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Antioxidative and enzymatic profiling of radish (*Raphanus sativus*) seeds under salinity stress

Kritika Sonkusale[1], Shivali Tiwari[1], Dr. Anju Meshram[2*]
[1] Amity Institute of Biotechnology, Amity University Chhattisgarh, Raipur, Chhattisgarh, India
[2] Assistant Professor, Amity Institute of Biotechnology, Amity University Chhattisgarh, Raipur, Chhattisgarh, India
Corresponding Author: Dr. Anju Meshram

Abstract

Salinity stress is a major abiotic factor that adversely affects seed germination and plant development, particularly by inducing oxidative stress through excessive production of reactive oxygen species (ROS). This study investigates the enzymatic antioxidant response in *Raphanus sativus* (radish) seeds subjected to varying concentrations of saline solutions (NaCl). Seeds were germinated under controlled laboratory conditions with incremental salinity levels (0, 10, 50 and 100 mM NaCl) and the activity of key antioxidant enzymes— catalase (CAT) and Ascorbate peroxidase (APX) as well as protein content was quantified. The results revealed a concentration-dependent modulation in enzyme activity, with moderate salinity (up to 10-50 mM) triggering enhanced antioxidant defence mechanisms, while higher concentrations (50–100 mM) led to reduced enzymatic activity, indicating possible oxidative damage. This enzymatic profiling underscores the role of antioxidative enzymes in mitigating salinity-induced stress during seed germination and suggests a threshold of salt tolerance in *Raphanus sativus* seeds. The findings contribute to understanding the biochemical resilience of radish under salt stress, providing a basis for breeding or engineering salt-tolerant cultivars.

Keywords: *Raphanus sativus*, salinity stress, antioxidant enzymes, oxidative stress, enzymatic profiling, seed germination, salt tolerance

Introduction

Soil salinity is a major global constraint on agriculture, affecting nearly 20% of irrigated land and expected to increase due to climate change and poor land management (Munns and Tester, 2008) [5]. Salinity imposes osmotic stress that restricts water uptake and ion toxicity caused by excessive Na^+ and Cl^- accumulation, disrupting nutrient balance and metabolic functions (Munns and Tester, 2008; Parida and Das, 2005) [5]. A key secondary effect is oxidative stress, resulting from overproduction of reactive oxygen species (ROS) such as O_2^-, H_2O_2 and •OH, which damage cellular components and reduce plant performance (Mittler, 2002; Sharma and Dubey, 2005) [4, 6]. Plants counteract ROS through an antioxidant defence system comprising enzymatic components—SOD, CAT, POD/APX, GR—and non-enzymatic antioxidants. SOD converts O_2^- to H_2O_2, which is subsequently detoxified by CAT and peroxidases, while GR sustains the reduced glutathione pool essential for the ascorbate–glutathione cycle (Ahmad and Prasad, 2012; Mittler, 2002) [1, 4].

Radish (*Raphanus sativus* L.), a fast-growing Brassicaceous vegetable, is particularly sensitive to salinity during germination and early growth. Elevated NaCl levels reduce germination, seedling length, biomass and overall crop quality (Khan *et al*., 2015) [2]. Moderate salinity (60–100 mM NaCl) stimulates SOD and CAT activities in radish seedlings, reflecting activation of defence pathways, whereas higher levels (≥150 mM) suppress antioxidant enzymes due to oxidative damage (Kumari and Kaur, 2017) [3]. Considerable cultivar-dependent variation exists, with round red varieties showing stronger antioxidant responses and greater tolerance (Sanoubar *et al*., 2020) [22]. Ion homeostasis mechanisms, particularly the Na^+/H^+ antiporter RsSOS1, further contribute to salt tolerance, with its overexpression enhancing Na^+ efflux and resilience (Li *et al*., 2022).

Protein profiling through SDS-PAGE complements enzyme assays by detecting qualitative changes such as isozyme induction under stress. GA_3-primed radish seedlings exhibit altered esterase and peroxidase isozyme patterns correlating with improved tolerance (Kasim and Dowidar 2006) [11]. Beyond antioxidative responses, salinity strongly influences germination and early seedling growth in radish. High salt levels restrict water uptake and enzyme activation, thereby reducing germination percentage, seedling vigour and early metabolic activity (Gupta and Huang, 2017). Moderate salinity (60–100 mM NaCl) slows germination yet does not completely inhibit it, while higher concentrations (150–200 mM NaCl) impose severe osmotic and ionic stress, leading to sharp declines in growth and survival (Jamil *et al*., 2006; Khan *et al*., 2015) [2, 8]. These early responses are important because initial salt exposure often determines later physiological and biochemical tolerance.

At the biochemical level, radish seedlings exhibit marked induction of SOD, CAT and APX under moderate salinity (50–100 mM), reflecting activation of ROS-scavenging pathwaysl (Bungala *et al*., 2024; Abeed *et al*., 2023) [20, 21]. Proteomic studies further reveal extensive changes in stress-related proteins, including those involved in antioxidative defence, ion transport and energy metabolism (Sun *et al*., 2017) [10]. SDS-PAGE and native-PAGE analyses also show isozyme shifts in peroxidases and esterases, which increase under mild stress but decline at higher salinity (Kumari *et al*., 2015) [19]. Such cultivar-specific biochemical and proteomic variations form the basis for screening radish genotypes with improved salt tolerance. This study integrates germination assessment, antioxidant enzyme analysis and protein profiling to identify biochemical indicators of salt tolerance and support radish improvement for saline environments.

Fig 1: Radish Plants in Pots

Materials and Methods

1. Preparation of Hoagland Nutrient medium

Hoagland and Arnon's nutrient solution (Hoagland and Arnon, 1950) [13]. is a widely used hydroponic medium supplying all essential macro- and micronutrients in readily available ionic forms. It minimizes soil variability and provides precise control of nutrient supply, making it ideal for plant physiology experiments, hydroponics, greenhouse studies and nutrient-deficiency research (Salisbury and Ross, 1992) [14]. The solution contains N, P, K, Ca, Mg and S as macronutrients, along with Fe, Mn, Zn, Cu, B and Mo as micronutrients, and is also used in tissue culture systems for consistent growth responses (George *et al*., 2008) [12].

Preparation Steps

1. Preparation of Stock Solutions (1 L each)

Separate macronutrient, micronutrient and iron (Fe-EDTA) stocks are prepared.

2. Preparation of Working Solution

Required volumes of each stock are added to distilled water in the order: macronutrients → micronutrients → iron source.

3. pH Adjustment

Final solution adjusted to pH 5.8–6.0 using dilute NaOH or HCl for optimal nutrient uptake.

4. Sterilization

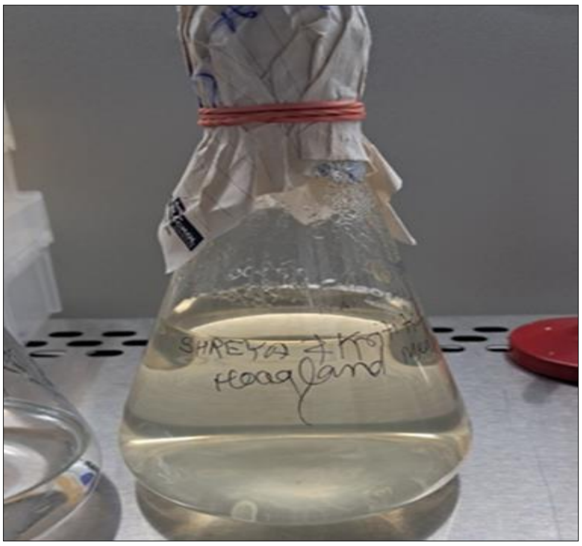
Hydroponics: usually not required.

Tissue culture: sterilize by autoclaving (when applicable) or membrane filtration for heat-sensitive components.

5. Storage

Stock solutions stored in clean, sealed bottles at room temperature or refrigeration to prevent contamination.

Before use, all glassware is rinsed with 2% NaOCl and washed thoroughly with distilled water (3–5 times).



Hoagland Media

2. Catalase Estimation

Catalase is a key antioxidant enzyme that decomposes hydrogen peroxide (H_2O_2) into water and oxygen, protecting cells from oxidative damage (Chelikani *et al*., 2004) [16]. Catalase activity is commonly measured using titrimetric or spectrophotometric methods. The spectrophotometric assay is widely preferred; it monitors the decrease in absorbance of H_2O_2 at 240 nm, which is directly proportional to catalase activity (Aebi, 1984; Goth 1991) [15, 17].

Materials and Methods

Materials

Fresh radish tissue, 50 mM phosphate buffer (pH 7.0), 0.1 M H_2O_2, spectrophotometer, cuvettes, ice bath, mortar–pestle, filter paper/cheesecloth, centrifuge

Methods

Preparation of Reagents

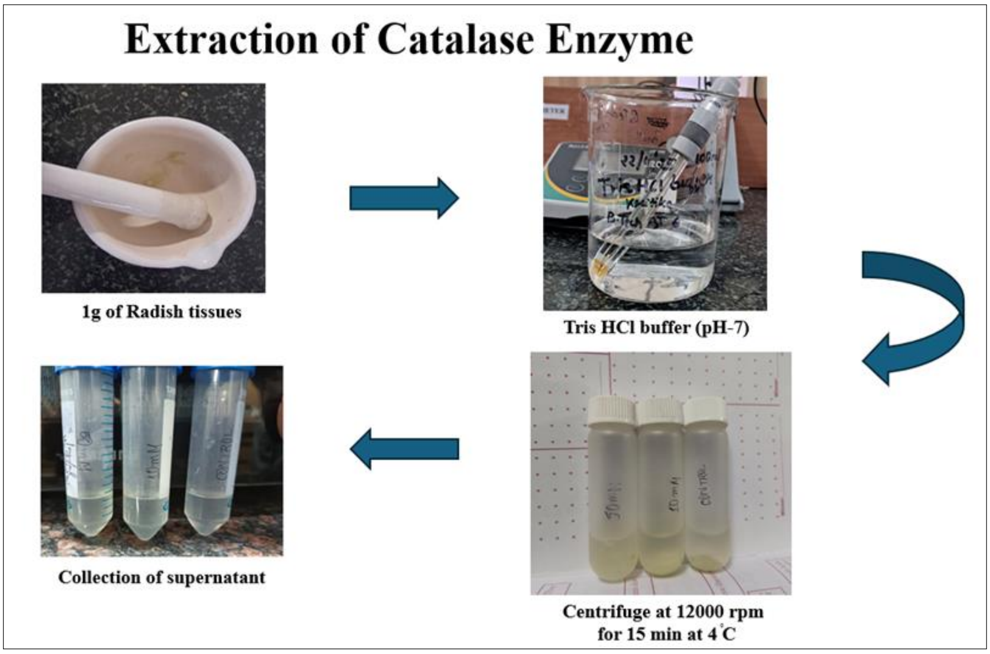
a. 50 mM Tris-HCl Buffer (pH 7.0)

1. Dissolve 0.79 g Tris-HCl in 80 mL distilled water.

2. Adjust pH to 7.0 using 1 M NaOH.
3. Make up to 100 mL and store at 4°C.

b. M H_2O_2 (10 mL)

1. Add 0.10 mL of 30% H_2O_2 to a 10 mL volumetric flask.
2. Make up to volume with distilled water.



Procedure

a. Enzyme Extraction

1. Homogenize 2–5 g radish tissue in chilled phosphate buffer (10 mL/g).
2. Filter and optionally centrifuge at 10,000 rpm, 10 min, 4°C.
3. Collect supernatant as enzyme extract.

b. Catalase Assay (Spectrophotometric)

Reaction mixture (in cuvette):

1. 2.9 mL phosphate buffer
2. 1.0 mL 0.1 M H_2O_2
3. 0.1 mL enzyme extract
4. Mix and record decrease in absorbance at 240 nm for 1 min.
5. Use buffer + H_2O_2 as blank.

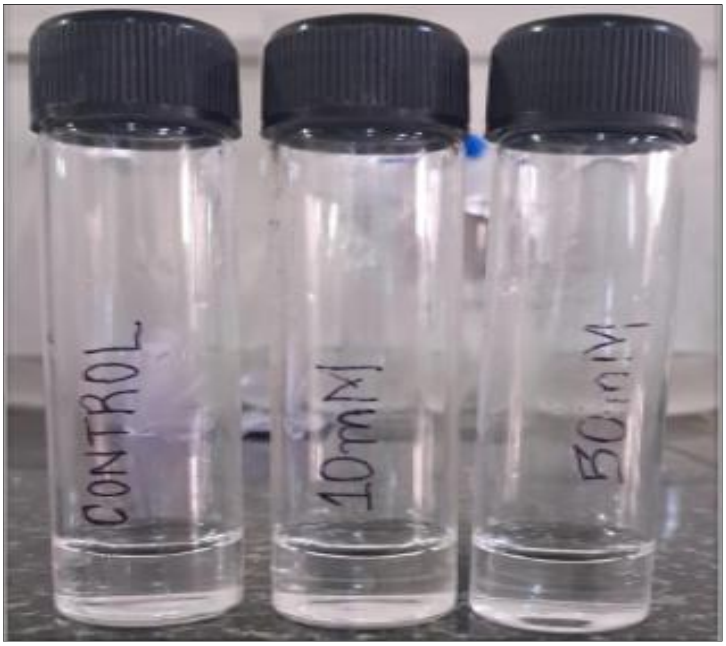


Fig 2: Solution mix

Calculation

Using $\varepsilon (H_2O_2) = 43.6\ M^{-1}\ cm^{-1}$:

Catalase Activity (U/mL) = $(\Delta A/\text{min} \times V\_\text{total}) / (\varepsilon \times d \times V\_\text{enzyme})$

Where d = 1 cm.

Table 4: Values of Catalase Activity (μmol/min/mL) of 7th and 14th day from seed sown

Salt Concentration	7th Day (U/mL)	14th Day (U/mL)
Control	0.0591	0.0579
10 mM	0.0499	0.0615
50 mM	0.0477	0.0601
100 mM	0.0000	0.0580

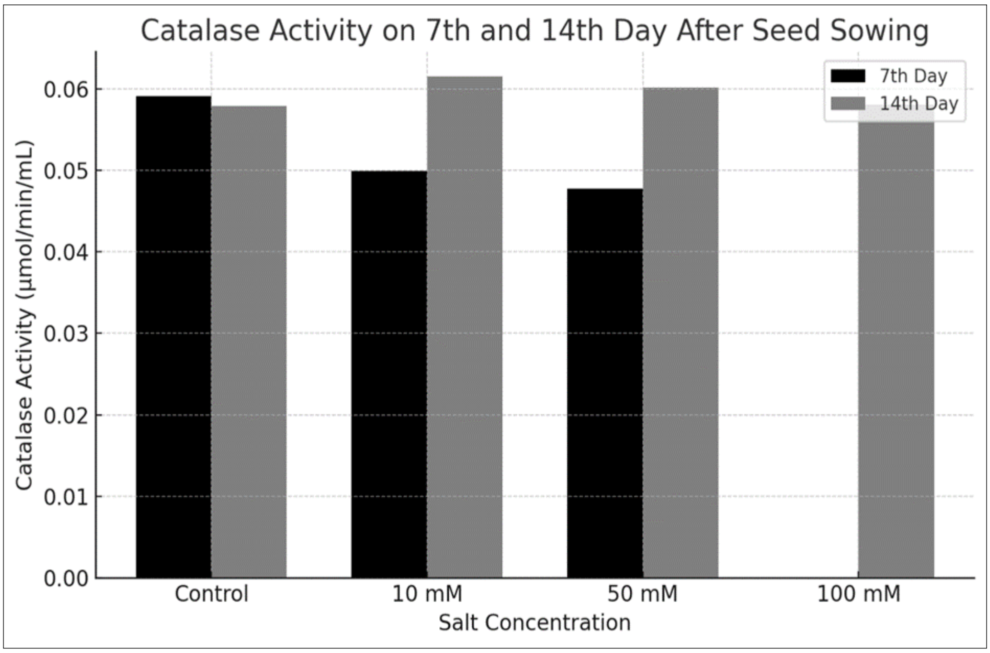

Catalase Activity on 7th and 14th Day After Seed Sowing

Fig 3: Graph of catalase activity on 7th day of sowing the seeds

3. Ascorbate Peroxidase Estimation

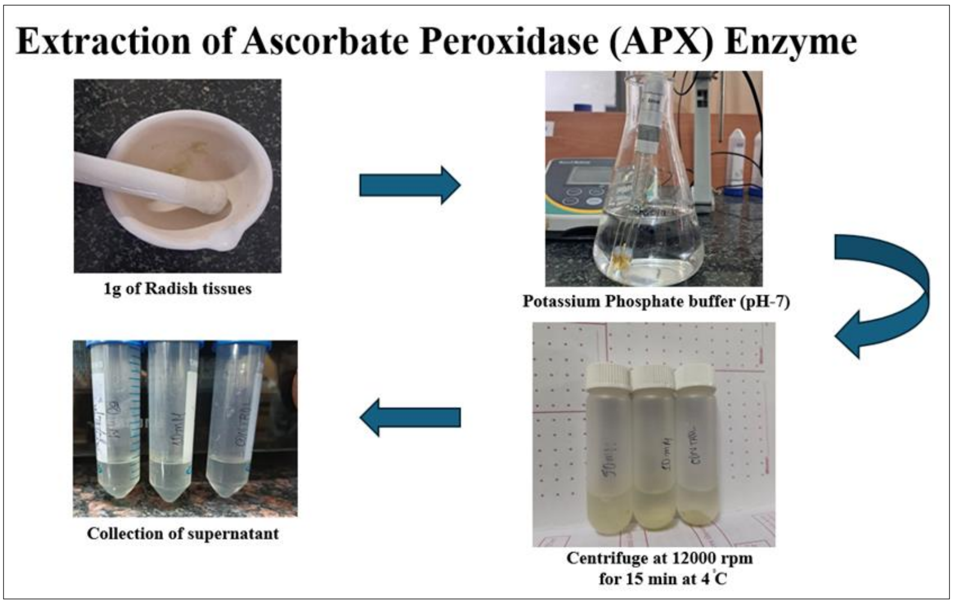
Ascorbate peroxidase (APX) is an antioxidant enzyme involved in detoxifying hydrogen peroxide (H_2O_2) using ascorbate as the electron donor. APX converts H_2O_2 → H_2O, simultaneously oxidizing ascorbate → monodehydroascorbate. This oxidation causes a decrease in absorbance at 290 nm, which is measured spectrophotometrically. The rate of decrease in A_{290} is directly proportional to APX activity. (Reference method: Nakano & Asada, 1981) [18]

Materials

- Fresh radish tissue
- 50 mM phosphate buffer (pH 7.0)
- Ascorbate (0.5 mM)
- H_2O_2 (0.1 mM; fresh)
- EDTA (0.1–1 mM, optional)
- UV spectrophotometer (290 nm), quartz cuvettes
- Ice, homogenizer, centrifuge

Preparation of Enzyme Extract

1. Homogenize 0.5–1 g fresh radish tissue in 5–10 mL ice-cold phosphate buffer + ascorbate + EDTA.
2. Centrifuge at 10,000–12,000 rpm, 15 min, 4°C.
3. Collect supernatant → crude APX extract.


Extraction of Ascorbate Peroxidase (APX) Enzyme

Assay Procedure (Total volume: 3 mL)

In a quartz cuvette:

- mL phosphate buffer
- mL ascorbate
- mL H_2O_2
- mL enzyme extract (added last)

Immediately measure absorbance at 290 nm every 15 seconds for 1–2 minutes.

Calculation of APX Activity

$$\text{APX Activity} = \frac{\Delta A_{290}/\text{min} \times \text{Total Volume}}{\epsilon \times \text{Path Length} \times \text{Enzyme Volume}}$$

Where:

- ε (extinction coefficient) = **$2.8\ mM^{-1}\ cm^{-1}$**
- Path length = **1 cm**
- Total volume = **3.0 mL**
- Enzyme volume = **0.1 mL**
- ΔA/min = slope of absorbance decreases at 290 nm

Reported as µmol ascorbate oxidized/min/mL enzyme.

Table 5: Values of APX Activity (µmol/min/mL) of 7th and 14th day from seed sown

Salt Concentration	7th Day (U/mL)	14th Day (U/mL)
Control	8.06	6.81
10 mM	7.76	6.80
50 mM	8.02	6.87
100 mM	0.00	6.98

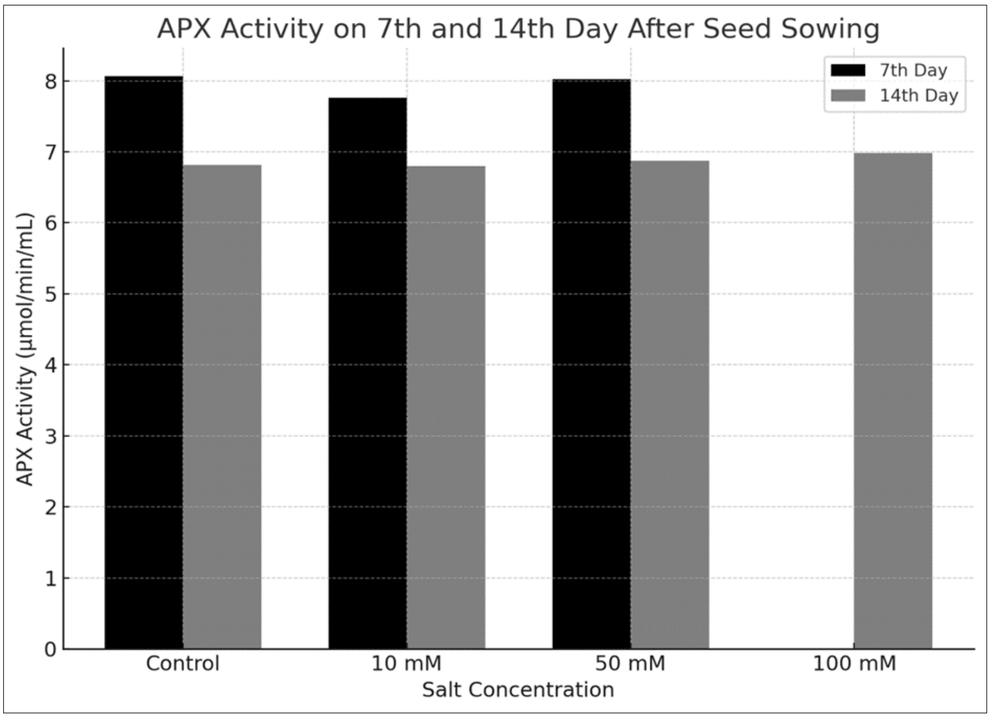


Fig 4: APX Activity on 7th and 14th day after seed was sown

4. **SDS-PAGE for Protein Estimation:**

Theory

SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) is a protein-separation technique based on molecular weight. Polyacrylamide gels provide uniform pore size. SDS acts as a denaturing detergent, binding uniformly to proteins and imparting a constant negative charge-to-mass ratio, forcing proteins into linear form. Thus, separation depends only on size, not charge (Laemmli, 1970) [9]. It is widely used for protein profiling, molecular weight estimation, purity checking, and biochemical analysis.

Principle

Proteins treated with SDS and β-mercaptoethanol become denatured and uniformly negatively charged. When an electric field is applied, proteins migrate through the polyacrylamide gel:

Smaller proteins travel faster → farther; Larger proteins → slower.

Materials

- **Gel reagents:** Acrylamide/Bis, Tris-HCl buffers (resolving pH 8.8; stacking pH 6.8), SDS, APS, TEMED
- Sample loading buffer (with SDS, β-mercaptoethanol, glycerol, bromophenol blue)
- Running buffer (Tris-Glycine-SDS)
- Staining & destaining solutions (Coomassie Brilliant Blue)
- Electrophoresis chamber, glass plates, combs, power supply, micropipettes

Gel Preparation

Resolving Gel (10 mL)

- Water, Acrylamide (30%), 1.5M Tris-HCl (pH 8.8), SDS, APS, TEMED

Stacking Gel (10 mL)

- Water, Acrylamide (30%), 0.5M Tris-HCl (pH 6.8), SDS, APS, TEMED

APS + TEMED initiate polymerization.

Sample Preparation

Mix equal volume of protein sample + 2× loading buffer.
Heat (optional) at 95°C for 5 minutes to denature proteins.
Samples include radish plants under different salinity treatments (Control, 10 mM, 50 mM, 100 mM).

Procedure

1. Clean plates and set up casting frame. Seal bottom with agarose.
2. Pour resolving gel → allow to polymerize.
3. Add stacking gel → insert comb → allow to set.
4. Assemble gel in electrophoresis tank; add running buffer.
5. Load 20 µL sample per well.
6. Run electrophoresis at constant voltage for ~1 hour until dye front reaches ¾ of the gel.
7. Remove gel and rinse 3–5 times with water to remove SDS.
8. Stain with Coomassie Blue until bands appear (~1 hour).
9. Destain to visualize clear protein bands.

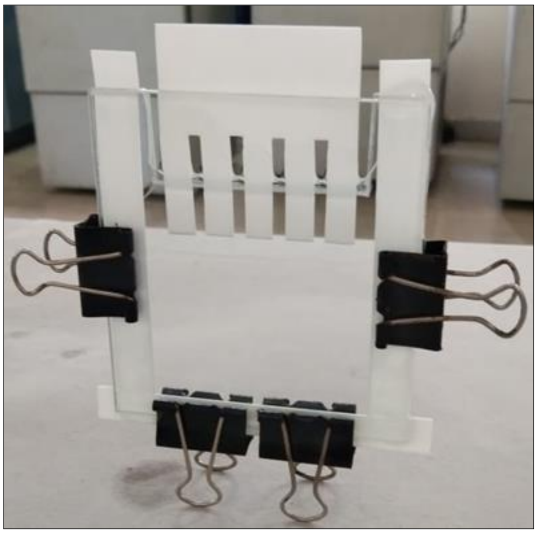


Fig 5: Glass slab containing resolving gel, stacking gel and comb

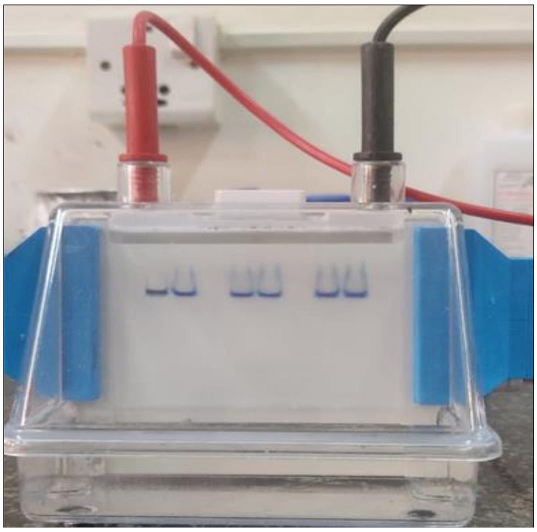


Fig 6: Glass slab is placed in the chamber and sample solution is placed in the wells

Table 6: Protein estimated at varied salt concentrations on 7th day of sowing the seeds

Salt Concentration	Approx. Protein Content (mg/g FW=Fresh Weight)	Observation
0 mM (Control)	7 mg/g FW	Normal protein biosynthesis without salt-induced stress.
10 mM NaCl	16 mg/g FW	Slight increase due to mild stress-induced activation of protein synthesis pathways.
50 mM NaCl	6 mg/g FW	Decline begins; oxidative stress starts affecting metabolic processes.
100 mM NaCl	-	-

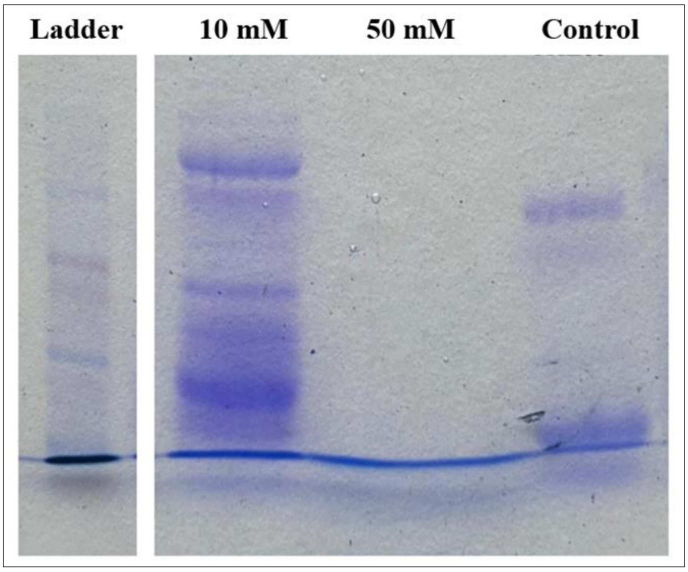


Fig 7: Result of Protein separation using SDS-PAGE at 7th day after seeds sown

Table 7: Protein estimated at varied salt concentrations on 14th day of sowing the seeds

Salt Concentration	Approx. Protein Content (mg/g FW=Fresh Weight)	Observation
0 mM (Control)	9 mg/g FW	Normal protein biosynthesis without salt-induced stress.
10 mM NaCl	17 mg/g FW	Slight increase due to mild stress-induced activation of protein synthesis pathways.
50 mM NaCl	8 mg/g FW	Decline begins; oxidative stress starts affecting metabolic processes.
100 mM NaCl	6 mg/g FW	Marked reduction in protein content; severe ionic and osmotic stress inhibits growth.

Results and Discussion

Antioxidant Enzyme Response under Salinity Stress

Salinity significantly influenced the activity of catalase (CAT) and ascorbate peroxidase (APX) in radish seedlings, with clear variations observed across treatments and growth stages. On the 7th day, CAT activity showed a marked decline with increasing salt concentration, decreasing from 0.0591 U/mL in the control to 0.0499 U/mL (10 mM), 0.0477 U/mL (50 mM), and complete suppression at 100 mM. This sharp reduction at higher salinity suggests that severe ionic and osmotic stress inhibit the synthesis or functioning of CAT. However, by the 14th day, CAT activity increased under mild to moderate salinity, reaching 0.0615 U/mL at 10 mM and 0.0601 U/mL at 50 mM, while remaining comparable to the control at 100 mM. This recovery indicates that seedlings attempt to re-establish antioxidative balance as they adapt over time.

APX activity displayed a similar trend. On the 7th day, APX was highest in the control (8.06 U/mL), followed closely by 50 mM (8.02 U/mL) and 10 mM (7.76 U/mL), while 100 mM exhibited zero activity, mirroring the inhibition observed for CAT. By the 14th day, APX activity decreased overall (6.80–6.98 U/mL), but the narrow range suggests partial stabilization and adaptation after prolonged exposure. These findings align with previous studies reporting enzyme activation under moderate salinity and suppression under severe stress (Kumari and Kaur, 2017; Abeed *et al*., 2023) [3, 21].

Together, CAT and APX trends demonstrate that mild to moderate salinity (10–50 mM) activates antioxidative defence mechanisms, while higher salinity levels surpass the stress tolerance threshold, leading to enzymatic inhibition due to ROS overload.

Protein Content and SDS-PAGE Profile

Protein estimation and SDS-PAGE analysis further confirmed the biochemical changes induced by salinity. Protein content increased substantially at 10 mM NaCl, the 7th and 14th day showing 16 mg/g FW and 17 mg/g FW, respectively, compared to the control (7 mg/g and 9 mg/g). This enhancement under mild stress indicates induction of stress-related proteins and activation of metabolic pathways supporting early tolerance.

At moderate salinity (50 mM), protein content decreased to 6 mg/g FW (day 7) and 8 mg/g FW (day 14), reflecting the beginning of metabolic inhibition caused by oxidative stress. High salinity (100 mM) caused severe reductions, particularly evident on the 14th day (6 mg/g FW), while the 7th day sample showed undetectable levels—indicating that early-stage seedlings are highly vulnerable to severe salt stress.

SDS-PAGE banding patterns supported these results. Distinct and intense protein bands were observed at 10 mM, indicating higher protein synthesis and stress-induced isozyme activation. Moderate salinity produced weaker bands, whereas high salinity led to faint or absent bands, suggesting protein degradation or inhibited protein biosynthesis. Similar observations were reported in radish proteomic studies, where stress-responsive proteins declined sharply beyond the tolerance limit (Sun *et al*., 2017; Kasim and Dowidar, 2006) [10, 11].

Conclusion

The present study evaluated the physiological, biochemical, and protein-level responses of radish (*Raphanus sativus*) seedlings to salinity stress. Seeds were subjected to varying NaCl concentrations (0, 50, 100, 150 mM) to assess the impact on germination and early growth. Increasing salinity caused a progressive decline in germination percentage, root and shoot length, and vigour index, with moderate salinity inducing partial inhibition and higher salinity resulting in severe osmotic and ionic toxicity.

Biochemical analyses of key antioxidative enzymes—catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD)—showed enhanced activity under moderate salinity, indicating activation of the antioxidative defence system in response to ROS accumulation. However, enzyme activities declined at higher salt levels, suggesting inhibition or destabilization of defence mechanisms under extreme stress.

Protein profiling through SDS-PAGE revealed distinct changes in banding patterns across treatments. Upregulated and stress-induced protein bands were observed under moderate salinity, indicating increased expression of stress-responsive proteins. Conversely, reduced or absent bands at high salinity suggested repression of protein synthesis or protein degradation due to cellular damage.

Overall, the study demonstrates that salinity stress affects radish at multiple biological levels, with moderate salinity eliciting adaptive biochemical and molecular responses, while higher salinity surpasses the plant's tolerance limits. Antioxidant enzymes emerge as potential biomarkers for salinity tolerance. These findings contribute to understanding salinity-stress biology in radish and may support future breeding, molecular studies, and genetic improvement programs aimed at enhancing salt tolerance in crop plants.

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