IMPACT OF REDUCED GRAPHENE OXIDE ON MITIGATING LEAD STRESS IN WHEAT PLANTS: MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSES

Master of Philosophy

In

PLANT SCIENCES

By

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DEPARTMENT OF PLANT SCIENCES
FACULTY OF BIOLOGICAL SCIENCES
QUAID-I-AZAM UNIVERSITY
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A Dissertation Submitted in Partial Fulfilment of the Requirements for the

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CERTIFICATE

This is to clarify that this thesis dissertation entitled "**Impact of reduced Graphene oxide on mitigating Lead stress in wheat plants: Morphological and Physiological responses**" submitted by **Mr. Ashfaq Ahmad** S/O **Mushtaq Ahmad** registration number **02042113037**, is accepted in its present form by the **Department of Plant Sciences**, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the **Degree of Master of Philosophy** (M.Phil.) in **Plant Sciences.**

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It is certified that **Mr. ASHFAQ AHMAD** S/O **MUSHTAQ AHMAD** registration number **02042113037** completed his M.Phil. On **14th Sep 2023**. The title of his thesis is **"Impact of Reduced Graphene oxide on Mitigating Lead Stress in Wheat Plants: Morphological and Physiological Responses"** from Department of Plant Science, Faculty of Biological Science, Quaid-I-Azam University Islamabad. His thesis has been checked on plagiarism software for similarity index and found **14%** Turnitin result that lies in limit provided by Higher education commission (HEC) is **19%.**

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I M. Ashfaq Ahmad hereby state that my M.Phil. thesis title "**Impact of Reduced Graphene oxide on Mitigating Lead Stress in Wheat Plants: Morphological and Physiological Responses"** is my own work and has not been submitted previously by me for taking any degree from Quaid-I-Azam University or anywhere else in the country/ world.

At any time, if my statement is found to be incorrect even after my Graduation, University has the right to withdraw my M.Phil. Degree.

Ashfaq Ahmad

DEDICATION

I dedicate this success to my beloved

Parents and my elder brother

For their endless love, support, and encouragement throughout my life.

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Ashfaq Ahmad

Table of Contents

Chapter 1

List of Abbreviations

Abbreviations	Description
ABA	Abscisic acid
APX	Ascorbate peroxidase
ave.	Average
CAT	Catalase
Cd	Cadmium
Cu	Copper
FTIR	Fourier transform infrared
Fe	Iron
GO	Graphene oxide
rGO	Reduced graphene oxide
HMs	Heavy metals
L	Liter
mg/gm	Milligram/Gram
mg/kg	Milligram/Kilogram
mg/L	Milligram/Liter
ml/L	Millilitre/Liter
mM	Milli Molar
Pb	Pb
POD	Peroxidase
Ppm	Part Per Million
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SOD	superoxide dismutase
µgm/ml	Micro Gram Per Millilitre
µM	Micro Molar
UV-R	Ultraviolet radiation
XRD	X-ray Diffraction

ABSTRACT

Rapid global industrialization and population increase have increased the risk of heavy metals accumulation in plant body. Lead (Pb) is regarded as the 2nd most toxic heavy metal causing toxicity in plants, animals as well as in human beings. Pb can accumulate in plant tissues and disrupt the morphological, biochemical, and physiological processes of plants, which in turn results in substantial variation in plant functions. In this study, Graphene oxide was synthesized through graphite oxidation by Hummers methods in a stepwise manner and was greenly reduced through the use of *Tecoma stans* plant leaves extract. Uv-spectroscopy, FTIR, XRD, Zeta potential, and SEM were done for the characterization of reduced graphene oxide (rGO) nanoparticles. In the current study, a pot experiment was conducted in which different concentrations (15, 30, 60, 120 mg/L) of rGO were applied. Three different concentrations (300, 500,700 mg/L) of Pb stress were applied. To observe the mitigative effects of rGO, 30 mg/L of rGO and 700 mg/L of Pb were used in combination. Changes in morphological and biochemical characteristics of wheat plants were noted for both Pb stress and rGO treatments. Pb was found to inhibit the morphological and biochemical characteristics of plants. rGO, alone as well as in combination with Pb stress, were found to enhance root/shoot growth, and biomass of the plants over control treatment. rGO was found to increase the chlorophyll content of wheat plants, while Pb was found to reduce it. In a combined treatment of rGO and Pb, an increase in chlorophyll content was observed in comparison to control and Pb stress plants. Under Pb stress conditions antioxidant enzyme activities like SOD and CAT were found to decrease in comparison to control treatment while they were greatly enhanced by rGO NPs alone and in combination with Pb. Enhancement in the activities of POD and APX were found for both rGO NPs and Pb separately and in combination as compared to the control. The current findings revealed that greenly reduced graphene oxide NPs can effectively promote growth in wheat plants under the stress conditions of Pb by elevating the chlorophyll content of leaves, reducing the Pb uptake, and suppressing ROS produced due to Pb toxicity. The application and use of graphene oxide for the improvement of plant growth is dose-dependent and may vary from species to species in plants.

1. Introduction

Heavy metals (HMs) as a soil constituent naturally exist in the Earth's crust (Aslam et al., 2021). Due to the stability and non-degradability of heavy metals, they persistently accumulate in the environment for a long time (Aslam et al., 2021). The toxicity of heavy metal has become a major threat to our environment all over the globe and is threatening the ecosystem by several means. The threatening effects of HMs extend to plants and animals and are ultimately direct and indirect concerning human health. The increase in urbanization and industrialization day by day has led to the emission of heavy metals into the environment on a large scale (Alamri et al., 2018). Considering essentialness to plant growth, development, and production some of the metals amongst heavy metals are regarded as essential for plants but can cause phytotoxicity and adversely affect plants biomass and yield if in excess hence resulting in food insecurity (Huang et al., 2020). Non-essential HMs i.e., Pb, cadmium, Mercury, and silver enter the cell producing reactive oxygen species (ROS) which distort cellular functions. Pb is considered to be one of the most toxic among all heavy metals which can adversely affect plant growth, development, yield, and metabolic activities (Alamri et al., 2018). Among the hazardous heavy metals, Pb is considered to be the second most hazardous HM due to its non-degradability and stable nature in the environment US environmental protection agency (EPA) has listed it as a human carcinogen (Hossain et al., 2023). Agricultural soils are being contaminated with Pb which is mainly due to anthropogenic activities and is a widespread problem in agroforest land (ur Rehman et al., 2017). Pb Contamination of soil depends on; the concentration of Pb pollutants in soil, inhibition of plant growth, decrease in chlorophyll and photosynthetic activity, and change in anti-enzymatic activities and accumulation of acid-reactive substances of thiobarbituric and H_2O_2 excessively in plant tissues (Semenova et al., 2019). Pb enters like other heavy metals into the plant cells and induces oxidative stress in plants due to the production of reactive oxygen species (ROS) in the growing parts of the plant which in turn results in a reduction of productivity of the plant. Antioxidant systems of the plant protect plants from corrosive and degraded effects caused by any means. All these complex detoxification are highly compartmentalized processes in plant cells (Lamhamdi et al., 2011).

Both enzymatic such as peroxidase (POD), ascorbate peroxidase (APX), Catalase (CAT) and superoxide dismutase (SOD) and non-enzymatic such as oxidized glutathione (GSSG) and reduced glutathione (GSH) as antioxidants are involved directly and indirectly in detoxification of reactive oxygen species (ROS) in plants (Ashraf et al., 2017). Pb accumulation can occur in plants through various routes which may be water, air, or soil. The most prominent harmful effects of Pb include; interfering with soil nutrient uptake by plants, reduction in germination rate, reduced photosynthetic activity of plants, occurrence of plant growth delay, disturbance in respiration, metabolic alteration, alteration in enzymatic activities, change in root morphology, enlargement of cell vacuoles, inhibited nucleolus, increase in plasmolysis and damaging the thylakoids of photosynthetic bodies plastids (Kanwal et al., 2020). Pb, which is a contaminant of almost every ecosystem for example; soil, water, air, and living organisms, enters through contaminated soil into the plant tissues (Gurpreet et al., 2015). Concerning the origin and nature of the source heavy metal, Pb reaches the soil environment through pedogenic and anthropogenic processes. Primarily anthropogenic activities are linked with industrial processes, manufacturing, and non-treated disposal of industrial and domestic waste materials or effluent, which are coined the major sources of contamination of soils through Pb (Patricia et al., 2009). An increase in exogenous Pb levels increases the accumulation of Pb in plant tissues. Several physiological and biochemical changes are being caused by Pb and can equally effect seed germination, plant growth, water status of the soil, and nitrate assimilation (Mostafa., et al 2013). In the past three centuries, environmental Pb concentration has increased up to 1000 fold mainly due to large industrial demands and almost no recycling or less recycling of the Pb to reduce it, which results in an accumulation in the plants and animals bodies (Mumtaz et al., 2020).

Nano materials in recent times emerged as a hot topic in the research area due to their extraordinary properties and applications in scientific and technological applications all over the globe. Its utilization and applications have made it possible to use appropriate nanomaterial in agriculture, agroforestry, and other field of science of keen interest. It has been experimentally conformed that certain specific NPs in low doses can stimulate physiological processes in plants (Chakravarty et al., 2015). Graphene oxide (GO) is one of the graphene-based nanomaterials with oxygen-

containing functional groups, which makes its dispersibility in water and other organic-based solvents. Graphene oxide at the same time has some extraordinary physical and chemical properties which make it of more interest (Ren et al., 2020). Graphene oxide (GO) is a nanomaterial that has a small size, with a specified shape, and geometry, and has distinctive physiochemical properties, which make it widely to be used in biomedicine, nan-electronics, energy, and other fields of science all over the world. Presently, there are more than 1000 manufacturers who are producing graphene-based materials or products globally, some of the well-known are IBM and Intel. By the end of 2020, the market value for GO products was projected to touch the value of 675 million US dollars with approximation and is expected to be more in the coming years due to its high demand, which is expected to cause a considerable amount of graphene-based waste addition and accumulation in the ecosystem (Minling et al., 2019).

Concerns about, the environmental safety of GO have been provoked. Plants the critical primary producers in terrestrial environments have an ecological role and function to control and maintain the ecosystem and food supply. Therefore the interaction between graphene oxide and plants is vital to assess the ecological risks and scientific applications of graphene (Ren et al., 2020). Recently, researchers have experimentally indicated that graphene oxide can be used as a carrier for fertilizer to reduce its release rate and improve nutrient utilization efficiency, which enables these materials enable to the development of slow-release fertilizers. Being the essential primary component of the ecosystems, plants are continuously in contact with the graphene which is being released into the soil regularly. The long-lasting effects of graphene oxide on plants should be considered and understood before applying it on a large scale to improve agroforestry and the agriculture sector (Zhang et al., 2021). Some of the researchers have found significant effects of graphene oxide on the growth and development of plants. These effects are variable and can be influenced by certain factors i.e. concentration of graphene oxide and genotype (Shen et al., 2018). It increases plant mineral micronutrient uptake of plants as a carrier in soil. It positively affects soil nutritional traits and has a positive impact on plant growth and physiology and benefits by protecting against other phytotoxic agents (Hammerschmiedt et al., 2023). However, the impact of GO on plants is dose-dependent. From some of the experimental work, it has been shown that functional

graphene oxide in certain plants, at low concentrations, can stimulate physiological processes. Germination of aged seeds and root differentiation of wheat plants could be promoted by treating them with 200 mg/L of hydrated graphene ribbon (Zhang et al., 2021).

Wheat botanically named *Triticum aestivum* L. is one of the overwintering herbaceous Gramineae plant species and is considered one of the most important food crops around the globe (Jiang et al., 2019). Wheat is the most particularly cultivated food crop in the world. It has the significance of all cereals and is ranked as 1st globally among the grain-producing crops consumed by humans all over the world. Around 36% of the population of world is dependent on wheat which is considered a staple food (Sabagh et al., 2021). Wheat is amongst the three Leading food crops including maize and rice. Wheat covers about 651 million hectares of agricultural land all over the globe (Iqbal et al., 2021). Wheat is one of the Leading crops produced, consumed, and traded in the world, China is considered the largest wheat-producing and consuming country in the world with a production value of 17% and a consuming value of 19% in the first two decades of 21st century (Zhai et al., 2016). In Pakistan, its contribution value added to the agriculture sector is 10.3% and has a share of about 2.2% of the GDP (Ahmad et al., 2016). Wheat is a rich source of protein and calories about 82 to 85% of the world's population depend on wheat for their basic protein and calorie uptake worldwide (Taie et al., 2019). About 700 million tons of production of wheat all over the world, and it provides 20% of the total calories consumed by humans to fulfill their needs. However decreases in productivity have been found due to certain biotic and abiotic stress conditions in the environment (Cabral et al., 2020). All the different environmental conditions i.e. salinity, water logging, drought, lack of proper nutrients, and certain pathogenic microbes and insect attacks led to a decline in wheat production to a considerable level (Mostafa et al., 2016).

1.1 Heavy Metals

Heavy metals are classified as a heterogeneous group of elements that can be differentiated based on their functions and the chemical properties they each bear separately. Heavy are mostly placed in the block which consists of transitional elements in the periodic table of the elements. Heavy metals weigh more than 5 gm cm^3 (Kiran et al., 2022). Around the world agricultural soils are contaminated with

heavy metals which include cadmium, Pb, mercury, chromium, arsenic, and many other heavy metals. Heavy metals in higher concentrations produce toxicity to all forms of life from the micro level to humans (Goyal et al., 2020). With a half-life of more than 20 years unlike organic matter, heavy metals are non-biodegradable which persists for a long and only changes their oxidation sate. Of all the elements fifty-three elements are documented and considered universal pollutants which are causing toxicity in different means (Ashraf et al., 2019). Heavy metals reside for a long time in soil because of the interaction of heavy metals with the particular components of soil. Transport of heavy metal in any soil depends on the chemical form of the metal and the nature in which it exists which ultimately affects the quality of the aquifer as well (Al-Taani et al., 2021). Depositions of heavy metals at the atmospheric level accumulate in the soil. Accumulation of heavy metals is one of the major problems caused by inorganic contaminants in soil and water ecosystems. Heavy metals in higher quantities in agri-based products have an impact on food security and disturb human health (Oladoye et al., 2022). Rapidly growth in industrialization and related activities such as mining, tanneries, and development in modern agriculture and farm management in which fertilizers and pesticides are practiced in excess which in turn negatively impacts human health with the release of heavy metals into the environment more particularly heavy metals which contaminate soil, water bodies, and air (Thakur et al., 2022).

Plants are continuously exposed to environmental stresses due to their sessile nature in their life cycle. Over previous years heavy metal stresses have attained significant importance among all the other stresses (Rahman et al., 2022). Heavy metals in higher concentrations cause' a reduction in overall plant growth more specifically affect leaf growth which causes wilting and bluish-purple coloring of the leaf. Roots are the first plant parts that are exposed to heavy metals among all others and hence root extension and proliferation are caused by heavy metals (Kumar et al., 2022). Generally, some common toxic effects are produced on plants by both essential and non-essential heavy metals including, inhibition in growth and photosynthesis, chlorosis, low biomass accumulation, change in water balance and nutrient assimilation in plant parts, and senescence which can Pb to plant death (Singh et al., 2015). Exposure to plants for a long time to heavy metals also results in the inhibition of enzymes, disrupting metabolic activities and inactivating photosystems of plants

(Rizvi et al., 2017). Contamination caused to plants by heavy metals is critical to all forms of life as these heavy metals route through plants. Exposure of plants to heavy metals induces oxidative stress in plants and alters cellular ionic homeostasis which results in damage to cellular components (Dubey et al., 2018). Heavy metals directly affect the quality of the environment and hence prove their significance in terms of negative impact on the environment. Heavy metals in higher concentrations in the human population cause severe health-related issues that affect the reproductive system, nervous system, cardiovascular system, and renal system and cause some sort of behavioral abnormalities in humans (Abbasi et al., 2020).

1.2 Pb as a heavy metal

Anthropogenically contamination of soil with Pb is due to industrial-based sources likely as battery recycling, mining, agricultural pesticides, and from tetraethylPb ($(CH_3CH_2)_4Pb$), as an additive formerly added and found in automotive fuels. Pb fallout from the fuel containing tetraethyl lead into the atmosphere through burning and smelting deposited into the topsoil (Beavers et al., 2021). Relatively Pb is a denser heavy metal, low in electrical conductivity in comparison to other metals. Weathering of rocks with Pb loaded in it could be coined as one of the main sources of Pb contamination with the inclusion of Pb-loaded gasoline usage in vehicles, paint chips shedding, disposal of waste, and sewage water usage for irrigation (Saleem et al., 2018). Generally, Pb exists in 4^+ and 2^+ oxidation states for inorganic species. In different series reactions such as adsorption binding, ion exchange reactions, and complexion precipitation type reactions Pb acts differently which in turn affects solubility and its bioavailability. Through the processes of oxidation/reduction and alkylation/dealkylation with the help of microorganisms in soil, plants, and the aid of animals Pb species mutual conversion can happen which may vary by considering soil composition, potentials of a redox reaction to occurs, reaction surface, reaction binding site (Liu et al., 2020). In plants, Pb is coined as a non-essential element, which causes significant alteration in plant's metabolic processes by damaging metabolic pathways. The growth and development of plants can be negatively or positively affected by Pb treatment and the degree of alteration in plant morphological parameters is concentration-dependent (Vasilachi et al., 2021). Due to the non-biodegradability and toxic nature of Pb known for a long time in history as trace

heavy metals adversely affect photosynthesis, plant growth, and morphology (Alkhatib et al., 2019).

Pb among other heavy metals is the most toxic damaging heavy metal to pants (Shehzad et al., 2023). Plants absorb Pb from soil and the concentration of Pb in roots remains high while a small portion of it is translocated to the stem and leaf part of the plant. Pb ions produce toxicity in plant tissues which directly damage the photosynthetic pathway, In turn plant's developmental processes are distorted i.e. mineral absorption, plant growth, and germination of seeds are badly affected (Zhou et al., 2018). Pb harms the living system and its devastating effects are known to cause damage. Through anthropogenic and natural sources Pb accumulates into soil which affects soil milieu and soil biota, leaving the scientific society in an immense concerning position. Plant growth and the safety of food are disturbed by the accumulation of Pb in vegetation through both isotopic and stable means naturally existing in the environment (Mitra et al., 2019). Heavy metals in higher concentrations intervene photosynthetic process, respiration, imbalance in plant mineral nutrition, membrane dysfunction, disturbing enzymatic activities, and hormonal imbalance in plants. Plants act as reservoirs of intermediate levels for transferring heavy metals from soil to humans and other living organisms, which may make the situation worse (Yahaghi et al., 2019).

1.3 Plants' Responses to Pb Stress

Plant exposure to Pb inhibits plant growth and exhibits other symptoms (Lyu et al., 2020). Pb alters membrane permeability, disturb mineral uptake and distribution, damage photosynthetic machinery, and disturb and reduce enzyme activities which in turn generate oxidative stress All these directly or indirectly affect the morphology of plant and their photosynthesis process All these changes are different to different plant species (Hu et al., 2014). Heavy metal ions (HMs ions) bind to the sulfhydryl groups in protein molecules which replace the essential cations to bind in specific sites in the molecule, This results in the inactivation of enzymes and producing reactive oxygen species (ROS), hence causing oxidative damage to proteins, lipids, and nucleic acids molecules (Shehzad et al., 2023). Oxidative stress is induced by three means due to Pb excessiveness: (a) Fenton reaction and autoxidation, (b) replaced essential metal of metalloproteins and its deficiency effects, (c) suppression of protein's functional groups (Zhang et al., 2019). Pb metal

translocation in plantss occurs by apoplastic and symplastic movement. Pb metallic ions are being translocated through the apoplastic movement which results in deposition of it into endodermis cells and further transportation occurs through symplastic movement. Exposure of plants to Pb in higher concentrations through the Fenton-Haber-Weiss reaction resulted in the overproduction of reactive oxygen species following the appearance of toxic symptoms in plants (Kohli et al., 2019). Pb acts as an inducing factor, which can continuously alleviate the accumulation of reactive oxygen species in plants, causing chain polymerization of proteins, disrupting membrane permeability, and reducing functional binding enzyme activities on cell membranes (Cai et al., 2021). Sulfhydryl compounds, including NPT, GSH, and PCs, cause ionic chelation of heavy metals to less toxic or non-toxic substances; so, they can be then used to detoxify Pb. Sulfhydryl compounds play an important role in the mitigative response to HM stress in plants; GSH binds to free metallic ion of heavy metal in the cytoplasm directly and immediately start detoxification (Chen et al., 2020). Pb stress induces changes in the amino acid levels such as lysine, arginine, proline, and glutamine. Which then affects the synthesis and deposition of amino acids to disrupt the Pb stress response in maize plants (Zhang et al., 2021).

1.3.1 Pb Uptake in Plants

Apart from the specified conditions and circumstances where plants are grown in metal-concentrated proximities, the assessable route through which plants take in metals is absorption by plants from soil through roots (Ashraf et al., 2021). Plants are the first to take in the released environmental Pb and then accumulate it in the human body via the soil-plant-human system through the food chain. To limit the entry of anthropogenically produced Pb into the environment and plant roots to reduce its accumulation in areal edible parts of plants if of keen desire (Gong et al., 2022). Plants take Pb from the environment by two means it may be directly absorbed in a free ionic state from soil through roots by capillary action or may take it through cellular respiration from atmospheric air (Collin et al., 2022). In general, it has been reported that vegetable crops especially leafy crop varieties are taking up more amount of Pb as compared to the grain and fruit crops (Hossain et al., 2021). Exposure of plants to Pb stress impacted negatively which may include susceptibility of seed germination and establishment of seedlings, disturbed nutrient uptake, alteration in chlorophyll biosynthesis in green parts of plants which ultimately disturb

photosynthesis, change in respiration pattern, cell membrane permeability and activity of Calvin cycle enzyme got effected (Shu et al., 2012). Pb stress alters sugar metabolism, and nitrogen metabolism, disturbs water balance, change in hormonal status, enhance leaf senescence, and change in oxidative stress (Khab et al., 2016). Fungi that are actively involved in symbiotic relationships with almost 80% of terrestrial plants commonly called arbuscular mycorrhizal fungi (AMF), are thought to have an active part in resistance against contaminations of heavy metals like that of Pb (Sánchez et al., 2021).

1.3.2 Effect of Pb on seed germination

In the life cycle of plants, germination of seeds and seedling growth are considered to be the important stages of plant growth (Liu et al., 2011). Pb inhibits seed germination under Pb stress which may result from the interference of Pb with some of the important enzymes needed for seed germination (Atta et al., 2020). According to the research work of Gupta et al. (2022) reduction of 72.8% was noted in seed germination for Pb stress of 20 mM. Another study work done by Shafiq et al (2008) shows that compared to the control increasing the concentration of Pb to 75 ppm, significantly decreased seed germination, while seedling and root growth was found to show a significant reduction at 50 ppm Pb treatment. During Pb stress, Pb ions have direct effects and prevent germination of seeds, seedlings growth, parameters of length like root/shoot, tolerance index, and reduced biomass of shoot and root.

Pb is concentration-dependent and reduces seed germination if in higher concentrations (Anwar et al., 2022). An increase in the concentration of Pb was found to cause a high reduction in the germination of seeds, root/shoot length, and seedling length, decrease in root shoot ratio and plant dry biomass was noted (Ghani et al., 2016). The average germination percentage valve for control was found to be 46.66%, which showed significant reduction at 250 mg/L of Pb stress and for 1000 mg/L concentration of Pb stress it was found 5%. Thus, it showed 9 times reduction in percentage germination of L. perenne at 1000 mg/L concentration of Pb stress. For germination index significant differences were found in between control and other treatments. However, the differences found in between 250 and 500 mg/L of Pb concentrations and that of in between 500 and 750 mg/L of Pb were noted to have no such significance (Gholinejad et al., 2020). Metals cause toxicity when tested crops

are exposed to them during germination. Under copper treatment highest percentage reduction of 51.2% was noted in wheat plants for Cu 175, which is then followed by Pb with a reduction of 47.5% and then at the third slot is cadmium with 35.3% when exposed at 220 ppm in both tomato and pea respectively (Baruah et al., 2019). The obstruction in germination may be due to the antagonistic activity of Pb with enzymes like amylase and protease of higher plants, Pb induces stress which in turn boosts NADH- dependent extracellular synthesis of H_2O_2 in the growing seeds and hence it resulted into the arrest of seed growth in wheat plants (Osman et al., 2023).

1.3.3 Effect of Pb on plant morphology

Naturally plants are susceptible to various stresses which include biotic and abiotic stresses. Chemical based agricultural fertilizers and industrial effluents have increased the toxic heavy metals level in agricultural soils which is negatively impacting plant-soil environment system (Gadallah et al., 2014). Among metals, Pb is counted as one of the heavy metals which are highly toxic and when in excess it slow down the germination rate and reduce height and biomass of maize plants (Rizvi et al., 2018). All the stresses impact plant but heavy metals stress negatively affect development and crop productivity, which make it of great concerning interest to researchers (El-Okkiah et al., 2022). Pb is one of the non-essential element for plant metabolism, is toxic to all the physiological processes, and disturb the normal functions of plants from cellular to organ levels, including disturbed seed germination and retarded growth, water imbalance, nutritional disturbance, and reduction in photosynthesis, transpiration, and respiration (Islam et al., 2008). Pb reduces the growth of plants, biomass, pigments necessary in the photosynthesis of plants, and the antioxidant enzyme capacity of plants to protect plants from damage done by ROS (Kanwal et al., 2008). The research work of Shakoor et al (2014) shows that when B. napus plants were exposed to increasing Pb levels of 50 and 100 µM, various plant growth attributes would be significantly inhibited like that of plant height, length of root and shoot, number of leaves per plant and leaf area. For both of the concentrations of Pb significant reduction was noted in fresh and dry biomass of plants but inhibitory effects were found severe for 100 µM concentration of Pb. The research work of Sha et al. (2019) showed that when plants were treated for 10 days with different concentrations of Pb, for 80 µM Pb treatment chlorosis and necrosis appeared in fronds and hence resulted in morphological changes in plants. From a

research point of view, it can be concluded that Pb contamination adversely affects plant growth, physiology, and yield of sunflower plants (Saleem et al., 2018).

1.3.4 Photosynthetic activity

Photosynthesis in plants is a basic fundamental process for the plants to capture light energy from the sun and change it into a usable form for plants, Photosynthesis is considered one of the most heavily metals sensitive processes (Bayçu et al., 2018). The amount of chlorophyll content in plants directly reflects the plant's photosynthetic capacity and growth (Mani et al., 2014). Higher chlorophyll content is necessarily needed by plants to maintain normal photosynthetic processes under stress. However most of the experimental work has shown that stress reduces chlorophyll contents in the leaves of plants (Huihui et al., 2020). Heavy metals equally affect the biosynthesis of chlorophyll to substitute magnesium ions from chlorophyll molecules. Many of the heavy metals affect the enzymes that are involved in chlorophyll synthesis which tends to disturb electron transport in light reactions and change some of the functional enzymes involved in dark reactions inside plants (Rai et al., 2016). Pb has a direct effect on plants when applied to plants it reduces photosynthesis in the whole plant and is believed to result in the closure of stomata rather than directly affecting the photosynthesis process (Zhang et al., 2018). Furthermore, chlorophyll synthesis is inhibited by Pb by inhibiting the uptake of essential elements such as Fe or Mg by plants. Pb in higher concentrations causes an imbalance of mineral nutrients in plants (Giannakoula et al., 2021). Pb replaces Mg_2^+, $Ca_2^{+,}$ and Fe_2^+ ions in the photosynthesis apparatus or interacts with SH groups or essential metal ions that are involved in the photosynthesis process. Consequently, Pb affects ROS production through impairment processes of photosynthesis involved in oxygen consumption such as photorespiration, Mehler reaction, and chlororespiration (Dao et al., 2016). Reactive oxygen species (ROS) are also produced in peroxisomes and mitochondria which also have an impact, caused by Pb to create disturbance in plant cells (Apel et al., 2004).

1.3.5 Mineral Uptake

For the growth and healthy development of plants mineral nourishment is required. The connection between Pb and nutrient availability in soil to plant shows negativity (Lamhamdi et al., 2013). Some of the results of numerous researchers have shown that Pb has a significantly negative impact on the nutritional absorption of

plants from soil (Gopal and Rizvi., 2008). According to Singh et al. (2015) under Pb stress, a trend of reduction in Ca, Mg, Na, and K levels was noticed in maize shoots and roots in relation to control. Plants when exposed to a higher concentration of Pb will ultimately affect the mineral nutrient content available to plants from soil. Pb in Higher concentration competes with other cations and inhibits the uptake of divalent ions in soil by plants such as iron (Fe_2^+), magnesium (Mg_2^+), manganese (Mn_2^+), and calcium (Ca_2^+), and that of monovalent cations like potassium (K^+). The research work of Zulfiqar et al. (2019) shows that, plants exposed to Pb reduced the divalent cations (Fe_2^+, Ca_2^+, Mg_2^+, Mn_2^+, and Zn_2^+) concentration in leaves of plants. Another research work done by Bhatti et al (2013) shows that plants of *T. aestivum* when treated with Pb of 40 and 60 ppm concentrations decreased Potassium and sodium ion levels in the roots and shoots of plants. In comparison to Spinacia oleracea, *T. aestivum* has shown a decrease in calcium, magnesium, potassium, and sodium levels when exposed to Pb in concentrations of 1.5, 3, and 15 mM (Lamhamdi et al., 2013). Pb toxicity produces imbalances in the nutrient uptake of plants which is majorly disturbing plants' healthy growth.

1.4 Effect of Pb on Antioxidant Activities

Production of reactive oxygen species (ROS) is one of the important points of concern as it is damage caused by Pb and other stresses to the plants which destroy plasma membrane through different mechanisms like alteration in the composition of membrane lipids, thiol combination in the membrane, and alteration and destruction of membrane transporters. Studies have shown that Pb stress increases oxidative stress in some plant species under different experimental conditions (Fatemi et al., 2021). Oxidative stress in living organisms can be related to Pb toxicity as it increases the concentration of reactive oxygen species or reduces the antioxidant capacity of an organism (Kasperczyk et al., 2015). Plants directly or indirectly activate the production of reactive oxygen species (ROS), in response to Pb in excess which hampers the antioxidant defense machinery of plants, degrades the proteins of defense, and causes change in chloroplast structures, imbalance nutrient uptake, causing a decline in the photosynthetic efficiency of plants, causes inhibition in the division of cells and eventually retarded growth of plants (Zeng et al., 2021). The research study of Okant and Kaya et al. (2019) suggests that to scavenge H_2O_2 in plants to produce tolerance against Pb stress in maize. Plants increase in antioxidant

activity by the plant is one of the important strategies evolved by plants to withstand its harshness. Plants have evolved and settled various approaches to withstand heavy metal toxicity and increase their capabilities to suit its existence in heavy metals contaminated soil. Accumulation and production of malondialdehyde (MDA), a product of lipid peroxidation indicate the cellular damage caused due to the production of reactive oxygen species. Many of the studies have suggested that antioxidant enzymes can scavenge and remove reactive oxygen species to protect plants against the toxicity produced by heavy metals (Sharma et al., 2016). SOD is regarded as one of the key protective enzymes, which can remove excess anions of superoxide in plants generated through the Haber-Weiss reaction (Hou et al., 2018). POD and CAT have the function of removing the excessively produced H_2O_2 in plants (Goswami and Das, 2016). From an antioxidant perspective, toxic heavy metals, including Pb, interfere with the plant's cellular homeostasis by generating reactive oxygen species (ROS) to disturb the defense system to fight the harshness. In this way plants health is negatively affected, which consequently, retarded plant growth and may Pb to plant death (Usman et al., 2020).

1.4.1 Catalase (CAT) Activity

Catalase tetrameric heme protein molecule has an alternative divalent oxidation and reduction in its active site in the presence of hydrogen peroxide (H_2O_2) while acting as a substrate for GPx, which ultimately reduces glutathione to protect constituents of cells from the damaging effects of peroxides produced in the metabolic processes and other ROS producing reactions. A decrease in CAT activity could have an impact on the reduction in absorption or inhibition of heme biosynthesis (Gargouri et al., 2017). Consequently, CAT activity was found to be significantly high in the leaves in contrast to the roots. CAT is involved in the reduction of H_2O_2 into products such as water and oxygen to protect plant cells from its oxidative damage (Venkatachalam et al., 2017). The reason behind it might be either due to enhance production of hydrogen peroxide (H_2O_2) or over-expression of genes encoding CAT enzyme (Khan et al., 2020). Increase in CAT activity under Pb stress and its phyto-toxicity could be explained by the mechanism of substrate enhancement in plant to chop with the problem of excess of H_2O_2 and protect plants from its harmful effects ((Reddy et al., 2005). Decomposition of H_2O_2 to H_2O and O_2 is catalyzed by CAT enzymes. The results from research work of Hakeem et al., (2019) revels that

significant increase in CAT activity was noted for 15-day-old plants, but consequently it was observed that at 300 µM treatment with Pb significant decline in CAT activity occurred in 30-day-old plants. The research work of Rahbari et al., (2020) shows that Pb stress increases CAT activity and the highest activity was fund at 1000 mg/ kg treatment of Pb for plants in the leaves.

1.4.2 Superoxide Dismutase (SOD) Activity

An enzyme that accelerates and catalyzes the deprotonation of superoxide radicles into oxygen and hydrogen oxide molecules is known as superoxide dismutase (SODs). Mainly there are three kinds of SODs, based on the metals substituted in their binding sites: the one with copper and zinc (Cu, Zn-SODs), and the other two are iron (Fe-SODs) and manganese(Mn-SODs) (Río et al., 2018). SOD is one of the most potent antioxidants in the cell among others. It acts as one of the critical intracellular antioxidant enzymes and serves as a part of the initial line of defense against oxidative stresses in the body (Ghodaro and Akinloye., 2018). Many of the stresses such as abiotic stresses including heavy metal stress have been found to enhance SOD activity i.e. in *Pogonatherum cranium* under Pb stress the activities of SOD and malondialdehyde(MDA) were found at maximum to Pb (Hou et al., 2019). The SOD activity was significantly found to increase by 56 and 72 % for 48 and 96 h, to 50 µM Pb stress, in comparison to the control (Thakur et al., 2017). Evidence from the research work of Dalya et al., (2018) shows that Pb stress with 2 mM enhances the functions of the SOD and other antioxidant enzymes in *B. juncea* plants rhizome.

1.4.3 Ascorbate Peroxidase (APX) activity

APX is one of the crucial antioxidant enzymes APX belonging to the family of heme-containing peroxidases which catalyze the conversion of Hydrogen peroxide into water by using ascorbate as an electron donor (Pandey et al., 2017). Iron is regarded as an essential feature for the catalytic efficiency of heme-dependent oxidoreductases such as APXs, and activity will be lower for iron deficit oxidoreductases. The two other antioxidant enzymes i.e. glutathione and superoxide dismutase have been identified to enhance the activity of APX, demonstrating the interaction of antioxidant enzymes (Zhang et al., 2014). The work of Li et al., (2019) reveals the differential expression level of APX and in several other studies, it is documented that the fluctuation in different isoforms of APX in Arabidopsis thaliana under different circumstances was found. Similarly, the APX activity was found to be

elevated by Pb in the seedlings of Eichhornia crassipes (Malar et al., 2014). In *Arachis hypogaea* L. cultivars, APX and other antioxidant enzyme activities were found to be enhanced under Pb contamination (Nareshkumar et al., 2015). In another study by Hasanuzzaman et al., (2018), MDHAR and DHAR functions got lower under Pb stress in wheat plants, resulting in a drop in the concentration of ascorbate. Under Pb treatment, the APX activity did not differ from the standard Pb-free treatment or any other Pb-containing interventions for *Brassica juncea* L. plants, which means that Pb's impact is not sufficient to impact enzyme function (Soares et al., 2020).

1.4.4 Peroxidase Activity (POD) activity

POD and CAT enzymes are considered classical in plant systems which resist oxidative stresses and form a complete antioxidant chain that works together to scavenge or eliminate reactive oxygen species ROS (Zhang et al., 2023). Pb stress changes the antioxidant activities which may be variable with that of nutrient treatments, Pb stress induces SOD and POD activities in plants (Khan et al., 2018). According to the research work of Zhang et al., (2020) by increasing the Pb toxicity, the content of POD increases significantly and the maximum POD activity occurred at 40 μM Pb stress. Another study by Rathika et al., (2019) shows that POD activity increases with soil contaminated with pb of 135, 130, and 142 U/mg respectively. The POD activity was found to be recorded as highest at 400 and 800 ppm for Meixiangzhan-2 and Xiangyaxianzhan, respectively. And overall, the enzymatic activities were found to be comparatively higher at higher concentrations (Ashraf et al., 2017). The research work of Zhong et al., (2017) shows that the elevated treatment of Pb boosts the POD activity and may further increase it to the peak with 400 μM Pb treatment.

1.5 Nanotechnology

The study deals with materials having a size of less than 100 nm is termed nanotechnology. It is a novel technique that can play a dynamic role in crop improvement and production by enhancing the efficiency of input to minimize relevant loss in crops (Shang et al., 2019). Nanotechnology presents a wide range of solutions to the issues related to the agriculture sector and has effective methodologies to solve them (Lv et al., 2018). The advantages nanoparticles have by their name are

(a) Their high surface activity,(b)surface reaction sites, (c) catalytic effectiveness, (d) distinct optical and magnetic properties, and many more (Wang et al., 2019). Plants response to nanoparticles is influenced by both plant species and their growth stages and the nature, size, shape, and charge of nanoparticles (Burman and Kumar, 2018). Nanoparticle uptake by plants may have some sort of effectiveness due to the size, shape, and chemical composition of the nanoparticles, application methodologies, and interactions of NPs with the environment, the restriction to inflow caused by a cell wall, changes in plant physiology, and the anatomy of the different plant species (Sanzari et al., 2019).

Nanoparticles enter into the plants through the cuticle, stomata, cortex, stigma, and root tips that Pb to improve plant root and shoot length, chlorophyll contents, enzymes activities, and protein contents that can prevent the membrane from being damaged and enhancing tolerance to stress (Mishra et al., 2018). Nanoparticles not only increase agricultural output but due to their physiochemical characteristics, they are valuable in reducing stresses (Zhou et al., 2020). Enhancement to the tolerance and improvement in photosynthetic activity and metabolic process was recorded upon the entry of nanoparticles into the plant's bodies (Farooq et al., 2022). Nanoparticles can decrease heavy metal uptake in plants and wash adverse effects produced by heavy metals (Ragab and Saad 2020). Under heavy metal stress, some of the nanoparticles can enhance seed germination, plant growth, and development (Yadu et al., 2018). The use of NPs is regarded as a modern approach that has greatly improved plant growth and their activities to enhance agricultural output further to meet the demand of life.

1.5.1 . Reduced graphene oxide (rGO) Nanoparticle

Graphene is a new type of nanomaterial that has been discovered, which in itself is unique in its physical properties and has some biologically important potential applications in living systems (Li et al., 2018). Graphene oxide (GO) is a functionalized graphene bearing oxygen-containing functional groups, that has some superior properties i.e. mechanical stability, large surface area, and optical and tunable electrical properties ((Li et al., 2015). Surface functional groups of GO such as hydroxyl, epoxy, and carboxyl make it one of the extraordinary candidates in the world of nanomaterials for its striking properties (Siddiqui et al., 2019). Groups attached to GO have made it more useful than the other graphene derivatives through

physiological stability, biocompatibility, hydrophilicity, and interlayer spacing (Yin et al., 2018).GO can be prepared in the laboratory from graphite by using the modified Hummers and Offemans method (Hammerschmiedt et al., 2023) and can be further modified by the reduction of C=O groups with metal atoms (Zn, Cu, Ag), which further enables environmental adsorptive detox cation from metalloids (Sengupta et al., 2022). GO is one of the most popular two-dimensional carbon-based nanomaterials having wide uses in various fields (Zhou et al., 2023). GO is being widely used in energy and pharmaceutical industries, and environmental restoration, and is gaining attention in the field of agriculture as well (Guo et al., 2021).

As a significant expansion in manufacturing and its application in several industries, the global market value of graphene and graphene-based products is growing rapidly, in 2019 Its estimated value was thought to be $ 87.5 million and by 2030 it may rise to $ 646.8 million (Kazlauskas et al., 2023). Unique properties and applications of graphene oxide make it interact with the pollutants which bring changes in their behaviors and toxicity (Hu et al., 2018). However, the effects on plant uptake by GO nano-sheets co-occurring with heavy metals are scarce (Li et al., 2020). But as a carrier, it increases minerals micronutrient uptake of plants via controlled release (Carneiro et al., 2022). These effects of graphene oxides are positive for soil nutritional traits to plant growth and physiology (Juarez-Maldonado et al., 2019). It is thought that GO may also benefit against other toxic agents by protecting them from their harmful effects (Arikan et al., 2022). But GO is dose-dependent to plants and its application at higher levels up to 2000mg/L was found to increase reactive oxygen species in tomatoes, and cabbage and was found to decrease photosystem II activity in peas (Samadi et al., 2021).

1.6 Effect of rGO on Plants' Heavy Metals Uptake

Nowadays, nanomaterials or nanoparticles are considered an excellent means of removing heavy metals from waste water (Teik et al., 2020). In comparison to traditional materials, the use of non-toxic nanomaterial absorbents exhibits the highest efficiency rate in removing toxic heavy metals present in water media (Jeseung et al., 2020). Many of the nanomaterials i.e., carbon nanotubes, carbon-based materials, GO, metal oxides, or simple metal and in addition, polymeric absorbents have been exploited and widely used in aqueous solutions to remove the heavy metal ions

(Junxing et al., 2018). Sorbents of GO and GO-based materials are thought to remove contaminants, including organic pollutants and heavy metals from the media (Priya et al., 2021). Due to the high surface area of GO which makes it is an ideal substrate for metal nanoparticles to disperse (Moon et al., 2012). Furthermore, the presence of an oxygen-containing functional group enables it to form complexes with ions of heavy metals i.e., Pb, Cd, and As, etc on the GO surface, which further have an impact on the fate of the environment and ecotoxicologically induce effects of heavy metals and GO nanosheets in combination (Ren et al., 2018).

GO nanosheets are thought as good adsorbents in aquatic environments for Cd uptake, which show maximum adsorption of heavy metals from the environment (Li et al., 2020). Some of the studies indicate that GO bears better biocompatibility with living tissues in comparison to other nanomaterials (Hu and Zhou, 2013) which may have positive productive effects on crop plants to improve productivity (Younes et al., 2019). GO is being widely used to regulate plant loading with micronutrients to prevent phytotoxicity of plants in contaminated soils for the improvement of crop yield (Rizwan et al., 2019). However, few studies have investigated and found contradictions regarding the response shown by plants to GO in heavy-metal-contaminated environments (Cheng et al., 2016). Yin et al. (2018) have reported that under hydroponic conditions, GO in the concentration of 100–1500 mg/L causes a reduction in the growth of rice buds and seed germination in combined response with the detrimental effects of Cd_2^+, which somehow shows a similar effect as caused by zinc oxide nanoparticles when foliar sprayed on corn plants grown in the soil polluted with Cd (Rizwan et al., 2019). In the same way, Hu et al. (2014) have observed that 0.1-10 mg/L of GO treatment when applied through root enhances arsenic toxicity in wheat plants.

1.6.1 Effect of rGO on plant morphology

More recently, interest has been made in the potential toxicity caused in biological systems by graphene and graphene-based materials (Yang et al., 2013). For example, when strains of Staphylococcus aureus and Escherichia coli were treated with GO and its reduced form, rGO was found to result in membrane damage (Ghaderi and Akhavan, 2010). Results show that GO and rGO also induce persistent injury to animals and human cells (Duch et al., 2011). It is an important and

concerning point to assess ecological risks related to GO, especially in the case of plants to know plant cells' interactions with GO, before continuing with the application of GO-based products in any ecosystem. However, still very little is known about GO uptake or the effects on the regulation of cell morphology, cell organelles damage, cellular metabolism, and the redox balance of cells (Hu et al., 2014). Several studies have focused on plant species which show extremeness regarding the environment in which they grow and the effect of GO on them (Monica et al., 2009). In one of the study conducted on seed germination and seedling growth of tomato it was found that GO penetrate the seed husks and facilitate water uptake hence resulting in faster seed germination and increased germination rates (Zhang et al., 2015) Very few studies in green plants have inquired the mitigative impact of graphene and graphene-based materials. Begum et al. (2011) have found that RGO treatment of 500-2000 mg/L inhibited the growth of leaves in cabbage, red spinach, tomato, and lettuce after 20 days of treatment. However, the research work of Zhao et al. (2015) shows that the subjection of GO to the plants did not cause any changes in the root and shoot growth of Arabidopsis. But treating plants of *B. napus* L with GO in the concentration of 50-100 mg/L for 15 days, the length of the seminal root was found shorter, and a decrease in root weight was noted (Cheng et al., 2016). In the same way, Chen et al. (2018) research work shows that exposure of Triticum aestivum to suspension of GO for 9 days, seedling growth was found to be significantly restrained, which adversely affects root and shoot development and plant length, conversely causes reduction in biomass and morphological damage to the root cells of plants.

1.7 Effect of GO on anti-enzymatic activities

The principal defense system that intercepts or degrades reactive oxygen species (ROS) in the body is termed an antioxidant system (Li et al., 2011). In the antioxidant system SOD, CAT, and POD are important components against the stress in which free radicals are produced (Wan et al., 2014). The research work of González-García et al. (2019) shows the positive effect of graphene on plants growth by improving water and nutrient absorption, which act in the regulation of plants growth, activating the biosynthesis of abscisic acid and indole acetic acid, promoting the marker genes to express cell division and elongation of the cell wall and increasing antioxidant enzymes activity of enzymes as that of CAT, SOD, APX and

GP, which Pbs to the accumulation of proteins and finally improve plants growth. Excess ROS is removed by plants by the activation of antioxidant enzymes and another non-enzymatic self-defense system. SOD enzyme generation is induced by the production of $O_2^{\cdot -}$, which resulted in the dismutation of $O_2^{\cdot -}$ into H_2O_2 (Prasad et al., 2016). Subsequently, the CAT enzyme brick H_2O_2 into H_2O and APX enzyme of the ascorbic acid-glutathione cycle (Muneer et al., 2014). The research work of Hu et al. (2014) shows that oxidative stresses disturb the activity of certain key antioxidant enzymes. Compared to the controlled conditions, GO significantly inhibits POD activity. It was also found that CAT activity was inhibited for the treatments in comparison to control plants. No clear differences were noted for SOD activity in all the samples, where results show consistency with GO treatment by selectively inhibiting activity; however, due to similar concentrations of MDA in all groups, no remarkable oxidative damage was noted. Shen et al. (2018) reported that GO at treatments do not significantly increase POD, CAT, SOD, APX, or MDA which, therefore, didn't result in plant stress. Vochita et al. (2019) reported that SOD, POD, and CAT activities decrease for 1000 and 2000mg/L GO treatments and show a slight increase at 500mg/L GO treatment in comparison to control seedlings.

1.7.1 Superoxide dismutase (SOD)

In plants, enzymes are localized in different parts and work in different compartments of plant cells which work altogether to detoxify reactive oxygen species(ROS) generated through different biotic and abiotic stresses. SOD, is regarded as the first line of defense for the cell against ROS, activity recorded for it is higher for 500 mg/L and 1000 mg/L than that of control in plantlets which indicates a possible excess of oxygen radicals generated by GO treatment (Vochita et al., 2019) SOD activity got decrease first and then increased with the increase in concentration of GO compared to control from 0 to 50 mg/L GO treatment (Shen et al., 2018). Reduction in the level of H_2O_2 is noted due to the amino acids which shift the pathways of ethylene synthesis, and prevent oxidative damage to the cells (Zhu et al., 2016). Amino acids are regarded as precursors for the synthesis of antioxidant enzymes in the antioxidant system to protect plants against damaging oxidative stresses in plants (Foyer and Noctor, 2005). SOD enzymes are activated to control the production of H_2O_2 and protect plants from its poisoning effects (Hossain et al., 2015). Research work of Wang et al. (2022) shows that 400µg/ ml treatment of GO

significantly increased SOD activity in comparison to the control and other treatments in wheat plants. Ren et al. (2016) have shown that nanoparticles of GO and amino acids mitigate the negative effect of salt stress and hence increase SOD activity to decrease ROS content. The work of Zhao et al. (2022) also shows that by increasing GO concentration SOD activity significantly increased except 0.2% GO, and the increase was found greater for 100 days than that of 50 days of exposure.

1.7.2 Catalase (CAT)

CAT converts the resultant of SOD H_2O_2 into a non-toxic product of H_2O and O_2 in peroxisomes in plant cells, activity of CAT is higher at 500 mg/L treatment of GO than that of control while decreases for higher concentration and becomes minimum at a concentration of 2000 mg/L (Vochita et al., 2019). In comparison to control GO inhibits CAT activity and is concentration-dependent (Hu et al., 2014). From the research work of Zhao et al. (2022) it was also found that CAT activity significantly increases with increasing GO concentration but except 0.2% GO, and it was noted that the increase in CAT activity was greater for 100 days of exposure than that of 50 days exposure of plants to the treatment. The study work of Anjum et al. (2014) also revealed that a GO concentration of 800 mg/L significantly decreases H_2O_2 content and increases CAT activity to a maximum followed by 400 mg/L of GO treatment in comparison to 100, 200, and 1600 mg/L treatments of GO. It was also found that CAT content increased according to Zhao et al. (2022) while the content of H_2O_2 decreased with GO treatment.

1.7.3 Peroxidase (POD)

Oxidative stresses disturb the activity of certain key antioxidant enzymes. Compared to the control, GO significantly inhibits the activity of POD (Hu et al., 2014). Peroxidases are involved in the oxidation of many substrates, which are stimulated by H_2O_2 accumulation in the plant cells. As compared to CAT, POD has a higher affinity for H_2O_2 but processes it at a very slow rate. The activity of POD increases with a low concentration of GO and decreases at high concentrations of GO (Vochita et al., 2019). However, the results of Shen et al. (2018) revealed that POD activities in one plant specie increases with the increase in GO concentration but decreases in another plant species by increasing GO concentration. The study work of Mahmoud and Abdelhameed et al. (2021) shows that when modified nanographene oxide and amino acid were applied to pearl millet plants significant increase in POD

activity occurred in pearl millet leaves. Research work of Zhao et al. (2022) also shows that by increasing GO concentration except for 0.2% GO treatment POD activity significantly increased and the increase was greater for 100 days exposure of plant to GO than that of 50 days exposure.

1.7.4 Ascorbate peroxidase (APX)

H2O2 is metabolized by APX into H2O by reducing GSSG into GSH using NADPH as reducing power to drive this redox reaction to completion. So, the possible alteration in the AsA-GSH cycle may affect the scavenging of H_2O_2, which could adversely affect the growth and overall physiology of the living body (Prasad et al., 2016). In the defense mechanism of plants H_2O_2 plays a crucial role, an increase in its concentration causes toxicity and damages the biological membrane due to the induced oxidative stresses which could be scavenged by the antioxidant enzyme APX (Ganjavi et al., 2021). The APX enzyme activity could be noted to reach the maximum under GO treatment of 50µg m/L and then decreased further in comparison to the control treatment (Wang et al., 2022). The research study of Anjum et al. (2014) revealed that the activity of APX increases to a maximum at 800 mg/L of GO treatment with a decrease in H2O2 content and the exact is followed by the treatment of 400 mg/L in comparison to GO treatments of 100, 200 and 1600 mg/L with that of control. Nanoparticles of GO increase the activity of APX under both stress and non-stress conditions in plants (Fatehi et al., 2021). The study of Arikan et al. (2022) also shows that 250 mg/L GO treatment increases the activity of APX and the maximum activities observed were 87% and 127% for different treatments of GO to the plants.

1.8 Aims and Objectives

The main objectives of this study are:

- To find out the effects of Pb stress on the morphological parameters of wheat plants.
- To find and explore the effects of Pb stress on antioxidant enzymes of wheat plants.
- To evaluate the mitigative impacts of greenly reduced GO Nanoparticles (rGO-NPs) on wheat plants under Pb stress conditions.
- To evaluate the physiological and biochemical mechanisms induced by rGO NPs to ameliorate the stress of Pb in wheat plants.

2. MATERIALS AND METHODS

A pot culture was performed to evaluate the *Triticum aestivum* and its susceptibility index under heavy metals stress and NPs. The details of materials and methods used for experimentation and its evaluation are mentioned below.

2.1 Synthesis of GO

1 gram of graphite was added to 17.5 ml of H_2SO_4 which was stirred for 45 minutes. Afterward, 0.5 g of $NaNO_3$ was added and the solution was further stirred for 15 minutes. Then 3 grams of $KMnO_4$ were slowly added by keeping the temperature below 10℃ and continued stirring for 10 to 15 minutes. After that, the solution was placed on a hot plate with continuous stirring for 1 hour at 40℃. Distilled water was added to this mixture dropwise by keeping the temperature below 10℃. The colour of the mixture turned brown which indicated the formation of GO.

2.2 Green reduction of GO

Tecoma stans leaves were collected from Quaid I Azam University Islamabad. The leaves were washed to remove the dust and other contaminations. They were weighed 40 g, cut down into small pieces, and then ground with mortar and pestle by gradually adding 150 ml of water. The solution was heated on the hot plate until its color changed to yellowish. The plant extract was then filtered with Whatman No 42-filter paper. The plant extract was added to the mixture stirred and heated at 70℃ for 10 hours to evaporate the solvent and leave the reducing and capping agent behind. The color of the mixture turned black. 75 ml OF H_2O_2 was added drop by drop to stop the further reaction. The mixture was then centrifuged at 8000 rpm for 20 minutes, the supernatant, and washed the pellet with 3% HCl three times and then with distilled water 2 times. The blackish brown paste obtained was then kept in the oven for 24 hours at 50℃ and blackish flakes of dried rGO were obtained which were then crushed into powder form.

2.3 Characterization of rGO

2.3.1 UV analysis

For the determination of rGO, samples were collected at different intervals during preparation, and absorbance was measured using a UV-vis spectrophotometer at 200-600 nm wavelength.

2.3.2 Fourier-Transformed Infrared Spectrophotometer (FTIR) Analysis

The presence of different functional groups in rGO NPs was identified with the help of infrared spectra in transmission mode with the help of a Fourier-transformed Infrared Spectrophotometer (FT/IR-610, JASCO) keeping wavelength in the range of 400-4000 cm^{-1}.

2.3.3 X-Ray Diffraction (XRD) Analysis

X-ray diffractometer (Shimadzu Model: XRD 6000) was used for the XRD investigation of rGO NPs in the powder form in the range of 20-80̊ at 2 theta which is equal to 0.154 nm in wavelength.

2.3.4 SEM-EDX Analysis

The assessment of rGO NPs morphology with EDX analysis was done by using a scanning electron microscope (SEM: JEOL JSM-5800 LV/EDS).

2.4 Experimental Description

2.4.1 Experimental Site

The experimental works were carried out at the Plant Sciences Department, Quaid-i-Azam University, Islamabad. The experimental procedures were carried out at the Plant Proteomics Laboratory, Quaid-i-Azam University, Islamabad.

2.4.2 Experimental Design

A complete setup was designed to carry out the experimental work in a pre-planned manner. According to the need and importance of each experimental analysis, three replicates were used to get the mean values for all the parameters. The detailed work for each single parameter of all the replicates was kept on record for further analyses.

2.5 Experimental Detail

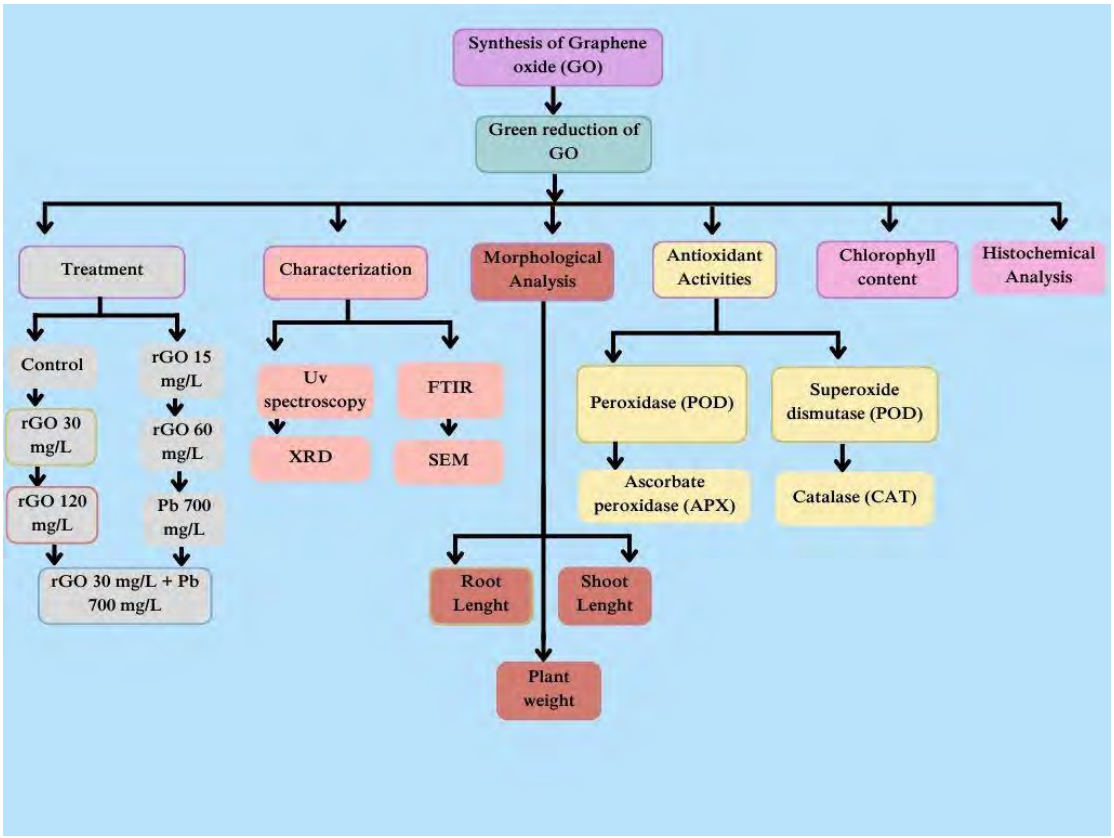

Figure 2.0 shows the work Layout of the current research work

2.5.1 Plant Specie

Triticum aestivum (Wheat plant), variety Akbar 2019, was selected for the experimental work.

2.5.2 Growth Medium

The sand was selected as the growth medium, which was properly bleached for 24 hours to remove all the impurities and then washed with tap water 15 times to remove the bleach and the mineral nutrients up to the maximum.

2.5.3 Seed Sowing

Triticum aestivum (Akbar 2019) seeds were obtained from the Pakistan Agriculture Research Council located at the National Research Centre Islamabad (NARC). Healthy seeds were selected through screening of seeds. The seeds were then sterilized with 3% sodium hypochlorite for 10 minutes. Seeds were then washed with distilled water three times, 20 healthy seeds were sown in each pot containing 800 ml of sand and 210 ml of water.

2.5.4 Growth Condition

Plants were grown in the laboratory by exposing them to 16 hours of light and 8 hours of dark and the temperature was kept at 20-25℃ under control conditions. Plants were irrigated daily with tap water.

2.5.5 rGO and Pb suspension preparation

Four different solutions of rGO were prepared: 12 mg of rGO were dissolved in 100 ml of distilled water to prepare a 120 mg/L solution, 6 mg of rGO was dissolved in 100 ml of water to prepare a 60 mg/L solution, 3 mg of rGO were dissolved in distilled water to prepare 30 mg/L solution and 1.5 mg of rGO was dissolved in 100 ml of water to prepare 15 mg/L solution as well and were sonicated for 30 minutes. For a 700 mg/L solution, 70 mg of Pb nitrate $Pb(NO_3)$ was dissolved in 100 ml of distilled water.

2.6 Metal and Nanoparticle Application

Heavy metal Pb nitrate $Pb\ (NO_3)_2$ was applied as a stress to induce phytotoxicity in wheat while rGO NPs were used for its mitigation. Four treatments of rGO were applied separately with concentrations of 15, 30, 60, and 120 mg/L, one was used for Pb stress, and one was kept as a combination of Pb 700 mg/L and rGO 30 mg/L on 8^{th} days. Plants were harvested by keeping a gap of one day for each treatment after the application of nanoparticles and Pb stress.

2.7 Growth Parameters

The morphological parameters of wheat plants were measured for three independent replicates by repeating the same treatments in the same condition.

2.7.1 Shoot length

Shoot length was measured in cm on the scale and their average values were calculated.

2.7.2 Root length

The average root length was measured from the tip of the root to the base where shoots were attached to roots using a centimeter scale.

2.7.3 Fresh weight

With the help of an electronic scale, the plant's different parts i.e. root and shoot's weight were measured in mg.

2.7.4 Chlorophyll content (SPAD value) determination

A chlorophyll meter (SPAD-502; Konica, Minolta sensing, Inc., Japan) was used for measuring The SPAD values for chlorophyll content in the leaves.

2.8 Histochemical Analysis

Stress-induced cell membrane damage was quantified with Evan's blue staining method. Fresh plant root parts were collected from control and differently treated plants and transferred into 2 ml Eppendorf tubes. After that, 2 ml of Evan's blue dye was added to each Eppendorf containing samples and was kept for 24 hours. Then after 24 hours of staining, they were properly washed with distilled water and the stained samples were then examined under a light microscope.

2.9 Antioxidant enzyme activity

125 mg of roots were ground with the help of an ice-cold pestle and mortar from each sample for each replicate. 1 ml of buffer was added to the extraction, the buffer consisted of 35 ml of 0.07 potassium phosphate buffer, 400 µl of 100 mM EDTA, 500 µl OF 200 mM ascorbic acid, and 2g Polyvinylpyrrolidone. The slurry was added to the Eppendorf tube and was centrifuged for 20 minutes at 15000 rpm after that the pellets formed were discarded and the supernatant was centrifuged for 20 minutes at 15000 rpm again. The supernatant was collected and used as an enzyme extract.

2.9.1 Catalase (CAT) Activity

The activity of CAT was done and measured according to Aebi, (1984) methodology.

Reagents for the estimation of CAT activity are in Table 1 below;

S.No	Constituents	Test	Blank
1	Potassium phosphate buffer (50 mM)	12.5 ml	12.5 ml
2	H_2O_2 (10 mM)	1.75 ml	1.75 ml
3	Water	2.38 ml	2.38 ml
4	Enzyme extract	10 µl	

Through spectrophotometer, the optical density for CAT was calculated at 240 nm wavelength

2.9.2 Ascorbate Peroxidase (APX) Activity

For the calculation of APX activity Nakano and Asada's (1981) methodology was used.

Table 2 Reagents for the estimation of APX activity are as under;

S.No	Constituents	Test	Blank
1	Potassium phosphate buffer (25 mM)	6.3 ml	6.3 ml
2	EDTA (0.1 mM)	18 µl	18 µl
3	Ascorbic acid (0.25 mM)	90 µl	90 µl
4	H_2O_2 (10 mM)	1.8 ml	1.8 ml
5	Water	8.92 ml	8.92 ml
6	Enzyme extract	10 µl	

At 25° C reduction in APX was noted successively giving the amount of APX at 290 nm wavelength for both blank and test solutions and was measured through a spectrophotometer.

2.9.3 Superoxide Dismutase (SOD) Activity

SOD contents investigation was conducted through Verma and Dubey (2003) methodology.

Table 3 shows Reagents for the estimation of SOD activity is given as;

S.No	Constituents	Test	Blank
1	Potassium phosphate buffer (200 mM)	3.5 ml	3.5 ml
2	NBT (250 µM	3.5 ml	3.5 ml

3	Riboflavin (10 µM)	3.5 ml	3.5 ml
4	Distilled water	6.4 ml	6.4 ml
5	TEMED	35 µl	35 µl
6	Enzyme extract	10 µl	

The blank and test solutions densities were measured at 560 nm wavelength for SOD activity with a spectrophotometer.

2.9.4 Peroxidase (POD) Activity

The activity of POD was estimated through Velikova et al., (2000) methodology.

Reagents for the estimation of POD activity are in Table 4.

S.No	Constituents	Test	Blank
1	Sodium phosphate buffer (100 mM)	5.25 ml	5.25 ml
2	H_2O_2 (1%)	3.5 ml	3.5 ml
3	H_2SO_4(5N)	3.5 ml	3.5 ml
4	P-Phenylenediamine (4%)	3.5 ml	3.5 ml
5	Distilled water	1.25 ml	1.25 ml
6	Enzyme extract	10 µl	

The optical densities for the activity of POD for test and blank solutions were measured at 485 nm with the help of a spectrophotometer.

Results

The result of GO nanoparticle synthesis and its green reduction, its characterization, as well as its effects on wheat plants are shown here. Furthermore, the effects of rGO NPs along with Pb stress on wheat morphological, physiological, and biochemical parameters are also results.

3.1 Synthesis of GO

17.5 ml of concentrated H_2SO_4 was taken in a flask and 1 gram of graphite was added to it and stirred. After 45 minutes of stirring, 0.5 grams of $NaNO_3$ were added to it and kept on stirring continuously for an hour. Now 3 grams of $KMnO_4$ were added slowly by keeping temperature below 10℃ on ice bath and stirred for 10 to 15 minutes. Stir it on a hot plate for 1 hour at 40℃ and then add 45 ml of distilled water drop by drop due to fume formation keeping the temperature below 10 °C the color of the suspension will become brown. The sample was taken and UV was done through a spectrophotometer.

3.1.1Green reduction of GO

A plant extract was prepared from *Tecoma stans* plants to reduce graphene oxide (GO) through a green reduction process. 15 ml of the plant extract was added to 150 ml of brown GO suspension and stirred on a hot plate for 10 hours at 70°C, as depicted in Figure 1.1. At this temperature, the solvent evaporated, leaving behind the reducing and capping agents, causing the suspension's color to change from brown to black. Subsequently, 75 milliliters of H_2O_2 were added drop by drop to halt the reaction. The suspension was transferred to 50 ml falcon tubes and centrifuged at 8000 rpm for 20 minutes. The supernatant was discarded, and the pellets were then washed three times with 2% HCl and two times with distilled water. The brown-black paste obtained was placed in an oven for 24 hours to dry and was subsequently ground into a black powder, resulting in reduced graphene oxide (rGO), as shown in Figure 1.1.

Figure 1.1 (a), (b), (C), (d), (e), (f), and (g) shows preparation of rGO

3.2 rGO NPs Characterization

The rGO NPs were characterized via several techniques such as UV-VIS spectroscopy, FTIR, XRD, and SEM. These characterization results predicted the green reduced synthesized nano rGO and are discussed below.

3.2.1 Color change

A colour change is a good indication of a reduction in GO. The brown GO paste before thermal reduction turned to black after drying in an oven at 45 °C for 24 hours, as shown in Fig, This indicates the successful conversion of GO into rGO as shown in Figure 1.2 (a),(b).

Figure 1.2 (a) and (b) shows the color change of rGO

3.2.2 UV-VIS Spectroscopy

The UV-VIS spectroscopy of rGO NPs was done at a wavelength of 150-600 nm in nine intervals of 50. Absorbance has shown two peaks for GO one peak occurred at 300 nm while the other at 250 nm For GO, it is at around 230 but due to particle size it may proceed to 250 nm which means our nanoparticles are synthesized as shown in figure 1.3.

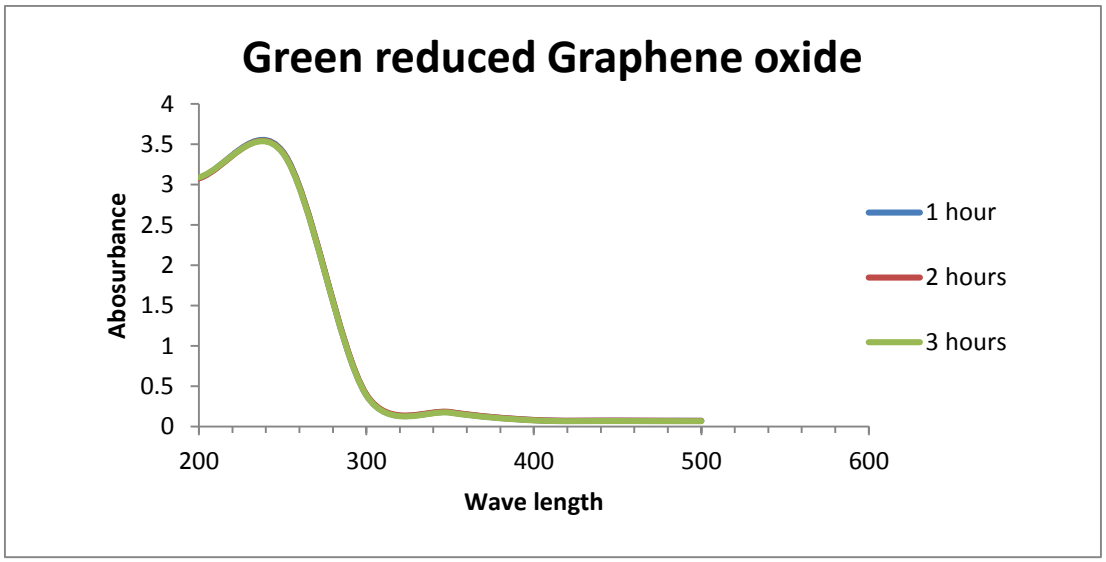

Figure 1.3 shows the UV graph for rGO

3.2.3 Fourier-Transformed Infrared Spectrophotometer (FTIR) Analysis

Typically FTIR spectra of greenly reduced GO are given in Fig. 1.4, which includes several distinct bands in the range of 422-3705 cm^{-1} wavenumbers. All these bands are due to certain different types of vibration, which specifically describe certain characteristics of functional groups of greenly rGO such as the sharp broadband at 3179.37 cm^{-1} representing hydroxyl (OH) functional group of rGO in which the OH group is in the intermolecular bonded, 1720.237 cm^{-1} represent C=O stretching, at 1615.88 cm^{-1} amine (N-H) functional group is present, at 1228.14 cm^{-1} alkyl aryl ether (C-O) functional group is residing, at 1032.463 cm-1 sulfoxide (S=O) functional group is present, while at 435.39 and 420.89 cm^{-1} monosubstituted C-H containing functional groups are found. The peaks in 1039.63, 1228.14, 1615.88, 1723.14, and 3171.89 cm^{-1} are found to decrease and disappear dramatically, which indicates the removal of oxygen-containing functional groups in GO this is an

indication of the removal of oxygen-containing functional groups in GO. From the rGO spectra, it is confirmed that most of the oxygen-containing functional groups are removed from GO and hence its reduction occurred. But still, some residual functional group of oxygen exists on the surface of rGO.

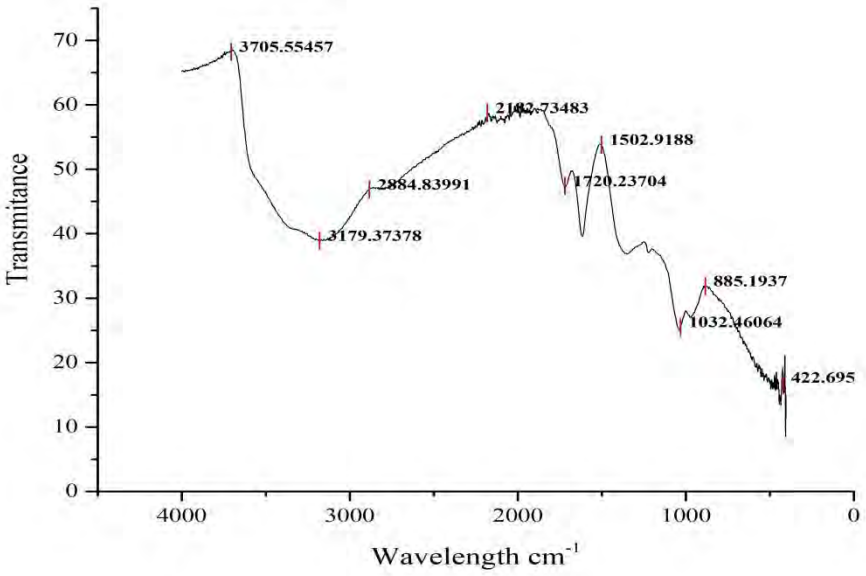

Figure 1.4 shows the FTIR spectra of rGO

3.2.4 X-Ray Diffraction (XRD) Analysis

XRD patterns for greenly rGO were analyzed through Origin pro 9.0 64-bit software. The XRD graph in Figure 1.5 has shown sharp peaks in the range of 11.26º to 42.43º starting from 2 theta. The narrow, sharp, and long peak which range at 11.26º indicates the crystalline nature of rGO NPs as shown in figure 1.5. The average size calculated for rGO was found to be 2.87 – 5.52 nm with the help of the Debye-Scherrer equation as given below in Table 5.

$$Lc = K\lambda/\beta \cos \theta$$

Position 2 theta	FWHM	Crystallite size D (nm)	Average size (nm)
11.26044	1.14198	6.923032027	4.227400773
29.94562	32.7502	0.234336548	2.879585146

42.43534	1.34045	5.524833744	5.524833744

Table 5 shows the average size of the rGO particle through XRD spectroscopy

Where 'k' represents the proportionality constant and its value is 0.9 λ representing the wavelength of X-rays. FWHM of diffraction is represented by β representing the FWHM, while the angle of diffraction is represented by θ in the above equation.

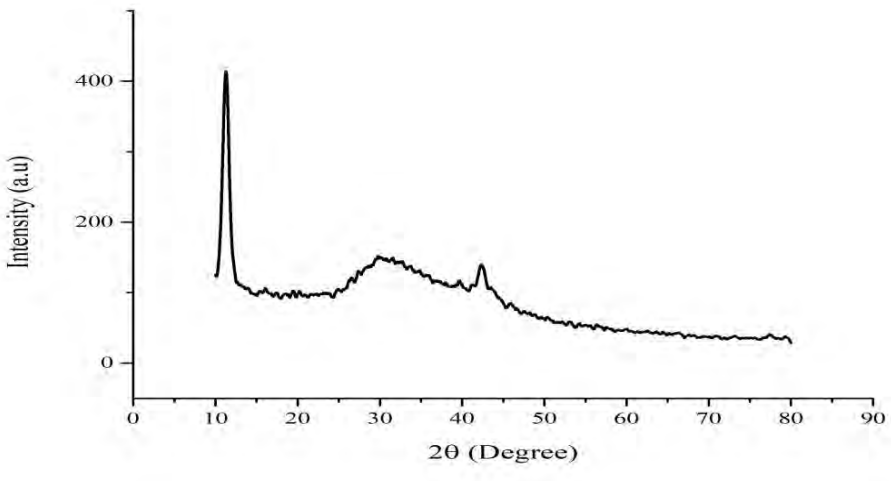

Figure 1.5, shows a graph of different peaks in the XRD spectra graph

3.2.5 Zeta potential

Zeta potential acts as a crucial indicator for surface potential charge and assigning stability to nanoparticles in aqueous suspensions. Greenly rGO NPs exhibit a potential value of -20.6 mV, as shown in Figure 1.6. symbolize the presence of a negative charge on nanoparticles. Understanding Zeta potential is necessary to comprehend the stability of nanoparticles in aqueous suspensions. Literature studies suggest that nanoparticles having zeta potential values higher than +30 mV and less than -30 mV are thought to be stable in the dispersion medium. The Zeta potential value of rGO highlights the long-term stability and compatibility.

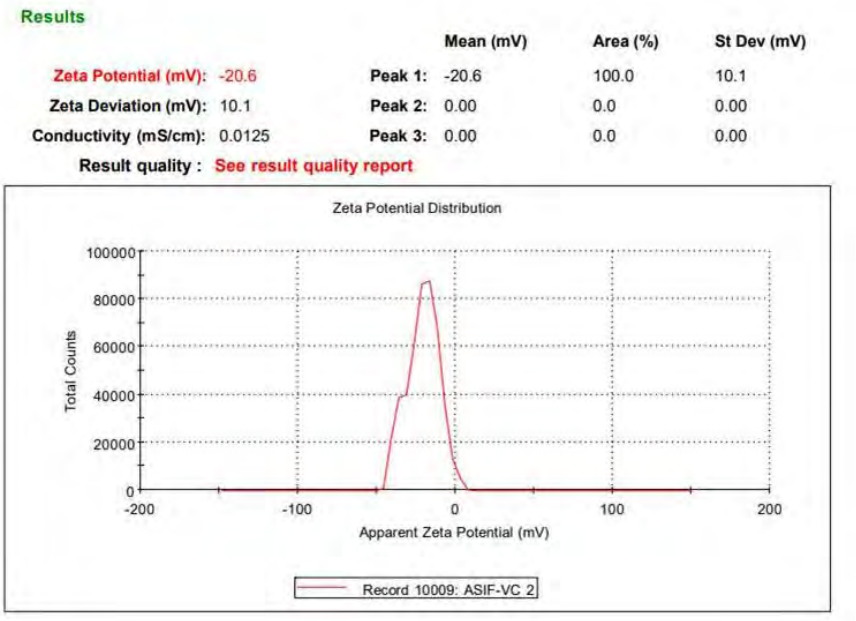

Figure 1.6 shows a graph of the zeta potential

3.2.6 SEM analysis of rGO

The SEM analysis shows that the greenly rGO NPs have polygonal spherical shapes with a particle size range from 9.67-11.57 nm (Fig.1.7 b, c). Aggregation of moderate levels is also shown in images. From EDS spectra presence of carbon and oxygen is confirmed with percentage values of 64.45 % for carbon and 35.55 for oxygen in greenly rGO NPs (Fig 1.7d). The elemental mapping was carried out with the EDS technique as shown in (Fig.1.7f) where carbon (kα) is represented with red color and oxygen (kα) is described with green color.

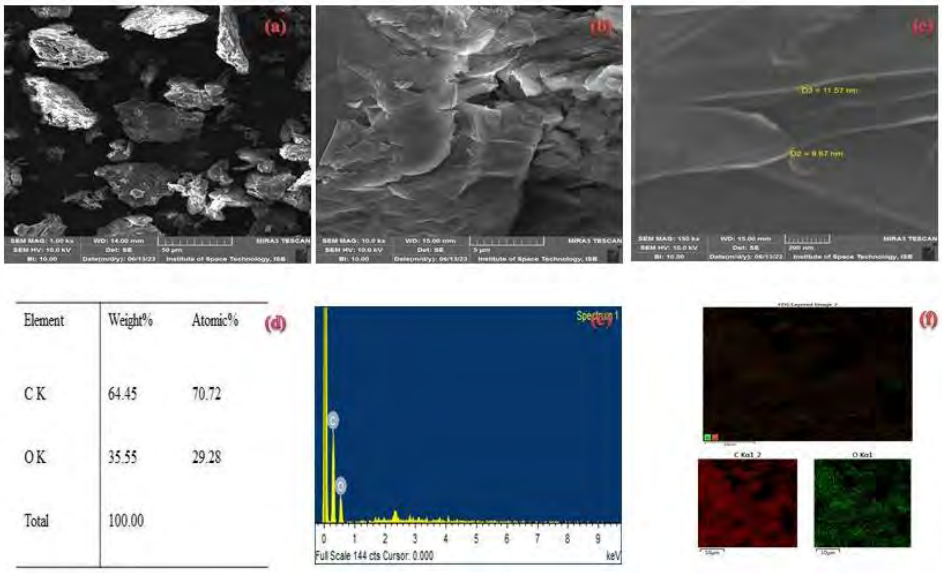

Figure 1.7 (a), (b), (c) show SEM images of rGO, (d) show EDS values, (e) shows EDS graph, and (f) shows ka of oxygen and carbon.

3.3 Effects of Pb on wheat morphologies

Morphological characterization of wheat plants was investigated at a concentration of 700 mg/L of Pb stress.

3.3.1 Effect of Pb on the wheat plant, root, and shoot length

To investigate the effect of Pb on plant length, shoot, and root length 8 days old plant of wheat was exposed to Pb stress of three different concentrations (300, 500, and 700 mg/L) Significant variations were noted in all three replicates of plant harvested with the gap of 2 days after treatment in comparison to control plants as shown in figure 2.1. Results demonstrate that Pb causes serious damage to the plant's shoot and root length and significant reduction was noted in all the lengths of plants. On days 2 and 4 significant reduction was noted for plants treated with 700 mg/L concentration of Pb in comparison to control plants and other treatments of Pb shown in Figure 2.1 (a and b). For days 2 and 4 reduction in plant shoot and root lengths was noted.

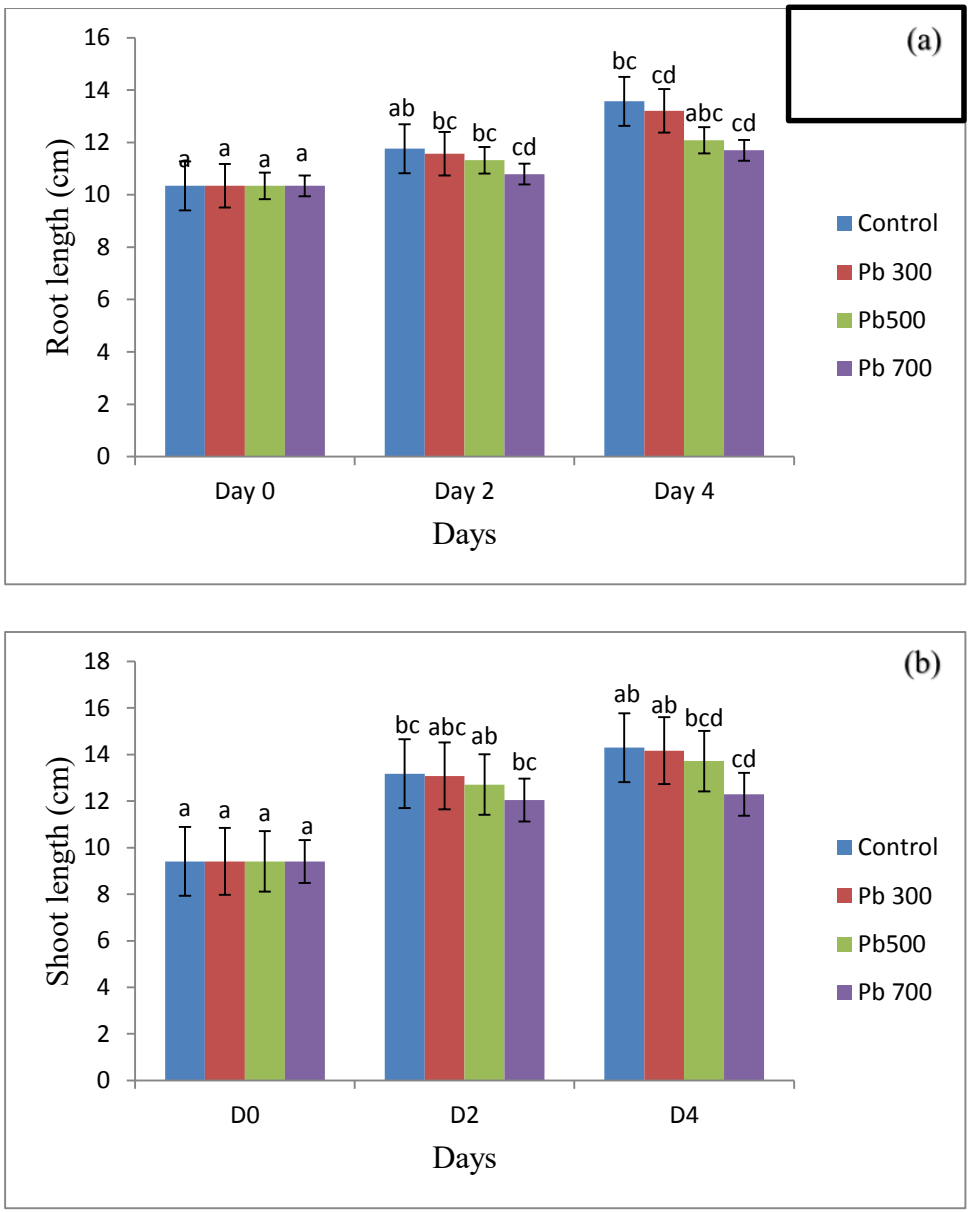

Figure 2.1(a) shows the root length of wheat plants against different concentrations of Pb. Figure 2.1(b) shows the shoot length of wheat plants against different concentrations of Pb.

3.3.2 Effect of Pb on wheat fresh weight

The fresh weight of the plant was investigated in response to the Pb stress. Three different concentrations (300, 500, 700 mg/L) of Pb were applied to wheat plants. significant variations were noted for Pb stress in fresh-weight plants for all the concentrations of Pb stresses. Results demonstrate that in comparison to the control plants, and other treatments of Pb, 700 mg/L was found to significantly reduce the total fresh weight of plants. This means that 700 mg/L of Pb causes more damage to

the plants and reduces their fresh weight for all day 2 and 4 plants in comparison to Pb 300 and 500 mg/L. For further application in our study to check matigation in response to Pb stress through rGO 700 mg/L of Pb stress was used.

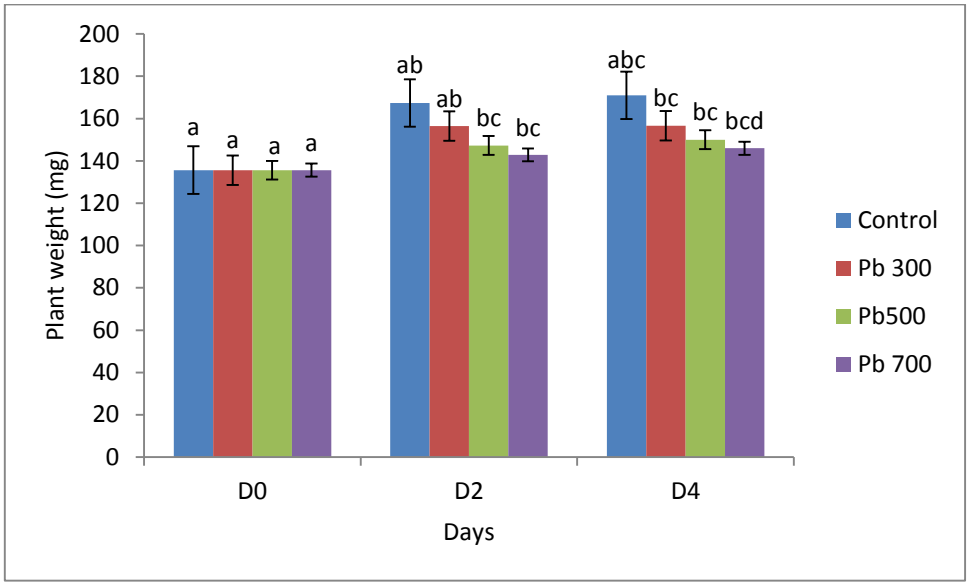

Figure 2.2 shows the plant weight of plant against different concentrations of Pb stresses.

3.3.3 Morphological Characterization of Wheat Plant under rGO NPs Application

Morphological characteristics of 8 days 8-day-old wheat plant were investigated at four different concentrations (15, 30, 60, and 120 mg/L) of greenly rGO NPs for three days with one day of gap. The statistically analyzed data show significant variation between all treatments concerning time exposure in terms of all parameters.

3.3.4 Effect of rGO NPs on root length of wheat plants

The root length of the wheat plant was investigated under four various treatments of rGO NPs (15, 30, 60, and 120 mg/L) shown in Fig 2.3. From the statistical analysis, a significant variation was noted in the root length at various concentrations mentioned above at different time intervals. The results show that rGO NPs caused significant promotion in root length. On days 2 and 4 after stress application, all rGO NPs caused root elongation positively in comparison with the

control except for rGO 15 mg/L which didn't carry the trend as the other did and had the same results as that for the control. All the treatments have promoted root length positively. Maximum increase in length of averaged 21.43 cm occurred at 60 mg/L of rGO on day 2 of the treatment followed by the other treatments. But at day 4 rGO of 30 mg/L have shown a maximum average length of 24.72 in comparison to control and other treatments. From the above results, it is concluded that 30 mg/L of rGO causes a maximum increase in root length of wheat plants in comparison to control and other rGO treatments as shown in Fig 2.3.

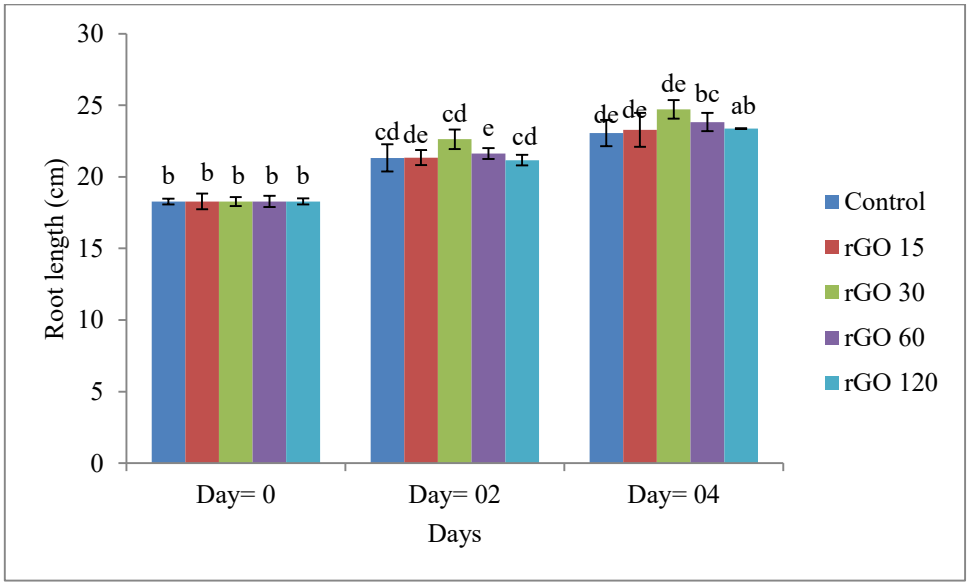

Figure 2.3 shows root length under different rGO treatments

3.3.5 Effect of rGO NPs on shoot length of wheat plants

The shoot length of the wheat plant was evaluated in response to four different concentrations (15, 30, 60, and 120 mg/L) of rGO NPs shown in Fig 2.4. A significant difference in shoot length of wheat plants was shown from the statistical analysis. According to the experimental findings, rGO NPs have a significant impact on the shoot length of wheat plants. At day 2 maximum increase in average shoot length of 16.80 cm was noted for 60 mg/L of GO in comparison to that of control and other rGO NPs treatments of the same day plants in all the replicates. However the trend was noted and on day 4, 30 mg/L of rGO treatment was found to have a maximum average shoot length of 17.98 cm in comparison to control and other rGO treatments. And same was the case with rGO 120 mg/L which showed an increase in average

shoot length in comparison with control on day 2, but on day 4 the average shoot values of rGO and control were found to be almost the same in a statistical comparison of all the three replicates. Hence results show that up to 120 mg/L of rGO treatments have a positive impact on wheat shoot length and improve wheat shoot length positively. In the case of the shoot length effect of rGO, 15 mg/L was almost the same as that of the control and wasn't as effective as that of others as shown in Fig.

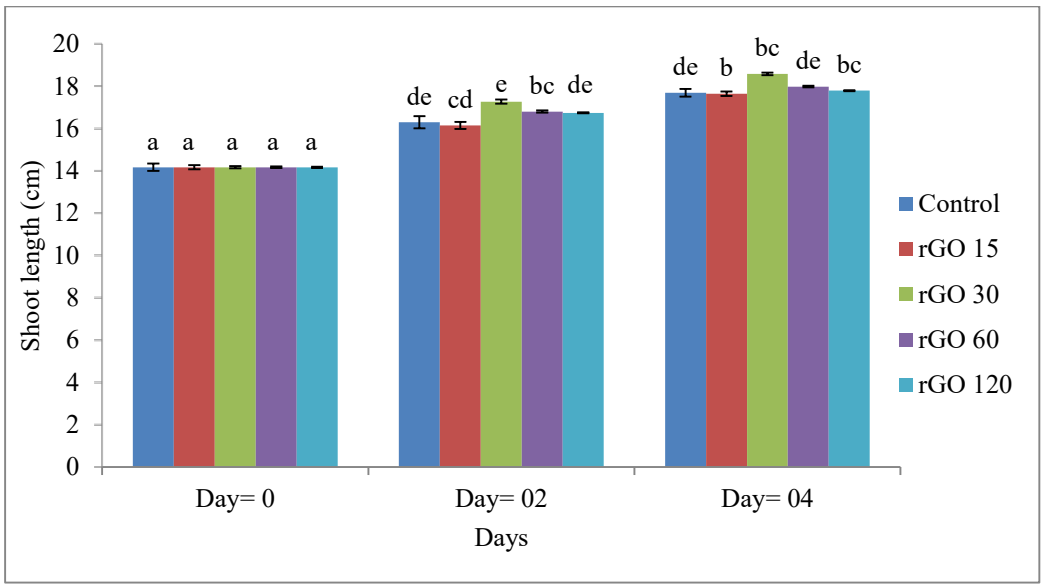

Figure 2.4 shows shoot length under different rGO treatments

3.3.6 Effects of rGO NPs on fresh weight of wheat plants

The fresh weight of wheat plants was investigated at four different concentrations (15, 30, 60, and 120 mg/L) of rGO NPs shown in Fig 2.5. Fresh weight of rGO NPs treated plants showed a positive response to all treatments. Results showed a significant enhancement in the plant weight subjected to 30 and 120 mg/L concentrations of rGO NPs. Some exceptional trend occurred at 60 mg/L treatment of rGO where an increase In plant fresh weight was noted on day 2 but a sudden decrease was shown on day 4 in all three replicates in comparison to control and other rGO treatments in wheat plants. The maximum average plant fresh weight was noted at day 4 of 30 mg/L treatment followed by 120 mg/L treatment of rGO for wheat plants. An increase in average plant root weight was noted for wheat plants in

all three treatments in all three replicates. The maximum average root weight was noted for 120 mg/L treatment of rGO at day 4 with an average value of 121.32 mg of weight. The shoot weight of wheat plants increases for all the rGO treatments in comparison with control, but a trend was noticed where the maximum value for shoot length was noted for 30 mg/L (116.45 mg) of rGO followed by 120 mg/L (114.21 mg) of rGO instead of 60 mg/L (106.7 mg) of rGO in a continuous manner as shown in fig 2.5. For both days there wasn't any significant difference that occurred for rGO 15 mg/L in comparison to control and other treatments of rGO. To observe the mitigation of Pb stress in wheat plants, rGO 30 mg/L was used in combination with Pb 700 mg/L stress.

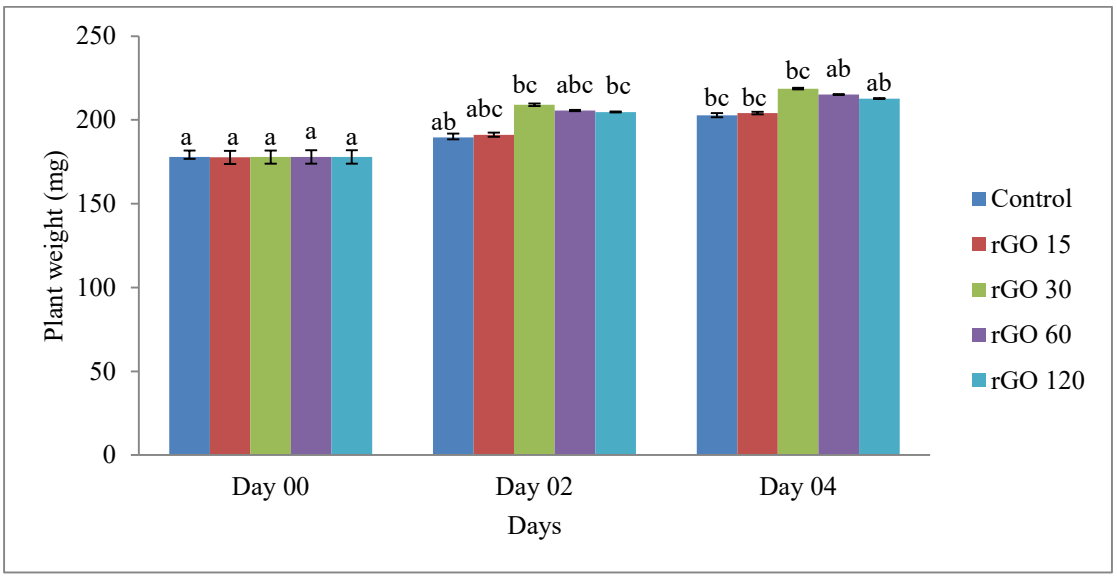

Figure 2.5 shows plant weight under different rGO treatments

3.3.7 Morphological characterization of wheat plant under Pb and rGO NPs stress

To investigate the effective influence of Pb on wheat plant morphology and their mitigation through rGO nanoparticles, 700 mg/L of concentrations of Pb, 30 mg/L concentration of rGO NPs, and a combination of Pb and rGO NPs (700 + 30 mg L−1) were used. The results demonstrate a significant variation in all parameters of plants at these concentrations over different time exposures.

3.3.8 Pb, rGO NPs, and Pb+rGO NPs effect on wheat shoot length

To investigate the influence of Pb and rGO NPs, the wheat plant was treated with Pb (700 mg/L), rGO NPs (30 mg/L), and co-exposure of Pb and rGO NPs (700 mg/L+ 30 mg/L). The statistical analysis exhibited a significant variation in shoot length for all treatments at all three days by keeping a gap of one day to find out the clear differences as shown in Fig 2.6. Plants were treated on day 8 after sowing and were harvested as day zero plants, day 2 and day 4 plants for all the treatments to measure and check the required parameters for plants. On day 2 significant differences were noted for Pb stress in comparison to control and rGO 30 mg/L treatment as a reduction in average for all the three replicate shoot lengths was noted for wheat plants. The average shoot length for all three replicate Pb treatments of 700 mg/L at day 2 was found to be 16.24 cm, for the control it was 16.52 cm, and for rGO 30 mg/L was 16.60 cm approximately the same as the control. For day 4 the differences were clearer than that of day 2 as shoot length under Pb stress of 700 mg/L was found to reduce in comparison to the control and rGO treatment, for Pb shoot length was found to be 16.64 cm, for control it was 17.69 cm and for rGO it was 17.98 cm as an average of all the three replicates. Shoot length for the combination of Pb 700 mg/L and rGO 30 mg/L was found that shoot length increase for rGO in comparison to Pb-stressed plants of the same day. On day 2 the average shoot length of all three replicates for Pb stress was found to be 16.24 cm while for Pb + rGO combination it was found to be 16.46 cm, and in the same way, the results remained the same for day 4 plants where average shoot length was recorded 16.64 cm for Pb stress and 17.71 cm for the combination of Pb and rGO as shown in figure 2.6.

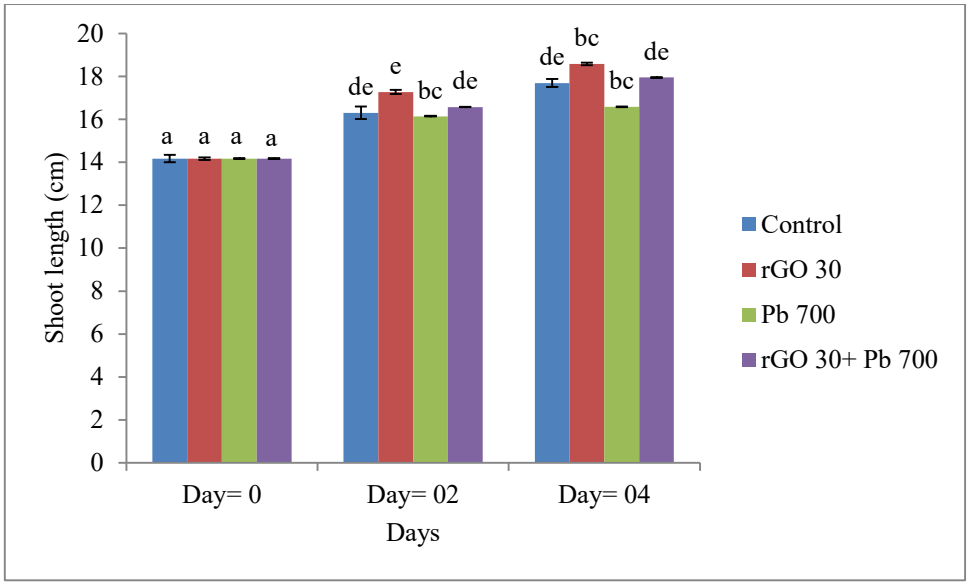

Figure 2.6 shows shoot length under rGO, Pb, rGO + Pb stress conditions

3.3.9 Pb, rGO NPs, and Pb+rGO NPs effect on wheat plants root length

The data of root length of the wheat plant under the concentrations of Pb 700 mg/L, rGO NPs 30 mg/L, and a combination of Pb and rGO NPs (700 mg/L + 30 mg/L) was statistically analyzed. A significant variation in root lengths was found for all 3 days in all treatments as shown in fig 2.7. A significant increase in root length was noted for both rGO 30 mg/L and the combination of rGO with Pb in comparison to only Pb-stressed plants for all days plants. For day 2 root length of control and rGO were almost the same at 21.32 cm for control and 21.33 for rGO-treated plants but root length for the Pb-stressed plants on day 2 was found to reduce with an average root length of 16.73 cm for all three replicates. and the same was observed on day 4 where average root length was found to be 23.06 cm for control, 24.72 cm for rGO, and 15.5 cm for Pb-stressed plants. In the same way in a combination treatment of Pb and Pb it was also noted that root length increased in comparison to only Pb-stressed plants for both day 2 and day 4 plants. In a combination treatment of Pb and rGO, the average root length of all three replicates was found to be 24.9 cm which shows an increase in term of 15.5 cm for Pb where reduction is clear in the root length of plants.

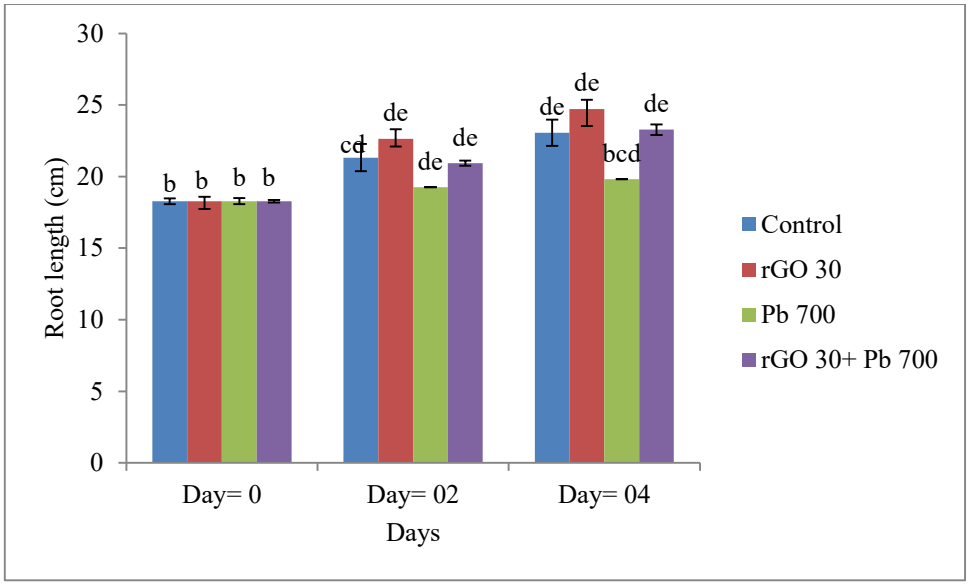

Figure 2.7 shows root length under rGO, Pb, rGO + Pb stress conditions

3.3.10 Pb, rGO NPs, and Pb+rGO NPs effect on fresh weight of wheat plant

From the statistical analysis, it was revealed that fresh weight of wheat plant under concentrations of Pb 700 mg/L, rGO NPs 30 mg/L, and a co-exposure or combination of Pb and rGO NPs (700 mg/L+ 30 mg/L) significantly varied at all time intervals after stress application as shown in fig 2.8(a). In comparison to the control, the fresh weight of the plant increased for rGO treatment of 30 mg/L, and the same was noted for Pb-stressed plants, where in comparison to Pb-stressed plants with a concentration of 700 mg/L of Pb, rGO-treated plants were found to have increased in fresh weight. On day 2 the average fresh weight of plants for all the replicates of control was found 189.49 mg, for rGO treated plant it was 198.99 mg and for Pb stressed plant it was 186.50 mg which show clear significant variation in plant fresh weight for all the treatments shown in figure 2.8. On day 4 Pb stress was found to further decrease the fresh weight of the plant and rGO 30 mg/L was found to increase plant fresh weight in comparison to control. In combination with Pb 700 mg/L and rGO 30 mg/L, it was noted that plants average fresh weight of all three replicates increases in comparison to only Pb stress plants. On day 2 of the treatment plant's average fresh weight was found to be 210.59 mg for the Pb+rGO NPs combination and for only Pb-stressed plants it was 186.50 mg. On day 4 it was noted that average fresh plant weight increased further for the Pb+rGO NPs combination and decreased for Pb stress, for Pb+rGO NPs it was found to be 225.91 mg, and for Pb it was 174.3 mg. For the combination of Pb and rGO, it was also noted that the average weight of

all three replicates of fresh root and shoot of wheat plants increased in comparison to that of only Pb-stressed plants.

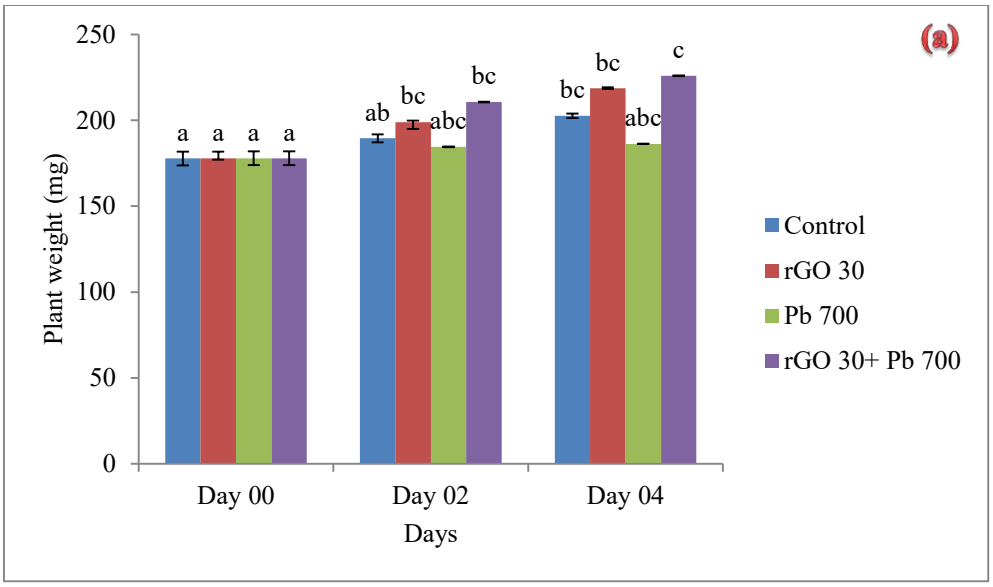

Figure 2.8 (a) shows plant weight under rGO, Pb, rGO + Pb stress conditions

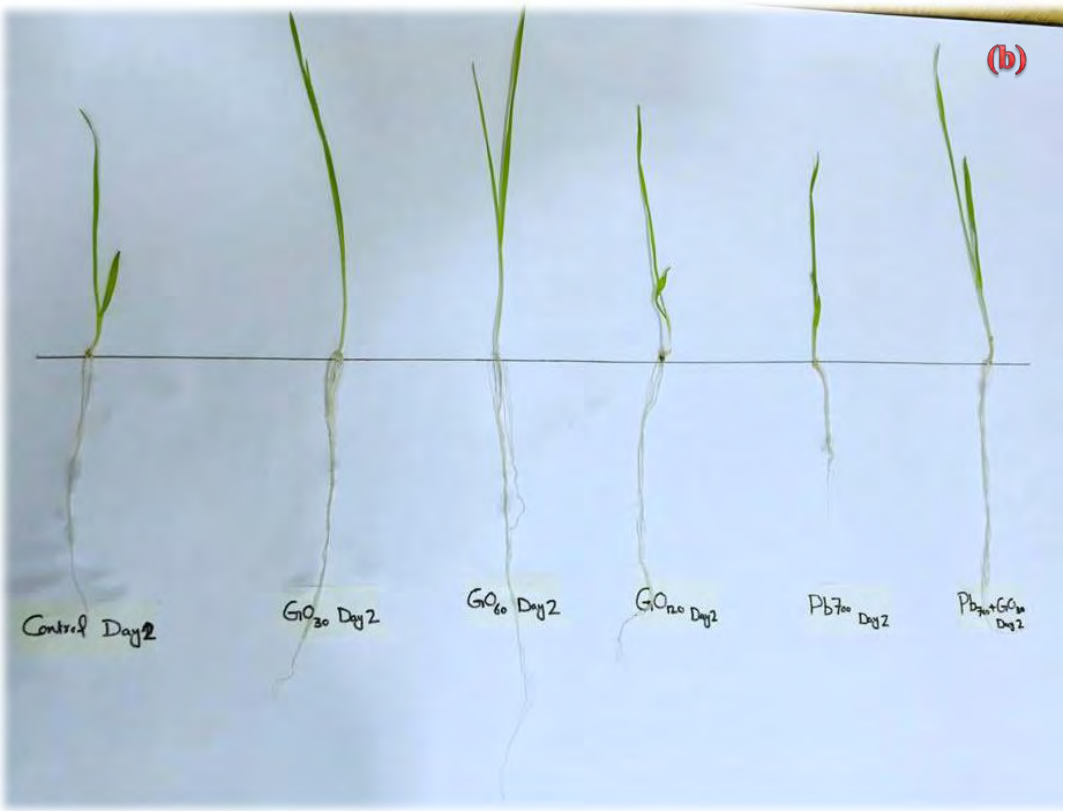

Figure 2.8 (b) shows the effect of rGO, Pb, and rGO + Pb stress on wheat plant morphology

3.4 Chlorophyll content

The chlorophyll content of fresh leaves of wheat plants exposed to different concentrations of Pb 700 mg/L, rGO NPs 30, 60, and 120 mg/L, a combination of Pb and rGO NPs (700 mg/L+ 30 mg/L) was measured with the help of SPAD meter and the data was statistically analyzed. Results in Figure 3 show significant variation in chlorophyll content at different concentrations exposed at different time intervals for all the plants. SPAD chlorophyll level was significantly enhanced by rGO NPs in comparison with control of all the days, while plants exposed to Pb stress were noted to show a reduction in chlorophyll content in all days plants of wheat. It was noted that the application of rGO NPs decreases the toxicity caused by Pb in all days of plants and increases the SPAD chlorophyll values over the Pb stress. Non-significant enhancement in the chlorophyll content of wheat plants to Pb+rGO NPs was present in all days plants in comparison to control plants as shown in fig. 3.

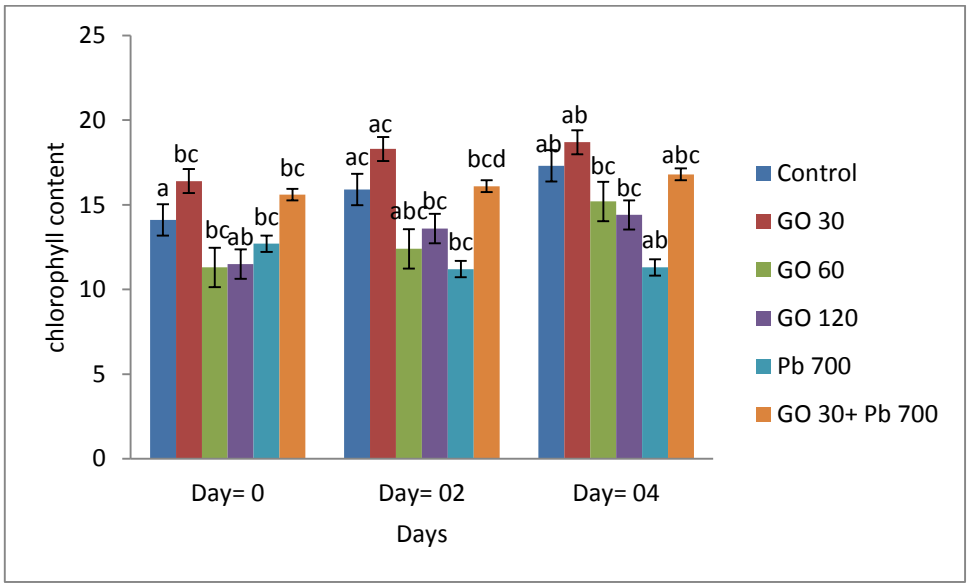

Figure 3 shows the graph for chlorophyll content

3.5 Histochemical analysis

Damage resulting from Pb stress and amelioration caused by rGO NPs was observed in the roots of wheat plants stained with Evan's blue dye as shown in Figure 4. Significant variations were observed in the root anatomy at day 2 and 4 plants after

treatment in comparison to the control. Damage was noted in the roots for Pb 700 while rGO 30 and rGO 60 showed almost no damage to roots but for rGO 120 damage was observed in roots cells. In comparison to Pb stress, the combined treatment of Pb and rGO showed less damage to root cells for both days shown in Figure 4.

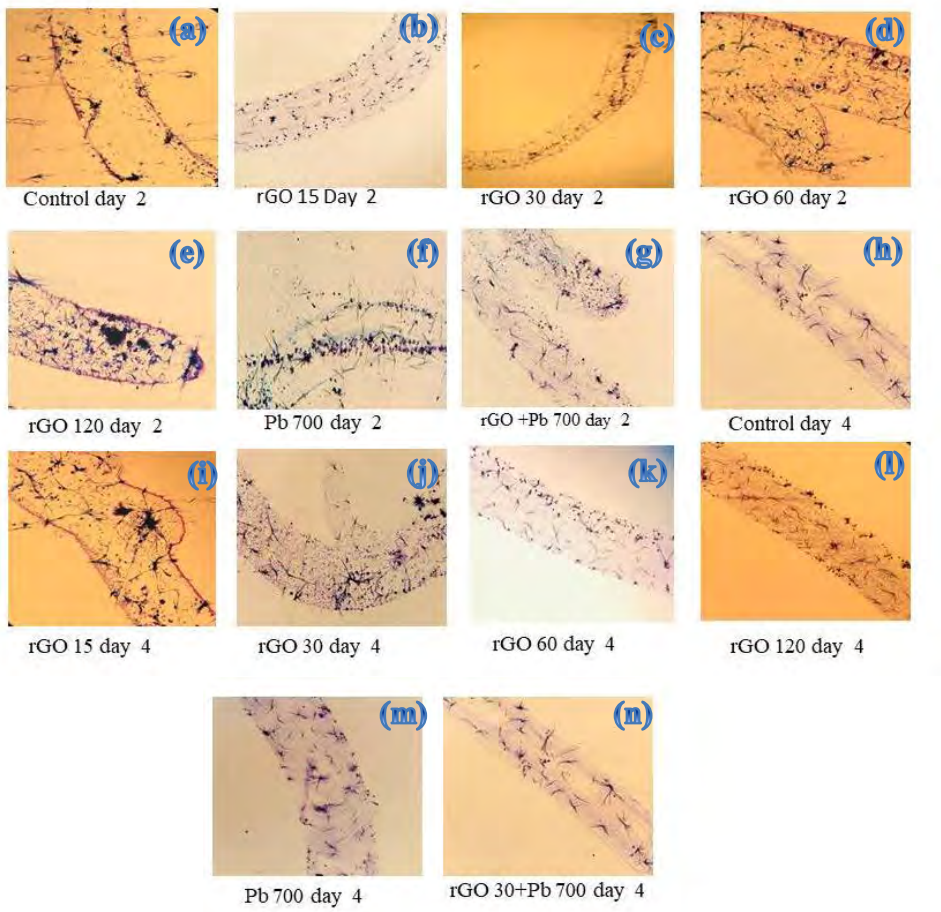

Figure 4 (a), (b), (c), (d), (e), (f), (g) show root anatomy of control, rGO and Pb stress at day 2 while Figure 4(h), (i), (j), (k), (l),(m), (n) show root anatomy of control, rGO and Pb stress at day 4.

3.6 Antioxidant enzyme activity

Measurement of different antioxidant enzymes of wheat plant produced in response to Pb stress of 700 mg/L, rGO NPs 30, 60, 120 mg/L, and Pb and rGO NPs in combination (700 mg/L+ 30 mg/L) stresses was done. From the statistical analysis, a highly significant variation was found for all the antioxidant enzymes in wheat plants between the different treatments exposed at different time intervals.

3.6.1 Catalase (CAT) activity

An antioxidant enzyme Catalase is produced by plants in response to counteract the toxicity caused by stress. The CAT activity was investigated in wheat plants in response to the stress produced by Pb and rGO NPs treatments. A significant variation in CAT activity was found in roots subjected to various treatments of Pb and rGO NPs alone and in combination which can be demonstrated from Fig 5.1.below. A substantial decrease in CAT activity was found for Pb treatment on day 2 in roots of wheat plants in comparison to control which was found to increase on day 4, while a significant increase was found in the activity of CAT in response to different concentrations of rGO NPs in roots of wheat plants of all days in comparison with control except rGO 120 mg/L where CAT activity was found almost same on both day 2 and 4 of the treatment. rGO NPs (30 mg/L) in combination with Pb (700 mg/L) stress condition have significantly enhanced the CAT activity in roots of wheat plants in comparison with the control and Pb treated plants, which illustrate the mitigative function of the rGO NPs against the damaging effects produced by the Pb in stress condition.

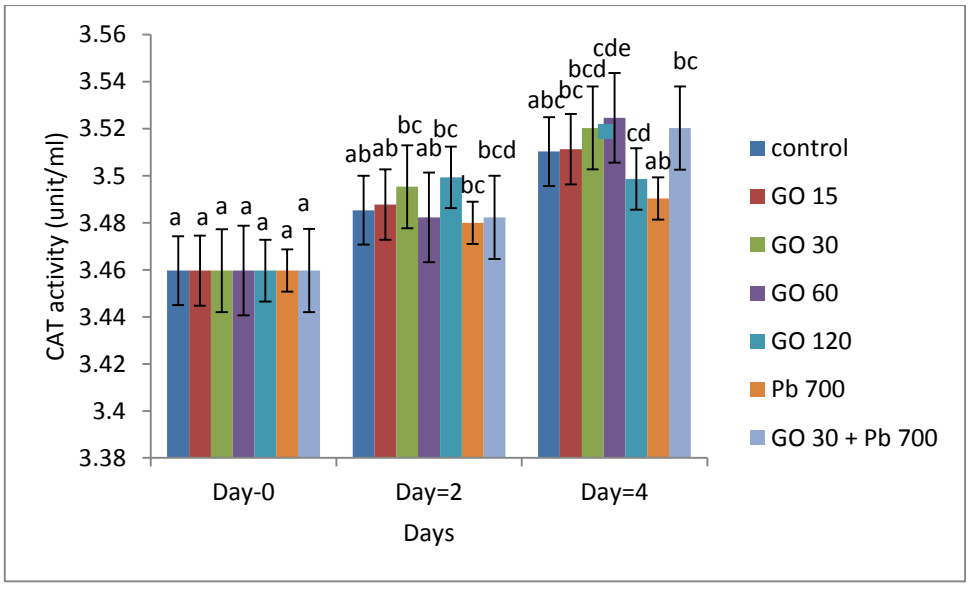

Figure 5.1 shows a graph for CAT activity of different concentrations of rGO and Pb stresses.

3.6.2 Ascorbate Peroxidase (APX) Activity

The activity of APX under different concentrations of rGO NPs and Pb separately and in combination was investigated. Significant variation was found in APX activity for different treatments at different time intervals in wheat plants The APX activity was substantially enhanced in the Pb, and rGO NPs treated plants in roots of all days plants in comparison with the control. rGO NPs and Pb stress in combination (30 mg/L+ 700 mg/L) were found highly significant, and an increase in the activity of APX was found which shows that rGO NPs can reduce the oxidative stress of Pb by stopping their damaging effects on the plants. The maximum APX activity was found in the plants that were subjected to Pb+ rGO NPs conditions. The APX activity was found to be more on day 4 of the Pb+ rGO NPs treatment in comparison to control and other treatments as shown in Fig. 5.2.

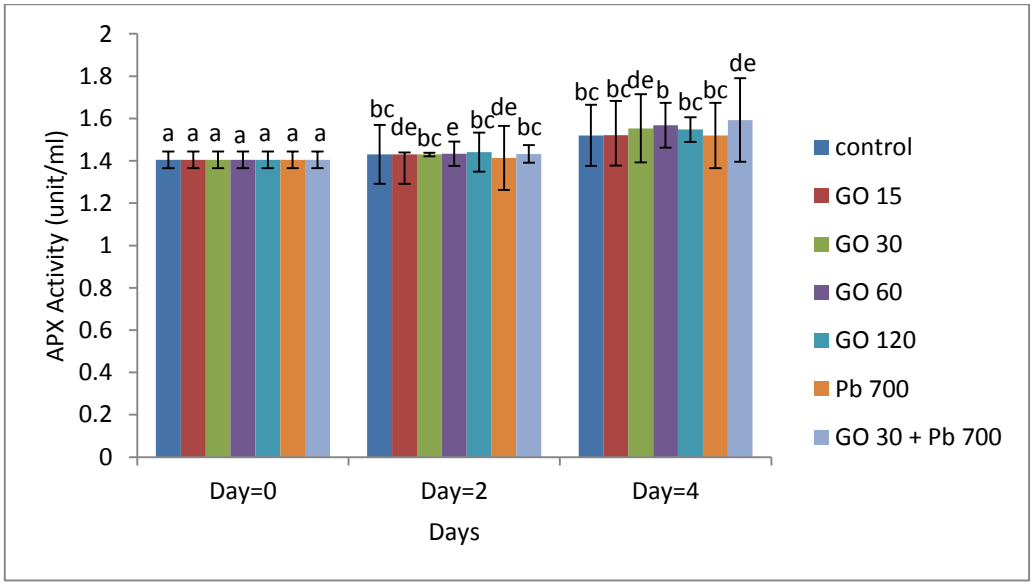

Figure 5.2 shows a graph for APX activity of different concentrations of rGO and Pb stresses.

3.6.3 Superoxide dismutase (SOD) Activity

The activity of SOD was evaluated in wheat plants root which were exposed to different stress conditions of Pb 700 mg/L, rGO NPs (30, 60, and 120 mg/L) separately, and in combination with rGO 30 mg/L+ Pb 700 mg/L. Data was statistically analyzed and a significant variation was noted for different concentrations at different time intervals in wheat plants as shown in Fig. 5.3. Reduction was noted

in SOD activity in the roots of Pb-treated plants in comparison to control of all days wheat plants. NPs of rGO alone and in combination with Pb stress showed a significant enhancement in the activity of SOD in root in comparison to the plants only just treated with Pb stress. SOD is an antioxidant enzyme that withstands the damaging effects caused by Pb to protect plants. From the figure 5.3. it is more evident that SOD activities were found to increase to help plants in the time of stress.

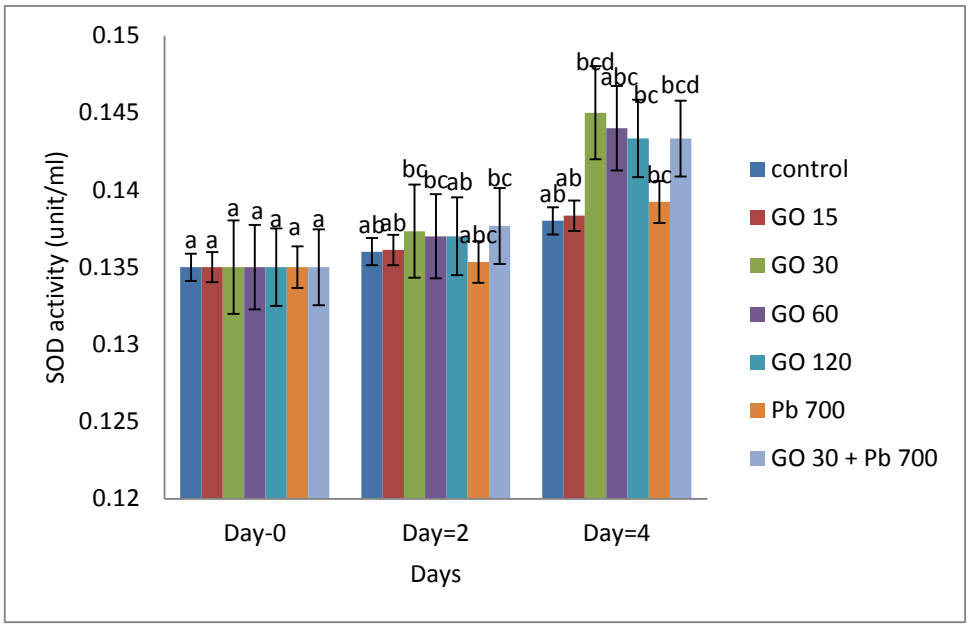

Figure 5.3 shows a graph for the SOD activity of different concentrations of rGO and Pb stresses.

3.6.4 Peroxidase (POD) Activity

The activity of POD activity in root of wheat plants exposed to different treatments of rGO NPs (30, 60, and 120 mg/L), Pb (700 mg/L), and combined treatment of Pb and rGO NPs (700 mg/L+ 30 mg/L) was evaluated. Significant variations were observed for all the treatments after statistical analysis of data as shown in Fig 5.4. A significant enhancement in the activity of POD was observed for all treatments of rGO and Pb separately and in combination when compared to the control plants of wheat. The activity of POD was enhanced in the Pb-treated root of wheat plants but POD activity was maximally enhanced by rGO treatments alone or in combination with Pb stress in comparison to that of control as evident from fig 5.4. below.

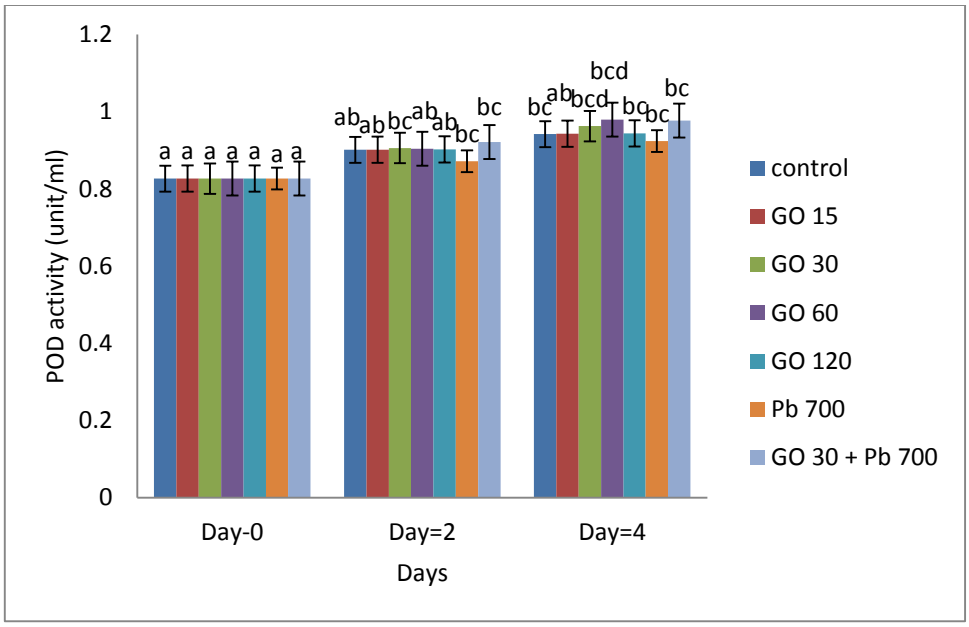

Figure 5.4 shows a graph for the POD activity of different concentrations of rGO and Pb stresses.

Discussion

There are two main synthesis methods of GO (Jagiełło et al., 2020), the one named "Hummers and Offeman method" in which potassium magnate ($KMnO_4$) is added as oxidant into the reaction mixture which consists of concentrated sulphuric acid and sal of $NaNO_3$ (Hummers and Hoffman., 1957) and the second one called as Marcano and Tour method which is an improved method of graphite "oxidation synthesis" which involve elimination of sodium nitrate from the reaction mixture to increase potassium manganate amount by carrying out reaction under the presence of concentrated H_2SO_4 and H_3PO_4 acids in the ratio of 9:1. As a result GO of high content of oxygen functional group can be obtained (Marcano & Tour et al., 2010). Based on both these two methods numerous modifications are made to synthesize GO, including partial oxidation to produce GO with a C/O atomic ratio between 12 and 3 (Lojka et al., 2019), double oxidation to synthesize a high concentration of carboxyl groups (Jankovsk et al., 2016), and the use of various nitric acid concentrations (from 50% to 98%) to synthesize GO with a C/O atomic ratio of 6.5 and 2.8 and utilizing one-hour fast oxidation (Jankovský et al., 2017). In alternative to these green reducers such as organic acids, biochemical substances, amino acids bacteria, fungus, plant extracts, and cellulosic compounds are being applied to synthesize rGO from GO which are thought to be less corrosive, carcinogenic, and toxic than the chemically or thermally reduced one. (Aunkor et al., 2016).

4.1 Synthesis of rGO NPs its green reduction and characterization

Chemically the most widely commonly used method to prepare graphene oxide is the "Hummers method" in which potassium permanganate is added to solution mixture of sodium nitrate, sulfuric acid, and graphite to prepare GO (Chen et al., 2013). Reduction of GO can be obtained by removing oxygen-containing functional groups attached to GO (Xu et al., 2015). Reduction of GO employing chemical reduction is the most versatile method used which can be performed both in acidic and alkaline medium to synthesize economically cheap and scalable GO (Stankovich et al., 2007). Anyhow chemical synthesis of GO is poisonous to the ecosystem consequently we need easy, ecofriendly, and cheap scalable preparation of GO (Adhikar et al., 2013). Plant extracts are thought to be the sustainable solution to rGO in the future due to their low economical cost, renewable and proper

optimization synthesis process, and a comparable or higher reduction rate than that of conventional hydrazine reduction (Ismil et al., 2019). *Tecoma stans* leaves extract was used as a reducing/capping agent to rGO by green mean into rGO, which was investigated and confirmed by using three different concentrations of plant extract (Mahmoud et al., 2022). From the recent research work of many of the researchers, it was indicated experimentally that graphene oxide can be used as a fertilizer carrier to improve nutrient utilization by reducing its release rate. Plants as a primary essential component of the ecosystem are in continuous contact with graphene-based products released into soil regularly. A researcher before applying GO on a large scale to improve agriculture and agroforestry the everlasting effect of GO on plant health should be considered and implemented (Zhang et al., 2021).

Characterization of rGO NPs was done by several of the researchers (Smith., 2011, Acik et al., 2011 Andrijanto et al., 2016). UV-Vis spectroscopy was confirmed from the research work of (Wijaya et al., 2020 and Zhang et al., 2019) where two different peaks were noted, one at 300 nm and the other one at 250 nm which confirmed our findings of UV analysis for GO. The absorbance of UV–Vis at 265 nm peak for nanosheets of graphene, indicates a graphitic structure, and in general it could be regarded as the excitation of graphitic structure through π-plasmon of the graphitic. For GO UV-Vis there are two characteristic features used as means of identification, the one maximum at 231 nm which corresponds to $\underline{\pi} \rightarrow \pi^*$ transition of aromatic carbon molecular structures, and the shoulder occurs at approximately 300 nm corresponding to $n \rightarrow \pi^*$ type transition between C=O bonding (Zhang et al., 2019). Presence of hydroxyl groups (OH) at 3000-3700 cm^{-1}, carboxyl functional group at 1650-1750 cm^{-1}, sp2 hybridized C=C (plane stretching) at 1500-1600 cm^{-1}, and epoxides at 1280-1320 cm^{-1} (Acik et al., 2010), sp^{-2} hybridized C=C (plane stretching) founded at ~1550-1650 cm^{-1}, and epoxides found at approximately 1350 cm^{-1} (Zhihao et al., 2019) peaks confirm our FTIR results for rGO. SEM-EDS of rGO was confirmed from the work of (Jagiełło et al., 2020) according to which rGO has a planner hexagonal lattice structure with a particle size of 9.67 to 200 nm. In X-ray diffraction analysis sharp peak initiall occurred at 29.94° with spacing of 2.87 nm spacing after synthesis of GO peak at 29.94° disappeared and appearance of new peak at 11.26° which show conversion of graphene into GO due to oxidation reduction of epoxy and hydroxyl groups during intercalation (De et al., 2018). The change in

spacing which initially was 2.87 nm to 4.22 nm indicates the exfoliation of GO. After reduction, it was found that rGO's peak point of 11.26° became broad, and graphitic intensity of 29.94° increased further. This indicates reduction and degradation of C-O and restoration of C=C occurred (Wijaya et al., 2020) which confirms our results for XRD analysis of greenly reduced GO as shown in fig 3.4.4. zeta potential calculated was found to be -20.6 for greenly reduced GO and is in cationic form with a stable and comfortable structure which is according to the work (Azizighannad & Mitra., 2018). In general, particles having zeta potential values more than negative of -30 mV and more than +30 mV can produce stable dispersions due to electrostatic repulsions. rGO form stable dispersion in basic media with a pH range of 8- 11.5 and the highest ζ was -44.2 which resulted from a compressed double layer at higher ionic strength (Konkena et al., 2012).

4.2 Morphological characterization

Pb is considered to be one of the most frequently occurring damaging toxic heavy metals to plants (Shehzad et al., 2023). Most of the Pb ions are absorbed by plants from the soil which remain concentrated in the roots, and a small portion of Pb is transferred to the stems and leaves through translocation. Pb ions exert toxic effects on the tissues of plants and damage the photosynthetic system of the plant (Zhou et al., 2018). Pb one of the heavy metal environmental pollutants is extremely toxic to plants' health and reduces their productivity which in turn consequently threatens the health of humans and all the biota. There is no biological function of Pb, but on the contrary, it can disrupt the physiological, morphological, and biochemical functions of wheat plants (Souahi et al., 2021). Pb in higher concentration was found to decrease seed germination in rice and reduce other growth parameters of plants (Mesmar et al., 1991). Similar results were reported for wheat plants where Pb stress was found to reduce plant biomass by contaminating the soil with Pb (Rabey et al., 2005). However, contrary to our study, an apparent increase in plant parts was noted for corn seedlings which was due to an increase in the synthesis of cell wall polysaccharides under Pb stress. Root cells are in direct contact with the Pb stress in the soil so elongation of the root can be very sensitive to Pb which inhibits root growth and elongation, It was reported by several of the workers that Pb stress inhibits root growth by inhibiting cell division in the root tips, with mitotic abnormalities and hence damages microtubules and destabilizing cellular membranes of the root cells (Gopal et al., 2008). Under Pb stress reduction in shoot length may be

due to disruption in micronutrient (Mg, K, Ca, and Na, etc.) absorption by plants. An increase in ROS level may be the other possible reason for it as a higher level of ROS causes damage to cellular metabolism which in turn can result in the reduction of plant growth (Soares et al., 2020).

Roots are the first parts of a plant that comes in direct contact with soil and interact with different components present in soil. It takes heavy metals directly from the soil. From previous investigations, it was revealed that transporter cells are present in roots which release different chelators that are implicated in the uptake of Pb in plants (Xia et al., 2019). From the study work of Kohli et al. (2019), it was found that the accumulation of Pb in roots is high when compared to shoots which may be due to the obstacle caused by the endodermis cell of the roots toward the transportation of Pb from roots to shoots of the plant. As a result, roots are thought to be more suffered and will have more damage than the shoots due to higher uptake of Pb. From the study work of Fatemi et al. (2020) and Soares et al. (2020), it was observed that the root length of the *B. juncea* plant was found to be more adversely affected due to the uptake of Pb in higher levels by roots. Our results are consistent with the study of Amin et al. (2018) where a decrease in root length was noted which may be due to impairing water capacity and nutrient absorption caused by Pb stress. Micronutrient deficiency in the tissues of plants could be attributed to a reduction in cell division in apical meristems of roots which may in turn result in a decrease in total root length. Thus, it can be concluded that mitosis will be unable to proceed if there is a lack of the above-mentioned requirements.

A decrease in plant weight has been reported in various earlier research studies under Pb stress by (Youssef et al., 2021) and Pirzadah et al. (2020)). In the current research work, it was observed that Pb stress has significantly reduced the fresh plant weight of wheat plants. Similar results were observed in the research work of Agnihotri and Seth et al. (2020), and Sanaei et al. (2022), The main cause for the reduction in biomass could be, as biomass of plants and photosynthesis are thought to be closely related to each other so heavy metals stress reduce photosynthesis and cell division which conversely led to reduction in biomass of plant. Pb interferes with photosynthesis by altering the photosynthetic pigment's synthetic process, blocking the electron transport chain reaction and stopping the dark reaction by inhibiting important enzymes of the Calvin cycle, and limiting CO2 availability by causing

damage to stomatal cells (Fatemi et al., 2020; Keller et al., 2015). All these factors are responsible for stunted growth in plants and hence resulted in reduction of total plant weight.

The most important and concerning point is the ecological risks evaluation of GO, in particular to find the interaction of plant cells with GO before applying it to any of them but anyhow till now very little is known about the uptake or effects of graphene-based products to cell morphology, cellular damage or the cellular metabolic and redox balance of plant cells to GO (Hu et al., 2014). Several studies have been done to focus on the impact of GO especially on those plant species, which regarding their growth environment show extreme sensitivity (Monica et al., 2009).). In one of the studies conducted on tomato seed germination and seed growth, GO was found to penetrate the seed husk and facilitate water uptake which in turn resulted in fast germination of the seed (Zhang et al., 2015). Very few studies have been done in which the mitigative impact of graphene-based products has been investigated in green plants. In the research work of Begum et al. (2011) it was found that after 20 days treating plants with 500-2000 mg/L of rGO inhibited the growth of leaves in cabbage, red spinach, tomato, and lettuce. However, the research work of Zhao et al. (2015) has shown that GO exposure did not cause any considerable changes in the shoot and root growth of *Arabidopsis* plants. But for 15 days of treatment with GO (50–100 mg/L) the length of the seminal root was found shorter and a decrease in root weight was noted for *B. napus* L. (Cheng et al., 2016). In the same way, Chen et al. (2018) research work has shown that by exposing *Triticum aestivum* to GO suspension for 9 days, significant restrain was noted in seedlings growth, which may Pb to adverse effects on the development of root/shoot length and biomass as well as morphological damages to the root cells of plants.

Several researchers have found that GO is promoting plant growth which has sparked the interest of many others in its potential applications in agriculture and agroforestry to increase crop productivity. Appropriate concentration of GO can be found to have positive effects on plants and the concentration may change from specie to species, the particle size of GO should be considered before application in agriculture (Yang et al., 2022). The results of Zhang et al. (2021) have demonstrated that GO shows positive effects at 50 mg/L, and enhances the capacity of photosynthesis in leaves, which in turn improves plant yield and root/shoot

morphological characters of plants, and improves plant nutrients. In comparison to leaves effect of GO was found more obvious in roots than in leaves (Zhang et al., 2021). Zhu et al. (2020) have found that a small amount of GO was found to promote plant growth and significantly increase the biomass of stem and leaves in alfalfa (*Medicago sativa)*. In *Festuca arundinacea* a small concentration of GO such as 0.2 mg/L was found could have increased the height and biomass of the plant (Wang et al., 2018). Our results are in accordance with the research work of Guo et al. (2021) where it was observed that treating tomatoes with 50 and 100 mg/L GO concentrations of GO could promote the plant's growth, and increasing the dose of GO up to 200 mg/L didn't cause any significant alteration in the stem diameter and weight of plants. Park et al.(2020) have shown that GO in an appropriate amount has a positive effect on the growth of *A. thaliana* L., as an increase in root/shoot length, increase in the number of leaves, and the formation of flower buds indicates a positive impact of GO on plants. From all these findings it can be concluded that GO should be used in an appropriate amount and the amount may change from species to species to promote growth in plants. Cao et al. (2021) have found that 10-100 mg/L of GO in *Populus alba* L. promoted the aboveground parts growth rate. He speculated that the promotion in the growth of plants with the use of GO was due to the improvement of soil fertility. Growth in tomato plants may be effectively promoted by GO in a concentration-dependent manner by stimulating cell division of root/shoot cells (Gao et al., 2021).

All the essential and non-essential elements can be easily absorbed by roots from the soil in a dissolved form with water from the soil as roots are in close contact with the soil minerals and soil water. In the plant growth and development process, root elongation plays an important role. In Aloe vera plants 10-100 mg/L of GO show great influence on root growth in comparison to aerial parts. An elevation was noted in total root length, total surface area of the root, root volume, and fresh weight of plants at different concentrations of GO (Zhang et al., 2021).100 mg/L concentration of GO was found to promote root growth in wheat seedlings (Ren et al., 2020). and elongation was noted in the rhizome of rice (Gao., et al. 2019). In comparison to control, 50 mg/L of GO was found to promote root length, volume, and total number of root tips and forks in maize seedlings (Chen et al., 2021). In tobacco plants, 20 mg/L of GO treatment was found to promote the number of adventitious roots (Jiao et

al., 2016). On the other hand, 0.1 mg/L of GO such a low concentration could achieve the same effects in Gala apple plants (Li et al., 2018). Results suggest that GO-promoting effects on root growth vary from species to species in plants and depend on GO doses used to treat plants. All the above results are consistence with our findings for GO.

Similarly most of the growth regulators, the effect of GO on plant growth is concentration-dependent, and an optimal range could induce such effects. For example, in the research work of Zhang et al. (2021) maize plants were treated with different concentrations (0, 25,50,100, 200 mg/L) of GO to analyze the growth state on day 14 to determine the optimal (50 mg/L) concentration of GO for plant growth. In raspberry seedlings increasing concentration of GO trend was noted Firstly increase in root length, surface area, and root tip number was noted but later on, reduction occurred, and the optimal concentration noted was 2 mg/L to promote plant growth (Hu et al., 2020). According to the same study at 4 mg/L or higher concentration formation and development of adventitious roots were inhibited which probably indicates toxicity of GO to raspberry. Results of the study demonstrate that plant growth could be promoted only at appropriate concentrations of GO. In the work study of Guo et al. (2021) in comparison to control, 50 mg/L and 100 mg/L of GO were found to increase the surface area of the root tips and hair in tomato plants. The increase in surface area and the total projected area were found to be 31% and 27%, respectively, in comparison to the control treatment (Guo et al., 2021). In addition, Guo et al. (2021) have found that in quinoa 4 and 8 mg/L of GO have induced root morphology of quinoa seedlings in comparison to control, which indicates that this concentration of GO can promote growth and other morphological development of quinoa plants (Guo et al., 2019). Our findings follow all the above research work in the promotion of plant growth in wheat plants. From the above-mentioned research, we can conclude that GO can be used in appropriate amounts to promote plant growth soon in agricultural sectors.

GO was also found to promote plant height, in alfalfa plants, Significant increase in stem and leaf biomass was noted for GO treatment (Zhu et al., 2020). In the *Festuca arundinacea* plant, 0.2 mg/L of GO in such a small concentration has increased plant height and biomass (Wang et al., 2018). In the research study of Guo et al.(2021) 50 and 100 mg/L of GO treatment was found to increase plant growth in

mature tomato plants, but consequently, no significant effect was noted in the diameter and weight for increasing dose up to 200 mg/L in plants (Guo et al., 2021). Park et al. (2021) in particular pointed out that GO in an appropriate amount could have a positive effect on the plant growth in *A. thaliana* L., as an increase was noted in root length, leaf surface area, total number of leaves, and floral bud formation. In addition, 10–100 mg/L of GO was found to enhance the growth rate of *Populus alba* L., for above-ground parts in a concentration-dependent manner. They speculated that the promotion of growth rate due to GO is due to the improvement of soil fertility. Growth promotion in tomato plants is due to the application of GO, which may stimulate cell division in the root and shoot parts of the plant (Guo et al., 2021). Zhang et al. (2021) revealed that GO promotes the growth of *Aloe vera* plants by stimulating the photosynthesis process. In research work they demonstrated that GO at 10–100 mg/L of concentration, by showing best efficiency at 50 mg/L, which was found to exhibit positive effects on the plant growth of *Aloe vera* L. by causing an enhancement in the photosynthetic capacity of leaves, inducing yield and leaf morphological characteristics, an improvement in nutrient contents of leaves i.e. protein and amino acids. Similarly, Zhang et al. (2020) have found that in elm-cut seedlings 50 mg/L of GO treatment could promote the rate of growth by increasing the density of stomata in leaves, stomatal conductance, and the concentration of CO_2 inside leaves, to improve the photosynthetic efficiency of plants. From all the above findings one thing is clear GO if used in an appropriate concentration promote plant growth but the dose may vary from specie to specie in different plants. Our results are consistence with all the above-mentioned results.

4.3 Chlorophyll content

In plant leaves the amount of chlorophyll content is an important indicator for stressful conditions in plants. Heavy metals show a negative impact on plant chlorophyll during stress conditions (Rizwan et al., 2016). Changes in chlorophyll content in response to changes in environmental conditions, deliberately have an impact on a plant's health. In our study, a significant reduction was noted in leaf chlorophyll content when exposed to Pb stress (Priyanka et al., 2021). Meanwhile, when rice was exposed to 2–10 µg/mL of GO concentrations (Chen et al., 2019) and carrots were exposed to 0.05 and 0.1 mg/mL concentrations of GO (Siddiqui et al.,

(2019), an increase in chlorophyll content was noted. In addition, Hazeem et al. (2016) have shown that GO at 0.5 mg/L of concentration have improved the pigment content of algae *Picochlorum sp*., however at higher concentrations of GO up to 5 mg/L, was noted to have a negative impact on the concentration of photosynthetic pigmentation. In our study by applying different concentrations of rGO to wheat plants in accordance with Chen et al. (2019) chlorophyll content was found to increase for rGO treatment of 30 mg/L in comparison to the control which is in accordance to Hazeem et al. (2016) higher treatments of rGO was found to decrease chlorophyll content and according to Priyanka et al (2021), Pb was found to reduce total chlorophyll content of wheat plants in comparison to control plants. From the research work of Zhang et al. (2016) it is evident that 500 mg/L of GO was found to decrease chlorophyll content which means that higher doses of GO have a negative impact on the chlorophyll content of plants which may alter species to species. GO is altering chlorophyll and plant photosynthetic processes which may have enormous impacts on the environment, possibly impacting carbon fixation and agricultural productivity. We demonstrated that GO had a stronger impact on photosynthesis in hydroponic cultivation. As a result, GO might have further effects on plants, which need additional investigative research.

4.4 Antioxidant Enzymes Activity

Heavy metal stress results in excess production of reactive oxygen species (ROS) which in plants may either directly or indirectly cause cellular damage. Anti-oxidative defense systems comprising CAT, APX, SOD, POD, and many other enzymatic and non-enzymatic defenses always play an important role, while safeguarding plants from adverse oxidative injuries during stress conditions (Santos et al., 2017). Nanoparticles (NPs) were found to enhance antioxidant enzyme activities identified in earlier research (Sharma et al., 2019). In the current study, a significant reduction and enhancement in the antioxidant enzyme activities under rGO NPs stress was observed in wheat plants. Our results are in accordance with the research work of Zhang et al. (2016) who demonstrated the changes in antioxidant enzyme activities in wheat plants exposed to rGO NPs treatments, while reduction in wheat growth and biomass due to induction in oxidative stress caused by Pb stress in wheat plant is in

accordance to the research work of Kaur et al. (2012). Some of the activities are given below;

4.5 Catalase (CAT) Activity

CAT is one of the important antioxidant enzymes that scavenge reactive oxygen species (ROS) to break excess H_2O_2 into water and oxygen to protect the cells from oxidative and corrosive effects of heavy metals stress (Nandi et al., 2019; Hussain et al., 2019). Reduction in CAT activity due to heavy metals stress has been reported in earlier research works (Ogunkunle et al., 2020; Venkatachalam et al., 2017).GO according to the work of Anjum et al. (2012) at a concentration of 800 mg/L followed by 400 mg/L in comparison to control and other treatments reduced H_2O_2 content and increased CAT activity in the root of *V.fabula* plant which is in accordance with our results. According to the work of Cheng et al. (2016), it has been observed that CAT activity increases by increasing GO concentration in the root of *B. napus* L. which supports our results, and similar results were observed from the research work of Chen et al. (2018) for naked oat plants root in hydroponic condition. The work of Lamhamdi et al. (2011) shows that by increasing Pb stress CAT activity decreases in the roots of wheat plants which is in accordance with our results. Our results are also supported by the work of Hosseini et al. (2007) that Pb stress decreases catalase activity in the roots of *B. napus* L. The research work of Gao et al. (2019) shows that GO in combination with heavy metal increases the activity of catalase enzymes in plants which is in accordance with our results.

4.6 Ascorbate Peroxidase (APX)

APX is an important antioxidant enzyme that scavenges H_20_2 by using ascorbate as an electron donor. (Gheshlaghpour et al., 2021) observed that APX activity was enhanced by Pb stress in basil plants. GO according to the work of Anjum et al. (2012) at a concentration of 800 mg/L followed by 400 mg/L in comparison to control and other treatment reduced H_2O_2 content and increased APX activity in the root of V.fabula plant which is in accordance to our results. In the current work, APX activity was found enhanced by GO alone and in combination with Pb in comparison to control treatment in wheat plants to scavenge H_2O_2 production. Our results are in accordance with Cheng et al. (2016) for APX activity which increased for GO in *B. napus* L. plant roots. The possible reason for

enhancement in APX activity might be related to the higher level of reactive oxygen species (ROS) produced due to Pb stress. This increase in APX activity can combat H_2O_2 produced in cells, to decrease the cellular membrane damage caused by oxidative stress (Venkatachalam et al., 2017). All the above results strongly support our findings.

4.7 Superoxide Dismutase (SOD)

The heme-containing antioxidant enzyme SOD serves as the first line of defense which prevents the bioactive molecules of cells from oxidative damage caused by ROS by oxidizing substrates using H_2O_2 and preventing excess accumulation of H_2O_2 (Santos et al., 2017). The activity of SOD was found to elevate in response to GO treatment in the root of wheat plants as a result of earlier research by Gao et al. (2019). In the research work of Zhang et al. (2016) increase in SOD activity was observed for wheat plants treated with GO. Our results are in agreement with both Gao et al. (2019) and Zhang et al. (2016) for GO. An increase in SOD activity was observed for Pb stress in the roots of wheat plants in comparison to control these results of Navabpour et al. (2020) show consistency with our findings. Further increase was noted for SOD activity in combined treatment of rGO and Pb stress, SOD act on superoxide radicles produce in a different compartment of the cells and acts as a precursor for other ROS, therefore it is considered the first line of defense against reactive oxygen species produced in plants.

4.8 Peroxidase (POD)

To catalyze and break down H_2O_2, POD is termed as another important antioxidant enzyme. Enhancement in the activity of POD was found under Pb stress was observed by Abdelkrim et al. (2023) in grass pea plants. An increase in the activity of POD against Pb stress was also observed in the research work of Zulfiqar et al. (2019) in plant roots. Our results are in accordance with both Abdelkarim et al. (2023) and Zulfiqar et al. (2019) for Pb stress in wheat plants. GO was found to increase the POD activity in the roots of wheat plants according to the work of Gao et al. (2019) same was perceived in the work of Yang et al. (2022), where an increase in POD activity was discerned in plant roots. Our results are consistent with Gao et al. (2019) and Yang et al. (2022). From our research work, it was observed that Pb in

combination with rGO induces the activity of POD. These results indicate that a higher dose of GO can induce oxidative stress in plants.

Conclusion

In conclusion, the application of greenly reduced graphene oxide nanoparticles (rGO NPs) proved effective in mitigating the toxic effects of lead (Pb) on wheat plants. Our investigation demonstrated the positive impact of rGO NPs on plant morphology and various biochemical factors, promoting plant growth, development, and biomass accumulation. This, in turn, helps counteract the adverse effects of heavy metal exposure. Additionally, we have observed significant improvements in chlorophyll levels and antioxidant activity, along with a reduction in Pb absorption when rGO is used in conjunction with Pb in wheat plants. Collectively, our findings highlight the promising role of rGO in alleviating Pb-induced toxicity in plants. Importantly, our research identifies an optimal concentration of 30 mg/L of rGO as effective in reducing Pb toxicity in wheat plants.

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