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Six-Well Plate Water Agar Assay for Assessing Chitosan-Mediated Salinity Tolerance during Tomato Germination

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ABSTRACT

Salinity is one of the major agricultural global issues, which causes extensive crop loss and economic damage which affecting 8.7% of global land and projected to impact as much as 50% of arable land by 2050. Tomato (Solanum lycopersicum) is classified as moderately salt-sensitive, with moderate salinity (25-50 mM NaCl) slowing germination and seedling growth, which severely reduces stand establishment. Seed priming is a process of controlled hydration that is known to improve stress tolerance. Here, we demonstrate that the seed priming with chitosan improved seed germination, and early seedling growth under salinity stress in tomato. We then optimized the agar medium (0.5% w/v) and chitosan priming dose (0.2% w/v) in a rapid six -well agar plate assay for maximal vigor. At 50–200mM NaCl concentration, the germination percentage and biomass showed steep declines in unprimed seeds. Conversely, chitosan-primed seeds exhibited significantly improved germination rates and seedling vigor indices across all salinity levels. Priming alleviated the salt stress effects well under salt stress: primed seedlings showed high relative water content (RWC), and total chlorophylls increased antioxidant capacity, and increased friendly pressure of osmo-protectants on osmo-protectants (proline) in relation to non-primed control plants. PCA of 12 physiological traits highlighted separation between primed and unprimed treatments when evaluated under a high salinity treatment. To conclude, seed priming with 0.2% chitosan represents an easy and scalable method to enhance early-stage salt tolerance in tomato. Moreover, the novel six-well agar assay platform described in this work is a sturdy and high-throughput platform for screening seed treatment responses against NaCl salt stresses.

Keywords: *Solanum lycopersicum; seed germination; salinity stress; chitosan; seed priming; six-well plate assay.*

Introduction

Soil salinization is a growing threat to modern agriculture and global food security. More than 830 million hectares (8.7% of global land) are already affected by salt, and up to half of all arable land is likely to face this problem by 2050 (Bose and Pal, 2025). Salinity, aggravated by irrigation with low-quality water and climate change, leads to multi-billion-dollar losses in crop production every year. When major vegetable crops such as tomato are grown on saline soils, yield reductions can be significant. Tomato (*Solanum lycopersicum*) is one of the most known vegetables amongst both experts important vegetables worldwide, with production rates from 180–250 million tons every year. As a result, it is designated as moderately salt-sensitive [2, 10]. Furthermore, tomato seed germination is inhibited at moderate levels of salt (25-50 mM NaCl), which also reduce early seedling growth and affects stand establishment and quality yield [15, 22, 29]. The high soil salinity affects the germinating seeds mainly by two stresses: osmotic stress and ionic stress.

Osmotic stress results from high solute levels that impede water uptake, which leads to cell dehydration [25], reducing seed-imbibition rates and exposing seedling roots to water deficits that lead to a decrease in turgor and growth retardation [24], this reduced turgor is counteracted by an accumulation of compatible osmolytes such as soluble sugars and proline, allowing the plants maintain osmotic balance. Simultaneously, through ionic stress, higher concentrations of Na^+ and Cl^- ions interfere with nutrient homeostasis as seen in the competition of Na^+ for K^+ uptake leading to cytotoxicity to cellular structures [8]. Oxidative stress is considered to be a key downstream effect of osmotic and ionic stress. Salt stress leads overproduction of (ROS) reactive oxygen species, which react with and damage membranes, proteins and DNA. Tissues suffering from salt stress usually have high malondialdehyde (MDA) level, which is the marker of lipid peroxidation, as a result. Plants respond by upregulating antioxidant enzymes (e.g., catalase, peroxidases, superoxide dismutase) to scavenge ROS [28, 30, 1]. Additionally, salinity distorts hormonal communication generally raising abscisic acid while reducing the levels of gibberellins and auxins which maintain seed dormancy and discourage germination [12]. In fact, salt stress is as a combination of osmotic dehydration, ion toxicity and oxidative damage that impede seedling establishment [23]. This applies to tomato seeds as well: their germination rates and seedling vigor decline dramatically with increasing salinity [2, 3]. In this regard, seed priming has recognized as a promising tool for enhancing germination and early vigor in adverse conditions [9, 17, 27]. Priming is the preparation of seeds by controlled hydration to initiate metabolic processes, but not germination (radicle emergence), and followed by drying back to a storable moisture content [11]. Chitosan, a biodegradable polysaccharide obtained from chitin, is one of the natural priming agents that has been found to be plant growth-promoting and stress-protective [16]. Chitosan and its oligomers serve as biostimulants, inducing expression of stress-responsive genes and regulating osmotic adjustment [26]. Chitosan and its oligomers are excellent elicitors, evoking effective responses in plants such as improved seed germination, vigor of seedlings, and activation of defence action against abiotic and biotic stress [21, 13, 19]. Furthermore, while there is a lot of literature on chitosan's advantages for humans and plants, information related to how applied in tomato germination improvement under salt stress via rapid screening methods is scarce.

We designed a six-well agar plate germination assay for the study with precise control over salt and priming treatments. Using this system, we performed four targeted experiments: agar optimization to determine the appropriate gel strength; chitosan dose–response to identify a suitable priming concentration; salinity dose–response to define baseline sensitivity and a priming versus salinity response to examine how priming increases salt tolerance. We aimed to enhance early-stage salinity tolerance in tomato using chitosan seed priming and to develop a loss-of-function trait for systematic, high-throughput screening of amelioration strategies under stress.

Materials and Methods

Tomato seeds (*Solanum lycopersicum* L., cv. Arka Rakshak) exhibiting >70% viability from a commercial supplier. For priming treatments, a stock solution of medium-viscosity chitosan (>75% deacetylation, MW 280 - 400 kDa) was prepared by dissolving in 1% (v/v) acetic acid and each working concentration from 0.05 to 0.8% (w/v). Before treatment, seeds were 1 min surface-sterilized using 1.5% sodium hypochlorite, thoroughly rinsed and air-dried. The priming of 100 seeds per treatment in the respective chitosan solutions for 5 min at 25°C with occasional swirling was performed to initiate metabolism without radicle emergence and then air dried on sterile filter paper for 24 h to restore moisture content close to the original. Primed seeds were stored at 4°C until use and unprimed control seeds were treated with distilled water exactly the same as primed seeds (5 min).

Agar Medium and Six-Well Plate Assay Setup

We used a six-well plate assay to set up a small-scale, reproducible germination system with controlled salt levels. A 0.5% (w/v) aqueous agar purified water concentration was selected as optimal based on preliminary trials testing concentrations ranging from 0 to 2% (w/v), as it created an environment that remained soft enough for root penetration while providing sufficient moisture for absorption [14].

Analytical-grade NaCl (i.e., salt) was added to reach final salinities of 0, 25, 50, 100, 150 and 200 mM; media were then autoclaved and allowed to cool (vented check around 50°C), dispensing 10 mL into each well with a diameter of 35 mm. After solidification, 10 and seeds were evenly distributed and embedded into the agar matrix at a depth of 3–5 mm. Plates were sealed with Parafilm to reduce desiccation, incubated at 25 ± 2 °C, and kept in the dark for the first three days of incubation to stimulate germination, after which plates were incubated under continuous cyclical conditions of 16-hours light/8 h dark for a further four days.

Experimental Design

Experiment 1: **Optimization of agar concentration (without salt).**

A medium firmness assay was performed under salt-free conditions using six different agar concentrations in the range 0-2 % (0, 0.125, 0.25, 0.5, 1, and 2 % w/v). Germination percentage and seedling length were measured after 7 days for each agar level. Subsequent experiments were carried out on 0.5% agar as this was the optimal consistency.

Experiment 2: **Dose–response of chitosan priming (no salt).**

To evaluate the biostimulant effect of chitosan under no stress, seeds were subjected to priming in 0 (water control), 0.05%, 0.1%, 0.2%, 0.4% or 0.8% (w/v) chitosan (5 min each), then dried and sown on 0.5% plain agar (0 mM NaCl). Plates were grown for 7 days. We measured final germination %, shoot-length, root-length, fresh biomass weight, and dry biomass weight, and calculated a Seedling Vigor Index (SVI) = (% germination) × (mean seedling length), where mean seedling length is the sum of shoot + root in mm. These trials determined the concentration of chitosan that improved germination and vigor (optimal: 0.2% chitosan) under these non-stress conditions.

Experiment 3: **Salinity dose–response unprimed seeds.**

Salinity tolerance of seeds was determined by germinating unprimed seeds on 0.5% agar containing 0, 25, 50, 100, 150, and 200 mM NaCl. The final germination % 7 DAS was determined per level of salt. At 7 DAS, length of the shoot, root, fresh weight and dry weight of seedling (10 to plot representative seedlings per replicate). To calculate the dry weight, the seedlings were oven-dried at 60°C for 48 h; relative water content (RWC) of the fresh seedlings was determined as follows: RWC = [(fresh weight − dry weight)/ (turgid weight − dry weight)] × 100%. Fully hydrated sampled seedlings were used to measure turgid weight. The ratio of tissue water contains the information for stress, which is known as RWC. This experiment found the salinity threshold that significantly affects tomato germination and growth.

Experiment 4: Priming × salinity interaction

Based on 1- 3 experiment, we chose the optimal priming dose of 0.2% chitosan. Seeds primed with 0.2% chitosan and not primed (water control) were germinated on 0.5% agar containing NaCl doses at 0, 25, 50, 100, 150, and 200 mM. This models the sowing of primed versus non-primed seeds in soils with increasing salinity. Germination and growth parameters were assessed at 7 DAS as previously described in Exp. 3. Furthermore, seedlings from these treatments were employed for physiological and biochemical assays to compare stress indices in primed versus unprimed plants.

Biochemical assays in seedlings
In an attempt to explain physiological and biochemical changes induced by priming during salt stress, we measured several markers in 7-day-old seedlings from Experiment 4. For each treatment, 0.5 g of fresh seedling tissue (composed shoots and roots from one replicate) was sampled for assays (two analytical replicates per treatment).

Photosynthetic pigments. Extraction of total chlorophyll and carotenoids from 100 mg fresh tissue in 80% acetone and absorbance was recorded at 645 nm, 663 nm, (chlorophyll b and a) and 470 nm for (carotenoids). Chlorophyll a and b contents (mg/g FW) were calculated by using standard equations, total chlorophyll (a + b), carotenoids (mg/g FW).

Proline. The free proline were assayed through acid-ninhydrin method. 100 mg of tissue was homogenized in sulfosalicylic acid 3%; the extract was treated with ninhydrin (acidic) at 95°C for 1 h. The reddish chromophore was extracted into toluene and absorbance recorded at 520 nm. Proline concentrations (μmol g^{-1} FW) were extrapolated from the standard graph curve.

Reducing sugars. Reducing sugars solubility were quantified by the dinitrosalicylic acid (DNS) assay. 100 mg tissue was extracted in boiling water, then reacted with DNS reagent and heated to develop orange-red color. Results are expressed as mg reducing sugars/g FW after comparing absorbance at 540 nm with a glucose standard.

Ascorbic-acid (AsA). AsA content was estimated by following the Folin-Ciocalteu method. 5% metaphosphoric acid was used to homogenize 100 mg of the tissue and then filtered. Folin reagent and sodium carbonate was added to the extract, and absorbance was recorded at 760 nm. AsA levels (μg/mg FW) were determined from an ascorbate standard curve.

Total antioxidant capacity. The DPPH radical scavenging assay was used to assess overall antioxidant power. 10μL of the seedling extracts was mixed with 100μL of DPPH in methanol (0.1 mM) and incubated for 30 min in the dark. The glutathione concentration was calculated by measuring the decrease in DPPH at 517 nm. The antioxidant capacity was reported as % inhibition of DPPH (compared to a control without the extract). % inhibition was converted to μmol Trolox equivalents g^{-1} FW according to a Trolox standard curve for quantification purposes.

Statistical Analysis
Experiments were conducted in a completely randomized design with 10 replicates of each treatment. One-way ANOVA were conducted for single-factor experiments (agar/chitosan dosage). Two-way ANOVA assessed main effects and interactions for the Priming vs Salinity experiment. Tukey's HSD ($P < 0.05$) was used for post-hoc comparisons. Goods indexes were computed by performing a Principal Component Analysis (PCA) on the correlation matrix of all parameters. Analyses were conducted using GraphPad Prism 9 and R software.

Results and Discussion

Method development for assessment of seed vigor
We also optimized the agar concentration for a system that would have sufficient moisture availability while still providing physical support.

The results (Table 1) showed that the ideal agar concentration was 0.5% (w/v). This firmness showed the maximum germination percentage (82.1%) and seedlings length (62.2 mm), resulting in the highest SVI. Gels at lower concentrations were too colloidal to hold the plant upright whereas those at higher concentrations (1%) inhibited root penetration and dried out by 14-day [14]. And then, determine the optimum priming concentration of chitosan. Seeds were germinated on non-saline agar as described (Table 2). All treatments with chitosan significantly increased performance compared with the control treatment primed in water. The most effective was 0.2% chitosan solution which significantly increased the germination rate to 92.1%, compared with control plants, and increased SVI by >30%. However, increased vigor was slightly inhibited at higher doses (0.8%), indicating potential phytotoxicity. Therefore, chitosan (0.2%) was chosen for salinity stress assays.

Germination and growth inhibition at various salinity stress levels
To observe the effects of increased NaCl in the medium, non-primed tomato seeds were subjected to different concentration levels which showed a successive decline in both germination and growth (Fig. 2A, Table 3). Germination success dropped sharply from 81% at 50mM NaCl to 48% at the next salinity level; only 26% germinated at either 150 or 200 mM (Fig. 2A). This classic salt-inhibition is attributable to osmotic effects (lowering water availability) and ionic toxicity disrupting metabolism [23, 31]. These findings are consistent with studies on tomato, which document a significant reduction in germination at NaCl concentrations above 50–100 mM [2, 10, 18]. Seedling growth was much more sensitive. 200 mM NaCl severely reduced shoot and root lengths, while the SVI, which combines germination and length 33 decreased by 87 %. The near-complete loss of vigor at high salinity is well documented in tomato and other crops [7, 4]. Stressed seedlings exhibited classic drought physiological traits, as relative water content (RWC) fell to 64% at 200 mM (compared to 90% for controls), manifesting severe tissue dehydration. In high salt, total chlorophyll greatly declined to almost half the amount compared to control conditions, indicating that oxidation or a disruption of de novo synthesis occurred. Seedlings responded by inducing defense pathways: leaf proline (osmo-protectant) rose > 4-fold with high NaCl. Proline and other compatible solutes reduce osmotic potential of cells, maintaining cell turgor and shielding the structures from salt stress in tomato. similarly, total antioxidant capacity increased under moderate stress, suggesting the mobilization of defense systems. However, at extreme salinity, these defenses were exceeded as indicated by a plateau or decline in capacity indicating metabolic limits were reached. In brief, unprimed tomato seedlings exhibited dose-dependent decreases in germination, growth, and water status under salinity stress, as also previously reported [2, 10, 18, 7, 4, 25, 33, 20].

Chitosan Priming Mitigates Salinity Stress
Seed priming with 0.2% chitosan significantly enhanced salinity tolerance (Fig. 2B, Table 4). Primed seeds exhibited significantly greater germination than unprimed ones at each concentration of NaCl.. The effect was strongest under the most severe stress: at 200 mM NaCl, primed germination (38.7%) was ~50% more than unprimed (26.1%).

This indicates that priming enabled the seeds to counteract this initial osmotic shock, keeping them metabolically active enough for germination. Improvement of germination under stressful conditions is also consistent to prime treatments [4]. Chitosan treatments were also correlated with increased vigor at the seedling stage (similar results have been reported elsewhere where exogenous chitosan was found to increase tomato tolerance to salinity) [32]. The mechanism seems to be a general strengthening of seedling physiology. Primed seedlings maintained similar RWC throughout all salinities when compared to control seedlings including osmotic stress conditions, indicating increased water uptake/retention activity. They accumulated much higher proline accumulation for osmotic adjustment and cellular protection. Additionally, primed seedlings exhibited enhanced antioxidant defense: ascorbic acid content and DPPH scavenging capacity were both higher. Chitosan's mode of action could be similar as an elicitor, pre-activating these defense pathways [21] and producing a "primed" state. Such readiness allows quicker, more vigorous reactions to ROS in response to salt challenge [1]. The strong priming × salinity interaction (two-way ANOVA, $P < 0.05$) support the idea that chitosan provisions work better under higher stress. That is, the action of chitosan priming as a stress tolerance up-regulator means primed seedlings that are physiologically more similar to moderate-stressed non-primed than to severe stressed unprimed seedlings. This priming effect is desirable for the improvement of crop establishment as soils become progressively salinized [5].

Multivariate Analysis (PCA)

To reduce the dimensionality of complex dataset, we performed principal component analysis (PCA) on all 12 measured parameters. The two first components explained 93.2% of variance (Fig. 3A). PC1 (70.3% variance) divided treatments by salt severity: low/no salt samples clustered with high growth/vigor variables on the negative side of PC1, while high-salt treatments (150–200 mM) clustered with stress-response traits (proline, antioxidant) on the positive side (Fig. 3B). PC2 (22.9% variance) separated primed vs unprimed samples. Primed treatments occupied different areas from unprimed controls at all salinities. Primed seedlings under high salinity shifted toward the moderate-stress cluster. For instance, primed-150 mM samples (P-150) cluster closer to the 100 mM cluster compared with unprimed-150 (U-150), in the PC1–PC2 plane. This "repositioning" indicates that chitosan priming holistically mitigates high salt stress, resulting in a more similar overall physiological status of stressed seedlings to those exposed to lower stress conditions. Such a multivariate shift of germination, growth, water status and biochemical defenses is an indicator of successful priming. It also show-cases how this six well assay, with PCA steps, can swiftly screen and visualize the effectiveness of a priming or other treatments to abiotic stress [21, 1].

Table 1: Effect of the water–agar concentration on the seed germination, and seedling vigor parameters of the tomato (cv. Arka Rakshak) 7 DAS (no salt)

Agar (%)	Germination (%)	Seedling length (mm)	Root length (mm)	Fresh biomass (mg)	Dry biomass (mg)	Vigor Index
0.00	61.4 ± 4.6 d	32.21 ± 4.1 e	21.44 ± 2.31 d	94.29 ± 21.13 e	11.06 ± 2.1 d	3254 ± 127 e
0.13	68.6 ± 4.1 c	44.07 ± 6.01 d	47.18 ± 4.09 c	180.42 ± 32.16 d	16 ± 4.3 c	6242 ± 141 d
0.25	73.4 ± 3.2 B	47.11 ± 5.37 c	49.35 ± 6.21 c	210.11 ± 38.31 c	20 ± 4.2 b	7046 ± 134 c
0.50	82.1 ± 4.8 a	62.20 ± 7.03 a	52.31 ± 6.07 a	260.28 ± 41.40 a	23 ± 5.1 a	9357 ± 162 a
1.00	80.5 ± 5.3 a	58.41 ± 9.15 ab	51.42 ± 8.11 ab	240.36 ± 33.20 ab	22 ± 6.1 ab	8774 ± 179 b
2.00	79.6 ± 5.2 ab	56.28 ± 11.03 b	50.27 ± 8.23 b	230.27 ± 29.37 b	21 ± 5.2 b	8436 ± 198 b

Values are mean ± SE (n=10). Means within the same column with different letters are significantly difference based on Tukey's HSD test, P≤0.05 Means in the same row with same letter are not significantly different (p < 0.05); means sharing the letter "a" have highest mean value, further letters denote falling mean value.

Table 2: Chitosan priming concentration effect on tomato seed germination, and seedlings growth (7 DAS, on 0.5% agar + no salt)

Chitosan (%)	Germination (%)	Seedling length (mm)	Root length (mm)	Fresh biomass (mg)	Dry biomass (mg)	Vigor Index
0	80.87 ± 3.02 c	64.59 ± 8.2 d	48.02 ± 4.64 e	254.40 ± 21.2 d	21.05 ± 2 c	9224 ± 136 d
0.05	84.30 ± 1.47 bc	67.85 ± 4.64 c	55.18 ± 4.08 c	261.21 ± 32.3 c	22.4 ± 4.3 bc	10181 ± 104 c
0.1	90.74 ± 1.57 ab	70.43 ± 4.53 b	57.24 ± 3.86 bc	270.41 ± 38.1 b	24 .33± 4 a	11184 ± 169 b
0.2	92.09 ± 3.2 a	71.37 ± 3.49 a	61.05 ± 6.05 a	284.11 ± 41.1 a	24.54 ± 6 a	12189 ± 174 a
0.4	91.0 ± 4.2 ab	70.08 ± 4.91 b	56.14 ± 8.11 c	281.04 ± 33.1 a	23.4 ± 6 b	11471 ± 162 b
0.8	86.7 ± 5.2 bc	68.08 ± 6.31 c	52.37 ± 6.05 d	268 ± 29	24 ± 5	10404 ± 168

Values are mean ± SE (n=10). Means within columns with different letter(s) are significantly different (P≤0.05); Tukey's HSD test. Treatments denoted with the same letter "a" have the highest mean, and letters descend in descending order.

Table 3: Germination, growth, and physiological parameter of unprimed tomato seed sand seedlings growth under different NaCl concentrations (7 DAS).

NaCl (mM)	Germ (%)	Shoot L (mm)	Root L (mm)	Fresh Biomass (mg)	Vigor Index	Dry Weight (mg)	RWC (%)	Chl (µg/mg FW)	Proline (µmol/g)	Red. sugar (mg/g)	AsA (µg/mg)	Antiox (µmol Trolox/g FW)
0	81.98 ± 1.3 b	65 ± 3.3 ab	58.3 ± 2.7 ab	288.8 ± 14.6 a	11180 ± 259 a	24.8 ± 1.2 b	80 ± 4.1 b	1.86 ± 0.1 b	9.62 ± 0.5 d	1.8 ± 0.1 c	1.6 ± 0.1 c	16.2 ± 0.8 c
25	87.59 ± 3.1 a	68 ± 3.4 a	61.1 ± 3.1 a	292.1 ± 14.1 a	11352 ± 267 a	26.1 ± 1.3 b	81 ± 4.1 a	1.91 ± 0.1 a	16.09 ± 0.8 c	2.3 ± 0.1 b	2.1 ± 0.1 b	23.9 ± 1.1 b
50	80.91 ± 3.0 c	63 ± 3.6 bc	54.2± 2.9 b	276 ± 13.8 b	9758± 287 b	24.2 ± 1.3 b	79 ± 4.0 c	1.84 ± 0.1 b	34.77 ± 1.5 b	2.7 ± 0.2 a	2.4 ± 0.1 a	34.8 ± 1.5 a
100	76.55± 3.1 d	61 ± 3.1 c	43.1 ± 2.2 c	240.4 ± 12 c	8008 ± 201 c	23.2 ± 1.2 bc	77 ± 3.9 c	1.69± 0.1 c	39.21 ± 2.1 ab	2.1 ± 0.2 b	2.6 ± 0.1 a	36.6 ± 1.9 a
150	50.32 ± 1.0 e	41 ± 2.1 d	34.3 ± 1.7 d	170..6 ± 8.5 d	3600 ± 180 d	18 ± 0.9 c	68 ± 3.7 d	1.40 ± 0.1 d	42.82 ± 2.5 a	1.6 ± 0.1 c	1.2 ± 0.1 d	35.2 ± 2.1 a
200	26.08 ± 0.9 f	33 ± 1.7 e	21.4 ± 1.1 e	112 ± 5.6 e	1404 ± 70 e	11 ± 0.6 d	64 ± 3.5 d	0.96 ± 0.1 e	41.68 ± 3.0 b	1.6 ± 0.1 c	1.1 ± 0.1 d	31.8 ± 1.7 a

Data are presented as mean ± SE (n = 10). Values in the same column with different letters are significantly different at P ≤ 0.05 level, according to Tukey's HSD test. Treatments that share the same letter "a have max mean value, and letters decrease in order. RWC = Relative Water Content; Chl = Total Chlorophyll. Red. = Reducing sugars; AsA = Ascorbic Acid; Antiox = Total Antioxidant Capacity.

Table 4. The influence of 0.2% chitosan seeds priming treatment on tomato germination and seedling parameters under different NaCl concentration (7DAS).

NaCl (mM)	Germ (%)	Shoot Length (mm)	Root Length (mm)	Fresh Biomass (mg)	Vigor Index	Dry Weight (mg)	RWC (%)	Chl (µg/mg FW)	Proline (µmol/g)	Red. sugar (mg/g)	AsA (µg/mg)	Antiox (µmol Trolox/g FW)
0	94.6 ± 4.7 a	76 ± 3.8 a	63 ± 3.2 a	314 ± 15 a	13149 ± 357 ab	29 ± 1.5 a	80.1 ± 4.1 ab	1.9 ± 0.1 a	31.2±1 d	2.2±0.19 c	2.6 ±0.18 b	28.1 ± 1.4 d
25	96.7 ± 4.8 a	78 ± 3.9 a	66 ± 3.3 a	321 ± 12 a	13925 ± 396 a	29 ± 1.5 a	82 ± 4.1 a	1.9 ± 0.1 a	46.4±2 c	3.7 ± 0.2 a	2.9 ± 0.2 ab	42.6 ± 1.6 c
50	92.2 ± 4.6 ab	74 ± 3.7 a	61 ± 3.1 ab	301 ± 10 b	12447 ± 322 b	28 ± 1.4 ab	80.3 ± 3.9 ab	1.8 ± 0.1 b	46.1 ± 2 c	3.9 ± 0.3 a	3.1 ± 0.2 a	47.3 ± 1.8 a
100	84.8 ± 4.2 b	68 ± 3.4 b	51 ± 2.5 b	296 ± 14 b	10091 ± 304 c	27 ± 1.3 b	79.7 ± 3.8 ab	1.6 ± 0.1 c	51.2 ± 3 b	3.1± 0.18 b	2.3 ± 0.16 b	44.1 ± 2.21b
150	66.9 ± 3.3 c	54 ± 2.7 c	43 ± 2.2 c	238 ± 12 c	6487 ± 324 d	22 ± 1.1 c	77.2 ± 3.7 b	1.4 ± 0.1 d	59.3 ± 4 a	2.1 ± 0.2 c	2.3 ± 0.2 b	44.2 ± 2.4 b
200	48.7 ± 2.4 d	41 ± 2.1 d	31 ± 1.6 d	177 ± 9 d	3504 ± 175 e	14 ± 0.7 d	76.1 ± 3.5 c	1.1 ± 0.1 e	56.7 ± 4 b	2.0 ± 0.1 c	1.8 ± 0.2 c	40.6 ± 2.2 bc

Values are mean ± SE (n = 10). Means with the same letters in the same column are not significantly different at P ≤ 0.05 according to Tukey's HSD test. Treatments with the same letter "a" have the highest mean, followed by letters in descending order. Notes: RWC = Relative Water Content; Chl = Total Chlorophyll; Red. = Reducing sugars; AsA = Ascorbic Acid; Antiox = Total Antioxidant Capacity

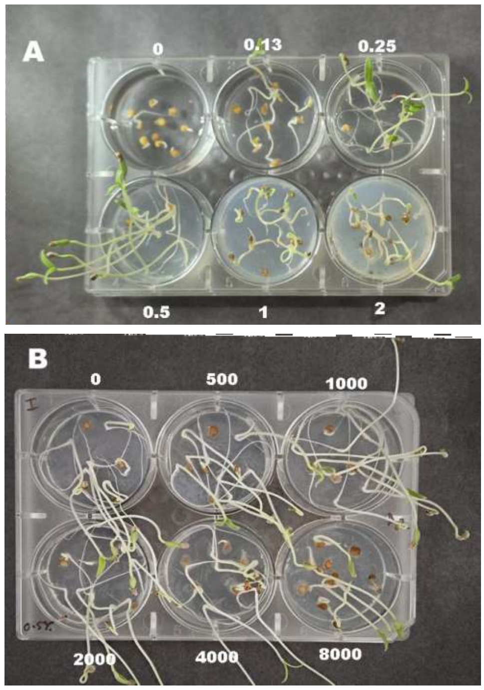

Fig. 1: Chitosan and Agar-Effects of Concentration on Tomato Germination and Seedling Vigor in a 6 Well Plate Assay

(A) Effect of agar concentration on seed germination and seedling growth. (B) Influence of chitosan concentration on percentage germination and vigor index, at 4 DAS.

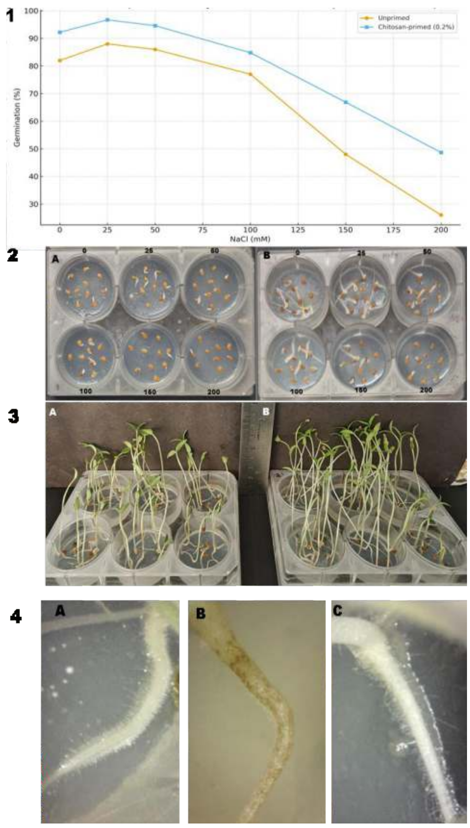

Fig. 2: Response of tomato seeds germination and seedling growth under NaCl stress (0–200 mM).

*(2.1) Germination percentage of seeds without priming and with chitosan-priming (2.2) Six-well plate images illustrate germination under NaCl stress: (**2A**) unprimed (left) at 3 Days after Sowing (DAS), and (**2B**) chitosan-primed (right). Response of 7 DAS seedling growth: (**3A**) unprimed and (**3B**) chitosan-primed. (2.4) Root architecture on exposure to salt stress:(**4A**) control (0 mM NaCl)_revealed lower hairy roots, (**4B**) 100 mM NaCl stressed seedlings reflected lesser hairy root formation and scorching, while (**4C**) chitosan-primed seedlings showed profuse hairy root development immersed under 100 mM NaCl with mucilaginous secretions.*

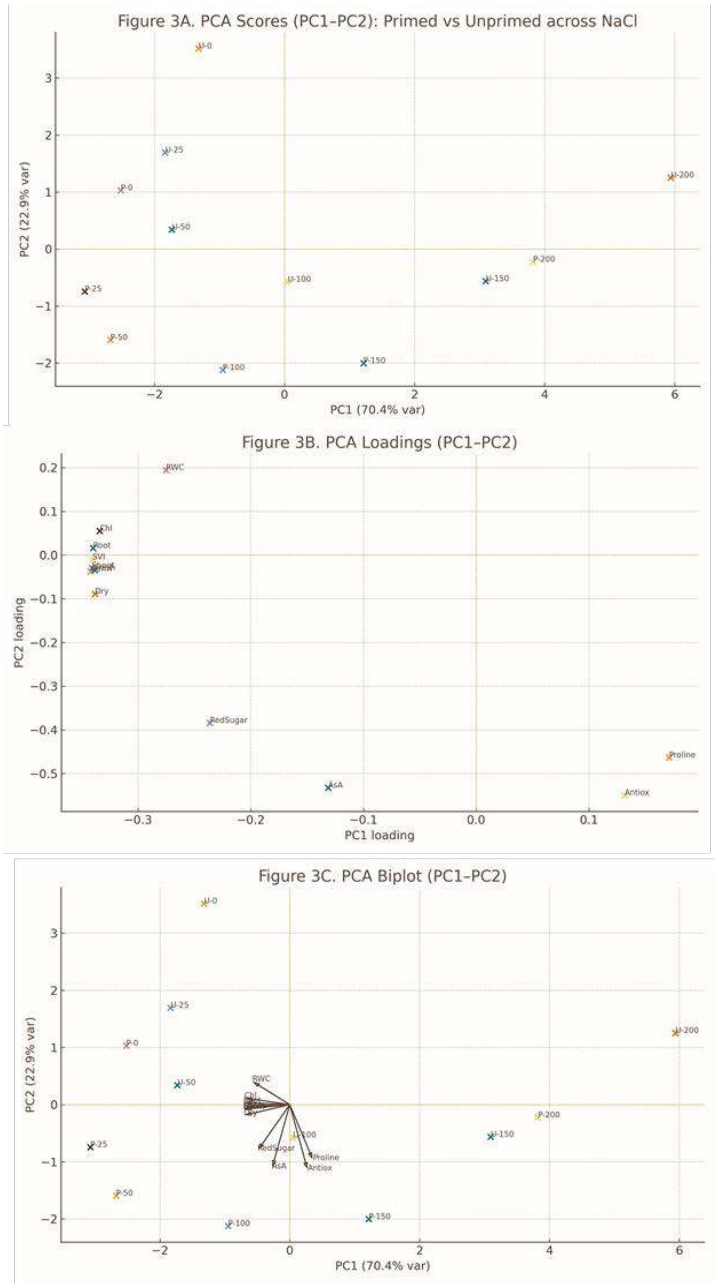

Fig. 3: PCA of tomato seedling responses under NaCl stress (0–200 mM)
(A) *PCA score plot (PC1–PC2) of treatments; data points are labeled as U mM (unprimed), P mM (chitosan-primed). PC1 (70.4%) describes the main salinity-tolerance axis, where primed samples remain close to high-vigor sector at high salinity levels.* ***(B)*** *PCA loading plot (PC1–PC2).* ***(C)*** *Variable-contribution plot indicating that vigor- and growth-related traits (germination, shoot/root length, SVI, biomass and RWC), present positive association with PC1; whereas stress-associated characters (proline, reducing sugars, antioxidant capacity and AsA), load in opposite array, thereby defining the tolerance axis.*

Conclusion

The six-well agar plate assay presented here is a rapid, high-throughput amenity for screening seed priming treatments against abiotic stresses that can easily be adapted to other crops. Simple and effective chitosan-mediated seed priming enhances tomato germination and early vigor under saline conditions. Pretreatment with 0.2% chitosan solution was enough to make the plants salinity resistant. Primed seeds showed enhanced germination and vigorous seedling growth at NaCl levels that severely inhibited unprimed seeds. Mechanism included better water status and enhanced antioxidant defenses (proline, ascorbate, radical-scavenging capacity) which helped seedlings to quickly neutralize salt-induced oxidative damage. This intervention can boost agriculture in sodium-disordered areas more, particularly the earliest stage of growth stands.

Declaration of Competing Interest

No competing financial interests or personal relationships that could have seemed to colour the work reported in this paper.

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