m asl; District Kinnaur, Himachal Pradesh). Following air drying for a fortnight, they were stored in polyethylene bottles under ambient conditions until subsequent studies.

Seed germination and seedling growth

Seeds, selected for uniformity, were surface sterilized with 0.1% $HgCl_2$ for 5 min. followed by thorough washing with water. Seeds were soaked in solutions containing 100, 250, 500, 1000 µM Zn (as $ZnSO_4$) and 50, 100, 250, 500 µM Cu (as $CuSO_4$) for 24 h at 25±2ºC. Seeds soaked simultaneously in water constituted the control. After 24 h, the seeds were transferred to Petri plates lined with two layers of Whatman filter paper made wet with 5 ml of respective solutions of stated concentrations of Zn or Cu. Appropriate amounts of water were added subsequently to maintain the substrate wet. Seeds were allowed to germinate in an incubator at 25±2ºC under illumination (PAR: 40 µmol $m^{-2}s^{-1}$). The emergence of radicle (2-5 mm) was taken as seed germination. Seed germination was recorded at regular intervals until the final count. Seedling growth was measured in terms of root length, shoot length and seedling fresh weight after 30 d.

Biochemical/Metabolic parameters

α-amylase **assay**

α-amylase activity was determined following the method of Filner and Varner (1967). The seeds treated with the stated Zn and Cu concentrations for 72 h (24 h soaking followed by 48 h incubation on filter papers made wet with the respective HM solutions) were homogenized with 2 ml chilled 0.05 M Tris-HCl buffer (pH 7.2) and homogenate centrifuged at 10,000 rpm for 10 min at 4 ºC. Supernatant served as enzyme extract. 1 ml enzyme was incubated with 1 ml substrate (0.15% starch in Tris-HCl buffer; 0.2 mM $CaCl_2$) for 10 min. at 25±2ºC. The reaction was quenched by adding 3 ml of IKI reagent (0.6% iodine in 6% KI; 1 ml of this diluted to 50 ml with 0.05 N HCl). In control, IKI was added prior to the addition of enzyme. Absorbance was read at 620 nm and the amount of starch degraded was determined with the help of a calibration curve.

TTC reduction assay

The surface sterilized seeds were imbibed with water (control) or solutions containing Zn (250, 1000 µM) or Cu (100, 500 µM) for 24 h at 25±2ºC. Thereafter, the seeds were cut into halves. For each treatment, 6 halves in triplicate were incubated with 0.1% aqueous TTC (triphenyl tetrazolium chloride) solution for 72 h in dark at 25±2ºC. At the end of incubation, seed tissue was homogenized with 2 ml MetOH and the homogenate centrifuged at 10,000 rpm for 10 min. The absorbance of supernatant was recorded at 485 nm and the TTC reduction, a measure of tissue dehydrogenase activity, was expressed as A485/3 seeds.

Determination of lipid peroxidation

Lipid peroxidation was determined in terms of malondialdehyde (MDA) contents according to Dhindsa *et al.* (1981). Briefly, the seed tissue (approx. 100 mg), following HM treatment as in case of α-amylase assay, was homogenized with 2 ml of 0.1% TCA (trichloroacetic acid) and the homogenate centrifuged at 10,000 rpm for 10 min. One ml supernatant was reacted with 2 ml 0.5% TBA (thiobarbituric acid) at 95ºC in a water bath for 45 min. The reaction was terminated by cooling the reaction mixture in ice for 5 min. The absorbance was read at 532 nm and the measurements corrected for unspecific turbidity by subtracting the absorbance at 600 nm. MDA contents were determined by using the extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Catalase assay

Catalase activity was determined polarographically using a Clarke type O_2 electrode (Hansatech, UK). The seed tissue (approx. 100 mg) was homogenized with 1 ml of 100 mM HEPES-KOH buffer (pH 7.4) and homogenate centrifuged at 10,000 rpm for 10 min. The supernatant served as enzyme extract. The reaction mixture contained 890 µl HEPES-KOH buffer, 100 µl H_2O_2 (100 mM) and 10 µl enzyme extract. The enzyme activity was calculated from the slopes recorded on graph paper using the value of dissolved O_2 at 25ºC.

Ascorbate peroxidase assay

Ascorbate peroxidase activity was determined according to Hossain and Asada (1984). The seed tissue (approx. 100 mg) was homogenized with 1 ml 100 MM HEPES-NaOH buffer (pH 7.6) containing 5 mM ascorbate and homogenate centrifuged at 10,000 rpm for 5 min. (4 ºC). Supernatant served as enzyme extract. The reaction mixture contained 50 µl 5 mM ascorbate, 1 ml 50 MM HEPES-NaOH (pH 7.6) buffer, 50 µl enzyme extract and 100 µl H_2O_2 (3 mM). Following the addition of substrate, change in absorbance at 290 nm was monitored. The enzyme activity was determined using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Protein estimation

Protein was estimated according to Lowry *et al.* (1951).

Statistics

All experiments were performed in triplicate and repeated once. Data are represented as arithmetic means ± S.E.

RESULTS

Seed germination

A time-course of germination of seeds of *D. deltoidea* revealed that in control germination started after 9 d of incubation that gradually increased to a maximum of 67% after 27 d. Zn (100-1000 µM) and Cu (50-500 µM) treatment differentially influenced the seed germination in a concentration-specific manner. Thus, Zn at lowest concentration (100 µM) did not influence the germination much. However, the higher Zn concentrations (250, 500, 1000 µM) promoted the seed germination. The germination promotion due to 250 µM Zn was observed until 15 d when it was 138% of control; beyond 15 d there was no promotion. In contrast, germination promotion due to higher Zn concentrations (500 and 1000 µM) was evident throughout the incubation period of 27 d. However, the extent of promotion generally diminished with the lapse of time. For example, Zn (1000 µM) promoted the seed germination by 69 and 20% after 15 and 27 d, respectively (Fig. 1 A).

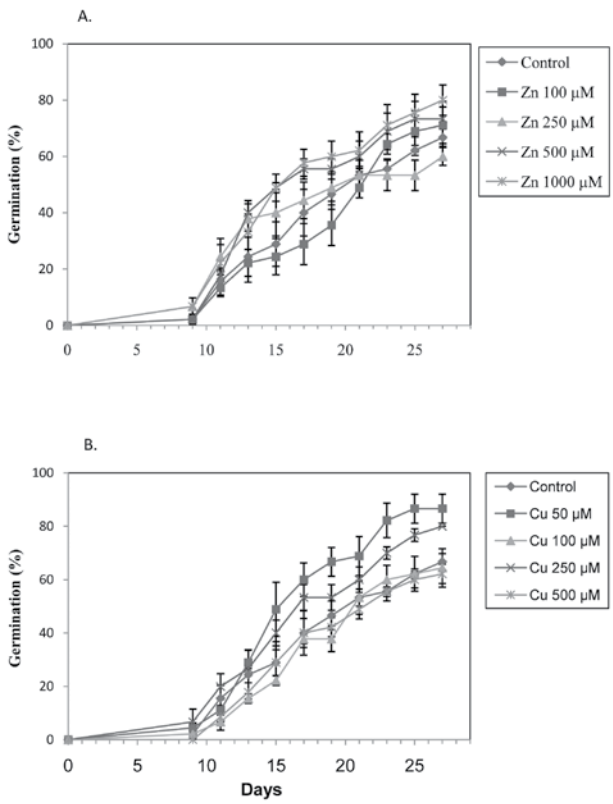
Cu was found to promote seed germination at 50 and 250 µM concentrations, the former being more effective. The magnitude of promotion was greater initially and gradually reduced with the lapse of time. Thus, the magnitude of promotion of seed germination due to 50 µM Cu was 69 and 30% after 15 and 27 d, respectively. Similar observations were obtained with 250 µM Cu. 500 µM Cu did not affect the germination much (Fig. 1 B).

Seedling growth

Both Zn and Cu promoted the seedling growth of *D. deltoidea*, measured after 30 d, with distinct organ-specific differences. The root length promotion was greater than that of shoot length particularly in case of Zn. Thus, Zn enhanced the root length by 23% at 250 µM that increased to 123% at 1000 µM (Fig. 2 A). Cu enhanced the root length by 54% at 500 µM; the lower concentrations (100, 250 µM) were only marginally promotory (Fig. 2 D). The shoot length was promoted by 18 and 24% at 100 µM Zn and 50 µM Cu, respectively. No further increase in the extent of shoot growth promotion was observed at higher metal concentrations (Fig. 2 B, E). Seedling fresh weight was increased

both by Zn and Cu, former being more effective than the latter. Thus, an increase of 55 and 69% in seedling fresh weight at 500 and 1000 µM Zn, respectively was observed (Fig. 2 C). These values were 37 and 43% in case of 250 and 500 µM Cu, respectively (Fig. 2 F).

Figure-1: Time-course of seed germination of *D. deltoidea* as affected by Zn (A) and Cu (B) treatment. Values are arithmetic means of three replicates comprising 15 seeds each ± S.E.



Metabolic parameters

TTC reduction ability and α-amylase activity

The TTC reduction ability, a measure of dehydrogenase activity, of *D. deltoidea* seeds was consistently enhanced by the applied Zn and Cu concentrations for 24 h. Thus, an increase of 39 and 65% was observed at 250 and 1000 µM Zn, respectively. These values were 52 and 85% at 100 and 500 µM Cu, respectively (Fig. 3 A). Likewise, the α-amylase activity in the seeds was enhanced by Zn and Cu treatment for 72 h. For example, a 2.12- and 2.51-fold increased α-amylase activity was evident at 250 µM Zn and 100 µM Cu, respectively (Fig. 3 B).

Lipid peroxidation and activity of catalase (CAT) and ascorbate peroxidase (APX)

The malondialdehyde (MDA) contents, an index of lipid peroxidation, was found to marginally increase in the seeds of *D. deltoidea* due to a 72 h Zn and Cu treatment. For example, they increased by 18 and 27% due to 250 µM Zn and 500 µM Cu, respectively (Fig. 4 A). Simultaneously, the activity of two antioxidative enzymes, namely, CAT and APX was measured. These two enzymes responded differentially to the treatment with Zn and Cu. Thus, the CAT activity in the seeds remained generally unaffected by Zn and Cu (Fig. 4 B). In contrast, the APX activity was strongly promoted both by Zn and Cu. For example, an increase of 89 and 127% in the APX activity was evident at 1000 µM Zn and 500 µM Cu, respectively (Fig. 4 C).

DISCUSSION

The aim of present study was to evaluate the influence of Zn (100 -1000 µM) and Cu (50-500 µM) on seed germination and seedling growth performance of a high altitude population of *D. deltoidea* from Himachal Pradesh. Seed treatment with both Zn and Cu, at applied concentrations, led to improved germination and seedling growth. Indeed, several HMs have been reported to stimulate seed germination at certain low concentrations. For example, alfalfa seeds were able to germinate and grow efficiently in the presence of 5-40 ppm Zn (Peralta *et al.* 2000). Similarly, Pb and Zn significantly promoted the seed germination in sage (*Salvia officinalis*) (Jeliakova and Craker 2000). Stimulation of seed germination by low levels of Cd and Pb in certain forest tree species and by those of Ni in *Brassica juncea*

Figure-2: Seedling growth of *D. deltoidea* after 30 d as affected by Zn (A, B, C) and Cu (D, E, F). Values are arithmetic means ± S.E. n = 15.

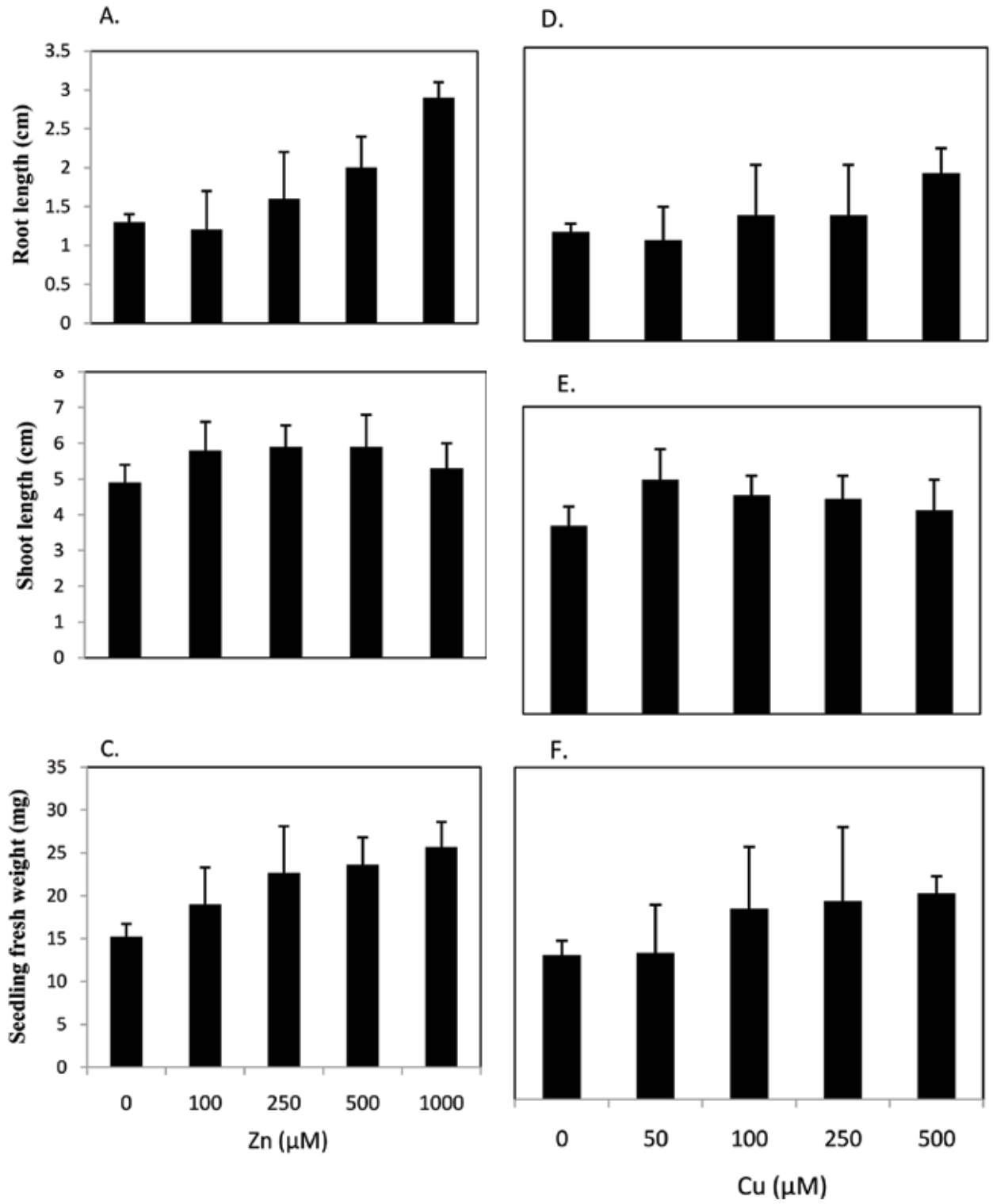


Figure-3: Effect of Zn and Cu on TTC reduction ability (A) and α-amylase activity (B) in *D. deltoidea* seeds. Values are arithmetic means ± S.E., n = 3.

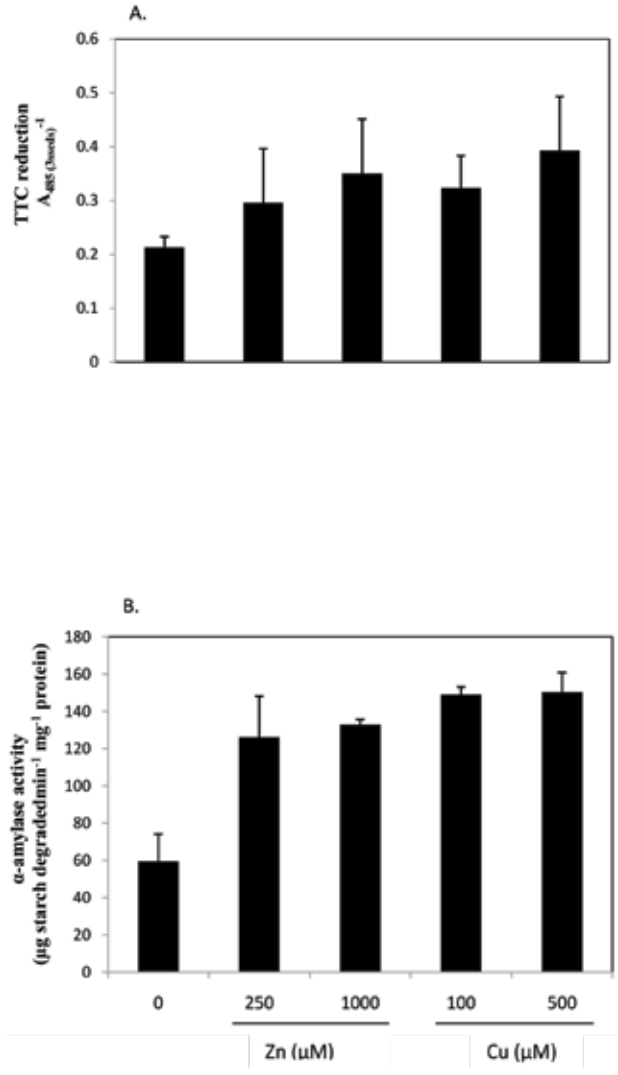
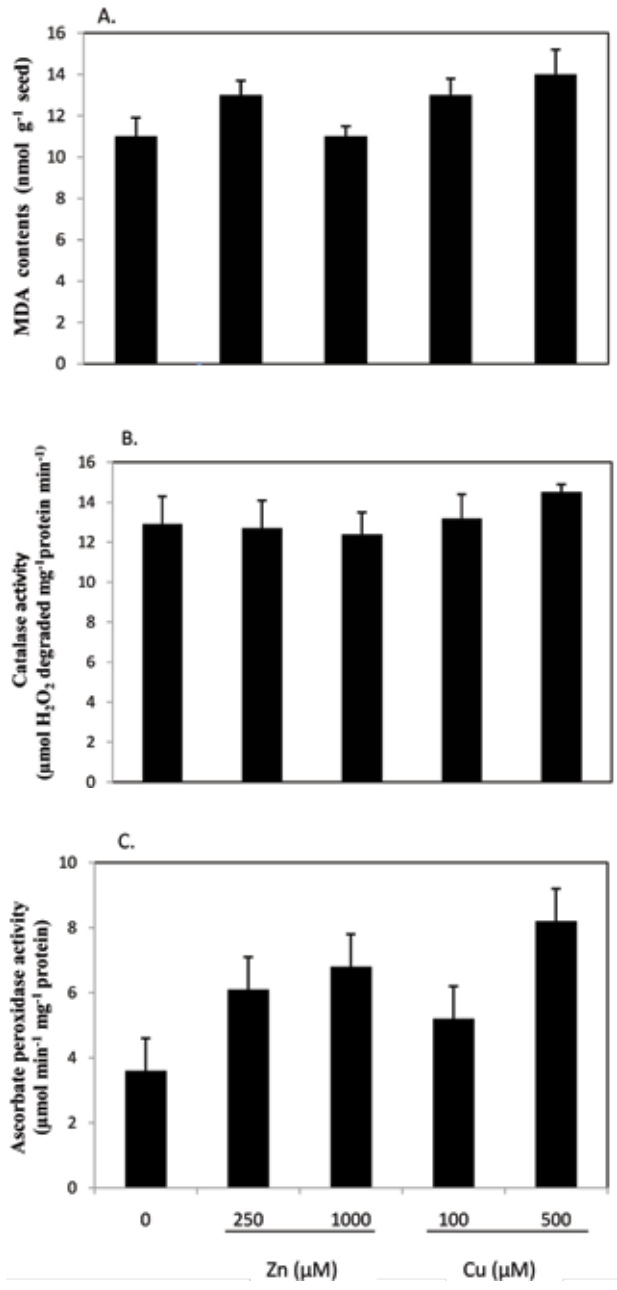


Figure-4: Effect of Zn and Cu treatment (72 h) on MDA contents (A) and catalase activity (B) and ascorbate peroxidase activity (C) in *D. deltoidea* seeds. Values are arithmetic means ± S.E., n = 3.



has been shown (Thakur and Sharma 2016). Hg promoted the seed germination at low concentration but inhibited the same at higher one in *Phaseolus vulgaris* (Sharma 1982) and *Pisum sativum* (Sharma 1983). Zn and Cu had no significant effect on seed germination of corn (Mahmood *et al.* 2005) and *Bowiea volubilis* (Street *et al.* 2007), respectively. In fact, the type and magnitude of the effect of HMs on seed germination appears to be determined by the degree of seed coat permeability to metal ions in different species (Wierzbicka and Obidzinska 1998).

The Zn and Cu concentrations used in this study are apparently in a higher range that could be expected to be inhibitory for plant processes. The fact that they promoted seed germination and seedling growth indicates that seed coat, acting as a barrier, hampered their uptake in a manner that seed tissue Zn/Cu concentrations remained at promotory levels. It is likely that *D. deltoidea* seeds contained insufficient Zn and Cu that were raised to optimum concentrations upon treatment with exogenous Zn and Cu. However, we did not measure the seed metal contents. Zn and Cu, present as seed reserve, are involved in seed germination as they are metabolized and translocated to various sites in germinating seeds (Ozturk et al. 2006). Seeds with low Zn contents have been reported to show poor seedling vigour and seedling establishment (Welch, 1999). Alternatively, *D. deltoidea* seed germination process might be tolerant to excess Zn and Cu concentrations. It would be interesting to analyse the studied *D. deltoidea* population from this view point.

With a view to get insight into the possible mechanism of Zn/Cu-induced stimulation of *D. deltoidea* seed germination, we monitored certain metabolic parameters. In response to Zn and Cu treatment, the TTC reduction ability of seeds, a measure of the activity of dehydrogenases including those from respiratory metabolism, was substantially enhanced. This obviously enhanced the generation of energy involved in processes leading to seed germination. A similarly enhanced α-amylase activity in the Zn- and Cu- treated seeds signifies the importance of starch degradation to yield sugars for mobilization to growing embryo in enhancing the seed germination and seedling growth. Zn and Cu might influence the metabolism of GA_3, involved in induction of α-amylase. Atici *et al.* (2005) demonstrated Zn to induce GA_3 biosynthesis in chickpea seeds. Finally, the seed MDA contents were marginally enhanced at certain concentrations of Zn and Cu indicating a low/no oxidative damage. Besides being generally damaging, different reactive oxygen species (ROS) also serve as signals influencing the course of metabolic events (Noctor *et al.* 2002). The involvement of redox metabolism in the regulation of seed germination events is established (Bailly *et al.* 2002). Of the two H_2O_2 decomposing enzymes, CAT remained generally unaffected by Zn and Cu whereas APX was stimulated. APX, with a lower substrate Km than CAT, is likely to have been involved in regulation of H_2O_2 levels. In conclusion, Zn and Cu treatment improved the seed germination and seedling growth performance of *D. deltoidea* that is likely a consequence of diverse altered metabolic events associated with seed germination.

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