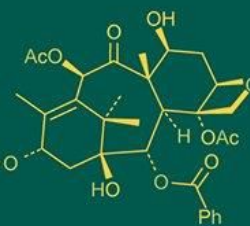


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; 8(9): 579-583
www.biochemjournal.com
Received: 20-06-2024
Accepted: 29-07-2024

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Impact of zinc sulfide nanoparticle in mitigating drought stress in *Brassica juncea*

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DOI: <https://doi.org/10.33545/26174693.2024.v8.i9h.2239>

Abstract

Drought stress challenges food grain productivity and triggers oxidative stress in plants due to ROS. Plants have mechanisms to counteract ROS, with antioxidant enzymes aiding stress tolerance. *Brassica juncea* was studied for drought stress alleviation using zinc sulfide nanoparticles. Various concentrations of ZnS NPs were applied via foliar spray on 18-day-old seedlings. Following treatment, seedlings endured three days of drought stress. Analysis showed a reduction in redox molecules and increased activity of stress-related enzymes. ZnS NP enhanced the activity of enzymes, effectively mitigating stress in *Brassica juncea*. Among concentrations tested, 25 ppm was most effective.

Keywords: Zinc sulfide nanoparticles, stress, *Brassica juncea*

Introduction

In the 21st century, increased food demand due to a growing global population requires more production from limited land resources. Abiotic stresses like drought, salinity, and extreme temperature affect crop yields, with approximately 70% of reductions linked to such stresses (Acquaah, 2007) [1]. Creating stress-tolerant plants through genetic engineering may offer a solution, despite global concerns about GM crop acceptance for environmental and health reasons.

It is almost certain that the problem requires to innovate and consider out-of-the-box policy options. Use of nanotechnology in agriculture offer a viable alternative to GM crops, for mitigating abiotic stresses, drought stress.

Various abiotic stresses, such as drought, salinity, high temperature, and high light stress, are interconnected. These stresses result in the generation of reactive oxygen species (ROS), known as oxidative stress, which is a common secondary response to abiotic and biotic stresses. Oxidative stress occurs when plants produce excessive ROS, including free radicals (e.g., superoxide anion, hydroxyl radical) and non-radical molecules (e.g., hydrogen peroxide, singlet oxygen). Interestingly, while ROS can damage cells at high concentrations, they also serve as signaling molecules to activate cellular defense mechanisms at lower levels. (Smirnov, 1998) [2]. However, an interesting fact is that all these ROS can also act as signalling molecules to activate the cellular defense machinery (Arora *et al.*, 2012; Kumar *et al.*, 2021) [3, 14] i.e. at lower concentrations, these reactive oxygen species act as signalling molecules while at higher concentrations, when these species accumulate beyond a critical level, they cause cellular damage.

In literature, there are reports that priming or prior exposure of a plant to mild stress inducers, increases the stress tolerance potential of the treated plants (Zhang *et al.*, 2005) [4]. Also, treatment of plants with specific engineered metal nanoparticles can help activate the antioxidant machinery of the treated plants (Sharma *et al.*, 2012) [5]. According to recent reports transition metal nanoparticles have the ability to perform interactions with various bio molecules inside cells through electron exchange due to their incompletely filled d-orbital (Sharma *et al.*, 2012) [5]. Such interactions have been shown to activate specific enzymes, in a dose dependent manner (Zhang *et al.*, 2005) [4]. The up-regulated antioxidant enzymes can potentially provide protection against reactive oxygen species.

We hypothesize that similar to the metal derived nanoparticles, the micronutrient based nanoparticles will be able to induce the antioxidant response as well as supplement the levels of the specific nutrients in the treated plants. Micronutrients like boron (B), copper (Cu), iron

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(Fe), manganese (Mn), molybdenum (Mo) and zinc (Zn), play a significant role in plant growth via metabolic processes associated with photosynthesis, chlorophyll synthesis, cell wall development, respiration, water absorption, nitrogen fixation, resistance to stresses (biotic & abiotic) and regulating enzyme activities involved in the synthesis of primary and secondary metabolites *etc.* (Adhikary *et al.*, 2010) [6]. Among the micronutrients, Zinc plays a pivotal role in metabolic processes, as it is involved in several enzymes as a co-factor and is also essential for the production of various metabolites. Therefore, in order to test the ability of the metal derived nanoparticles to activate the cellular antioxidant machinery of the treated plants, for the purpose of providing protection from drought stress; experiments were designed wherein *Brassica* plants subjected to drought stress were pretreated with zinc sulfide nanoparticles, and tested for their ability to withstand the onslaught of drought stress.

As *Brassica* is highly sensitive to Zn deficiency (Gupta and Kaushik, 2006) [16] and also has comparatively higher requirements for sulphur, therefore in the current experiments we propose to explore the role of zinc sulfide nanoparticles in providing protection against stress induced generation of reactive oxygen species.

Materials and Methods

Optimization of Drought Stress

Experiments were conducted for finding out the magnitude of drought stress which hampers plant growth, but is not deleterious for the affected plants. For this, the seeds were sown in pots and kept in a poly-house under controlled condition (26 ± 1 °C temperature, 70% relative humidity and 16/8 h day/night cycle). Each pot contained 3 seedlings. 20-day-old seedlings of *Brassica juncea* were used for imposing drought stress. Drought stress was given by withholding irrigation for six constitutive days with the control plant being irrigated daily, so as to maintain the soil at 75-85% of its water holding capacity. Sampling was done on each day between 10 and 11 am and various biochemical assays were performed immediately. Fig. 1.

Synthesis and characterization of zinc sulfide nanoparticles

Zinc sulfide nanoparticles were synthesized through chemical reduction of zinc sulfate as per the protocol given by Gayou *et al.* (2010) [8]. ZnSO_4 and Na_2S were used as zinc and sulphur source respectively. The size and shape of synthesized zinc sulfide nanoparticles were characterized through UV-Visible spectrophotometer and transmission electron microscope. Formation of zinc sulfide nanoparticles was determined by plotting the absorption spectra of the synthesized particles. The absorption maxima (λ -max) for zinc sulfide nanoparticles was found at 230 nm. According to literature, an absorption peak occurring between 225 and 235nm is associated with the formation of nano-sized zinc sulfide particles. The nanoparticles were characterized through transmission electron microscopy for deducing their shape and size. The TEM picture showed that the synthesized zinc sulfide nanoparticles are spherical in shape & have a size between 30 to 50 nm.

Nanoparticle pre-treatment on drought stress tolerance potential

Brassica juncea seedlings were grown in a poly-house under

controlled condition for 18 days, with three seedlings per pot. Eighteen day-old healthy and well irrigated seedlings were treated with different concentrations of zinc sulfide nanoparticles (0, 5, 15, 25 & 50 ppm) through foliar spray. Spraying was done with the help of a small sprayer to prevent spray drift into other treatments. Equal volume of nanoparticle formulation was sprayed on the treated plants and all the foliar sprays were done between 10 to 11am of a particular day.

After two days of zinc sulfide nanoparticle treatment, 20-day-old seedlings were subjected to drought stress by withholding irrigation for three consecutive days. Samples from the treated seedlings were collected three days after exposing the seedlings to water deficit and various standardized stress markers like, proline, MDA, hydrogen peroxide, electrolyte leakage and specific antioxidant enzyme activities were analysed.

Stress regulated markers

Free proline content

Proline content in leaf samples was determined following a method by Bates *et al.* (1973) [9]. Samples were homogenized with sulfosalicylic acid, then centrifuged. The supernatant was mixed with glacial acetic acid and acid ninhydrin reagent, incubated, and the reaction stopped with an ice bath. Toluene was added, vortexed, and the absorbance measured at 520nm to calculate proline content per gram of fresh weight using a standard curve.

Malondialdehyde content (MDA)

Malondialdehyde content was measured spectrophotometrically following Heath and Packer's (1968) [10] procedure. Leaf material was homogenized in 0.25% 2-thiobarbituric acid (TBA) and 10% TCA (1:10 ratio), incubated at 95 °C for 30 min, then centrifuged at 12,000 g for 30 min. Supernatant's absorbance was measured at 532 nm and 600 nm, with the latter subtracted as non-specific absorbance. MDA concentration was calculated using an extinction coefficient of $155\text{mM}^{-1}\text{cm}^{-1}$ and expressed as M per gram fresh weight.

Measurement of electrolyte leakage

Electrolyte leakage was assessed by washing 1 gm of fresh leaf samples, cutting them into small pieces, and suspending them in de-ionized water for 25 min. The samples were then incubated at 25 °C for 2 h, after which EC1 was measured. Next, the samples were heated to 100 °C to release electrolytes, and EC2 was measured. The percentage of electrolyte leakage was calculated using the formula $1 - (\text{EC1} / \text{EC2}) \times 100$. Antioxidant Enzyme Assay.

Results

Effect of zinc sulfide nanoparticle pre-treatment on drought stress tolerance

These experiments were carried out to study the effect of pre-treatment of different concentrations of zinc sulfide nanoparticles concentration (0ppm, 5ppm, 15ppm, 25ppm & 50ppm) on *Brassica juncea* exposed to drought stress. Zinc sulfide nanoparticles were synthesized through chemical reduction of zinc sulfate as per the protocol given by Gayou *et al.* (2010) [8]. ZnSO_4 and Na_2S were used as zinc and sulphur source respectively. The size and shape of synthesized zinc sulfide nanoparticles were characterized through UV-Visible spectrophotometer and transmission

electron microscope (Fig.2). Formation of zinc sulfide nanoparticles was determined by plotting the absorption spectra of the synthesized particles (Fig. 2a). The absorption maxima (λ -max) for zinc sulfide nanoparticles was found at 230 nm. According to literature, an absorption peak occurring between 225 and 235 nm is associated with the

formation of nano-sized zinc sulfide particles (Calandra *et al.*, 2003) [11]. The nanoparticles were characterized through transmission electron microscopy for deducing their shape and size. The TEM picture showed that the synthesized zinc sulfide nanoparticles are spherical in shape & have a size between 30 to 50 nm (Fig.2.b).



Fig 1: Effect of increasing magnitude of drought on Brassica juncea. a) 0 day b) 1 day c) 2 day d) 3 day e) 4day f) 5day g) 6 day

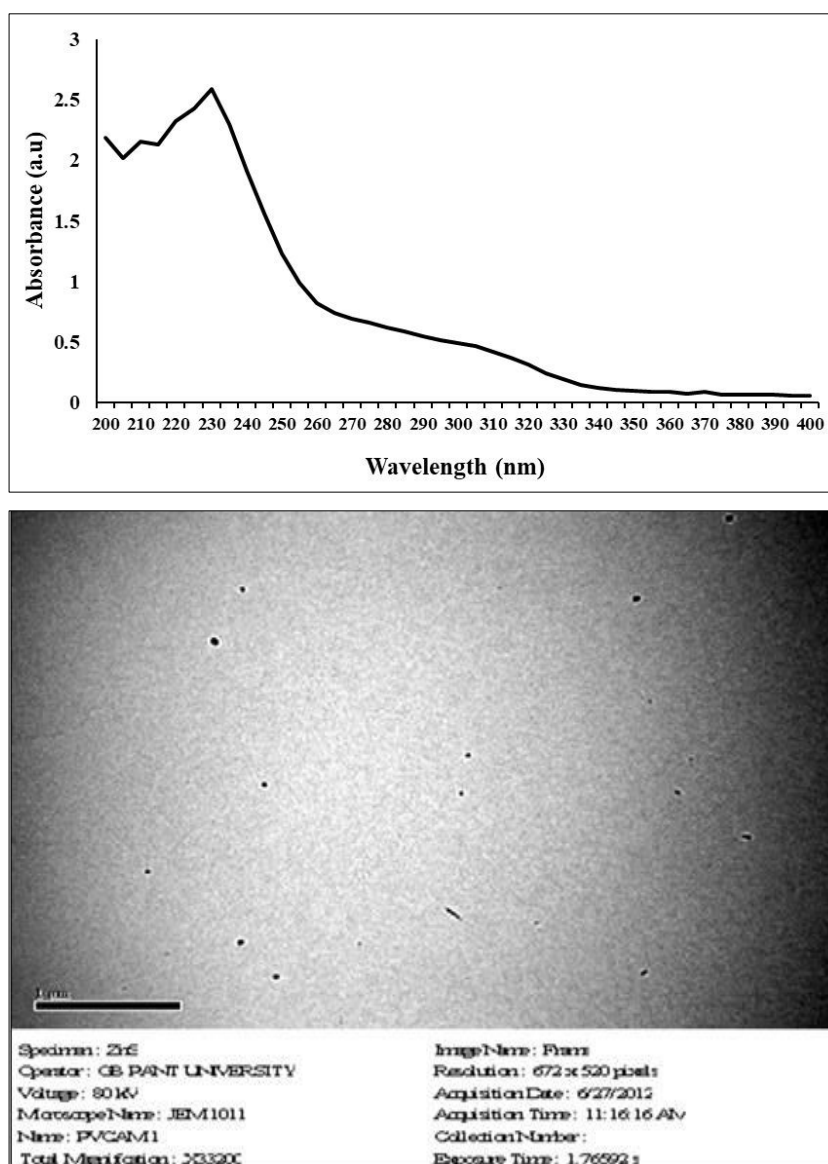


Fig 2: Characterization of zinc sulfide nanoparticles: a) Absorption spectra of zinc sulfide nanoparticles b) Transmission electron micrograph of Zinc sulfide nanoparticles

Drought stress analysis of zinc sulfide nanoparticle treated seedlings

Different zinc sulfide nanoparticles concentration (0 ppm, 5ppm, 15ppm, 25ppm & 50ppm) were applied through foliar spray to 18-day-old seedlings. After two days of nanoparticle treatment, seedlings were subjected to drought stress for three consecutive days, by withholding irrigation. Analysis of various redox regulatory molecules was done after third day of drought stress.

Malondialdehyde (MDA)

Malondialdehyde is stress marker and it is a quantitative index of free oxygen radical induced oxidative damage. In present experiment it was observed that malondialdehyde (MDA) content of *Brassica juncea*, pretreated with different concentration of zinc sulfide nanoparticles, was lower in comparison to untreated seedlings (Fig.3). At 5 ppm and 15 ppm zinc sulfide nanoparticles treatment, no statistically significant difference in MDA content was observed in the treated seedlings as compare to the untreated seedlings. However treatment of 25 and 50 ppm zinc sulfide nanoparticle treatment significantly lowered the MDA content, as compared to the untreated seedlings. These results indicate that zinc sulfide nanoparticle could successfully reduce the oxidative load in the treated seedling Sharma *et al.*, (2012) [5] have reported that metal nanoparticle treatment reduces the oxidative damage in treated seedling.

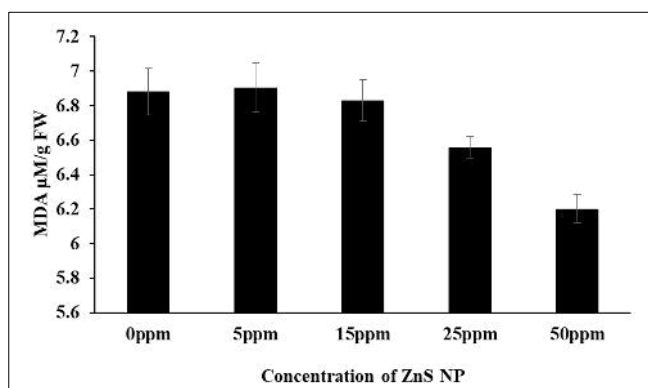


Fig 3: Effect of zinc sulfide nanoparticle pre-treatment on Malondialdehyde content in *Brassica juncea* subjected to 3 days of drought stress. Data represent Mean + SE

Proline

Proline accumulation is one of the most widely studied response of plants subjected to drought stress. Proline is reported to accumulate in plants exposed to a variety of abiotic stresses and protects the plant from the harmful effect. Higher proline levels are associated with higher degree / magnitude of stress experienced by the plants (Joshi *et al.*, 2010) [12]. In the current experiments it was observed that zinc sulfide nanoparticle treatment lowered the proline levels of stressed seedlings, at all the nanoparticle treatments tested. Proline content of *Brassica juncea* seedlings pretreated with different concentration of zinc sulfide nanoparticles, were found to be significantly lower at 5 ppm (24%), 15ppm (54%) and 25p pm (19.5%), as compared to untreated plants (Fig.4). Maximum decrease in proline level was recorded at 15ppm zinc sulfide nanoparticle pretreatment. However there was no significant difference between proline content of untreated and 50 ppm zinc sulfide nanoparticle treated seedlings. Decrease in

proline content indicates that the stressed seedlings are experiencing lesser magnitude of oxidative stress after zinc sulfide nanoparticle pre-treatment, as compared to the untreated seedlings.

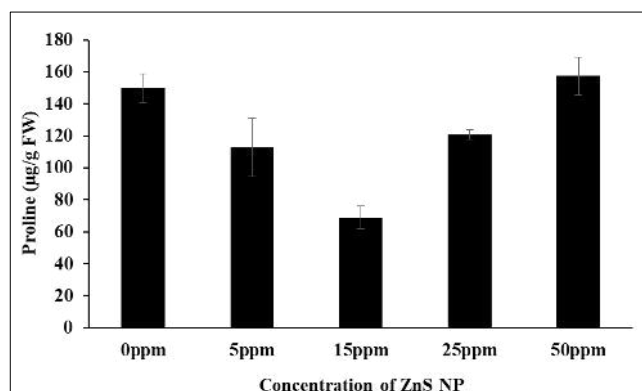


Fig 4: Effect of zinc sulfide nanoparticle pre-treatment on proline content in *Brassica juncea* subjected to 3 days of drought stress. Data represent Mean + SE

Electrolyte leakage

Electrolyte leakage of *Brassica juncea* seedlings pretreated with different concentration of zinc sulfide nanoparticles was significantly lower than the untreated seedlings, at all the tested concentrations of the nanoparticles (Fig.5). Maximum decrease of 27% in electrolyte leakage was observed at 5 ppm zinc sulfide nanoparticle pre-treatment. Drought stress imposes alteration to the cell membranes such as increase in permeability and decrease in selectivity, which results in increased electrolyte leakage (Blokhina *et al.*, 2003) [13]. In the current study also, a reduction in electrolyte leakage of *Brassica juncea* seedlings pre-treated with zinc sulfide nanoparticles under drought stress indicates that the nanoparticle treatment has enhanced the membrane stability by reduces the production of ROS species. The results are in agreement with the levels of MDA as recorded in the nanoparticle pre-treated seedlings, wherein the MDA level was lower in the seedlings exposed to drought stress after zinc sulfide nanoparticle pretreatment, as compared to the non-pre-treated seedling.

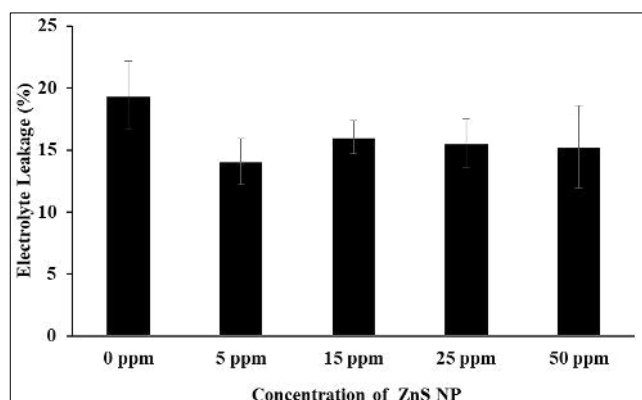


Fig 5: Effect of zinc sulfide nanoparticle pre-treatment on electrolyte leakage in *Brassica juncea* subjected to 3 days of drought stress. Data represent Mean + SE

Conclusion

Our experiments show that zinc sulfide nanoparticles can enhance antioxidant defenses in *Brassica juncea* against drought stress. Pre-treated seedlings exhibited improved

redox status and reduced oxidative damage markers such as malondialdehyde and hydrogen peroxide. Chlorophyll a/b ratio also increased, indicating better photosynthetic efficiency. Nanoparticle treatment primed antioxidant enzymes like ascorbate peroxidase, while catalase activity remained unaffected. Ascorbate peroxidase played a key role in metabolizing stress-induced hydrogen peroxide. Further research is needed to understand the underlying molecular mechanisms of this protective effect.

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