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RESEARCH ARTICLE

Assessing the role of EDTA and SA in mustard under Cd and Pb stress

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Abstract

Phytoextraction is an economically viable and environmentally sustainable method for approaching heavy metal-polluted soils. Pollen grains are notably more susceptible to pollutants compared to other parts of the plant. However, in polluted environments, certain metals like lead (Pb) and cadmium (Cd) may have limited bioavailability, posing significant challenges to the phytoextraction process. One way to overcome this limitation is by employing chelators. The experiment was conducted in a randomized block design to investigate the effects of two chelators, EDTA and salicylic acid (SA), in enhancing the plant water status, biochemicals and reproductive parameters of *Brassica juncea* seedlings. These seedlings were sown in pots and subjected to various treatments of Cd and Pb of concentrations 10 and 20 mM, either alone or in combination with 0.5 mM EDTA and SA. The toxicity induced by Pb and Cd in *B. juncea* was recorded through decreased levels of osmotic potential in leaves and roots and decreased leaf chlorophyll content. Additionally, various reproductive parameters, including pollen viability, pollen germination, and *in-vitro* pollen germination, were highly affected by stress. Conversely, the supplementation of EDTA and SA to Cd, and Pb-treated plants effectively mitigated the negative impact of heavy metal stress by increasing osmotic potential, chlorophyll content, and reproductive parameters. Notably, EDTA and SA also significantly increased Pb and Cd accumulation. The result demonstrates the potential of EDTA and SA in improving plant reproductive biology.

Keywords: Chlorophyll, Pollen germination, Pollen viability, Phytoremediation, Chelators.

Introduction

The issue of metal toxicity in both air and agriculture has become a significant global concern, escalating due to both natural phenomena and human activities (Thounaojam T.C. *et al.*, 2012). In recent years, numerous scientists worldwide have investigated the toxicity, accumulation, and adverse effects of metal stress on crops. Research endeavors have predominantly centered around comprehending biochemical and physiological mechanisms such as seed sprouting, the process of photosynthesis, water transpiration, and the aging of leaves. Elevated production of reactive oxygen species (ROS), known to cause damage to proteins and lipid membranes, serves as a key indicator of oxidative stress in plants (Panda & Choudhury, 2005; Sabrine *et al.*, 2010) and (Hassan & Mansoor, 2014). Pollen grains serve as the male reproductive cells of plants, dispersing through the air to facilitate pollination. The rise in industrial activities and the widespread use of petroleum-based products have contributed to escalating air pollution levels, particularly in urban and industrialized regions. Pollen grains are notably more susceptible to pollutants compared to other parts of the plant. Monitoring pollen tube growth and pollen germination serves as a sensitive indicator for detecting various pollutants, including air pollution, pesticides, heavy metals, and acid rain (Gür & Topdemir, 2005). Exposure to air pollutants during pollination can compromise pollen viability, particularly due to metal toxicity, ultimately impacting their fertility. *Brassica* are important crops throughout the world and they produce 3-celled pollen. Previous investigations into *in-vitro* pollen germination have highlighted challenges such as inconsistent and genotype-dependent responses (Chiang, 1974). A media was developed characterized by elevated pH levels (8.0-9.0) and concentration of sucrose (20%) to address these

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issues (Roberts *et al*., 1983; Hodgkin, 1983). *Brassica napus* is known for its capacity to accumulate high levels of cadmium (Cd), (Selvam & Wong, 2009). This accumulation has been linked to inhibited growth, decreased levels of photosynthetic pigments, antioxidant enzymes activity, and increased levels of MDA and ROS within *B. napus* plants (Ali *et al*., 2014). Furthermore, Cd exposure has been observed to cause severe damage to leaves and root. Due to high mobility within plant tissues, cadmium (Cd) can easily move to reproductive organs like flowers and pollen, causing disturbances in development and hindering seed formation. As per a literature review, Cd^{2+} demonstrates significant toxic effect on *in-vitro* germination of pollen and pollen tube growth in Easter Lily (Zhang *et al*., 1999). Additionally, Cd^{2+} was identified as the sole metal among those screened to induce intracellular interactions and alter organelle distribution within pollen tip regions (Sawidis & Reiss, 1995). Numerous studies have demonstrated the inhibitory effects of cadmium on reproductive parameters (Sabrine *et al*., 2010; Wang *et al*., 2014). Furthermore, Cd negatively impacts plant reproduction by impeding pollen germination and ovule growth, resulting in the development of inviable flowers and shrunken grains (Kumar S. & Dhingra H. 2005). The common visible effects of stress produced by cadmium (Cd) in plants consist of chlorosis, necrosis, leaf curling, hindered pollen germination and tube elongation, and a general slowdown in growth. Heavy metals negatively impact plant metabolism by hindering nutrient absorption, interrupting photosynthesis through disturbances in chlorophyll metabolism and damage to chloroplast structure, and changing nitrogen and sulfur metabolism. (Benavides, 2005), and (Hasanuzzaman *et al*., 2012). The use of chelating agents has been observed to mitigate cadmium (Cd) toxicity by modulating Cd uptake in various plant species, including *Vigna unguiculata* (Agbadah *et al.,* 2016), *Oryza sativa* (Xu *et al*., 2010), and *Phaseolus vulgaris* (Hardiman & Jacoby, 1984). Furthermore, chelating agents have demonstrated efficacy in enhancing antioxidant defense mechanisms and promoting overall plant growth under Cd stress conditions (Farid *et al*., 2015) and Xu *et al*., 2009).

Fertilization of the pistil following flower pollination is imperative for fruit set. The viability, tube growth, and morphological consistency associated with pollen quality are crucial traits that are of interest to plant breeders, geneticists, and growers. Consequently, numerous researchers have explored methods to inhibit viability, tube growth, and alter pollen morphology using various bioregulators such as kinetin, gibberellic acid and brassinolide, in almond (Maita & Sotomoyar, 2015), jasmonic acid, ACC, and lead (Pb) and cadmium (Cd) in *Picea wilsonii* (Sheng *et al.,* 2012; Wang *et al.,* 2014). While numerous studies have investigated the influence of heavy metals on morpho-physiological parameters in plants, despite the crucial significance of sexual reproduction in plants, there is still a lack of extensive research on the impact of heavy metal toxicity on this process, along with the associated physiological and biochemical mechanisms, particularly under the influence of multiple heavy metal stresses.

In current investigation, we assessed how pollen germination and its length were affected under heavy metal stress and chelators help to improve the reproductive biology of *Brassica napus* pollen. The phytoremediation method employs plants, either alone or in conjunction with associated microorganisms, to mitigate the presence of harmful contaminants, a technique that has gained widespread acceptance and application in recent years. This approach is valued for its cost-effectiveness, sustainability, environmental friendliness, and non-intrusive nature, making it poised to play a pivotal role on an industrial scale provided proper considerations are taken into account, such as the type of pollutant, waste composition, seasonal variations, and plant diversity (Lin *et al*., 2002; Yang *et al*., 2017). Phytoremediation functions by leveraging the inherent ability of certain plants to absorb and accumulate metals, thereby sequestering them within plant biomass. The aim of this remediation strategy is to reduce heavy metal concentrations in contaminated soil, rendering the plants suitable for various beneficial uses such as forestry, horticulture, agriculture, and grazing. Numerous plant species utilize a variety of strategies to alleviate metal toxicity, such as binding metals to cell walls, blocking their transport across cell membranes, actively removing them, segregating them into compartments, expelling them, and internally chelating metals. (Singh & Singh, 2017).

Material and Methods

The present study was conducted at the Herbal Garden, Maharshi Dayanand University (Rohtak), India (latitude 28° 54' 0" N, longitude 76° 34' 0" E, Altitude 220 m). *Brassica juncea* L. healthy seeds of genotype (RH0725) were procured from the Chaudhary Charan Singh Haryana Agriculture University (HAU) Hisar, India. Six (kg) of sand was placed into the 12-inch pots lined with polyethylene bags. The sand underwent cleaning with 0.1 N HCl to remove fungal contamination, followed by rinsing with DDW before being transferred into the pots. The study was designed according to a Complete Randomised Design (CRD) methodology, with three replications. The aim of this study was to examine the response of Indian mustard (*B. juncea*) to heavy metal (Pb and Cd) at 0 mM (Control), 10, 20 mM, and chelators application, i.e., EDTA and SA (0.5 mM) to the phytoaccumulation capacity of *B. juncea* for different concentration of heavy metals (Pb and Cd). *Brassica* seeds were grown for a period of 30 days in soil that had been artificially treated with various levels of heavy metals. After an interval of thirty days, the initial sowing process occurred. The chelator EDTA and SA (0.5 mM) were added, and the plants were later grown for an additional fifteen days. Prior to the sowing procedure, an extensive soil sample was collected to determine the concentration of heavy metals. The experiment was carried out in adhering to normal agronomic procedures. After two weeks of seeding and thinning, they were placed in each pot. The pots were then subjected to various treatments involving heavy metal stressors (Cd and Pb) and chelating agents (EDTA and SA) (Table 1):

The plants were recorded using Wilson and Reisenauer's (1963) nutrient solution, with 200 ml added per pot. At 50 days following seeding, the plants were tested.

Tetrazolium chloride test for pollen viability

Pollen grains were harvested from plants exposed to varying concentrations of heavy metal, Cd, and Pb treatments, following the protocol outlined by (Hauser and Morrison, 1964). Pollen samples were placed in a drop of 0.5% TTC solution and immediately shielded to prevent oxygen exposure, which can hinder dye reduction. The prepared slide was then placed in a petri dish with moist filter paper and kept at room temperature for 2 hours. The viability was assessed as a percentage by counting the number of stained pollen grains out of 100 randomly selected pollen in each replicate.

Pollen tube length and pollen germination

A semi-solid medium was prepared using sucrose, boric acid, calcium nitrate, and agar. Use a brush to evenly spread the pollens onto the semi-solid medium in petri dishes. Incubate the plates at a temperature of 25 ± 2°C for 3 hours in darkness. Following incubation, stop germination by flooding the medium with killing and fixing solution as described by Sass (1951). Observe and document the germination of pollen grains and the length of their tubes using a light microscope.

Table 1: Treatments involving heavy metal stressors (Cd and Pb) and chelating agents (EDTA and SA)

S. No.	*Treatments*	*Chemical combination*
1.	T_0	Control
2.	T_1	10 mMCd
3.	T_2	20 mMCd
4.	T_3	10 mMCd + EDTA
5.	T_4	10 mMCd + SA
6.	T_5	20 mMCd + EDTA
7.	T_6	20 mMCd + SA
8.	T_7	10 mMPb
9.	T_8	20 mMPb
10.	T_9	10 mMPb + EDTA
11.	T_{10}	10 mMPb + SA
12.	T_{11}	20 mMPb + EDTA
13.	T_{12}	20 mMPb + SA

Results

Osmotic potential

The osmotic potential of the leaf and root decreased with heavy metal stress from 10 to 20 mM lead and cadmium concentration. The osmotic potential of leaves and root showed a significant reduction by 18.54, 25.50% in cadmium stress and 21.59, 32.88% in lead stress as compared to control treatments. The co-application of EDTA and SA at 0.5 mM with cadmium and lead stress induces a significant elevation in osmotic potential in leaves and roots as compared to the application of cadmium and lead stress alone (Figure 1 A, B). The exogenous application of EDTA was found to increase its activity by 17.82 and 16.84% in cadmium and 21.62, 15.65% in lead-treated plants, respectively. Similarly, SA increased osmotic potential by 19.10 and 15.50% in root-treated 20 mM Cd stress. In addition, it was found that an increased amount of Pb led to an increase of osmotic potential by 21.62 and 16.16% in the shoot and roots.

Chlorophyll (SPAD)

The results show that treating *Brassica* plants with Cd and Pb led to toxicity symptoms, such as reduced chlorophyll content of leaves. The results suggest a reduction in tolerance throughout all parameters when subjected to

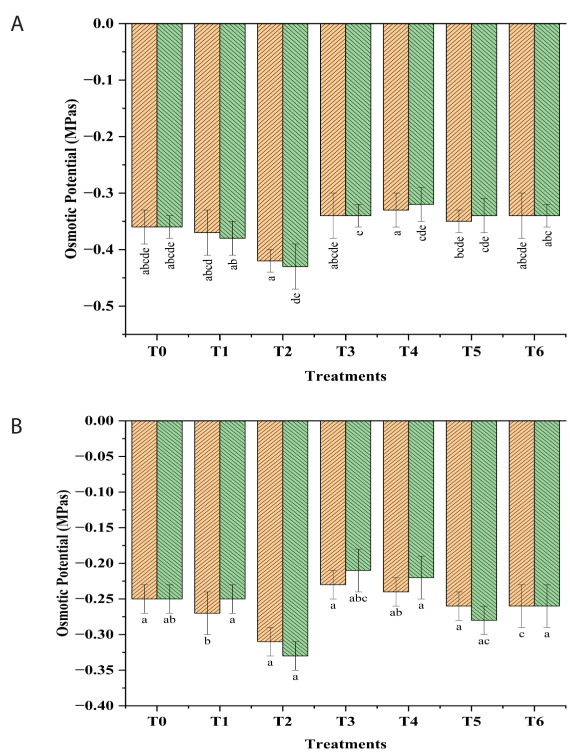

Figure 1: The effect of EDTA and SA on the osmotic potential of leaves (A), and roots (B) of *Brassica* under heavy metal stress, subjected to 0, 10, 20 mM Cd and Pb, respectively. Each value represents the average of three replicates, with standard error (SE) calculated. [Control (T0),10 mM (T1), 20 mM (T2), 10 mM+EDTA (T3), 10 mM+SA (T4), 20 mM+ EDTA (T5), 20 mM+SA (T6)]

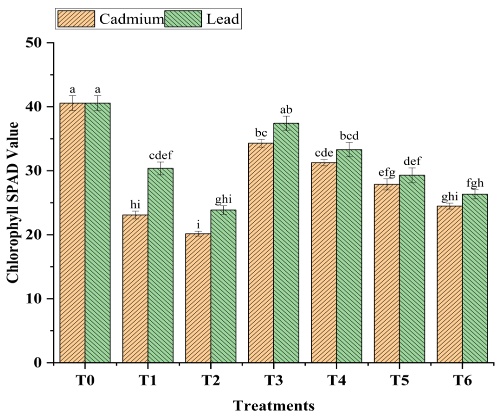

Figure 2: The effect of EDTA and SA on chlorophyll content (SPAD value) of *Brassica* under heavy metal stress, subjected to 0, 10, 20 mM Cd and Pb respectively. Each value represents the average of three replicates, with standard error (SE) calculated. [Control (T0),10 mM (T1),20 mM (T2), 10 mM+ EDTA (T3), 10 mM+SA (T4), 20 mM+EDTA (T5), 20 mM+SA (T6)]

raising the levels of Cd and Pb (20 mM). The exogenous application of EDTA and SA to plants treated with Cd and Pb has improved their tolerance to heavy metal stress, enhancing various parameters, including chlorophyll of leaves. The application of Cd at an amount of 20 mM brought about a substantial decrease in chlorophyll content in 50.28% of leaves when compared to the control group (Figure 2). The findings of this investigation indicate that the treatment of plants to Pb (20 mM) resulted in similar results. Notably, the introduction of lead stress led to a decrease in (41.16 %) of leaves when compared to the control group. Applying EDTA and SA has an essential effect on the photosynthetic pigments compared to plants subjected to heavy metals. The outcomes of the study indicate that the utilization of EDTA and cadmium treatment led to an essential improvement in the chlorophyll content of the plants compared to those treated merely with cadmium. The treatment of 20 mM Cd+EDTA showed a higher rise in chlorophyll (38.18%). Similarly, results were obtained when 20 mM Pb + EDTA application occurred, which led to a higher increase (22.76%) in comparison with the Cd-treated plant (Figure 2). The application of both EDTA and SA chelators led to significant rises in the photosynthetic pigments (Figure 2). Similar trend was observed with SA application with 20 mM Pb and Cd treatment, it increased its level form 10.33 to 21.32% chlorophyll content. The results indicate that cadmium displayed more significant toxicity than lead between the two heavy metal stressors.

Pollen Germination and Pollen viability

The results of TTC tests are shown in Figure 3. The interaction of genotypes x staining test was significantly different in the pollen viability percentage of genotypes in the staining test. One-way ANOVA revealed heavy metal stress and EDTA and SA significantly affected pollen viability ($p < 0.05$; Figure 3).

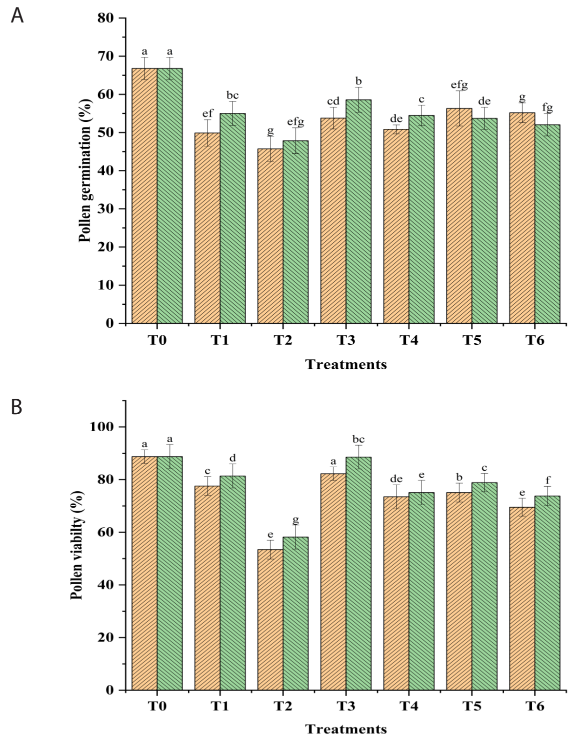

Figure 3: The effect of EDTA and SA on pollen germination (A) and pollen viability (B) of *Brassica* under heavy metal stress, subjected to 0, 10,20 mM Cd and Pb, respectively. Each value represents the average of three replicates, with standard error (SE) calculated. [Control (T0),10 mM (T1),20 mM (T2), 10 mM+EDTA (T3), 10 mM+SA (T4), 20 mM+EDTA (T5), 20 mM+SA (T6)]

The pollen germination and pollen viability were decreased with increased heavy metal stress from 10 to 20 mM lead and cadmium concentration. The pollen germination and pollen viability significantly reduced 31.55, 39.79% in cadmium stress, 28.36, and 34.45% in lead stress compared with the control treatments. The co-application of EDTA and SA at a concentration of 0.5 mM with cadmium and lead stress induces a significant elevation in pollen germination and pollen viability as compared to the application of cadmium and lead stress alone (Figure 3 A, B). The exogenous application of EDTA was found to increase its activity by 23.16 and 40.55% in cadmium and 12.13 and 35.04% in lead-treated plants, respectively. Similarly, SA increased pollen germination and pollen viability by 20.71 and 39.35% in plants subjected to 20 mM Cd stress. In addition, it was found that an increased amount of Pb with SA led to an increase of pollen germination and pollen viability by 8.73 and 19.49%.

Pollen tube length

Pollen tube length was decreased with the increase of heavy metal concentration. One-way ANOVA revealed heavy metal stress and EDTA and SA had significant effects on pollen germination ($p < 0.05$; Figure 4). The pollen tube length decreased with the increase of heavy metal stress from 10 to 20 mM lead and cadmium concentration. The

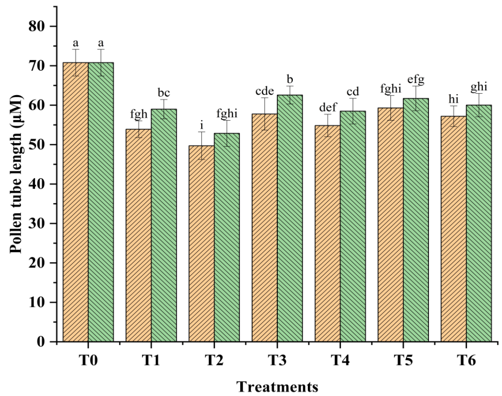

Figure 4: The effect of EDTA and SA on pollen tube length (A) of *Brassica* under heavy metal stress, subjected to 0, 10, 20 mM Cd and Pb respectively. Each value represents the average of three replicates, with standard error (SE) calculated. [Control (T0), 10 mM (T1), 20 mM (T2), 10 mM+EDTA (T3), 10 mM+SA (T4), 20 mM+EDTA (T5), 20 mM+SA (T6)]

pollen tube length was significantly reduced by 29.77, and 25.53% in cadmium and lead stress compared with the control treatments. The co-application of EDTA and SA at a concentration of 0.5 mM with cadmium and lead stress induces a significant elevation in pollen tube length as compared to the application of cadmium and lead stress alone (Figure 4). The exogenous application of EDTA was found to increase its activity by 20.11% in cadmium and 17.05% in lead-treated plants, respectively. Similarly, SA increased pollen tube length by 15.02% in plants subjected to 20 mM Cd stress. In addition, it was found that an increased amount of Pb with SA led to increase of pollen tube length by 13.87%.

Correlation

Figure 5 depicts the a correlation graph based on Pearson's correlation coefficient was created to analyze the relationships among different parameters, such as biochemical and reproductive parameter of *Brassica juncea* under heavy metal stress Cadmium (A) and Lead (B).

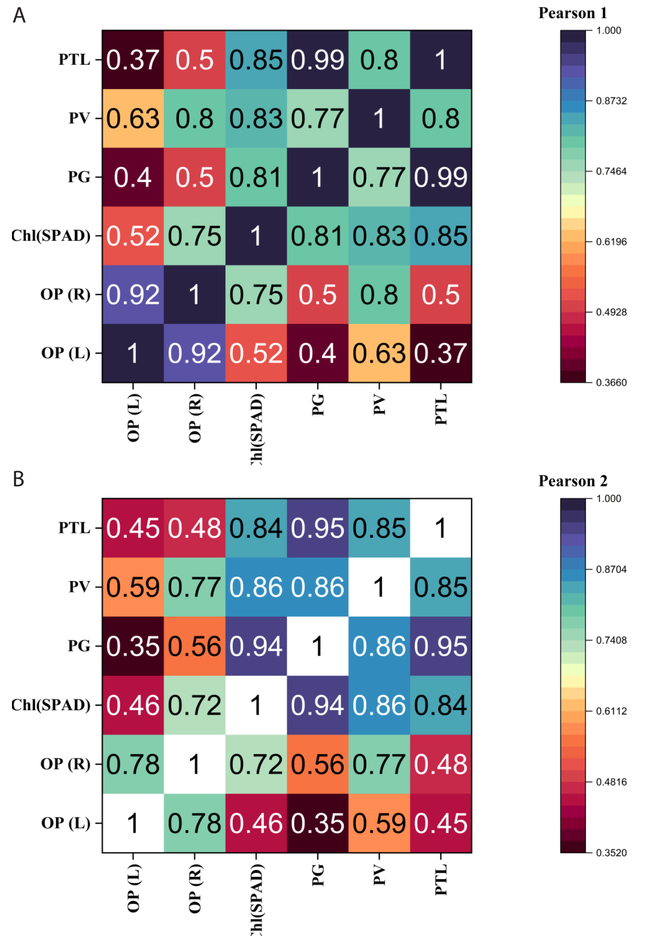

Figure 5: A correlation graph based on Pearson's correlation coefficient was created to analyze the relationships among different parameters, such as biochemical and reproductive parameter of *Brassica juncea* under heavy metal stress Cadmium (A) and Lead (B). The mean values of different variables were standardized and grouped together in this investigation. The color scale illustrates the intensity of the normalized mean values across different factors. The abbreviation used in figure are as follows: OP(L): Osmotic Potential in Leaves; OP(R): Osmotic Potential in Roots ; Chl (SPAD): Chlorophyll SPAD; PG: Pollen germination; PV: Pollen Viability; PTL: Pollen tube length.The lines extending from the central area of the biplots indicate either negative or positive correlations with different parameters, while their proximity to each other indicates the strength of the correlation between that parameter and the line

Discussion

Chlorophyll content (SPAD)

The present investigation revealed a significant decrease in photosynthetic pigment was in leaves of *Brassica* genotypes subjected to different levels of lead and cadmium heavy metal stress. The chlorophyll content was decreased owing to the heavy metal stress and more decline was observed in the *Brassica* genotype with different heavy metal levels (10, 20 mM) in comparison to non-stressed plants (Figure 2). Earlier reports by Kanwal *et al*., 2014 revealing that a marked decline in chlorophyll levels and gas exchange metrics in *B. napus* L. leaves due to toxicity of Pb in comparison to plants under normal conditions. However, the introduction of EDTA externally demonstrated a notable improvement in chlorophyll levels and gas exchange parameters in the presence of lead-induced stress. Our finding suggested that EDTA and SA relatively reversed the heavy metal stress-induced impairment in chlorophyll content. EDTA stands out as a widely recognized and effective chemical agent due to its robust chelating properties, recoverability, and relative biochemical stability, making it an instrumental tool in soil remediation efforts (Meers *et al.*, 2005). The incorporation of EDTA is recognized as a pivotal factor in regulating metal leaching processes (Luo *et al*., 2005). The use of SA showed a notable rise in chlorophyll levels in rice seedlings exposed to lead-induced stress (Jing, *et al*., 2007). SA's effects, influenced by factors such as time and concentration, exhibit dual outcomes depending on the plant species, yet at optimal concentrations, they positively impact chlorophyll levels and photosynthesis (Belkhadi *et al*., 2010). The augmentation of chlorophyll content is likely attributed to enhancing cell permeability, and the formation of novel proteins (Popova *et al*., 2003). Several studies have reported SA-induced enhancements in photosynthesis in corn and soybeans (Kaydan *et al*., 2007; Khatibi *et al*., 2008). Additionally, SA treatment has been observed to elevate chlorophyll levels in spinach (*Spinacia oleracea*) (Eraslan F. *et al*., 2008). Similarly, the application of SA in barley seedlings has been shown to mitigate cadmium toxicity (Metwally A. *et al*., 2003).

Osmotic potential

Osmotic potential of leaves and roots in *Brassica napus* genotypes decreased with increasing of heavy metal lead and cadmium stress from 10 to 20 mM (Figure 1A,B). As per Kramer P.J. and Boyer J.S. 1995, the hyperaccumulation of heavy metals resembles the osmotic adjustment mechanism, which entails the accumulation of compatible organic solutes and/or inorganic ions like Na^+, K^+, and Cl^- during water or salt stress. This accumulation helps decrease the osmotic potential within cells, maintaining turgor pressure and cellular function. Similarly, Baker and Walker(1989) proposed that metals might be hyperaccumulated to elevate cellular osmolarity. Heavy metal stress negatively impacted various water-related attributes. However, the addition of EDTA and salicylic acid to the growth medium resulted in a significant rise in osmotic potential. The chelators create complexes with heavy metals, thereby boosting water potential. The reduction in water potential may be linked to the accumulation of osmolytes, which helps alleviate the negative impacts of metal stress (Kavi Kishor & Sreenivasulu, 2014). In our observation, *B. juncea* plants treated with EDTA and salicylic acid, either alone or in combination, showed increased water potential when subjected to lead and cadmium stress. This increase in water potential could contribute to maintaining a high photosynthetic rate and biomass production.

Pollen germination, pollen viability and pollen tube length

The present investigation revealed a significant decrease in reproductive parameters of *Brassica* genotypes subjected to different levels of lead and cadmium heavy metal stress. The pollen germination, pollen viability and pollen tube length (Figure 4) were decreased owing the heavy metal stress and more decline was observed in the *Brassica* genotype with different heavy metal levels (10, 20 mM) in comparison to non-stressed plants (Figure 3A, B). The *in-vitro* impact of heavy metal reveals its diminishing effect on pollen tube growth due to the adverse influence on treated pollen grains. Prior research has suggested that heavy metals can diminish pollen germination rates and pollen tubes' growth, though the precise mechanisms involved are not fully understood (Sawidis, 2008) and (Breygina *et al*., 2012). In general, the existence of heavy metals hindered the germination of pollen grains and the growth of pollen tubes. However, there was an observed stimulating effect in the early stages at concentrations below the inhibitory level (10–10–10–12 M). Numerous researchers have noted that low doses of heavy metals can stimulate both pollen germination and tube growth (Sawidis, 2008). In this investigation, *B. juncea* treated with EDTA and salicylic acid, both individually and in combination, exhibited elevated pollen germination, pollen viability and pollen tube length under lead and cadmium stress, potentially sustaining a high photosynthetic rate and biomass production. This phenomenon, called hormesis, associated with the sensitivity of organisms, describes the beneficial effects of a small dose of a toxic substance (Xiong & Peng, 2001) (Stebbing, 1998). Tedeschini *et al.,* 2015 noted a comparable result, finding that applying chelators to the leaves decreased levels of reactive oxygen species in pollen grains and increased pollen viability. The cuticle of the rice anther comprises a thin, hydrophobic layer covering its outer surface, providing protection for microspore development (Zhang *et al*., 2011).

Conclusion

Heavy metals adversely affect chlorophyll content, osmotic potential and reproductive parameters. EDTA and salicylic acid (SA) have been employed in numerous studies involving various species within the Brassicaceae family, serving as metal chelators to investigate a variety of physiological and biochemical parameters. EDTA shows more effectiveness in minimizing the adverse effects in *Brassica*. The phytoremediation potential of EDTA and SA mitigating the adverse impact of heavy metal stress on biochemical and reproductive parameters in *Brassica* was determined in the present study. Comparing various heavy metal treatments, either applied alone or combined with EDTA and SA, indicated that combining EDTA and SA with Cd and Pb was the most effective in alleviating the adverse effects of heavy metal stress. Synergistic effects were observed with the application of EDTA and SA, even under severe heavy metal stress (20 mM), leading to the amelioration of biochemical processes and reproductive parameters. EDTA and SA treatment significantly improved the osmotic potential, chlorophyll content and reproductive parameters.

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