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Research Article

# Trehalose-Induced Regulations in Physio-biochemical Attributes of *Helianthus annuus* L. under Lead Stress

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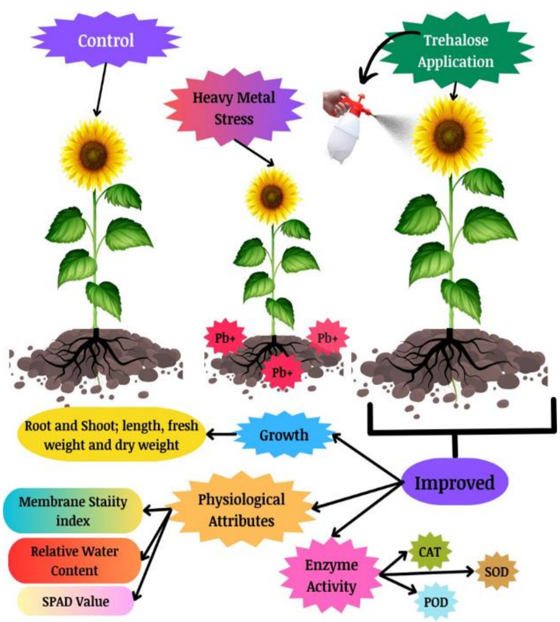
## Highlights

- Heavy metal stress significantly impairs various aspects of plant growth and physiology, including root and shoot length, biomass production, plant water relations, membrane stability, chlorophyll content, and antioxidant enzyme activities.
- The application of trehalose effectively mitigates the detrimental effects of heavy metal lead acetate stress on plants. Trehalose treatment leads to improvements in plant physiology, enhanced water relations, and strengthened antioxidant defense mechanisms.
- Trehalose treatment results in reduced levels of stress markers such as malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$), indicating a decrease in oxidative stress in plants subjected to heavy metal toxicity.

## Graphical Abstract

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**ABSTRACT**

Heavy metal is a substantially severe abiotic environmental stress that is rising in many regions of the world. Current climate change circumstances and rapid industrialization increased heavy metal accumulation in soil and water. Overall, worldwide agricultural production decreased. Trehalose, a well-known plant growth regulator, is crucial in plants' responses to a variety of abiotic conditions. The Morpho-physiological and biochemical parameters were analyzed in hydroponic research to identify the possible role of trehalose in alleviating heavy metal lead acetate stress in sunflower (*Helianthus annuus* L.) crops. In the sole and mixed form, two $Pb(C_2H_3O_2)_2$ (Control and 30 micron) and

one trehalose (50 M $L^{-1}$) levels were used, and a half-strength Hoagland solution was used as a nutritional medium. The current study found that heavy metal stress had a significant negative impact on plant growth (root and shoot length), fresh and dry biomass, and plant water relations, as well as poor plant water relations, membrane stability, chlorophyll content, and antioxidant enzyme activities such as superoxide dismutase (SOD), peroxide (POD), ascorbate peroxide (APX), and catalase (CAT). MDA and $H_2O_2$ are the stress markers reduced by the application of trehalose. Exogenous treatment of trehalose, on the other hand, significantly mitigates the detrimental effects of metal toxicity by altering plant physiology, enhancing water relations, and providing robust antioxidant defense against heavy metal lead acetate stress. The findings of this study provide a new dimension to scientists' understanding of the beneficial usage of trehalose in plant science and offer up new research opportunities.
**Keywords**: Metal toxic environment, Trehalose, Sunflower, Antioxidant Defense, RWC, Lead.

## INTRODUCTION

The sunflower, (*Helianthus annuus* L.), ranks as the 4th largest oilseed crop worldwide, following soybean, palm oil, and canola. In Pakistan, it ranks as the third most important oilseed crop, following rapeseed. Heavy metal pollution is a ubiquitous problem caused by anthropogenic activities [1]. These significantly impact Agri-production losses [2]. The first decade of this century perceived a substantial improvement in the crop yield of sunflowers; however, the average crop yield has gradually decreased since 2010 [3]. Heavy metals impose strong and detrimental effects on plants' morpho-physical, biochemical, and osmotic attributes. Some essential heavy metals i.e., Cu, Co, Fe, Mg, Mo, Ni, and Zn play their role in plant development but, lead (Pb) makes stable complexes such as lead phosphate $Pb_3(PO_4)_2$, lead carbonate $PbCO_3$, lead sulfate $PbSO_4$, and hydroxides $Pb(OH)_2$, which degrade fertility of soil and crop yields in the agricultural sector [4]. Under current climate conditions globally 70% of agricultural yield is affected by heavy metals while 3.5 % [5]. of the world's geographical area is unaffected by it [6]. United States, China, India, Japan, and Pakistan, almost around the world have reported that Pb concentration in soils has gradually increased and become a key factor in reducing crop yields [7] [8]. This study focused on lead acetate $Pb(C_2H_3O_2)_2$ as stress for the hydroponic experiment on sunflowers. Lead acetate $Pb(C_2H_3O_2)_2$ is not biocompatible and its exposure to plants for a long time has a deleterious impact on seed growth, development of seedlings, and transpiration. It affects root growth, synthesis of chlorophyll, and cell division in plants [9, 10]. In exposure to heavy metals, plants must respond to trigger changes in a wide range of physiological and biochemical attributes to develop or adopt a series of strategies that allow them to manage the toxic consequences of heavy metals i.e., $Pb(C_2H_3O_2)_2$.

Synthetic organic osmolytes like salicylic acid [11], Polyamine [12], abscisic acid [13]. glycine betaine [14] melatonin [15, 16] and trehalose [17] are known as plant growth regulators; exogenously applied to crops help them to become more stress-tolerant [18]. Trehalose plays a significant role in plant functioning and it is considered to be a potential Osmo protectant. It stabilizes the cellular membranes, enzymes, proteins and protects the biological system from the damaging impacts of stress on plants [19, 20]. Trehalose protects the biological structures from toxicity by lead $Pb(C_2H_3O_2)_2$ stress through preventing damage during desiccation, through serving as a signaling molecule as antioxidant molecules and enzymes [21].

Numerous species, produce trehalose (Tre), a non-reducing disaccharide of glucose composed of dual units of glucose [22]. Trehalose is assumed to function by removing reactive oxygen species (ROS) and safeguarding the machinery which has to take charge of protein synthesis [23]. By subjecting sunflowers, to increasing levels of heavy metals aim is to determine the relative tolerance capacity of sunflowers in the presence of trehalose and measure their stress tolerance utilizing morpho-physiological, biochemical, antioxidative, and photosynthetic attributes.

Sunflower is an oilseed crop grown for edible oil production in the world [24]. About 87 % of vegetable oil is produced by sunflower [25]. This crop is well-liked among farmers because of its short lifespan, adaptability to abiotic stresses, and high cash return. The 3rd most important oilseed crop in Pakistan after Cotton, Rapeseed, and Mustard [26]. Due to the lower latitude of Pakistan, the disastrous effects of climate change on agricultural productivity result in Pakistan's persistently getting worse production of edible oil. The sunflower crop was grown on 0.09 M hectares, producing 0.11 and 0.04 M tons of seed and oil, respectively [27]. Recent studies have provided evidence indicating that plants possess the ability to uptake trehalose from exogenous sources, leading to its subsequent accumulation in various plant tissues. The observed phenomenon has been found to yield advantageous results for agricultural production. This study investigates the impact of heavy metal toxicity on several sunflower cultivars. Investigating the morpho-physiological and biochemical reactions of these cultivars about the changing Agricultural sector and prevailing environmental circumstances. The research hypothesis, trehalose application may have the capacity to mitigate the negative effects caused by exposure to metals. The work is a comprehensive protective mechanism that incorporates a range of tactics. These strategies are designed to safeguard biological membranes, enhance plant biomass, fortify antioxidative capacity, optimize nutrient levels, and preserve chlorophyll pigments in sunflower crops. The primary objective of this research was as follows: to assess the efficacy of trehalose-induced tolerance in sunflower plants exposed to stress generated by lead acetate. Moreover, the primary aim of this research is to evaluate the influence of trehalose on many aspects such as morphology, physiology, biochemistry, and antioxidant enzyme activities. Overall, this study examines the degree of resistance demonstrated by different sunflower cultivars in response to the toxicity induced by lead acetate.

Our results emphasize the vital role of trehalose in enhancing sunflower plant development and resistance under heavy metal stress and use this knowledge and information to extract the potential yield of sunflower in soils that have been impacted by heavy metals. This study also revealed that sunflowers can be used potentially as a phytoextractor.

## MATERIAL AND METHODS

### Experimental Description

The hydroponic system was successfully implemented at wire house. The above-mentioned institution is situated at an elevation of 116 meters above sea level at The Islamia University of Bahawalpur, Pakistan. The sunflower cultivars were planted in a substrate consisting of sand, characterized by elevated levels of humidity. These cultivars were subjected to an average daily duration of sunlight ranging from 10 to 12 hours, accompanied by a temperature of 30 °C. Before the commencement of the planting process, the seeds underwent sterilization by a thorough washing procedure including a solution comprising 2.5 % sodium hypochlorite (NaOCl). The seedlings that exhibited robust health and possessed comparable genetic traits were carefully retrieved, with meticulous consideration paid to the preservation of their root systems, and subsequently rinsed using distilled water. Once the seedlings have reached the stage of developing two leaves, they are carefully transplanted onto Polystyrene sheets. These sheets are then placed within plastic tubs, each with a capacity of 20 L. Each tub possesses the capability to accommodate a maximum of nine plants. The research utilized a metal acute toxicity level of 30 µM, achieved using the precise combination of lead acetate $Pb(C_2H_3O_2)_2$. After the transplantation of the seedlings, the application of trehalose was subsequently administered at a concentration of 50 µmol $L^{-1}$, both in its individual and combined forms, following a two-week gap. The study was conducted using a fully randomized design, comprising three replications. The research employed three distinct treatment groups: the Control group, which did not receive $Pb(C_2H_3O_2)_2$ or trehalose supplementation; the $Pb(C_2H_3O_2)_2$ 30 µM lead stress group; and the Pb+ Tre group, which experienced 30 µM lead stress along with 50 µmol $L^{-1}$ trehalose supplementation. The delivery of oxygen to the plants was facilitated through the utilization of aeration pumps. At the same time, a nutritional medium consisting of a Hoagland solution at a concentration of 50 % was utilized. The nutritional media utilized in this investigation consisted of various constituents $L^{-1}$. The components used in the experiment consisted of

$KH_2PO_4$ at a concentration of 68 grams, $KNO_3$ at a concentration of 101 grams, Ca $(NO_3)_2{\cdot}4H_2O$ at a concentration of 118 grams, $MgSO_4{\cdot}7H_2O$ at a concentration of 61.5 grams, $H_3BO_3$ at a concentration of 2.86 grams, $MnCl_2$ at a concentration of 1.81 grams, $ZnSO_4$ at a concentration of 0.22 grams, $CuSO_4{\cdot}5H_2O$ at a concentration of 0.08 grams, $H_2MoO_4{\cdot}H_2O$ at a concentration of 0.02 grams and Fe-EDTA at a concentration of 18.6 grams. The replenishment of the nutrition solution occurred on a weekly frequency. The pH of the growth medium was consistently maintained at a value of 6.0±0.5 throughout the whole duration of the experiment.

Plant Growth

The plant growth is significantly increased by height and weight. There are various distinct standards used in scientific literature to determine plant growth rates. Plant harvesting was done after 4 weeks of the establishment of treatments with stress. Plants are washed with tap water and quickly transferred to the laboratory.

**Chlorophyll Content**

Leaf chlorophyll concentration (SPAD) is measured by using a portable chlorophyll SPAD meter (SPAD-502-Minolta Japan).

**Relative Water Contents (RWC)**

Fresh leaf tissues were selected and after obtaining their fresh weight (FW), leaves were soaked for 24 hours to determine their turgid weight (TW). Subsequent samples were dried at 80°C to get their dry weight (DW) (Lazcano-Ferrat and Lovatt, 1999).

$RWC\ \% = (FW - DW) / (TW - DW) \times 100$

**Membrane Stability Index (MSI)**

MSI was determined using the method described by [30]. Leaf disc (100 mg) was heated in a water bath for 30 minutes at 40 °C in distilled water (10 ml), and the EC was determined ($C_1$). The same samples were heated at 100 °C for ten minutes, and EC was calculated as $C_2$. The membrane stability index was computed as

$MSI\ \% = [1 - (C_1 / C_2) \times 100]$.

**Stress Marker MDA and $H_2O_2$**

The methodology developed by Velikova was utilized to evaluate the amounts of hydrogen peroxide ($H_2O_2$) [31]. The determination of $H_2O_2$ concentration was conducted by assessing the optical density at a wavelength of 390 nm, employing a standard curve.

The examined-on lipid degradation in leaves encompassed the evaluation of malondialdehyde (MDA) concentrations, employing a leaf sample size of 200 mg. The foliage was submerged in a solution including trichloroacetic acid at a concentration of 0.1%. Following this, the samples underwent centrifugation at a magnitude of 10,000 times the acceleration due to gravity for 10 minutes. A volume of 1 ml of the supernatant was mixed with a solution of TBA with a concentration of 0.5 %.

The combination obtained was subjected to heat treatment at a temperature of 95 °C for 30 minutes. Subsequently, the mixture recorded centrifugation. The measurement of absorbance in the supernatant was conducted at a wavelength of 532 nm, and any non-specific absorbance detected at 600 nm was subtracted to correct the results. The quantification of MDA content was conducted by employing the extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ and the results were reported in terms of nmol $g^{-1}$ fresh weight [32].

**Antioxidant Enzymes**

The collection of samples and the subsequent assessment of absorbance are conducted under controlled temperature conditions ranging from 0 to 4 °C. The leaf fragments were gathered and homogenized using an extraction buffer solution consisting of 10 mL, containing 50 mM phosphate with a pH of 7.8, 1 g of polyvinyl pyrrolidone (PVP), 1 mM of EDTA, and 0.5 % Triton X-100, following the procedure described by Kamran [33]. The solution underwent centrifuging at a rotating speed of 12,000 rpm for 20 minutes. Subsequently, the liquid component, with a volume of approximately 2-3 mL, supernatant, was collected and utilized for enzyme analyses. The supernatant that was collected was used to investigate the activity of superoxide dismutase (SOD) using the NBT approach, with absorbance measurements taken at 560 nm [34]. The assessment of peroxidase (POD) activity was conducted by employing guaiacol, hydrogen peroxide ($H_2O_2$), and a $KH_2PO_4$ buffer solution supplemented with EDTA at a pH level of 7. The measurements were conducted at a specific wavelength of 470 nm utilizing a spectrophotometer. The assessment of catalase (CAT) activity [35]. In the study conducted by [34], the evaluation of ascorbate peroxidase (APX) activity was carried out using an enzyme extract consisting of potassium phosphate buffer and EDTA. The quantification and documentation of changes in light absorption levels at a specific wavelength of 290 nm were performed.

**Statistical Analysis**

The data acquired from the present experiment underwent analysis utilizing the statistical program Statistics 8.1 (Statistics USA) and is displayed graphically. The bars depicted in the graph represent the mean value derived from three separate replicates, while the error bars indicate the standard deviation.

**RESULTS**

**Plant Growth and Biomass**

This study aimed to evaluate the effects of a substantial lead concentration (30 µM) on the morphological traits of many sunflower cultivars. The experimental procedure involved the inclusion and exclusion of trehalose (50 µmol $L^{-1}$) as an exogenous supplement. The results obtained from the experiment revealed a decrease of 27 % in shoot fresh weight, 34% in shoot dry weight, 26 % in root fresh weight, 36 % in root dry weight, 11 % in shoot length, and 30 % in root length. However, the use of trehalose led to a significant improvement in various growth metrics. The shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, shoot length, and root length demonstrated statistically significant enhancements of 23 %, 21 %, 22 %, 31 %, 5 %, and 21 % respectively, in comparison to plants grown in a metal-contaminated substrate without the application of trehalose (Pb) mentioned in figure 1 and 2.

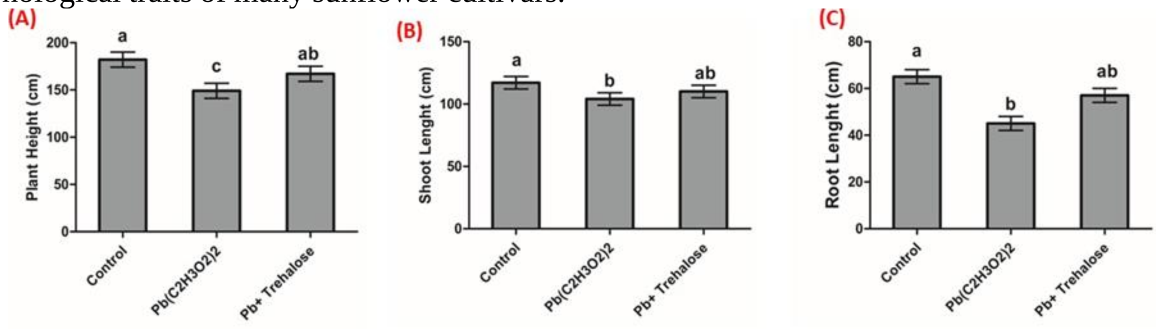


**Figure. 1** The effect of exogenous trehalose (Tre) supply on (A) Plant height (cm) (B) Shoot length (cm) (C) Root length (cm) of Sunflower (*Helianthus Annuus* L.) cultivars subjected to heavy metal stress.

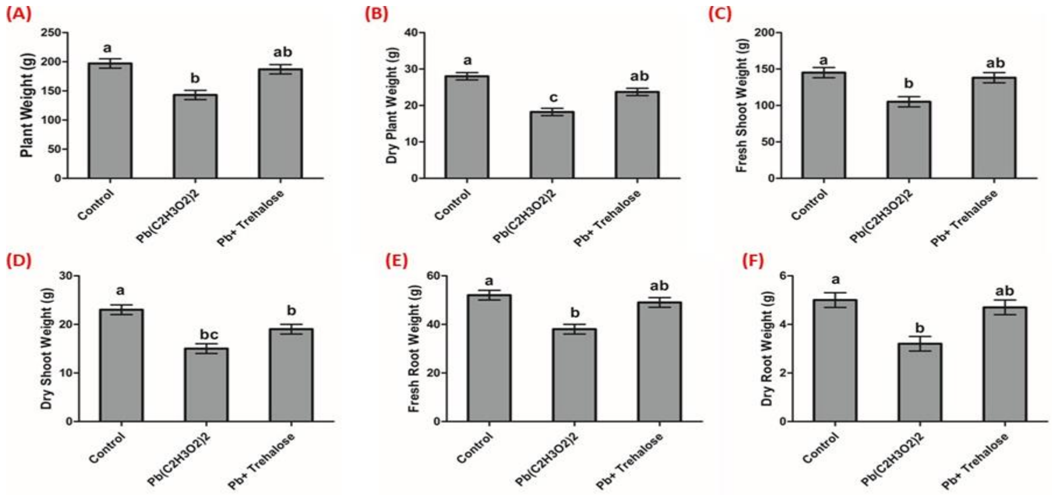


**Figure. 2** The effect of exogenous trehalose (Tre) supply on (A) Fresh plant weight (g) (B) Dry plant weight (g) (C) Fresh shoot weight (D) Dry shoot weight (g) (E) Fresh root weight (g) (F) Dry root weight (g) of Sunflower (*Helianthus Annuus* L.) cultivars subjected to heavy metal stress.

**Physiological Attributes**

The results about the comparative water levels, photosynthetic pigments, and membrane stability index of a hybrid sunflower variety when exposed to metal-induced stress conditions, specifically lead. In comparison to the control treatment, the relative water contents, membrane stability, and chlorophyll index exhibited a reduction of 38.16

%, 36.71 %, and 42.23 % respectively. The incorporation of trehalose in conjunction with metal stress (Pb + Tre) led to a notable augmentation in the levels of relative water contents (RWC), membrane stability index (MSI), and chlorophyll in sunflower seedlings. The introduction of trehalose in a metal-hazardous media resulted in significant increases in the RWC, MSI, and chlorophyll levels by 21.67 %, 19.35 %, and 29.73 % respectively, as compared to plants grown in the absence of trehalose mentioned in Figure 3.

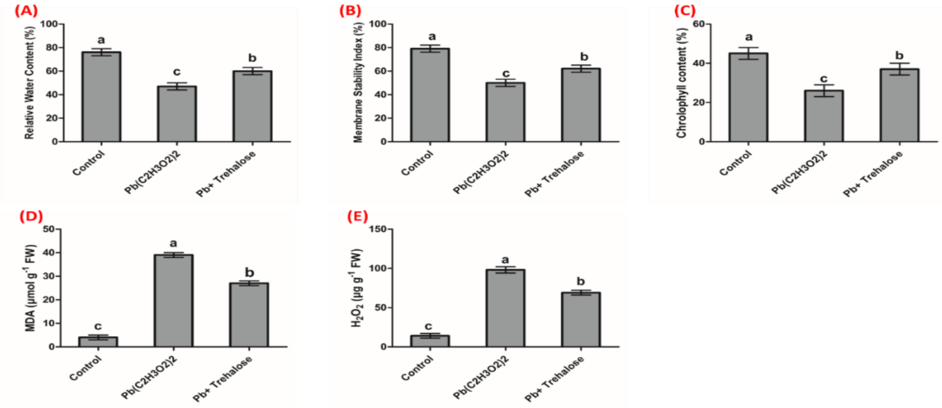


**Figure. 3** The effect of exogenous trehalose (Tre) supply on (A) Relative water content (%) (B) Membrane stability index (%) (C) Chlorophyll content (%) (D) MDA (µmol $g^{-1}$ FW) and $H_2O_2$ (µg $g^{-1}$ FW) of Sunflower (*Helianthus annuus* L.) cultivars subjected to heavy metal stress.

**Antioxidant Enzyme Activities**

The present study aimed to examine the impact of heavy metal toxicity, specifically at a concentration of 30 µM, on the enzymatic activity of several anti-oxidative enzymes in hybrid sunflower seedlings. The evaluation also encompassed the examination of the effects of trehalose supplementation at a concentration of 50 µmol $L^{-1}$. The findings indicate that the presence of heavy metals resulted in a reduction in the activity of antioxidant enzymes in sunflower plants. The investigation unveiled a significant decrease in the enzymatic functions of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX). The aforementioned decreases corresponded to percentages of 58.64 %, 26.09 %, 38.78 %, and 47.44%, respectively, about plants that were grown under standard conditions, which served as the control treatment. Nevertheless, previous studies have provided evidence indicating that the use of trehalose resulted in a significant augmentation in the enzymatic functions of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) by 18.75%, 15 %, 23.08 %, and 18 %, correspondingly. The reported augmentation was noted in plants cultivated in a substrate containing elevated concentrations of heavy metals, although lacking the inclusion of trehalose mentioned in Figure 4.

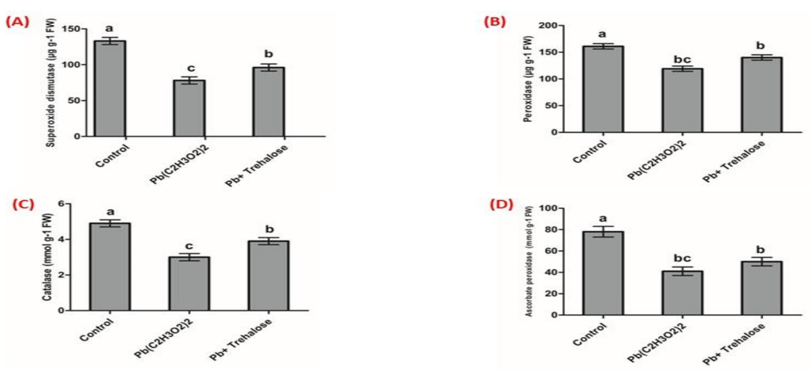


**Figure 4** The effect of exogenous trehalose (Tre) supply on (A) SOD (B) POD (C) CAT (D) APX of Sunflower (*Helianthus Annuus* L.) cultivars subjected to heavy metal stress.

## DISCUSSION

The sunflower, (*Helianthus annuus* L.), ranks as the 4th largest oilseed crop worldwide, following soybean, palm oil, and canola. In Pakistan, it ranks as the third most important oilseed crop, following rapeseed. The practice of growing crops in regions impacted by industrial activities is linked to efforts aimed at mitigating the issues related to the absorption and buildup of heavy metals in plants. The intricate interplay between metallurgical facilities and agricultural regions exerts a multifaceted impact on crops, mostly attributed to the uptake of heavy metals by plant roots and the infiltration of aerosol pollutants through the leaf epidermis. As per the findings of Aslam, the occurrence of heightened levels of metal toxicity in the growth medium has been associated with disruptions in morphological traits and physical attributes, alongside the suppression of antioxidant defense enzyme activity [39]. As a result, scientists have determined that this phenomenon harms the productivity of agricultural commodities [40]. A study that revealed the effectiveness of trehalose in mitigating the adverse effects of stress induced by heavy metals [41]. There is a direct association between increased concentrations of lead in the growth medium and a decline in certain plant growth attributes. The decline in performance can be ascribed to the instability of the membrane and the disturbance of photosynthetic processes. As a result, an imbalance arises in the course of nutritional intake, the discontinuation of cellular division, and the generation of reactive oxygen species (ROS). The combined effects of these factors have a substantial impact on the biomass of plants [40]. The exogenous administration of trehalose has been seen to elicit diverse impacts on plant physiology, both in the presence of stress and under non-stressful conditions. The molecule known as trehalose has been found to have multiple benefits in the mitigation of lead risks. The aforementioned benefits encompass the ability to acclimate to oxidative stress, augment the antioxidant defense mechanism, and elevate photosynthetic efficacy. According to the findings of Ashraf their analysis demonstrated that the existence of metal toxicity negatively impacts plant water relations and undermines the structural integrity of cellular membranes[38]. The observed decrease in performance was seen in the cultivated sunflower hybrids. The escalating occurrence of heavy metals in soil has a discernible influence on the relative water content (RWC) and membrane stability index (MSI), resulting in a decline in plant water absorption, leaf succulence, compromised membrane stability, and impairment of cellular membranes [39, 40, 43].

The measurement of malondialdehyde (MDA) is a dependable indicator used to assess the presence of lipid peroxidation mechanisms in the cellular membrane. Therefore, the observed elevation in malondialdehyde (MDA) levels resulting from heavy metal stress addiction indicates the presence of oxidative stress induced by excessive production of reactive oxygen species (ROS). In their investigation, observed a positive association between the presence of heavy metal-contaminated soil and the generation of hydrogen peroxide ($H_2O_2$), leading to an increase in lipid peroxidation. Their work observed a significant decrease in $H_2O_2$ levels and subsequent reduction in $H_2O_2$-induced lipid peroxidation.

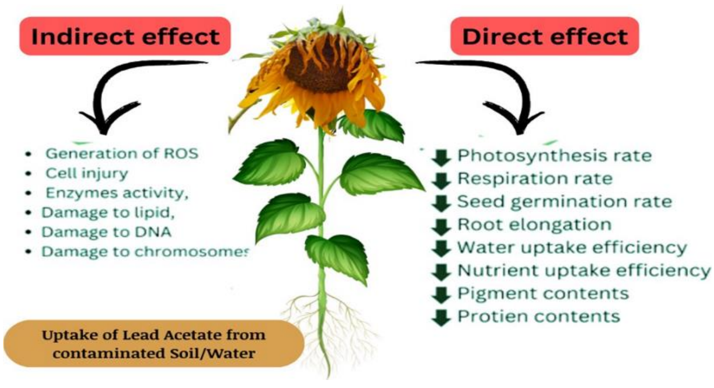


**Figure. 5** Uptake of $PbCH_3COOH$ from contaminated soil, water, or air and its effect on sunflower

Figure 5 illustrates the impacts of elevated levels of Pb on plant species, spanning both direct and indirect pathways. The immediate consequences encompass a reduction in the rates of photosynthesis and respiration, a diminished rate of seed germination, a decline in the elongation of roots, a decreased efficiency in the absorption of water and nutrients, and a decrease in the concentrations of pigments and proteins. The idea of indirect impacts encompasses various pathways, including the production of reactive oxygen species (ROS), cellular harm, modification of enzyme activity, and disturbance of lipid, DNA, and chromosomal integrity [40, 43].

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