STRESS ALLEVIATION POTENTIAL OF GRAPHENE OXIDE UPON EXPOSURE TO CADMIUM IN WHEAT

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(2025)

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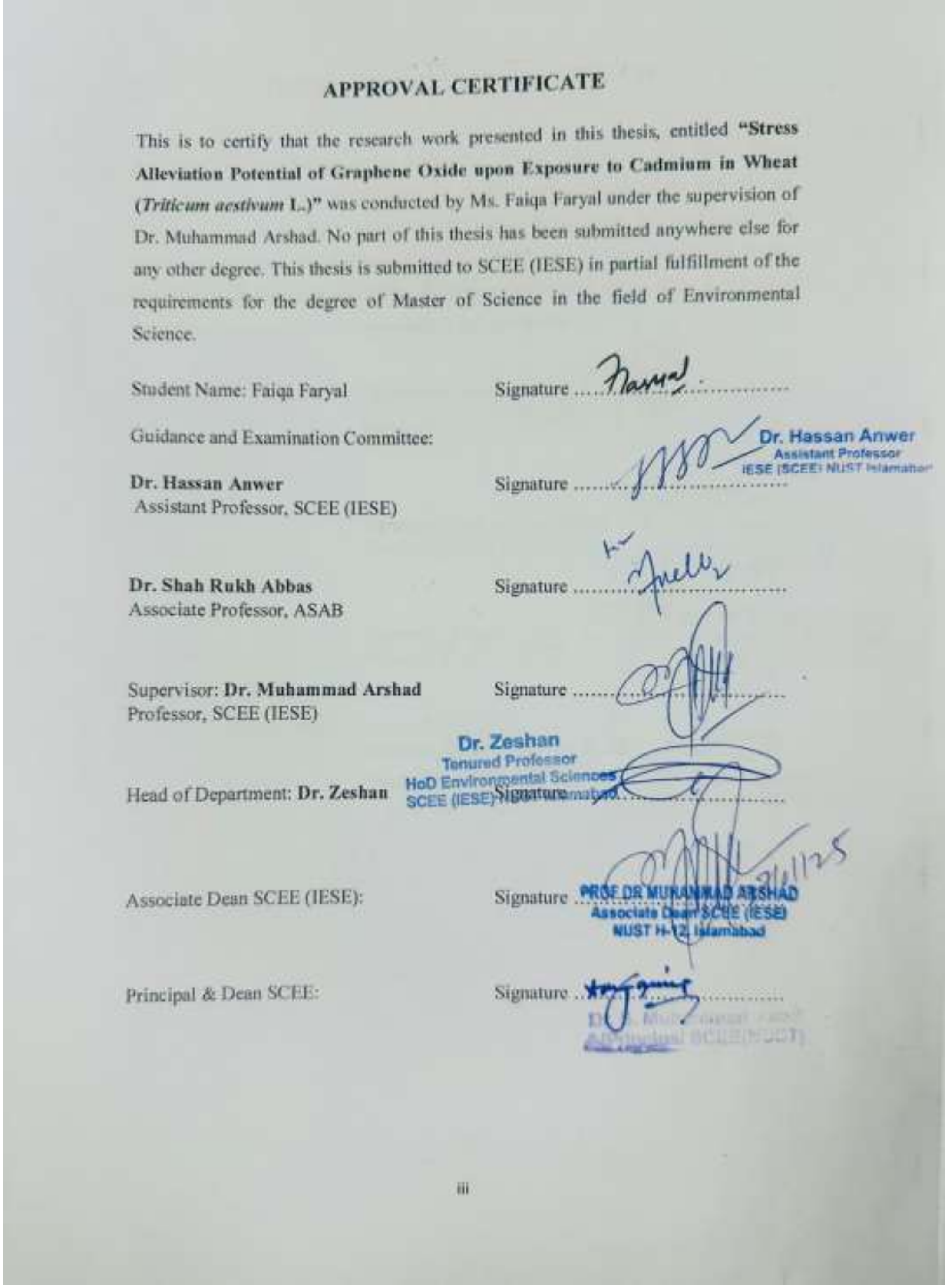
A thesis submitted to the National University of Sciences and Technology, Islamabad,

in partial fulfillment of the requirements for the degree of

Master of Science in
Environmental Science

Institute of Environmental Sciences and Engineering
School of Civil and Environmental Engineering
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
(2025)



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This is to certify that the research work presented in this thesis, entitled **"Stress Alleviation Potential of Graphene Oxide upon Exposure to Cadmium in Wheat (*Triticum aestivum* L.)"** was conducted by Ms. Faiqa Faryal under the supervision of Dr. Muhammad Arshad. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to SCEE (IESE) in partial fulfillment of the requirements for the degree of Master of Science in the field of Environmental Science.

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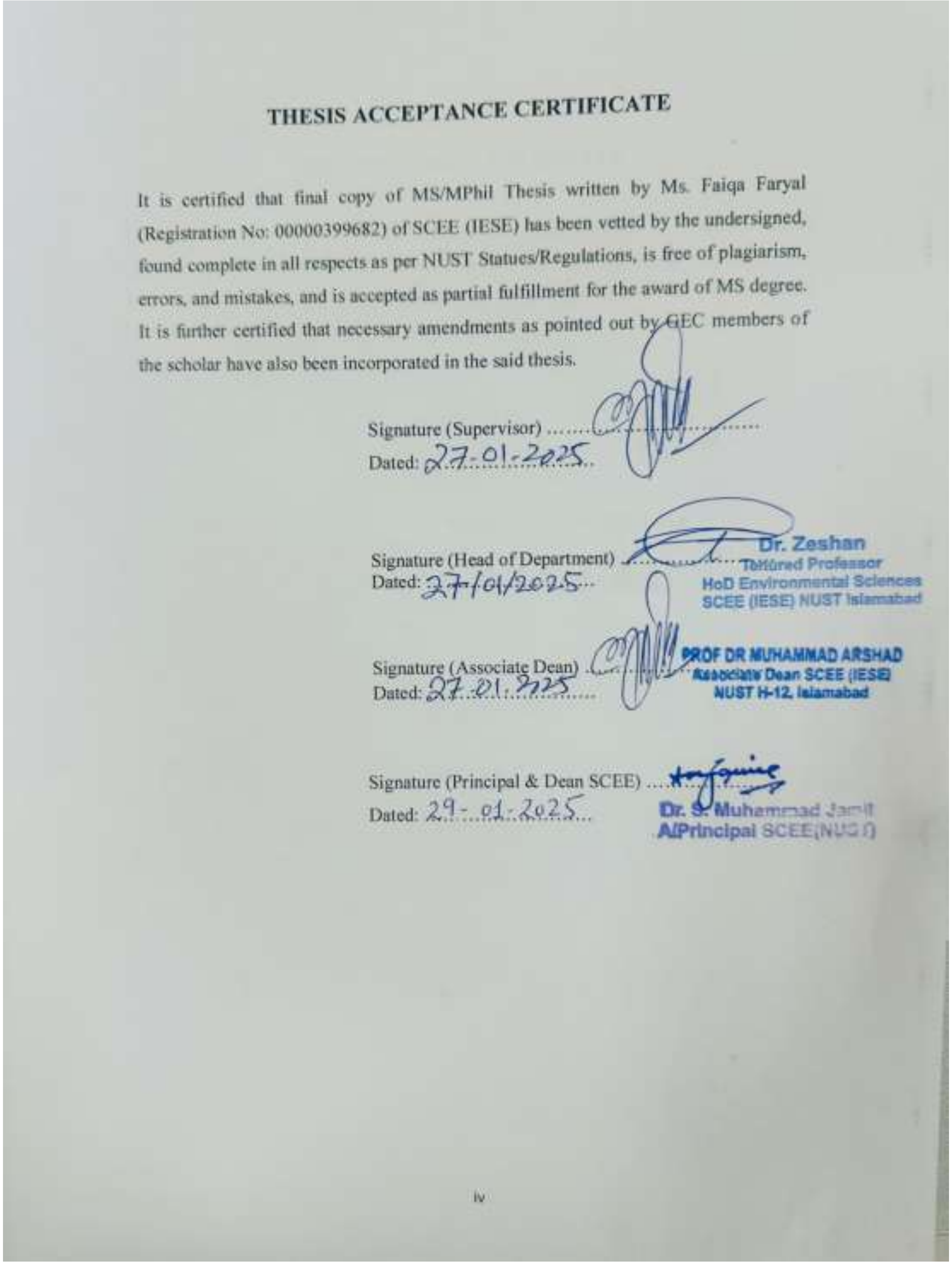

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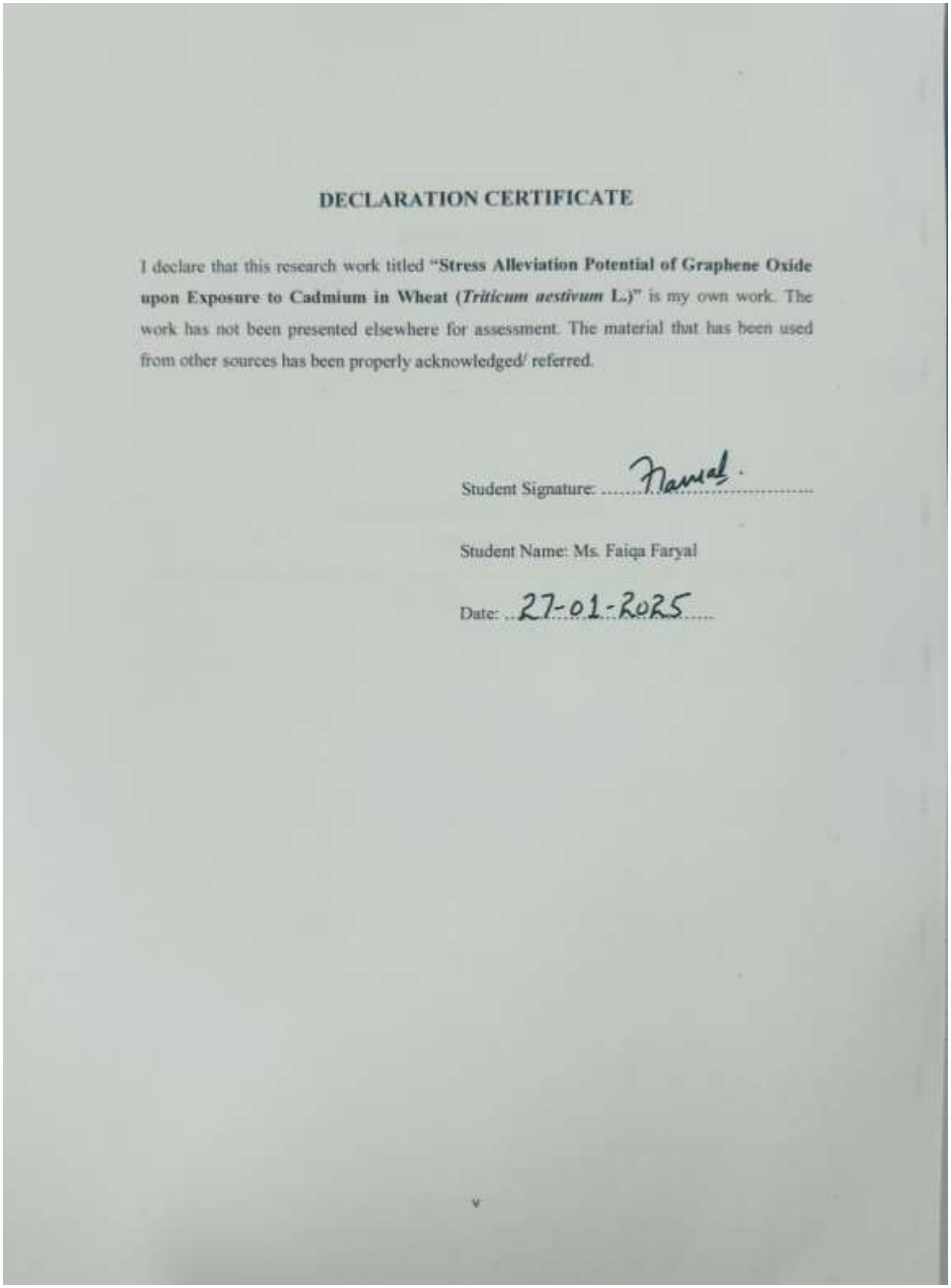

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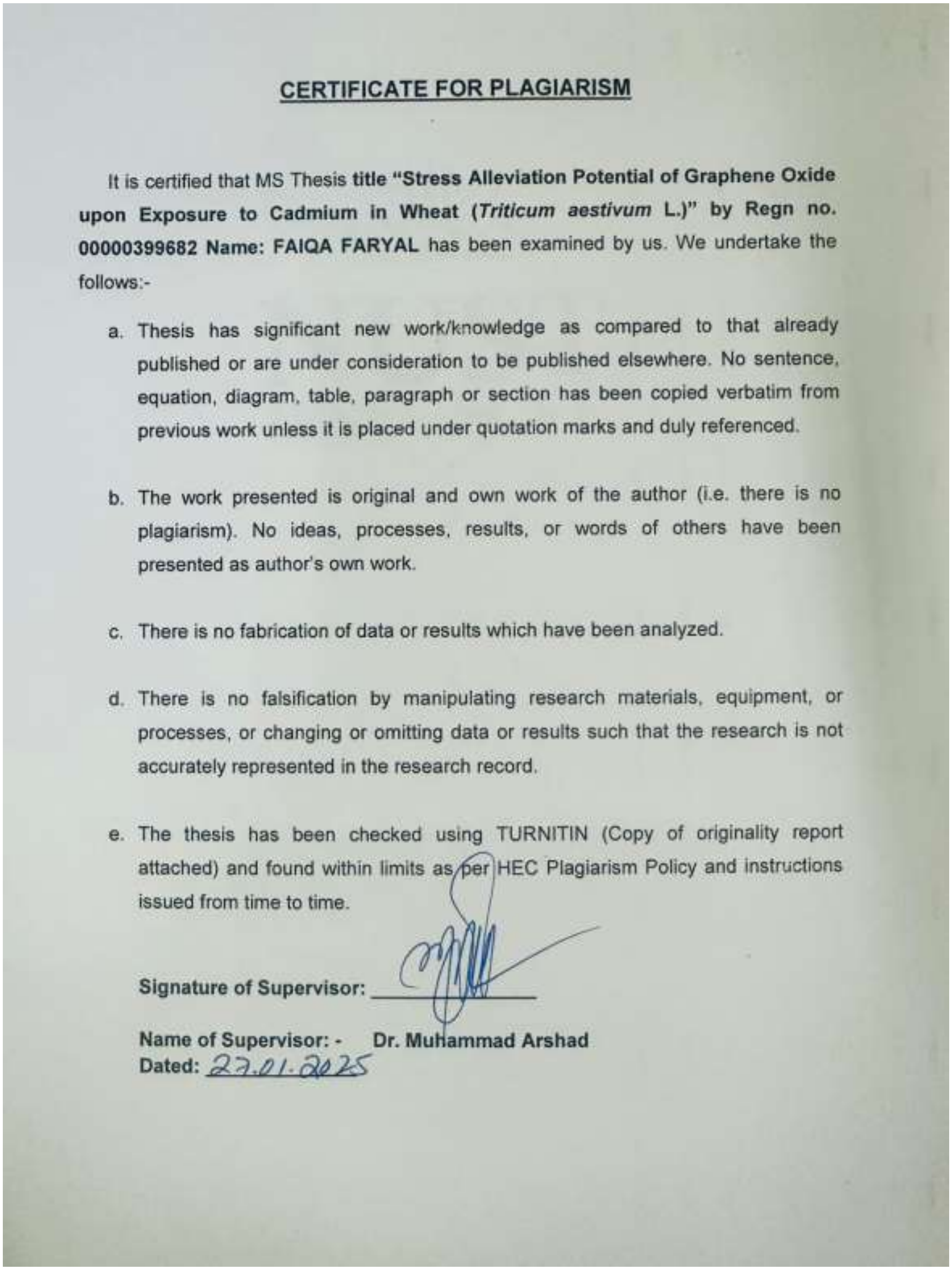

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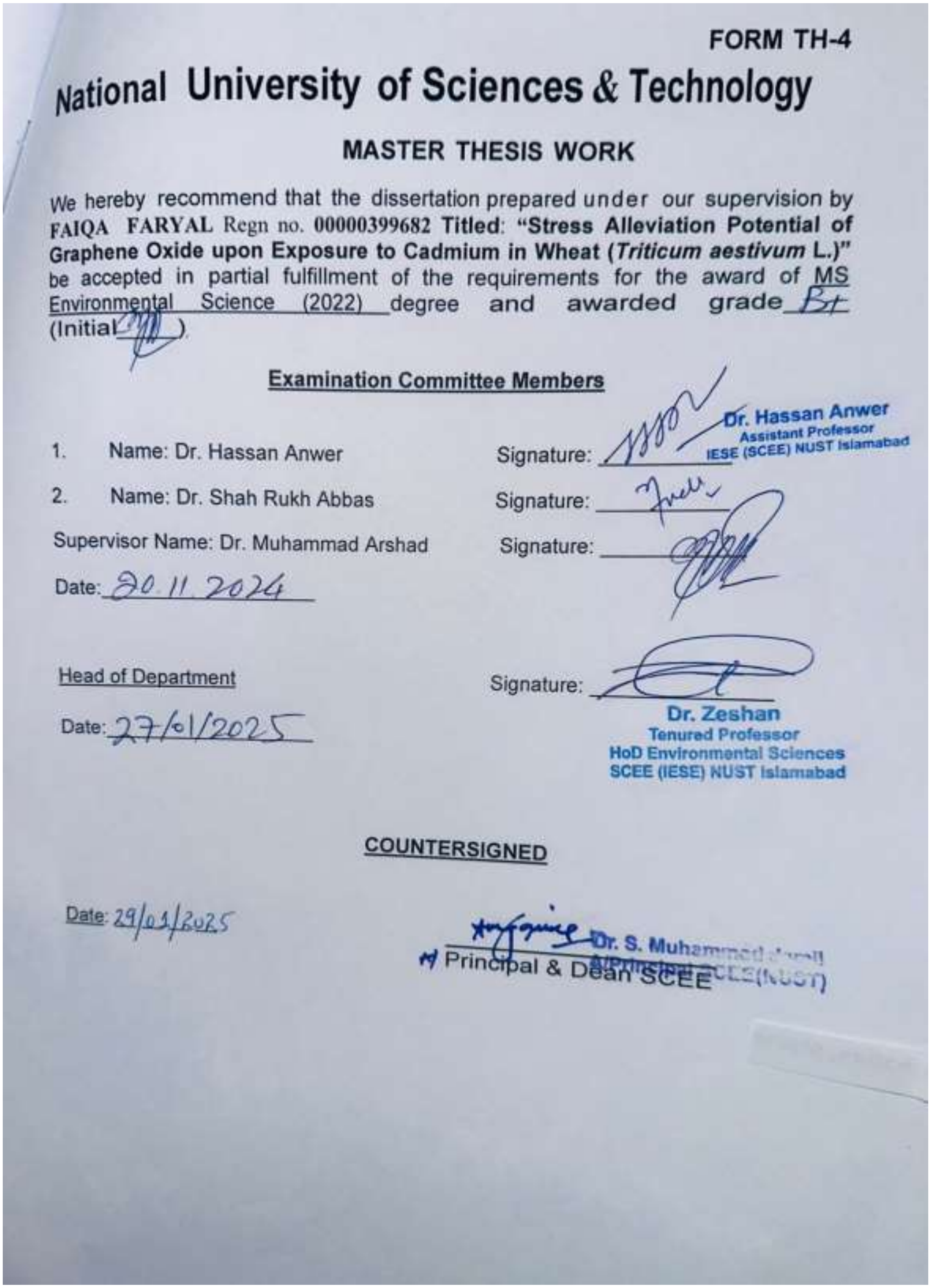

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We hereby recommend that the dissertation prepared under our supervision by FAIQA FARYAL Regn no. 00000399682 Titled: **"Stress Alleviation Potential of Graphene Oxide upon Exposure to Cadmium in Wheat (*Triticum aestivum* L.)"** be accepted in partial fulfillment of the requirements for the award of MS Environmental Science (2022) degree and awarded grade B+ (Initial).

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DEDICATION

This thesis is dedicated to my beloved

“Parents”

whose unwavering support, encouragement, and sacrifices have been the cornerstone of my academic journey.

ACKNOWLEDGEMENTS

First and foremost, Thanks to Allah Almighty, the "*Most Kind*" and "*Most Merciful*" Who blessed us with the aptitude, skills, and wisdom to accomplish this project. All respect to the Holy Prophet (P.B.U.H.) whose life is the model I am trying to base my life around. Secondly, I would like to extend my deepest gratitude to my parents for their unwavering trust and assistance in making this degree possible.

Dr. Muhammad Arshad, my incredible supervisor, his unparalleled expertise, unwavering support, and constant encouragement were the guiding lights that kept me focused. This thesis would not have been possible without his assistance and his knowledge. My deep gratitude goes to him for giving his valuable time in department discussion and concrete suggestions to improve the research work and thesis write-up. I will never forget the quality of his instruction and the profundity of his analysis.

My heartfelt thanks also go to my GEC members; **Dr. Hassan Anwer** and **Dr. Shah Rukh Abbas** whose insightful feedback and encouragement were invaluable throughout this journey. I am indebted to them for sparing time from their busy schedule for attending progress reviews and providing their beneficial suggestions and comments in the context of research and thesis. I am profoundly grateful to **Dr. Zeshan**, Head of the Department of Environmental Sciences for his unwavering support during my thesis journey. My appreciation goes to the entire faculty, the staff of IESE, and all my classmates for their help and support during the research phase.

Additionally, I would like to present deepest gratitude to Ms. Anisa Tahir for her support and encouragement during thesis write-up and overall experimental work. I am thankful to everyone out there who has offered kind words, advice, or even a moment of encouragement along the way—your impact has been significant, and I am truly grateful.

This work would not have been possible without each and every one of you.

Faiqa Faryal

TABLE OF CONTENTS

DEDICATION vi

ACKNOWLEDGEMENTS ix

ABSTRACT xvi

CHAPTER 1 1

INTRODUCTION 1

- 1.1. Cd Toxicity in Wheat 1
- 1.2. Nanotechnology in Heavy Metal Stress Alleviation 4
- 1.3. Graphene Oxide for Cd-Resilient Wheat 5
- 1.4. Application of Graphene Oxide in Agriculture 6
- 1.5. Significance of the Research 8
- 1.6. Aim of the Study 9
- 1.7. Research Objectives of the Study 9

CHAPTER 2 11

LITERATURE REVIEW 11

- 2.1. Heavy Metal Contamination in Soil 11
- 2.2. Effects of Cd on Plants 12
- 2.3. Nanotechnology in Agriculture and Stress Alleviation 13
- 2.4. Graphene Oxide: A Novel Nanomaterial for Soil Remediation 21
- 2.5. Significance of Graphene Oxide in Wheat Cultivation 22
- 2.6. Research Gaps in Graphene Oxide Applications for Crop Stress 22

Chapter 3 24

MATERIALS AND METHODS 24

- 3.1 Phase I 25
  - 3.1.1. Synthesis of GO 25
  - 3.1.2. Characterization of GO 27
  - 3.1.2.1. Scanning Electron Microscopy (SEM) Analysis 27
  - 3.1.2.2. Energy dispersive X-ray spectroscopy (EDX) Analysis 28
  - 3.1.2.3. X-Ray Diffraction (XRD) Analysis 28
  - 3.1.2.4. Fourier Transform Infrared Analysis (FTIR) Analysis 29
- 3.1.3. Preparation of required GO doses 29

3.2. Phase II 29
3.2.1. Soil Amendments and Characterization/ Soil Collection/Soil Preliminary Analysis 29
3.2.1.1. Soil pH and Electrical Conductivity 30
3.2.1.2. Water Holding Capacity (WHC) of Soil 30
3.2.1.3. Soil Texture 31
3.2.1.4. Soil Moisture Content 32
3.2.2. Soil Amendments 32
3.2.3. Plant Material and Seed Selection 32
3.2.3.1. Experimental Design and Treatment 32
3.3. Phase III 36
3.3.1. Harvesting and Storage 36
3.3.2. Measurement and Analysis of Physicochemical Parameters 37
3.3.2.1. Cd Concentration in Plant Tissues 37
3.3.2.2. Assessment of Chlorophyll Content 39
3.3.2.3. Relative Water Content (RWC) 39
3.3.2.4. Membrane Stability Index (MSI) 40
3.3.2.5. Reactive Oxygen Species (ROS) or H_2O_2 Content 40
3.3.2.6. Antioxidant Enzymes Assay 41
3.3.2.6.1. Superoxidase Dismutase (SOD) 41
3.3.2.6.2. Catalase (CAT) 43
3.3.2.6.3. Peroxidase (POD) 44
3.4. Statistical Analysis 46
CHAPTER 4 47
RESULTS AND DISCUSSION 47
4.1 Characterization of Soil 47
4.2 Characterization of Graphene Oxide 48
4.2.1 Scanning Electron Microscopy (SEM) 48
4.2.2 Energy-dispersive X-ray spectroscopy (EDS) 48
4.2.3 X-Ray Diffraction (XRD) Analysis 49
4.2.4 Fourier Transform Infrared Spectroscopy (FTIR) 50

4.3 Growth Parameters 51
4.3.1 Plant Height 51
4.3.2 Root length 53
4.3.3 Root Fresh Weight 54
4.3.4 Root dry weight 56
4.3.5 Shoot Fresh Weight 57
4.3.6 Shoot Dry Weight 58
4.4 Spike length 59
4.5 Husk Dry Weight 60
4.6 Grain Weight 61
4.7 Chlorophyll Content 62
4.8 Relative Water Content (RWC) 64
4.9 Membrane Stability Index (MSI) 65
4.10 Relative Oxygenated Species (ROS) 67
4.11 Antioxidant Enzymes Activity 68
4.11.1 Superoxidase Dismutase (SOD) 69
4.11.2 Catalase (CAT) 70
4.11.3 Peroxidase (POD) 71
4.12 Cd Concentration in Plant Tissues 73
4.12.1 Cd Uptake in Roots 73
4.12.2 Cd Uptake in Shoots 74
4.12.3 Cd Uptake in grains 75
CHAPTER 5 77
CONCLUSIONS AND RECOMMENDATIONS 77
5.1 Conclusions 77
5.2 Recommendations 78
REFERENCES 79

LIST OF FIGURES

Figure 1: Applications of Graphene Oxide Nanoparticles in the Agricultural Sector........................7
Figure 2: Indirect and direct effects of toxic metals on crop plants...12
Figure 3: Schematic illustration of research design. 24
Figure 4: (a) Mixture of H_2SO_4, H_3PO_4 and graphite flakes on hot plate (b) Formation of slurry ..26
Figure 5: Randomised completed block design (RCBD) of treatment groups 33
Figure 6: Experimental design of the study 35
Figure 7: Vacuum filtration of samples and 20ml prepared sample for AAS..................................38
Figure 8: Using chlorophyll absorbance meter for assessing chlorophyll content 39
Figure 9: Experimental setup for determination of SOD activity 43
Figure 10: Experimental setup for determination of CAT activity 44
Figure 11: Experimental setup for POD activity determination 45
Figure 12: SEM Images of graphene oxide at (a) 1 µm and (b) 10µm. 48
Figure 13: EDX spectrum of synthesized Graphene Oxide. 49
Figure 14: Structural Analysis of synthesized graphene oxide by XRD pattern. 50
Figure 15: FTIR spectrum of functional groups in synthesized GO. 51
Figure 16: Comparison of plant height across control and various GO treatment groups. 52
Figure 17: Comparison of root length across control and various GO treatment groups. 54
Figure 18: Comparison of root fresh weight across control and various GO treatment groups.......55
Figure 19: Comparison of root dry weight across control and various GO treatment groups. 56
Figure 20: Comparison of shoot fresh weight across control and various GO treatment groups. ...58
Figure 23: Comparison of husk dry weight across control and various GO treatment groups. 61
Figure 24: Comparison of grain weight across control and various GO treatment groups. 62
Figure 25: Comparison of chlorophyll content across control and various GO treatment groups...64
Figure 27: Comparison of membrane stability index across control and various GO treatment groups...66
Figure 28: Comparison of relative oxygenated species across control and various GO treatment groups...68
Figure 32: Cd concentration in wheat roots under various treatments...74
Figure 34: Cd concentration in wheat grains under various treatments...76

LIST OF TABLES

Table 1: Effects of different nanoparticle concentrations on various crop plants. 15
Table 2: Initial characteristics of soil for pot experiment. 47

LIST OF ABBREVIATIONS

Acronym	**Description**
AAS	Atomic Absorption Spectrophotometry
ANOVA	A One-Way Analysis of Variance
ATSDR	Agency for Toxic Substances and Disease Registry
CAT	Catalase
CCI	Chlorophyll Content Index
Cd	Cadmium
DNA	Deoxyribonucleic acid
EDX	Energy Dispersive X-ray spectroscopy
FTIR	Fourier Transform Infrared Analysis
GDP	Gross Domestic Product
GO	Graphene Oxide
MSI	Membrane Stability Index
POD	Peroxidase
ROS	Reactive Oxygen Species
RWC	Relative Water Content
SDG	Sustainable Development Goal
SEM	Scanning Electron Microscopy
SOD	Superoxidase Dismutase
SPSS	Statistical Package for the Social Sciences
WHC	Water Holding Capacity
XRD	X-Ray Diffraction

ABSTRACT

Agriculture is widely recognized as the core foundation of the economy, significantly influencing the Gross Domestic Product (GDP) of a country. Wheat is among nutrient-rich grain that is crucial for ensuring food security. It plays an essential role in advancing Sustainable Development Goal (SDG) 2, i.e., “Zero Hunger”, adopted by the United Nations in 2015. However, wheat may experience certain biotic and abiotic stresses within environmental settings, with particular emphasis on heavy metal toxicity. In this regard, owing to the highly accumulative and toxic characteristics of cadmium (Cd), it emerges as a heavy metal of primary concern as it acts as a yield-limiting factor for wheat growth. The present study aimed to integrate nanotechnology in agriculture to alleviate Cd toxicity in wheat through a multi-dimensional design. Graphene oxide (GO) was synthesized via the modified Hummer’s method and went under various characterization techniques (SEM, EDS, XRD, and FTIR) to confirm the synthesis of nanoparticles. GO foliar application was carried out subsequently on wheat grown under Cd induced stress conditions (at 20 mg/kg and 40 mg/kg). GO at two distinct concentrations (30 mg/L and 60 mg/L) were applied during critical growth stages of wheat growth, i.e., 8th, 9th and 10th weeks of germination. Effects on cadmium accumulation in wheat tissues, physiological and biochemical parameters, including growth, chlorophyll content, and anti-oxidant activities were recorded, revealing that exogenous application of GO at 30 mg/L significantly improved ($p < 0.05$) the plant growth (plant height, spike length, shoot fresh and dry weight), physiological parameters (membrane stability index, relative chlorophyll content SPAD, relative water contents) and reduced Cd accumulation in wheat grains, shoots and roots. The findings of this study revealed dose-dependent nature of GO i.e., lower concentration (30 mg/L) demonstrates positive effects, while leading to toxicity at higher doses (60 mg/L). This work highlights significance of determining the concentration range of foliar application of nanoparticles in agriculture for enhancing the crop yield under heavy metals stress conditions, leading to sustainable agricultural practices in the era of the expected widespread application of nanotechnology.

CHAPTER 1

INTRODUCTION

1.1. Cadmium Toxicity in Wheat

With the unprecedented world's population growth, the well-being of nations relies remarkably on the prosperity of the agricultural sector. In many developing countries, the economy is principally supported by agriculture, which significantly influences the Gross Domestic Product (GDP) of a country. In this regard, wheat (*Triticum aestivum* L.) being a vital cereal crop from the *Poaceae* family*;* also known as *Gramineae,* is extensively cultivated worldwide (Mir et al. 2019). As a nutrient-rich grain, wheat is crucial for food security as it significantly supports the Sustainable Development Goals (SDGs), i.e., SDG-2 (Zero Hunger), adopted by the United Nations in 2015. Promoting sustainable agricultural practices is key for ensuring food security and addressing global hunger challenges.

In Pakistan, wheat is considered an essential energy crop that meets the nutritional needs of the population. In Punjab, wheat serves as the primary diet and staple food, accounting for over 75 percent of the region's domestic crop yield. Recent data from the Economic Survey of Pakistan indicates that wheat production reached a record high of 31.4 million tonnes during the 2023-24 fiscal year. These statistics reveal that wheat is not only widely cultivated globally but also plays an incomparable role in fulfilling human dietary requirements. While wheat has the capability to endure harsh environmental conditions, heavy metal contamination in soil still hinders its productivity. As the global human population continues to grow rapidly, it is crucial to escalate wheat production to meet increasing food demands. Though the composition of wheat varies slightly by region, it consistently provides substantial amounts of proteins, fats, carbohydrates, vitamins, and minerals (Wieser et al. 2020). To ensure food security and meet nutritional requirements, priority should be given to wheat varieties that are resistant to changing climatic conditions. In addition to fulfilling nutritional requirements, wheat is also employed in the manufacturing of diverse industrial products. Wheat straw is a valuable resource for biofuel production, such as bioethanol and biogas. The construction industry uses it in eco-friendly straw bale buildings and as raw material for particleboard. Additionally, it is an essential input in paper production and bio-plastic manufacturing, contributing to sustainable industrial practices (Huang et al., 2023)

Despite of the fact that wheat is extensively cultivated across all regions of Pakistan, the country's wheat production is still relatively lower than in other regions of the world. Notably, numerous challenges associated with the inadequate agricultural infrastructure and lack of focus on modern farming strategies are apparent in Pakistan. Several factors contribute to this low productivity of wheat, including inefficient fertilizer use, nutrient deficiencies, soil salinity, and the limited adoption of advanced agricultural technologies (Kalhoro 2016). In light of these issues, wheat has become a primary focus of agricultural research, aimed at reducing hunger, ensuring food security, promoting sustainability, boosting productivity, and driving economic growth.

Heavy metals are indigenous to the earth's lithology and are found across various environmental spheres, including soil, air, and water (Aslam et al. 2020). In nature, heavy metals are stable and non-degradable, which allows them to persist in the environment (Ghnaya et al. 2013). The synergy of rapid globalization and unprecedented anthropogenic activities, including industrial emissions, outdated agricultural practices, and mining are major contributors to heavy metals contamination in the biosphere (Zhan et al. 2024). Elevated heavy metal concentrations negatively impact various morphological, physiological, and biochemical properties of the crops (Arshad and Mustafa 2023). In recent times, agroecosystems have been susceptible to heavy metal contamination, threatening global food security (Hussain et al. 2018). Thus, it is crucial to meet the permissible limits of heavy metals to warrant environmental sustainability.

The severe toxicity of cadmium (Cd) to living organisms has gained widespread recognition in recent decades (Gallego et al. 2012). Soil contaminated with Cd presents a pervasive problem for environmental health. Previously, studies have affirmed that Cd-induced toxicity in plants, as it severely affects plant's metabolism, is a major reason for limiting productivity (Saif et al., 2023, Ren et al., 2020, Rizwan et al. 2016, Rizwan et al., 2019). Due to its high bioaccumulation potential, Cd is readily uptaken by plant roots, followed by its translocation to aerial components of plants, hindering plant development through its toxicity (Shoeva and Khlestkina 2018). Several studies demonstrated that Cd induces oxidative stress, ultra-structural changes, and decreased antioxidant activities, leading to reduced growth in plants (Rizwan et al., 2019, Rehman et al. 2017; Yousaf et al. 2016, Arshad et al., 2022). Research also signifies that high level contamination of Cd diminishes the plants' uptake capability linked to essential nutrients, such as zinc, manganese, and iron, which are essential to regulate metabolic activities (Murtaza et al. 2017; Qaswar et al. 2017).

In soil medium, Cd contamination is mainly originated from diverse industrial activities, unregulated fertilizer use, incineration, improper disposal of sewage waste, and atmospheric deposition (Qayyum et al. 2017). Currently, it is challenging to eliminate heavy metal contaminants, particularly Cd, from soil, as these metals are not easily transformed into less harmful forms (Venkatachalam et al. 2017). Cd directly impacts human health and has proven to be carcinogenic at certain concentrations in nature, making its contamination particularly in the agricultural sector - a global concern (Srivastava et al. 2017). Numerous diseases, including cardiovascular issues, renal disorders, bone demineralization, and cancer, have been linked to the consumption of Cd-contaminated diets(Clemens and Ma 2016). Wheat is particularly susceptible to heavy metal stress due to frequent exposure to hazardous soils and contaminated water, which endangers both food security and human health. Several studies demonstrated inhibition of wheat crop growth, nutrient availability, and grain yield associated with Cd contamination (Hussain et al. 2019a; Rizwan et al. 2019). Heavy metals upon entering food chains, disrupt the balance of ecosystems. In wheat, bioconcentration factors of heavy metals are widely reported in research studies (Al-Othman et al. 2016; Bezie et al. 2021). Nevertheless, as Cd is a non-essential metal, certain organic and inorganic treatments have been actively utilized to alleviate its harmful impacts on cereal crop growth (Qayyum et al. 2017; Rehman et al. 2017). To address the needs of the ever-increasing population, various approaches have been adopted to enhance agricultural productivity while simultaneously reducing Cd contamination.

1.2. Nanotechnology in Heavy Metal Stress Alleviation

Fortunately, nanotechnological innovations aided to reduce the hazardous impacts of heavy metals on crop yield. Several techniques have proven effective in mitigating the adverse effects of heavy metals on crop; however, the integration of nanotechnology in agriculture has shown extraordinary outcomes. Nano-agriculture not only boosts productivity but is also proves environmental friendly (Mustafa et al. 2021). In the twenty-first century, nanotechnology has gained significant importance in addressing various industrial and agricultural challenges (Iavicoli et al. 2017; Van Koetsem et al. 2017; Van Koetsem et al. 2018). It is a novel approach for alleviating heavy metal stress in agriculture, with nano-pesticides and nano-fertilizers being broadly utilized in many agricultural countries. In the modern times, nanotechnology emerged as advanced solution for addressing the nutritional deficiencies of crops under heavy metal stress, contributing to agricultural sustainability (Jain et al. 2018). Nanoparticles, which ranged in size between 1 and 100nm, have gained significant attention owing to their exceptional characteristics, including those of high surface-to-volume ratio and enhanced sorption capacity (Irshad et al. 2020). Additionally, nanoparticles demonstrate the potential to reduce nutrient losses, improving plant growth (I Khan et al. 2019), and thereby enhancing global food production, including those in wheat crops (Hussain et al. 2018; Rizwan et al. 2019). The use of nanoparticles to mitigate Cd stress in cereal crops is an eco-friendly approach to promote agricultural productivity and safeguarding public health (Mohamed et al. 2017).

1.3. Graphene Oxide for Cd-Resilient Wheat

There are numerous nanoparticles, but graphene oxide (GO) has found vast applications across diverse scientific fields, including chemistry, physics, materials science, and biology (Zhao et al. 2015). GO is an emerging nanomaterial with unique geometry, shapes, and sizes. Owing to its distinctive characteristics, it emerges as a popular nano-treatment material for its diverse applications across various sectors, including industrial and agricultural sectors (Yadav et al. 2022). Beyond its utilization in solar cells, GO has also found applications in the biomedical field. Its ferromagnetic properties make it versatile in all fields (Park et al. 2020). Due to its exceptional characteristics, GO is widely employed in broad applications, including nanoencapsulation, water treatment, innovative packaging, pesticides and insecticide analysis, and as a plant growth stimulant in agricultural settings(Bullock and Bussy 2019). Elgengehi et al. (2020) demonstrated the successful adsorption of Cd on GO, which is principally due to dispersive forces. These exceptional absorption abilities exhibited GO significant applications in environmental protection, particularly in drinking water and wastewater treatment (Zhang et al. 2022), and soil remediation (Liu and Lal 2015).

1.4. Role of Graphene Oxide in Agricultural Practices

The GO application at certain concentrations in the agricultural sector has proven beneficial, as it facilitates heavy metal stress alleviation. Some of the applications of GO oxide in the agricultural sector are demonstrated in Figure 1. GO supports micronutrients influx from soil and protects crops from numerous phytotoxic agents (Hammerschmiedt et al. 2023). Liu et al. (2023) demonstrated beneficial impacts of using variable GO concentrations in Cd phytoremediation, employing *L. japonica* as a hyperaccumulator. Similarly, Rassaei (2024) reported the significance of employing GO in lessening the toxicity of Cd in maize plants and improving nutrient uptake by acting as a chelating agent.

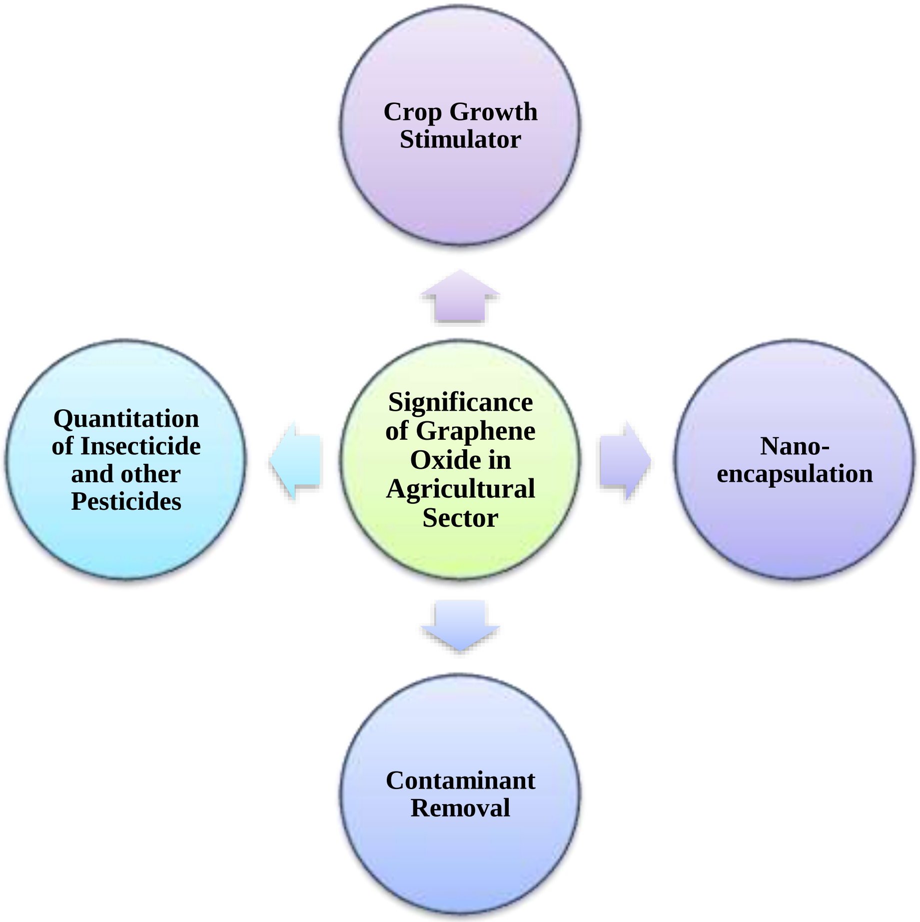


Figure 1*: Applications of Graphene Oxide Nanoparticles in the Agricultural Sector.*

The extensive use of nanoparticles for alleviating heavy metal stress is a promising strategy that ultimately paves the way toward sustainability. However, several factors influence the efficacy of nanoparticles. Numerous research studies have reported the significant contribution of GO in promoting plant growth across various species (Ahmadi et al. 2024; Hatami et al. 2019). These research studies concluded that effects of GO are concentration-dependent (Al-Rawashdeh et al. 2020; Guo et al. 2021). Experimental research in different mediums has demonstrated that low doses of GO can stimulate various physiological and chemical parameters (Hassan et al. 2022). Conversely, high doses have been shown to cause adverse effects on crops, potentially leading to reduced productivity (Javed et al. 2023). To ensure food security, comprehensive research studies should be conducted before the direct application of nanoparticles in agricultural fields, addressing various factors such as concentration, application methods and their environmental fate.

1.5. Significance of the Research

This research study holds vital value in demonstrating the stress alleviation potential of GO upon exposure to Cd in wheat. The application of green synthesis of nanoparticles yields highly significant results in mitigating heavy metal stress, paving the way for a cleaner ecosystem. The results of this study imply meaningful implications for efforts in environmental remediation. This work stands out by evaluating the efficiency of Graphene Oxide (GO) in promoting wheat growth. Additionally, this work supports sustainability by adopting environmentally friendly agricultural practices in order to ensure food security. The mechanism through which nanoparticles mitigate oxidative stress in wheat is of paramount importance. Investigating the nanotechnology applications in the agriculture bridges the gap among nano-science, agriculture and biology. This work highlights the dose-dependent nature of GO, leading to future research studies aimed at identifying safe concentration of nanoparticles that can enhance crop productivity. This research will foster interdisciplinary collaboration between fields like agriculture, nanotechnology, environmental science, and toxicology, advancing scientific knowledge. Government organizations and policy makers can use the findings of this study to develop effective strategies for managing Cd contamination in agricultural fields. This research can help mitigate the environmental impacts of cadmium pollution on wheat crops and thus will have contribution to cleaner ecosystems.

1.6. Aim of the Study

The present research aims to contribute in achieving sustainability and ensuring food security by proposing environmentally friendly methods to enhance crop productivity. The research focuses on advancing the nanotechnology in the agricultural sector, with particular emphasis on the significance of GO in mitigating heavy metal stress in wheat. Developing efficient strategies to address these stresses is critical for restoring ecological balance and improving agricultural outcomes. By understanding the dose-dependent nature of nanoparticles, this research provides insight that facilitates economic stability and sustainable growth in agricultural nations. The research highlights the potential of nano-fertilizers to manage abiotic and biotic stresses, ultimately contributing to the country's Gross Domestic Product (GDP). Through the optimization of nanoparticle dosage, high efficacy in the agricultural sector is achievable. The main aim of this work is in line with the national goals of enhancing wheat productivity through utilizing cost-effective and sustainable strategies.

1.7. Research Objectives of the Study

After a comprehensive literature review, the following objectives were set for this research:

1) To synthesize Graphene Oxide using a customized Hummer's method, followed by it characterization.
2) To study the impacts of GO varying concentrations on the plant growth, chlorophyll content, membrane stability, water holding capacity and antioxidant activity of wheat crop plant.

3) To analyse the viability of GO nanoparticles to mitigate Cd-stress in wheat plants by assessing key morphological parameters, including shoots/ roots length and fresh and dry weight.

CHAPTER 2

LITERATURE REVIEW

2.1. Heavy Metal Contamination in Soil

The contamination of soil with toxic trace metals is a global concern. Among various obstacles hindering sustainable agricultural production, heavy metal accumulation in agrarian soil stands out, posing a considerable risk to food security (Fajardo et al. 2019). Whereas, Cd, among these trace metals, is known to be notorious for its carcinogenic, and non-biodegradable properties. Notably, Cd has been classified as a hazardous metal by the Agency for Toxic Substances and Disease Registry (ATSDR) (Rafati Rahimzadeh et al. 2017). Globally, agricultural soils are predominantly contaminated by Cd originating from various sources, including excessive application of fertilizers, inappropriate disposal of sludge as well as atmospheric settings from mining, smelting, and other anthropogenic activities (Rizwan et al. 2016). In the past, studies have highlighted the adverse impacts of Cd on various crops. Some of these effects are illustrated in Figure 2. Toxic metals alter cellular structures by damaging to lipids and deoxyribonucleic acid (DNA). Additionally, Cd enters the food chain and food web, thus disrupting ecosystem balance and causing various hazardous diseases. Cd not only accumulates in the vegetative parts of crop plants but also translocates to grains of food crops, ultimately posing a risk to public health. Similarly, Cd negatively impacts a plant’s cell metabolism, reducing its ability to uptake necessary micro- and macronutrients from the soil, which consequently leads to decreased anti-oxidant activities and damages cellular ultrastructure (Qaswar et al. 2017). The threshold level for Cd in food crops is 0.2 mg/kg (i.e., ppm), but this limit can be exceeded without visible changes in vegetative growth. However, it adversely affects human health if Cd-contaminated food grains are consumed in dietary uptake (Rehman et al. 2017).

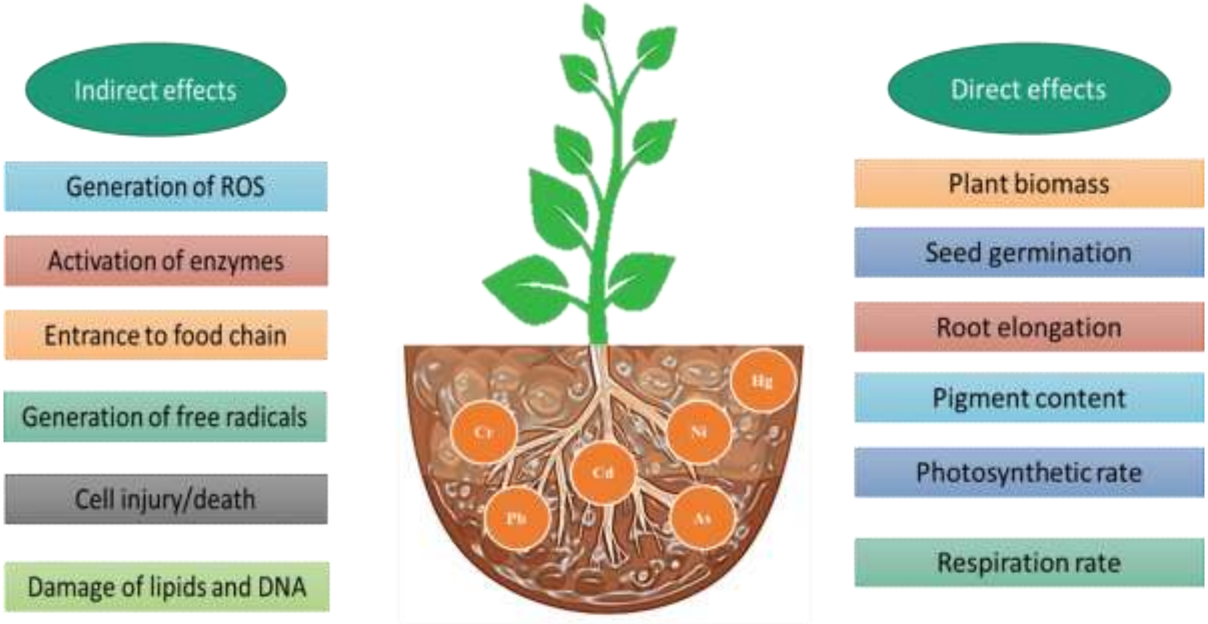


Figure 2: *Indirect and direct effects of toxic metals on crop plants.*

2.2. Effects of Cd on Plants

Cd is widely acknowledged as an inessential element for living organisms (Gallego et al. 2012). Cd reduces plant biomass via enhanced formation of reactive oxygen species (ROS). Although plants have built-in defense systems to quench ROS, however, it fail to operate effectively in the co-existence state of elevated Cd concentrations. Cd stress compromises the cell wall, cell membrane, chloroplast, and other cellular structures, thereby inhibiting photosynthetic activities in green crops (Ali et al. 2019). It also impairs nutrient uptake from plant roots, leading to homeostatic imbalances within the plant body. Considerable efforts are underway to reduce toxic metal accumulation by adopting advanced and environmentally friendly strategies.

2.3. Nanotechnology in Agriculture and Stress Alleviation

In the last decade, nanotechnology has garnered significant attention for addressing the growth needs of a rapidly increasing population, resulting in its application within the agricultural sector. Nanotechnology is a notable tool to enhance the development of food crops under various environmental stress conditions. In recent years, nanotechnology has been intensively utilized to achieve milestones in sustainable agriculture (Jain et al. 2018). Nanoparticles utilization facilitates the alleviation of heavy metal stress and is considered a practical approach compared to traditional remediation methods. Additionally, nanotechnology plays a crucial part in controlling disease spread, and enhancing crop yield quality. Nanotechnology provides innovative strategies to remediate heavy metal contamination in soil and ensure food security. With the extensive use of fertilizers, the agronomic productivity of various crops is limited. Plants require only a small quantity of fertilizers, and excessive traditional fertilizers application constitutes a significant risk to environmental health. The application of nano-fertilizers emerges as a promising solution as they provide essential growth nutrients to crops without degrading the ecosystem quality (Liu and Lal 2015; Raliya et al. 2018). Therefore, the nano-fertilizers application is crucial, as they not only provide micronutrients but also have the potential for soil remediation.

It has been well demonstrated that nanoparticles can effectively alleviate various stresses, including heavy metal toxicity, disrupting plant growth (Awad et al. 2019; Dimkpa et al. 2017; Mohamed et al. 2017). Numerous investigations have elaborated on the use of iron nanoparticles in mitigating the heavy metal-contaminated soil, supporting the crops' growth. Previously, iron oxide nanoparticles exhibited not only enhanced growth of wheat but also reduced heavy metal accumulation (Konate et al. 2017). Similarly, Michálková et al. (2017) demonstrated that the effective use of nano zero-valent iron diminished the hazardous impacts of heavy metals in sunflowers, highlighting its potential to promote healthy plant growth. It has also been revealed that low concentrations of nano zero-valent iron decrease oxidative stress compared to higher concentrations (Gong et al. 2017), demonstrating the dose-dependent nature of nanoparticles. These studies further indicate that nanoparticles could also be effectively used to remediate heavy metal contamination in soil. The outcomes of various nanoparticle treatment on different crops, along with their modes of application, are illustrated in Table 1. For instance, zinc oxide nanoparticles (Zn-NPs) have vast applications in the industrial sector and are also being used at lower concentrations to fulfill the zinc requirements of plants in agriculture (Mukherjee et al. 2015). The application of Zn-NPs has previously demonstrated an enhanced yield in mungbean and chickpea crops (Mahajan et al. 2011). Similarly, low concentrations of Zn-NPs significantly promote cotton growth while reducing oxidative damage (Venkatachalam et al. 2017). The association of zinc oxide with heavy metal decreases the toxic metals uptake, thereby protecting crops from their harmful effects. Nevertheless, the specific mechanism underlying the heavy metals remediation through the usage of nanoparticles remains unclear.

Table 1*:* Effects of different nanoparticle concentrations on various crop plants.

Crop studied	**Nanoparticles Doses**	**Application medium of NPs**	**Heavy Metal Stress Level**	**Key Findings**	**References**
Wheat (*Triticum aestivum* L.)	Iron Oxide nanoparticles (Fe-NPs) (0, 5, 10, 15, and 20 ppm)	Soil + Foliar	Cd – 7.38 ppm	• Fe-NPs alleviated the Cd toxicity in wheat tissues and crop grains • Exogenous application of Fe-NPs enhanced the plant dry biomass and grains count. • Fe-NPs increases dose-incremental Fe-biofortification.	Hussain et al. (2019a)

Wheat (*Triticum aestivum* L.)	Zinc oxide nanoparticles (ZnO-NPs)
	(0, 25, 50, 75, and 100 ppm)
		Soil + Foliar	Cd – 7.38 ppm	• ZnO-NPs improved the plant development (i.e., growth and crop yield).
				• Decrease in Cd levels;
				  • foliar application (30-77%)
				  • soil application (16-78%).
				• ZnO-NPs reduced the electrolyte leakage, and improved SOD and POD in leaves
					Hussain et al. (2018)

Wheat (*Triticum aestivum* L.)	Silicon nanoparticles (Si-NPs) (0, 25, 50, and	Soil	Cd – 7.67 ppm	• Si-NPs improved the plant development (i.e., growth and photosynthesis). • Reduction in Cd levels, particularly in grains, under both drought-stressed and normal-conditions. • Enhancement of wheat growth,	ZS Khan et al. (2019)

	100 ppm)			antioxidant activity and suppression of oxidative stress. Cd concentrations in plant tissues were peaked at the maximum level of Si-NPs application.	
Wheat (*Triticum aestivum* L.)	Si-NPs (0, 300, 600, 900, and 1200 ppm)	SFA*	Cd – 7.38 ppm	• Si-NPs significantly boosted plants overall dry biomass. • Si-NPs improved the foliar gas exchange attributes, as well as concentrations of chlorophyll a and chlorophyll b. • Decrease in Cd levels; • foliar application ○ shoots (16–58%) ○ roots (19–64%) ○ grains (20–82%) • soil application ○ shoots (11–53%) ○ roots (10–59%) ○ grains (22–83%).	Ali et al. (2019)
Wheat (*Triticum aestivum* L.)	ZnO NPs (0, 25, 50, and 100 ppm)	Foliar	Cd – 7.67 ppm	• ZnO-NPs enhanced the foliar chlorophyll, and wheat yield. • Minimum Cd and maximum Zn concentrations were recorded with highest level of foliar exposure of ZnO Nanoparticles used under normal moisture level. • Cd levels were reduced by; • 25 ppm at ZnO-NPs	Adrees et al. (2021)

				○ 26% (at normal moisture) ○ 35% (at water deficit) • 50 ppm at ZnO-NPs ○ 81% (at normal moisture) ○ 66% (at water deficit) • 100 ppm at ZnO-NPs ○ 87% (at normal moisture) ○ 81% (at water deficit)	
Maize (*Zea mays* L)	Graphene oxide nanoparticles (GO-NPs) (0, 5, 15, and 30 ppm)	Soil	Cd – (0, 10, 20, and 30 ppm)	• Results highlighted that effectiveness of GO as a chelating agent in mitigation of hazardousness of Cd on biomass and improve nutrient uptake in maize plants. • GO-NPs increases foliar chlorophyll content.	Rassaei (2024)
Wheat (*Triticum aestivum* L.)	ZnO-NPs (0, 25, 50, and 100 ppm)	Soil	Cd – 7.67 ppm	• ZnO-NPs lessen the oxidative stress, including Cd stress in synergy with drought-stress. • ZnO-NPs diminished the bioavailability of Cd, and increased dry biomass of plant. • ZnO-NPs application proven to facilitate wheat productivity, under either Cd-stressed alone or in conjunction with drought-stress conditions.	ZS Khan et al. (2019)
Wheat (*Triticum aestivum* L.)	reduced Graphene Oxide nanoparticles (rGO-NPs)	Sand	Lead (Pb) – (300, 500, 700 ppm)	• Greenly reduced graphene oxide nanoparticles (rGO-NPs) promote chlorophyll levels and growth in wheat plants.	Zhan et al. (2024)

	(15, 30, 60, and 120 ppm)			• rGO-NPs, antioxidant enzyme activities, including APX, SOD, POD and CAT were stimulated, and ROS production was suppressed under Pb-stressed conditions.	
Wheat (*Triticum aestivum* L.)	ZnO-NPs (10, 20, and 30 ppm)	Foliar	Chromium (Cr) and Arsenic (As) – (10ppm)	• ZnO-NPs reduced the hazardous effects of As and Cr contaminations on physiological and antioxidative metrics in two wheat species. • ZnO-NPs foliar application demonstrated the toxicity lessening by reduction in heavy metal uptake in wheat.	Iqbal et al. (2024)
Wheat (*Triticum aestivum* L.)	ZnO-NPs (25ppm)	Petri dishes	As – (1 and 10 ppm)	• ZnO-NPs facilitated in alleviation of arsenic adverse effects by germination rate, shoot and root length, seedling health, RWC, membrane stability index (MSI), and levels of chlorophyll, carotenoid as well as protein.	Kumar et al. (2021)
Duckweed (*Lemna minor* L.)	GO-NPs (1 and 5 ppm)	200-mL plastic cups	Copper (Cu) – (5–20 µM)	• Cu at >10 M concentration stress condition inhibited the percentage growth, chlorophyll content, plant biomass, and phosphorous uptake. • GO-NPs at 5 ppm application diminished Cu stress in L. minor	Hu et al. (2018)

				plants, alleviating adverse effects of high Cu concentrations.	
Persian clover (*Trifolium resupinatum* L.)	GO-NPs (0, 125, 250, and 500 ppm)	Substrate paper	sodium chloride (NaCl) – (0, -0.1, -0.2, -0.3, or -0.4 MegaPascal (MPa))	• GO-NPs exhibited resistance to salt stress as reflected by the improvement in seed germination. • Findings supported the potential for GO nanomaterials application in saline stressed conditions in agricultural practices	Stefanello et al. (2024)
Rice (*Oryza sativa* L.)	GO-NPs (0, 1, and 10 ppm)	Hydroponic system	Cd – (1 ppm)	• Results revealed concentrations dependent behaviour of GO-NPs. ▪ GO (at 1 ppm) - no apparent effects, ▪ GO (at 10 ppm) - enhanced seed sprouting, and rhizospheric growth. However, membrane permeability also accelerated, facilitating Cd uptake by rice plants. • In contrast, Cd exhibited negative impacts on seed germination in rice crops, which can be mitigated by application of GO-NPs in Cd contamination.	Li et al. (2020)
Lettuce (*Lactuca sativa* L.)	GO-NPs (0, 30, and 60 ppm)	Foliar	Cd – (0 and 2 ppm)	• GO-NPs foliar application (at 30 ppm) pronounced to lessen toxicity of Cd in lettuce plant. • GO-NPs promoted	Gao et al. (2020b)

				photosynthesis percentage, stomatal permeability, evapotranspiration flux, PSII quantum yield, foliar chlorophyll, quantum efficiency, electron transport efficiency, RuBisCO activity, biomass content, MDA content, and antioxidant enzymatic activity.	
Lettuce (*Lactuca sativa* L.)	GO-NPs (0, 30, and 60 ppm)	Hydroponic conditions Lettuce growth and Foliar spray of GO on Lettuce leaves	Cd – (2 ppm)	• GO-NPs (at 30 ppm) notably raised the morphological parameters, trichome density, vitamin C content. However, the foliar application at 60 ppm may impart adverse impact on lettuce growth. • Thus, optimal concentrations of GO-NPs may reduce Cd tolerance, promoting plant growth.	Gao et al. (2020a)

2.4. Graphene Oxide: A Novel Nanomaterial for Soil Remediation

Among emerging nanoparticles, GO stands out as a novel engineered material with unique size and specific geometry. It is a carbon nanomaterial with two-dimensional structure that features epoxy, hydroxyl, and carboxyl functional groups, and hydrophobic sp^2 domains embedded in a hydrophilic sp^3 C–O network (Li et al. 2017). GO has been extensively used for remediating soil pollution due to its unique physiochemical properties, and it is highly effective in mitigating heavy metal stress. As an environmentally friendly material, GO has high efficiency and is extensively utilized in industries such as polymer manufacturing, advancing flow reactor technology, biotechnology, and environmental protection (Dreyer et al. 2014). Despite its broad applications, the eco-toxicological risks of GO need to be assessed before applying it in agricultural fields.

Numerous studies have elucidated the influence of GO on plant productivity (Ahmadi et al. 2024; Ghorbanpour et al. 2018; Hatami et al. 2019), showing that the impacts of GO are concentration-dependent (Al-Rawashdeh et al. 2020; Guo et al. 2021). GO also enhances the essential nutrient uptake in the soil. GO has positively influenced the on physiology of plants and supports optimal growth (Hammerschmiedt et al. 2023). In a study on *A. thaliana*, Park et al. (2020) found that treating the plants with GO solution increased the root length and leaf count in plants. Watermelons treated with GO solution exhibited higher carbohydrate content and larger dimensions. Yin et al. (2018) demonstrated that GO reduced the toxic effects of Cd on rice buds at 100–1500 ppm concentrations under hydroponic environmental conditions.

Various studies have highlighted the positive impacts and biocompatibility of GO (Bagri et al. 2010; Hu and Zhou 2013; Hu and Zhou 2014; Younes et al. 2019). Anjum et al. (2014) reported that GO caused a reduction in the production of ROS at the 400 and 800 mg L^{-1} concentrations in *Vicia faba* L. However, lower concentrations of GO have also been shown to improve the tomato seedlings growth (Zhang et al. 2015).

2.5. Significance of Graphene Oxide in Wheat Cultivation

Globally, wheat is the second most widely cultivated crop that holds significant economic importance. Approximately 36 percent of the global population is wheat dependent to meet their energy needs (Mottaleb et al. 2023). However, attributing to various biotic and abiotic factors, wheat yield is declining. In this regard, heavy metal contamination in soil emerges as one of the critical threats (Poole et al. 2021). As mentioned earlier, Cd contamination poses hazardous risks to wheat productivity. Additionally, GO has been extensively utilized to treat heavy metal's stressed phytotoxicity in soil, enhancing the crop productivity (Kabiri et al. 2017; Rizwan et al. 2019). Nevertheless, studies also reveal the GO's toxic potential under heavy metal stress conditions (Cheng et al. 2016; Gurunathan 2015; Hu and Zhou 2014; Rizwan et al. 2019).

Hu et al. (2014) reported enhancement of arsenic accumulation in wheat, when GO is applied through the root pathway at 0.1–10 ppm concentration levels. Another study highlighted the toxic nature of GO, causing cell damage and Cd accumulation in wheat seedlings (Gao et al. 2019a; Gao et al. 2019b). These results depicted that the impacts of GO are highly dependent on its concentration, the route of nanoparticles application, the toxicity of the heavy metal involved, and the crop species. Therefore, it is imperative to investigate the efficacy as well as safety of the GO before its field application.

2.6. Research Gaps in Graphene Oxide Applications for Crop Stress

Currently, the scientific knowledge on the foliar application of GO on food crops exposed to heavy metals stress is limited, which highlights the critical need to understand the concentration-dependent plant response to ensure the optimal dosage of GO for certain crop species, an area that has previously been unclear. Additionally, exploring the foliar application of GO under heavy metal stress is imperative, as most studies in the literature focus on soil or rhizospheric applications of GO. At the same time, to investigate the potential approaches of stress alleviation, such as ROS scavenging at various concentrations of GO application is critical. Finally, employing the indigenous crop with considerable local as well as global influence on food security and economic stability helps to address scientific gaps by providing valuable data on the effects of GO applications. These contributions are essential for promoting sustainable agriculture through the safe and effective use of GO in mitigating heavy metals stress in crops.

Chapter 3

MATERIALS AND METHODS

Experiments for the research study were carried out at the Environmental Biotechnology Lab at the Institute of Environmental Sciences and Engineering (IESE), National University of Sciences and Technology (NUST), Islamabad, Pakistan. The experimental design was planned to effectively assess the effects of foliar application of GO nanoparticles on various biochemical and morphological parameters of wheat (*Triticum aestivum* L.), cultivated in Cd-stressed soil. Keeping in view the objectives of the study, the experimental setup was designed in three phases as illustrated in Figure 3.

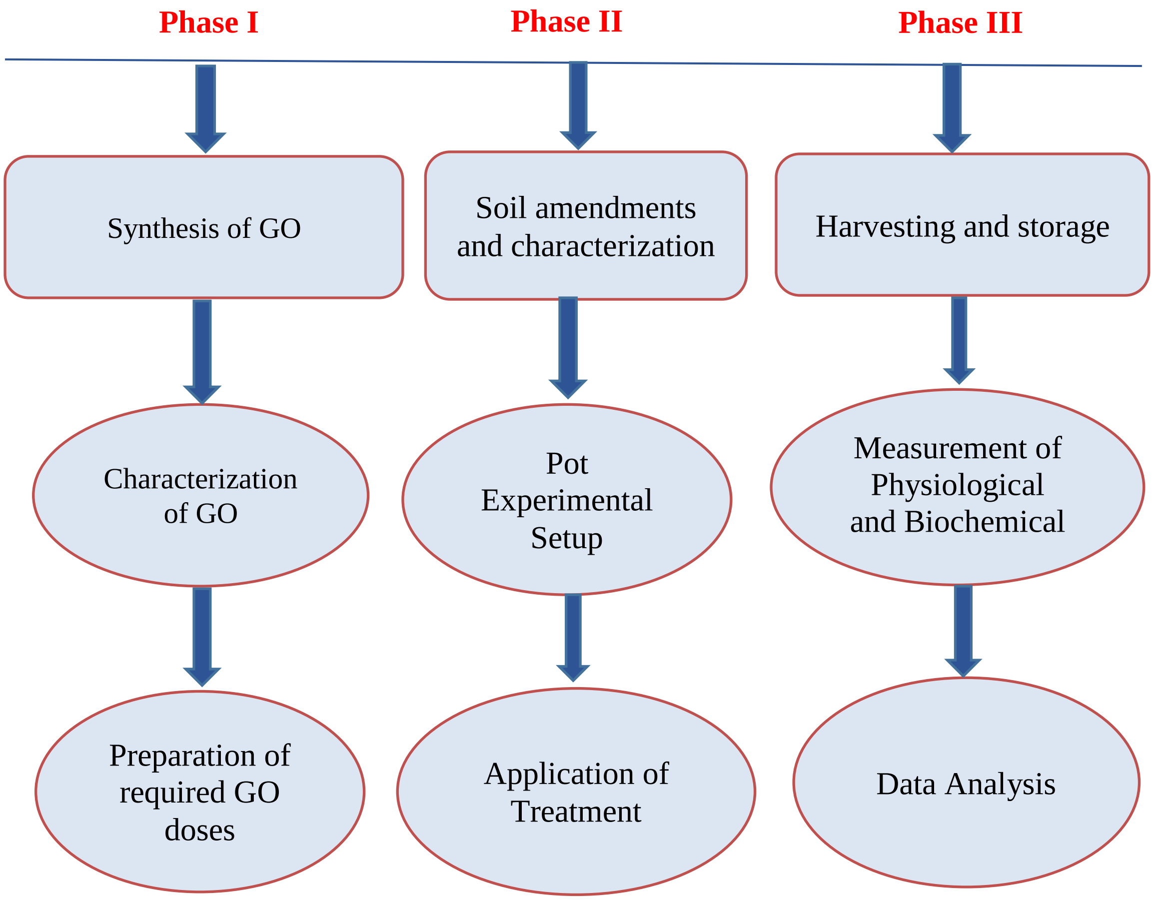


Figure 3: *Schematic illustration of research design.*

Among three major phases, the ***Phase I*** focused on the synthesis of GO, its characterization, and the preparation of desired GO concentration solutions used to evaluate its mitigating effects on wheat plants under cadmium stress. ***Phase II*** of the experiment included soil amendments and characterization study, followed by the foliar spray of nanoparticles on wheat plants. In the final phase (***Phase III***), measurement and analysis were performed on harvested plant samples, followed by data analysis. Detailed description of each step of the study design is described as follows;

3.1 Phase I

3.1.1. Synthesis of GO (Graphene Oxide)

GO was prepared using a modified Hummer's method (de Oliveira et al. 2023; Li et al. 2017). Initially, a mixture solution of 360 ml conc. sulphuric acid (H_2SO_4) and 40 ml of phosphoric acid (H_3PO_4) were mixed with 3 g of graphite flakes. The mixture was magnetically stirred at 3000 rpm for 15 minutes. Considering the exothermic nature of the reaction, the ice bath was employed, and 18 g of potassium permanganate ($KMnO_4$) was added at keeping the temperature below 15°C. The mixture was then stirred again for 15 minutes at 1000 rpm in order to ensure homogeneity of the mixture. After forming a slurry, the mixture was covered with aluminum foil, with 3 to 5 holes and placed on a stirrer for 24 hours in a fume hood. Subsequently, 3ml of 30% hydrogen peroxide (H_2O_2) was poured into a beaker that contained a mixture of already mentioned chemicals while keeping the beaker in an ice bath, and mixture was stirred continuously until it turned yellowish. The mixture was left to settle for another 24 hours in a dry place. The next step involved draining the excess liquid and refilling the beaker with deionized water. After settling, 300 ml dionized (DI) water was poured into the beaker, and the mixture was aliquoted into falcon tubes, which were then centrifuged at 6000 rpm for 15 minutes. In the next stage, falcon tubes were drained, then each tube was filled with 50ml of 1 M HCL and subjected to additional centrifugation at 6000 rpm for 15 minutes. After draining the HCL, each falcon tube was filled with DI water and centrifuged again.

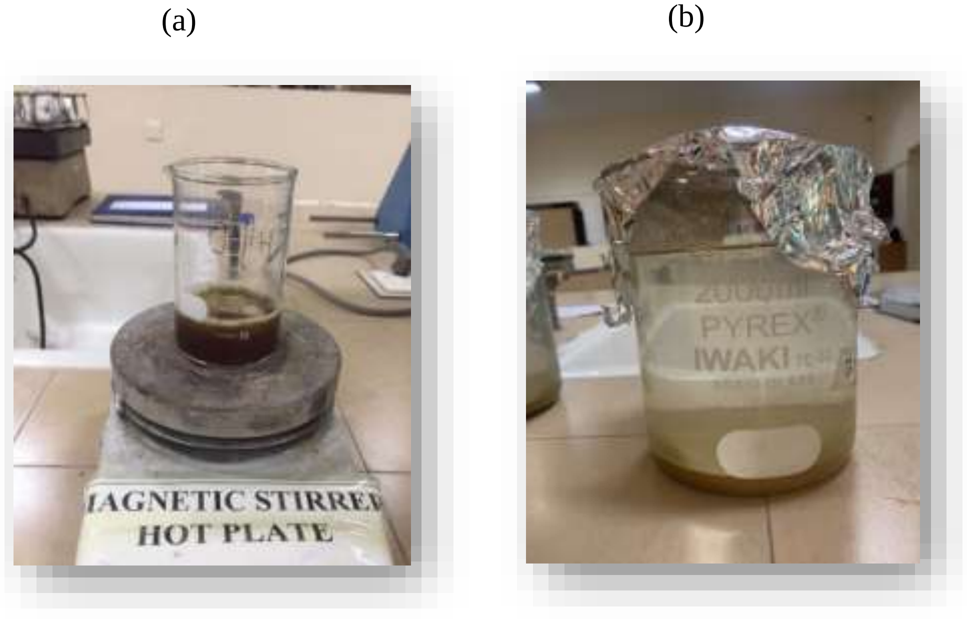


Figure 4: *(a) Mixture of H_2SO_4, H_3PO_4 and graphite flakes on hot plate (b) Formation of slurry*

Following the removal of excessive liquid, the falcon tubes were again filled with DI water, and sonicated for 40 minutes. The entire mixture was then transferred into a petri-dish and subjected to an oven drying for 24 hours at 60 ^{o}C temperature. The graphene sheet was crushed into powder and subjected to vacuum filtration, a crucial step for balancing its pH. Finally, the resultant GO product was again oven dried for an additional 24 hours at 60 ^{o}C temperature, and using a mortar and pestle, the product material was crushed into powder form and stored in a dark place.

Figure 5: *Final stage of Graphene Oxide used in the experiment*

3.1.2. Characterization of GO

GO is a carbon-based nanomaterial that possesses a diverse range of chemical and physical characteristics. In order to elaborate its attributes, characterization was performed using various techniques, including Scanning Electron Microscopy (SEM), Energy Dispersive X-ray spectroscopy (EDX), X-Ray Diffraction (XRD), and Fourier Transform Infrared Analysis (FTIR). The samples for analysis were transported in Eppendorf tubes to the School of Chemical and Materials Engineering (SCME) Laboratory at the National University of Sciences and Technology (NUST), Islamabad.

3.1.2.1. Scanning Electron Microscopy (SEM) Analysis

SEM is a highly sensitive tool used for the structural analysis of various nanoparticles. In SEM, an electron beam is generated using high voltage in a vacuum. When these accelerated electrons strike the sample, various signals are produced. An image is formed at the magnetic lens when the electron collector gathers backscattered electrons. Due to the high efficiency of

SEM in confirming nanoparticles, the morphological confirmation of the prepared GO sample was performed using this method. The SEM images provided visual evidence of the size distribution and morphology of the GO product synthesized. For this study, SEM images were captured at 0.5 µm and 1 µm, operating at 20kV.

3.1.2.2. Energy dispersive X-ray spectroscopy (EDX) Analysis

EDX is recognized as a vital analytical technique in the determination of the elemental composition across diverse samples, providing detailed information about the elemental occurrence. Thus, for determining the occurrence profile of various elements in the synthesized GO product sample, EDX analysis was conducted using the Compact Detector Unit (CDU) in the SEM. The spectrum of EDX for the GO sample was collected at an acceleration voltage of 20 kV for fifty seconds.

3.1.2.3. X-Ray Diffraction (XRD) Analysis

XRD is a robust method technique employed to evaluate the atomic spacing and chemical characteristics in a sample. XRD provides reliable data values due to which it is being rapidly used for the determination of phase in a sample. In it a bean X-ray is utilized that hits a sample and this ray is diffracted at a specific angle. All the diffracted rays are collected and they give an exceptionally high peak that elaborates the crystalline nature of the sample. In this study, the crystal structure of synthesized GO was characterized using this technique. Using X'Pert Highscore software, the average crystalline size of GO was calculated. GO Analysis was observed at area of the Two-Theta (2θ) bin between 10 to 90 degrees of XRD.

3.1.2.4. Fourier Transform Infrared Analysis (FTIR) Analysis

FTIR analysis is a valuable method for identifying specific functional groups present in a sample. The quantification of infrared radiation absorption at specific wavelengths, and the presence of unique chemical bonds can be easily detected using this technique. This analysis confirms the synthesis of specific nanoparticles by showing characteristic functional groups at specific wavelengths Accordingly, FTIR analysis was performed on the GO sample to verify the occurrence of functional groups, including hydroxyl, carbonyl, and epoxy groups. The X-rays wavenumber range was from 4000 to 400 cm^{-1} at 1 cm^{-1} resolution applied to the GO sample.

3.1.3. Preparation of required GO doses

To prepare the required doses of GO, 30 mg and 60 mg of GO were weighed separately using a sensitive balance. The measured amounts were added to separate beakers, and 1 liter of DI water was poured into each beaker. Both mixtures were stirred using a magnetic stirrer for 30 minutes, followed by one-hour ultra-sonication, one at a time, to ensure proper dispersion of the GO in water. To achieve uniform solutions, the mixtures were filtered using a filtration assembly. Later, the prepared GO doses were then transferred to spray bottles to ensure even distribution of GO on the foliage and stems of the wheat plants. To ensure the effectiveness of the GO, fresh GO solutions were prepared before each application.

3.2. Phase II

3.2.1. Soil Amendments and Characterization/ Soil Collection/Soil Preliminary Analysis

In the present study, soil was purchased from a local nursery in Islamabad. All stone masses and gravel particles were manually removed from the soil. To ensure the elimination of excessive moisture content in soil, it was allowed to air-dry under natural conditions for 15 days.

Afterward, the soil was screened through a sieve of 2 mm pore size. To ensure research integrity, a comprehensive analysis of the soil properties was conducted, which included determining soil pH, electrical conductivity (EC), water holding capacity (WHC), soil texture, organic matter content, and soil moisture content. All parameters were evaluated in the Environmental Biotechnology Lab at IESE, NUST.

3.2.1.1. Soil pH and Electrical Conductivity

Soil pH is a critical factor that significantly influences plant growth. A multi parameter instrument was employed to analyze the EC and pH of soil samples. Three samples of 10 g soil were collected and placed in 100 ml beakers. Afterwards, 20 ml of DI water was poured to each beaker, using a graduated cylinder. All the beakers were wrapped in aluminum foil to inhibit evaporation and placed in shaking incubator for 15 minutes to ensure a homogeneous mixture. Afterwards, the pH was determined by pH meter and readings were recorded for all three samples (Brandt et al. 2017).

For measuring EC of the soil, three 25 g soil samples were placed in 250 ml beakers, and 100 ml of DI water was poured to each beaker. The EC measurements were conducted in triplicates. To ensure the homogeneity of the soil mixtures, the beakers were placed in a shaking incubator for 15 minutes. The EC meter electrode was then immersed in each mixture, and readings were recorded separately.

3.2.1.2. Water Holding Capacity (WHC) of Soil

For determination of the WHC, three 25g soil samples were collected, filtered through Whatman filter papers, with the help of 100 ml of DI water through each funnel. After a while, the soil filtrates were collected, and volume of the filtrates was measured (Horne and Scotter, 2016). Finally, the WHC for each sample was calculated by using the following the equation (1).

$$WHC\ (\%) = 100\, ml\ of\ DI\ Water - Filtrate\ collected\ in\ the\ beaker\ (ml) \quad \text{Eq. (1)}$$

3.2.1.3. Soil Texture

The United States Department of Agriculture (USDA) formulated the textural triangle methodology to evaluate soil texture (Barman and Choudhury 2020; Jensen et al. 2017). This system determines soil texture by evaluating the composition ratios of sand, silt, and clay within a soil sample. Based on the percentage composition of these particles, soil can be classified as loamy, clay loam, silty clay, and sandy loam. This method is crucial for determining soil properties, which hold significant value in plant growth. To determine soil texture using this method, 100 g of soil was homogenized with a sufficient quantity of DI water to form a homogenous solution. The solution was then allowed to settle for a certain period. A hydrometer was utilized to measure the settling speed of the various soil particles, thus determining soil texture. By analyzing the settling velocity, the percentage of sand and silt present in the soil sample was revealed. The obtained percentages were compared with the USDA textural triangle. Additionally, the sand percentage was calculated using the subtraction method, where the sum of the percentages of silt and clay was subtracted from 100. Ultimately, based on the calculated percentages, the soil texture class was determined by locating the corresponding value on the textural triangle.

3.2.1.4. Soil Moisture Content

To evaluate the moisture content in soil, a 10 g of soil sample was oven-dried for 24 hours at 105 °C temperature. Afterward, the soil sample was kept in a desiccator for 30 minutes before weighing. The soil moisture content was then determined using equation (2) as follows.

$$Soil\ moisture\ (\%) = \frac{Wet\ weight\ of\ soil\ (g) - Dry\ weight\ of\ soil\ (g)}{Wet\ weight\ of\ soil\ (g)} \times 100 \qquad \text{Eq. (2)}$$

3.2.2. Soil Amendments

In line with the objectives of this study, Cd was artificially induced into the soil as a soil amendment. Two different concentrations of Cd were added to the sieved soil using Cd chloride ($CdCl_2$) analytical solutions. Based on previous studies, two levels of Cd contamination were selected: 20 mg/kg and 40 mg/kg. Soil without the Cd concentration solution served as the control group for this research study. The Cd solutions were systematically mixed with the soil in a split-plot design, with application per kilogram of soil. The treated soil was then applied in pots (dimension: 20 x 14 cm). Afterwards, pots were kept at room temperature for two weeks to ensure uniform Cd distribution. To maintain equilibrium conditions, the pots were also sprayed with DI water.

3.2.3. Plant Material and Seed Selection

3.2.3.1. Experimental Design and Treatment

Wheat was selected for this experimental study as it is a Rabi crop with a short life cycle and ease of cultivation. This crop is vital due to its nutritional value and it possesses potential for economic growth. Wheat seeds were acquired from the National Agriculture Research Council (NARC), Islamabad. Before sowing, the seeds underwent sterilization with a 2% (v/v) sodium hypochlorite solution, and rinsed with tap water, followed by soaking in DI water. Initially, in each pot, moist soil was sown uniformly with 8 seeds. The pot experiment was conducted in the greenhouse of the IESE under ambient conditions following an entirely randomized design, with each treatment was replicated thrice. The experimental setup of the research study is illustrated in Figure 5.

Figure 5: *Randomised completed block design (RCBD) of treatment groups*

In order to meet the nitrogen, phosphorus and potassium requirements of wheat, each pot was fertilized with urea, ammonium phosphate, and potassium sulphate. After 10 days, only 5 seedlings were kept, and others were manually removed. Throughout the experiment, pots were irrigated with DI water. The experiment involved the foliar application of GO NPs at concentrations of 30 and 60 mg/L, using a spray bottle. The first nanoparticle treatment was applied at the 7th week of wheat seed germination, ensuring the foliage was completely moistened. The second and third applications were conducted in the 9th and 11th weeks, respectively. Each pot in the NPs treatment group received 200 ml of GO solution. Before the application of foliar spray, the soil was covered with polyethylene sheets to prevent contact between NPs and the soil. Foliar treatments were administered early in the morning, followed by irrigation in the evening.

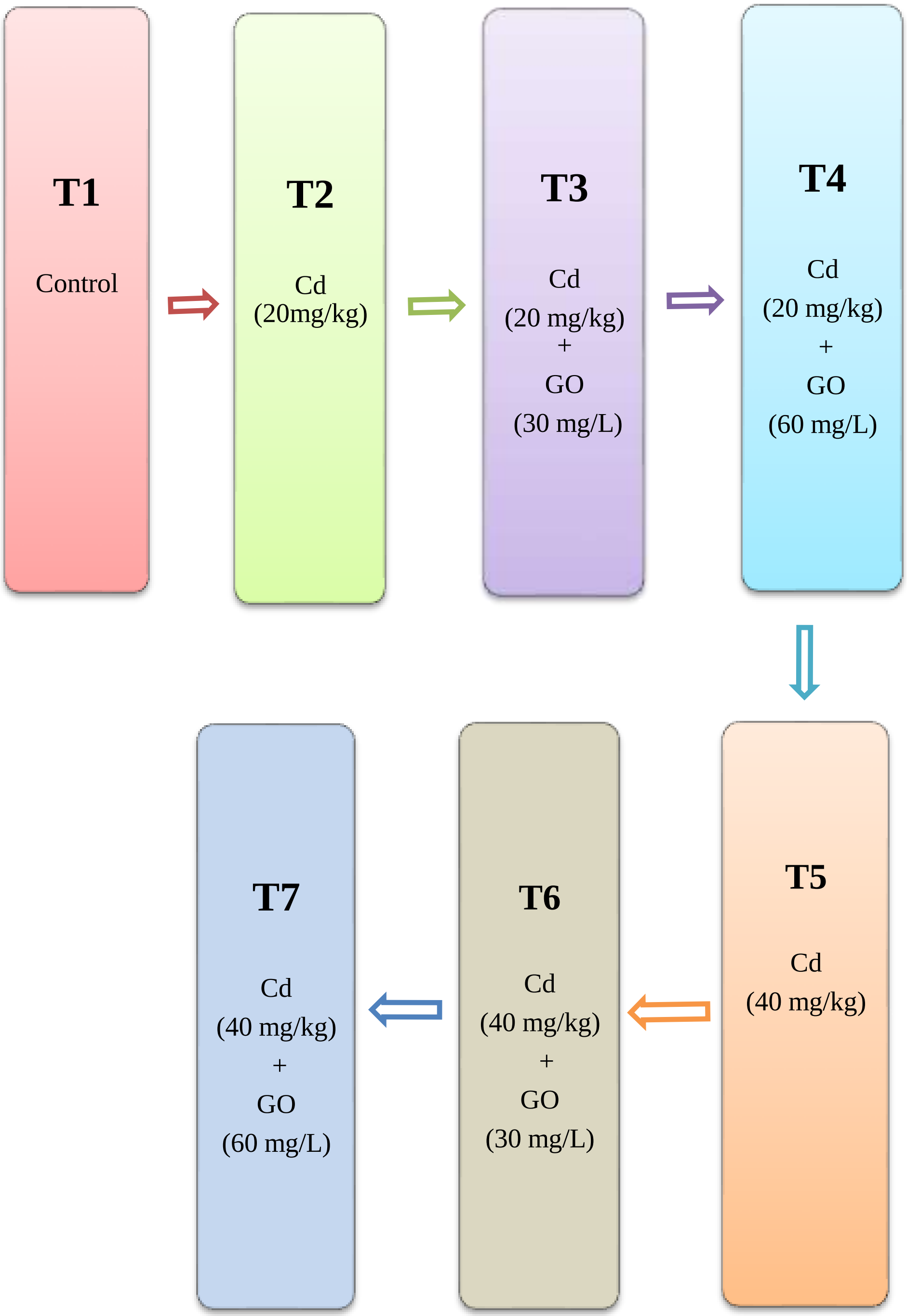


Figure 6: *Schematic layout of treatment groups*

3.3. Phase III

3.3.1. Harvesting and Storage

After one week of final NPs spray, plants were harvested in the 14th week of treatment. Physiological measurements, including shoot length, number of spikes, and leaves, were recorded before harvesting. To measure root length, one healthy plant from each pot was delicately removed from the soil and sterilized. Impurities and soil particles were removed from it. Using a stainless meter scale its length was determined. Readings were taken in triplicates for each treatment group. In addition, chlorophyll content and photosynthetic parameters were evaluated before harvesting the plant samples. Harvesting was carried out carefully from the pots of different treatment groups. Plant sections, comprising of roots, shoots and grains were delicately separated, and stored in properly labeled polythene bags. To maintain the integrity of the treatment, the post-harvesting fresh and dry mass of the plant samples were immediately measured. For determination of fresh weight, one healthy plant from each group was selected and readings were taken in triplicates for each treatment group. For dry weight calculations, plant samples were oven-dried at 60 °C for 24 hours, after which their dry mass was determined using the weighing balance. These analyses are essential for evaluating the efficiency of GO by providing in-depth information about the plant's health. Additionally, other biochemical analyses were also performed on fresh plant tissues. Estimation of ROS as well as antioxidant activities assessment were carried out on fully expanded leaves of plants. For this purpose, the sample leaves were crushed in the presence of liquid nitrogen to halt their metabolic processes. To determine Cd content in roots, shoots, and grains; the sample leaves were dried and stored safely for further analysis.

3.3.2. Measurement and Analysis of Physicochemical Parameters

A comprehensive analysis of biochemical and physiological parameters of harvested samples was also conducted at the Environmental Biotechnology Laboratory at IESE, NUST.

3.3.2.1. Cd Concentration in Plant Tissues

To evaluate the alleviation potential of GO, Cd concentration in plant sections was measured. For this purpose, samples were oven-dried at 60 °C for 24 hours, followed by crushing them into a fine powder. Each sample of wheat grains, shoot, and root was carefully weighed at 0.5g for the wet digestion process. For this task, weighted samples were placed in conical flasks were heated on a hot plate, at 120 °C, to perform acid digestion using a nitric acid and perchloric acid mixture in a fume hood. The acid digestion process began when brown fumes emerged from the reaction mixture. The hot plate temperature was gradually increased, and over time brown fumes decreased, turning the color of the mixture to light yellowish. Continuous heating resulted in a transparent solution, indicating the digestion process endpoint as portrayed in Figure 7.

Reaching the end point, flasks were then taken off the hot plate and left to cool down. Vacuum filtration was used for filtering the samples through the filter paper (Whatman No. 42), followed by adding DI water, making the sample to a volume of 20 ml. All samples were examined in triplicates under controlled conditions, and an atomic absorption spectrophotometry (AAS) was employed to quantify the Cd content in samples, with results expressed in milligrams per gram, dry weight (mg/g, dw).

Figure 7: *(a): Sample preparation for AAS analysis through wet digestion method*

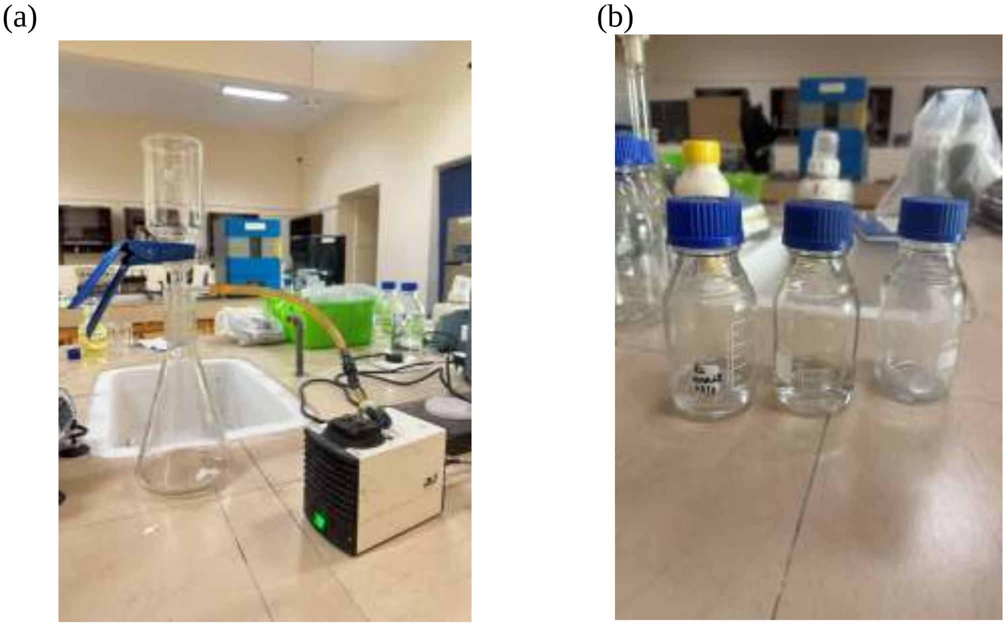


Figure 8*: (a) Vacuum filtration of samples   (b) 20ml prepared sample for AAS*

3.3.2.2. Assessment of Chlorophyll Content

For assessment of chlorophyll content, a portable chlorophyll absorbance meter (CCM-200 Plus) was used. Before taking measurements, the chlorophyll meter was calibrated. The measurements were taken from wheat leaves twice a week in the afternoon during the experiment. The Chlorophyll Content Index (CCI) calculations were based on absorbance measurements from the leaves. Readings were recorded from every plant in a pot, and the average readings were reported. The readings were specifically taken along the midrib and life margins of the wheat leaves.

Figure 7: *Assessment of Chlorophyll Content of wheat plants*

3.3.2.3. Relative Water Content (RWC)

For the estimation of RWC, 0.5 g fresh leaves (each) were sampled from each individual treatment pot upon reaching maturity. The leaves were cut into 1 cm segments, which were immersed in DI water in beakers and left to soak for one hour. Afterward, the turgid weight was determined by gently blotting the leaf discs with a paper towel. These leaf discs were then oven-dried for 24 hours, after which their dry weights were recorded (Soltys-Kalina et al. 2016). The RWC was computed using the formula given below:

$$\boldsymbol{RWC}\ (\%) = \frac{(\boldsymbol{Fresh\ Weight - Dry\ Weight})}{(\boldsymbol{Turgid\ Weight - Dry\ Weight})} \times \mathbf{100} \qquad \text{Eq. (3)}$$

3.3.2.4. Membrane Stability Index (MSI)

In order to determine the MSI, three distinct leaves were collected taken from each treatment pot and cut into 1 cm segments. The segments were immersed in 10 ml DI water at a room temperature for one hour, and the EC_0 of the aqueous mixture was recorded. Then, leaf samples were then autoclaved, initially at 40°C for 20 minutes to measure EC_1, and later at 100°C for 10 minutes to record $EC_{2,}$(Dewir and Alsadon 2022). The MSI measurements were carried out in triplicates, and the MSI was determined using the formula (as given in equation 4).

$$\boldsymbol{MSI}\ (\%) = \frac{(\boldsymbol{EC_1 - EC_0})}{(\boldsymbol{EC_2 - EC_0})} \times \mathbf{100} \qquad \text{Eq. (4)}$$

3.3.2.5. Reactive Oxygen Species (ROS) or H_2O_2 Content

Fresh leaves (0.5 g each) were collected from the treatment plants, and of these leaves were frozen using the liquid nitrogen to determine the ROS in wheat plant leaves. The leaves were then simultaneously crushed using a pre-chilled pulverizing set, followed by the addition of 5 ml of 0.1% trichloroacetic acid (TCA) solution to the crushed samples. To obtain a pure leaf extract, the sample centrifugation was performed at 14,000 rpm for 15 minutes at refrigeration temperature (i.e., 4°C). The resulting decantate was pipetted into Eppendorf tubes for further analysis.

For H_20_2 determination, 0.5 ml of potassium phosphate (K_3PO_4) buffer was stirred with 0.5 ml leaf extract in a cuvette. Afterwards, potassium iodide (KI) was injected dropwise to the solution, and the absorbance of resultant mixture was analyzed at a 390 nm wavelength, using a UV-Vis spectrophotometer. All measurements were performed in triplicates.

3.3.2.6. Antioxidant Enzymes Assay

Keeping in view objectives of the study, activities of enzymes were also evaluated to assess the GO's foliar spray efficiency on wheat plants under Cd-stress conditions. Superoxidase dismutase (SOD), Catalase (CAT), and Peroxidase (POD) activities were determined in this research. For the determination of these antioxidants, the initial step involved the preparation of a leaf extract by isolating total protein from leaves. The protocol for preparation of enzymatic assay adopted by Asif et al. (2023) was implemented, wherein 200 mg of fresh leaves were chilled with nitrogen stream, and grounded concomitantly using a pre-chilled pulverizing tool. During grinding, 1.2 ml of buffer solution, consisting of 0.1 mM EDTA and 0.2 mM potassium phosphate buffer (pH 7) was added, and the solution was centrifuged twice at 15,000 rpm at 4 °C, collecting the supernatant in Eppendorf tubes, which were then stored at low temperatures for further analysis. All measurements were carried out in triplicates to ensure data integrity.

3.3.2.6.1. Superoxidase Dismutase (SOD)

For the determination of SOD, the protocol described by Asif et al. (2023) was followed, using a 3 ml reaction mixture, comprising of 50 mM phosphate buffer (pH: 7.8), 0.1 mM EDTA, 60 μM riboflavin, 13 mM methionine, and 75 μM nitro blue tetrazolium (NBT), with 200 μL of the enzyme extract. A control sample was also prepared by mixing all these mentioned solutions with DI water. Duplicate sets of samples were prepared, one set of tubes consisted of mixture were placed under fluorescent light for 15 minutes in order to initiate the process, while the other set of tubes was placed in dark environmental conditions. After incubation, the optical density of both sets was determined at a 560 nm wavelength using UV spectrophotometer, with sample sets compared against the control sample. SOD activity was calculated as the quantity of plant enzymes needed to cause NBT reduction. Readings were recorded individually and quantified in units per gram (Unit g^{-1}, fresh weight (FW)).

$$\boldsymbol{SOD\ Activity} = \frac{(\mathbf{A_{ck}} - \mathbf{A_c}) \times \mathbf{V}}{(\mathbf{0.5} \times \mathbf{A_{ck}} \times \mathbf{W} \times \mathbf{V_t})} \qquad \text{Eq. (5)}$$

Where,

Ack = Absorbance of the control sample (without SOD activity)

Ac = Absorbance value of the sample with SOD activity in dark

V = Volume consumed of enzyme extract (ml)

W = Fresh weight of the plant tissue (g)

V_t = Total volume of the reaction mixture (ml)

***Figure 8**: Experimental setup for determination of SOD activity*

3.3.2.6.2. Catalase (CAT)

The protocol formulated by Aebi and Lester (1984) for CAT determination was followed in this study. A 3 ml reaction mixture was formulated in triplicates for each treatment group, consisting of 15 mM of H_2O_2, 50 mM phosphate buffer (pH of 7.8), and 200 µL of enzyme extract, and the absorbance was immediately quantified at a 240 nm wavelength for 45 seconds at 25 °C, to assess the enzymatic breakdown of H_2O_2. The CAT activity was expressed in unit g^{-1}, FW by following the Eq. (6).

$$\boldsymbol{CAT\ Activity} = \frac{(\Delta \mathbf{A_{240}} \times \mathbf{V}/\mathbf{a_{enz}})}{(\text{€}_{\mathbf{mM}} \times \mathbf{W})} \qquad \text{Eq. (6)}$$

Where,

ΔA_{240} = Variation in optical absorbance reading at 240 nm wavelength

V = Volume of the reaction mixture (ml)

a_{enz} = Amount of enzyme extract used (ml)

$€_{mM}$ = Absorption constant (assumed to be 26.6)

W = Fresh weight of the plant tissue (g)

Figure 9: *Experimental setup for determination of CAT activity*

3.3.2.6.3. Peroxidase (POD)

The procedure used for determining POD activity was adapted from Asif et al. (2023). The chemical solution mixture was formulated using a 50 mM of phosphate buffer (pH of 7.8), 12 mM of guaiacol, 15 mM of H_2O_2 and 200 µL of enzyme extract, and the absorbance was quantified at a wavelength of 470 nm for 90 seconds at 25 °C. Readings were carefully recorded for all samples. POD activity demonstrates the micro-moles of oxidized guaiacol and is expressed as units per gram (FW). POD measurement provides technical insight into the catalytic efficiency of guaiacol oxidation by H_2O_2, following formula as described in equation 7.

$$\boldsymbol{POD\ Activity} = \frac{(\Delta \mathbf{A}_{\mathbf{470}} \times \mathbf{V}/\mathbf{a}_{\mathbf{enz}})}{(€_{\mathbf{mM}} \times \mathbf{W})} \qquad \text{Eq. (7)}$$

Where,

ΔA_{470} = Change in optical absorbance value at 470 nm wavelength

V = Volume of the reaction mixture (ml)

a_{enz} = Amount of enzyme extract used (ml)

$€_{mM}$ = Absorption constant (assumed to be 26.6)

W = Fresh weight of the plant tissue (g)

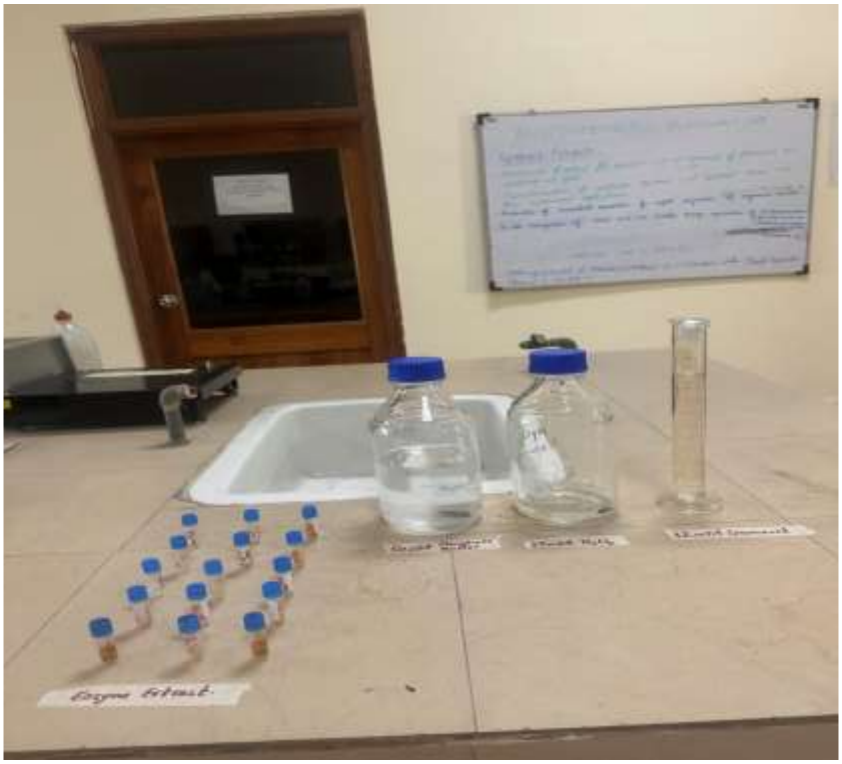


Figure 10: *Experimental setup for POD activity determination*

3.4. Statistical Analysis

The resulting data underwent statistical analysis by employing the Statistical Package for the Social Sciences (SPSS, v. 16.0) software. Average values of triplicates were reported along with the standard error of the mean. A one-way analysis of variance (ANOVA) was carried out on data, and later a post hoc test was performed to compare the groups. In order to highlight statistical differences between the control and treatment groups, the significance level was set at 0.05 ($p < 0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of Soil

The baseline attributes of the soil employed in the greenhouse pot experiment are outlined in Table 2. Soil comprised balanced composition of clay, sand, and silt that was indicated by its sandy loam texture. The soil had alkaline pH, acceptable ranges of water holding capacity, and electrical conductivity. All these parameters suggested that soil was good for wheat growth. Therefore, a pot experiment was conducted using this soil.

Table 2: *Initial characteristics of soil for pot experiment.*

Parameters	**Unit**	**Value**
Texture	-	Sandy Loam
Sand	%	62
Silt	%	24
Clay	%	14
pH	-	7.8
Electrical Conductivity (EC)	dS m^{-1}	1.5
Water Holding Capacity (WHC)	%	29.5
Organic matter content (OMC)	%	0.35

4.2 Characterization of Graphene Oxide

4.2.1 Scanning Electron Microscopy (SEM)

To analyze the GO texture and morphology, SEM facility available at the SCME, NUST was employed. These images revealed that GO possessed sheet-like structure with great flexibility as SEM images showed an overall crumpled structure. GO had a high surface area characterized by wrinkled and rippled structure (Priliana et al. 2022; Vargas et al. 2023). Well morphology of GO was indicated by SEM images as displayed in Figure 12, that demonstrated a larger surface area with a crumpled structure.

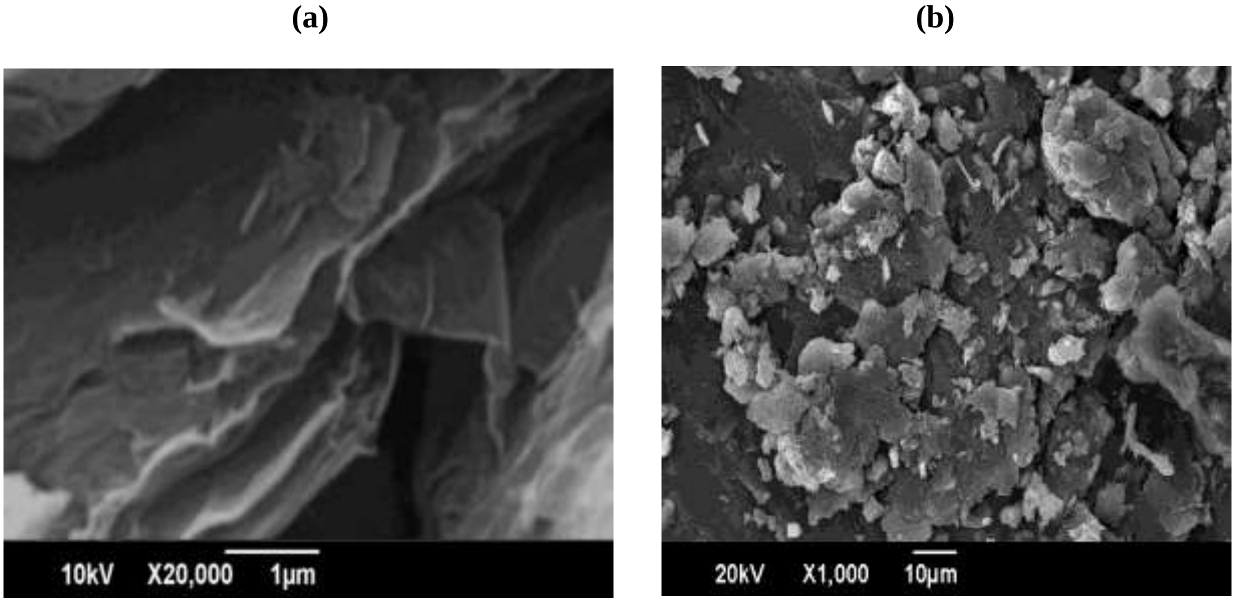
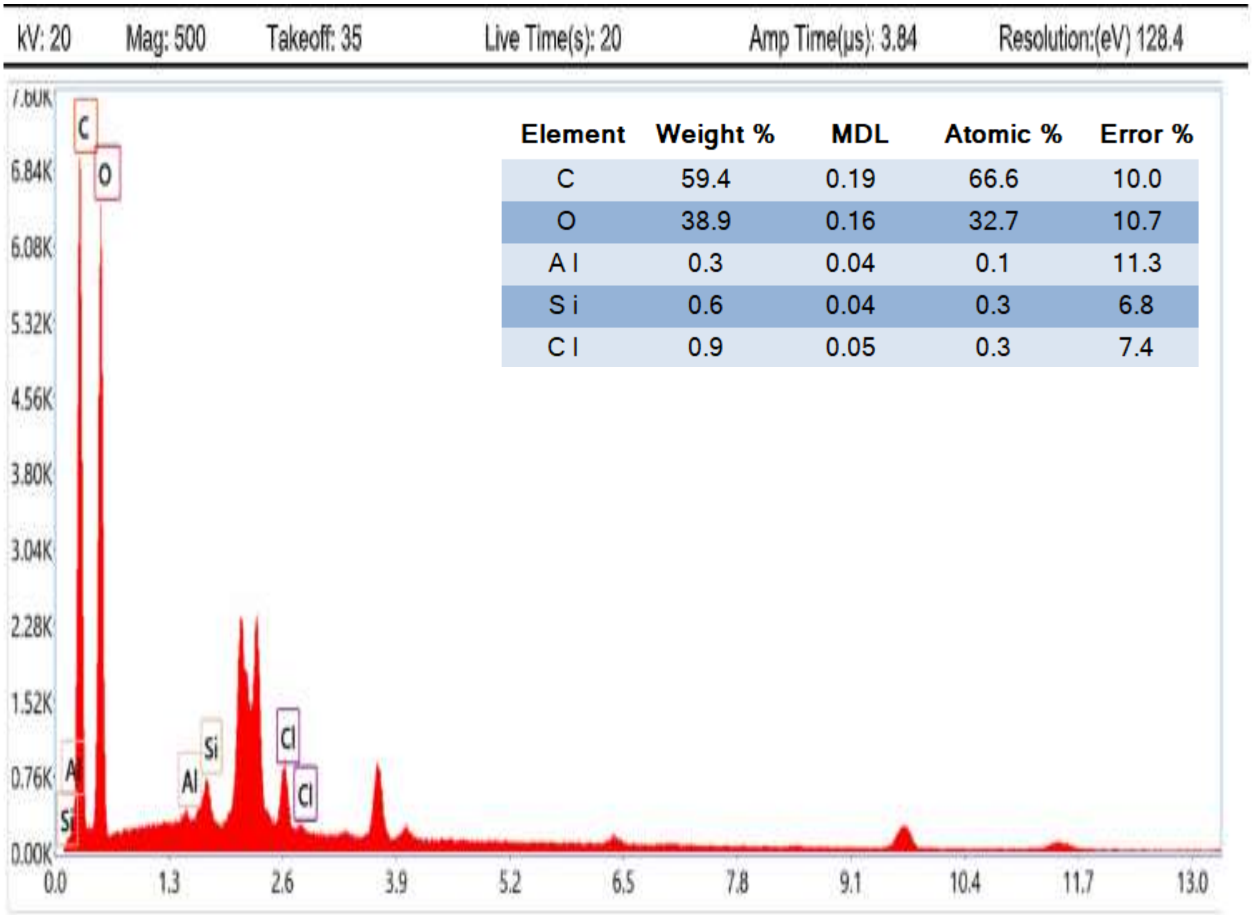


Figure 11: *SEM Images of graphene oxide at (a) 1 µm and (b) 10µm.*

4.2.2 Energy-dispersive X-ray spectroscopy (EDX)

EDX is a widely used technique to identify specific nanoparticles through their elemental composition. EDX of GO revealed a high abundance of carbon and oxygen (C is 59.4$^{\%}$ and O is 38.9$^{\%}$) in its composition that ultimately confirmed the preparation of GO. The validation of the current study is also confirmed by matching results with already reported research on synthesis of GO (Huang et al. 2023a; Huang et al. 2023b; Mahmood et al. 2023; Saif et al. 2023).



kV: 20 Mag: 500 Takeoff: 35 Live Time(s): 20 Amp Time(µs): 3.84 Resolution:(eV) 128.4

Element	Weight %	MDL	Atomic %	Error %
C	59.4	0.19	66.6	10.0
O	38.9	0.16	32.7	10.7
Al	0.3	0.04	0.1	11.3
Si	0.6	0.04	0.3	6.8
Cl	0.9	0.05	0.3	7.4

Figure 12: *EDX spectrum of synthesized Graphene Oxide.*

4.2.3 X-Ray Diffraction (XRD) Analysis

XRD of GO was conducted to analyze its phase composition and crystallographic structure. Results illustrated a wide range of narrow peaks that indicated complete oxidation of GO and its defined structure. In addition, sharp narrow peaks also pointed out smaller and uniform size of GO as shown in below figure.

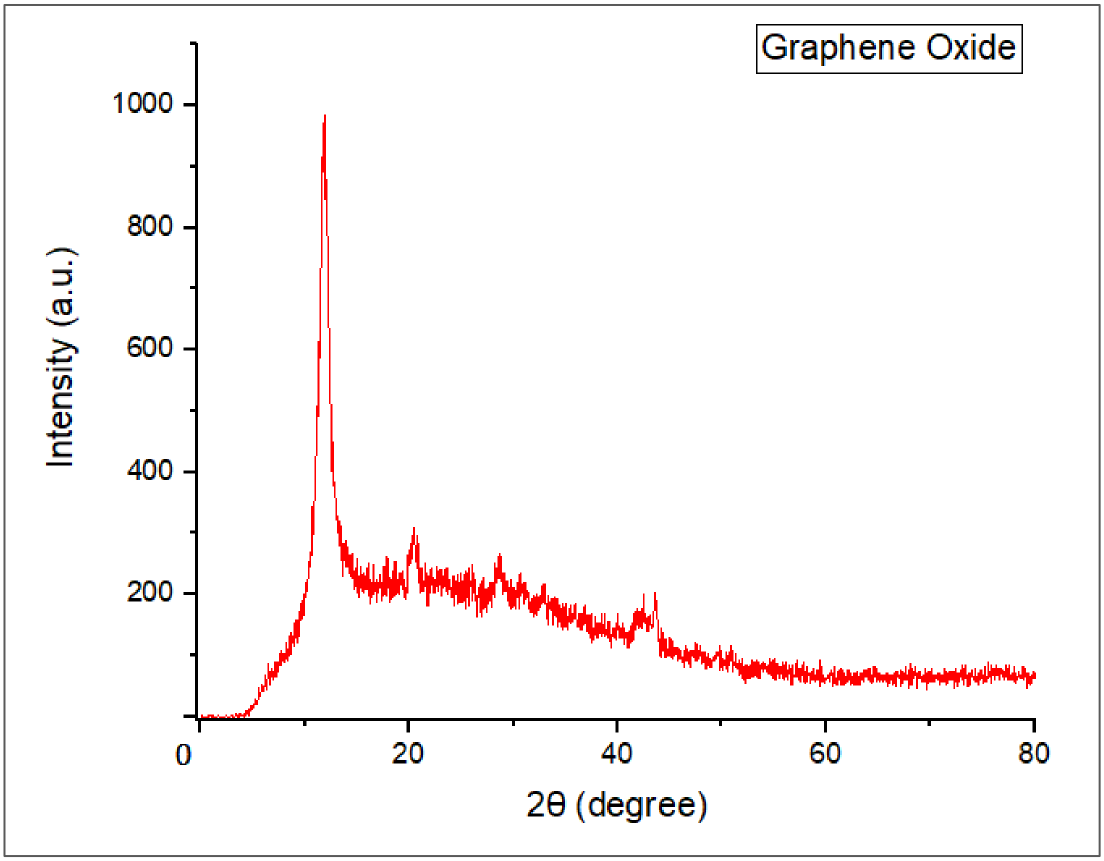


Figure 13: *Structural Analysis of synthesized graphene oxide by XRD pattern.*

As portrayed in Figure 14, the distinct appearance of the sharpest peak was observed at 11.4 degrees, almost equal to 10.4 degrees, which is the distinguishing characteristic peak of GO (Harres et al. 2023). This specified crystalline plane of GO in which carbon atoms are in a lattice plane so that interlamellar spacing is increased during the complete oxidation process (Marcano et al. 2010).

4.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was utilized to recognize the occurrence of various functional groups that could confirm the chemical structure and oxidation state of GO. FTIR of GO showed a pronounced characteristic spike at 3397 cm^{-1}, representing the presence of a hydroxyl group (Nunes et al.

2023). As shown in Figure 15, the peaks at 1725 cm^{-1}, 1614 cm^{-1}, 1115.71 cm^{-1} represented carboxyl, carbonyl, and epoxy groups respectively. These peaks directed the unique chemical and hydrophilic nature of synthesized GO (de Oliveira et al. 2023).

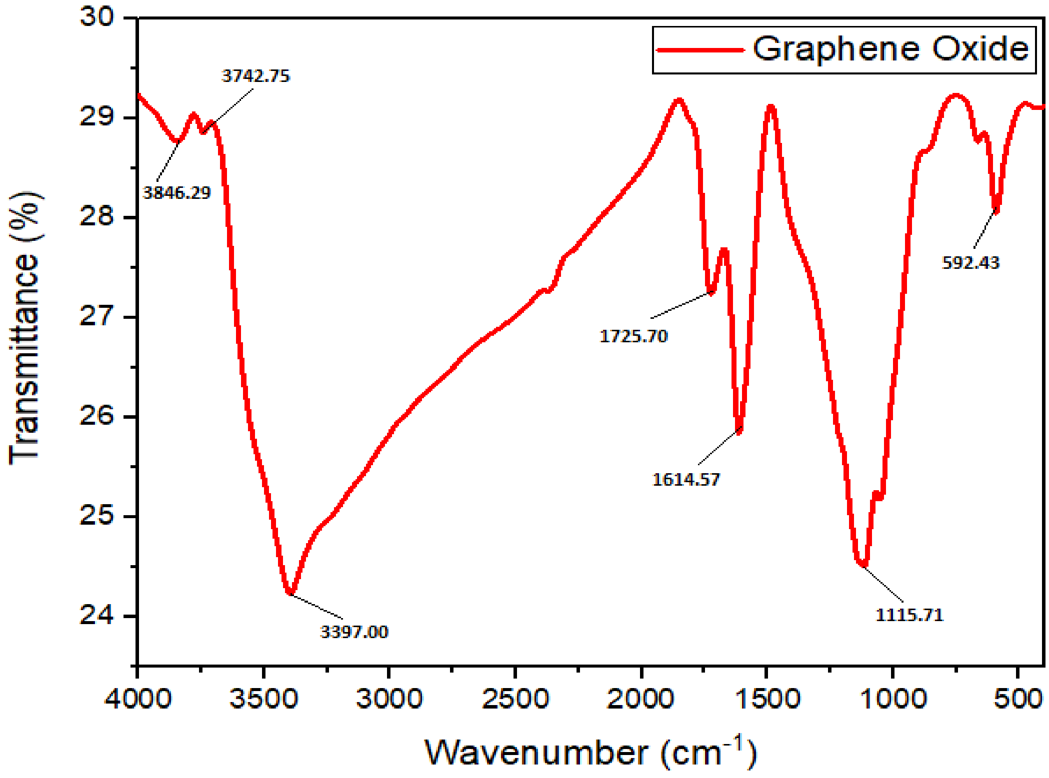


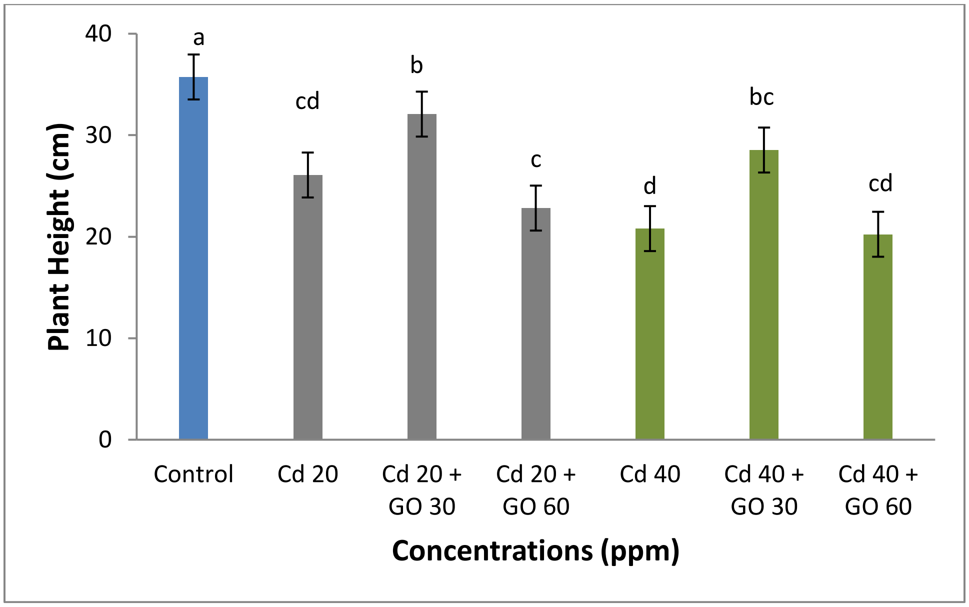
Figure 14: *FTIR spectrum of functional groups in synthesized GO.*

4.3 Growth Parameters

4.3.1 Plant Height

Under Cd stress, wheat plant height was severely suffered. In the current experiment, shoot length faced a major reduction with increasing concentration of Cd stress as shown in Figure 16. Results showed that the percentage deduction in plant height was 27% in Cd 20 and 41.8% in Cd 40 treatment group as compared to the control. The main reason behind such a high rate of plant height reduction is due to the high rate of translocation of Cd to aerial parts of the plant which ultimately poses a threat to normal functioning of plant cells (Rizwan et al. 2019). In nature, Cd has the potential to halt the growth of plants by disturbing metabolic activities (Younes et al. 2019). There are various studies that have elaborated toxic effects of Cd on the growth of plants (Rossi et al. 2018; Yin et al. 2018; Zhao et al. 2011).

To mitigate the hazardous impacts of Cd on crop plants, nanoparticles are extensively utilized. In the present experiment, foliar application of GO at 30 mg/L and 60 mg/L concentration was carried out under Cd-stress conditions. Analysis revealed that GO treatment helped in alleviating heavy metal stress. Compared to the control group, plant height was significantly improved in Cd 20 + GO 30 and Cd 40 + GO 30 groups. The observations from this study align with recent studies in which carbon-based nanoparticles enhanced the growth of plants (Reddy Pullagurala et al. 2018; Younes et al. 2019).



Error Bars indicate Standard Error (± SE) of three replicates and significant difference is indicated with distinct letters, at a P-value ≤ 0.05.

Figure 15: *Comparison of plant height across control and various GO treatment groups.*

GO at higher concentrations showed negative impacts on plant height which shows dose dependent nature of GO. There was a clear reduction in plant height when GO was applied at 60 mg/L at Cd 20 and Cd 40 groups as compared to Cd stress groups. GO at low concentrations proves significant for plant growth while higher doses negatively impact plants. Gao et al. (2019a) demonstrated that a higher concentration of nanoparticles interferes with cell division and stops plant growth while at lower doses they induce a high rate of formation of indole acetic acid, promoting plant health. Rizwan et al. (2019) also showed that nanoparticles at lower concentration ranges promote the release of growth hormones that are effective for morphology of crop plants.

4.3.2 Root length

Wheat roots are severely affected by heavy metal stress as they are directly in contact with contaminated soil. They serve as a major barrier for the transport of toxic contaminants to aerial vegetative parts (Rizwan et al. 2019). Therefore, heavy metal imparts hazardous impacts on the roots of plants, impacting their capacity to uptake essential nutrients from the soil (Gao et al. 2019a). Root biomass severely declined in the wheat plant groups that are under Cd stress in the present study. It was noticed that the application of GO caused alleviation of heavy metal stress on roots at concentration of 30mg/L as elaborated in Figure 17. GO possesses high rate of mobility and it has a hydrophilic nature due to which harmful impacts of Cd on root length lessened (Gao et al., 2023, Zhao et al., 2022)

It is estimated that Cd at 20 mg/kg and 40 mg/kg concentrations considerably inhibits the root length by 20 % and 28%, respectively. These results align with previous studies demonstrating a reduction in root length of plants in heavy metals contaminated soil (Nunes et al.

2023; Qayyum et al. 2017; Rassaei et al., 2024). Notably, the application of GO at 30mg/L showed positive impacts on wheat root length in Cd-stressed soil at 20 mg/kg and 40 mg/kg concentrations. However, a noticeable reduction in root length was observed when the GO was applied at 60mg/L, which elaborates on the detrimental attributes of nanoparticles at elevated concentrations. Similar findings were also previously reported that elaborated harmful impacts of nanoparticles at higher ranges on plant growth that faced heavy metal stress (Li et al. 2020; Malina et al. 2022; Mir et al. 2019; Mustafa et al. 2021; Park et al. 2020).

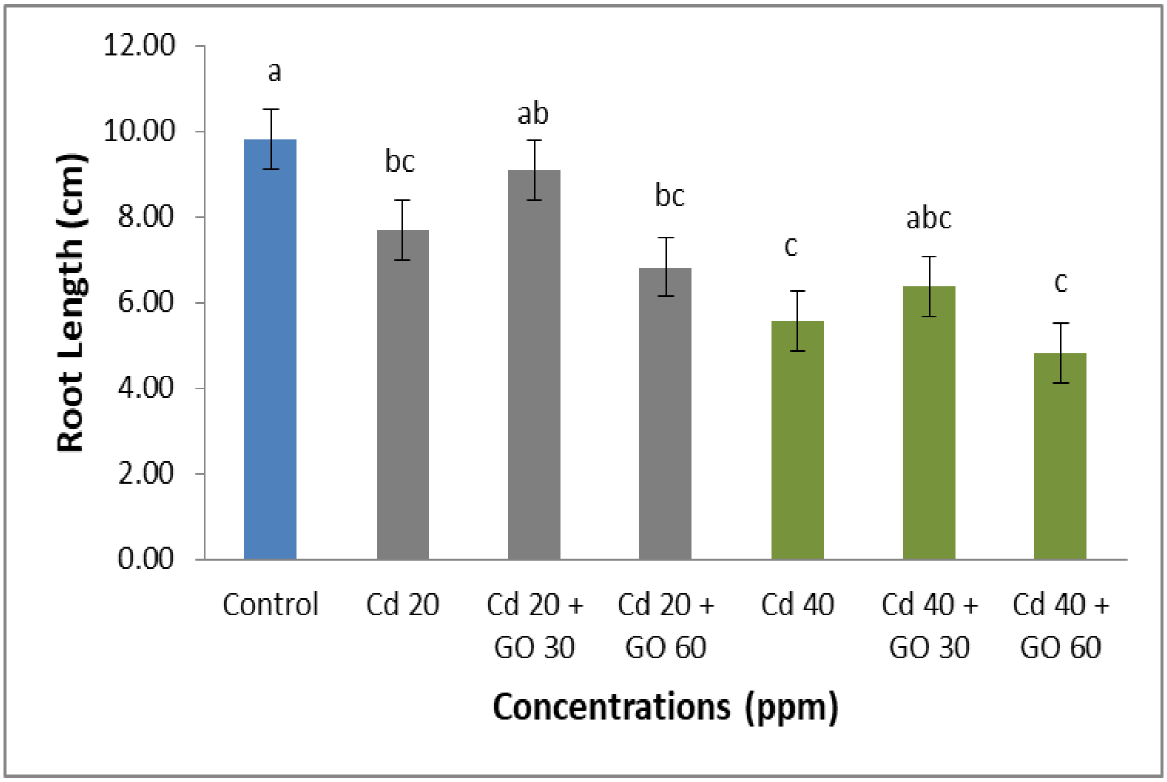


Figure 16: *Comparison of root length across control and various GO treatment groups.*

4.3.3 Root Fresh Weight

Cd also has an impact on root fresh weight as described by different studies (Abbas et al. 2017; Adrees et al. 2021)(Ali et al. 2019) (Anjum et al. 2008). The toxic nature of Cd causes reduction in plant's wet weight by stopping release of plant growth regulators. In the present study, roots of wheat plants were in direct exposure to heavy metal stress that ultimately caused reduction in root fresh weight in Cd 20mg/kg and Cd 40 mg/kg group. However, foliar spray of GO at 30 mg/L alleviated heavy metal stress on root fresh weight as indicated in Figure 18. The findings of this study correspond to previous studies, that exhibited the application of nanoparticles at 30 mg/L of GO to mitigate arsenic stress on root fresh weight under hydroponic on root fresh weight (Qaswar et al. 2017; Rossi et al. 2018; Shoeva and Khlestkina 2018; Yang et al. 2020).

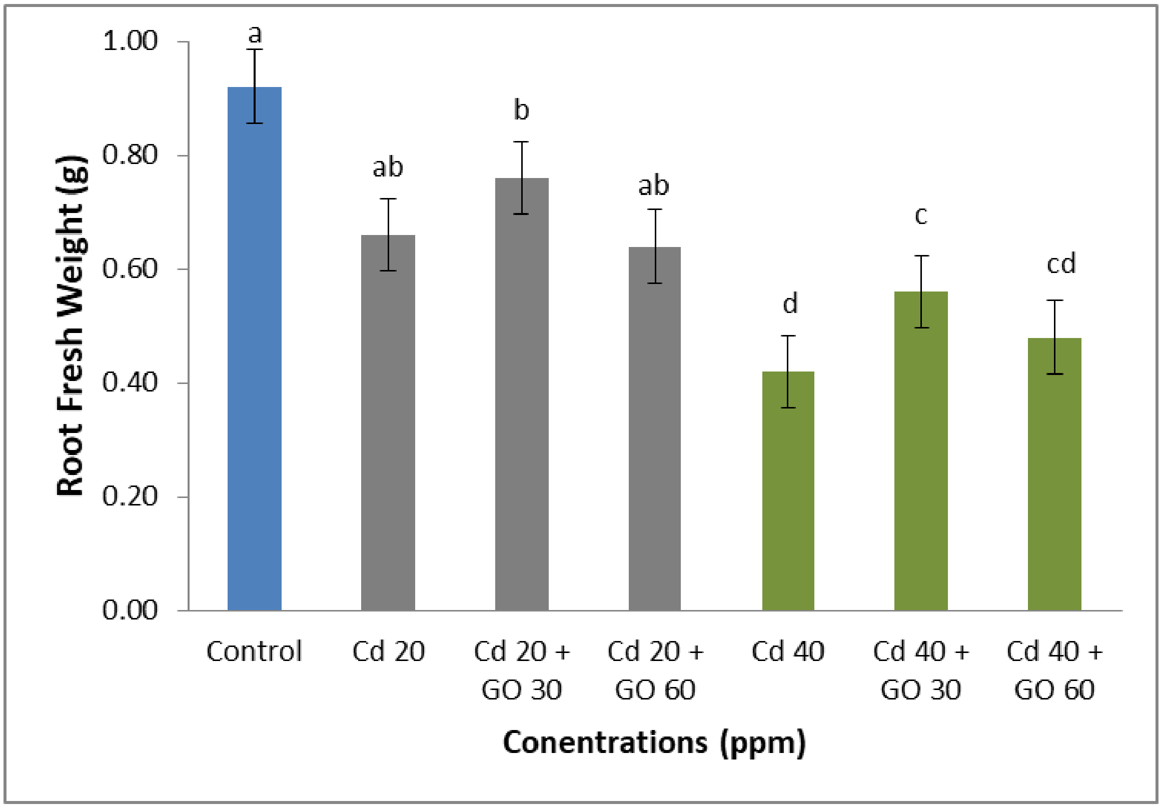


Figure 17*: *Comparison of root fresh weight across control and various GO treatment groups.

4.3.4 Root dry weight

Wheat root dry weight was impacted in the Cd-stressed soil conditions. As stated earlier, roots are directly facing a toxic environment due to which their biomass is negatively impacted. However, graphene oxides at 30 mg/L caused alleviation of Cd stress on wheat root dry weight as shown in Figure 4.8. It was noted that GO treatment at higher ranges causes further reduction in root dry biomass. The reason behind this is explained by different studies that reported nanoparticles at higher ranges pose irregular cellular functions that stop the development of cells which results in reduced biomass which ultimately weakens plants' strength to cope with environmental stresses. (Iqbal et al. 2024; Li et al. 2020; Mahmood et al. 2023; Rassaei 2024).

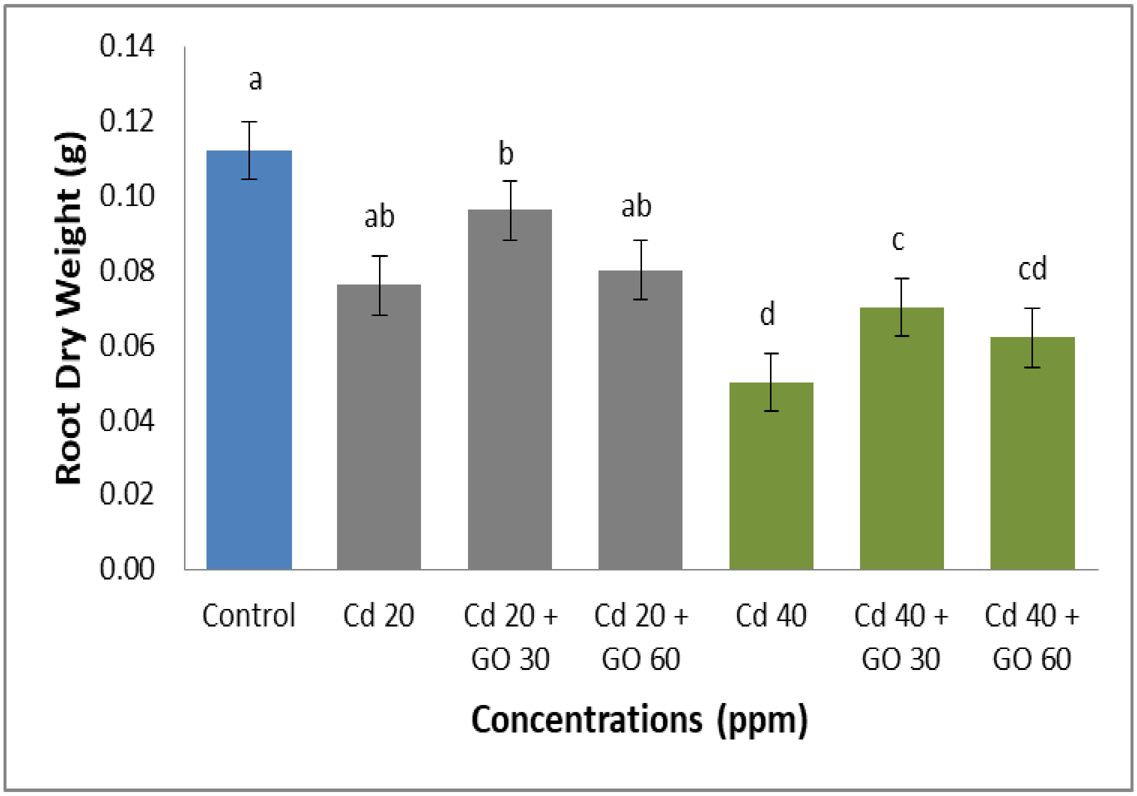


Figure 18: *Comparison of root dry weight across control and various GO treatment groups.*

4.3.5 Shoot Fresh Weight

In the current study, it was revealed that Cd posed negative impacts on shoot biomass as elaborated in Figure 20. Shoot fresh weight severed as a major indicator of harmful impacts of Cd as least production of abscisic acid, which is the main reason behind minimum shoot fresh weight is typically induced by Cd-stress conditions. Fresh weight of wheat was reduced by 30 % in Cd 20 mg/kg, and by 41.3 % in Cd 40 mg/kg groups, respectively. However, the application of foliar spray of GO on Cd stressed wheat caused alleviation from heavy metal stress. The fresh weight was enhanced by the application of GO at 30 mg/L in 20 mg/kg and 40 mg/kg Cd stress groups. Meanwhile, the GO treatment at 60 mg/L caused negative impacts on shoot biomass as indicated in Figure 20. The observations from this experiment are aligned to past investigations, showing a higher concentration of nanoparticles proved detrimental for agricultural plants (Iqbal et al. 2024), (Li et al. 2020; Mir et al. 2019; Nunes et al. 2023).

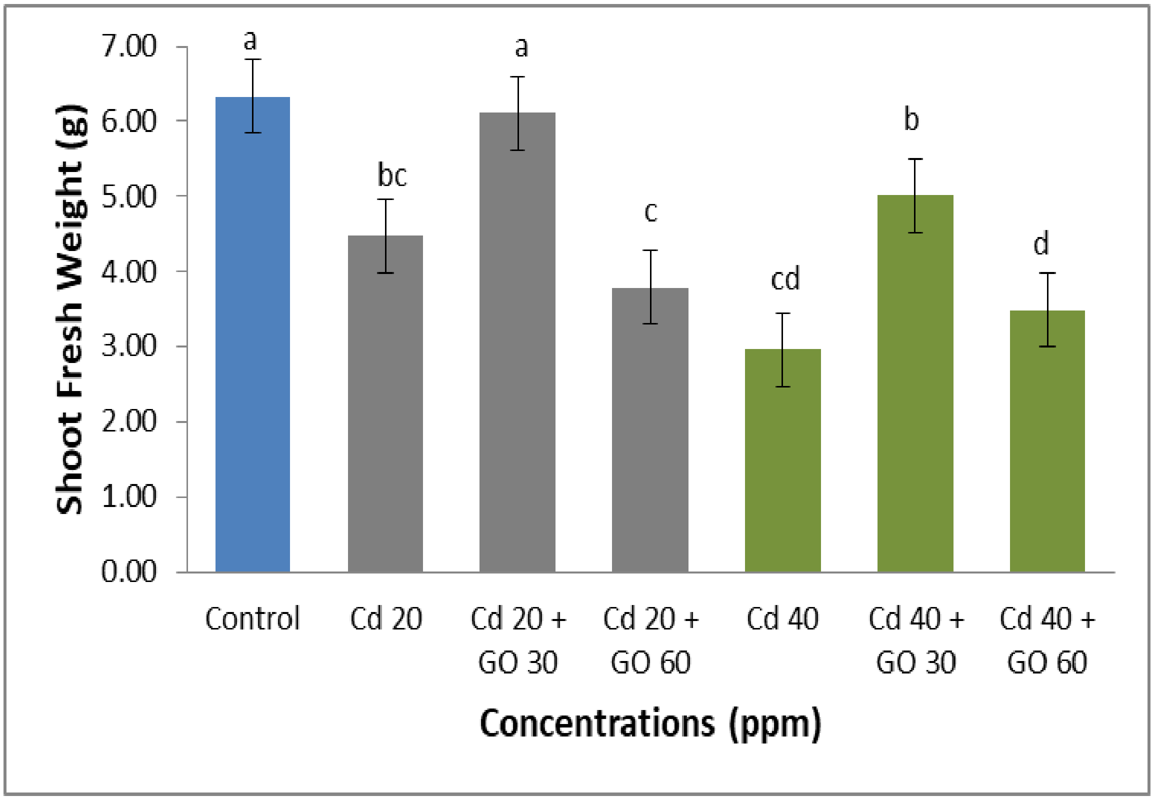


Figure 19: *Comparison of shoot fresh weight across control and various GO treatment groups.*

4.3.6 Shoot Dry Weight

For shoot dry weight, the same trend has been observed as that for shoot fresh weight as elaborated in Figure 21. It is noteworthy that the application of GO at lower concentrations tends to reduce the toxic impacts of Cd on shoot dry weight. The results of this study are in agreement with previous studies that demonstrated application of higher doses leads to further reduction in shoot dry biomass (Li et al. 2020; Mahmood et al. 2023; Rassaei 2024).

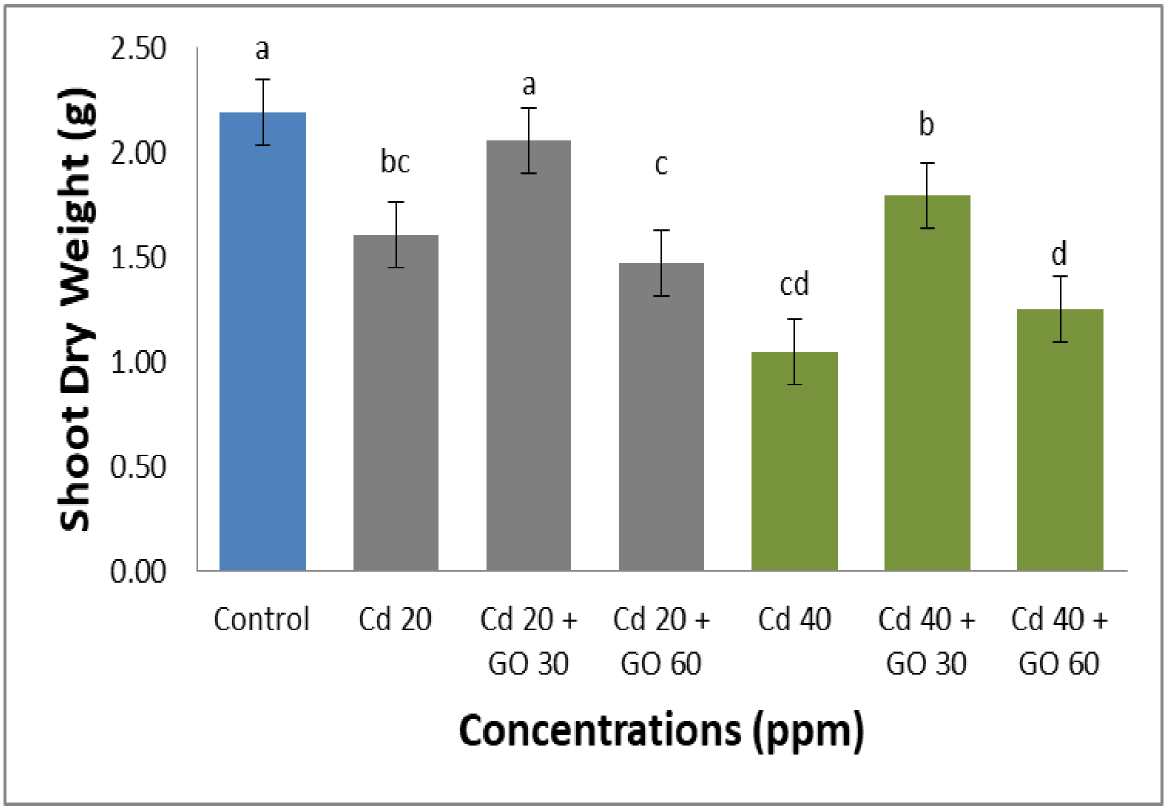


Figure 21: *Comparison of shoot dry weight across control and various GO treatment groups.*

4.4 Spike length

Under Cd stress, spike length of wheat plant is reduced as compared to control group that is elaborated in Figure 22. Cd toxicity causes reduction in division of meristematic cells due to which spike length ultimately decreases. However, GO application at 30 mg/L showed alleviation from stress that was evident from increased spike length. Rizwan et al. (2019) elaborated that nanoparticle application can alleviate metal stress from crop plants at suitable ranges. Hence, the results of this study align with previous research on the promotion of wheat spike length as nanoparticles strengthen plants (Iqbal et al. 2024; Reddy Pullagurala et al. 2018; Rossi et al. 2018).

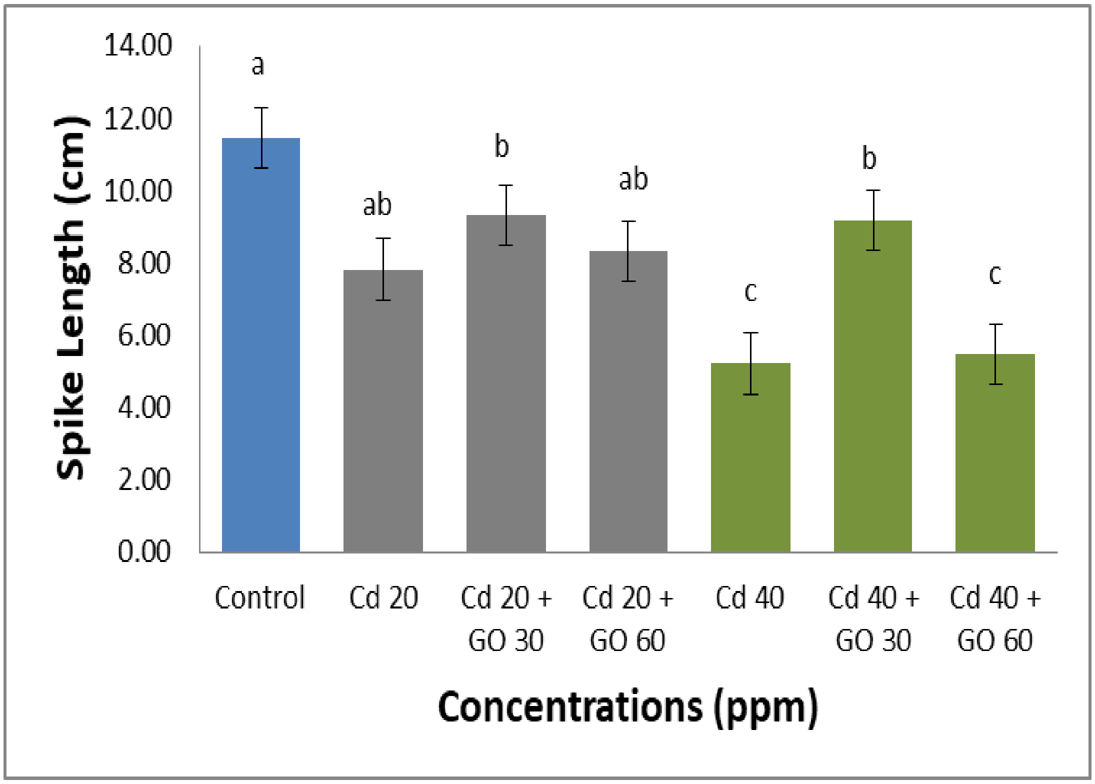


Figure 22: *Comparison of spike length across control and various GO treatment groups.*

4.5 Husk Dry Weight

Husk dry weight was also affected by different concentrations of Cd. In Figure 23, it is shown how the foliar spray of GO helped in alleviating heavy metal stress on husk dry weight. GO at 30 mg/L showed positive impacts while treatment with higher doses of nanoparticles showed negative effects under two different concentrations of Cd. The results are in line with recent studies that elaborated the toxic nature of nanoparticles at higher doses (Ahmadi et al. 2024; Li et al. 2024; Rassaei 2024).

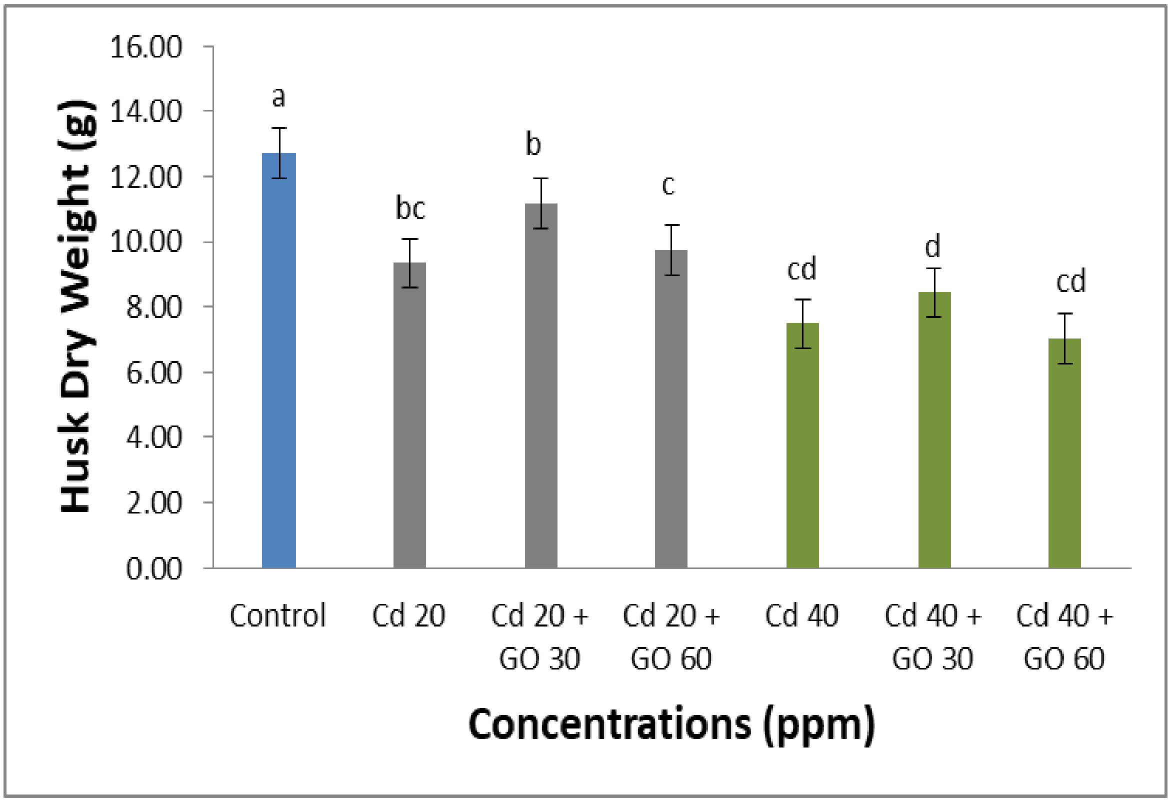


Figure 20: *Comparison of husk dry weight across control and various GO treatment groups*.

4.6 Grain Weight

Impacts of GO at 30 mg/L and 60 mg/L were studied on wheat crop under varying concentrations of Cd stress. It is resulted that the application of lower doses of GO caused enhancement in wheat grain weight as compared to higher doses of nanoparticles (Iqbal et al. 2024). Cd caused a reduction in plant's ability to uptake essential nutrients from soil that ultimately became the reason of the least grain weight. However, using advanced technology to alleviate heavy metal stress, it is revealed that GO at 30mg/L proves beneficial for increasing grain weight. On the other hand, higher doses of GO caused further reduction as evident from Figure 24. These observations are consistent with previous investigations in which foliar application of nanoparticles helps in alleviating plant stress (Affrald 2024; Ahmadi et al. 2024; Hussain et al. 2019b).

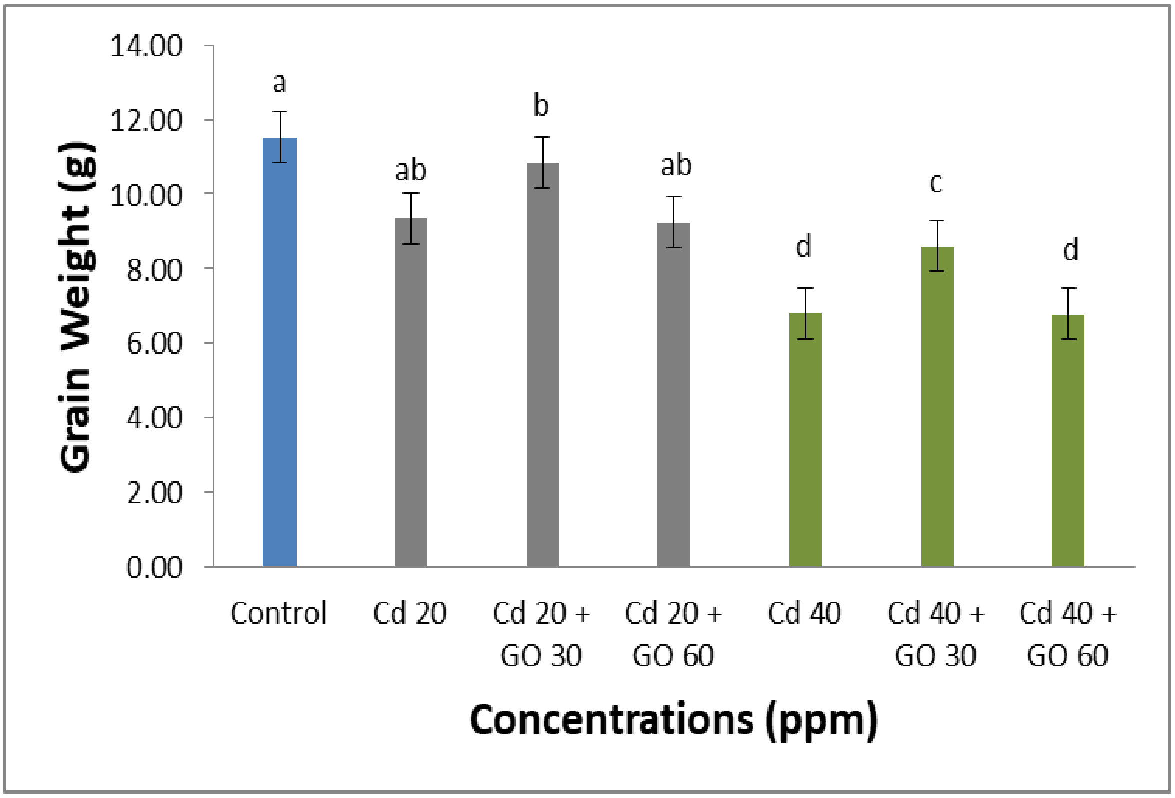


Figure 21: *Comparison of grain weight across control and various GO treatment groups.*

4.7 Chlorophyll Content

Photosynthetic activities are crucial for plant development and growth and these activities play a vital role in combating various stresses on a plant's intracellular structure (Alp-Turgut et al. 2024). Photosystem activities and the electron transport chain are severely shaped by the heavy metal stress conditions in the environment (Gao et al. 2020a). Through enhanced rate of photosynthesis, the biomass of plants could also be increased which yields higher gains of productivity (Zhou et al. 2023). It has been reported that Cd disturbs cell metabolism by affecting various essential organelles especially chloroplast disrupting major elements of chlorophyll (Al-Rawashdeh et al. 2020; Huang et al. 2023a; Mohamed et al. 2017).

In this research study, foliar spray of GO tends to enhance photosynthetic capacity on Cd stressed wheat plants. As GO was directly applied to wheat leaves, therefore it directly affected the photosynthetic activity of wheat plants. GO possesses the potential to directly flow into the plant's cell and reduce oxidative impairment posed by Cd stress (Affrald 2024). Results imply that GO foliar spray at 30 mg/L reduces adverse impacts of Cd on wheat plants. GO enhanced the ability of the wheat plant to capture more light energy as the nature of carbon-based nanoparticles is to increase the efficiency of chloroplast in plants (Baragaño et al. 2020). In crops, GO serves as an artificial antenna in order to capture the wavelength of light that plays a beneficial role in promoting light reaction in the chloroplast. GO application proves vital for enhancing the activity of Ribulose bisphosphate Carboxylase-Oxygenase (RuBisCO) that can be suppressed by the accumulation of Cd in plant cells. In this study, 30 mg/L GO application exhibits the raise in chlorophyll content of plants under Cd stress conditions. The main reason behind this rise is the exceptionally high surface area of GO that enhances the Cd absorption from cells, enhancing the efficiency of photosynthetic pigments (Ahmadi et al. 2024). However, GO at 60 mg/L caused further reduction in chlorophyll content in Cd stressed leaves showing the phytotoxic nature of nanoparticles at higher concentrations (Chen et al. 2018; Ghorbanpour et al. 2018).

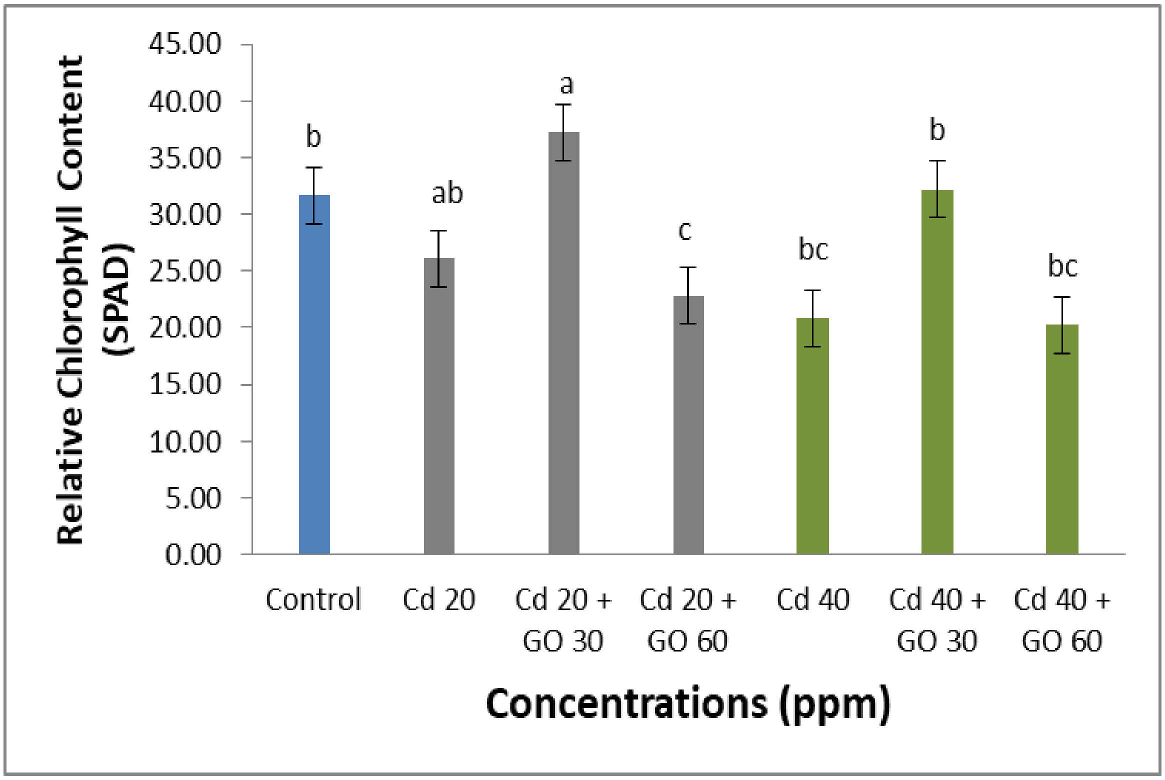


Figure 22: *Comparison of chlorophyll content across control and various GO treatment groups*.

4.8 Relative Water Content (RWC)

In the experiment, it was noted that the toxicity of Cd became the main reason in the decrease of water uptake by plants relative to the control group. In Figure 26, it is depicted that relative water content is decreased by 30% in Cd 20 mg/kg group while a reduction of 42% is observed in Cd 40 mg/kg group. Cd causes root ability to conduct water uptake besides damaging root cells (Iqbal et al. 2024; Nunes et al. 2023). This leads to an impairment in water uptake and transportation to vital organelles of plant cells. Moreover, Cd toxicity also impacts normal stomata conductance leading to irregular water losses. In addition, Cd also disrupts the functioning of vascular tissues especially the xylem that leads to disturbing water transport system in studied plants (Li et al. 2020; Mir et al. 2019).

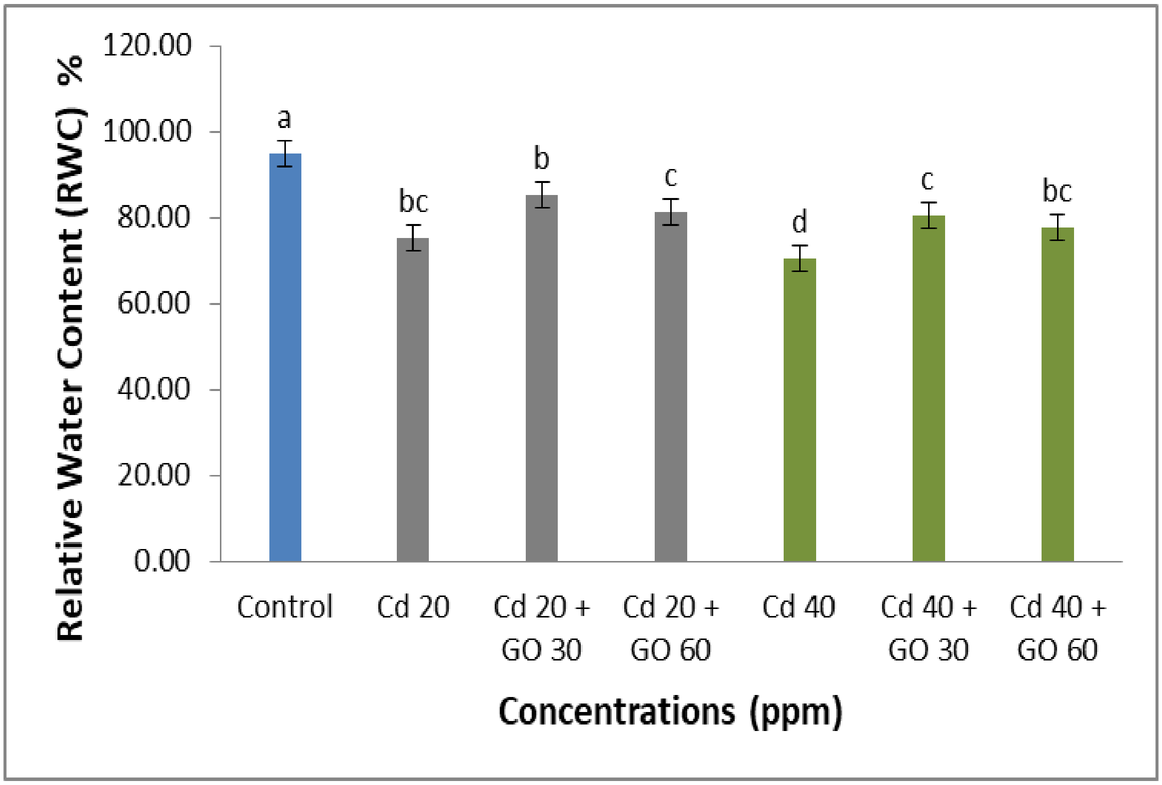


***Figure 26**: Comparison of relative water content across control and various GO treatment groups.*

However, the application of GO at 30 mg/L helped in increasing the relative water content of wheat plants under Cd stress. GO at this concentration helps in bringing back normal functions of plant's cells thus improving water uptake from roots. On the other hand, it was revealed that foliar spray of GO at 60 mg/L gave negative results in aspect of relative water uptake in wheat plants under stress. The results are aligned with previous findings in which it was elaborated that higher concentrations of GO caused decrease in RWC (Rassaei 2024).

4.9 Membrane Stability Index (MSI)

Cd negatively impacts the Membrane stability index of wheat plants because its toxicity induces harmful impacts on the cell membrane. It causes damage to cellular permeability that becomes the main reason in the loss of several significant ions for normal functioning of cells.

Through cellular leakage, wheat plants physiological activities are also disturbed due to high concentration of Cd. It is depicted from the experiment of the current study that Cd at 20 and 40 mg/kg concentrations resulted in a reduction of the membrane stability index of wheat plants up to 26 % and 32.1 % respectively. The recorded observations are consistent with literature that reports that heavy metals induce decrease in MSI among crop plants (Iqbal et al. 2024).

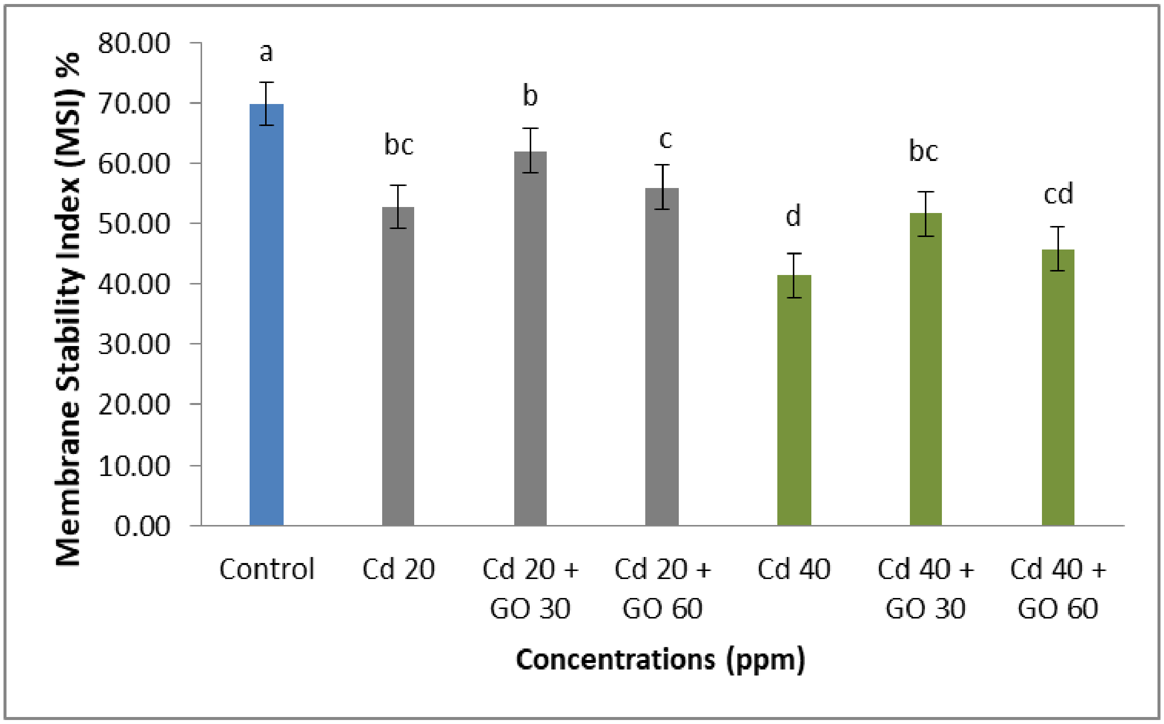


Figure 23: *Comparison of membrane stability index across control and various GO treatment groups.*

To alleviate heavy metal stress, a foliar spray of GO was employed on plants under Cd stress. GO at 30 mg/L was found to cause an increase in membrane stability index by reducing EC of wheat plants. GO at 30 mg/L played the role of barrier against Cd toxicity compared to controls. In contrast, GO at 60 mg/L caused further reduction as higher concentration of GO caused excess ROS in plant cells that became the main reason for MSI reduction. GO at higher concentrations proved to be phytotoxic which caused further cellular membrane damage. The results of this experiment are also aligned with previous findings that elaborated nano-toxicity nature of GO at higher concentrations on crop plant's metabolism (Mahmood et al. 2023).

4.10 Relative Oxygenated Species (ROS)

Heavy metals, specifically Cd, trigger an abrupt increase in ROS in crop plant cells. In wheat, Cd toxicity disturbs the normal functioning of chloroplast and mitochondria thereby damaging cellular metabolism. Cd is attributed as an external agent that acts as an inhibitor of antioxidant enzymatic activity that results in the accumulation of H_2O_2 in cells. This results in cellular injury that may lead to DNA damage if heavy metal concentration exceeds a certain limit. In this study, it is revealed that Cd caused a huge production of ROS as depicted in Figure 28. The production of H_20_2 increased in Cd 20 and Cd 40 mg/kg groups by 86% and 92% respectively. This huge overproduction is attributed to high toxic nature of Cd that results in cellular damage to wheat plant cells (Nunes et al. 2023). $O_2\bullet^{-}$ and $OH\bullet^{-}$ radicals were excessively generated that became the leading reason causing oxidative damage.

GO at 30 mg/L proved vital in lightning oxidative damage to wheat plants by significantly decreasing ROS levels in Cd-stressed plants relative to the control group. The findings demonstrated that nanoparticles at lower concentrations lessen ROS levels by following a potential mechanism by improving antioxidant enzymatic activity. While GO at 60 mg/L did not show any improvement in maintaining cellular activity. The observations of this experiment are well in line with previous research outcomes that showed nanoparticles at higher ranges prove phytotoxic agents (Qayyum et al. 2017; Rassaei 2024).

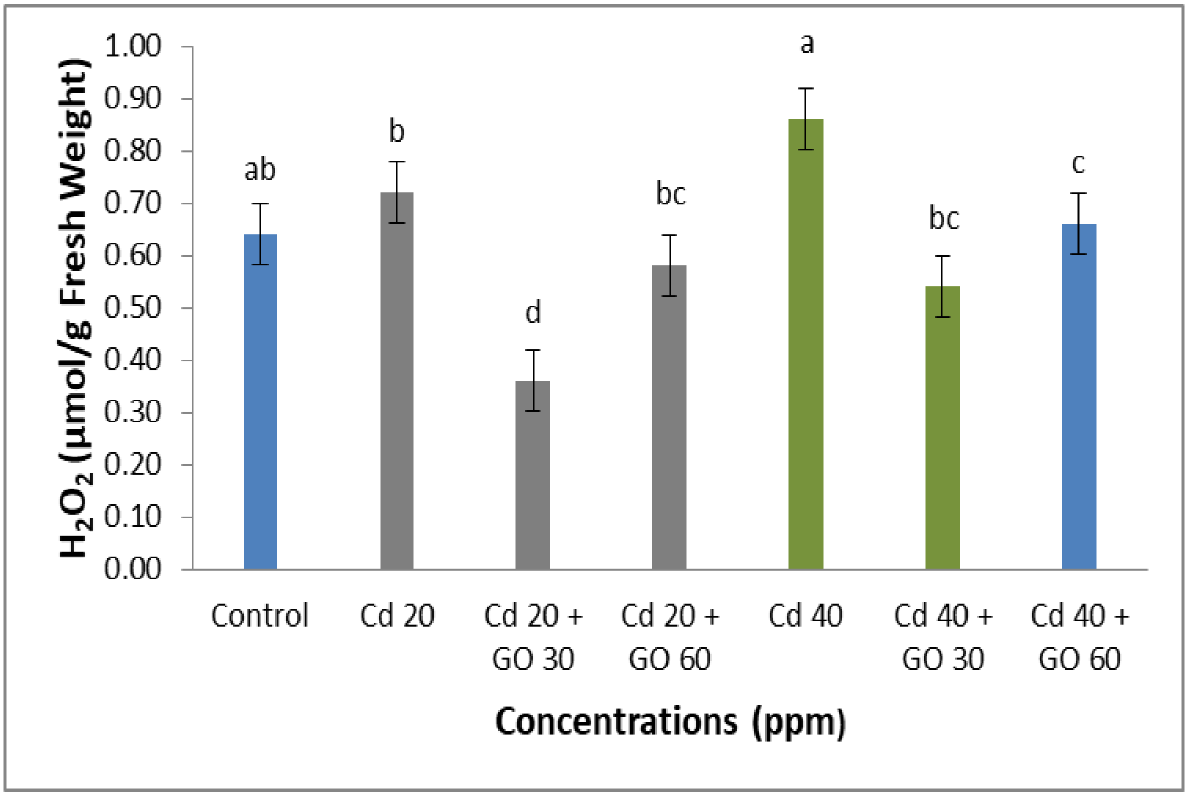


Figure 24: *Comparison of relative oxygenated species across control and various GO treatment groups.*

4.11 Antioxidant Enzymes Activity

In wheat, Cd toxicity activates intrinsic defense response to mitigate the hazardous impacts of heavy metal. SOD, CAT, and POD are major antioxidative enzymes that mainly manage ROS amount at the cellular scale. All these enzymes are primarily influenced by certain environmental stresses, including heavy metal toxicity. Heavy metals significantly damage the physiological attributes of plants that may result in least gains of productivity from crop plants. In the recent decade, it has been resulted from several studies that nanoparticles play an effective role in mitigating crop toxicity due to heavy metals (Nunes et al. 2023; Rassaei 2024). Nanoparticles play their defensive role by increasing the concentration of antioxidants leading to lessening oxidative damage. However, nanoparticles at certain ranges have proven to be advantageous for plants growth, while higher concentrations of NPs may prove harmful for crop plants. Activities of the three key antioxidants were studied in this research, with the findings presented as follows;

4.11.1 Superoxidase Dismutase (SOD)

SOD acts as the primary protective mechanism in mitigating the hazardous impacts of Cd. It is involved in the oxidative conversion of O^{2-} (superoxide anion) into H_2O_2 and O_2. In times of high Cd concentration, the SOD level escalated to neutralize oxidative damage by increasing the aforementioned conversion of superoxide anions. In the present experiment, SOD was significantly enhanced in Cd 20 and Cd 40 mg/kg groups by 44 % and 35% respectively. When foliar spray of GO at 30mg/L and 60 mg/L was conducted, SOD activity was further enhanced by 67% and 55% respectively. It was observed that GO spray at 60 mg/L did not show any significant improvement in SOD activity (Rassaei 2024).

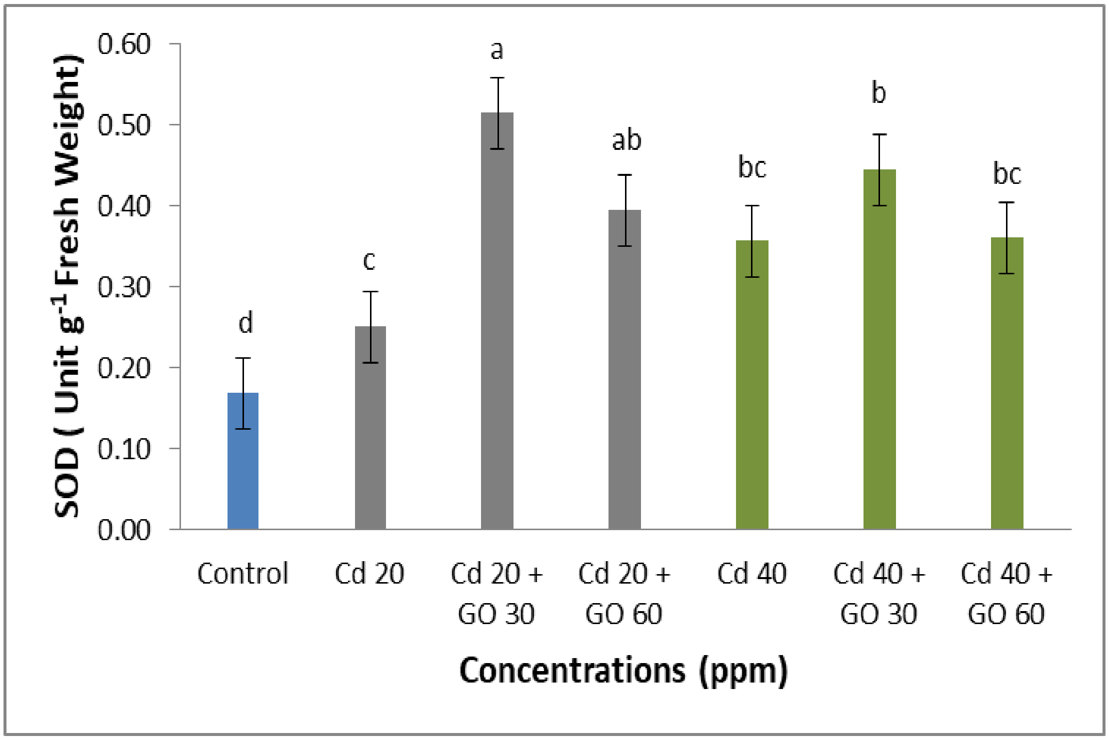


Figure 29**:** *Comparison of superoxidase dismutase (SOD) activity across control and various GO treatment groups*.

4.11.2 Catalase (CAT)

CAT enzyme plays a vital role in maintaining the homeostatic condition of plant cells under heavy metal stress conditions by increasing the reduction of H_2O_2 into water and oxygen. Catalase activity increases in order to reduce the accumulation of H_2O_2. Moreover, the application of GO at certain ranges aids in effectively mitigating oxidative damage under Cd stress at crop plants. In the present experiment, compared to the control, an increase in CAT activity was observed up to 37% and 25% in Cd 20 and Cd 40 mg/kg groups respectively. The findings are consistent with past research investigations, reporting enhanced levels of CAT activity under heavy metal stress among crop plants (Iqbal et al. 2024; Li et al. 2020; Mahmood et al. 2023). The foliar spray of GO at 30 mg/L further enhanced CAT activity among wheat plants that were in Cd stress conditions. While 60 mg/L GO showed no considerable improvement in CAT activity.

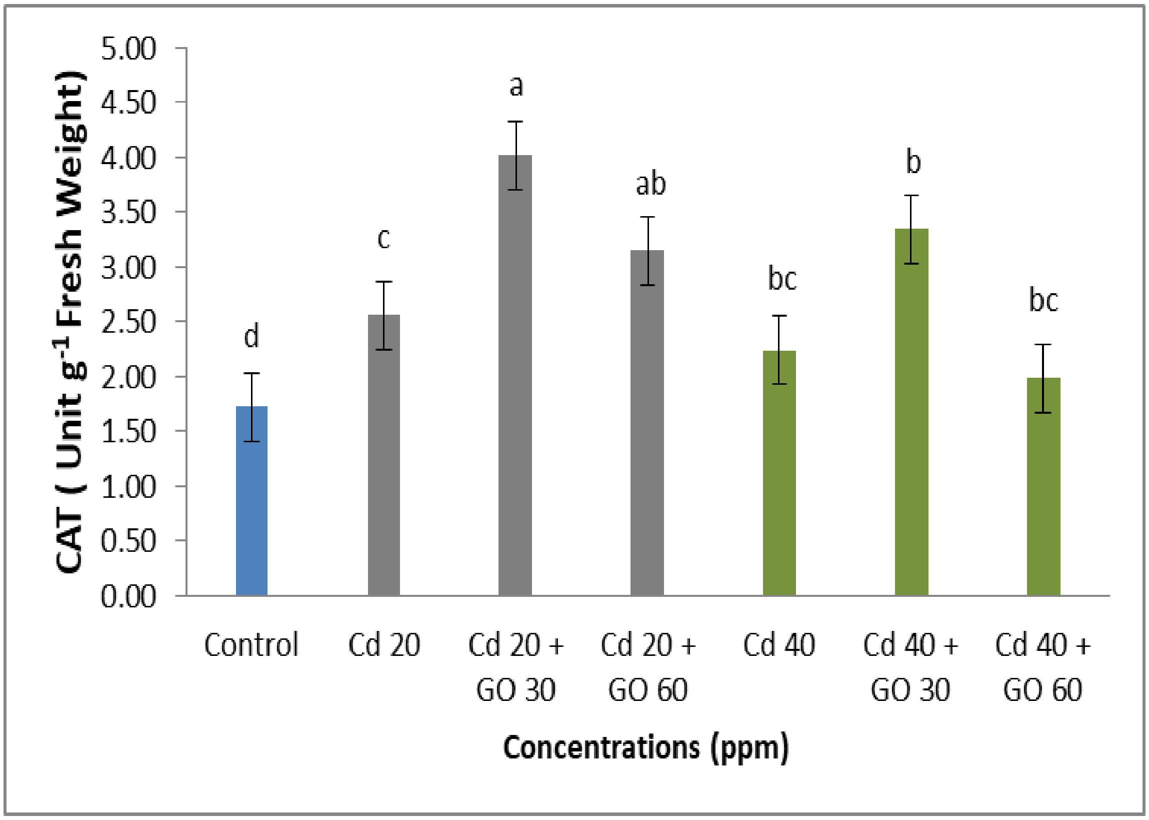


Figure 30: *Comparison of catalase (CAT) activity across control and various GO treatment groups*.

4.11.3 Peroxidase (POD)

POD plays its role in mitigating heavy metal stress by oxidizing substrates with the help of H_2O_2. Complete detoxification of H_2O_2 is achieved with the enhancement of POD activity. Similar to CAT, peroxidase activity can be increased by using a certain range of nanoparticles to combat heavy metal stress. It is depicted in Figure 31, POD activity compared to control was increased in Cd 20 and Cd 40 mg/kg groups by 33 % and 29% respectively. GO at 30 mg/L provided aid in the instant detoxification of H_2O_2 by oxidizing phenolic compounds. In this concentration, it was found that POD helped in protecting cellular metabolism from the hazardous impacts of Cd (Li et al. 2020; Mahmood et al. 2023). However, 60 mg/L concentrations of GO did not reveal any significant improvement in POD activity.

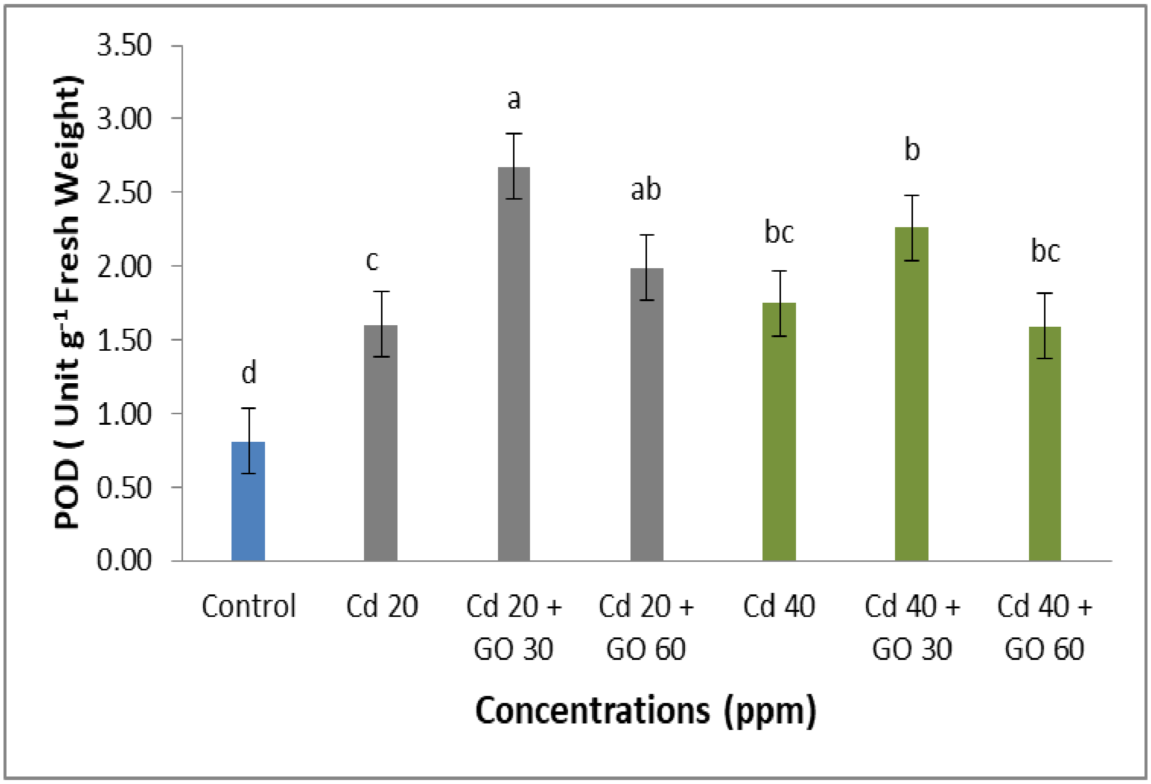


Figure 31: *Comparison of peroxidase (POD) activity across control and various GO treatment groups.*

Through the antioxidant enzymatic activities, it is revealed that GO has the potential to mitigate heavy metal stress as it acted as chelating agent with Cd owing to its superior surface area that helped in enhancing enzymatic activity (Li et al. 2020). Moreover, GO is reported to alter genes in a way that cells easily endure stress conditions through scavenging ROS. In addition, GO has the ability to enhance stomata conductance as well as nutrient uptake thus maintaining a suitable environment for plants.

However, effects of GO are totally dependent on its concentration suggesting its dose dependent nature. GO at low concentrations in this study demonstrated boosted antioxidant enzymatic activity thereby proving nanoparticles are beneficial for crop plants at lower ranges while at higher concentrations they act as synergetic agents along environmental pollutants to pose harmful impacts on primary producers of the ecosystem (Li et al. 2020; Mahmood et al. 2023).

4.12 Cd Concentration in Plant Tissues

Cd has the translocation ability from the rhizosphere to aerial parts of plants. Along with water uptake, it gains entry into plant's cells and tries to concentrate in various parts of plants. In the recent decade, it has been revealed that the application of certain nanoparticles helps in alleviating heavy metal stress by reducing the uptake of them from roots. In the present study, GO at different concentrations was foliar applied to Cd stressed wheat plants to evaluate the stress alleviation potential of GO.

4.12.1 Cd Uptake in Roots

Atomic absorption spectroscopy (AAS) was conducted on a wheat roots sample and the results of all treatment groups are elaborated in Figure 32. It was observed that GO at 30 mg/L caused reduction in heavy metal uptake up to 24 % and 31 % among Cd 20 and Cd 40 mg/g groups relative to control group. In contrast, Cd uptake was not significantly reduced in stressed wheat plants when they were treated with higher GO concentration that was 60 mg/L. The observations of this investigation are in agreement with previous researches, reporting higher ranges of NPs prove to phytotoxic agents (Huang et al. 2023a; Huang et al. 2023b; Mahmood et al. 2023; Saif et al. 2023).

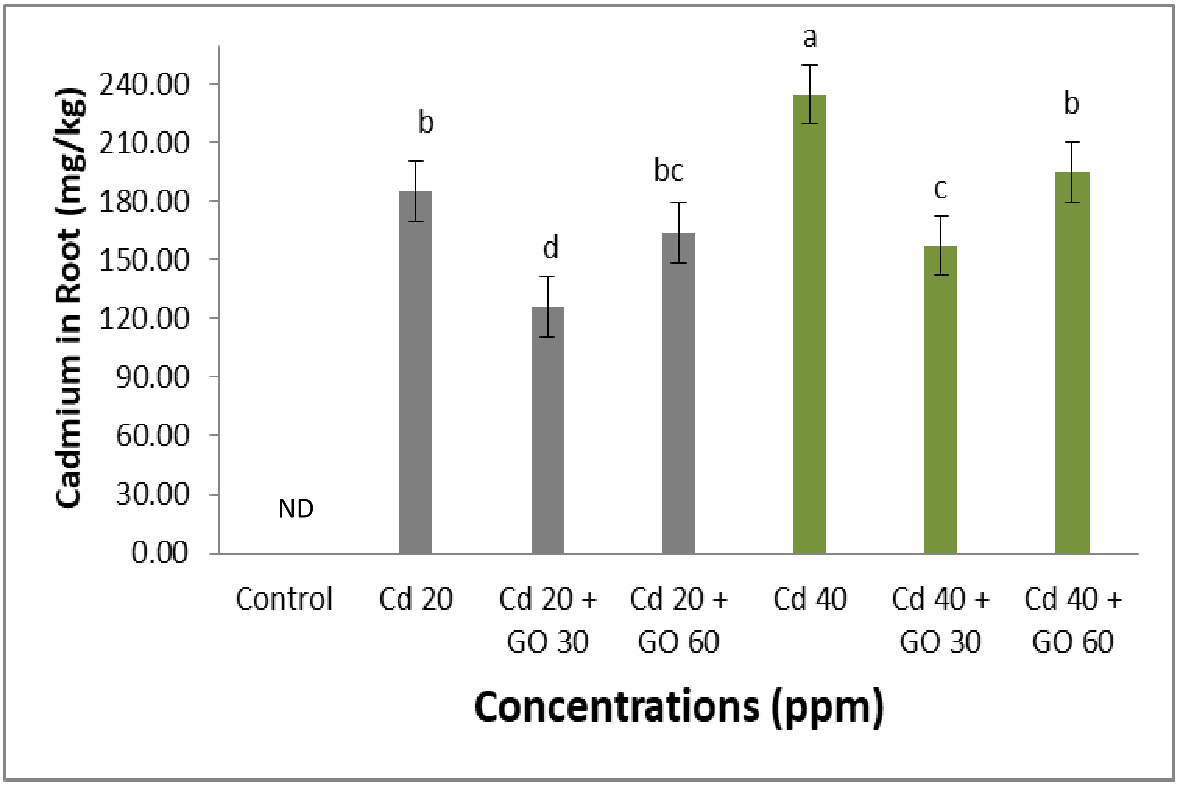


Figure 25**:** *Cd concentration in wheat roots under various treatments.*

4.12.2 Cd Uptake in Shoots

Cd can also be transported to shoots when its concentration is high in the plant's environment. To mitigate the toxic impacts of Cd on crop plants, foliar spray proved significantly important. Cd uptake in shoots and subsequent application of GO results are presented graphically in Figure 33. It is clearly illustrated that GO at 30 mg/L played a key role in decreasing Cd accumulation in shoots of wheat. Among the Cd 20 and Cd 40 groups, the percentage reduction of Cd accumulation was 54 % and 67 % respectively. GO has a large number of binding sites that chelate with Cd thus resulting in its decreased accumulation in aerial parts (Huang et al. 2023b; Saif et al. 2023). Conversely, Cd accumulation was found to be increased when GO at 60 mg/L was applied. This shows the damaging effects of nanoparticles at their higher ranges.

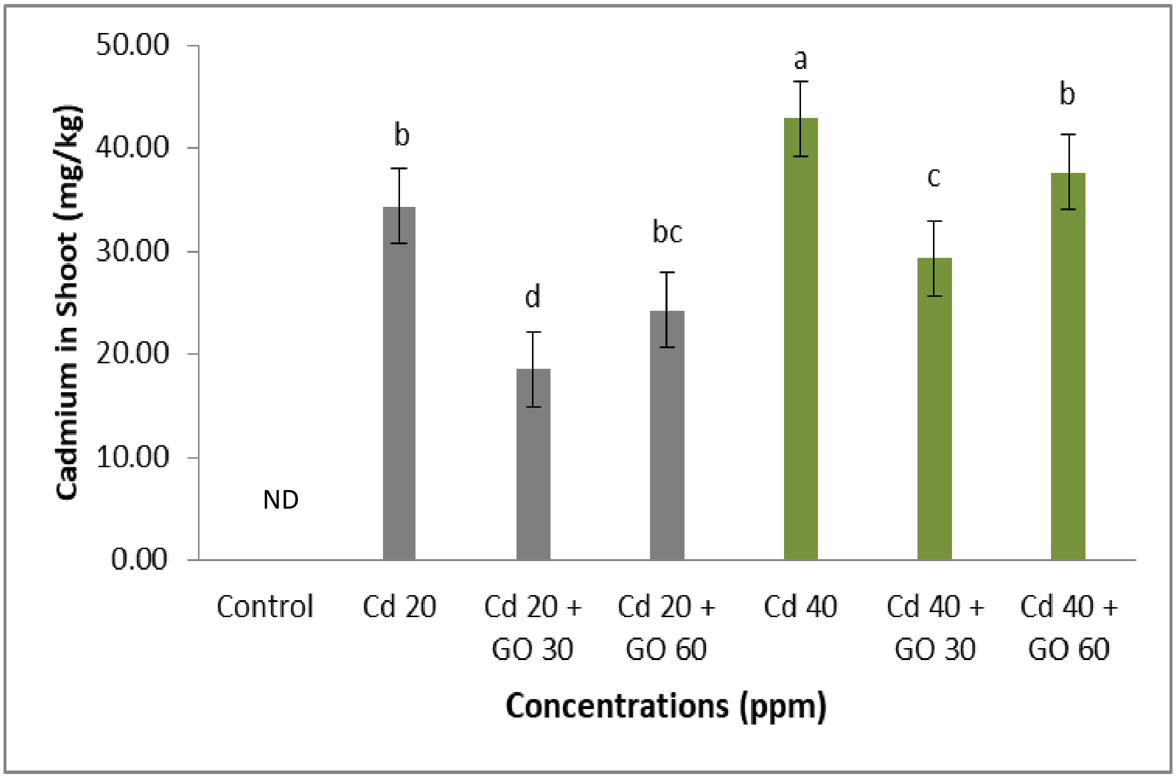


Figure 33: *Cd concentration in wheat roots under various treatments.*

4.12.3 Cd Uptake in grains

In cereal plants, heavy metal accumulation can lead to toxic impacts on human health. Cd has high solubility which causes its intense movement from soil to aerial parts of plants. Cd affects nutrient uptake by grains thereby affecting the productivity of crop plants. In recent decades, it has been found that the application of nanotechnology in agriculture helps in mitigating heavy metal stress. In this experiment, it is revealed that application of GO at 30 mg/L helped in decreasing Cd uptake in grains upto 36 % and 30 % in Cd 20 and Cd 40 mg/kg groups as compared to control. This demonstrated stress alleviating potential of GO at a lower concentration range. While it came to view that GO at 60 mg/L caused no significant improvement in mitigating Cd toxicity The results of this study concur with previous findings that nanoparticles at higher ranges are not suitable in the agricultural sector (Mahmood et al. 2023).

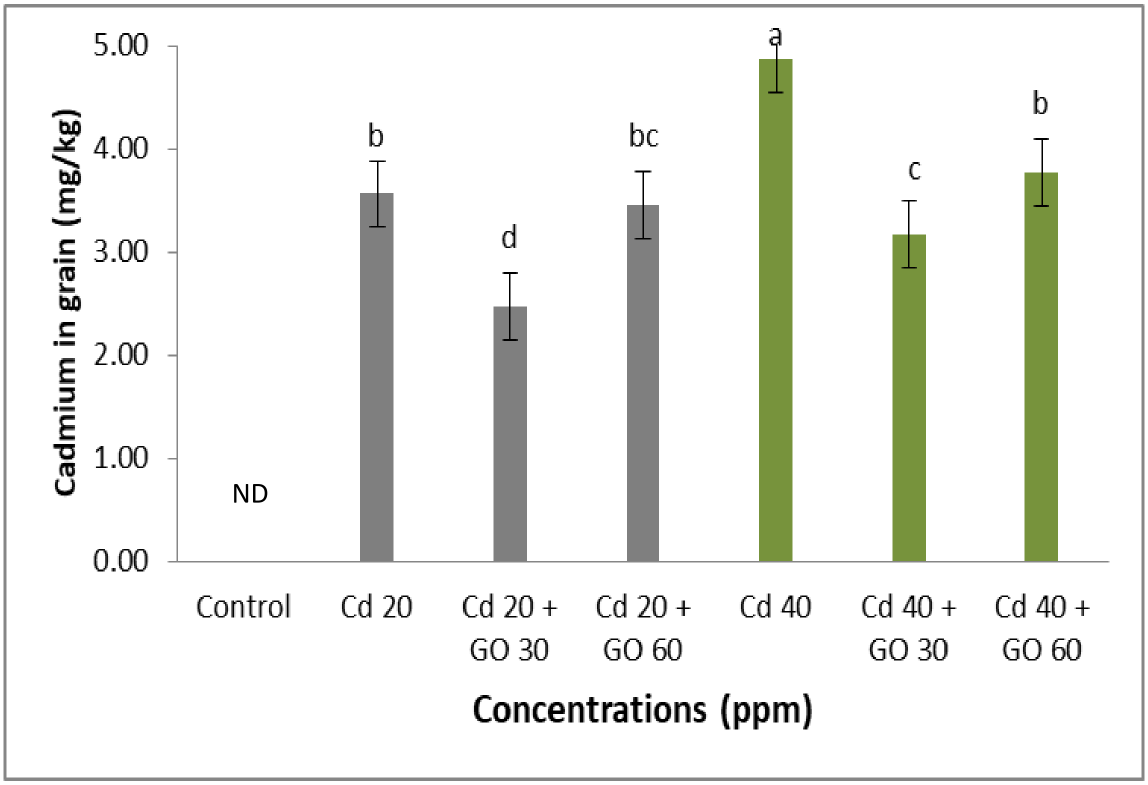


Figure 26**:** *Cd concentration in wheat grains under various treatments.*

From the above discussion, it is concluded that the structural composition of GO has helped in mitigating heavy metal stress from wheat plants. Due to the large surface of GO, it can easily bind Cd ions to it which results in removing Cd accumulation in plant tissues. Moreover, GO helps in nutrient uptake from the roots which prove crucial in maintaining a homeostatic environment. It also aids in repairing cellular damage that has occurred to plant cells due to heavy metal toxicity by altering gene expressions in a way that plants become resistant to further negative harms of heavy metals especially Cd. On the other hand, GO at higher doses prove phyto-toxic agent that may lead to further damage to crop plants. Thus, application of GO should be carried out in appropriate dosage.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

It is concluded from the present study that Cd has hazardous impacts on crop plants' health especially altering physiological and biochemical attributes. However, integrating nanotechnology in the agricultural sector leads to high productivity gains. Nanoparticles demonstrated the ability to mitigate heavy metal induced stress in plants when employed appropriately in suitable ranges. Preparation of specific nanoparticles is a complex and expensive process however using appropriate modified methods of preparation that are environmentally friendly is need of hour. GO preparation has the least effects on the environment as it does not contributed any negative change in air composition and modified Hummer’s method provides cheap as well as economical synthesis and ready to use application. In the present experiment, GO was prepared using Hummer’s method and foliar application of required doses proved vital for extracting valuable information about the nature of NPs and heavy metals. In this investigation, GO at 30 mg/L has proven an optimal dose for nanoparticle-based treatment in wheat plants under Cd stress. GO has alleviated the impacts of Cd by inducing enhanced activity of antioxidant enzymes and reducing the concentration of Cd in plant tissues. Conversely, 60 mg/L GO exhibited negative impacts on physiological parameters (wheat plant’s fresh and dry weight) that led to the least productivity. This study revealed that higher concentrations of GO have negative impacts while lower doses promote growth (Grain weight, root, and shoot biomass) exhibiting dose-dependent nature of nanoparticles. In conclusion, GO nanoparticle mode of application and concentration ranges hold significant importance on the fate of the ecosystem.

5.2 Recommendations

Future studies are required to demonstrate the complete life cycle of plants treated with nanoparticles under heavy metal stress in field conditions. Additionally, research investigations are imperative to explore variations of nanoparticles application modes, ultimately impacting plants in real-world environments subjected to heavy metal stress. Investigation on the fate of nanoparticles in the environment is required in order to determine their suitable dosage concentrations and their toxicity nature. Additionally, the mechanism through which GO alleviates heavy metal contamination is not yet fully elucidated. It is critical to understand the comprehensive pathway of nanoparticles treatment to ensure its safe and effective usage in the agriculture sector. For practical implementation of nanoparticles on crop plants, there is a need to study their whole life cycle. Future advancement of nanoparticles should be taken by educating farmers about their suitable usage in fields. There is a need to conduct comprehensive field-based research on the usage of nanoparticles for the health and safety of the environment. Last but not least, nanoparticle synthesis and subsequent appropriate mode of application is an expensive process. It is the need of the hour to explore new pathways of their cost-effective synthesis that may lead to economic benefits for developing countries. If the gap between the usage of advanced technology and traditional activities in the agricultural sector is lessened, then a country can easily achieve sustainable development goals.

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