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EVALUATION OF CALCIUM OXIDE NANOPARTICLES TO ENHANCE HEAVY METAL STRESS TOLERANCE IN PLANTS

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Abstract: Heavy metal contamination is a severe environmental problem affecting global food production and safety. Heavy metal stress due to its toxicity, bioaccumulation, and non-biodegradability, it become quite serious in nature. The available strategies for preventing heavy metal contamination are not frequently used because of their inefficient and time- or money-consuming properties. Recent developments in nanotechnology have been made based on ameliorative strategies which have a potential alternative to physic-chemical methods. Under heavy metal stress, the application of calcium oxide nanoparticles (CaO-NPs) significantly boosts plant biomass, anti-oxidative enzyme activities (such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and glutathione reductase (GR)), and the level of non-enzymatic antioxidants (ascorbate and glutathione). Additionally, CaO-NPs enhance the gene expression linked to anti-oxidative enzymes. It can be suggested that CaO-NPs could be used as a potential chemical to reduce heavy metal uptake and toxicity in the plants grown under heavy metal contaminated soil. This review provides an overview of plant-CaO-NPs research in increasing heavy metal stress tolerance in plants.

Keywords: Heavy metal, abiotic stress, calcium oxide, plant, tolerance

1. Introduction

Globally, abiotic stresses such as drought, salt, extremely high temperatures, and heavy metal stress are reducing crop productivity. Furthermore, extreme and/or prolonged abiotic stresses have the potential to cause the death of individual cells and perhaps the entire plant, as well as completely jeopardizing the yield (He et al., 2018; You and Chan, 2015; Choudhury et al., 2013). Reactive oxygen species (ROS) induced by abiotic stresses cause programmed cell death in various plant species (Petrov et al., 2015). The regulation of growth, developmental processes, and stress adaptation are all influenced by ROS (Mhamdi and Van, 2018; Mittler et al., 2011; Gechev et al., 2006). Abiotic stresses cause plant damage, cell death, and growth of plants, which have significant negative impact on food yields globally. Abiotic stresses at extreme level also leads to oxidative stress.

Moreover, heavy metal (HM) contamination is frequently found in food and blood, it has recently gained public attention. HM stress has an impact on plant growth and, indirectly on human health through the food chain (Wang et al., 2019). One of the main elements limiting crop output and jeopardizing food security is HM pollution(Ogden et al., 2020; Dong et al., 2020). In order to limit crop production, HM primarily affects normal structure of cell, the antioxidant system, as well as plant growth. More significantly, it is predicted that by 2030 and 2050, the world's population would have increased to 8.54 billion and 9.73 billion, respectively, necessitating a 70–100% increase in present food production (Li et al., 2020; Guo et al., 2019). Therefore, HM removal/immobilization technology needs to be improved in polluted fields.

Since the last few years, nanotechnology has emerged as an extremely powerful discipline that is revolutionizing a wide range of fields such as medicine, agriculture, industrial, environmental, and electronics. Nanotechnology is a new discipline of science that deals with nanoparticles and how they are synthesized (Ziauddin et al., 2014). As a means to overcome nutritional poverty and food scarcity, nanotechnology is emerging as a tool for agriculture. NPs typically refer to substances that have at least one dimension less than 100 nm. According to the requirements, NPs with various particle sizes, geometries, and functionalities can be produced (Adeel et al., 2020). NPs have a number of benefits over conventional materials, including high surface activity, an increased number of surface reaction sites, strong catalytic efficiency, and special optical and magnetic properties (Wang et al., 2019; Yang et al., 2018; Yang et al., 2017). NPs are also used as nanofertilizers (Rui et al., 2016; Rui et al., 2018; Li et al., 2020) and nanopesticides (Adeel et al., 2021; Hao et al., 2019; Zhao et al., 2018; Hao et al., 2018), which have the advantage of being easily absorbed by plants and slowly released in the environment compared to traditional fertilizers (Lowry et al., 2019). Therefore, through nanotechnology, we can improve crop productivity, minimize losses and enhance yields in the future, enabling sustainability, crop productivity, and overcoming abiotic stresses (Tariq et al., 2020). NPs (such as CaO NPs, CeO2, and TiO2 NPs) can boost antioxidant enzyme activity, which can lower the increased level of reactive oxygen species (ROS) in plants, reducing plant stress and thus, enhance quality and production of plants (Nazir et al., 2022a, b; Wang et al., 2020; Usman et al., 2020).

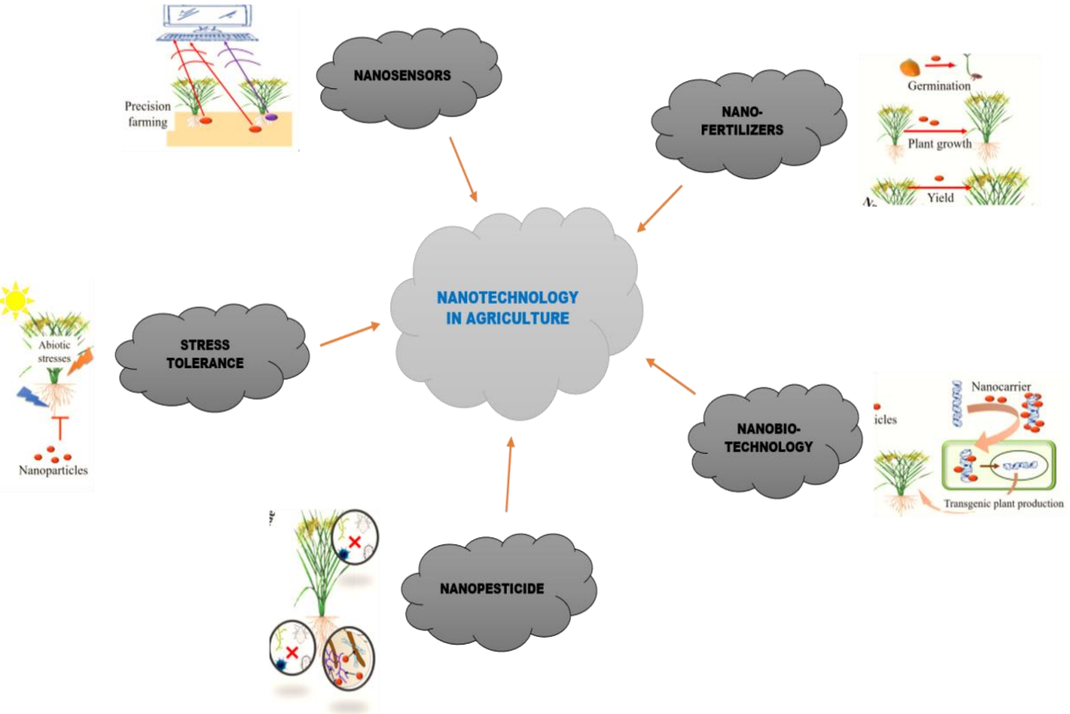

Figure 1. Role of nanotechnology in Agriculture

Calcium (Ca) is crucial for the development of plant tissues and for the improvement of plant growth. The calcium in plants helps to hold plants cell walls together. Additionally, it is essential in activating various enzymes to coordinate with several cellular activities. It is also essential for the regulation of growth of the root system, and also enhance defense against outside attack, as well as increase the value of forage crops as feed for cattle (White and Broadley, 2003). Calcium oxide nanoparticles, have an advantageous impact on plant growth, plant biomass, anti-oxidative enzyme activities (such as APX, GR), and the content of non-enzymatic antioxidants (such as ascorbate and glutathione), along with a considerable decrease in the levels of malondialdehyde (MDA) and hydrogen peroxide. It also has various essential properties such as adsorption, antibacterial properties, catalysis, and absorption (Gandhi et al., 2021). However, due to their superior efficacy in eliminating heavy metals, micronutrient-based nano-fertilizers have recently attracted a lot of interest in the agriculture sector (Nazir et al., 2022b). It is widely known that micronutrient-based NPs, such as calcium oxide NPs, promote seed germination and plant growth by triggering oxidative defense mechanisms and preserving ionic homeostasis. Furthermore, due to their distinct qualities and eco-safe characteristics, calcium oxide (CaO) nanoparticles are increasingly being used as a method of choice for environmental remediation. According to Davarpanah et al. (2018), CaO NPs have a positive effect on plants. Recently, Nazir et al. (2022a, b) also demonstrated the positive role of CaO-NPs in alleviating heavy metal toxicity in plants.

In this review, we focus on the new strategies and different responses seen in plants under HMs stress along with the positive role of calcium oxide nanoparticles in enhancing heavy metal stress tolerance in plants.

2. Plant responses to heavy metal stress

Heavy metal stress is increasing throughout the world due to anthropogenic, technological, and geogenic activities. Reactive oxygen species, which obstruct the majority of cellular functions at different levels of metabolism, are produced as a result of HMs exposure, and these ROS may cause a variety of harmful effects in plants. Due to their extreme instability, ROS have the potential to damage cellular components as well as serve as an essential secondary messenger for activating the plant defense system. To prevent this harm, cells have both enzymatic and non-enzymatic defense mechanisms. Some are constitutive, whereas others are only engaged in response to a perceived stress-related signal. Further, SOD, CAT,

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and GR are enzymatic scavengers of ROS, whereas anthocyanins, glutathione, ascorbic acid, carotenoids, α-tocopherol, organic acids and flavonoids are non-enzymatic antioxidants. Organic acids like citric, malic, and oxalic acid, among others, are linked to the intracellular and extracellular chelation processes of HMs. Metal complexation with glutathione, amino acids, the production of phytochelatins, and sequestration in vacuoles are all crucial components of the detoxification process. Excessive stress results in a cascade, the MAPK (mitogen-activated protein kinase) pathway, and the production of ligands that detoxify metals (Sytar et al., 2013).

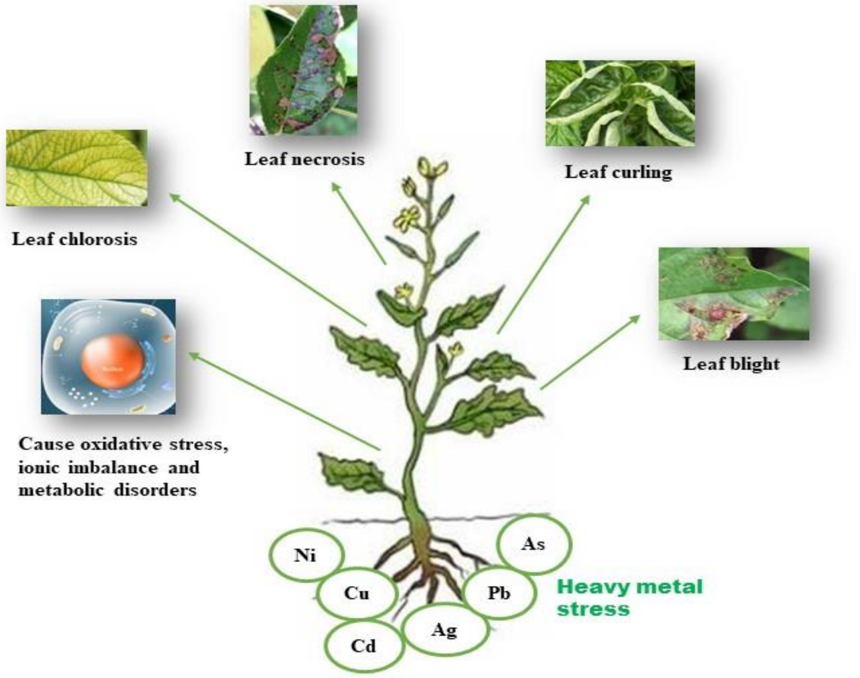

Figure 2: Heavy metal (HMs) stress induced toxicity in plants

2.1 Heavy metal uptake in plants

Due to the sessile nature of plants, they are unable to migrate from one location to another to escape from various environmental stresses that may affect their productivity, growth, or development due to changes in the innermost concentrations of bio-reactive metals (Chatterjee et al., 2011; Schützendübel and Polle, 2002). Further, plants absorb various micronutrients from the surrounding terrestrial or aquatic environments through their root systems, including zinc (Zn), iron (Fe), copper (Cu), and other elements. These sources might also contain low to high concentrations of non-essential elements like lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As). In order to combat heavy metal toxicity, plants use a variety of strategies, such as compartmentalizing the metal ions, immobilizing them, excluding them, and chelating them (Cobbett, 2000).

Various plants have extraordinary capability to survive in toxic environments in heavy metal-contaminated areas and have also been proven to acquire a significant amount of such metals within their biomass (hyperaccumulators). For instance, various reports demonstrated that naturally occurring hyperaccumulators, viz., the nickel hyperaccumulating species *Thlaspi caerulescens* (Freeman et al., 2004), and the arsenic hyperaccumulating fern species *Pteris vittata* (Gumaelius et al., 2004), may tolerate high levels of metal accumulation without suffering serious harm to their internal organs. Plants are the only organisms that can physically transfer nutrients like Ca, Mg, Co, and Mn. Moreover, through occupying transmembrane nutrient transporters, cadmium competes with these necessary nutrients during transportation (Papoyan and Kochian, 2004; Curie et al., 2000; Thomine et al., 2000; Clemens et al., 1998). Cadmium enters into the roots via cortical tissue and also presumably accumulated in the roots. However, it travels to the xylem via a symplastic and/or apoplastic pathway, for further transport to shoots, and may be complexed by a number of ligands including organic acids and/or phytochelatins (PCs) (Salt et al., 1995; Cataldo and Wildung, 1983). When Cd enters the root, it damages cells, particularly nucleoli, and impairs a number of enzymatic reactions like nitrate reductase (NR) and ribonuclease activity (Hernandez et al., 1997; Shah and Dubey, 1995). Cadmium damages the light harvesting complex II, & photosystems I and II, and increases non-photochemical quenching, which has an impact on photosynthesis which leads to Fe(II) deficiency in plant shoot tissues (Larsson et al., 1998; Alcantara et al., 1994; Siedlecka and Krupa, 1996; Krupa, 1988). Even at low concentrations, heavy metals are harmful to plant cells and does not appear to have any biologically beneficial effects. For example, according to various reports, most plants are poisoned by Cd concentrations of 5 to 10 mg per gram of dry leaf mass (Lux et al., 2011; White and Brown, 2010). There are reports, though, that some genera of plants' ecotype roots spread widely in Cd-enriched soils (Liu et al., 2010).

Additionally, these plants have a protective mechanism that limits Cd entrance to the xylem and inhibits metal accumulation in shoot tissues, particularly through the formation of Cd-chelators at the root zone (Lux et al., 2011). It is indicated that the cells are exposed to different metals and metalloids including Cu^{2+}, As^{2+}, and Ag^{+} can also synthesize chelators like PC (Tennstedt et al., 2009; Inouhe, 2005; Cobbet, 2000; Gekeler et al., 1989). Cadmium content in plant shoots vary greatly in nature, for which phylogenetic diversity and environmental factors are responsible (Watanabe et al., 2007). Moreover, caryophyllales and lamiales plants acquire Cd at substantially higher rates in shoots than other species (Broadley et al., 2001). However, Cd concentrations are often lower in seeds, fruits, and tubers and higher in roots than in shoots, indicating that most plants have limited ability to transfer Cd to the xylem and phloem (Lux et al., 2011; Conn and Gilliham, 2010; Seregin and Kozhevnikova, 2008). The most crucial aspect of a plant's strategy is the selection and optimal uptake of heavy metals, which are necessary for growth, and rejection of those elements that are not beneficial (Perales-Vela et al., 2006; Cobbett and Goldsbrough, 2002). Heavy metal stress in plants involves a complex system of signal transduction, which is a two-step process, where the activation process starts by sensing the heavy metals. Reduced availability of essential nutrients will reduce plant's vigor and ability to withstand heavy metal stress (Huang et al., 2008). Once the plant detects the presence of metals, proteins and stress-related signaling molecules, then it results in the explicit activation of metal-responsive genes to combat the toxicity induced by heavy metal stress (Maksymiec, 2007). Consequently, the development of metal-specific legands (chelation) and subsequent compartmentalization of the ligand-metal complexes in the cells can be the typical defense mechanisms for heavy metal detoxification in plants and other organisms (Cobbett, 2000).

2.2 Heavy metal detoxification mechanism in plants

Further, once a heavy metal enters the plant cell, mechanisms for its sequestration into the vacuole are activated so that it can be taken out of the active cellular compartments and the cytosol, where sensitive metabolic processes occur (Hossain et al., 2012; Dalcorso et al., 2010). As a result, a major vacuole in plant cells appears to be a reliable location for accumulating heavy metals. According to various reports, the vacuolar proton pumps, particularly the vacuolar proton ATPase (V-ATPase) and the vacuolar proton pyrophosphatase (V-PPase), aid in the vacuolar uptake of the majority of solutes. A few well-known heavy metal transporter proteins include the iron-regulated transporter (IRT)-like protein ZIP family, ATP-binding cassette (ABC) transporters, P-type metal ATPases, mitochondrial ABC transporters (ATM), copper transporter (COPT) family members, multidrug resistance-associated proteins (MRP), and cation diffusion facilitator (CDF). Isolation of vacuole or its compartmental flux studies on HMs accumulation, specifically on Cd-exposed tobacco seedlings (Nicotiana rustica var Pavonii), revealed vacuoles that held nearly all of the Cd-binding peptides and Cd observed in protoplasts (Huang et al., 2012; Vögeli-Lange and Wagner, 1990). In plants, PC-metal complex is sequestered at the vacuole. Phytochelatins, particularly in response to heavy metals viz., cadmium and arsenic, involve the accumulation of metal-complexes in the vacuole by producing high molecular weight (HMW) compound after incorporation of sulfur (S2-) (Salt et al., 1998). PC-Cd complexes are transported into the vacuole through ATP-dependent ABC transporters and Cd/ H+ antiporters in the tonoplast (Salt and Rauser, 1995; Salt and Wagner, 1993).

Moreover, Ortiz et al. (1992) discovered that a Cd-sensitive mutant of *Schizo-saccharomyces pombe* can synthesize PCs but not accumulate the Cd-PC-sulfide complexes. Another significant action of PCs is the movement of Cd from root to shoot. When wheat gene TaPCS1 was expressed in transgenic *Arabidopsis*, it improved the effectiveness of Cd transport from the root to the shoot (Gong et al., 2003). PCs chelate Cd^{2+} more efficiently than glutathione (GSH) molecules, and PCs and GSH complexes may both be reabsorbed into vacuoles (Huang et al., 2012; Pal and Rai, 2010; Li et al., 1996; Howden et al., 1995; Kneer and Zenk, 1992). Research on Cd-sensitive *S. pombe* mutants has provided the most convincing evidence of this pathway. Ortiz et al. (1992, 1995) first demonstrate hmt1, is a Cd^{2+}-sensitive mutant of S. pombe that prevents the accumulation of vacuolar PC-Cd. HMT1 is an ATP-binding cassette (ABC) transporter that can detect PCs and PC-Cd. Similar to this, YCF1, a full-molecule ABC transporter that aids in the sequestration of a GS2-Cd complex into vacuoles, was found in a Cd^{2+} sensitive mutant of *Saccharomyces cerevisiae* (Huang et al., 2012; Li et al., 1996, 1997). Further, in overexpressed *S. pombe* mutants that lack PCs for substrate, Preveral et al. (2009) described the role of SpHMT1 for the transport of GS2-Cd conjugates.

Higher plants recently revealed two ABCC subfamily members of ABC transporters, which enhances vacuolar PC-As (III) trafficking (Song et al., 2010). Again, a significant decrease in vacuolar Cd^{2+} was seen in the atABCC1 atABCC2 mutant, indicating that AtABCC1 and AtABCC2 are important for vacuolar Cd^{2+} sequestration (Huang et al., 2012; Park et al., 2012). Moreover, PC-deficient mutant cad1-3 has showed no influence on this process, indicating the SpHMT1 function that needs PCs. Ectopically produced SpHMT1 in *Arabidopsis* has demonstrated that PCs play a fundamental role in vacuolar Cd^{2+} sequestration (Huang et al., 2012).

2.2.1 Metal binding ligand’s essential role in plants

The optimal concentration of essential bio-metals is maintained in plants by metal-binding ligands, which also lower the toxicity thresholds for non-essential metals. There are now known metal-binding ligands that enable plants to survive under harsh conditions (Callahan et al., 2006; Rauser, 1999). For instance, malate & citrate, which aid in the extracellular chelation of aluminum (Al), are known to be connected with tolerance to Al in plants and are also visible in Al-resistant mutants of *Arabidopsis* (Delhaize and Ryan, 1995). Additionally, amino acids like histidine (His) support the chelation of metal ions in cells and xylem sap (Rauser, 1999). The most prevalent peptide ligands are phytochelatins (PCs) and metallothioneins (MTs). These cysteine-rich polypeptides offer thiols to bind various metal types, aiding in cellular metal homeostasis and detoxification (Inouhe et al., 2012; Cobett, 2000; Rauser, 1995). To counteract the harmful effects of non-essential heavy metals like As (arsenic) or Cd (cadmium), however, PC production is likely one of the most complex enzyme-catalyzed defense mechanisms known to protect plants (Rea, 2012).

2.2.2 Phytochelatins (PCs) role in oxidative stress induced by heavy metals and its detoxification mechanism

Plants respond to HMs stress by chelation and subsequent ion sequestration mechanisms. Phytochelatins are thought to be a part of the mechanism in higher plants that detoxifies heavy metals because immobilized metals are less toxic than free ions (Cobbett and Goldsbrough, 2002). Such metal-binding peptides appearing in plants may be significant biochemical indication of heavy metal contamination under several environmental conditions (Gupta et al., 2002a, b). According to various reports, plants may detoxify a variety of metal ions, including Cd, As, Hg, Cu, Zn, Ag, & Ni, by forming PC-metal complexes (Manara, 2012; Ha et al., 1999; Mehra et al., 1996; Rauser, 1999; Maitani et al., 1996). With isolated cad1 mutants, the stress response of plants was examined for various heavy metals with regard to the function of phytochelatins. The cad1-3 mutant of *A. thaliana* was shown to be more sensitive to Cd and arsenate than wild-type plants (Ha et al., 1999). Using the PCs (PCS-deficient) mutant of cad1-3, *S. pombe* was found to be only moderately sensitive to Cu and Hg, and intermediately sensitive to Ag (Manara, 2012; Ha et al., 1999; Maitani et al., 1996). Several investigations show that Cu activates PC production both in vivo and in vitro. Mutants lacking PC exhibited relatively reduced Cu sensitivity. Salt et al. (1989), illustrated that the copper-tolerant plant *Mimulus guttatus*, corroborated the function of PCs in Cu tolerance. In contrast, both the Cu-tolerant and Cu-sensitive ecotypes of *Silene vulgaris* produced roughly the same amount of PCs when the root tips were exposed to Cu. It is also known that PC-Cu complexes are comparatively fleeting and poorly sequestered to the vacuole. This phenomena suggests a varied level of tolerance, which could be brought on by different mechanisms (Cobbett and Goldsbrough, 2002; De Knecht et al., 1994; Schat and Kalff, 1992). Another study using the plant *Rubia tinctorum* discovered that when roots are exposed to certain heavy metals, PC-metal complexes are produced. Although PC complexes with Cd, Ag, and Cu ions were found in vivo, and Ag, Cd, Hg, As & Pb ions proved to be the most effective at inducing PCs (Cobbett and Goldsbrough, 2002; Maitani et al., 1996). It was discovered that the PC complexes produced by Pb and arsenate include Cu ions rather than the metal ion needed to initiate synthesis. However, this may indicate that some metal has been exchanged in complexes with PCs (Cobbett and Goldsbrough, 2002). Despite this, there are a lot of evidence that suggests PCs play a significant part in how plants react to various heavy metals and detoxify them. In addition to the well-known functions of PCs in the cell, such as the homeostasis of metals, antioxidant properties, and sulfur metabolism (Cobbett, 2000; Dietz et al., 1999), PCs are also in charge of the development of heavy metal hypersensitivity. According to reports, high PC levels in transgenic plants encourage the plant to accumulate more heavy metals without increasing tolerance or developing a hypersensitivity to heavy metals (Manara, 2012; Pomponi et al., 2006; Lee et al., 2003).

3. Role of calcium oxide nanoparticles (CaO-NPs) in the enhancement of heavy metal (HMs) stress tolerance in plants

In plants, calcium is crucial for growth, photosynthesis, reducing stress, and mediating hormonal responses (Knight et al., 2010; Liang et al., 2009; Brand and Becker, 1984). It also plays a key role in a variety of physiological and biochemical activities in plants (Shi et al., 2002). Nanotechnology is a very fascinating area of science and technology that may lead to new uses in agriculture and biotechnology (Siddiqui and Al-Whaibi, 2014). Nanoparticles can enhance plant metabolism and exhibit a variety of physicochemical properties (Giraldo et al., 2014). In order to improve the biological roles of various species, metal oxide nanoparticles are a better resource than metal oxide salts (Kadar et al., 2012; Johnston et al., 2010). There is very little research available on the essential role of calcium oxide nanoparticles (CaO-NPs) in the literature in alleviating heavy metal stress in plants.

Nazir and his co-workers, (2022a) investigated the positive role of CaO-NPs in alleviating arsenic stress induced toxicity from the two genotypes of barley (LJZ and Pu-9). They illustrated that the exogenous application of calcium oxide nanoparticles reduce the toxic effect of As stress and enhance plant growth, chlorophyll and calcium content as well as lower the ROS (reactive oxygen species) production and MDA (malondialdehyde) level in both barley genotypes. In the preceding year itself another study was reported by Nazir et al. (2022b) regarding the exogenous application of calcium oxide nanoparticles in alleviating cadmium (Cd) induced oxidative stress from barley seedlings. As a result, they revealed that

CaO-NPs when applied exogenously increase photosynthetic rate, antioxidant enzyme activity [(superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX)], plant biomass and as well as the level of non-enzymatic antioxidants (glutathione and ascorbate) which leads to the reduction of H_2O_2 (hydrogen peroxide) and MDA level in barley seedlings. In the same year Khalaf and his colleagues, demonstrated the role of calcium oxide nanoparticles in *Lupinus termis* plants grown under cadmium stress which resulted in the enhancement of antioxidant enzymatic [(CAT) and peroxidase (POD)] activity and enhance Cd stress tolerance along with the decrease in total soluble carbohydrate content in *Lupinus* plants.

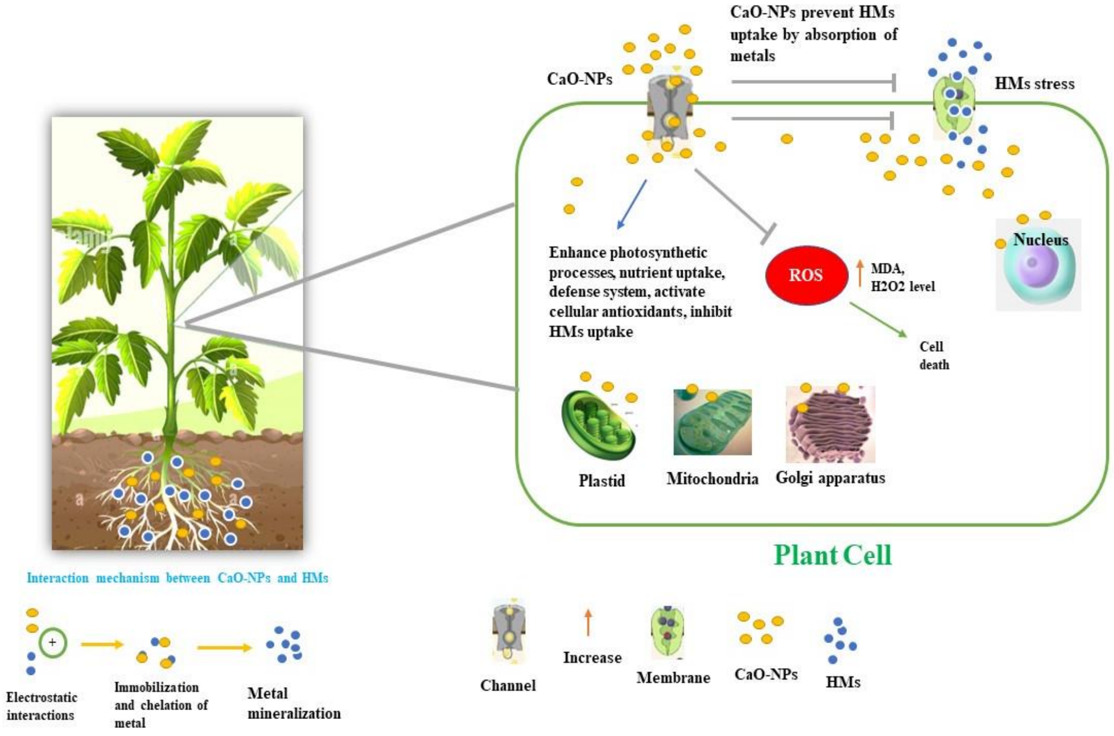

Figure 3: Schematic representation of calcium oxide nanoparticles (CaO-NPs) and heavy metal (HMs) interaction to alleviate heavy metal stress in plant.

Table 1: Role of calcium oxide nanoparticles to alleviate heavy metal induced oxidative stress in plants

S.No	Plant name	CaO-NPs concentration	Type of abiotic stress	Abiotic stress concentration	Method of application	Experimental setup	Effects of CaO-NPs	References
1.	*Hordeum vulgare* L.	25 mg/l	Arsenic (As)	0, 25, 50, 100, 150 µM	CaO NPs directly added in soil	Pot culture	Enhance Ca uptake, ROS scavenging ability, reduce As uptake and its transportation from roots to shoots.	Nazir et al., 2022a
2.	*Hordeum vulgare* L.	-	Cadmium (Cd)	-	CaO NPs directly added in soil	Pot culture	Increase antioxidant enzyme activity (APX, CAT,GR, SOD), plant biomass, and non-enzymatic antioxidants (glutathione, ascorbate). Reduce MDA, H_2O_2 level and alleviate toxicity induced by Cd stress.	Nazir et al., 2022b
3.	*Lupinus termis* L.	50, 100µM	Cadmium (Cd)	0, 20, 60mg/l	CaO NPs added in a medium	Petri dishes	Reduce oxidative stress induced by Cadmium stress. Increase antioxidant enzyme activity (POD, CAT).	Khalaf et al., 2022

4. Conclusion

The toxicity of heavy metals is a serious issue since both plants and animals consume them. Plant productivity is being hindered by the toxicity of heavy metal stress. It is currently unclear that how HMs are absorbed, effluxed, accumulated, translocated, and detoxified in plants. According to several findings, it has been examined that the heavy metal toxicity induces growth inhibition and oxidative stress in plants. The roles of CaO-NPs in alleviating HMs toxicity could be attributed to its enhancement of Ca uptake and ROS scavenging ability as well as reduction of HMs uptake and its transportation from roots to shoots in plants. Furthermore, the mechanisms and pathways by which heavy metals cause oxidative stress should be taken into consideration. According to the findings, CaO-NPs can help to reduce the detrimental effects of heavy metals on plants. However, there are still a lot of dark, grey regions exists that need to be clarified in order to apply different nanomaterials strategies that can be helpful for plant response mechanisms and resistance to HMs stress.

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