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# VARIABLE SEED GERMINATION AND SEEDLING GROWTH RESPONSES OF *HIPPOPHAE RHAMNOIDES* AND *H. SALICIFOLIA* TO CADMIUM

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The phytotoxic effects of heavy metals (HMs) and metalloids in crop plant species are well documented. There is, however, a dearth of information concerning HM toxicity in wild plants of medicinal and economic importance particularly those from high altitude regions. Here, we have assessed the effects of a range of Cd concentrations on seed germination, seedling growth and certain associated metabolic parameters of *Hippophae salicifolia* (Kinnaur) and two populations of *H. rhamnoides* namely, Kinnaur and Lahaul (Himachal Pradesh, India). The stated *Hippophae* (seabuckthorn) species, distributed in Himalayan states of India including Himachal Pradesh, possess tremendous medicinal value, fix atmospheric nitrogen and reduce soil erosion. Higher Cd concentrations differentially suppressed the seed germination of the tested *Hippophae* species/populations with the lowest effect being in case of *H. salicifolia.* The lower Cd concentrations stimulated the germination in *H. salicifolia* and *H. rhamnoides* Kinnaur. The seedling growth was suppressed by Cd with organ- and species/population specific differences. The magnitude of root growth inhibition was invariably greater than that of shoot and *H. salicifolia* proved more tolerant to Cd than both the *H. rhamnoides* populations. The TTC reduction ability of embryos excised from Cd treated seeds was marginally suppressed by Cd while α-amylase activity was substantially inhibited. Due to Cd treatment, the MDA contents, activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) and proline contents increased in the seedling tissue. No marked species/population specific differences were evident in these parameters except that MDA contents did not change due to lower Cd concentration in *H. salicifolia.* The relative Cd tolerance of *H. salicifolia* has implications for its better survival in an HM-contaminated scenario.

**Key words:** Cadmium, *Hippophae rhamnoides*, *H. salicifolia*, Seabuckthorn, seed germination, seedling growth

Owing mainly to diverse anthropogenic but also to natural sources, the concentrations of heavy metals (HMs) such as Cd, Pb, Hg etc. have tremendously increased in soil and water (Sharma and Dietz 2006; Marschner 2012). HMs are taken up by plants often via transport pathways meant for essential metal ions. For example, Cd employs $Ca^{2+}$ channels for entering the cell (Perfus-Barbeoch *et al.* 2002). Even the essential HMs such as Zn, Cu, Ni etc. turn toxic as and when their concentration exceeds certain threshold values. HMs eventually pose a threat to human and animal health through food chain. HM-dependent phytotoxicity and associated yield losses are well documented (Sharma and Dietz 2006). Following uptake, HMs interfere with multiple cellular and metabolic activities through various mechanisms. The latter mainly include (i) disruption of enzyme functions due to thiol affinities of HMs, (ii) imposition of redox imbalance and resultant oxidative stress and (iii) displacement of essential metal ions from respective functional sites (Sharma and Dietz 2009).

In last some years, phytoremediation, a plant based technology aimed at reducing the environmental HM burden, has emerged as an environment friendly way out (Pilon-Smits, 2005). A clearer understanding of plant responses to HMs is expected to facilitate an optimized realization of phytoremediation. In this context, effects of HMs on crop plant species have often been reported (Sharma 1985a,b, Sharma *et al.* 2004; Thakur and Sharma 2015). Diverse plant processes are suppressed by heavy metals including seed germination (Thakur and Sharma 2015), photosynthesis (Bazzaz *et al.* 1974; Kumar *et al.* 2019), transpiration (Bazzaz *et al.* 1974) and essential ion homeostasis (Sharma *et al.* 2004). Occasionally, lower concentrations of non-essential HMs have been reported to stimulate processes like seed germination (Sharma, 1985a). There is, however, a dearth of information concerning the responses of wild

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plants of medicinal and economic importance to HM stress. Such information holds significance as the HM tolerance status of the concerned plant species is revealed. This has clear implications for expanding the cultivation of wild economically important plants to newer locations where soils might be contaminated with toxicants including HMs.

Seabuckthorn (Genus-*Hippophae*; Family-Elaeagnaceae), an $N_2$ fixing, deciduous, perennial plant, is widely distributed, ranging from Himalayan regions including those in India to several European countries (Rousi 1971, Ruan *et al.* 2013). It is well known for its strong medicinal properties all over the world due to various biologically active compounds (Michel *et al.* 2012). For example, the beneficial effects of seabuckthorn seed oil have been reported in cancer therapy and in the treatment of cardiovascular diseases, gastrointestinal ulcers, skin disorders and as a hepato-protective agent (Cheng *et al.* 2003). It also contributes to the enrichment of soil due to its nitrogen fixing ability and to the reduction of soil erosion due to robust and elaborate root system (Zhang and Chen 2007).

In the present study, a comparative analysis of seed germination and seedling growth responses of two populations of *H. rhamnoides* one each from Kinnaur and Lahaul (Himachal Pradesh, India) and *H. salicifolia* from Kinnaur to a range of cadmium concentrations has been carried out. Several associated metabolic parameters relevant to seed germination process have also been monitored. Cd is a common and one of the most toxic HMs that is routinely studied as an example of HM stress (Verbruggen *et al.* 2009).

## MATERIALS AND METHODS

**Source of seeds:** The ripened fruits of two species of seabuckthorn (*Hippophae*) were collected from different locations in Himachal Pradesh. In case of *H. rhamnoides,* they were collected from Kinnaur (Pooh, 2700-2800 masl) and Lahaul (Pattan valley, 2900-3000 masl) whereas those of *H. salicifolia* were collected from Kinnaur (Sangla, 2600-2700 masl). Following de-pulping of the fruits, the seeds were air dried for about a fortnight and stored in polyethylene bottles under ambient conditions.

**Determination of triphenyl tetrazolium chloride (TTC) reduction ability of seeds:** The surface sterilized (0.1% $HgCl_2$ for 5 min.) and thoroughly washed seeds were soaked in distilled water (control) or solutions of $CdCl_2$ (100, 250, 500 µM) for 24 h. These seeds were cut into equal halves and incubated with 0.1% aq solution of TTC at 25±2° C in dark for 72 h. Thereafter, the seed tissue was homogenized with 2 ml MetOH and the homogenate centrifuged at 10,000 rpm for 10 min. at 4° C. The absorbance of supernatant was read at 485 nm and the TTC reduction ability of seed tissue expressed as $A_{485}$ $(10 \text{ seeds})^{-1}$.

**Seed germination and seedling growth assays:** The uniform surface sterilized (0.1% $HgCl_2$; 5 min) and thoroughly washed seeds were soaked in distilled water (control) or the solutions (20 ml) of $CdCl_2$ (50,100, 200 µM Cd) at 25±2°C for 24 h. They were then transferred to petri dishes lined with three layers of filter papers made wet with 5 ml solution of respective Cd concentrations with subsequent addition of water to maintain the filter papers wet. The petri dishes were placed in a seed germinator at 25±2° C under continuous illumination (PAR: 40 µmol $m^{-2}$ $sec^{-1}$) and seed germination recorded at periodic intervals until the final count. For each treatment, 15 seeds in triplicate (15×3) were used. The seedling growth was measured after 15 and 30 d in *H. rhamnoides* and *H. salicifolia,* respectively in terms of root length, shoot length and seedling fresh weight.

**α-amylase assay :** The α-amylase was assayed in the Cd-treated seeds as above following the method of Filner and Varner (1967). In brief, the seeds were homogenized with chilled 0.05

M Tris-HCl buffer (pH 7.2) and the homogenate centrifuged at 10,000 rpm for 10 min. The supernatant served as the enzyme extract. One ml of enzyme was incubated with 1 ml of substrate (0.15% starch containing 0.2 mM $CaCl_2$) for 10 min. at 25°C. After addition of 3 ml of IKI reagent, the absorbance was read at 620 nm and α-amylase activity calculated using a calibration curve prepared with starch.

**Determination of lipid peroxidation**: Lipid peroxidation was determined following the method of Dhindsa *et al.* (1981). The seedling tissue was homogenized with 2 ml of 0.1% trichloroacetic acid (TCA) and the homogenate centrifuged at 10,000 rpm for 10 min. Two ml supernatant was reacted with 4 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA. The reaction mixture was incubated in a water bath at 95°C for 45 min and then the reaction was terminated in ice and absorbance read at 532 nm. The measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The MDA contents were calculated by using the extinction coefficient (155 $mM^{-1}$ $cm^{1}$) (Heath and Packer 1968).

**Determination of proline contents:** The free proline contents were measured according to Bates *et al.* (1973). The seedling tissue was homogenized with 3% sulphosalicylic acid and the homogenate centrifuged at 10,000 rpm for 10 min. One ml of supernatant was reacted with 1 ml freshly prepared ninhydrin reagent and 1 ml of glacial acetic acid in a boiling water bath at 95° C for 1 h and then the reaction terminated in an ice bath. The reaction mixture was extracted with 2 ml toluene that was aspirated from aqueous phase and absorbance read at 520 nm using toluene as blank. The proline contents were determined using a calibration curve.

**Superoxide dismutase (SOD) and ascorbate peroxidase (APX) assay:** The SOD activity in the seedling tissue was measured following the method of Beauchamp and Fridivich (1971) and that of ascorbate peroxidase (APX) activity was measured according to Hossain and Asada (1984). The details are provided by Thakur and Sharma (2015).

**Protein estimation:** Protein contents in the extracts were measured by Bradford method (Bradford 1976). The reaction mixture contained 790 µl $H_2O$, 10 µl sample extract and 200 µl Bradford reagent. After 5 min, that absorbance was read at 595 nm and protein contents determined with using a BSA calibration curve.

Statistical analysis

All experiments were carried out in triplicate and repeated at least once. Data are presented as arithmetic means ± standard error (SE).

## RESULTS

**Effects of Cd on seed germination and seedling growth:** In a time-course study of seed germination, the tested *Hippophae* species and populations showed a strong variability of pattern and extent. Thus, the seed germination *H. rhamnoides* Kinnaur and *H. rhamnoides* Lahaul started after 2 and 6 d of incubation, respectively that increased to 96 and 78% after 15 d. The seed germination was much slower in *H. salicifolia*; it started after 16 d of incubation and increased to 96% after 25 d.

The seed germination was differentially affected by Cd in different *Hippophae* species and populations. In *H. rhamnoides* Kinnaur, seed germination was promoted by Cd in a concentration dependent manner upto 5th day of incubation. An enhancement of 4, 10 and 25% was evident at 100, 250 and 500 µM Cd, respectively on 5th day (Fig. 1A). Subsequently, seed germination was inhibited by all Cd concentrations to varying extents. In *H. rhamnoides* Lahaul, Cd inhibited the seed germination initially, for example, by 43 and 57% at 100 and 500 µM Cd, respectively on 8th day. However, the

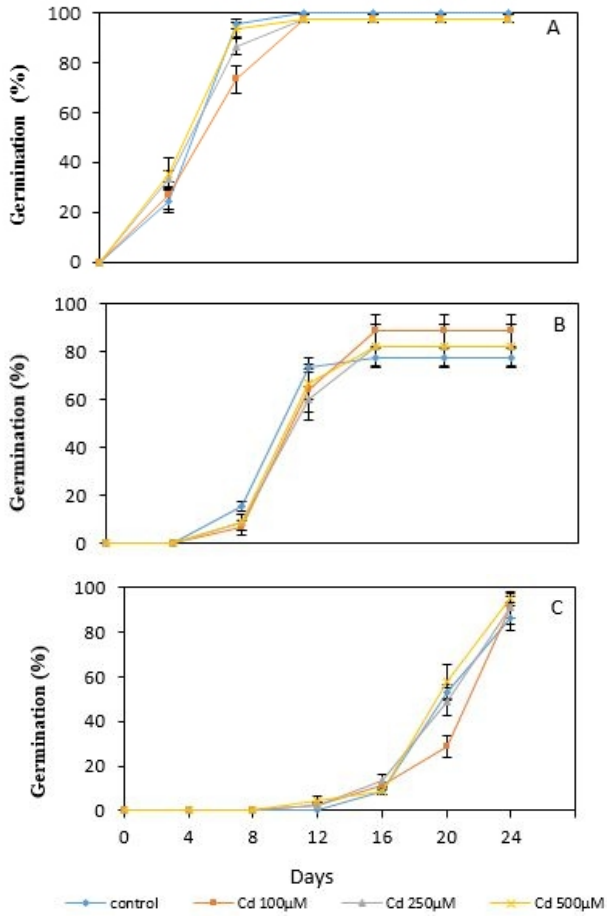


**Figure 1.** Time-course of seed germination of *H. rhamnoides* and *H. salicifolia* as affected by Cd: **A**. *H. rhamnoides,* Kinnaur, **B**. *H. rhamnoides*, Lahaul, **C**. *H. salicifolia*. Values are arithmetic means of three replicates each comprising 15 seeds ± S.E.

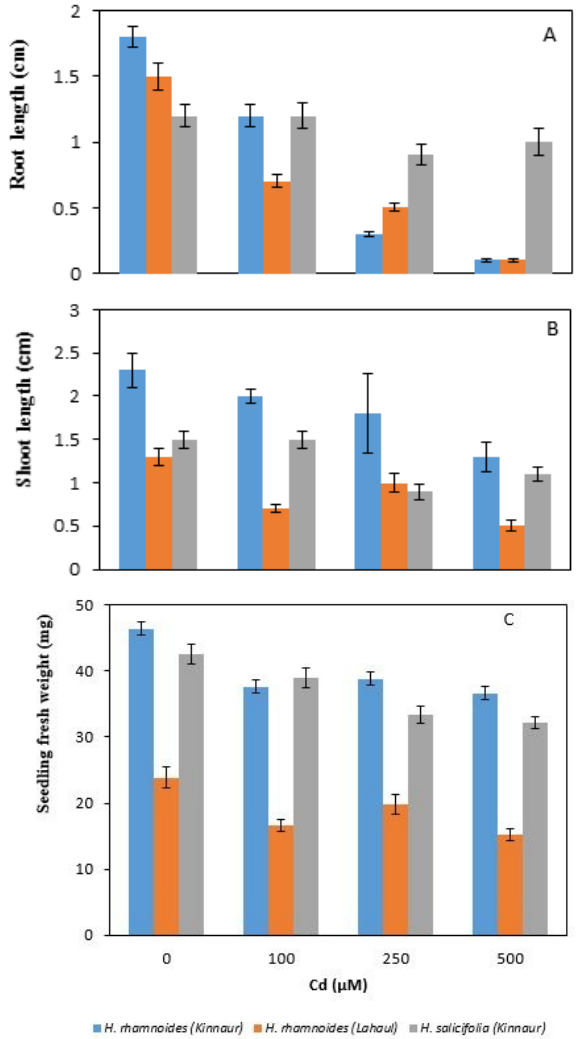


**Figure 2.** Effects of Cd on seedling growth **A.** root length, **B.** shoot length, **C.** seedling fresh weight) of *Hippophae rhamnoides* populations (Kinnaur, Lahaul) and *H. salicifolia*. Data after 15 and 30 d in *H. rhamnoides* and *H. salicifolia,* respectively. Values are arithmetic means ± S.E.; n = 20.

inhibitory effect of Cd was completely lost after 12 days (Fig. 1B). The promotory effect of Cd on seed germination was also noticed in *H. salicifolia*; an enhancement of 11 and 20% was evident at 250 and 500 µM Cd, respectively on 20$^{th}$ day that was lost after 22 d and all the tested Cd concentrations remained without any effect (Fig. 1C).

Due to the difference in time course pattern of seed germination, the seedling growth was measured after 15 and 30 d in *H. rhamnoides* populations and *H. salicifolia,* respectively. Irrespective of the effect on seed germination, Cd inhibited the seedling growth in a concentration-dependent manner in both the *H. rhamnoides* populations and *H. salicifolia*. Organ-specific differences in response to Cd were observed with root length being suppressed by a greater magnitude than the shoot length. The magnitude of Cd-induced root length suppression was less in *H. Salicifolia* than in the two *H. rhamnoides* populations. Thus, the root length in *H. salicifolia* was inhibited by 20 and 17% at 250 and 500 µM Cd, respectively. These values were 33, 83 and 94% in *H. rhamnoides* Kinnaur and 53, 67 and 93% in *H. rhamnoides* Lahaul at


**Figure 3.** Seedlings of *H. rhamnoides* populations **A.** Kinnaur; **B.** Lahaul) and *H. salicifolia* **C.** showing the effect of Cd concentrations (100, 250, 500 µM).

100, 250 and 500 µM Cd, respectively (Fig. 2A). Likewise, the Cd-induced shoot length inhibition was of lower magnitude in *H. salicifolia* than in the *H. rhamnoides* populations. At 250 µM Cd, a 40, 43 and 61% inhibition was observed in *H. salicifolia, H. rhamnoides* Kinnaur and *H. rhamnoides* Lahaul, respectively. At the highest Cd concentration (500 µM), the extent of inhibition was comparable (Fig. 2B). Due to 500 µM Cd, the seedling fresh weight was suppressed by 21, 21 and 36% in *H. rhamnoides* Kinnaur, *H. rhamnoides* Lahaul and *H. salicifolia*, respectively (Fig. 2C). Based on the seed germination and seedling growth response, *H. salicifolia* proved more tolerant to Cd than both the *H. rhamnoides* populations (Fig. 3).

## Effects of Cd on metabolic parameters

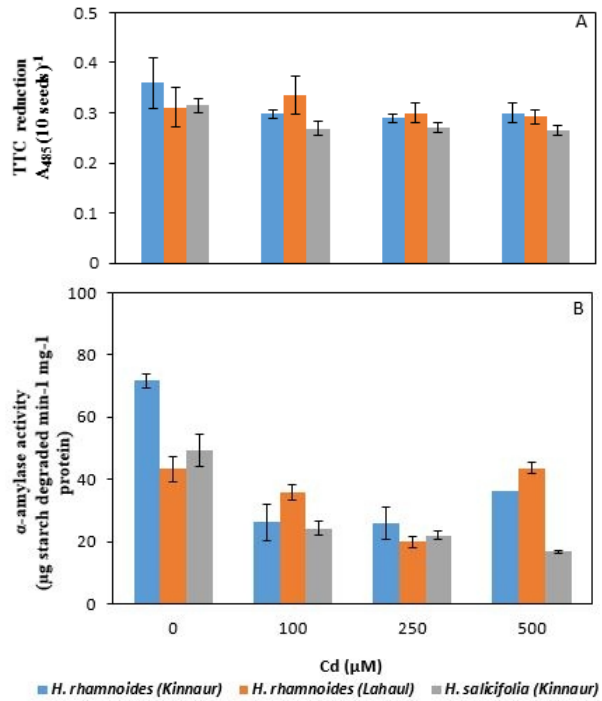


**Figure 4:** Effects of Cd on TTC (2,3,5 triphenyltetrazolium chloride) reduction ability (A) and α-amylase activity (B) in the seeds of *H. rhamnoides* Kinnaur and Lahaul populations and *H. salicifolia*. Values are arithmetic means± S.E.; n = 3.

**TTC reduction ability of seeds:** Due to the treatment of seeds with Cd, the TTC reduction ability, a measure of dehydrogenase activity, was slightly suppressed particularly at the higher concentrations. For example, a decrease of 17, 6 and 15% was observed at 500 µM Cd in *H. rhamnoides* Kinnaur, *H. rhamnoides* Lahaul and *H. salicifolia,* respectively (Fig. 4A).

**α-amylase activity:** Cd treatment of seeds for 24 h led to the concentration dependent suppression of α-amylase activity in all *Hippophae* species/populations. In *H. rhamnoides* Kinnaur, an inhibition of 63 and 50% was observed at 250 and 500 µM Cd, respectively (Fig. 4B). In *H. rhamnoides* Lahaul, the inhibition values were 18 and 53% respectively, at 100 and 250 µM Cd with no inhibition at 500 µM. In case of *H. salicifolia.* the magnitude of inhibition was 51, 55 and 65% at 100, 250 and 500 µM Cd, respectively (Fig. 4B).

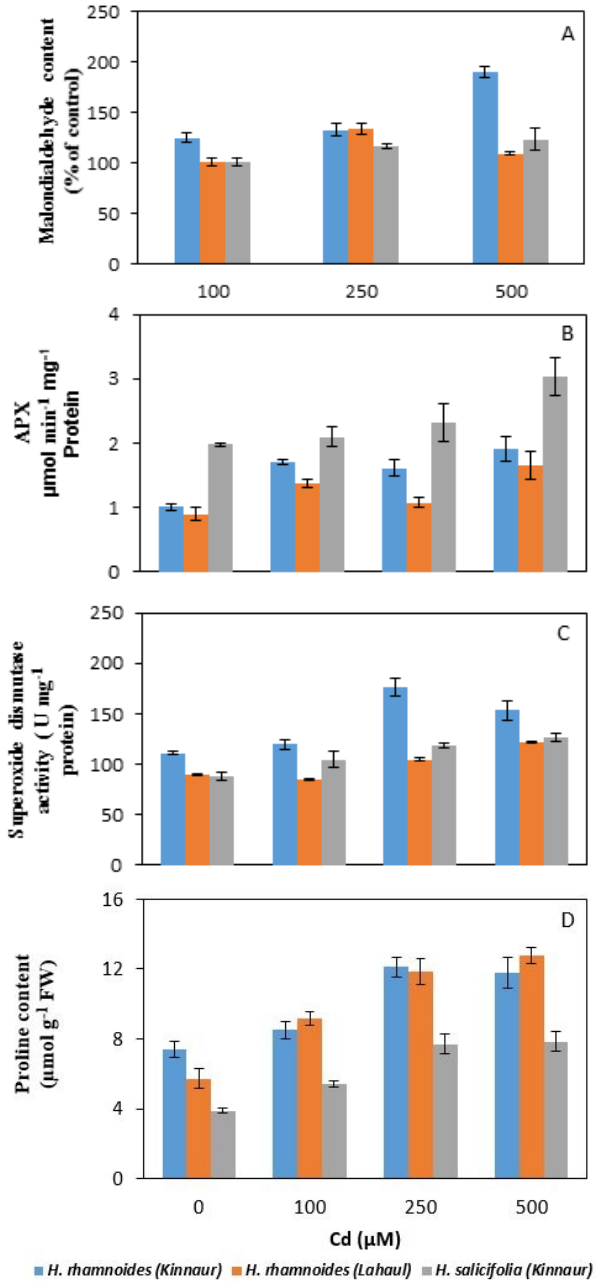


**Figure 5:** Effects of Cd on MDA contents **A.** APX activity **B.** SOD activity **C.** and free proline contents **D.** in 15-d-old seedlings of *H. rhamnoides* Kinnaur and Lahaul populations and 30-d-old seedlings of *H. salicifolia.* Values are arithmetic means± S.E.; n = 3.

**Lipid peroxidation and activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) :** Lipid peroxidation (MDA contents) and activities of SOD and APX were measured in 15 and 30 d old seedlings of *H. rhamnoides* populations and *H. salicifolia,* respectively. Due to Cd treatment, MDA contents were generally found to increase with an exception of no change in *H. salicifolia* at 100 µM. Thus, the MDA contents were 100, 116 and 123% (*H. salicifolia*), 125, 132, and 138% (*H. rhamnoides* Kinnaur) and 101, 133 and 109% (*H. rhamnoides* Lahaul) of control at 100, 250, 500 µM Cd, respectively (Fig. 5A).

Cd treatment led to enhanced APX activity in the seedlings. The activity increased by 70, 60, and 89% (*H. rhamnoides* Kinnaur), 35, 17 and 46% (*H. rhamnoides* Lahaul) and 17, 29 and 43% (*H. salicifolia*) at 100, 250 and 500 µM Cd, respectively, (Fig. 5B). The constitutive SOD activity was found to be highest in the seedlings of *H. rhamnoides* Kinnaur followed by that in case of *H. rhamnoides* Lahaul and *H. salicifolia*, respectively. Due to Cd treatment, SOD activity increased in a concentration dependent manner. The magnitude of increase was17, 58 and 38% in *H. rhamnoides* Kinnaur, 0, 16, 35% in *H. rhamnoides* Lahaul and 19, 33, 43% in *H. salicifolia* at 100, 250 and 500 µM Cd, respectively (Fig. 5C).

**Free proline contents:** Due to Cd treatment, the seedling proline contents increased in a concentration dependent manner in all three seabuckthorn populations with some quantitative differences. Proline contents were found to be 1.1-, 1.6-, and 1.6- fold those of control in *H. rhamnoides* Kinnaur at 100, 250 and 500 µM Cd, respectively (Fig. 5D). The proline contents doubled in *H. rhamnoides* Lahaul seedlings at 500 µM Cd. In case of *H. salicifolia*, the seedling proline contents were 1.4-, 1.9- and 2-fold those of control at 100, 250 and 500 µM Cd, respectively (Fig. 5D).

## DISCUSSION

The aim of study was to assess the seed germination and seedling growth responses of two populations of *H. rhamnoides* (Kinnaur and Lahaul) and one that of *H. salicifolia* to a range of Cd concentrations. The idea was to get insight into the species- and population-

specific differences in the response so that relative tolerance status could be assigned. The studied *Hippophae* species are distributed in Himalayan states including Himachal Pradesh (Singh 2003). In view of the tremendous importance of *Hippophae* species in economic, medical and ecological context, attempts are being made for their commercial cultivation beyond the current locations. Since the soil conditions in the newer locations might differ in terms of different parameters including the prevailing abiotic stress factors such as water-deficit, salinity, toxic metal ions etc. The stress tolerant populations/genotypes would apparently be preferred for better survival rates over the sensitive ones.

In a time-course pattern analysis, seed germination initiation was found to be delayed by 10-14 d in *H. salicifolia* than that in the two *H. rhamnoides* populations. Such a difference is attributable to variations in the seed characteristics such as seed coat features (Baskin and Baskin 2004) and could also be related to differential levels of seed germination inhibitors, if any (Sharma *et al.* 1986, 1988). The magnitude of inhibitory Cd effect on seed germination was found to be lower in *H. salicifolia* than in *H. rhamnoides*. Certain Cd concentrations, in fact, marginally stimulated the seed germination in the former. This is comparable to the promotion of seed germination due to low HM concentrations, for example, in *Pisum sativum* (Sharma 1985a) and *Stylosanthes humilis* (Delatorre and Barros 1996). The precise significance of such promotion by a toxicant is elusive.

Essentially due to a difference of time period in germination initiation (as above), 30 and 15 d old seedlings of *H. salicifolia* and *H. rhamnoides* appeared developmentally comparable. Therefore, the seedling growth responses to Cd were compared at these stages of the two species. Cd suppressed the seedling growth with organ- and population-specific differences in response to Cd. The magnitude of Cd-dependent root growth suppression was invariably greater than that of shoot growth. A straight forward explanation for such difference is retention of a larger part of Cd burden in the roots following uptake (Schat and Kalff 1992). As in case of seed germination, on the basis of seedling growth parameters also, *H. salicifolia* proved more tolerant to Cd than *H. rhamnoides*.

In order to get insight into the metabolic basis of Cd-induced inhibition of seed germination and seedling growth in the tested *Hippophae* species, certain associated metabolic parameters were determined in the Cd-treated seeds or seedlings. To begin with, the TTC reduction ability that indicates the dehydrogenase activity, of the embryos excised from Cd treated seeds was marginally suppressed to a comparable extent in all cases. This signifies the Cd interference with respiratory activity and is comparable to Ni effects in *Brassica juncea* (Thakur and Sharma 2015). Similar Cd inhibitory effects were observed on the α-amylase activity in the Cd-treated seeds of the studied species/population. This would be expected to restrict the availability of mobilizable sugars to the developing embryo that in turn inhibited the seed germination. The hydrolytic enzymes are reported to be inhibited by HMs (Sharma 1985a).

The Cd concentration-dependent increase in MDA contents of the seedling tissue of the tested species/populations implies the production of reactive oxygen species (ROS). The latter could be assumed to contribute to the cellular damage and in turn inhibition of germination and seedling growth. Similar findings have often been reported with other plant species e.g., *Hordeum vulgare* (Sharma *et al.* 2004) and *Pisum sativum* (Pandey *et al.* 2009). It was interesting that at low Cd concentration (100 μM), there was no increase in the MDA contents in *H. salicifolia* that is relatively more Cd tolerant and consequently suffered lesser damage. The enhanced SOD and APX activities in Cd-treated seedlings occurred in order to scavenge $O_2^-$ and $H_2O_2$. The degree of enhancement, however, was comparable across the species/populations. Altered activities of antioxidative enzymes in

response to HM stress are often reported (Sharma *et al.* 2004, Thakur and Sharma 2015). Accordingly, the HM- imposed redox imbalance comprises a major factor in development of HM toxicity. Therefore, the HM/metalloid tolerance is partly due to stronger antioxidative defense (Thakur and Sharma 2015, Kumar *et al.* 2021). The increased free proline contents observed in the seedlings of all plants is consistent with its role in Cd tolerance. Besides being an osmoregulant (Kavi Kishor *et al.* 1995), proline could scavenge free radicals (Kaul et al., 2008) and protect enzymes against HM-induced inhibition (Sharma *et al.* 1998).

Since the measured metabolic parameters are influenced similarly by Cd in the tested *Hippophae* species/populations, the observed differential Cd tolerance of *Hippophae* species could not be explained by them. The latter might be ascribed to differential Cd uptake and/or its cellular management including vacuolar compartmentalization (Sharma *et al.* 2016, Sharma *et al.* 2017). In conclusion, Cd proved toxic to the *Hippophae* species/populations with *H. salicifolia* being tolerant than others. It could survive better on soils with elevated HM concentrations.

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