

**ROLE OF *LAMINARIN* POLYSACCHARIDE IN GERMINATION AND SEEDLING
GROWTH OF *MEDICAGO SATIVA* UNDER SALT STRESS**

Submitted in partial fulfillment of the requirements for the award of Bachelor of
Science degree in Biotechnology

BY

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SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

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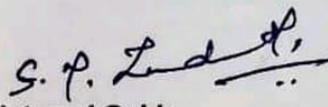
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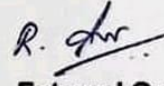
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of B. SANDHIYA (41770027) who carried out the project entitled "ROLE OF LAMINARIN POLYSACCHARIDE IN GERMINATION AND SEEDLING GROWTH OF *MEDICAGO SATIVA* UNDER SALT STRESS" under my supervision from September 2022 to April 2023.


Internal Guide

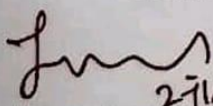
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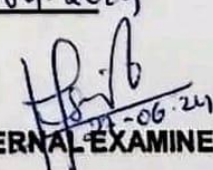
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27/4/24
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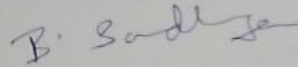
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DECLARATION

I B. SANDHIYA(41770027) hereby declare that the Project Report entitled "ROLE OF LAMINARIN POLYSACCHARIDE IN GERMINATION AND SEEDLING GROWTH OF *MEDICAGO SATIVA* UNDER SALT STRESS " done by me under the guidance of Dr. R. SRINIVASAN, Assistant Professor, Centre for Ocean Research, Col. Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology and Dr. INDHUMATHI S M Assistant Professor, department of biotechnology is submitted in partial fulfillment of their requirements for the award of Bachelor of Science degree in Biotechnology.



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ACKNOWLEDGMENT

I am pleased to acknowledge our sincere thanks to the Board of Management of **Sathyabama Institute of Science and Technology** for their kind encouragement in doing this project and completing it successfully. I am grateful to them.

I convey my sincere thanks to **Dr. REKHA CHAKRAVARTHI, Dean, School of Science and Humanities** and **Dr. V. RAMESH KUMAR, Head of the Department, Department of Biotechnology**, for providing me with the necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my internal project guide **Dr. INDUMATHI S.M** and external project guide **Dr. R. SRINIVASAN** for their valuable guidance, suggestions and constant encouragement that paved way for the successful completion of my work.

I wish to express my thanks to all **Teaching and Non-teaching staff** members of the Department of Biotechnology who were helpful in many ways for the completion of the review work.

ABSTRACT

This study investigates the role of *laminarin* nanoparticles in enhancing the germination of *medicago sativa* seeds under salt stress. Salinity stress poses a significant threat to crop productivity, and finding effective strategies to mitigate its adverse effects is crucial for sustainable agriculture. *Laminarin*, a polysaccharide derived from brown algae (*laminaria digitata*), has been reported to possess bioactive properties with potential applications in plant stress tolerance. In this research, *medicago sativa* seeds were subjected to 250mM of NaCl concentration in the presence and absence of *laminarin* nanoparticles(LNP). Germination rates, seedling growth parameters, and biochemical markers of stress were evaluated to assess the efficacy of *Laminarin* nanoparticle in alleviating salt-induced inhibition of germination. Preliminary results indicate that the *laminarin* nanoparticles positively influence seed germination and seedling vigor under salt stress conditions. It is found that the highest germination rate is found in 5 μ g and 50 μ g LNP concentrations. Decreased ROS accumulation is observed in high concentration of LNP coating. Treatment with 250mM NaCl brought about a significant decrease in the activity of CAT, SOD and APX when compared to control condition. It was found that a significant increase in the CAT, SOD and APX activity in control and slight increase in its activity under LNP. These findings suggest the potential of LNP as a novel approach to enhance crop resilience to salinity stress and contribute to the development sustainable agricultural practices.

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LIST OF ABBREVIATIONS

Se – Selenium

Fe – Iron

NaCl – Sodium Chloride

CAT – Catalase

SOD – Superoxide

APX – Ascorbate Peroxidase

POD – Peroxide

CRD – Completely randomized design

K – Potassium

N – Nitrogen

P – Phosphorus

Ca – Calcium

Zn – Zinc

GP – Germination Percentage

CGRI – Corrected Germination Rate Index

PEG – Polyethylene Glycol

ASA – Ascorbic Acid

NaOH – Sodium Hydroxide

RPM – Revolution per minute

TPP – Triphenylphosphine

ML – Milliliter

LNP – *Laminarin* Nanoparticle

ROS – Reactive Oxygen Species

DCFDA – Dichloro Dihydrofluorescein Diacetate

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CHAPTER-1

INTRODUCTION

1.1 ALFALFA

Alfalfa which is also known as 'King of forages', is an excellent perennial leguminous grass for forage, agriculture, and environment (*Huifang et al.,2020*)(*Zhao chen et al.,2021*). It is an important forage crop with good quality, high water saving potential and moderate saline tolerate legume which is widely cultivated around the world (*Huifang et al.,2020*)(*Yanzhe Hu et al.,2021*) . This legume forage is rich in high protein, valuable nutrient include amino acids, minerals, vitamins and dietary fiber that can be harvested for several times per year, which leads to the high biomass yield in terms of forage yield(*Huifang et al.,2020*)(*Yinping Feng et al.,2022*) (*Zhao chen et al.,2021*). In addition to highly nutritious, alfalfa is also highly adaptable to warm and cold climates (*EB Carpycy et al.,2009*). The highest planting area of alfalfa is in china, which is about 3.77 million hectares compared to other artificial grasslands (*Huifang et al.,2020*) . Animal husbandry is the area where the alfalfa is extensively used as a direct fed, or processed into haylage, silage (*Yinping Feng et al.,2022*) .

1.2 SALINITY

Agents of environmental and abiotic stress which have gained more impacts in last three decades are salinity, drought, chilling, high temperature, resulting from the use of pesticides, fertilizers etc (*Murat Dikilitas et al.,2020*). These abiotic stresses have undesirable effects on quality, crop productivity and negative growth trends in sustainable agriculture (*Muhammad Adhan Shahid et al.,2020*). Among these abiotic stresses salinity is the most significant and harmful agent that negatively influences crop yield and degradation (*R Yu et al.,2021*) (*Surendra Bhattarai et al.,2020*). In root zone excess concentration of soluble salts such as sulfate, chloride, calcium carbonate, sodium carbonate, magnesium carbonate, potassium carbonate making plants difficult to extract water and nutrients from soil and causing plant injury (*Dilip Biswas et al.,(2020)*). Decreased photosynthetic rate due to osmotic salt stress induced partial stomatal closure (*Rana Munns et al.,2008*). It is estimated to be over 1030 million

hectares in the world are the soil area affected by salt stress (*Bambang Suwinbnyo et al.,(2023)*). Germination and seed vigor of alfalfa can be reduced significantly under salt stress (*EB Carpycy et al.,2009*). Under three different growth stages Smith described at which alfalfa plants be affected by salinity: Germination, seedling growth, mature plant growth (*SE Smith et al.,1993*). Salinity affects soil, ground water and limits agricultural production at extreme conditions (*Boling Liu et al.,2019*).

1.3 β -GLUCAN

Indispensable components of many foods are polysaccharides (*Ramandeep Kaur et al.,2019*). For normal metabolic processes in body, digestible polysaccharides are needed source of calories and glucose. Moreover, a large amount of indigestible dietary fiber exerts beneficial health activities in the body (*David S Ludwig et al., 2017*) (*F Qiao et al.,2017*). β -glucan is one of the most widely known and documented bioactive polysaccharides (*Ramandeep Kaur et al.,2019*) . β -glucan is an non-starch polysaccharides composed of β -D-glucose monomer unit. β -glucan is a polymer linked holding linkage at $\beta(1\rightarrow3)$, $(1\rightarrow4)$ and /or $(1\rightarrow6)$, either in a branched or in an unbranched manner (*Artur Mykhalevych et al.,2022*). β -glucan could be found in a variety of natural sources such a yeast, mushroom, bacteria, algal, barley and oat (*Zhu Du Bian et al.,2015*). β -glucans are major bioactive compounds known to have biological activates including anti-cancer, anti-inflammatory and immune-modulating properties (*F Zhu t al.,2016*).

1.4 LAMINARIN NANOPARTICLE

There is a increasing attention owing to their abundance, reduced extraction cost, unique physiochemical and biological properties for polysaccharides derived from marine flora i.e seaweed (*Mehrzaad Zargarzadesh et al.,2020*). These marine polysaccharides, which include *fucoidon*, *laminarin*, *alginate*, *carrageenan*, *agar* and *amylopectin*, have been broadly used for pharmaceutical and biomedical purposes (*Aja Aravamudhan et al.,2014*).Of particular from the marine plant *laminarin digitata* the active ingredient *laminarin* polysaccharide is extracted o exhibit distinct therapeutic functions such as anti-tumor, antioxidant, anticoagulant activities, etc (*Dongxiao Cui et*

al.,2019). *Laminarin* is also known as *laminaran* is a biodegradable and non-toxic polysaccharide (*Mehrzaad Zargarzadesh et al.*,2020). *Laminarin* is One of the major carbohydrates in marine algae, is composed of β -1,3 glucan backbone and some β -1,6 intra-chain links (*Shanmugapriya Karuppusamy et al.*,2024) (*SU Kadam et al.*,2015). The content of *laminarin* increases in the day but decreases in night shows a diel variation trend in marine algae (*SU Kadam et al.*,2015).

To reach in good plant stand, different critical stages was faced by the life cycle of plants such as uneven seed germination, poor and early seedling growth which ultimately results in low crop yield(*MS Rhaman et al.*,2020). An pre-sowing technique in which seeds are moderately hydrated o the point is known as seed priming, where pre-germination metabolic processes begin without germination (*Muhammad Farooq et al.*,2020). To improve a diverse range of morpho-physiological, biochemical and molecular responses in plants(*Faisal Zulfiqar et al.*,2021). Increased germination rate under a broader range of environments and improved seedlings vigor and growth(*Miller B McDonald et al.*,2000). Seed priming is also associated with tolerance against major abiotic and biotic stress (*Faisal Zulfiqar et al.*,2021).

1.5 SEED PRIMING IN ALFALFA

1.5.1SELENIUM ON SEED VIGOR OF ALFALFA

For higher plants, animals and human selenium (Se) is an trace essential trace element. The seed vigor of alfalfa is improved with low concentration of Se (0.5 and 1.0mmol L⁻¹), But was inhibited by higher concentration (>2.0mmol L⁻¹). This study states the optimal manner of Se priming in alfalfa seeds be at 1.0mmol L⁻¹ concentration for 9 hrs (*FS Xia et al.*,2021).

1.5.2SALICYCLIC ACID ON IMPROVING TOLERANCE TO SALINITY AND IRON DECIFIENCY OF ALFALFA

Medicago sativa when subjected to salinity(NaCl) and iron(Fe) deficiency either separately or combined to investigate the role of salicylic acid seed priming on plant growth, chlorophyll content, photosynthesis and ionic composition. NaCl and Fe

deficiency reduces shoot and root growth, photosynthetic and transpiration rates, chlorophyll concentration, stomatal conductance and ionic uptake under unprimed condition. On all studied physiological parameters under different stress, seeds primed with 100µm salicylic acid had the best impact (*Nadia Boukari et al.,2019*).

1.5.3 THE EFFECT OF HYDRO AND OSMOPRIMING ON ALFALFA UNDER SALT STRESS

To check the hydro and osmo-priming, *Hamedani* and *Yazdi* are the two varieties of alfalfa used. Mannitol (4%) and water are used to prime the seeds. From primed seeds ten-days-old seedlings were obtained. The germination rate was higher in primed seeds, significantly enhances the activities of CAT, POD, SOD, and proline content, and reduced the MDA accumulation and electrolyte leakage under salt stress(*R Amooaghaie et al.,2011*).

1.5.4 SAND PRIMING OF ALFALFA UNDER SALT STRESS

Seeds where primed at 15°C for 48h with sand containing 4% (v/w) water, sealed in plastic box. The result showed that sand priming was an effective method to improve seed germination, enhance ability of salt tolerance and under higher salt concentration stress condition, improves seed germination and seedling growth of alfalfa (*J Hu et al.,2006*).

1.5.5 THE EFFECT OF SEED PRIMING OF ALFALFA UNDER SALT STRESS

Seeds are primed under salinity concentrations of 0,5 and 10ds.m⁻² Nacl solution with salicylic acid, vitamin B12 and distilled water were selected under intro condition. Seedling growth and germination indexes of alfalfa are enhanced by priming. Also Hypocotyl and radicle lengths are improved by applying salicylic acid (*M Jorjandi et al.,2021*).

CHAPTER-2

LITERATURE SURVEY

2.1 ALFALFA UNDER SALT STRESS

As alfalfa's response to salt stress is genetically and physiologically complex, its genetic improvement for salt tolerance is challenging. This review states that it is clear that alfalfa salt tolerance can be better characterized, genes conditioning salt tolerance be identified, new marker-based tools be developed to accelerate alfalfa breeding for salt tolerance (Surendra Bhattarai et al., 2021).

2.2 STRATEGIES TO IMPROVE THE YIELD UNDER SALT STRESS

The main aim of this review was to suggest different tolerance mechanisms, to highlight the effects of salt stress on the performance of *Medicago sativa* and also to suggest the management strategies for improving its yield under salt stress. This study says that the *Lucerne* performance under salt stress can be improved by the use of *rhizobial* and *Non-rhizobial* plant growth-promoting bacteria, *Arbuscular mycorrhizal fungi*, exogenous application of osmoprotectants and seed priming with Brassinolide, gibberellic acid and salicylic acid (Safaa Mohammed Al-Farsi et al., 2020).

2.3 EFFECT OF SALT TOLERANCE ON ALFALFA

This paper shows that salt tolerance degree of different alfalfa varieties was positively correlated by basal content of MT. The partially ameliorated seedling growth of salt-stressed alfalfa is fully recovered by MT and its precursor 5-HT. In summary, this study analyzed the regulatory mechanism of MT during alfalfa seed germination under salt stress and demonstrated the importance of MsPMTR1 in MT-mediated salt tolerance in alfalfa (Ruonan Yu et al., 2021).

2.4 ALFALFA POTENTIAL FOR CULTIVATION IN SALINE AREAS

For this study, morphological and forage yield response to different salinity levels thirty-four alfalfa landraces were evaluated. This study showed the variation of alfalfa

landraces potential for cultivation in saline areas and /or use in breeding programs aimed to develop salt tolerant alfalfa genotypes(*Saleem K Nadaf et al.,2020*).

2.5 GENOME-WIDE ASSOCIATED STUDY

Two types of analysis were done in this study , Genome-wide associated study(GWAS) involving 875,023 single-nucleotide polymorphisms was conducted on 220 alfalfa varieties, Transcriptome analysis 2,097 and 812 differentially expressed genes(DEGs) that were up-regulated and 2,445 and 928 DEGs that were down-regulated in the leaves and root under both normal and salt stress. The result revealed that two SNPs located within the upstream region of MsAUX28, which encodes an auxin response protein, were significantly associated with tolerance to salt stress. In the alfalfa domestication and improvement process, the two significant SNPs within upstream region of MsAUX28 existed as three different haplotypes in this panel Hap 1(G/G,A/A) was under selection(*Fei He et al.,2022*).

2.6 ACTIVITIES OF ANTIOXIDANT ENZYME

Analyzing the activity of several antioxidant enzymes, including superoxide dismutase (SOD), peroxide (POD), ascorbate peroxidase (APX), and catalase (CAT), in alfalfa shoots and roots subjected to salt and drought stresses during germination to understand the adaptability of alfalfa to environmental stresses. Under 200mM NaCl or 35% PEG treatment, the germination rate of six alfalfa cultivars was comparatively studied (*Wen-Bin Wang et al.,2009*).

2.7 EXPERIMENT PERFORMANCE IN CRD

An experiment was performed in factorial scheme having a completely randomized design (CRD) with three replication, to select the most tolerant alfalfa genotypes to salinity stress. The cultivars and four levels of salinity stress, they are control, 75, 150, and 225mM with NaCl respectively. Results of the cluster analysis using the data all measured traits and salinity levels clustered into two groups. First cluster were found to be tolerant to salt and second cluster were sensitive to salt. To confirm

their tolerance to salt waiting creation of new hybrid varieties, all the cultivars were used in large field trails (*Asieh Soltani et al.,2012*).

2.8 EFFECTS OF SALT AND ALKALI STRESS

This experiment was done to quantify the effects of salt and alkali stresses on germination, growth, photosynthesis and ion accumulation in alfalfa. The result showed that germination and radicle elongation is significantly reduced by both stresses, indicating that alfalfa was relatively sensitive to both stresses during seed germination and early seedling growth stages. These studies found were more severe than those of salt stress (*Ruili Li et al.,2010*).

2.9 PROTEOMIC ANALYSIS

During the germination stage alfalfa is sensitive to salt stress. A comprehensive proteomic analysis was performed to investigate the response of alfalfa to salt stress by comparing alfalfa cultivars that differ in salinity tolerance in early stages of seedling. The most salt-tolerant cultivar is *zhongmuno.3*, and most salt-sensitive cultivar is *daxiyang*, among the five cultivars examined in terms of their physiological and proteomic responses. For ten days two alfalfa cultivars were exposed to 0mmol⁻¹ or 200mmol⁻¹ NaCl salt treat. This results shows that the seedling growth and the cotyledons, chlorophyll content is significantly reduced by salt stress (*Arafet Manaa et al.,2011*).

2.10 SAND BASED HYDROPONIC SYSTEM

Under five salinity levels (0dsm⁻¹, 4dsm⁻¹, 8dsm⁻¹, 12dsm⁻¹ and 16dsm⁻¹) *Halo*, '*Brigevue*', '*Rugged*', and two intolerant cultivars '*Rangelander*' and '*vernal*' in a sand based hydroponic system in the greenhouse. '*Halo*' under salt stress has the highest germination percentage among the cultivars. In 16dsm⁻¹ EC '*Rugged*' and '*Halo*' had highest seed vigor. '*Rugged*' had highest chlorophyll content at 0-12ds⁻¹. This study demonstrated that seed vigor in the germination stage cannot be used to predict salt tolerance of alfalfa at mature growth stages, however physiological markers like plant

height and leaf chlorophyll serves for the high shoot biomass selection at mature growth stages under salt stress(*Seth Lundell et al.,2022*).

2.11 EFFECT OF RHIZOBIUM SYMBIOSIS

Alfalfa germination and biological nitrogen fixation is negatively affected by salt stress. This study investigated whether *rhizobium* symbiosis has an effect on host plant tolerance to salt stress. Higher survival rate was showed by alfalfa with active nodules. Through this study it is concluded that rhizobium symbiosis has an positive effect on salt tolerance alfalfa by improving the activity of antioxidant enzymes and osmotic adjustment capacity (*Yafang Wang et al.,2016*).

2.12 EFFECT OF THREE SALINITY LEVELS

Under salinity condition, yield and nutrient contents of alfalfa may be affected. To determine the effect of three salinity levels 60, 120, 180mM Nacl, this experiment was conducted. Under all of the salinity levels *Rehnani*, a tolerant cultivar, had the lowest Na and mg contents and the highest K, N, P, Ca, Zn, and Cu contents and dry weights. This study suggesting that leaf dry weight and leaf P content had the highest correlation with salt tolerance are the traits may be used as marker for selecting salts that are tolerant among genotypes in alfalfa9*Abdelkarim Hassan Awd Elkarim et al.,2010*).

2.13 FIVE CONCENTRATIONS OF SODIUM CHLORIDE

The salinity tolerance of 12 Alfalfa cultivars in-vitro using five concentrations of Sodium chloride ranging from 0 to 250mmol L⁻¹ were evaluated by this study. The results obtained in current study revealed that highest salinity tolerance in terms of germination percentage (GP), corrected germination rate index (CGRI), days to reach 50% germination (GT50) and ability to form cotyledonary and true leaves are acquired by *Hassawi*, *Saudi* cultivars, *Kasimi*, and the German cultivar (Berlin) (*Walid Soufan et al.,2013*).

2.14 PEG-MEDIATED SALT AND DROUGHT STRESS

To examine the *ZhongmuNo3* alfalfa seed germination stage with 200mM NaCl and 180g.L⁻¹ Polyethylene glycol (PEG) physiological and proteomic analyses were applied. Through two-dimensional electrophoresis (2-DE), more than 800 proteins were detected by proteomic technology. Discovered that under PEG-mediated drought stress and salt stress, some proteins related to carbohydrate, metabolism and energy production increased in abundance. Overall this study demonstrated a common mechanism of energy consumption during abiotic stress (*Qiaoli Ma et al.,2017*).

2.15 SALT RESISTANCE WAS PROMOTED

This study investigated about the effects of AsA applications on seed germination and seedling salt-tolerance of alfalfa. The results revealed that germination, amylase, and protease, as well as seedling growth, fresh weight (FW), dry weight (DW) were increased by 0.1 and 1mmol L⁻¹ of exogenous AsA and endogenous AsA both in shoot and root, except the 1mmol L⁻¹ AsA reduced the activities Of amylase, β -amylase and protease on day 3. In summary, the optimum concentration of 0.1mmol L⁻¹ AsA can be implemented to improve the seed germination and seedling growth of alfalfa under salt stress (*Zhao Chen et al.,2021*).

2.16 THE EFFECTS OF MELATONIN

In this reports to explore the effects of melatonin on alfalfa growth and salt tolerance, melatonin was applied exogenously. The result demonstrated that alfalfa seed germination and salt resistance was promoted, and reduced oxidative damage by melatonin application under salt stress. This study indicates that melatonin enhances the activities of antioxidant enzymes and plays a primary role as an antioxidant in scavenging H₂O₂ to improve the salt tolerance of alfalfa plants. Further application in research found that salt injury in alfalfa under salt stress alleviated by melatonin(*Huifang Cen et al.,2020*).

2.17 USE OF BIOSTIMULANTS

The use of biostimulants or osmoprotectants has been one of the most practical approaches so far to overcome environmental and abiotic stress. Not only stressed plants but also their transgenic expression would be beneficial with the use of osmoprotectant such as proline, glycine betaine, or amino acids. In this chapter, under abiotic stress conditions as well as their significance and expressions under biotic stress conditions, the role of osmoprotectants has been evaluated (*Murat Dikilitas et al.,2020*).

2.18 SALT-TOLERANT CULTIVAR

In both proteomic and physiological levels the response of soya bean to salt stress during germination was investigated. Under 100mmol/L NaCl salt-tolerant cultivar lee68 and salt-sensitive cultivar n2899 were exposed until radicle protrusion from the seed coat. In control the proteins were present in lower levels and were up-regulated under salt stress. The result indicates that during soya bean germination, these proteins might have important roles in defense mechanism against salt stress (*Yongqi Yin et al.,2015*).

2.19 IMPACT OF SALICYLIC ACID

The aim of this study is to explore the impact of salicylic acid (SA) seed priming on the growth, chlorophyll, photosynthesis, and ionic composition of two *medicago sativa* ecotypes(*Gabes and presmenti*) under salinity(75mM) and iron deficiency (1 μ M), both individually and in combination. Results of this study revealed that without priming, both salinity and iron(Fe) deficiency led to diminished shoot and root growth, lowered photosynthetic and transpiration rates, decreased chlorophyll concentration, reduced stomatal conductance, and hindered ionic uptake(*Nadia Boukari et al.,2019*).

CHAPTER-3

AIMS AND OBJECTIVE

3.1 AIM

To determine the effects of seed priming with nano-particles in alfalfa during salt stress.

3.2 OBJECTIVES

- Priming the *Medicago sativa* seeds with *Laminarin* nanoparticle of different concentration.
- Introducing the primed seeds to the stress condition (Here we use, salt stress) for germination.
- Analysing the germination rate, seedling growth and other characteristics of the primed seeds with unprimed seed(control)
- Overview the effects of LNP in overcoming the applied stress condition.

CHAPTER-4

MATERIALS REQUIRED AND METHODS

4.1 MATERIALS

The materials required for this experiment are Alfalfa, *Laminarin* from *Laminaria digitata* 100mg(0.1%), 40ml Beaker, 15ml Falcon tubes, Magnetic stirrer, water(50)ml, Sodium hydroxide(0.5g), Triphenylphosphine (TPP) (4.17ml), Centrifuge, Ethanol(double the amount), 4 Concentrations of *Laminarin*, Sterilized alfalfa seeds, Sterile water, 15 ml falcon tubes, Water -30ml, 25ml Nacl, Germination papers, Distilled water, 100ml Beaker, Ethanol, Rubber band, 2`Dichlorodihydrofluorescein diacetate, Buffer, Root tips from germinated plants, 0.1 gm leaves, 5.0 ml of acetone and 0.1 Potassium phosphate buffer(pH 7.0).

4.2 METHODS

4.2.1Seed collection

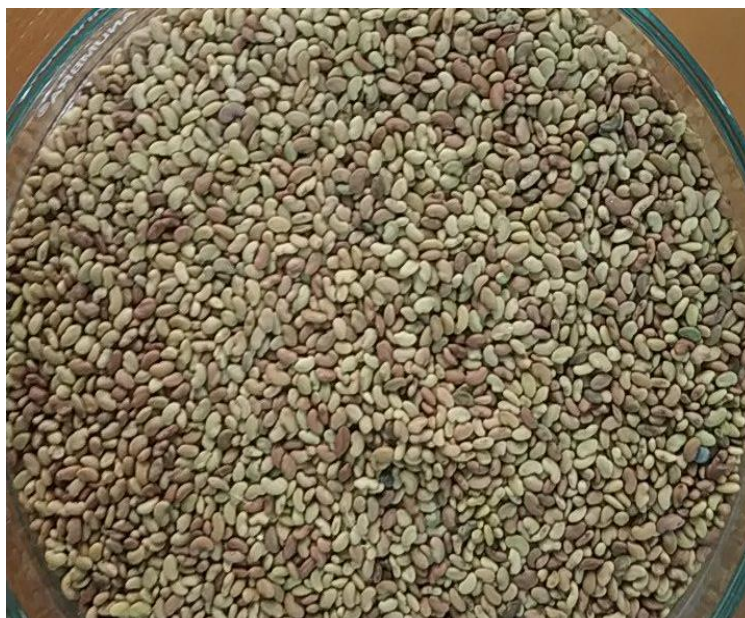


Fig 4.1 Alfalfa seeds

Alfalfa seeds were procured from S.K. internationals which is Non GMO and chemical free, 100% organic in nature. This alfalfa seeds are extensively used in Animal husbandry as a direct fed or processed into haylage, silage.

4.2.2 Preparation of nanoparticle

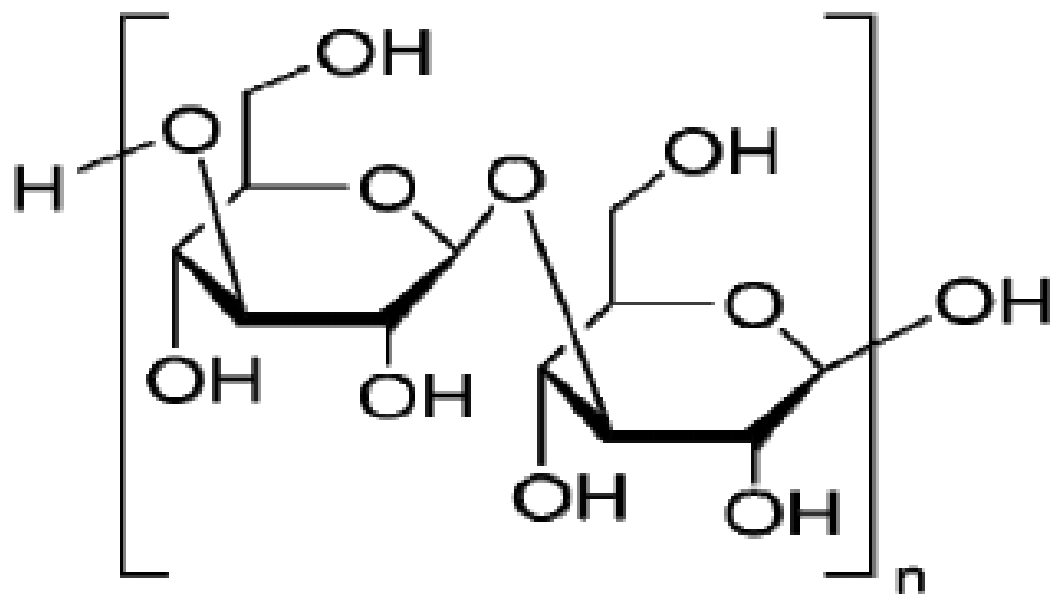


Fig 4.2 Structure of LNP

In 40ml beaker, 25ml of water is mixed with 2% (0.5g) of sodium hydroxide (Naoh).100 mg (0.1%) of *Laminarin* dissolved in 25ml of 2 % (0.5g) sodium hydroxide.Then kept for 2 hours in magnetic stirrer at 90°C with 420rpm.After 2 hrs of incubation in magnetic stirrer, precipitated by using double the amount of ethanol.To obtain the precipitate, the solution is kept in magnetic stirrer for 1hr, after the addition of ethanol.In 40ml beaker, 25ml of water was mixed with 2% (0.5g) of sodium hydroxide (Naoh).Then distribute the solutions equally in two falcon tubes for centrifugation. Once filled, both tubes should have the same volume of solution and can be placed in the centrifuge for further processing.Centrifuge the solutions at 7000 RPM for 15 minutes at 11°C, for isolating and concentrating the nanoparticle from solution.

After centrifugation, a pellet is obtained from the solution. TPP is prepared at a concentration of 2mg/ml for the experiment. The pellet was dissolved in 8.33ml of solution by adding 4.16ml of sterile water followed by 4.17 ml TPP drop wise. After being isolated for 30 minutes, the mixture is centrifuged again at 7000 rpm for 15 minutes to separate any remaining particles or impurities. The obtained pellet is washed several times and then stored in cold conditions for 24 hours to maintain its stability. The initial concentration of LNP is 5mg/ml, and after several steps, the final concentration decreases to 4mg/ml.

4.2.3 Seed priming

Before priming, seeds are sterilized by immersing them in a 4% sodium hypochlorite for 10 minutes to ensure cleanliness and prevent microbial contamination. Seed priming in alfalfa involved soaking the seeds in LNP solution for 24 hours to enhance germination and early seedling vigor. Four distinct concentration of LNP are used to conduct priming in alfalfa seeds.

After the completion of seed priming, the seeds are for the next process. Germination, which was initiate their growth into plants.

4.2.4. NaCl solution preparation

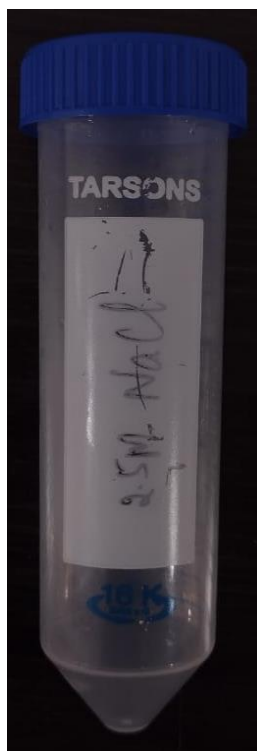


Fig 4.3 Stock solution of NaCl

The selected NaCl concentration is 250mM for the experiment. For the stock solution the NaCl solution prepared by dissolving 4.383 grams of NaCl in 30ml of water. Working standard is prepared from the NaCl stock solution, 25ml of NaCl is combined with 250ml of water creating a solution that was then used for germination.

4.2.5. Incubation of seeds for germination

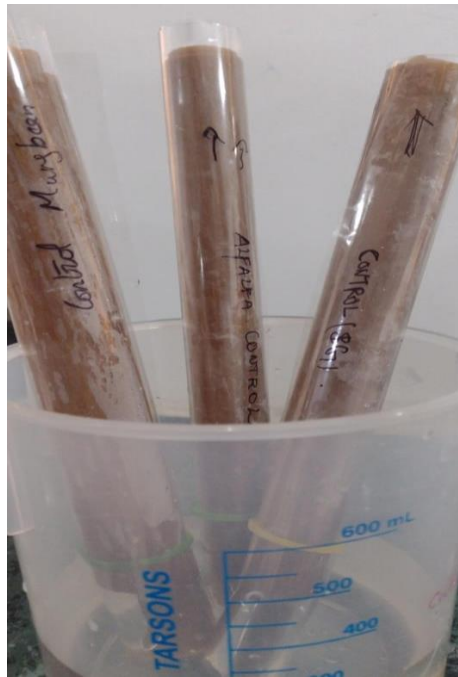


Fig 4.4 Seedlings of alfalfa without LNP treatment



Fig 4.5 Alfalfa seed priming with LNP under salt stress

After 24 hours of priming, the seeds are now prepared and ready to be placed for germination. For the germination process, a total number of 6 rolls of germination paper

have been prepared, encompassing a control group, stress conditions, and seed from four different concentrations of nanoparticle. IN the control group, 10 unprimed alfalfa seeds after sterilization with 2% hypochlorite, are positioned within the germination paper to facilitate germination, then bundled with a rubber band and positioned in a beaker containing only 250ml of water. IN a beaker containing with 250ml of distilled water combined with 25ml of Nacl stress as mentioned in working standard, 10 unprimed alfalfa seeds after sterilization with 2% hypochlorite, are positioned within the germination paper that facilitate the germination, then bundled with rubber band to avoid opening of germination paper.

The primed seeds (10) with a concentration of 5µg LNP are positioned within germination paper to facilitate germination and are placed in the beaker under 250mM of Nacl stress. From the seeds are primed with a concentration of 25µg LNP, 10 seeds are positioned within germination paper to facilitate germination and are placed under 250mM of Nacl stress. The primed seeds (10) with the concentration of 50µg LNP are positioned within germination paper to facilitate germination and placed in the beaker under 250mM Nacl stress. Under 250mM Nacl stress, from the seeds primed with the 100 µg LNP, 10 seeds are positioned within germination paper to facilitate germination, then bundled with rubber band.

These seeds are kept for germination for 7 days at a temperature range of 27⁰ C - 30⁰C. The water and stress levels in the beaker, where the seeds are positioned, are changed at the interval of 2 days to ensure there is no contamination and to maintain a consistent stress concentration. After 7 days of germination, further analysis including germination rate, root and shoot length, dry weight, fresh weight and seed vigor etc are conducted.

4.2. 6. ROS Accumulation



Fig 4.6 Separated root tips of Alfalfa treated with LNP under salt stress

- 2'-7' - Dichlorodihydrofluorescein diacetate

Dilute DCFDA stock solution in a suitable buffer or medium, typically, a final concentration of 10-50 μM is used. Excise the root tips from the each group of alfalfa plants (control, stress, four distinct concentration). Ensure they are intact and healthy. Place the root tips in a small container of well plate and cover them with the prepared DCFDA working solution. Incubate them in the dark for about 30 minutes to allow the DCFDA to penetrate the cells and be cleaved by intracellular esterases, resulting in the formation of the fluorescent compound DCF (2171- dichlorofluorescence. After the incubation period, wash the root tips with a buffer or medium to remove excess DCFDA. Mount the root tips a microscope slide and observe them under a fluorescence microscope equipped with appropriate filters for detecting DCF fluorescence. ROS accumulation was be indicated by the intensity of green fluorescence emitted by DCF. Quantify the fluorescence intensity using image analysis software to compare ROS levels among different samples or conditions.

4.2. 7.CHLOROPHYLL CONTENT

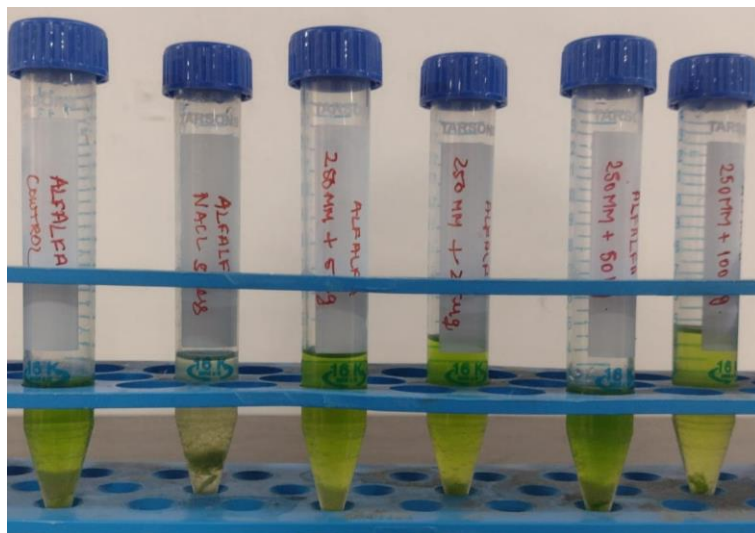


Fig 4.7 chlorophyll extract of Alfalfa treated with LNP under salt stress

Chlorophyll content was estimated following the protocol. Briefly, 0.1 gm leaves were homogenized in 5.0 ml of acetone and centrifuged at 4000 rpm for 20 min. The supernatant was collected, and the residue was re-extracted twice with 5ml of acetone and centrifuged. The supernatants were combined and volume was made to 20 ml with 80% acetone.

4.2.8. Antioxidant assay

The leaves of alfalfa seedlings were homogenized on ice using a motor and pestle in a 0.1M potassium phosphate buffer (pH 7.0), followed by the centrifugation at 12000 rpm for 15 minutes at 4°C. The resulting supernatant was utilized for enzyme assays (CAT, SOD, and APX).

CHAPTER-5

RESULT AND DISCUSSION

5.1. FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPY RESULT OF LAMINARIN NANOPARTICLE

The FTIR spectrum of *laminarin* nanoparticles extracted from *laminarin digitata* would display characteristics peaks corresponding to the functional groups present in *laminarin*. Since *laminarin digitata* is a brown seaweed rich in *laminarin*, the FTIR spectrum would likely show peaks associated with polysaccharides.

➤ These may include

- O-H stretching vibration typically observed around 3200-3600 cm^{-1} .
- C-H stretching vibration seen around 2800-3000 cm^{-1} .
- C-O stretching vibration manifests around 1000-1200 cm^{-1} .
- β – glycosidic linkage characteristic peak around 900-1000 cm^{-1} .
- β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 6) glycosidic bonds peak in the fingerprint region below 1500 cm^{-1} can provide information about the specific bonding pattern and branching in the *Laminarin* structure.

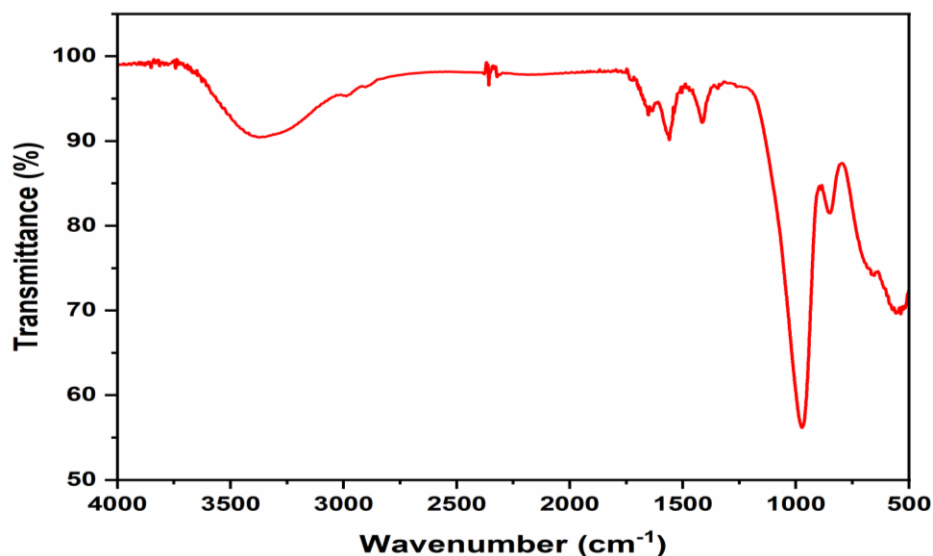


Fig 5.1 FTIR Spectrum of Laminarin nanoparticle

5.2. SCANNING ELECTRON MICROSCOPY RESULT OF *LAMINARIN* NANOPARTICLE

SEM (scanning electron microscopy) result provides insights into their size, shape, surface characteristics, and distribution. It could help to understand their physical properties, which are crucial for various applications such as drug delivery, biomedical imaging, and environmental remediation. SEM images can reveal the size distribution and shape of the nanoparticles, whether they are spherical, rod-shaped, or, irregular. Here in this SEM image the size is spherical. SEM image shows the homogeneity of the nanoparticle population, indicating the uniform in size and shape or if there are variations.

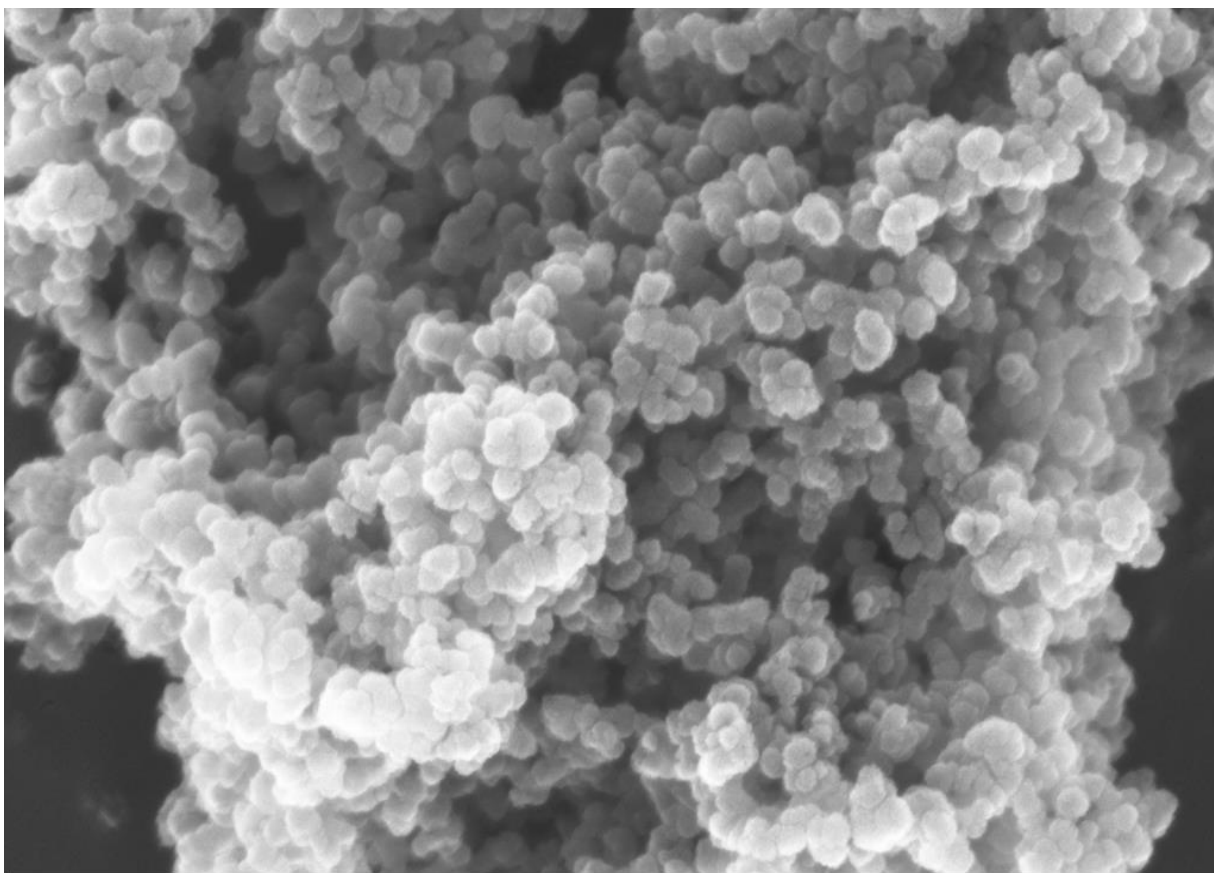


Fig 5.2 SEM Image of Laminarin nanoparticle

5.3. ROOT LENGTH

The table 5.3 represents the root length of the germinated alfalfa plants, which is taken after the seven days kept for germination. The measuring scale is used to determine the lengths of the plant's roots in centimeters. Fig 5.3 shows the graphical representation of root length and its standard deviation. The highest root length is 11.5 cm observed in 250mM NaCl+100µg LNP and the lowest root length is observed in NaCl stress.

ALFALFA	LENGTH (CM)
CONTROL	6 ± 1.63
NACL STRESS	4 ± 0.81
5µg LNP	6.5 ± 0.84
25 µg LNP	8 ± 1.63
50 µg LNP	8.5 ± 1
100 µg LNP	11.5 ±1.23

Table 5.1 Root length of alfalfa seedlings treated with LNP under salt stresss

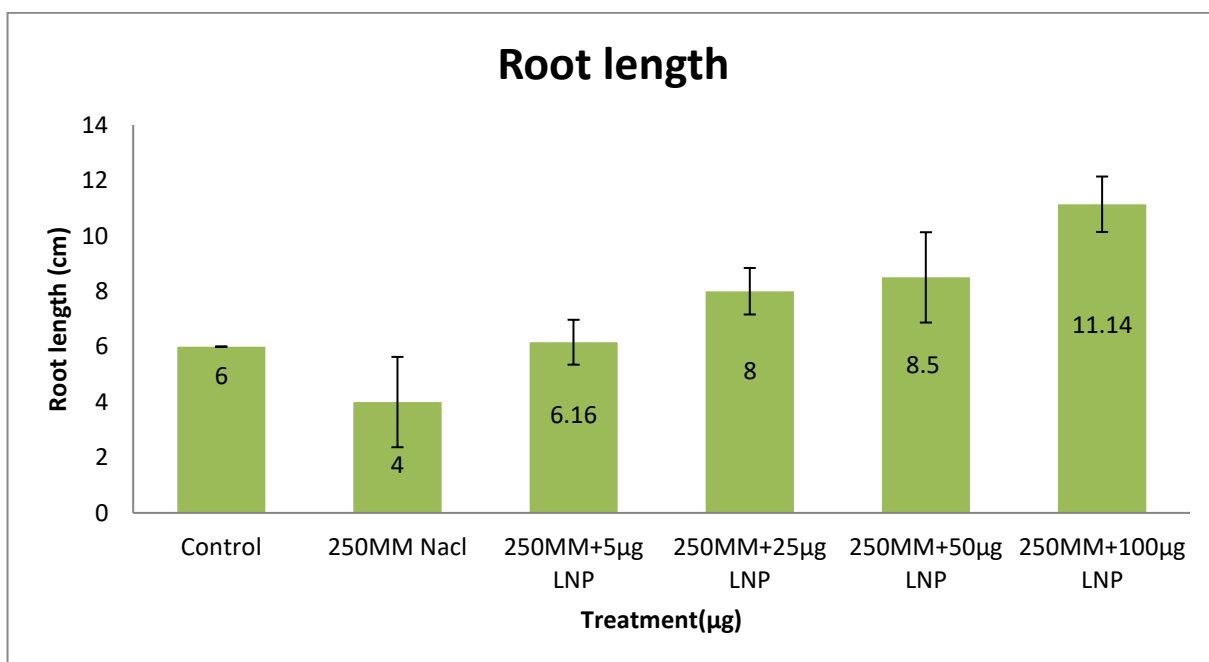


Fig 5.3 Effect of LNP priming on root length of alfalfa under salt stress

5.4. SHOOT LENGTH

The table represents the shoot length of germinated plants, which is taken after 7 days of germination. The measuring scale is used to determine the plant's shoots length in centimeters. Figure 5.5 shows the graphical representation of standard deviation of shoot length. The highest shoot length is 5cm observed in 250mM+50 μ g *Laminarin* and the lowest shoot length is observed in 250 mM NaCl+100 μ g LNP.

ALFALFA	SHOOT LENGTH(CM)
CONTROL	4 $\pm$ 1.24
NACL STRESS	3.5 $\pm$ 0.84
5 μ g LNP	4 $\pm$ 1.63
25 μ g LNP	3 $\pm$ 1.24
50 μ g LNP	5 $\pm$ 1.63
100 μ g LNP	2 $\pm$ 1.24

Table 5.2 Shoot length of alfalfa seedlings treated with LNP under salt stress

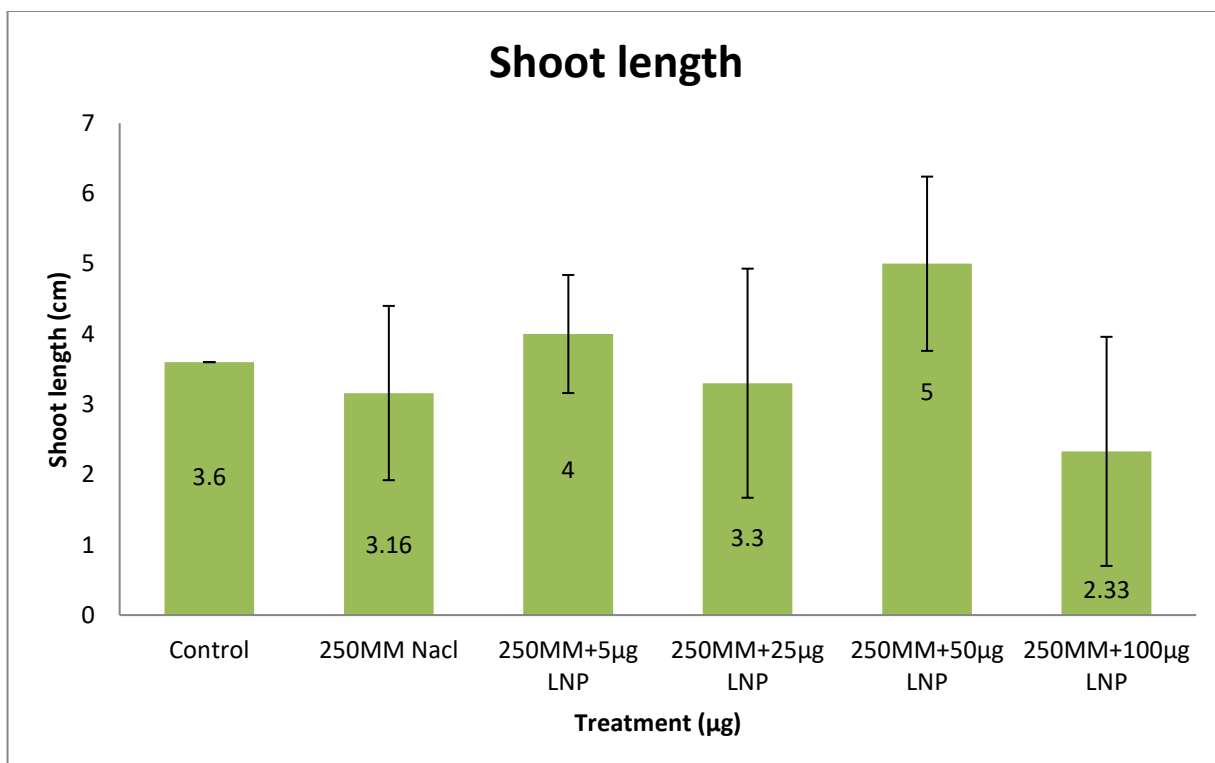


Fig 5.4 Effect of LNP priming on shoot length of alfalfa under salt stress

5.5. DRY WEIGHT

The alfalfa plants are kept in incubator for 48 hours at 80°C to make the plants dry completely. Then the dry weight is measured using the weighing machine in milligrams(mg). The table 5.3 represents the dry weight of primed plants under salt stress. With the graphical representation of S.D of dry weight , the highest dry weight 3.2 ± 0.16 is observed in 250 mM NaCl+25 μ g LNP and the lowest dry weight 2.1 ± 0.07 is noted in 250mM +5 μ g of germinated plants.

ALFALFA	DRY WEIGHT(MG)
CONTROL	2.4 ± 0.16
NACL STRESS	2.7 ± 0.12
5 μ g LNP	2.1 ± 0.07
25 μ g LNP	3.2 ± 0.16
50 μ g LNP	3.0 ± 0.12
100 μ g LNP	1.5 ± 0.07

Table 5.3 Dry weight of Alfalfa seedlings treated LNP under salt stress

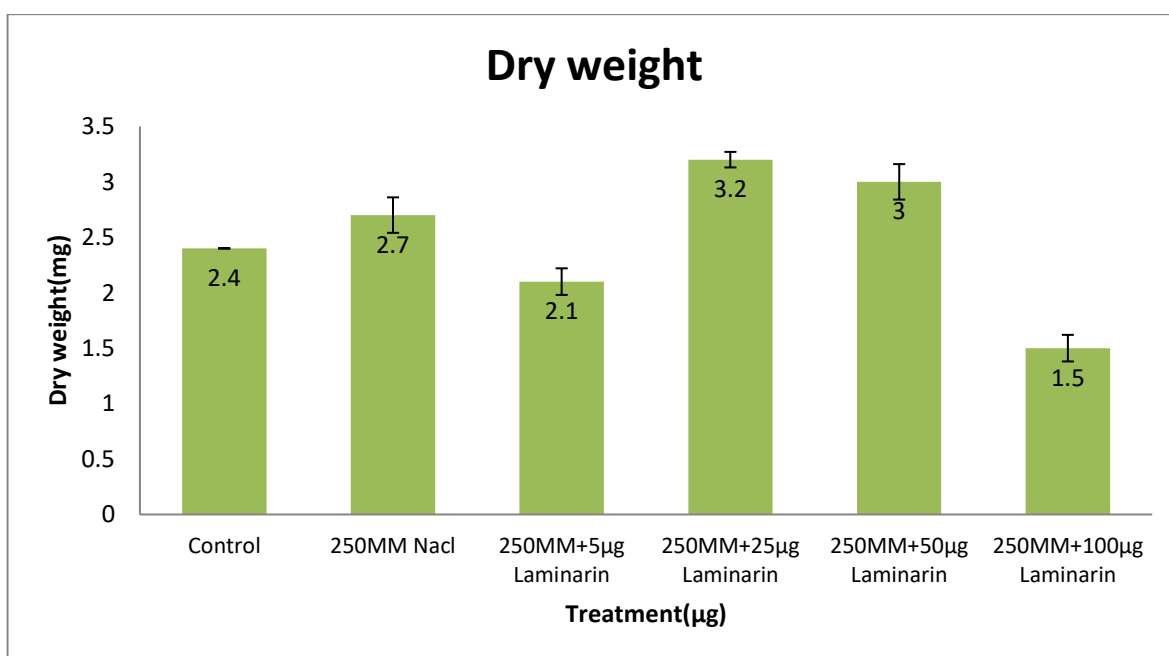


Fig 5.5 Effect of LNP priming on dry weight of Alfalfa seedlings under salt stress

5.6. FRESH WEIGHT

After 7 days of germination the plants, firstly the plants weight are observed and it is known as fresh weight. Fresh weight of alfalfa plants is measured using the Weighing machine in milligrams. With this results Table 5.3 and figure 5.6 that shows the difference between the dry weight of plants under salt stress, the highest fresh weight 36.8 ± 0.18 is observed in 250 mM NaCl + 50 μ g LNP and the lowest fresh weight 15.5 ± 0.12 is observed in NaCl stress.

ALFALFA	FRESH WEIGHT(mg)
CONTROL	36.9 ± 0.11
NACL STRESS	15.5 ± 0.12
5 μ g LNP	20.2 ± 0.07
25 μ g LNP	26.7 ± 0.16
50 μ g LNP	36.8 ± 0.18
100 μ g LNP	19.7 ± 0.24

Table 5.4 Fresh weight of alfalfa seedlings treated with LNP under salt stress

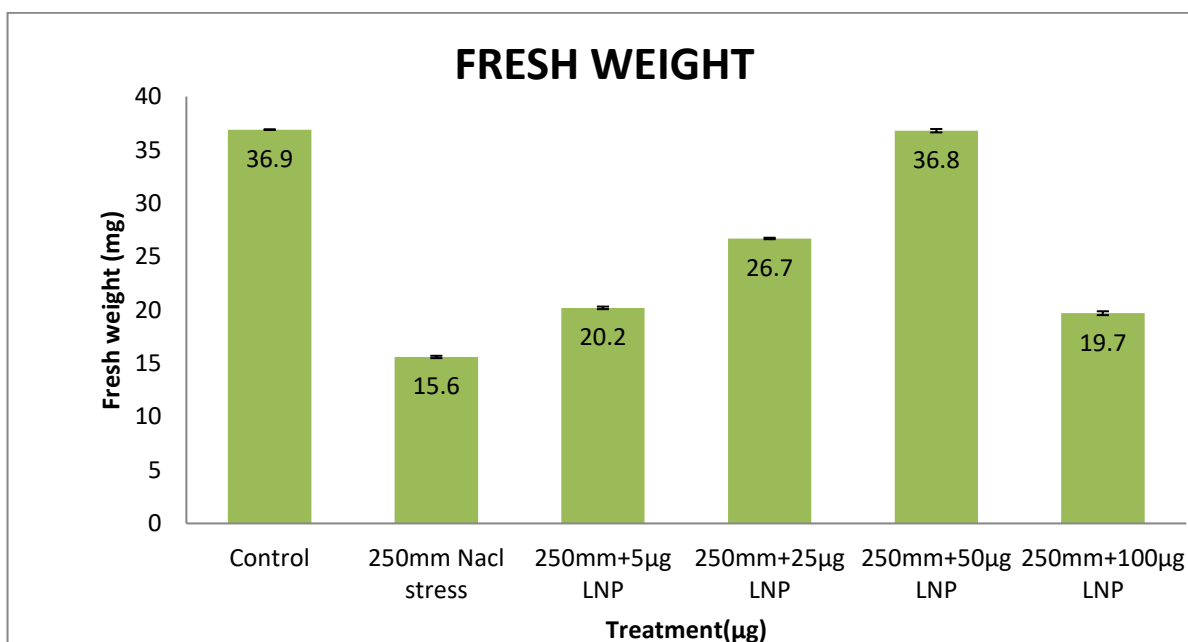


Fig 5.6 Effect of LNP priming on fresh weight of Alfalfa seedlings under salt stress

5.7. GERMINATION RATE

Germination rate of seeds are observed and noted in the intervals of 2 days. Germination rate is calculated with formula of

$$\text{No. of. Seeds Germinated} \div \text{Total no. of. Seeds in per plate} \times 10$$

The table 5.5 and figure 5.7 represents the germination percentage of alfalfa seeds. 90% is the highest germination rate observed in 5 µg and 50 µg concentrations of LNP under salt stress.

ALFALFA	GERMINATION RATE (%)
CONTROL	95 ± 0.2
NACL STRESS	30 ± 0.01
5µg LNP	35 ± 0.03
25 µg LNP	40 ± 0.08
50 µg LNP	77 ± 0.1
100 µg LNP	75 ± 0.12

Table 5.5 Germination rate of alfalfa seedlings treated with LNP under salt stress

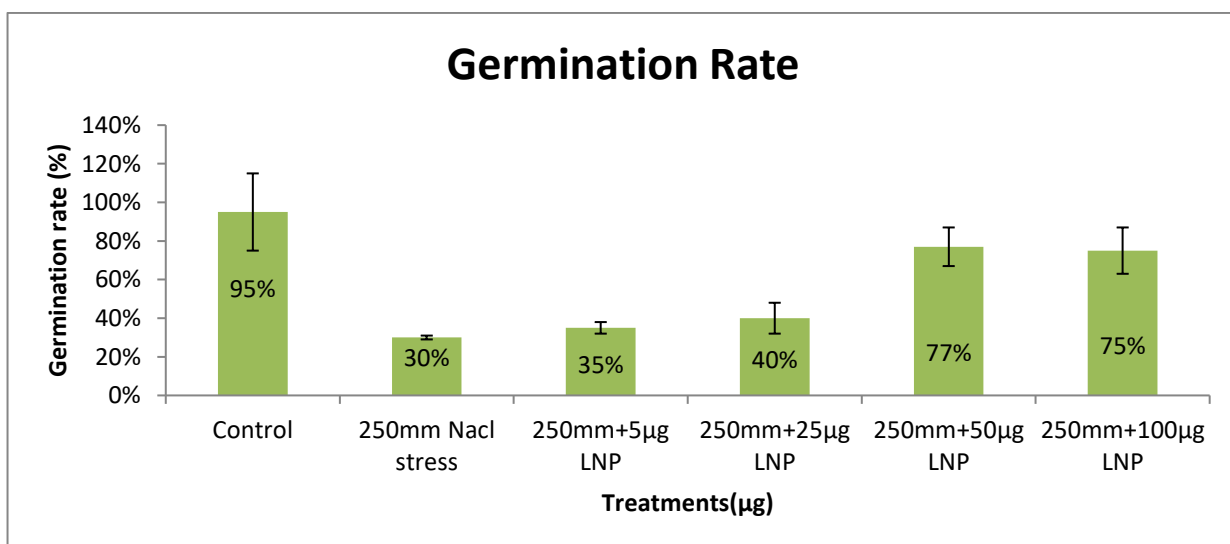


Fig 5.7 Effect of LNP priming on germination rate of Alfalfa seedlings under salt stress

5.8. VIGOR INDEX

This table and graph represents the vigor index of the germinated seeds. The vigor rate was calculated with the formula

$$\frac{\text{Root length} + \text{shoot length} \times \text{germination rate}}{100}$$

The highest seed vigor was found in the 50 μ g concentration of LNP under 250mM salt stress.

ALFALFA	VIGOUR RATE
CONTROL	7 $\pm$ 2.7
NACL STRESS	6 $\pm$ 1.9
5 μ g LNP	9.45 $\pm$ 2.1
25 μ g LNP	7.7 $\pm$ 2.08
50 μ g LNP	12.1 $\pm$ 2.6
100 μ g LNP	10.6 $\pm$ 2.2

Table 5.6 Vigor index of Alfalfa seedlings treated with LNP under salt stress

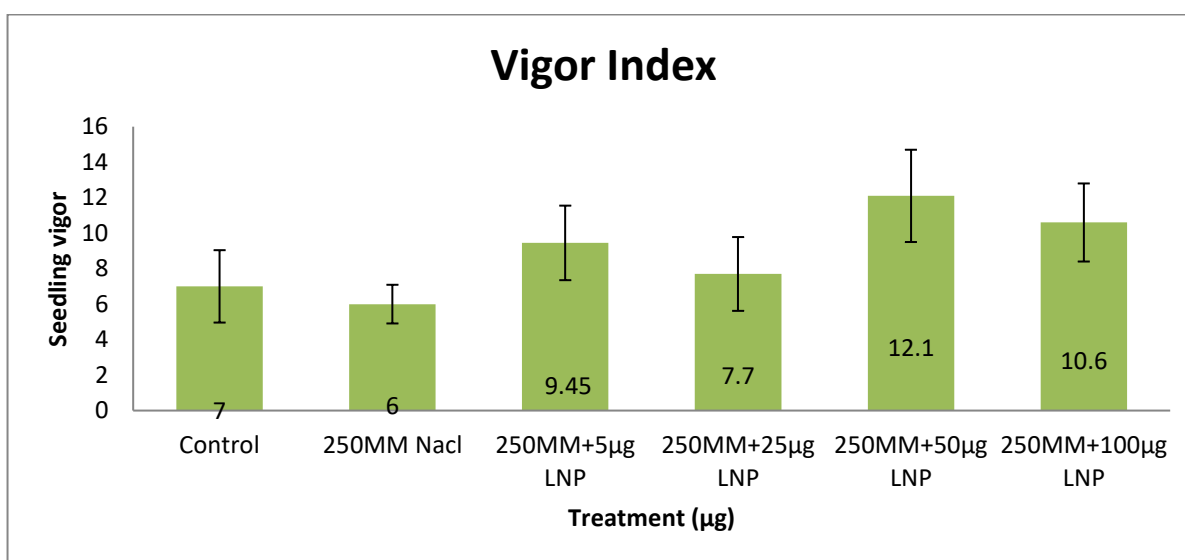


Fig 5.8 Effect of LNP priming on Vigor index of Alfalfa seedlings under salt stress

5.9. THE RESULT OF ROS ACCUMULATION

Root tips are stained with Dichlorodihydrofluorescein diacetate (DCFDA) to measure the ROS accumulation on the root tips in response to stress condition. DCFH-DA Dye enables us to visualize the ROS accumulation in the early seedlings. The bright fluorescence green in the root tips indicates the ROS accumulation in the tips. High level of ROS accumulation was observed at unprimed seedlings grown under salt stress, due to induced stress damaged roots. The root tips of seedlings primed with $100\ \mu\text{g L. np}$ concentration have no ROS accumulation. The other three concentrations ($5\ \mu\text{g L. np}$, $25\ \mu\text{g L. np}$, $50\ \mu\text{g L. np}$) have low level of ROS accumulation.

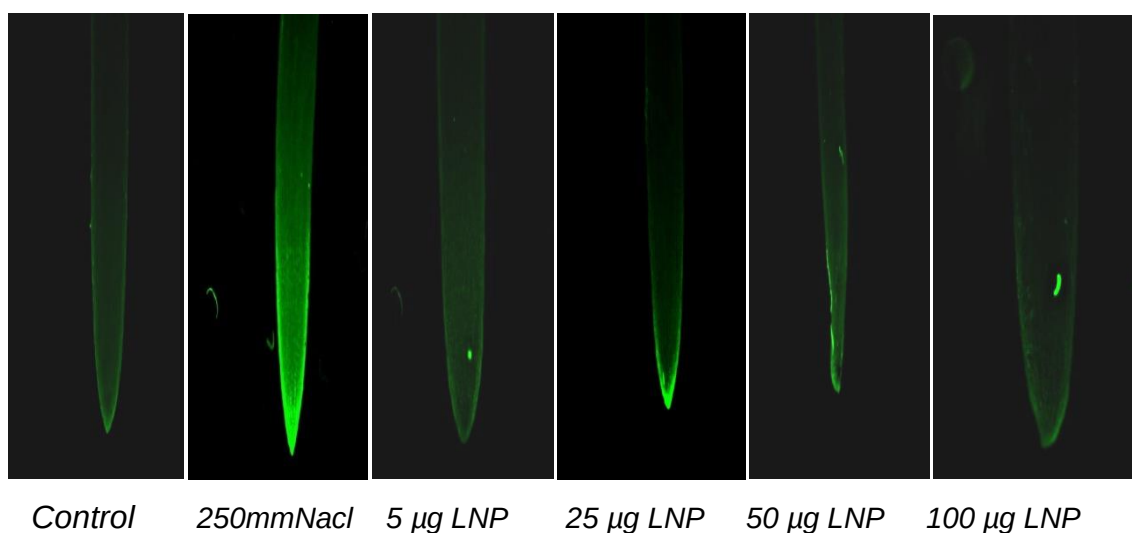


Fig 5.9 ROS accumulation in root tips of alfalfa treated with LNP under salt stress

5.10. Antioxidant enzyme activity

5.10.1 Catalase Activity

CAT activity would indicate the ability of alfalfa seedlings to scavenge hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) produced under stress condition. Treatment with 250 mM NaCl brought about a significant decrease in the activity of CAT when compared to control conditions. It was found that a significant increase in its activity under *Laminarin* nanoparticle treatment.

ALFALFA	CAT ACTIVITY(Units/mg)
CONTROL	4 ± 2.07
NACL STRESS	2 ± 0.7
5 μg LNP	5 ± 1.2
25 μg LNP	6 ± 1.4
50 μg LNP	8 ± 1.6
100 μg LNP	15 ± 3.3

Table 5.7 Effects of LNP priming on CAT activity of alfalfa under salt stress

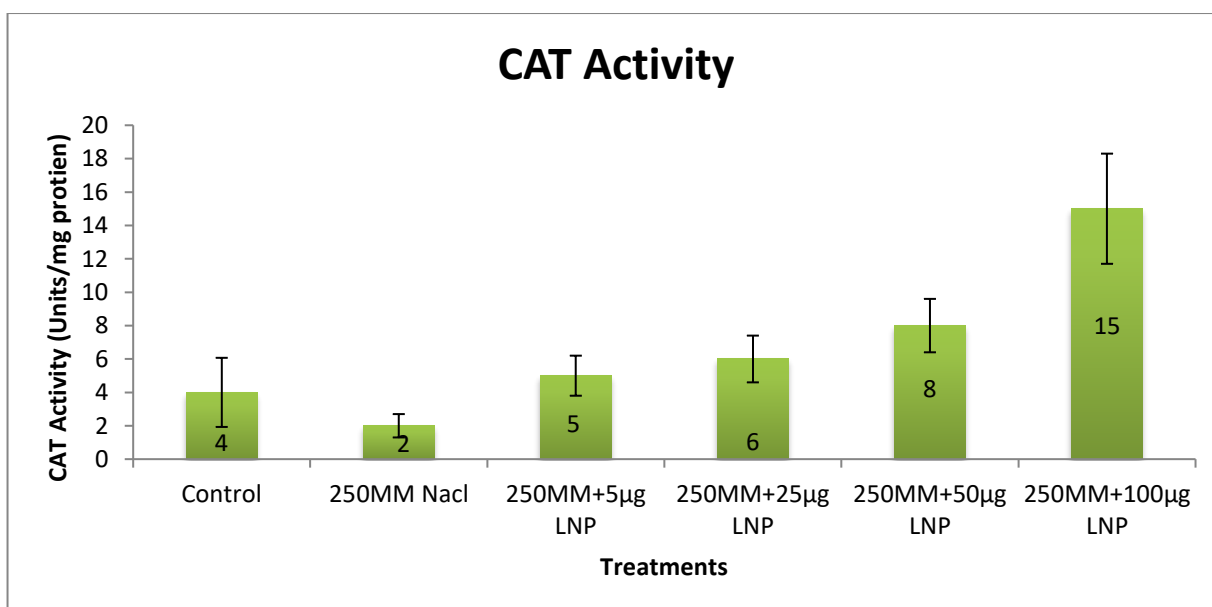


Fig 5.10 Effect of LNP priming on Catalase of Alfalfa seedlings under salt stress

5.10.2 Superoxide Dismutase(SOD) Activity

SOD activity would reflect the capacity of alfalfa to dismutate Superoxide radicals into oxygen(O_2) and H_2O_2 . Increased SOD activity would indicate enhanced ROS scavenging capacity and decreased damage to lipids of the plasma membrane under stress. Treatment with 250 mM NaCl brought about a significant decrease in the activity of SOD when compared to control condition. It was found that a significant increase in SOD activity in control and slight increase in its activity under *Laminarin* nanoparticle treatment.

ALFALFA	SOD ACTIVITY(Units/mg)
CONTROL	80 $\pm$ 2.44
NACL STRESS	13 $\pm$ 1.63
5 μ g LNP	64 $\pm$ 2.64
25 μ g LNP	70 $\pm$ 2.8
50 μ g LNP	55 $\pm$ 3.08
100 μ g LNP	90 $\pm$ 3.3

Table 5.8 Effects of LNP priming on SOD activity of alfalfa under salt stress

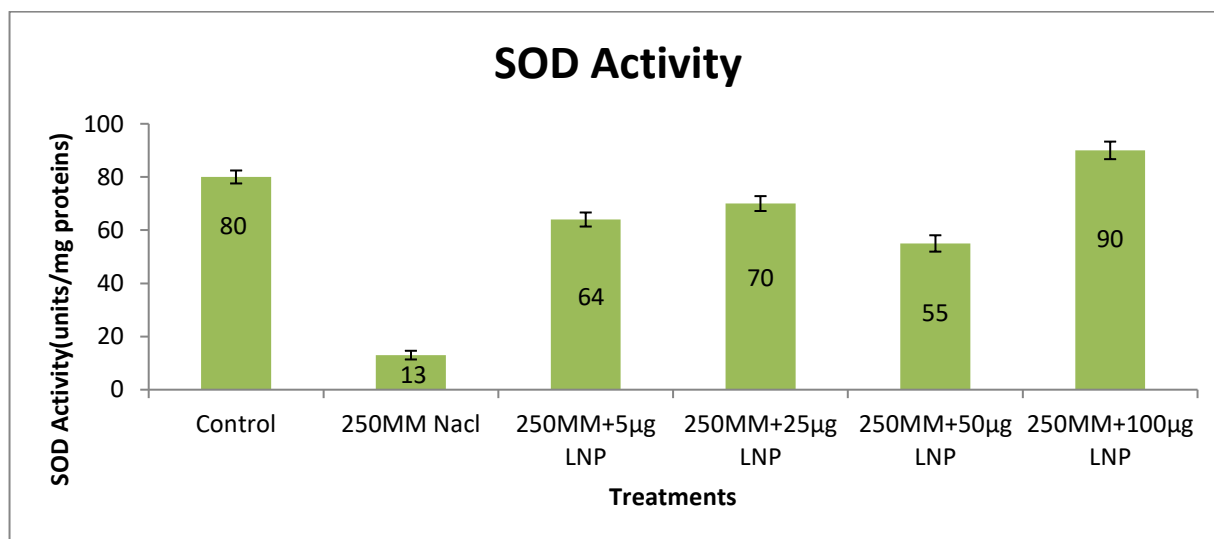


Fig 5.11 Effect of LNP priming on Superoxide dismutase of Alfalfa seedlings under salt stress

5.10.3 Ascorbate Peroxidase(APX) Activity

APX activity would show the ability of alfalfa to detoxify H_2O_2 by utilizing ascorbate as a specific electron donor, thereby preventing oxidative stress. Elevated APX activity would imply improves ROS scavenging and tolerance to salt stress. Treatment with 250 mM NaCl brought about a significant decrease in the activity of APX when compared to control conditions. It was found that a significant increase in APX activity in control and slight increase in its activity under *Laminarin* nanoparticle treatment.

ALFALFA	APX ACTIVITY(Units/mg)
CONTROL	3.3 ± 2.03
NACL STRESS	2 ± 0.7
5µg LNP	3.6 ± 1.2
25 µg LNP	6 ± 1.6
50 µg LNP	7 ± 1.8
100 µg LNP	7.6 ± 2.4

Table 5.9 Effects of LNP priming on APX activity of alfalfa under salt stress

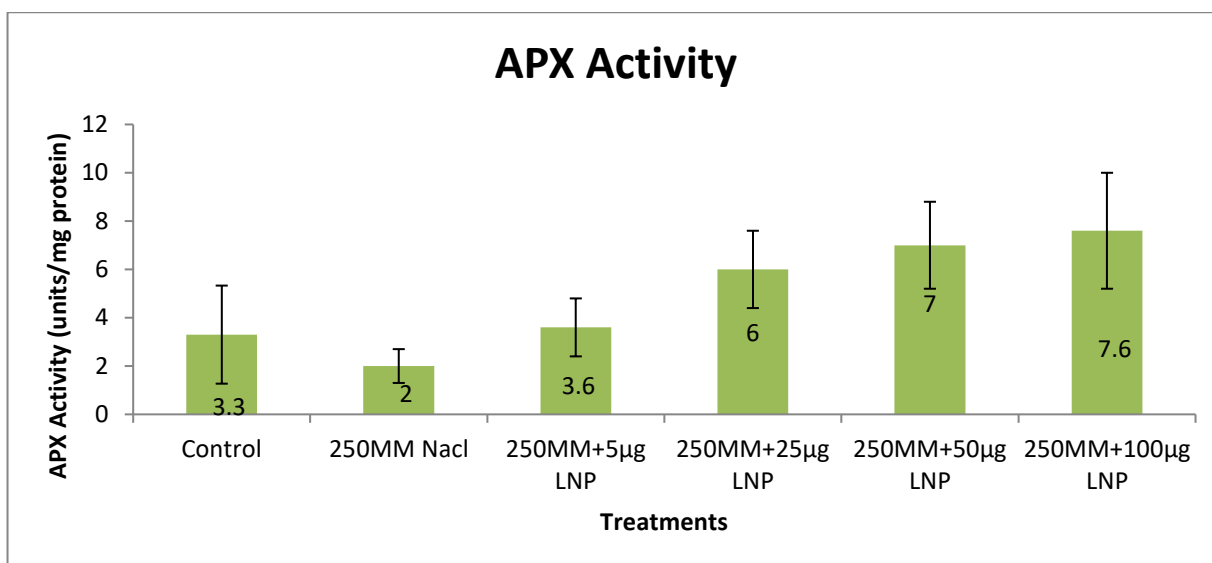


Fig 5.12 Effect of LNP priming on Ascorbate Peroxidase of Alfalfa seedlings under salt stress

5.11 CHLOROPHYLL CONTENT

Chlorophyll, a green pigment responsible for photosynthesis, plant tissue. In this experiment, measuring chlorophyll content can help us to assess how the presence of LNP affects the ability of alfalfa plants to photosynthesize and grow under salt stress. The highest chlorophyll content is found in the 250 mM NaCl + 100µg LNP concentration and the lowest chlorophyll content is observed under stress condition.

ALFALFA	CHLOROPHYLL CONTENT(mg/g)
CONTROL	0.8 ± 0.2
NACL STRESS	0.36 ± 0.03
5µg LNP	0.5 ± 0.07
25 µg LNP	0.6 ± 0.09
50 µg LNP	0.8 ± 0.1
100 µg LNP	1.9 ± 0.48

Table 5.10 Effects of LNP priming on Chlorophyll content of alfalfa under salt stress

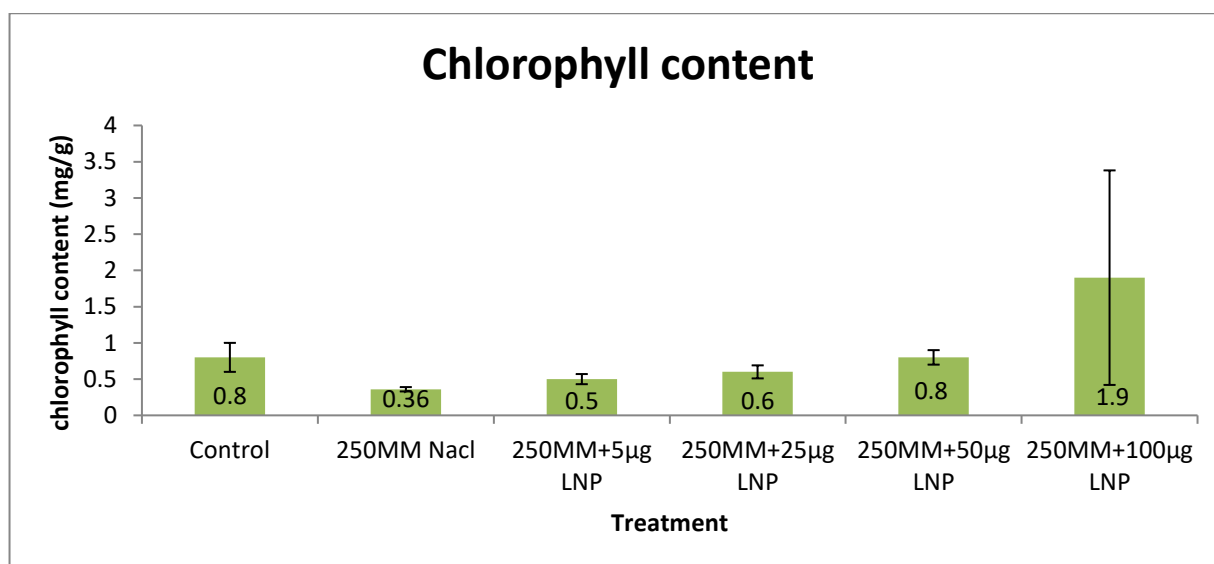


Fig 5.13 Effect of LNP priming on Chlorophyll content of Alfalfa seedlings under salt stress

CHAPTER-6

SUMMARY AND CONCLUSION

This study demonstrates the potential of *Laminarin nanoparticles* (LNP) extracted from *laminaria digitata* in mitigating the adverse effect of salt stress on the germination of *Medicago sativa* seeds. The seeds were primed with four concentrations of LNP (5µg, 25µg, 50µg, 100µg) to grow under 250mM NaCl stress. LNP was subjected to FTIR and SEM analysis. The germination period was taken over 7 days. After germination period statistical analysis such as root and shoot length, dry and fresh weight, vigor index, germination rate were observed and noted. Through this experimentation, it was observed a significant improvement in germination rates and seedling growth when primed with LNP compared to unprimed seeds under salt stress condition. Also seed priming as a effective result in overcoming abiotic stress condition. The ROS result demonstrates that lowest ROS accumulation is found in primed seeds under 5µg, 25µg, 50µg concentration of LNP and no ROS accumulation in 100µg concentration. It was found that under NaCl stress there is a increased activity of CAT, SOD and APX compared to control and decreased activity under 100µg LNP concentration. So the primed seeds contain high chlorophyll content at 100µg LNP concentration. These findings suggest that *laminarin* nanoparticles could serve as a promising tool for enhancing crop resilience to environmental stressors, such as salinity.

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