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Research Article

Evaluating the influence of salinity on antioxidant enzymes and phytase activity in germinating *Trigonella foenum graecum* L. (fenugreek) seeds and its immobilization by chitosan coated microspheres

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) an herbaceous legume known for its cholesterol-lowering, antidiabetic, mild antimicrobial, and emollient properties. This study was aimed in understanding the effect of exogenous treatment of sodium chloride (NaCl) levels (25, 50, 100, 150, and 200 mM) impact on seed germination, seedling length, seed vigour index, and antioxidant enzyme activities for ten days. The results revealed that increasing NaCl concentrations reduced seed germination percentage, germination index, and root and shoot lengths. The maximum activities of antioxidant enzymes like superoxide dismutase and catalase were 7.81 x 10^2±0.47 µmole/min and 3.9 x 10^2±0.38 Units/mL on 6th day, ascorbate oxidase 0.42±0.198 Units/mL on 8th day. Acid phosphatase activity peaked on the 8th day with 13.60±0.47 Units/mL at NaCl of 100 mM treatment, whereas the activity of phytase was maximum at NaCl of 25 mM with 15.66±0.46 Units/mL on the 6th and 8th day leading to a significant rise in total phosphorus 68±0.52 µg/mL on 6th day followed by 8th day with 64±0.52 µg/mL. Phosphorus concentration remained balanced across all days and salt treatments, reflecting its indispensable role in biomolecule biosynthesis that is essential for seed development. This equilibrium was likely facilitated by the coordinated activities of phytase and acid phosphatase. Phytase had an approximate molecular weight of 66 kDa. Microencapsulated phytase characterized using scanning electron microscopy, revealed spherical microspheres with diameter averaging 10.52 µm. The surface morphology of microspheres was resembling leaf veins with a uniform pore size of 1.64 µm. Fourier transform infrared spectra confirmed the functional groups shared by chitosan and alginate. Under salt stress, increased activities of enzymes like phytase and acid phosphatase, together with balanced phosphorus levels are vital for successful seed germination ensuing successive plant growth.

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INTRODUCTION

Salinity affects about 7% of the global landmass. About 20% of the land covering nearly about 230 million hectares under cultivation are impacted by salt deposition [1]. The prevalence of salinity may rise in the future due to factors like mismanaged irrigation systems, changes in the climate, and inadequate and unsustainable land management. Currently, about 90% of the global plant-derived food supply is obtained from nearly 30 different crop species, and most of them are sensitive to salt. They have experienced substantial loss in yield even under moderate salt environmental conditions and fail to meet the worldwide food demands [2, 3].

Salinity stress is a major abiotic factor that can impair germination of seeds, leading to decreased germination rates by slowing their germination periods, [4, 5] and reduce the seedling growth, as observed in rice and wheat varieties [6]. It lowers the osmotic potential of rhizic water, thus impeding uptake of water by dry seeds. Additionally, excessive Na^+ and Cl^- ions can cause ionic salt stress and toxicity, disintegrating biochemical pathways including the metabolism of proteins, nucleic acid, that can indirectly affect energy production, and cellular respiration [3, 5]. To adjust and manage with these stresses, plants recruit different mechanisms such as altering their physical characteristics, growth patterns, and physiological and biochemical functions simultaneously altering the activity of biologically significant enzymes [7-9].

In salinity stress circumstances, ROS generation rises, leading to oxidative damage and disruption of normal metabolism [10, 11]. It can affect vital cellular macromolecules and cell structure hindering seed germination [12, 13]. Hence, to mitigate stress-induced accumulation of reactive oxygen species (ROS), plants activate ROS-detoxifying enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate oxidase (AO), peroxidase, polyphenol oxidase (PPO), and glutathione reductase (GR) [14]. These collectively act as a defensive enzyme barrier whose goal is to shield cells from oxidative harm. A plethora of studies have revealed that increased SOD, AO, and CAT activities are closely associated with the plants› ability to withstand salinity [15, 16].

Among the various enzymes produced during germination, phosphatase enzymes play a significant role in regulating the availability of soluble inorganic phosphorus (Pi). A significant portion of the phosphorus in seeds is stored as phytic acid, accounting for 1-5% dry weight of edible legumes, cereals, oil seeds, nuts and pollens. Phytate can occur in various forms, ranging from soluble monovalent cations (E.g., Sodium phytate) [17] to insoluble polyvalent cation (E.g., Iron phytate) [18] and from dissolved to precipitated states [19]. The metabolic cost of producing phytic acid is high, as it requires six distinct ATP- requiring phosphorylation's per mol of product. It is a salt of *myo*-inositol hexakisphosphate or IP_6. [19, 20]. Plant seeds sequester phosphate as phytic acid during post-maturation, which is frequently localized within the vacuolar compartment. It is mobilized from the reserves during the initial stages of germination, growth of the seedling and, post-germination and is viewed as an antinutrient and an antioxidant [20-22].

In seeds and seedlings, phosphatases provide inorganic phosphate during germination that supports their growth. Various forms of phosphate esters are stored in the seeds and seedlings must be broken down by hydrolysis [23]. The acid phosphatase enzyme could resist salt stress and simultaneously maintain adequate levels of inorganic phosphate [24]. However, the role played by plant phosphatases is not yet fully understood [24 - 26].

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) a phosphomonoester, is capable of hydrolysing phytic acid and various other phosphoester compounds [27]. It is extensively distributed in microbes, tissues of plants and animals [28 - 30]. During germination, the possible increase in phosphates may be attributed to phytase activity resulting in the breakdown of phytates to release inositol and orthophosphate [31]. Very few studies have reported that, the phytase activity reaches maximum during germination [32]. Whereas in animals, nutrient absorption and bioavailability are reduced because of elevated phytic acid levels. Though it is resistant to the human digestive system, phytic acid is susceptible to dietary plant phytases and to phytases that are synthesized by enteric microbes [33].

This study used, Fenugreek seeds (*Trigonella foenum-graecum* L.) a versatile yearly flowering leguminous plant. It belongs to Fabaceae family, that produces autogamous blooms cultivated globally for their medicinal properties. This crop is cultivated widely in China, India, Egypt, Ethiopia, Morocco, Ukraine, Greece, Turkey, and other places. The nativity of this plant species extends from Iran through northern India. The two most prominent fenugreek species within India are *Trigonella foenum-graecum* (commonly named as Methi) and *Trigonella corniculate* (commonly called as Kasturi methi) [34]. The growth habitat of fenugreek is ambiguous; it persists with its growth properties even during severe frost or during desiccation. The nutritional content of fenugreek seeds includes 32% and 13% of insoluble and soluble dietary fibre, 36% protein, and 6%, 3%, 1.6% and 0.4% of oil, ash, starch and sugar respectively [35].

Fenugreek seeds therapeutic benefit is due to the availability of various biochemical phytoconstituents precisely studied in different research. Numerous pharmacological properties, including those related to fenugreek, are hypoglycaemia, hypocholesterolaemia, gastroprotection, chemoprevention, antioxidant, anti-inflammatory, antipyretic, and hunger stimulation [36]. Fenugreek has diverse applications beyond its medicinal properties. Various components are also used as flavouring and stabilizing agents in the food manufacturing industry, and as ingredients in adhesives, beauty aids, paper, and in paint products.

Recently, fenugreek has been recommended as a feed additive for fish in aquaculture [37]. The sustainability of aquaculture is dependent on fish growth, body composition, and reproductive ability in enhancing its productivity and maximizing its breeding ability [38]. Dietary interventions in aquaculture can significantly influence its growth efficiency, fecundity, fertilization, hatching rates, and disease prevention. Nutritional formulations with fenugreek can optimize fish health and development rather than hormonal applications which can create an imbalance in the ecosystem and sustainable food production systems [39].

Its wide distribution across diverse climates suggests significant genetic variation. Furthermore, as an under-bred crop, fenugreek likely possess a few more promising sets of genes for coping with environmental stresses. Despite rapid scientific advancements, fenugreek has received limited research attention. Hence, to unlock its full potential and facilitate improvements, more crucial studies at the biochemical and molecular level are essential. Identifying valuable genes for breeding programs, enabling site-specific mutations for genetic enhancement of ROS scavenging enzymes and specific phosphatases to overcome abiotic stresses can be explored. Furthermore, the use of fenugreek as green manure, fodder, and for soil reclamation, particularly in arid agricultural systems, warrants further investigation and promotion [40].

Immobilized phytase has been explored for diverse applications, including its use in amperometric biosensors for phytate detection in fertilizers [41], as a resource of exogenous inorganic phosphate (Pi) [42], as feed [43], as bioreactor [44], and to reduce phytate levels in soymilk [45]. Relatively few investigations have been conducted on the immobilization of plant phytase isolated from germinating fenugreek seeds. The process of immobilization requires natural biopolymers like cellulose, dextran, xanthan, agarose, chitosan, alginate, pectin, and various other polymers. Natural biopolymers are used regularly as they are non-toxic, abundant, biocompatible, biodegradable, flexible and have multifunctional applications. Below Table 1 explains the advantages and limitations of a few biopolymers obtained naturally [46-51].

Compared to other gels like pectin, cellulose, agarose, polyacrylamide, and gelatin, alginate offers superior biocompatibility and a more controlled release of enzymes. Agarose gels provide good mechanical strength but may lack the gentle encapsulation properties when compared to alginate. Polyacrylamide gels, although exhibit high porosity, they may pose potential toxicity risks, limiting their application in food and pharmaceutical industries. Gelatin is biodegradable and cost-effective but lacks the structural integrity required for long-term immobilization. Thus, alginate›s ability to retain enzyme activity while providing a protective microenvironment makes it a preferred choice for immobilization studies [52, 53]. To enhance immobilization, chitosan derived from chitin can be used. Hydrophilicity and biodegradability of chitosan, renders it a promising material for enzyme immobilization [54].

The observations and findings of this investigation can have broad significance and applications in agricultural biotechnology, enzyme technology, for a sustainable agriculture. The demonstrated resilience of fenugreek seeds under saline stress, with the enhanced stability of phytase via chitosan-coated alginate microspheres, opens the possibilities for improving crop production in saline soils. Additionally, the successful enzyme immobilization method can exhibit potential applications in the food, pharmaceutical, and environmental sectors for biocatalysis and nutrient management [55]. Fenugreek seeds have shown significant

Table 1. Advantages and limitations of naturally obtained biopolymers

Biopolymer	Advantages	Limitations	Reference
Chitosan	Non-toxic, biocompatible, biodegradable, antimicrobial activity, increases the shelf-life of fruits, has efficient mucoadhesive properties	Soluble in mild acidic solution	46
Alginate	Non-immunogenic, non-toxic, biodegradable, can be used as a gelling, emulsifying, and stabilizing material in the food manufacturing industry, antioxidant and has anti-inflammatory properties	Possibility to lose the immobilized compound due to pores, unstable at high pH	46
Pectin	Soluble in water, used as thickening and gelling agent, emulsifier and as a stabilizer	Less moisture barrier, low thermal stability, poor mechanical stability	47
Carrageenan	Increased bioavailability, improved retention rate of bioactive compound	Preparation and use in the delivery system require stability	48
Agarose	Thermostable	Denaturation of thermolabile compounds	49
Cellulose	Controlled release, and delivery	Requires a stable cross-linking agent	50
Cyclodextrin	Good encapsulation efficiency, bioavailability of the compound	Need a cross-linking agent	51

efficiency in tolerating salinity stress among the plant varieties that are experimented. To enhance seed germination and growth of plants under increasing salt conditions, more understanding on the activity of acid phosphatase and phytase is essential.

The present investigation was aimed to examine the impact of salt stress on the germination of fenugreek seeds by measuring the percentage of seed germination, seed vigour index (SVI), and the activities of free radical scavenging antioxidant enzymes, along with acid phosphatase, and phytase. This study was further extended to include the isolation, purification and immobilization of phytase from germinating seeds of fenugreek. A comprehensive understanding of this study could facilitate the optimization of germination conditions under salt stress and immobilization strategy, thereby enabling largescale production of fenugreek plants for its multiple benefits.

MATERIALS AND METHODS

Chemicals

The analytical grade chemicals to be employed for the investigation were procured from HiMedia and Merck.

Plant Material and Stress Treatments

Trigonella foenum graecum L (Fenugreek seeds) employed for the experiment were purchased from the local vendor. Seeds of uniform morphology were chosen and treated with ethanol (70% (v/v)) for two minutes for surface sterilization. This step was followed by second sterilization with 3% NaClO (sodium hypochlorite) solution for 20 minutes. The seeds were washed with double distilled water two or three times to remove the leftover traces of NaClO. Sterilized seeds were spread uniformly on a sterilized, labeled petri dish layered with filter paper. Each of the plates were treated with different concentrations of the salt (25, 50, 100, 150, and 200 mM NaCl) as labeled. A comparison of salt-treated seeds was made with control (untreated seeds). Petri plates were undisturbed and incubated at room temperature (RT). Regular monitoring of all the plates was done, and the plates were wetted with the solution of NaCl depending on the concentration labeled. Germination was observed for 10 days and noted at an interval of 48 hours. Each plate contained approximately 90 fenugreek seeds [56].

Germination Parameters

The emergence of a radicle indicates the seeds are germinating. Periodically, germinated seeds were enumerated for ten days with an interval of 2 days. We opted to determine enzyme activities during germination every other day instead of daily. This approach can effectively record biologically critical changes in metabolic pathways, preventing the detection of only minor, less important data. After the 10^{th} day, using a ruler growth of the seedling was monitored by noting the length of shoot and root in cm. The germination percentage [57] and seed vigour index (SVI) [58] of the seeds were determined per treatment using the formula

$$Germination\ percentage = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds}\ X\ 100$$

$$Seed\ Vigour\ Index\ (SVI) = Mean\ shoot\ length\ X\ Percentage\ of\ Seed\ Germination$$

Histochemical Staining

Germinated seeds were surface-sterilized using a one percent sodium hypochlorite (NaClO) and were subsequently incubated in a solution comprising 0.5 mM Nitro Blue Tetrazolium (NBT) prepared in phosphate buffer (100 mM, pH 7.0). All the tubes were sealed and left overnight for incubation [59].

Preparation of Enzyme Extract [Crude]

To prepare the crude extract, 2.0 g of germinating seeds from control and salt-stressed seeds were homogenized in a pre-chilled mortar and pestle with 20 mL phosphate buffer (0.1 M, pH 7.0). To get the extracts, the homogenates were filtered individually using two layered cheesecloths, and the filtrates were centrifuged at 10,000 rpm for 15 minutes at 4 °C. The clear supernatants were collected into clean, dry vials and stored at −20 °C until further use [60].

Assay of Antioxidant Enzymes

Catalase assay (CAT; EC 1.11.1.6)

For CAT assay, 8.0 mL volume of 0.05% H_2O_2 prepared in phosphate buffer (0.1 M) was treated with 50 µL of enzyme extract, mixed thoroughly, and incubated at RT for 60 seconds. The enzyme activity was terminated by adding 2.0 mL of 2% sulphuric acid. The remaining H_2O_2 was titrated against standardized potassium permanganate (0.02 M) until a pale pink colour appeared. Blank titration was performed with H_2O_2 and H_2SO_4 at zero time. One unit of CAT enzyme activity was defined as the amount of CAT enzyme required to decompose 1 µmol hydrogen peroxide per minute under specified assay conditions [61].

Superoxide dismutase assay (SOD; EC 1.15.1.1)

SOD activity was studied by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), a method of Yu & Rengel. To 500 µL of the seed extracts, add 1.0 mL of 125 mM Na_2CO_3, 400 µL of 24 µM NBT, and 200 µL of 0.1 mM EDTA and mixed thoroughly. To the prepared reaction mixture, add 400 µL of hydroxylamine hydrochloride (1 mM) to initiate the reaction. Change in the colour intensity was noted initially at zero minute and after 5 minutes at 560 nm spectrophotometrically. A control was run simultaneously without the seed extract. Activity of SOD was determined by measuring the quantity of enzyme required to inhibit 50% of the reaction under specified assay conditions [62, 63].

Ascorbate oxidase assay (AO; EC 1.10.3.3)

Ascorbate oxidase activity was assayed by spectrophotometric stop rate determination reaction where its molar coefficient value at λ max of 245 nm was used for calculation. Ascorbic acid standard with concentration ranging from 25 - 200 mM were treated individually with 0.1 mL of extracts and maintained at 25 ° C for five minutes. An enzyme blank was run simultaneously for each concentration of ascorbate solution where the enzyme was added into the blank after incubation. Change in absorbance was noted spectrophotometrically at 245 nm for test and blank. A control was simultaneously run without seed extract. One unit of ascorbate oxidase is explained as the quantity of AO required to catalyse the consumption of 10 µL of oxygen/ minute [64].

Acid phosphatase assay (EC 3.1.3.2)

Acid phosphatase acts on its substrate *p*-nitrophenyl phosphate (*p*NPP) to release its products namely *p*-nitrophenol (*p*NP) and free inorganic phosphate. Enzyme activity was recorded spectrophotometrically at 410 nm where the amount of product *p*- nitro phenol was determined using its molar extinction coefficient at λ max of 410 nm [65, 66]. To 500 µL of citrate buffer (100 mM) pH 4.8, 500 µL of 15 mM *p*-nitrophenyl phosphate was added, mixed, followed by the addition of 500 µL of the crude extract. Incubate the assay mixture for 10 minutes at 37 °C. Reaction was terminated by gradually adding 3.9 mL of 0.2 N sodium hydroxide turning it to alkaline condition. Blank was run simultaneously. One unit of acid phosphatase activity was to hydrolyse one micromole of the substrate *p*- nitrophenyl phosphate to its product per minute at pH 4.8, 37 ° C.

$$Enzyme\ activity = \frac{(Absorbance\ of\ Test - Absorbance\ of\ Blank)\ X\ 5.0}{10\ X\ 18.3\ X\ 0.5\ X\ Dilution\ factor}$$

where,

5.0=Assay volume (Total)

10=Assay time in minutes

18.3=Millimolar Extinction Coefficient (ε) of *p*-nitro phenol at 410 nm

0.5 mL=Enzyme extract volume used

Phytase assay

The activity of phytase enzyme was determined by estimating the concentration of phosphate (inorganic) released after the breakdown of the substrate phytate (commercially available in sodium form). Initiate the reaction by adding crude extract to 0.05 mL of 44.1 mM freshly prepared sodium phytate solution incubated for 30 minutes at 37 ° C. The reaction was arrested by the addition of 4.0 mL of CRS reagent [comprising of 5% (w/v) ammonium molybdate, 5N sulphuric acid, acetone in 1:1:2]. The mixture was swirled, and readings were noted at 400 nm spectrophotometrically. One unit of enzyme activity (U) was expressed as the quantity of enzyme phytase that liberates one millimole of phosphorus in 60 seconds under specified assay conditions [67-69].

Assay of Inorganic Phosphorus

Under acidic conditions, ammonium molybdate combines with inorganic phosphorus to make phosphomolybdate. In the presence of 1-amino-2-napthol 4-sulphonic acid [ANSA - Reducing reagent], phosphomolybdate reduces to a blue coloured complex of molybdenum with an absorption maximum at 660 nm. The blue colour intensity obtained is proportional to the concentration of phosphate. Standard phosphorus with concentration ranging from 6.2 to 31µg/mL was treated with one mL each of 5N sulphuric acid and ammonium molybdate followed by 100 µL of reducing reagent. Tubes were mixed thoroughly and the total volume of 10 mL was made with double distilled water [70]. All the extracts were treated in the same manner with appropriate dilutions.

PARTIAL PURIFICATION OF PHYTASE

Ammonium Sulphate Precipitation

Germinating fenugreek seeds were homogenized with sodium acetate buffer (100 mM, pH 5.0) and filtered through two layered muslin cloth. To separate the debris, centrifuge the homogenate at 4°C for 30 minutes at a speed of 10,000 rpm. The obtained clear supernatant was collected in a clean, dry, fresh vial. 30-60% ammonium sulphate precipitation was performed with the supernatant at 4°C. Centrifuge the saturated extract at a speed of 10,000 rpm for 20 minutes. The pellet was resuspended in ten mL of sodium acetate buffer. The resuspended pellet was dialyzed against the Tris HCl buffer (50 mM, pH 7.0) [71].

Anion Exchange Chromatography

Dialysate was layered on a Diethylaminoethyl (DEAE) Cellulose 52 column pre-equilibrated with Tris-HCl buffer (50 mM, 7.0). The flow rate was adjusted to 1.0 mL per minute. Collected fractions were subjected to spectrophotometric analysis by measuring each eluate absorbance at 280 nm. The fraction with maximum absorbance at 280 nm was subjected to determine the phytase enzyme activity.

Molecular Weight Determination by Page (SDS)

The separated proteins molecular weight was estimated by comparison with a protein marker using SDS-PAGE. 12% resolving gel with 8.8 pH was used to separate the proteins present in the samples [71,72].

Immobilization of Partially Purified Phytase Using Sodium-Alginate Coated Chitosan

200 mg of sodium alginate was prepared in 10 mL of Tris-HCl buffer (0.1 M, pH 8.0) by placing the beaker on a magnetic stirrer preheated to 30 – 40 ° C for 30 minutes. Once the alginate was dissolved, the partially purified phytase enzyme (0.5 mg/mL) was slowly mixed into

the alginate solution with continuous stirring. The mixture was stirred for nearly one hour on a magnetic stirrer for thorough homogenization of the enzyme and the alginate. In the following step, 0.5 gm of commercially available chitosan was added to 2% acetic acid solution (v/v). The beaker containing the solution was placed on a preheated hot plate set at 50 ° C to obtain a clear solution. To this 0.7 M calcium chloride solution was added slowly and kept for agitation at 50 to 55 ° C. To produce chitosan coated beads, the alginate-phytase mix was dropped, dropwise into the calcium chloride solution containing chitosan at a height of nearly one cm above the solution of calcium chloride. The appearance of white coloured beads indicates the formation of chitosan-coated encapsulated phytase beads. Beads were pooled together onto a petri plate. Immobilized phytase beads were further submitted for SEM and FTIR analysis to understand the surface morphology and to determine the type of functional groups respectively [43, 53, 72].

RESULTS AND DISCUSSION

One of the major environmental factors that hinders growth and development of plants is salinity. While salinity can negatively impact plants at all phases of their life cycle, germination and seedling emergence are particularly vulnerable to its effects [73]. It often disrupts germination of seeds and the seedling growth that is primarily attributed to osmotic stress and toxicity due to ions. The former can restrict water uptake while the latter disrupts cell processes [74, 75].

Fenugreek, a plant that thrives in the salty and dry regions of northeast India, is an effective model for investigating the mechanisms underlying salt-stress adaptation. To understand how antioxidant enzymes respond to increasing salinity, we examined the change in the activities of catalase, superoxide dismutase, and ascorbate oxidase in fenugreek seeds that were exposed to exogenous NaCl. The objective of this investigation was also to uncover the activity of acid phosphatase and phytase in reducing the adverse effects of NaCl stress.

Germination Percentage

In this experiment, fenugreek seedlings aged 2, 4, 6, 8, and 10 days were subjected to increasing NaCl concentrations, reaching up to 200 mM. It can be observed from the graph below (Fig. 1), that on the second day the seeds germinated with a germination percentage of 60% when treated with 25 mM sodium chloride and gradually the percentage decreased with a gradual increase in the NaCl concentration, whereas in the presence of tap water, the germination percentage was 95.6%. On day 4th, the germination percentage was 88.9%, 55.6%, 46.7%, 24.4%, and 96.7% in presence of 25, 50, 100, 150 mM and tap water respectively. The same response was noticed on the sixth day of germination with a low concentration of salt exhibiting germination maximum whereas, a high concentration of salt slowly inhibited the germination of fenugreek seeds. On the 8th and 10th day, there was maximum germination of seeds when exposed to 25 and 50 mM with 86.8% and 67.8%, 95.6%, and 77.8% respectively. It was also observed that, in these two low concentrations of salt the seeds were able to break their dormancy, and the seedlings have developed. As the concentration of salt increased, the seed's germination percentage decreased gradually as observed on all days.

The results exhibited significant differences in germination %, SVI, and seedling root and shoot elongation when compared to control. Fenugreek seedlings exhibiting similar responses to salinity stress have been observed by Souguir et al. [73] and by Ratnakar et al. [76], both noting suppression of seedling growth under salt stress. This could be due to the osmotic stress experienced by the germinating seeds on continuous exposure to high salt.

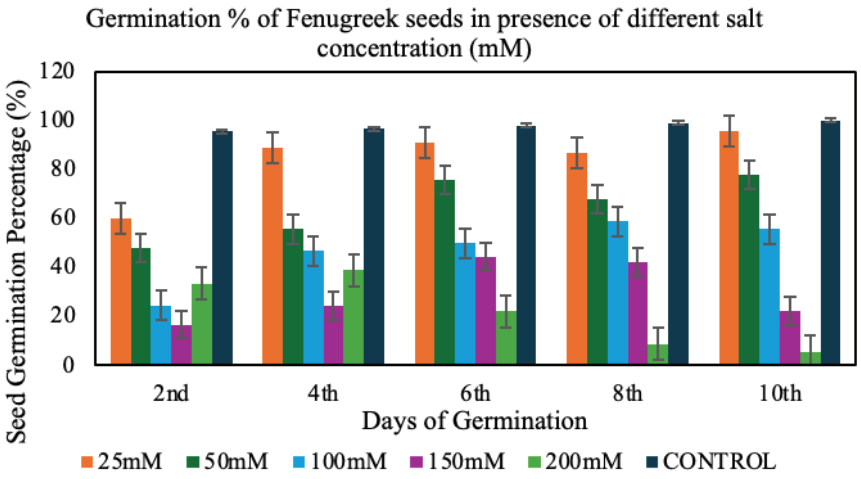


Figure 1. Germination percentage (%) of Fenugreek seeds in the presence of 25, 50, 100, 150, 200 mM NaCl and water on the 2nd, 4th, 6th, 8th and 10th day Mean±SE (n=2).

Seed Vigour Index

Seeds were regarded germinated when both the root and shoot had emerged to a length of at least two mm [59]. On the 10th day, the length of root and shoot were noted in cm using a ruler, and an average was taken. Seedling growth varied under different salt concentrations (Fig. 2). A sharp decrease in seedling shoots (0.74 cm±0.105) and root length (0.12 cm±0.084) was found when treated with 200 mM salt. Also, the number of leaves formed on the 10th day was found in 50 mM (2.01±0.0035) and 100 mM (2.01±0.005) of NaCl. Increasing salt concentrations led to a marked reduction in seedling elongation and hindered leaf development. Control with zero mM of NaCl exhibited maximum seedling growth with a shoot length of 2.59 cm±0.011, root length of 1.14 cm±0.0086 and an average number of leaves was 2.01±0.003.

The Seed Vigour Index (SVI) is employed to measure the capacity of seeds to develop into normal seedlings when exposed to stress. The seed vigour index was maximum in the presence of 25 mM 220.4±0.11 and 259.4±0.11 in control (Fig. 3). Whereas the SVI of germinating seeds was determined as 99.12±0.089, 37.18±0.031, 17.31±0.044 and 4.07±0.24 when exposed to 50, 100, 150, and 200 mM of sodium chloride concentrations respectively.

Stress due to salt, decreases germination percentage and seed vigour index of seeds maybe attributed due to osmotic and ionic effect, the former reduces water uptake and later accumulates the sodium and chloride ions [73]. Previous studies have demonstrated that, salinity can impact negatively on germination of seeds and embryonic growth by disrupting the mobilization and utilization of biomolecules stored in seeds thereby inhibiting the development of the embryo [77], alters the transport of electrons and contributing to unfavourable effects of ROS [78]. This becomes the starting point for oxidative damage in an organelle namely mitochondria, blocking respiration in seeds and

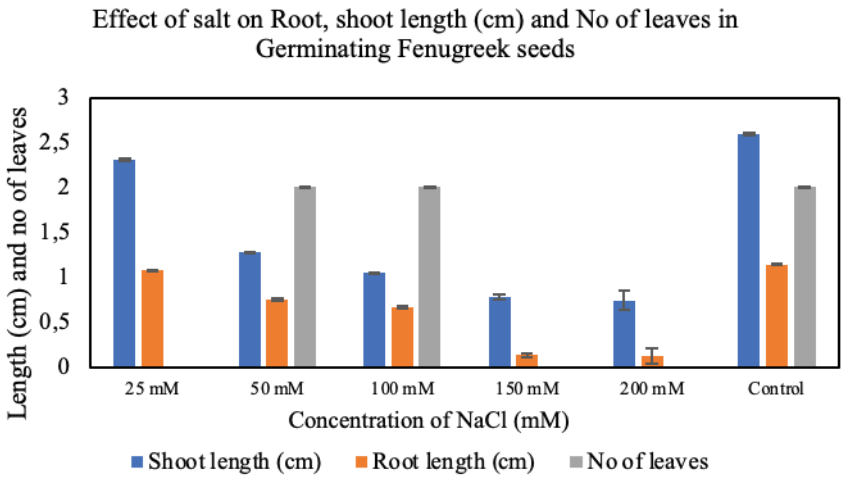


Figure 2. Effect of NaCl (mM) on Seedling length (cm) and no of leaves, Mean±SE (n=2).

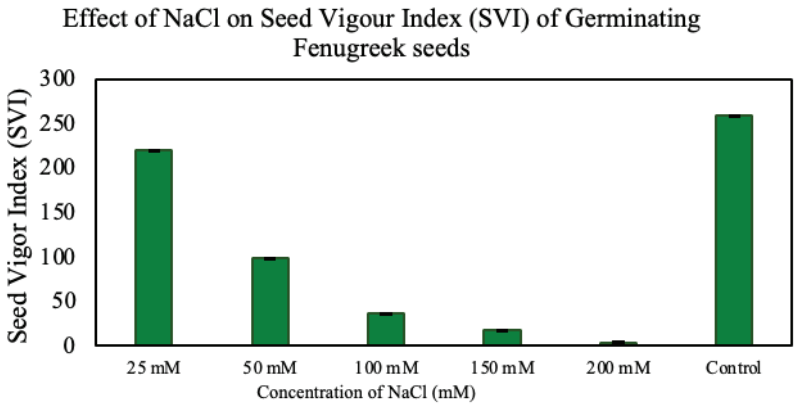


Figure 3. Effect of varying NaCl concentrations (mM) on the Seed Vigour Index (SVI) of germinating fenugreek seeds, expressed as Mean±SE (n=2).

simultaneously decreasing the production of energy [79]. These changes noticed from earlier studies could be the potential reason for the reduction or decreased percentage in the germination of seeds, seedling shoot and root length, and SVI of fenugreek seeds at all days attributed to salinity stress in our study.

Histochemical Staining

The two major reactive oxygen radicals that accumulate in the germinating seeds are H_2O_2 and O_2-. To reveal the *in-situ* accumulation of the ROS, the germinating seeds were stained with NBT from the 2nd to the 10th day that were incubated at different concentrations of NaCl. Histochemical staining showed that, the seed radicle and seed coat turned completely bluish black on the second day. Whereas, on the remaining other days the tip of the radicle only turned bluish black colour indicating the collection of free radicals mainly H_2O_2 and O_2- in the roots (Fig. 4). Salt stress damages biological membranes by lipid peroxidation. Various studies have reported that lipid peroxidation increases remarkably during salinity and disturbs membrane fluidity resulting in ion leakage from damaged tissues and accumulates free radicals [80]. Therefore, seeds subjected to high salt stained much darker thus indicating the localization of H_2O_2 and superoxide anion at the tip of the root. The intensity of the colour indicates the oxidative stress that the germinating seeds have experienced [81, 82].

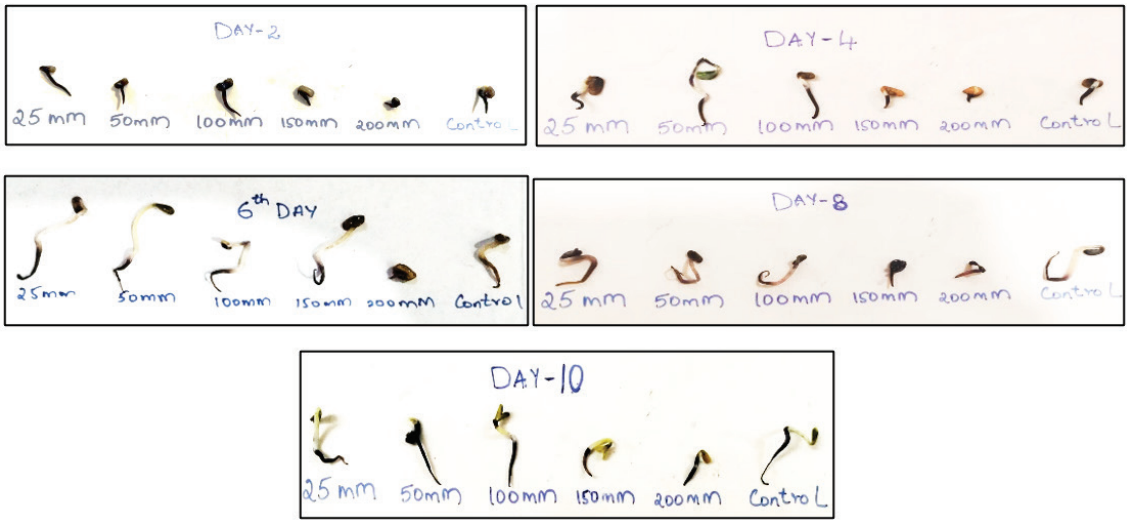


Figure 4. Histochemical staining using NBT on the Fenugreek seedling showing accumulation of H_2O_2 and O_2- under salt stress.

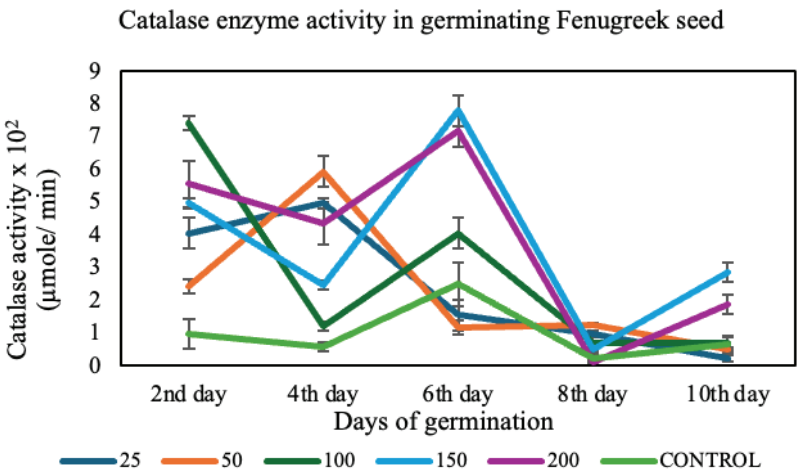


Figure 5. Catalase enzyme activity (µmole/min) in germinating seeds from day 2nd to 10th at different concentrations of NaCl, Mean±SE (n=2).

Antioxidant Enzymes

Salt stress significantly induces an upregulation of antioxidant enzyme profiles of germinating fenugreek seeds especially superoxide dismutase, catalase, and ascorbate oxidase. A significant response was observed during the interaction of salt and antioxidant enzymes studied here. Catalase [CAT] is generally localized in the microbody, mitochondria, and cytosol in a plant cell [83]. Germinating seeds break their dormancy and to develop into a healthy seedling requires continuous generation of free radical scavenging enzymes. From the graph above (Fig. 5), it can be observed that the CAT enzyme activity was maximum at 100 mM NaCl concentration initially with 7.44 x 10^2±0.21 µmole/min and 5.56 x 10^2±0.69 µmole/min at 200 mM NaCl on the second day of germination. It was observed that, the maximum activity of catalase was at 25 mM and 150 mM. As the incubation days increased, marked increase in CAT activity on the 6th day with 7.81 x 10^2±0.47 µmole/min and 7.18±0.47 µmole/min at 150 and 200 mM of salt respectively, when compared with control (2.5±0.66 µmole/min). On the 8th and 10th day, there was a remarkable decrease in CAT activity at lower to higher concentrations of NaCl. Despite the upregulation of catalase activity at elevated salt concentrations, the seeds remained susceptible to significant oxidative stress, resulting in delayed germination and stunted seedling growth, particularly at higher salt levels.

Salt stress in germinating seeds trigger the production of free radical scavenging enzymes to reduce ROS-induced oxidative damage, as noted by Zorov et al. [84]. In a study reported by, Baatour Olfa et al. [85] enhanced activities of catalase and peroxidase under 100 and 150 mM salt concentration, that align closely with the results of our study. Understanding the impact of salinity on germinating fenugreek seeds is critical, as ROS accumulation results in phytotoxic effects like peroxidation of lipids affecting the membranes, degradation of proteins involved in various metabolism, and damaging DNA by mutation [86]. To combat these free radicals, antioxidant enzyme activities increase in salt-stressed seeds to maintain homeostasis [87].

Superoxide dismutase is one of the key enzymes in cellular defense, as it converts radicals of superoxide into hydrogen peroxide (H_2O_2) and oxygen (O_2), thereby directly regulating the levels of these reactive oxygen species [67]. From Figure 6 it can be inferred that SOD activity increased in two-day old seedling of all NaCl concentration in the order of 25 mM (6.29 x 10^2±0.30 Units/mL), 150 mM (6.03 x 10^2±0.26 Units/mL), 200 mM (5.78 x 10^2±0.17 Units/mL), 50 mM (5.74 x 10^2±0.083 Units/mL), 100 mM (4.98 x 10^2±0.27 Units/mL) and 4.89 x 10^2±0.17 Units/mL in control. While its activity gradually decreased in 4-day old seedlings, but on the 6th day seedlings exhibited a slight change in SOD activity at 100 mM (2.64 x 10^2±0.26 Units/mL), 200 mM (3.9 x 10^2±0.38 Units/mL) and in control (3.74 x 10^2±0.28 Units/ mL). A decrease in SOD activity was observed on the eight day and again increased on the tenth day.

Ascorbate oxidase (AO) is an antioxidant enzyme, that helps to neutralize harmful compounds like free radicals or reactive oxygen species (ROS) that are released within the plant cells. Production of free radicals in plant cells is more significant during active biochemical processes and physiological activities [88, 89]. An alternative route to decompose hydrogen peroxide involves the ascorbate/ glutathione cycle, where AO catalyses the oxidation of ascorbate and the reduction of glutathione [87]. From Figure 7, it can be observed that the activity of ascorbate oxidase was minimum on 2nd day of germination in the presence of 25, 50, 150, 200 mM, and control was 0.011±0.017 Units/mL, 0.085±0.029 Units/mL, 0.092±0.034 Units/mL, 0.096±0.024 and 0.094±0.054 Units/mL respectively. The enzyme activity of AO reached maximum on the 6th day of germination with 0.209±0.024 Units/mL in control followed by

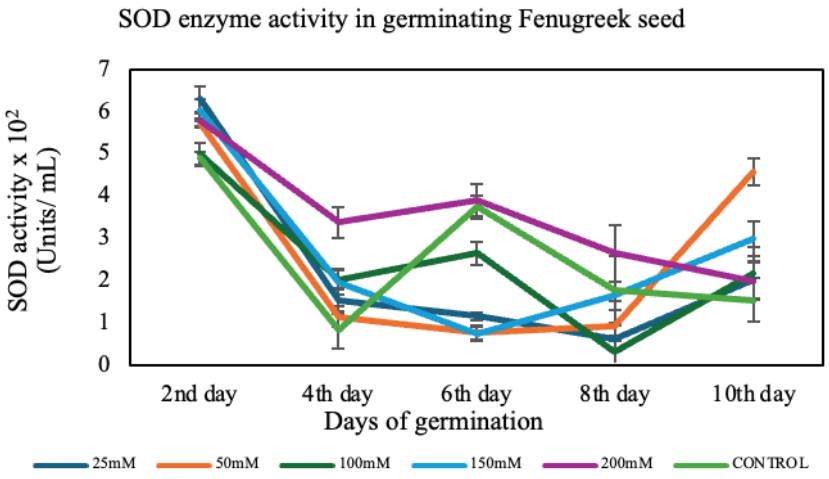


Figure 6. SOD enzyme activity (Units/mL) in germinating seeds from day 2 to 10 at different concentrations of NaCl, Mean±SE (n=2).

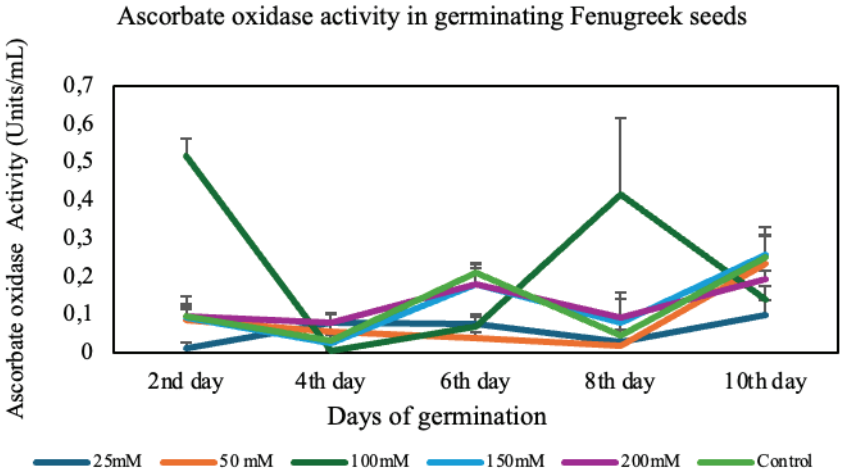


Figure 7. Ascorbate oxidase activity (Units/mL) in germinating seeds from day 2 to 10 at different concentrations of NaCl, Mean±SE (n=2).

150 mM NaCl with 0.18±0.054 Units/mL and 200 mM with 0.179±0.042 Units/mL enzyme activity. A decrease in enzyme activity on the 8th day and a slight increase in AO enzyme activity on the 10th day were determined. In contrast, the effect of 100 mM of NaCl showed maximum activity of 0.52±0.045 and 0.42±0.198 Units/mL on the 2nd and 8th day respectively.

In our current study, activities of CAT, ascorbate oxidase, and SOD increased when the salt concentrations were 100 mM, and 150 mM and these results were in agreement to Meloni et al. [90]. These results demonstrate that germinating seeds have developed a protective mechanism to decompose reactive species that are generated continuously. In a report by Scandalios [91] underlaid the significance of the cumulative effect of CAT and SOD in fenugreek seeds to scavenge the H_2O_2 under different salt stress.

Fenugreek seedlings in our study were able to efficiently counter the unfavourable role of NaCl stress during the early stages of development. This is possible only through the enhanced activity of ROS scavenging antioxidant enzymes, which play a crucial role in removing the harmful reactive oxygen species. This indicates that the germinating seeds could reverse the effect of salinity by maintaining the integrity on the plasma membrane.

Acid Phosphatase Activity

Acid phosphatase enzyme is involved in the metabolic processes of germinating seeds and in plant maturation. They are expressed continuously in germinating seeds, and the acid phosphatase activities increase with germination

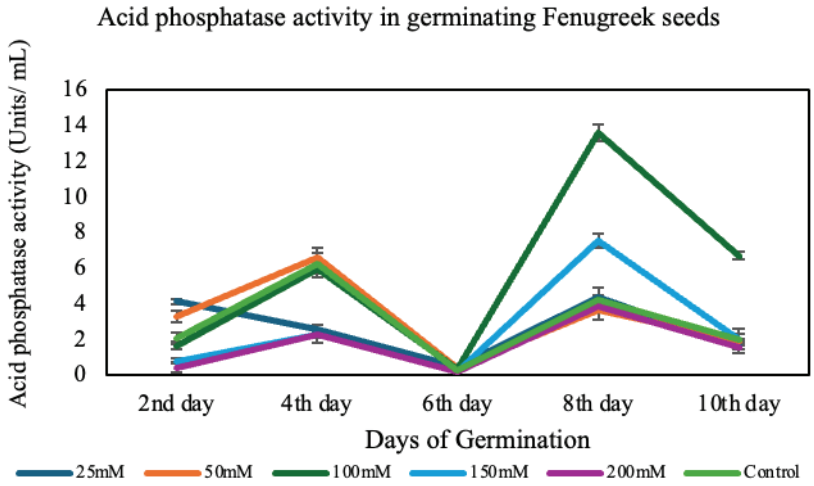


Figure 8. Acid phosphatase activity (Units/mL) in germinating seeds from day 2 to 10 at different concentrations of NaCl, Mean±SE (n=2).

of seeds to release the stored materials that are required by the developing embryo [66]. From Figure 8, it can be inferred that the activity of acid phosphatase increased on the 4th and 8th day with a drastic decrease on the 6th day. On 2nd day, there was maximum enzyme activity at 25 mM with 4.12±0.13 Units/mL and 3.3±0.32 Units/mL when treated with 50 mM of NaCl. On 4th day, the activity of acid phosphatase was 6.59±0.52 Units/mL at NaCl concentration of 50 mM, 6.28±0.57 Units/mL at zero mM of NaCl, 5.99±0.52 Units/mL at 100 mM of NaCl. The maximum activity of acid phosphatase was 13.60±0.47 Units/mL and 7.58±0.46 Units/mL on the 8th day when treated with 100 and 150 mM of NaCl salt respectively.

Studying the activity of acid phosphatase in germinating seeds will reveal its vital role in converting organic phosphorus to inorganic phosphorus. This conversion is a key process for transfer of energy, metabolic homeostasis and controlling many other functions during germination.

Phytase Activity

Phytase enzyme is active during seed germination and cleaves phosphate group from ortho position of phytates inositol ring, thereby liberating free phosphorus, Debnath et al. and Guzmán-Ortiz et al. [92, 93]. In the present study, the obtained result indicated that the phytase enzyme activity was maximum on the 6th and 8th day of germinating fenugreek seeds. This when corelated with the phosphorus content in the seed extracts, the content of phosphorus was maximum on the 6th and 8th day when treated with different concentrations of sodium chloride.

From Figure 9, it can be inferred that the phytase activity was maximum on the 6th and 8th day in germinating fenugreek seeds. At a minimum concentration of NaCl (25 mM) the enzyme activity was 15.66±0.46 Units/mL on the 6th and 8th day of germination. While less activity was found in 100 mM salt concentration with 1.18±0.59 Units/mL, and 1.258±0.49 Units/mL on the 2nd and 4th day of germination. At maximum concentration of salt (200 mM NaCl), the phytase activity was minimum of 0.73±0.18 Units/mL on day 2 of germination. From Figure 9, there is a trend observed where at all concentrations of salt, the phytase enzyme activity remained the same on the 6th and 8th day of germinating fenugreek seeds.

In a work reported by Marshall et al. [94], the activity of phytase enzyme increased and reached maximum on day 7th (rice), day 6th (maize), day 5th (millet), day 7th (sorghum), and 8th day (in wheat) respectively and later declined. Increased phytase activity during germination correlated with a significant surge in phosphorus concentration. These study reports are in agreement with our study on germinating fenugreek seeds.

To get a deeper insight on the physiological changes that emerge in germinating seeds under NaCl stress, we examined the acid phosphatase and phytase activities in fenugreek. The present investigation revealed that acid phosphatase activity was stimulated on 4th and 8th day of germination across different NaCl concentrations. These findings are consistent with a study as reported by Jain et al. and Sharma et al. [95, 96], as they also reported increased enzyme activity in pearl millet and sorghum. It is conceivable that, under stress conditions, germination of seeds is inhibited due to impairment in the transport and uptake of soluble phosphates. As a result, phosphatase enzyme activity is to act on stored insoluble phosphates to release free soluble phosphates that must be required for germinating seeds to overcome stress. This process regulates osmotic imbalance through the uptake of free phosphate as reported by El-Maarouf and Bailly [97]. The increased activity of phosphatase enzymes in salt-tolerant varieties under salinity suggests their direct involvement in providing the

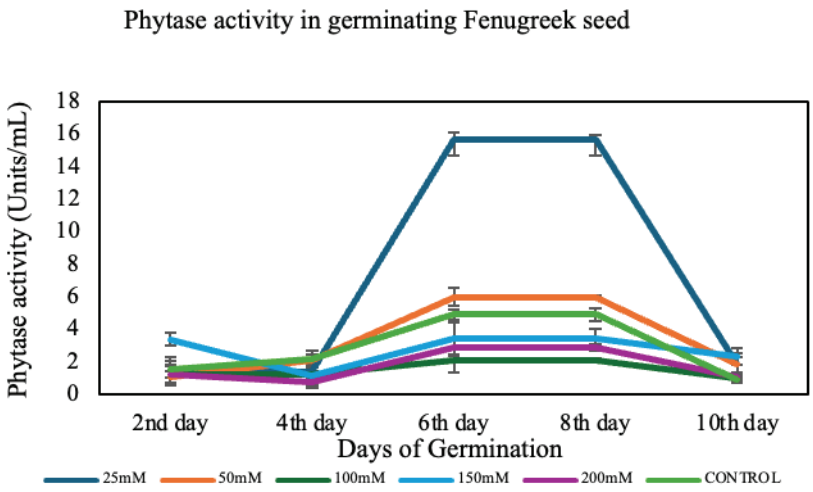


Figure 9. Phytase enzyme activity (Units/mL) in germinating seeds from day 2 to 10 at different concentrations of NaCl, Mean±SE (n=2).

necessary energy for cells to withstand the unfavourable effects of salinity, Nasri N et al. [98].

Protein Concentration by Lowry's Method

From Figure 10, it can be inferred that the concentration of protein was maximum at 25 mM of NaCl on the 2nd, 4th and 6th day of germination with 0.85±0.13 µg/mL, 0.54.7±0.13 µg/mL and 0.81±0.0 µg/mL respectively. The synthesis of proteins to counteract oxidative stress may be a prerequisite for seed dormancy alleviation and its subsequent germination. Protein concentration reached its maximum on sixth day when the seeds were treated with 100 mM of salt with 1.25±0.129 µg/mL, while the control showed 1.35±0.13 µg/mL. These findings are consistent with previously reported studies. Results showed that the antioxidant enzymes namely catalase, SOD and acid phosphatase enzyme activities were determined to be maximum on the 6th and 8th day of germination at 100 mM of NaCl respectively.

Phosphorus Concentration by Fiske Subbarow Method

A study by Yugandhar et al. [99] demonstrated that phosphorus in seeds is crucial in maintaining seed vigour, chlorophyll content, and in the photosynthetic process. Pi (phosphorus) is an indispensable macronutrient for plant growth, interferes with cellular energy transfer, photosynthesis, and respiration. Reduction or insufficiency of phosphorus can adversely affect growth and development of roots, finally leading to diminished yield of crops [100]. Therefore, understanding the role of phytase enzyme under salt stress in germinating seeds gives an insight into the role played by this enzyme [95]. Maximum phosphorus concentration was observed at 25 mM NaCl with 68±0.52 µg/mL on the 6th day followed by the 8th day with 64±0.52 µg/mL and at 50 mM NaCl was 54.7±0.52, 45.6±0.51, 54.7±0.52 µg/ml on 4th, 6th and 8th day respectively. Similarly, at 100 mM of NaCl,

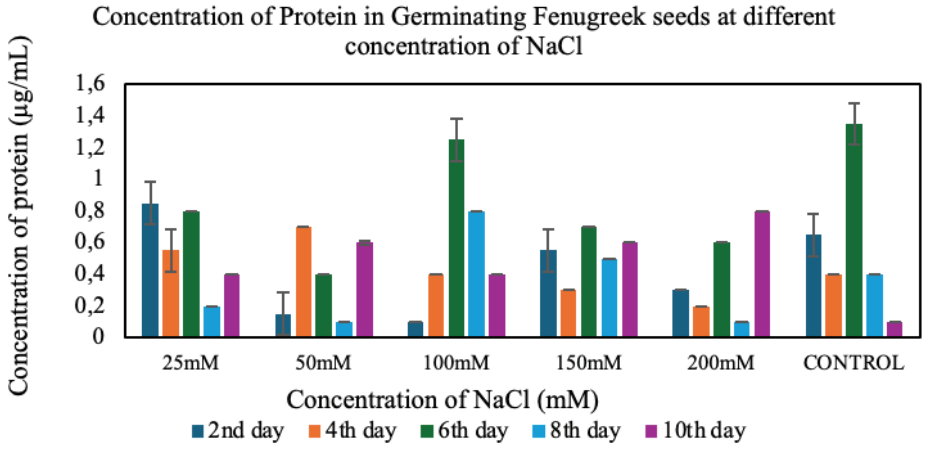


Figure 10. Protein Concentration in Germinating Fenugreek seeds under salinity, Mean±SE.

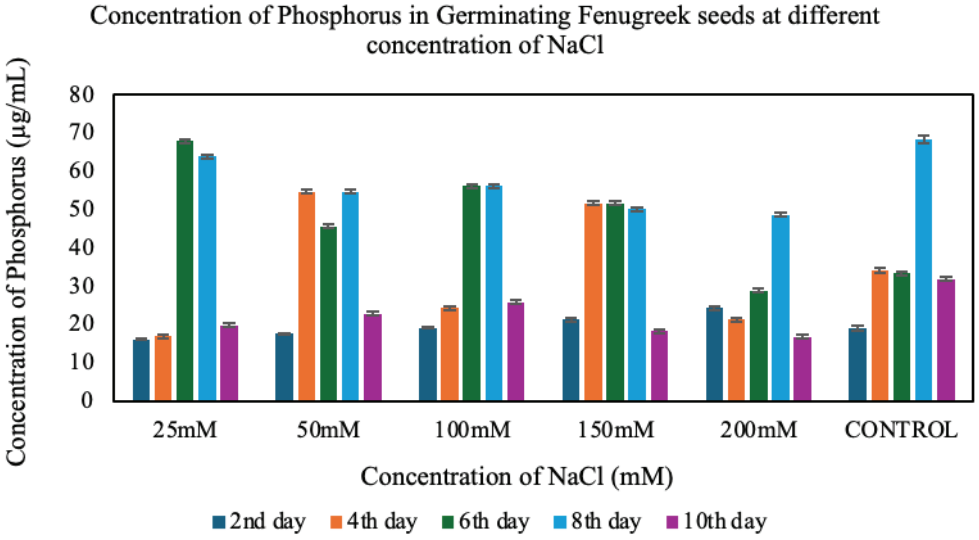


Figure 11. Concentration of phosphorus in Germinating Fenugreek seeds under salinity, Mean±SE (n=2).

phosphorus concentration was 56.2±0.51 µg/ml both on the 6th and 8th day of germination. At 150 mM NaCl, it was 51.6±0.51 µg/mL, 51.6±0.52 µg/mL, and 50.12±0.52 µg/mL on the 4th, 6th and 8th day respectively. Control on the day eight had the maximum concentration of phosphorus with 68.3±0.89 µg/mL. On the second day, the concentration of phosphorus reached its lowest level under varying NaCl concentrations (Fig. 11).

Seed germination is accompanied by numerous biochemical changes. Matured seeds contain approximately 60-90% of the phosphorus in total stored in the form of phytate, which decreases to nearly 40% upon germination [101]. Many reports suggest that there is a remarkable decrease in phytate content during the germination of seeds as observed by Rusydi [101] in soya bean, peanut, Bouajila et al. [102] reported in barley. Zeller et al. [103] reported that when the seeds germinate there is a synthesis of phytases or combined action of phosphatases and phytases are required in hydrolysing stored phosphate present in the form of phytate. Ali et al. [104] observed that action of phytase on stored phytic acid in germinating seeds would release phosphorus. This gets transported to the emerging radicle of germinating seeds for the synthesis of organic phosphates required for multiple purposes in germinating seeds.

Purification of Phytase from Germinating Fenugreek Seeds

Based on the above results, fenugreek seeds germinated in water was used for phytase enzyme purification. To precipitate the enzyme 30-60% $(NH_4)_2SO_4$ was used. The obtained precipitate was dialyzed against Tris- HCl buffer (pH 7.0, 50 mM). The dialysate was subjected to partial purification on anion exchange chromatography using DEAE Cellulose 52 column. Collected protein fractions were subjected to UV- Vis spectrophotometric analysis at 280 nm. Fractions showing maximum absorbance at 280 nm was used for determining the phytase enzyme activity as reported in Table 2.

Separation of Purified Phytase by SDS PAGE

The protein present in the crude extracts, ammonium sulphate precipitate, dialysate, and fraction obtained after ion exchange chromatography was subjected to SDS PAGE on 12% resolving gel. The results obtained were viewed in the electrophoresed gel as blue colour bands. From Figure 12, three blue colour bands in lane 3 and thin bands in lanes two and four were found. The bands obtained were compared with the molecular weight markers in lane 1, the blue band in lane 2 was found close to 66 kDa. From the available literature, studies have indicated the molecular weight of phytase could be between 50 – 70 kDa [105].

Table 2. Phytase activity and protein content across different fractions obtained during successive stages of purification

Procedure	Total phytase activity (Units/ml)	Total Protein (mg/ml)
Crude extract	59.6	1.5
Ammonium sulphate precipitation	2.18	0.5
Dialyzed precipitate	1.55	0.3
DEAE-Cellulose	8.11	1.2

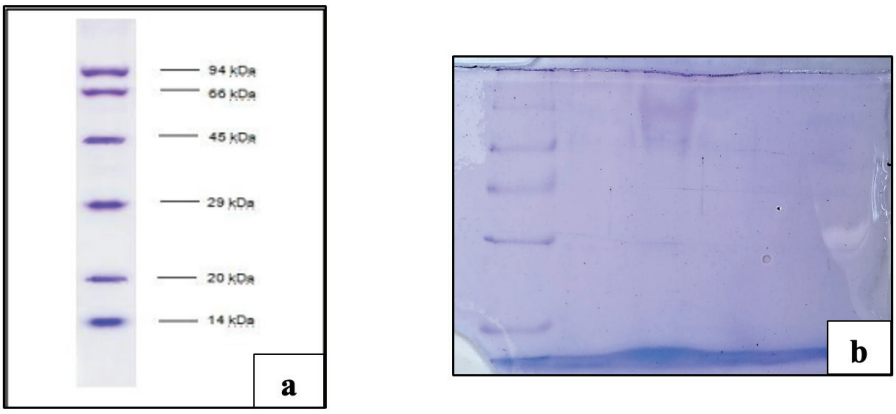


Figure 12. Electrophoresed gel after SDS-PAGE.

a) Protein molecular weight marker (Low range between 14 kDa to 94 kDa) and b) Gel with blue colour bands with lane 1 – Molecular weight protein marker, lane 2 fractions eluted from DEAE Cellulose resin, lane 3 ammonium sulphate precipitate and lane 4 is dialysate.

Fenugreek seeds phytase was partially purified using ammonium sulphate and fractionated through DEAE Cellulose 52 column. Molecular weight was reported to be between 60- 70 kDa, approximately 66 kDa from SDS PAGE. Reports by Ullah [106], Vats and Banerjee 2005 [107], and Greiner et al. [108] was 68 kDa of *A. ficuum*, 66 kDa of *A. niger* and of *A. niger* 11T53A9 strain was reported as 85 kDa respectively. Whereas Anukriti Verma et.al 2013 stated that the molecular weights of two plant phytase enzyme variants in the Indian wheat variety DBW 17 were PHY1 at 64 kDa and PHY2 at 62 kDa [109], phytase isolated from barley has been reported to exhibit a molecular weight of 66 kDa by Greiner et al. [110]. In contrast, Lung et al. [69] documented a phytase enzyme with 64 kDa isolated from tobacco root exudates.

Immobilization of Phytase *Via* Microencapsulation

Immobilization of phytase enzyme was experimented using alginate and chitosan by microencapsulation method. Based on the study performed by Belho and Ambasht [53], the optimized parameters shortlisted were used for the immobilization. Therefore, for this study, a matrix with 1.5% sodium alginate coated with 1% v/v acetic acid chitosan was used for encapsulation in the presence of calcium chloride to form microspheres. Crosslinks between Ca^{2+} ions from $CaCl_2$ interact with carboxylic acid groups of the alginate chain helping in the formation of a small spherical white colour bead (Fig. 13). SEM and FTIR techniques were employed to characterize the chitosan-coated microbeads.

Study done by Sastre et al. and Liu et al. [111, 112] signify the availability of different types of matrices that can be used for enzyme immobilization. Phytase immobilization has been done using microbial sources, such as *Aspergillus sps*, *Escherichia coli* (*E. coli*), *Staphylococcus sps*, as documented by Pragya et al. and El-Shora et al. [113, 114], Coutinho et al. 2020 [43] respectively and in mushrooms by Onem et al. [115]. Immobilizing phytase enzyme finds advantage and applications in detection of phytate by amperometric biosensors [41], to be used as a fertilizer along with application of inorganic phosphorus [42], bioreactor application [44], as animal feed [43] and in reducing the content of phytate in soymilk [45].

Very few studies as reported by Celem & Onal [116] and Chen et al. [45] have immobilized plant phytase using soymilk. Immobilization of phytase derived from germinating fenugreek seeds was experimented with in our laboratory using alginate and chitosan as very little information on plant phytase immobilization using chitosan has been reported. The results obtained in our study was in consistent with a study reported by Belho & Ambasht [53] where the microscopic analysis revealed that the chitosan- coated phytase microspheres exhibited a spherical morphology and appeared white in colour. Also, the FTIR spectral analysis results were determined to have wave numbers corresponding to the functional groups shared by chitosan, alginate, and the phytase enzyme.

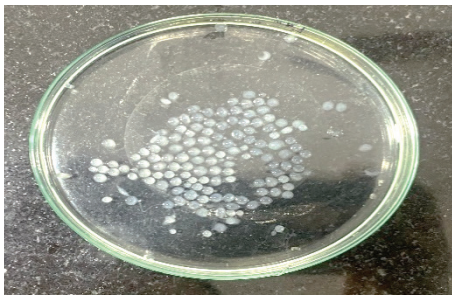


Figure 13. Photograph of microspheres prepared with alginate coated with chitosan.

Characterisation of Microspheres Using SEM

During microencapsulation, water is also captured due to molecular rearrangements and secondary forces like ionic, hydrogen bonds, and hydrophobic interactions. [111]. These interactions usually drive the formation of spherical and homogenous three-dimensional structures. The presence of excess chitosan may also be the cause for the formation of microcapsule clusters or aggregates. The average size of the microcapsules was calculated to be 10.52 µm with their shapes being spherical. From the micrographs obtained it can be understood that the surface morphology of the microbeads was uniform with regular patterns like the veins of the leaves (Fig. 14). SEM images dictate the surface morphology where it can be viewed on its surface with small pores averaging 1.64 µm spaced at regular intervals.

Characterisation of Microspheres Using FTIR

Infrared spectroscopy is a key technique for evaluating possible chemical interactions between various biomolecules and biopolymers. Figure 15 shows, the FTIR spectra for an extract containing microspheres. The spectra showed a broad and a deep band between 3300 to 3400 cm^{-1}, which is correlated with the existence of bending hydroxyl groups and stretching because of -OH groups. Significant bands at 2983.3 and 2856.67 cm^{-1} were also observed which correlate to C-H stretching of alkanes. [117]. An intense band near 1627.97 cm^{-1} may be caused by the C=O stretching of $NH.CO.CH_3$ typical to N acetyl D- glucosamine of Chitosan [53] with % transmittance of 69.2 - 69.9%. A stretch of C=C-C of aromatic bond was also observed at 1427.37 cm^{-1}.

Furthermore, a stretch within the range of 1000 - 1100 cm^{-1} indicates the axial deformation of the C-O bond. Bands at 1163.11 cm^{-1} and 1062.81 cm^{-1} may complement to secondary and tertiary alcohol respectively. Intense peaks in the range of 1700-1600 cm^{-1} may be due to the stretching of C=O of a secondary amide which may have formed due to attachment of the carboxylic group to the amine group in the microcapsule. [118]. Bands in the range of 1600 to 1200 cm^{-1} should be contributed by the phenolic compounds in the microcapsules as reported by de Souza et al. [119].

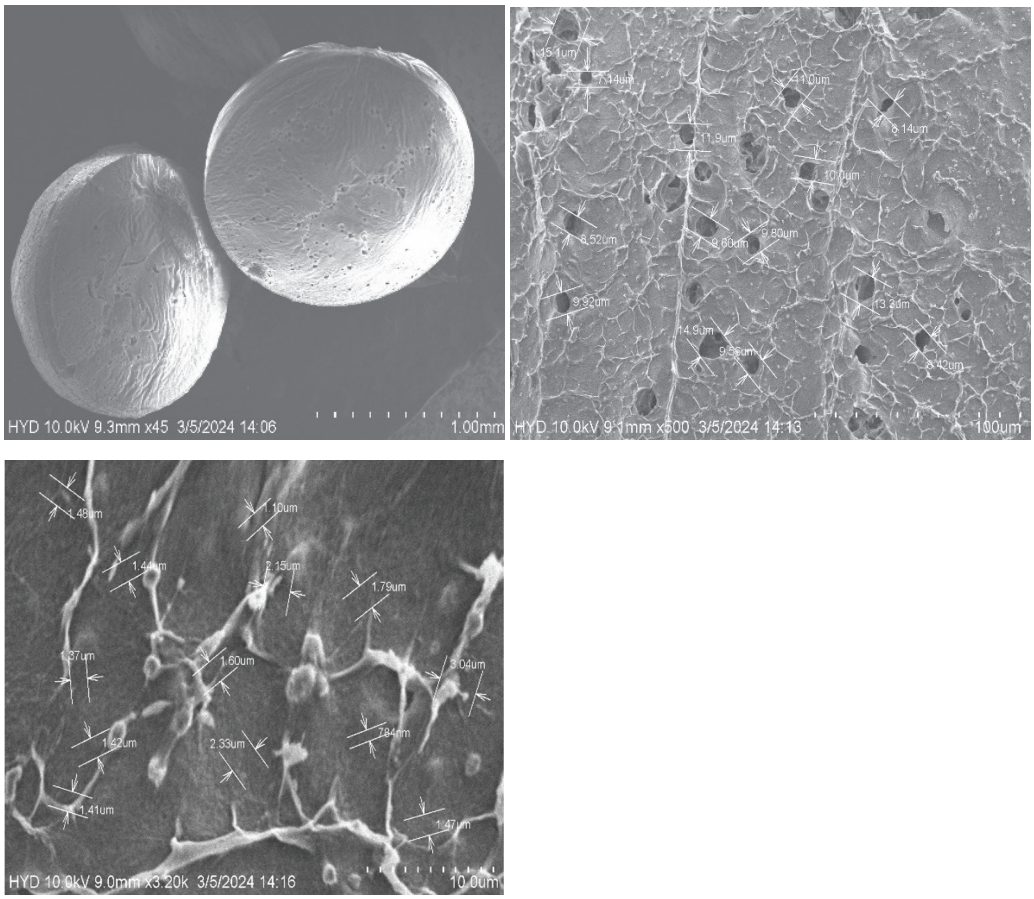


Figure 14. SEM micrograph images of Phytase enzyme immobilized (Microencapsulation) using Alginate and Chitosan

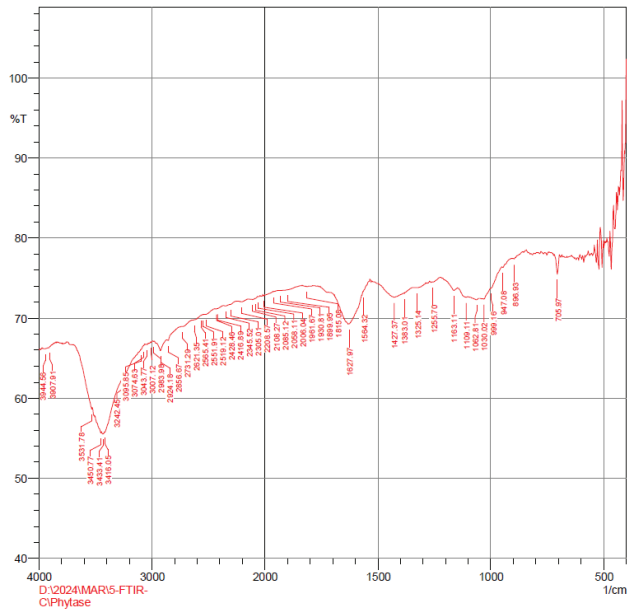


Figure 15. FTIR spectra of Phytase enzyme immobilized (microencapsulated) using Alginate and Chitosan.

CONCLUSION

Salinity a major abiotic stress that negatively impacts crop growth and development, productivity and subsequent harvest. It can disrupt various biological processes in plants, leading to oxidative damage in cellular components, impaired membrane function, compromised photosynthesis, nutrient imbalances, altered levels of free radicals, and can impair the functions of various biomolecules. From this study, results suggest that the seedlings of fenugreek exhibit a degree of salt tolerance during early germination, as indicated by their capacity to withstand high NaCl stress. This tolerance is likely mediated through the activation of free radical scavenging antioxidant enzymes. In vitro studies revealed enhanced activities of phytase and acid phosphatase, coupled with balanced phosphorus levels, suggesting that these enzymes are essential for facilitating seed germination. The findings of this study indicate that fenugreek seeds exhibit salt tolerance and are capable of regulating phosphorus release, even under saline conditions. This study pioneers an innovative approach to immobilise plant-derived phytase using alginate-chitosan

microspheres, a novel approach with potential applications across various industries.

Studying the impact of salinity on germinating fenugreek seeds can find applications in agriculture for sustainable crop yield. As fenugreek seeds are salt tolerant, understanding the role of phosphatase enzymes under salt stress helps the agriculturalist to supply only the recommended dosage of phosphate-based fertilizers during cropping. Consequently, further investigation is warranted to fully elucidate the efficiency of phytase mechanisms at molecular scale in enhancing stress tolerance and to evaluate its potential applications in sustainable agriculture.

Investigating plant acid phosphatase and phytase enzyme activities under abiotic stress especially is crucial in salt-sensitive seeds. While many studies have reported on the mechanism in salt-tolerant species like *Trigonella foenum-graecum L.*, a focussed approach on non-tolerant plants is essential to elucidate the molecular mechanisms underpinning phosphate homeostasis in response to stress by salinity. This approach prioritizes the improvement of agricultural yields both in quality and quantity, and in the expansion of sustainable practices to ensure plant cultivation and the production of their goods for long-term benefits.

Future prospect includes identification of novel genetic markers for breeding salt- tolerant crops and developing biotechnological interventions to enhance phosphorus use. This advancement will ultimately boost global food security through eco- friendly methods to unlock soil- bound phosphorus in saline environments.

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AUTHORSHIP CONTRIBUTIONS

VS and KB conceptualized and designed the idea, KB, RK and HM executed the experiments. KB and RK compiled and drafted the manuscript based on literature sources and experimental values. VS supervised and reviewed the manuscript. All authors have thoroughly read and approved the final manuscript for publication.

DATA AVAILABILITY STATEMENT

The authors confirm that the information and datasets generated or analysed during this work are included in the manuscript.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

This article does not contain any studies or work related to human participants or animals.

STATEMENT ON THE USE OF ARTIFICIAL INTELLIGENCE

Artificial intelligence was not used in the preparation of the article.

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