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Germination and Growth Responses of Trifoliate Orange (*Poncirus trifoliata* L.) Seeds to Glycerol-Chitosan-Based Biostimulant Treatments

Christina-Ioanna Ntouvika, Lejdina Hoxha, Magdalini Malliari, Charidimos Vermes, Paschalis Giannoulis * and Helen Kalorizou *

Department of Agriculture, Faculty of Agricultural Sciences, University of Patras, New Buildings, 30200 Missolonghi, Greece

* Correspondence: pasgiann@upatras.gr (P.G.); ekalorizou@upatras.gr (H.K.); Tel.: +30-2631058232 (H.K.)

Abstract

Trifoliate orange (*Poncirus trifoliata* L.), a citrus rootstock valued for its cold hardiness and resistance to broad biotic stress, produces desiccation-sensitive seeds whose successful germination requires optimized pre-sowing treatments for reliable sexual propagation. This study investigated whether chitosan-based coating systems, incorporating glycerol as a plasticizer combined with either an amino acid biostimulant (betaine-proline) or gibberellic acid (GA_3), could improve germination kinetics and seedling establishment. Seeds from mature fruits were subjected to 18 treatments across Petri dish and soil bioassays. In Petri dishes, the germination percentage remained uniformly high (86.67–100%), with GA_3-containing formulations promoting shoot elongation and proline-betaine combinations favoring root development. In soil, treatments primarily modulated germination kinetics: GA_3 combined with chitosan (1.5–2%) increased the germination speed by approximately 3.0–3.4 times relative to the control, while 1.5% chitosan with proline-betaine and glycerol yielded the fastest germination overall. Biomass responses were strongly formulation-dependent: 2% chitosan with GA_3 maximized shoot and total dry weight, whereas 2% chitosan with proline-betaine and glycerol substantially improved root dry biomass and overall seedling quality. Glycerol exerted context-dependent effects, enhancing amino acid formulations while reducing performance in GA_3 mixtures. These findings support the use of optimized chitosan-based treatments as effective tools for accelerating emergence and improving seedling robustness in rootstock production.

Keywords: seed management; macromolecular agents; seed coating; *Citrus* rootstock

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1. Introduction

The trifoliate orange (*Poncirus trifoliata* L.) is a deciduous species in the family Rutaceae with exceptional cold hardiness and resistance to virus infections (*Citrus tristeza virus*), bacteria (*Candidatus Liberibacter asiaticus*), nematodes (*Tylenchulus semipenetrans*), and insects such as *Diaphorina citri* (Kuwayama), achieved through the synthesis of selective secondary metabolites and phenylpropanoids [1–4]. In addition to cold resistance, which enables citrus cultivation in marginal climates, *P. trifoliata* rootstocks can induce dwarfing in commercial varieties of *Citrus* species, which is a suitable characteristic for high-density orchards [5–7].

In *P. trifoliata*, seeds with low moisture content (a reduction of over 50% relative to fresh seeds, from 52.1% to 24.8% following air-drying) show irreversible loss of viability and

germination capacity, ranging from 11.7% to 100%, depending on the drying technique [8]. Seed coat removal decreases the minimum time required for emergence by 47% and, at the same time, elevates the percentage of emergence by 18.75% [9].

Chitosan, obtained through the alkaline N-deacetylation of chitin, is a β-1,4-linked polymer of D-glucosamine and N-acetyl-D-glucosamine. Valued in agriculture for more than 50 years for its antifungal and elicitor activities [10], it is now commercially applied in seed coatings and nano-formulations that promote germination, regulate growth, and confer resistance to biotic and abiotic stress [11–14]. Chitosan applied as a thin coating or priming solution increases the rate and uniformity of water uptake during seed imbibition due to the presence of its hydrophilic amino and hydroxyl groups, while its film-forming behavior stabilizes the seed coat [12,15]. Chitosan priming reduced plasma membrane permeability and malondialdehyde (MDA) accumulation in wheat seeds, preserving membrane integrity through dehydration–rehydration cycles and improving resilience to heavy metal stress [16–18]. At later developmental stages, comparable membrane-stabilizing effects have been documented in the leaves of pretreated "Fuji" apple seedlings on *Malus sieversii* rootstock under drought conditions, where 100 mg/L chitosan minimized electrolyte leakage and MDA [19]. The in vitro combination of 120 mg/L chitosan and 0.5 mg/L benzyladenine was found to be optimal for the proliferation of M26 apple rootstock explants after 12 weeks [20].

Studies on exogenous proline and glycine betaine seed treatments in fruit-tree species under strictly non-stress germination conditions remain very limited. Exogenous proline applications (25 and 50 mM) improved photosynthesis, antioxidant enzyme activity, and Na^+ exclusion in salt-stressed two-year-old olive trees (*Olea europaea* L.), but did not significantly affect growth under non-stress conditions, as they were applied during the vegetative rather than germination stage [21,22]. Among wheat varieties, 15 mM proline priming maximized shoot and root biomass and plant height, alongside changes in photosystem II (PSII) performance and reserve mobilization [23].

Under non-stress conditions, 1 mM proline applied to MR269 rice seeds for 24 h yielded the highest germination percentage, germination rate index (GRI), seedling vigour index (SVI), α-amylase activity, and total soluble sugar content. By contrast, 20 mM proline was inhibitory, prolonging mean germination time and reducing GRI and SVI [24]. Similarly, 5 mM proline was the most effective dose for canola seed germination and seedling growth; however, the salinity alleviation effect was greater than in non-stressed seed lines [25].

Coating maize seeds with 2% chitosan and 0.30 g glycerol per gram of chitosan as a plasticizer improved germination rates and provided protection against *Penicillium* spp. [26]. The synthesized chitosan blends, incorporating polyethylene glycol and glycerol, were loaded with *Trichoderma* fungal cultures and applied as a coating on castor seeds. This hybrid microbial–polymer blend demonstrated improved germination rates and seedling vigour, while maintaining a highly viable population of beneficial fungi [27,28].

Few studies have specifically examined exogenous glycerol as a pre-sowing germination treatment in commercially important fruit, nut, oil, or timber trees. Glycerol is repeatedly listed among osmopriming agents (alongside polyethylene glycol, mannitol, sorbitol, and KNO_3) in seed priming [29]. In *Arabidopsis*, exogenous glycerol alters root architecture. Treatment with 1 mM glycerol markedly inhibits primary root elongation, an effect detectable by four days post-germination that intensifies with prolonged exposure [30].

Crude glycerin, as a biodiesel by-product, containing 80% glycerol, is strongly inhibitory to germination in both small-seeded weeds and crop seeds, acting as a bioherbicide [31]. Glycerol is also a standard cryoprotectant used in plant vitrification solutions to cryopreserve embryos of agriculturally important trees such as *Castanea sativa* [32].

The preparation of chitosan films incorporating various additives, along with the characterization of their resulting properties, has long been a challenging area of research. Chitosan films prepared with 0.52 M acetic acid, 3.5×10^{-5} M chitosan, 0.25 M NaOH, and 0.40 M ethanol reached a tensile strength of 749.235 N/m, while those with 0.17 M acetic acid, 2.5×10^{-5} M chitosan, 0.75 M NaOH, and 0.60 M ethanol gave an air permeability of 295.235 mL/min. These results demonstrate that adjusting the processing conditions and chemical composition can minimize chitosan film constringency and improve its elasticity and flexibility [33]. Glycerol incorporation enhanced the chitosan film's water vapor and carbon dioxide permeability but reduced its tensile strength, whereas stearic acid increased carbon dioxide permeability while lowering both water vapor permeability and tensile strength [34].

Using an alternative process, films were prepared from 3% chitosan in lactic acid (1% and 2.5%) plasticized with sorbitol or glycerol (0.25–0.50 mL/g). The plasticizer type did not significantly affect the thickness (27–81 μm for sorbitol; 25–60 μm for glycerol), which increased with acid concentration. Sorbitol films showed higher normalized breaking force but lower deformation than glycerol films, with elasticity declining at higher acid and plasticizer concentrations [35]. In general, chitosan concentrations in coating and film-forming solutions typically range from 0.5% to 3% (*w*/*w* or *w*/*v*) [36–40]. When glycerol is used as a plasticizer, its concentration ranges from roughly 0–3% (*w*/*w*) in simple systems [36], 0.5–1% (*w*/*v*) in more physiochemically complex formulations [37,40], and 10–30% (*w*/*w* of chitosan) in bio-composite films [41,42]. Higher chitosan content generally increases tensile strength, thickness, and density, and lowers moisture [37,40]. Higher glycerol concentration increases flexibility and elongation but lowers tensile strength and can raise permeability and moisture [37,40–42]. The chitosan–glycerol mixture may be supplemented with plant hormones or other biostimulatory molecules, offering considerable potential for application (Figure 1).

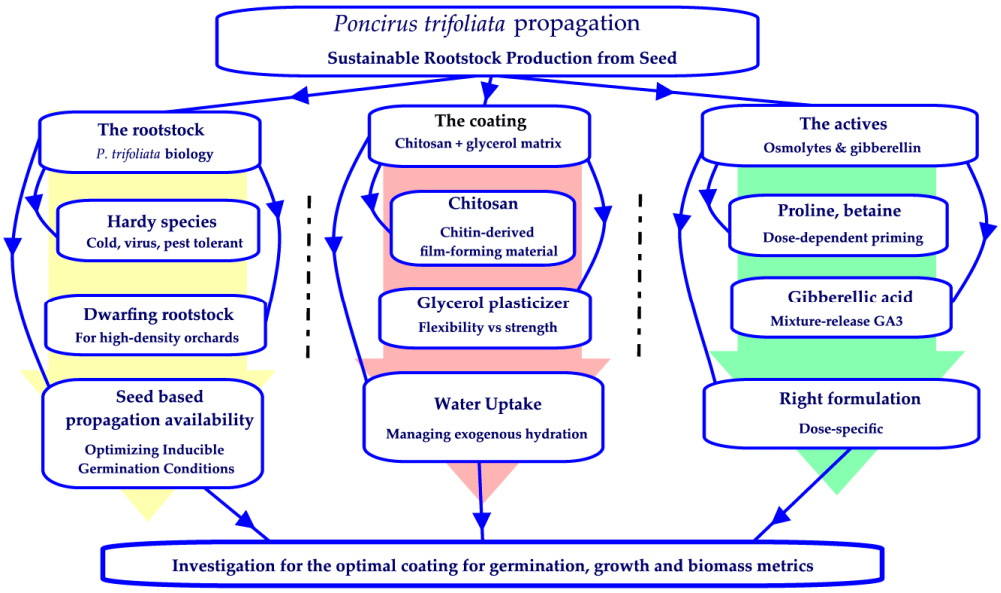


Figure 1. Applicability of chitosan, glycerol, osmolytes, and gibberellic acid on *P. trifoliata* seeds.

Encapsulation of GA_3 within chitosan nanoparticles or hydrogel matrices yields controlled-release formulations that protect the hormone from degradation and leaching while maintaining sustained bioavailability to seeds [43–48]. This approach leads to the following: (a) enhanced germination rates due to gibberellic acid, (b) increased root and shoot growth parameters, and (c) greater resilience under abiotic stress conditions. However, the effectiveness of chitosan–gibberellic acid combinations for seed germination

is based on the precise optimization of component concentrations and their mixing ratio. Some plant species responded best to nanoencapsulated gibberellic acid (GA_3)−chitosan formulations at low doses [47], while others demonstrated enhanced germination when exposed to high concentrations of GA_3 during the pre-soaking stage [49]. At high GA_3 concentrations, combining GA_3 with chitosan reduced treatment efficacy and growth parameters relative to single-agent applications, suggesting that the response may depend on species-specific genetic factors underlying the response [50].

Therefore, this study evaluates whether chitosan-based seed coatings, alone or combined with glycerol, proline-betaine, and GA_3, can improve germination performance, early growth, and biomass accumulation of *P. trifoliata* seedlings, aiming to identify suitable formulations for citrus rootstock propagation.

2. Materials and Methods

2.1. Collection and Processing of Trifoliate Orange Seeds

Mature fruits of trifoliate orange (*Poncirus trifoliata*) were collected from 16-year-old trees in the orchard of the Department of Agriculture, University of Patras, Greece. Seeds were manually extracted from the fruits and processed immediately after collection to minimize any storage-related effects on seed physiological status. Extracted seeds were soaked in distilled water at room temperature for 24 h, with periodic water replacement, to remove residual pulp (Figure 2). Subsequently, seeds were thoroughly rinsed with distilled water and air-dried before surface-sterilization with 1% (v/v) hydrogen peroxide (H_2O_2) for 5 min.

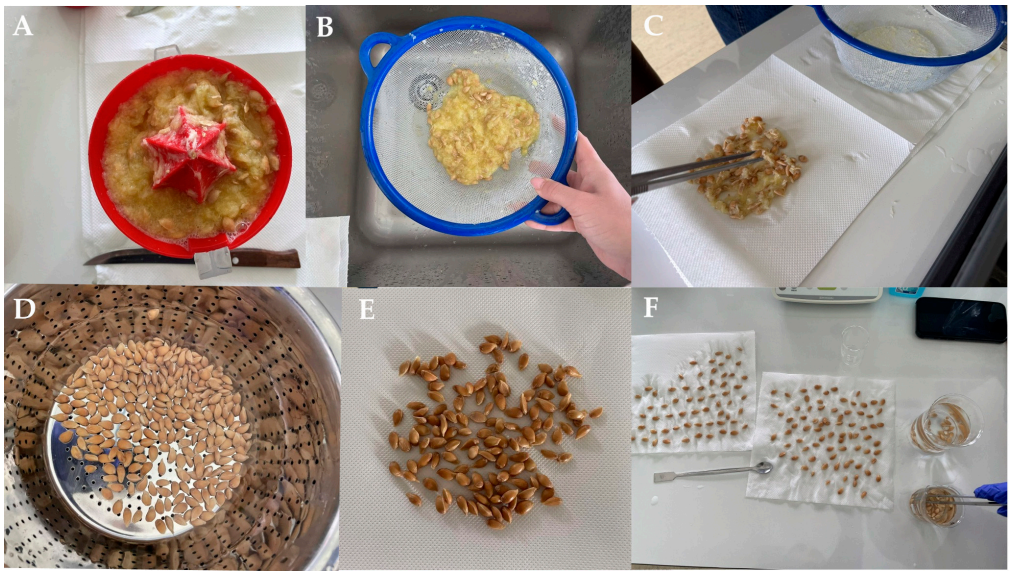


Figure 2. Extraction of *P. trifoliata* seeds from fruits: (**A**) manual extraction with a handheld juicing device; (**B**) excessive pulp removal; (**C**,**D**) residual pulp removal in cycles with water; (**E**) seed free of pulp, surface sterilized and dried; (**F**) inoculation of seeds with coating mixtures.

2.2. Preparation of Working Solutions

Chitosan (DAC > 95%, BLD Pharmatech GmbH, Reinbek, Germany) was dissolved in 1% (v/v) acetic acid (≥99.8%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) to prepare solutions at concentrations of 1%, 1.5%, and 2% (w/v) under continuous magnetic stirring at room temperature until complete dissolution. The pH was adjusted to 5.6 using 1 N NaOH. Glycerol (≥99%, CHEMSOLUTE®, Th. Geyer GmbH & Co. KG, Renningen, Germany) was added to the chitosan solutions as a plasticizing agent at final concentrations of 0.5%, 0.75%, and 1.0% (v/v), to the 1%, 1.5%, and 2% (w/v) chitosan formulations,

respectively. Each chitosan–glycerol treatment solution was prepared at a final volume of 30 mL. The mixtures were gently stirred until homogeneous chitosan–glycerol coating formulations were obtained.

A commercial proline-betaine-based biostimulant containing nitrogen (25%; 6.2% ammoniacal, 12.7% ureic, and 6.1% nitric), readily assimilable betaine (7%), and L-proline (3%) [Commercial name FYTOAMINO®-N produced by Karvelas S.A., Agrinio, Greece] was used. A stock solution was prepared by diluting 0.5 mL of the product in 100 mL of distilled water under gentle stirring. Subsequently, 10 mL of the stock solution was incorporated into the chitosan−glycerol formulations to a final FYTOAMINO®-N concentration of 0.125% (*v*/*v*). The mixtures were homogenized to ensure a uniform distribution of the biostimulant within the treatment solutions.

Gibberellic acid (GA_3) solutions were prepared using a commercial formulation containing 10% (*w*/*w*) active ingredient [Commercial name GIBAL produced by L. GOBBI Srl., Genova, Italy]. A GA_3 stock solution (10,000 ppm) was prepared, and 1 mL was added to 30 mL of the chitosan–glycerol treatment solution, resulting in a final GA_3 concentration of 300 ppm. The mixtures were gently stirred to ensure homogeneity before seed coating.

2.3. Seed Coating

Seed coating was performed by immersing seeds for 10 min in the following solutions: distilled water (water-treated control), chitosan solutions, chitosan–glycerol formulations, chitosan + biostimulant, and chitosan + GA_3 treatments, ensuring uniform exposure of seeds to the coating formulations. Seeds were then air-dried on sterile filter paper at room temperature before germination assays.

2.4. Experimental Design and Treatments

Following seed coating, seeds were subjected to two germination systems: a Petri dish assay and a soil-based assay.

2.4.1. Petri Dish Experiment

Seeds were placed in 9 cm diameter Petri dishes lined with cotton substrate moistened with distilled water at a density of 5 seeds per plate. The experiment was conducted with five replicates per treatment. Dishes were incubated in a germination chamber at 20 ± 1 °C with a 12 h photoperiod (Figure 3). Moisture was maintained throughout the experiment by periodic addition of sterile distilled water.



Figure 3. Germination of treated *P. trifoliata* lines on moist cotton substrate (**A**) and under soil conditions (**B**).

2.4.2. Soil-Based Experiment

Seeds were sown in trays containing a peat–perlite substrate (5:1, *v*/*v*) and incubated under the same conditions as the Petri dish experiment (20 ± 1 °C, 12 h photoperiod). Moisture was maintained by periodic irrigation with distilled water (Figure 3). Each treatment consisted of five replicates with five seeds per replicate.

Both germination systems included eighteen treatments (T1–T18), consisting of a water-treated control, chitosan-based treatments at three concentrations, proline-betaine biostimulant treatments, and GA_3 treatments, either alone or in combination with glycerol (Table 1).

Table 1. Composition of the seed coating treatments and final concentrations of the applied compounds.

Treatment	Priming Solution Composition	Final Concentration (s)
T1	Distilled water	Control
T2	Proline-betaine biostimulant	Biostimulant (0.125%, *v*/*v*)
T3	Chitosan	Chitosan (1.0%, *w*/*v*)
T4	Chitosan	Chitosan (1.5%, *w*/*v*)
T5	Chitosan	Chitosan (2.0%, *w*/*v*)
T6	Chitosan + proline-betaine biostimulant	Chitosan (1.0%, *w*/*v*) + biostimulant (0.125%, *v*/*v*)
T7	Chitosan + glycerol + proline-betaine biostimulant	Chitosan (1.0%, *w*/*v*) + glycerol (0.5%, *v*/*v*) + biostimulant (0.125%, *v*/*v*)
T8	Chitosan + proline-betaine biostimulant	Chitosan (1.5%, *w*/*v*) + biostimulant (0.125%, *v*/*v*)
T9	Chitosan + glycerol + proline-betaine biostimulant	Chitosan (1.5%, *w*/*v*) + glycerol (0.75%, *v*/*v*) + biostimulant (0.125%, *v*/*v*)
T10	Chitosan + proline-betaine biostimulant	Chitosan (2.0%, *w*/*v*) + biostimulant (0.125%, *v*/*v*)
T11	Chitosan + glycerol + proline-betaine biostimulant	Chitosan (2.0%, *w*/*v*) + glycerol (1.0%, *v*/*v*) + biostimulant (0.125%, *v*/*v*)
T12	GA_3	GA_3 (300 ppm)
T13	Chitosan + GA_3	Chitosan (1.0%, *w*/*v*) + GA_3 (300 ppm)
T14	Chitosan + glycerol + GA_3	Chitosan (1.0%, *w*/*v*) + glycerol (0.5%, *v*/*v*) + GA_3 (300 ppm)
T15	Chitosan + GA_3	Chitosan (1.5%, *w*/*v*) + GA_3 (300 ppm)
T16	Chitosan + glycerol + GA_3	Chitosan (1.5%, *w*/*v*) + glycerol (0.75%, *v*/*v*) + GA_3 (300 ppm)
T17	Chitosan + GA_3	Chitosan (2.0%, *w*/*v*) + GA_3 (300 ppm)
T18	Chitosan + glycerol + GA_3	Chitosan (2.0%, *w*/*v*) + glycerol (1.0%, *v*/*v*) + GA_3 (300 ppm)

2.5. Germination and Seedling Growth Parameters

In the Petri dish experiment, the number of germinated seeds, as well as shoot and radicle lengths, was recorded every two days. A seed was considered germinated when visible radicle protrusion occurred. Upon completion of the germination period, which occurred 30 days after seed placement in Petri dishes, the seedlings were separated into roots and shoots, and their fresh weights were determined. Root and shoot dry weights were measured after oven-drying at 70 °C for 48 h until constant weight was achieved.

Under soil-based conditions, germinated seeds were counted every two days based on visible seedling emergence. At the end of the experimental period, 48 days after sowing, the seedlings were carefully removed from the soil and washed free of adhering substrate particles. Excess moisture was removed using absorbent tissue paper. Shoot and root lengths, as well as fresh weight, were measured. Dry weight was determined after oven-drying at 70 °C until constant weight was achieved.

Based on observations of tissue development and expansion, the following parameters were calculated: (a) germination percentage, (b) mean germination time (MGT), (c) germination index (GI), (d) coefficient of velocity of germination (CVG), (e) mean daily germination (MDG), (f) germination speed (GS), (g) seedling vigour indices I and II (SVI I, SVI II), (h) root-to-shoot length and dry weight ratios, (i) daily root and shoot growth rates, and (j) mean root and shoot growth rate. The formulas used for these parameters are provided in Table 2 [51–53].

Table 2. Germination indices examined in this work.

Germination percentage (GP) $GP = \frac{Ng}{Nt} \times 100$ where Ng is the number of germinated seeds; Nt is the total number of seeds evaluated.	Mean germination time (MGT) $M.G.T. = \frac{\sum NiTi}{\sum Ni}$ where Ni is the number of seeds germinated on day i; Ti is the time in days from sowing to the i-th counting day.
Germination index (GI) $GI = \sum_{i=1}^{t} Ni \times wi$ where: Ni is the number of seeds germinated on day i; Wi is the weighting factor assigned to day i, decreasing from t (first day) to 1 (last day); T is the total number of observation days.	Coefficient of velocity of germination (CVG) $\%C.V.G. = 100 \times \frac{\sum Ni}{\sum NiTi}$ where Ni is the number of seeds that germinate per day; Ti is the time (days) of the experimental period.
Mean daily germination (MDG) $MDG = \frac{GP}{Ti}$ where GP is the germination percentage; Ti is the number of days required to reach maximum germination.	Germination speed (GS) $Gs = \sum Ni/Ti$ where Ni is the number of seeds germinated on day i; Ti is the number of days from the start of the experiment to the counting day.
Seedling vigour index I [SVI(I)] $SVI(I) = GP \times SL$ where GP is the germination percentage (%); SL is the mean seedling length (cm).	Seedling vigour index II [SVI(II)] $SVI(II) = GP \times DW$ where GP is the germination percentage (%); DW is the mean seedling dry weight (g).

2.6. Statistical Analysis

A completely randomized design with 18 treatments (five replicates each) was used. Data were subjected to one-way analysis of variance (ANOVA), and significant differences were separated using Tukey's test at $p \leq 0.05$. Results are presented as mean ± standard error (SE). Prism 8.0 (GraphPad, Boston, MA, USA) was used for statistical analysis, while heatmap clustering was performed using ClustVis (https://biit.cs.ut.ee/clustvis/ (accessed on 1 June 2026) [54]. The heatmap was generated using row-wise unit variance scaling, which is the default normalization method applied in ClustVis (Web version last updated 20 December 2018, Institute of Computer Science, University of Tartu, Tartu, Estonia); therefore, the color scale reflects standardized values rather than fold-expression levels.

3. Results

3.1. Petri Dish Experiment

3.1.1. Germination Indices

Across all six germination parameters, no statistically significant differences were observed among the treatments (Table 3). Germination percentage remained uniformly high across all treatments (86.67–100%), and the applied formulations did not significantly modify final germination percentage under the tested conditions.

Table 3. Effects of seed coating treatments on germination parameters in Petri dish environmental exposure [1].

Treatment	Coefficient of Velocity of Germination (CVG)	Mean Germination Time (MGT) (Days)	Germination Speed (GS)	Germination Percentage (%)	Mean Daily Germination %	Germination Index (GI)
T1	5.30 ± 0.42	19.10 ± 1.47	0.32 ± 0.04	93.33 ± 6.67	3.33 ± 0.21	54.67 ± 3.84
T2	7.01 ± 1.06	14.95 ± 2.31	0.37 ± 0.04	93.33 ± 6.67	4.47 ± 0.59	73.67 ± 7.42
T3	5.21 ± 0.65	19.80 ± 2.43	0.27 ± 0.08	86.67 ± 13.33	3.29 ± 0.66	51.33 ± 16.33
T4	5.32 ± 0.12	18.80 ± 0.40	0.29 ± 0.01	100.00 ± 0.00	4.01 ± 0.16	61.00 ± 2.00
T5	5.82 ± 0.26	17.25 ± 0.75	0.33 ± 0.03	93.33 ± 6.67	3.11 ± 0.22	63.67 ± 1.33
T6	5.25 ± 0.47	19.35 ± 1.62	0.28 ± 0.05	93.33 ± 6.67	3.77 ± 0.40	54.67 ± 9.74
T7	5.93 ± 0.37	17.00 ± 1.11	0.37 ± 0.02	100.00 ± 0.00	4.21 ± 0.31	70.00 ± 5.57
T8	6.79 ± 0.86	15.20 ± 1.91	0.37 ± 0.05	100.00 ± 0.00	5.19 ± 0.98	79.00 ± 9.54
T9	5.98 ± 0.76	17.30 ± 2.29	0.29 ± 0.04	93.33 ± 6.67	4.57 ± 1.06	63.67 ± 11.61
T10	6.29 ± 0.64	16.20 ± 1.51	0.37 ± 0.04	100.00 ± 0.00	4.35 ± 0.65	71.00 ± 9.17
T11	5.42 ± 0.35	18.60 ± 1.25	0.32 ± 0.06	93.33 ± 6.67	3.36 ± 0.34	55.00 ± 8.96
T12	6.80 ± 0.82	15.20 ± 2.03	0.43 ± 0.08	100.00 ± 0.00	4.01 ± 0.16	79.00 ± 10.15
T13	5.65 ± 0.53	18.00 ± 1.59	0.31 ± 0.03	100.00 ± 0.00	4.01 ± 0.16	65.00 ± 7.94
T14	6.78 ± 1.06	15.60 ± 2.75	0.37 ± 0.05	93.33 ± 6.67	4.57 ± 0.54	70.67 ± 10.73
T15	6.08 ± 0.43	16.60 ± 1.11	0.35 ± 0.04	100.00 ± 0.00	4.05 ± 0.34	72.00 ± 5.57
T16	5.16 ± 0.05	19.40 ± 0.20	0.29 ± 0.03	100.00 ± 0.00	4.17 ± 0.00	58.00 ± 1.00
T17	6.20 ± 0.27	16.20 ± 0.69	0.33 ± 0.02	100.00 ± 0.00	4.17 ± 0.00	74.00 ± 3.46
T18	7.13 ± 0.97	14.65 ± 2.89	0.36 ± 0.06	93.33 ± 6.67	4.60 ± 0.48	75.67 ± 10.48

[1] Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol.

The mean germination time ranged from 14.65 days (2% chitosan combined with gibberellic acid and glycerol) to 19.80 days (1% chitosan alone), although these differences were not statistically significant. Proline-betaine and the combined treatment of 1.5% chitosan with proline-betaine showed numerically shorter germination times than water-treated seed lines by 3.9–4.15 days. The coefficient of velocity of germination mirrored this pattern: 2% chitosan combined with gibberellic acid and glycerol, followed by proline-betaine, showed the numerically highest velocity (7.01) relative to water-treated lines (5.30), again without a statistically significant difference.

The germination index displayed the widest numerical spread, ranging from 51.33 (1% chitosan) to 79.00 (1.5% chitosan with proline-betaine and gibberellic acid); however, this variation did not reach the threshold of statistical significance. Treatments involving proline-betaine, 1.5% chitosan with proline-betaine, and gibberellic acid showed numerically favorable trends across several indices, although these effects were not supported by statistical evidence.

3.1.2. Shoot and Root Metrics

Gibberellic acid alone elicited the longest shoots, followed by the mixed application of 1.5–2% chitosan and gibberellic acid and 2% chitosan with gibberellic acid and glycerol. The shortest shoots were recorded in non-gibberellic acid application (1.5% chitosan, proline-betaine, and glycerol). Water-treated plant material (2.67 ± 0.62 cm) and most chitosan- or proline-betaine- based treatments occupied the intermediate range. These results suggest that gibberellic acid, whether applied alone or in combination with chitosan, was associated with greater shoot elongation under the tested conditions.

Proline-betaine supplementation recorded the highest rate of mean root growth rate (0.16 ± 0.01), while the combination of 1.5% chitosan with gibberellic acid and glycerol displayed the lowest. The majority of treatments, including the water-treated seeds (0.13 ± 0.02), fell within the overlapping group, indicating modest differentiation.

Gibberellic acid alone attained the highest mean shoot growth rate (0.19 ± 0.02), whereas 1.5% chitosan jointly applied with proline-betaine and glycerol exhibited the lowest (0.04 ± 0.02). All remaining treatments showed no clear separation.

No statistically significant differences were observed among treatments for root length, root-to-shoot ratio, daily root growth rate, and daily shoot growth rate (Table 4).

Table 4. Effects of seed coating treatments on shoot and root growth metrics while exposed in the Petri dish ambient environment [1].

Treatment	Root Length (cm)	Shoot Length (cm)	Root to Shoot Ratio	Daily Root Growth Rate	Daily Shoot Growth Rate	Mean Root Growth Rate	Mean Shoot Growth Rate
T1	3.47 ± 0.48	2.67 ± 0.62 ^{abc}	1.36 ± 0.17	0.15 ± 0.01	0.25 ± 0.06	0.13 ± 0.02 ^{ab}	0.10 ± 0.02 ^{ab}
T2	4.43 ± 0.39	3.57 ±0.22 ^{abc}	1.26 ± 0.16	0.19 ± 0.03	0.25 ± 0.06	0.16 ± 0.01 ^{b}	0.13 ± 0.01 ^{ab}
T3	3.00 ± 0.46	2.23 ± 0.32 ^{ab}	1.36 ± 0.14	0.18 ± 0.01	0.34 ± 0.10	0.11 ± 0.02 ^{ab}	0.08 ± 0.01 ^{ab}
T4	2.93 ± 0.47	2.63 ± 0.07 ^{abc}	1.12 ± 0.19	0.15 ± 0.03	0.32 ± 0.07	0.11 ± 0.02 ^{ab}	0.10 ± 0.00 ^{ab}
T5	2.57 ± 0.69	2.43 ± 0.50 ^{abc}	1.03 ± 0.06	0.09 ± 0.03	0.36 ± 0.11	0.09 ± 0.03 ^{ab}	0.09 ± 0.02 ^{ab}
T6	2.80 ± 0.53	2.43 ± 0.57 ^{abc}	1.31 ± 0.39	0.14 ± 0.03	0.29 ± 0.11	0.10 ± 0.02 ^{ab}	0.09 ± 0.02 ^{ab}
T7	3.27 ± 0.33	2.70 ± 0.45 ^{abc}	1.26 ± 0.17	0.14 ± 0.03	0.22 ± 0.04	0.12 ± 0.01 ^{ab}	0.10 ± 0.02 ^{ab}
T8	3.23 ± 0.47	3.00 ± 0.32 ^{abc}	1.07 ± 0.08	0.15 ± 0.01	0.25 ± 0.03	0.12 ± 0.02 ^{ab}	0.11 ± 0.01 ^{ab}
T9	2.73 ± 0.53	1.07 ± 0.55 ^{a}	0.93 ± 0.48	0.13 ± 0.02	0.22 ± 0.14	0.10 ± 0.02 ^{ab}	0.04 ± 0.02 ^{a}
T10	4.20 ± 0.15	3.40 ± 0.69 ^{abc}	1.33 ± 0.24	0.17 ± 0.01	0.25 ± 0.00	0.16 ± 0.01 ^{ab}	0.13 ± 0.03 ^{ab}
T11	3.50 ± 0.15	3.57 ± 0.38 ^{abc}	1.01 ± 0.13	0.16 ± 0.01	0.31 ± 0.05	0.13 ± 0.01 ^{ab}	0.13 ± 0.01 ^{ab}
T12	3.61 ± 0.38	5.25 ± 0.25 ^{c}	0.69 ± 0.07	0.15 ± 0.02	0.25 ± 0.03	0.12 ± 0.02 ^{ab}	0.19 ± 0.02 ^{b}
T13	2.73 ± 0.33	3.85 ± 0.08 ^{abc}	0.71 ± 0.09	0.13 ± 0.02	0.22 ± 0.02	0.10 ± 0.01 ^{ab}	0.15 ± 0.00 ^{ab}
T14	2.93 ± 0.29	3.83 ± 0.94 ^{abc}	0.85 ± 0.18	0.13 ± 0.02	0.40 ± 0.13	0.11 ± 0.01 ^{ab}	0.10 ± 0.06 ^{ab}
T15	2.90 ± 0.15	4.90 ± 0.95 ^{bc}	0.63 ± 0.09	0.10 ± 0.05	0.39 ± 0.09	0.11 ± 0.01 ^{ab}	0.11 ± 0.00 ^{ab}
T16	2.63 ± 0.22	2.92 ± 0.27 ^{abc}	0.92 ± 0.13	0.15 ± 0.03	0.46 ± 0.19	0.11 ± 0.01 ^{a}	0.07 ± 0.04 ^{ab}
T17	3.03 ± 0.28	4.67 ± 0.38 ^{bc}	0.67 ± 0.11	0.16 ± 0.02	0.27 ± 0.05	0.11 ± 0.01 ^{ab}	0.15 ± 0.03 ^{ab}
T18	2.87 ± 0.70	4.30 ± 0.95 ^{bc}	0.67 ± 0.10	0.13 ± 0.03	0.25 ± 0.04	0.11 ± 0.01 ^{ab}	0.15 ± 0.05 ^{ab}

[1] The different letters indicate a significant ($p < 0.05$) difference. Data in columns with no letters do not differ statistically. Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol.

3.1.3. Root and Shoot Weight Metrics

No statistically significant differences were detected in root fresh weight among treatments. Values ranged from 0.15 to 0.33 g, with the application of the following: (a) 1.5% chitosan combined with gibberellic acid and glycerol, (b) a proline-betaine-based biostimulant, and (c) 1% chitosan resulting in no significant changes in root fresh biomass compared with the water-treated control. Shoot fresh weight did not reveal significant differences; however, numerically higher shoot fresh weights were observed in gibberellic acid-containing treatments (e.g., gibberellic acid alone, 1% and 1.5% chitosan with gibberellic acid), whereas the water-treated seeds recorded a low average value. The narrow range

of values between treatments did not result in significant differences in root dry weight, indicating that this parameter was minimally or not affected by the exogenous treatments.

Water-treated seeds and seeds treated with 1% and 2% chitosan formed a group with the lowest shoot dry weights. In contrast, treatment with 1% chitosan, gibberellic acid and glycerol resulted in significantly higher shoot dry weights than all other treatments. Intermediate shoot dry weight-inducing responses were observed in treatments containing proline-betaine with or without 1.5% chitosan and gibberellic acid with or without 1% chitosan. However, their effect was lower than that observed for the 1% chitosan–gibberellic acid–glycerol treatment.

The treatment of 1% chitosan with proline-betaine exhibited the highest root/shoot dry weight ratio, indicating disproportionately greater root than shoot dry matter. Most other treatments, particularly those containing gibberellic acid in combination with other components displayed lower ratios (1.5% chitosan with gibberellic acid and 1% chitosan with gibberellic acid and glycerol). All other treatment regimens produced intermediate root-to-shoot dry weight ratios, falling between the upper and lower extremes of the parameter range (Table 5).

Table 5. Effects of seed coating treatments on shoot and root weight metrics while exposed in the Petri dish ambient environment [1].

Treatment	Root Fresh Weight (g)	Shoot Fresh Weight (g)	Root Dry Weight (g)	Shoot Dry Weight (g)	Root Dry/Shoot Dry Weight
T1	0.18 ± 0.03	0.13 ± 0.05	0.030 ± 0.006	0.018 ± 0.008 ^{a}	1.99 ± 0.52 ^{ab}
T2	0.33 ± 0.03	0.20 ± 0.03	0.055 ± 0.006	0.025 ± 0.006 ^{ab}	2.34 ± 0.28 ^{ab}
T3	0.33 ± 0.18	0.14 ± 0.03	0.034 ± 0.010	0.020 ± 0.005 ^{a}	1.67 ± 0.20 ^{a}
T4	0.23 ± 0.04	0.15 ± 0.04	0.036 ± 0.010	0.020 ± 0.006 ^{a}	1.87 ± 0.32 ^{ab}
T5	0.17 ± 0.02	0.12 ± 0.06	0.027 ± 0.004	0.017 ± 0.007 ^{a}	2.31 ± 1.02 ^{ab}
T6	0.25 ± 0.06	0.10 ± 0.06	0.038 ± 0.011	0.014 ± 0.008 ^{a}	4.86 ± 1.83 ^{b}
T7	0.25 ± 0.02	0.18 ± 0.06	0.043 ± 0.009	0.025 ± 0.009 ^{ab}	1.98 ± 0.30 ^{ab}
T8	0.24 ± 0.03	0.26 ± 0.05	0.042 ± 0.006	0.036 ± 0.007 ^{ab}	1.20 ± 0.15 ^{a}
T9	0.21 ± 0.02	0.13 ± 0.06	0.033 ± 0.004	0.019 ± 0.009 ^{a}	2.53 ± 0.71 ^{ab}
T10	0.28 ± 0.04	0.21 ± 0.01	0.045 ± 0.006	0.030 ± 0.002 ^{ab}	1.53 ± 0.22 ^{a}
T11	0.27 ± 0.03	0.21 ± 0.04	0.045 ± 0.006	0.029 ± 0.006 ^{ab}	1.64 ± 0.18 ^{a}
T12	0.19 ± 0.02	0.37 ± 0.10	0.033 ± 0.009	0.058 ± 0.020 ^{ab}	0.81 ± 0.39 ^{a}
T13	0.18 ± 0.02	0.37 ± 0.05	0.034 ± 0.004	0.054 ± 0.012 ^{ab}	0.69 ± 0.13 ^{a}
T14	0.20 ± 0.02	0.34 ± 0.09	0.039 ± 0.001	0.074 ± 0.006 ^{b}	0.52 ± 0.03 ^{a}
T15	0.17 ± 0.02	0.37 ± 0.07	0.032 ± 0.009	0.061 ± 0.013 ^{ab}	0.51 ± 0.03 ^{a}
T16	0.15 ± 0.02	0.14 ± 0.02	0.025 ± 0.006	0.022 ± 0.012 ^{a}	1.28 ± 0.47 ^{a}
T17	0.21 ± 0.01	0.32 ± 0.07	0.039 ± 0.003	0.048 ± 0.012 ^{ab}	0.90 ± 0.21 ^{a}
T18	0.19 ± 0.04	0.34 ± 0.11	0.027 ± 0.007	0.049 ± 0.015 ^{ab}	0.66 ± 0.19 ^{a}

[1] The different letters indicate a significant ($p < 0.05$) difference. Data in columns with no letters do not differ statistically. Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol.

3.2. Soil-Based Experiment

3.2.1. Germination Indices

The highest coefficient of velocity of germination was recorded in the presence of 1.5% chitosan–proline-betaine−glycerol, indicating the fastest overall germination velocity, followed by proline and betaine supplementation alone and the gibberellic acid−1.5% chitosan combinations with and without glycerol. The lowest value was observed in 2% chitosan–proline-betaine, significantly lower than most other treatments, achieving performance close to water-treated seeds.

Mixtures of the following ((a) 1.5% chitosan with proline-betaine and glycerol, (b) proline-betaine alone, and (c) 1.5% chitosan with gibberellic acid) achieved the shortest mean germination time in approximately 25 days. In contrast, the application of 2% chitosan with proline and betaine exhibited the longest time value of this parameter (33 days), indicating its inhibitory effect on germination speed. The water-treated seed lines performed better than the chitosan with amino acids treatment, providing an intermediate performance.

The most pronounced treatment effects were observed for germination speed. Gibberellic acid-containing 1.5% and 2% chitosan combinations achieved the highest germination speed values (0.433–0.439), followed by the combined application of 1% chitosan with gibberellic acid and glycerol, and the 1% chitosan–gibberellic acid mixture. These values represent a 3- to 3.4-fold increase compared with the water-treated seeds (0.128). Treatments containing gibberellic acid showed the highest germination speed values, suggesting a strong contribution of GA_3 to germination acceleration under soil conditions.

Combinations of 2% chitosan with gibberellic acid, 1% chitosan and 2% chitosan with proline-betaine and glycerol achieved the highest mean daily germination rates (2.48–2.57). The lowest values were recorded for 2% chitosan-combined applications with either gibberellic acid and glycerol, or amino acid supplementation. Similar performance was recorded for the combined application of 1% chitosan, proline-betaine, and glycerol. However, water-treated seeds did not exhibit major differences compared with the vast majority of applied treatments.

No significant differences were detected among treatments for either germination percentage or germination index. Germination percentage ranged from 60% (1.5% chitosan–gibberellic acid−glycerol, 2% chitosan–gibberellic acid−glycerol) to 93.33% (gibberellic acid), while germination index ranged from 16.00 (2% chitosan–gibberellic acid−glycerol) to 52.67 (gibberellic acid). Despite the numerical variation, no statistical separation was evident (Table 6).

Table 6. Effects of seed coating treatments on germination parameters under soil conditions [1].

Treatment [2]	Coefficient of Velocity of Germination (CVG)	Mean Germination Time (MGT) (Days)	Germination Speed (GS)	Germination Percentage (%)	Mean Daily Germination %	Germination Index (GI)
T1	3.46 ± 0.13 ^{abcd}	29.00 ± 1.09 ^{abc}	0.128 ± 0.007 ^{a}	73.33 ± 6.67	2.15 ± 0.18 ^{abc}	21.67 ± 1.76
T2	3.91 ± 0.14 ^{cd}	25.67 ± 0.88 ^{a}	0.159 ± 0.018 ^{a}	73.33 ± 6.67	2.10 ± 0.23 ^{abc}	37.67 ± 7.31
T3	3.65 ± 0.08 ^{bcd}	27.40 ± 0.59 ^{ab}	0.162 ± 0.012 ^{a}	86.67 ± 6.67	2.55 ± 0.07 ^{c}	34.33 ± 12.45
T4	3.34 ± 0.05 ^{abc}	30.00 ± 0.43 ^{abc}	0.127 ± 0.013 ^{a}	73.33 ± 6.67	2.03 ± 0.12 ^{abc}	26.67 ± 7.54
T5	3.42 ± 0.11 ^{abcd}	29.33 ± 0.94 ^{abc}	0.128 ± 0.015 ^{a}	73.33 ± 6.67	2.22 ± 0.20 ^{abc}	20.67 ± 2.19
T6	3.37 ± 0.15 ^{abc}	29.80 ± 1.37 ^{abc}	0.148 ± 0.005 ^{a}	86.67 ± 6.67	2.40 ± 0.10 ^{bc}	31.33 ± 3.76
T7	3.42 ± 0.17 ^{abcd}	29.42 ± 1.45 ^{abc}	0.121 ± 0.015 ^{a}	66.67 ± 6.67	1.70 ± 0.20 ^{a}	40.33 ± 15.90
T8	3.47 ± 0.07 ^{abcd}	28.83 ± 0.60 ^{abc}	0.136 ± 0.015 ^{a}	73.33 ± 6.67	1.98 ± 0.12 ^{abc}	34.67 ± 12.02
T9	4.01 ± 0.15 ^{d}	25.00 ± 1.00 ^{a}	0.176 ± 0.009 ^{ab}	80.00 ± 0.00	2.36 ± 0.07 ^{bc}	40.00 ± 6.93
T10	3.01 ± 0.13 ^{a}	33.33 ± 1.45 ^{c}	0.101 ± 0.007 ^{a}	66.67 ± 6.67	1.70 ± 0.04 ^{a}	23.33 ± 8.51
T11	3.53 ± 0.09 ^{abcd}	28.35 ± 0.69 ^{abc}	0.158 ± 0.011 ^{a}	86.67 ± 6.67	2.48 ± 0.16 ^{c}	33.33 ± 3.93
T12	3.47 ± 0.19 ^{abcd}	29.00 ± 1.71 ^{abc}	0.174 ± 0.013 ^{ab}	93.33 ± 6.67	2.43 ± 0.20 ^{ab}	52.67 ± 16.13
T13	3.54 ± 0.11 ^{abcd}	28.33 ± 0.85 ^{abc}	0.368 ± 0.028 ^{de}	73.33 ± 6.67	2.10 ± 0.23 ^{abc}	24.67 ± 1.76
T14	3.55 ± 0.08 ^{abcd}	28.22 ± 0.62 ^{abc}	0.379 ± 0.007 ^{de}	73.33 ± 6.67	2.11 ± 0.29 ^{abc}	29.67 ± 14.06
T15	3.83 ± 0.10 ^{cd}	26.18 ± 0.68 ^{a}	0.433 ± 0.007 ^{e}	73.33 ± 6.67	1.97 ± 0.23 ^{abc}	43.67 ± 11.72
T16	3.81 ± 0.09 ^{cd}	26.29 ± 0.59 ^{ab}	0.314 ± 0.017 ^{cd}	60.00 ± 11.55	1.79 ± 0.26 ^{ab}	22.00 ± 5.86
T17	3.68 ± 0.02 ^{bcd}	27.17 ± 0.17 ^{ab}	0.439 ± 0.010 ^{e}	80.00 ± 11.55	2.57 ± 0.30 ^{c}	20.33 ± 6.01
T18	3.19 ± 0.08 ^{ab}	31.39 ± 0.77 ^{bc}	0.246 ± 0.024 ^{bc}	60.00 ± 0.00	1.64 ± 0.11 ^{a}	16.00 ± 4.00

[1] The different letters indicate a significant ($p < 0.05$) difference. Data in columns with no letters do not differ statistically. [2] Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol.

3.2.2. Seedling Growth Metrics

The longest seedlings were produced by the following: (a) water-treated seeds, (b) proline-betaine-treated seeds, and c) 1.5% chitosan–proline-betaine-treated seeds, followed by mixtures of 1–1.5% chitosan with proline-betaine and glycerol. All gibberellic acid treatments yielded significantly lower plant length values relative to water-treated controls (Table 7). The most pronounced inhibitory effects were observed in the 1% and 2% chitosan treatments supplemented with gibberellic acid and glycerol (4.18–4.80 cm), corresponding to reductions of 57% and 51%, respectively. This indicates that, under the tested conditions, gibberellic acid-containing formulations resulted in lower seedling length when combined with certain chitosan concentrations.

Table 7. Seedling growth metrics while exposed in the soil environment [1].

Treatment [2]	Plant Length (cm)	Seedling Dry Weight (g)	Seedling Vigour Index (SVI)	Seedling Vigour Index (SVII)	Root Fresh Weight (g)	Shoot Fresh Weight (g)
T1	9.80 ± 0.01 ^{i}	0.772 ± 0.004 ^{g}	718.60 ± 65.14 ^{cde}	56.55 ± 4.89 ^{bcde}	0.749 ± 0.009 ^{ef}	1.791 ± 0.049 ^{L}
T2	9.80 ± 0.09 i	0.787 ± 0.007 ^{gh}	719.50 ± 69.52 ^{cde}	57.78 ± 5.53 ^{bcde}	0.906 ± 0.005 ^{j}	1.653 ± 0.008 ^{ij}
T3	8.50 ± 0.06 ^{ef}	0.711 ± 0.004 ^{e}	736.40 ± 55.06 ^{cde}	61.63 ± 4.94 ^{bcde}	0.664 ± 0.005 ^{d}	1.611 ± 0.005 ^{hij}
T4	8.30 ± 0.02 ^{ef}	0.562 ± 0.005 ^{c}	608.40 ± 53.84 ^{bcde}	41.15 ± 3.41 ^{ab}	0.730 ± 0.003 ^{e}	1.066 ± 0.036 ^{c}
T5	8.80 ± 0.05 ^{fg}	0.535 ± 0.008 ^{bc}	644.70 ± 55.58 ^{bcde}	39.22 ± 3.72 ^{ab}	0.684 ± 0.003 ^{d}	1.320 ± 0.000 ^{g}
T6	8.50 ± 0.08 ^{ef}	0.605 ± 0.009 ^{d}	737.80 ± 64.34 ^{cde}	52.45 ± 4.21 ^{bcd}	0.733 ± 0.005 ^{e}	1.275 ± 0.004 ^{fg}
T7	9.20 ± 0.06 ^{gh}	0.637 ± 0.004 ^{d}	612.70 ± 58.11 ^{bcde}	42.46 ± 4.30 ^{ab}	0.877 ± 0.004 ^{ij}	1.148 ± 0.005 ^{cde}
T8	9.80 ± 0.09 i	0.646 ± 0.007 ^{d}	718.80 ± 66.55 ^{cde}	47.48 ± 4.76 ^{abc}	0.851 ± 0.001 ^{hi}	1.102 ± 0.019 ^{cd}
T9	9.40 ± 0.05 ^{hi}	0.816 ± 0.006 ^{h}	752.00 ± 40.43 ^{de}	65.26 ± 0.45 ^{bcde}	1.091 ± 0.001 ^{L}	1.519 ± 0.005 ^{h}
T10	8.20 ± 0.04 ^{e}	0.377 ± 0.004 $_{a}$	546.20 ± 52.33 ^{abcde}	25.06 ± 2.19 ^{a}	0.555 ± 0.003 ^{b}	0.846 ± 0.005 ^{b}
T11	9.1 ± 0.08 ^{gh}	0.938 ± 0.007 ^{i}	788.10 ± 57.03 ^{e}	81.34 ± 6.79 ^{e}	1.068 ± 0.001 ^{L}	1.693 ± 0.008 ^{jk}
T12	6.10 ± 0.17 ^{d}	0.762 ± 0.012 ^{fg}	571.30 ± 54.36 ^{abcde}	71.28 ± 6.00 ^{cde}	0.837 ± 0.009 ^{hi}	1.907 ± 0.009 ^{m}
T13	5.50 ± 0.14 ^{c}	0.518 ± 0.005 ^{b}	405 ± 45.37 ^{ab}	38.03 ± 3.72 ^{ab}	0.603 ± 0.008 ^{c}	1.176 ± 0.003 ^{de}
T14	4.18 ± 0.05 ^{a}	0.637 ± 0.011 ^{d}	306.00 ± 25.27 ^{a}	46.60 ± 3.65 ^{abc}	0.858 ± 0.004 i	1.223 ± 0.005 ^{ef}
T15	6.35 ± 0.12 ^{d}	0.788 ± 0.009 ^{gh}	465.70 ± 43.33 ^{abc}	57.81 ± 5.31 ^{bcde}	0.786 ± 0.005 ^{fg}	1.569 ± 0.005 ^{hi}
T16	6.10 ± 0.12 ^{d}	0.722 ± 0.009 ^{ef}	363.30 ± 63.52 ab	43.10 ± 7.80 ^{ab}	0.810 ± 0.001 ^{gh}	1.754 ± 0.329 ^{kL}
T17	5.90 ± 0.12 ^{cd}	0.948 ± 0.014 ^{i}	470.70 ± 65.08 ^{abcd}	75.72 ± 10.57 ^{de}	1.011 ± 0.024 ^{k}	2.087 ± 0.038 ^{n}
T18	4.80 ± 0.17 ^{b}	0.375 ± 0.009 ^{a}	288.00 ± 10.39 ^{a}	22.49 ± 0.51 a	0.498 ± 0.016 ^{a}	0.661 ± 0.001 ^{a}

[1] The different letters indicate a significant ($p < 0.05$) difference. Data in columns with no letters do not differ statistically. [2] Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol.

Dry biomass accumulation showed a different pattern. The highest values of seedling dry weight were achieved by 2% chitosan with gibberellic acid or proline-betaine−glycerol mixtures, which both significantly exceeded the water-treated seed lines. Even lower chitosan levels (1.5%) in the proline-betaine and glycerol mixture outperformed the water-treated seed lines. In contrast, the absence of glycerol in the 2% chitosan–proline-betaine mixture and the gibberellic acid−glycerol enhancement of 2% chitosan yielded the lowest dry weight, less than 50% of the water-treated lines. The response of 2% chitosan formulations varied depending on the accompanying compound, with the highest dry biomass values observed when combined with gibberellic acid (without glycerol) or proline-betaine−glycerol.

Seedling vigour index I was highest in 2% chitosan–proline-betaine−glycerol and 1.5% chitosan–proline-betaine−glycerol seed supplementations, surpassing the water-treated plant material in mean values without statistical separation. The lowest SVI values were recorded for the 1% chitosan and 2% gibberellic acid and glycerol mixtures, reflecting an antagonistic effect on plant length effect in these formulations.

Seedling vigour index II provides a clearer picture. The combined supplementation of 2% chitosan with proline-betaine and glycerol yielded the highest index value, followed by

chitosan at the same concentration combined with gibberellic acid and finally gibberellic acid applied alone. The water-treated seed lines performed as an intermediate group with the lowest values to be observed in 2% chitosan mixtures with gibberellic acid, glycerol, or amino acids.

Chitosan (1.5–2%) combined with proline-betaine and glycerol produced the highest fresh root weight, 43–46% above that of water-treated seeds. Chitosan (2%) with gibberellic acid also showed strong root development followed by proline-betaine supplementation and, in all cases, performed better than water-treated seed lines. The weakest root systems were produced by the presence of gibberellic acid (2% chitosan with gibberellic acid and glycerol) or the absence of glycerol (2% chitosan with proline-betaine) in applicable formulas.

Chitosan (2%) with gibberellic acid achieved the highest shoot weight, followed by the standalone gibberellic acid application and the water-treated seed lines. Most chitosan−amino acid and chitosan−gibberellic acid combinations produced intermediate shoot weights that did not significantly differ from one another. Supplementation of 2% chitosan with gibberellic acid and glycerol ranked last followed by 2% chitosan with proline and betaine.

3.2.3. Seedling Biomass Metrics

Root dry biomass was highest in the 1.5–2% chitosan formulations supplemented with proline-betaine and glycerol, both significantly exceeding the water-treated lines (28–41%) (Table 8). Chitosan (2%)–gibberellic acid also performed well. Amino acid application alone or with 1.5% chitosan significantly favored root biomass accumulation, above the levels of water-treated seed lines. However, 2% chitosan with proline-betaine or with gibberellic acid and glycerol recorded the poorest root development, 40% below the water-treated seed lines.

Table 8. Seedling biomass metrics while exposed in the soil environment [1].

Treatment [2]	Root Dry Weight (g)	Shoot Dry Weight (g)	Root Dry/Shoot Dry Weight (g)	Plant Fresh Weight (g)	Plant Dry Weight (g)
T1	0.256 ± 0.005 ^{ef}	0.512 ± 0.005 ^{e}	0.507 ± 0.003 ^{bcd}	2.541 ± 0.058 ^{g}	0.772 ± 0.011 ^{f}
T2	0.285 ± 0.004 ^{g}	0.503 ± 0.004 ^{e}	0.567 ± 0.015 ^{de}	2.559 ± 0.012 ^{g}	0.788 ± 0.002 ^{fg}
T3	0.226 ± 0.003 ^{c}	0.485 ± 0.005 ^{e}	0.467 ± 0.009 ^{b}	2.275 ± 0.009 ^{f}	0.711 ± 0.005 ^{e}
T4	0.227 ± 0.005 ^{c}	0.335 ± 0.004 ^{b}	0.677 ± 0.009 ^{fghij}	1.797 ± 0.038 ^{c}	0.562 ± 0.009 ^{b}
T5	0.193 ± 0.002 ^{b}	0.342 ± 0.007 ^{bc}	0.563 ± 0.019 ^{de}	2.004 ± 0.003 ^{de}	0.535 ± 0.004 ^{b}
T6	0.230 ± 0.004 ^{cd}	0.375 ± 0.007 ^{d}	0.617 ± 0.013 ^{ef}	2.008 ± 0.006 ^{de}	0.605 ± 0.009 ^{c}
T7	0.245 ± 0.005 $_{de}$	0.377 ± 0.008 ^{d}	0.663 ± 0.018 ^{fgh}	2.025 ± 0.009 ^{de}	0.627 ± 0.010 ^{cd}
T8	0.276 ± 0.003 ^{fg}	0.370 ± 0.002 ^{cd}	0.743 ± 0.007 ^{j}	1.952 ± 0.020 ^{d}	0.646 ± 0.005 ^{d}
T9	0.329 ± 0.002 ^{h}	0.487 ± 0.003 ^{e}	0.677 ± 0.009 ^{fghi}	2.610 ± 0.005 ^{g}	0.816 ± 0.003 ^{g}
T10	0.154 ± 0.002 ^{a}	0.223 ± 0.003 ^{a}	0.690 ± 0.006 ^{ghij}	1.400 ± 0.002 ^{b}	0.377 ± 0.005 ^{a}
T11	0.362 ± 0.004 ^{i}	0.576 ± 0.004 ^{f}	0.630 ± 0.010 ^{efg}	2.760 ± 0.007 ^{h}	0.938 ± 0.001 ^{h}
T12	0.197 ± 0.003 b	0.566 ± 0.007 ^{f}	0.347 ± 0.007 ^{a}	2.744 ± 0.009 ^{h}	0.762 ± 0.009 ^{f}
T13	0.182 ± 0.002 ^{b}	0.513 ± 0.004 ^{e}	0.357 ± 0.007 ^{a}	1.779 ± 0.007 ^{c}	0.696 ± 0.005 ^{e}
T14	0.263 ± 0.003 ^{ef}	0.374 ± 0.007 ^{d}	0.700 ± 0.010 ^{hij}	2.082 ± 0.009 ^{e}	0.637 ± 0.009 ^{cd}
T15	0.275 ± 0.006 ^{fg}	0.513 ± 0.003 ^{e}	0.533 ± 0.015 ^{cd}	2.354 ± 0.003 ^{f}	0.788 ± 0.006 ^{fg}
T16	0.230 ± 0.005 ^{cd}	0.492 ± 0.002 ^{e}	0.467 ± 0.012 ^{b}	2.565 ± 0.007 ^{g}	0.722 ± 0.006 ^{e}
T17	0.310 ± 0.005 ^{h}	0.639 ± 0.005 ^{g}	0.483 ± 0.012 ^{bc}	3.098 ± 0.015 ^{i}	0.949 ± 0.004 ^{h}
T18	0.159 ± 0.001 ^{a}	0.216 ± 0.011 ^{a}	0.740 ± 0.035 ^{ij}	1.159 ± 0.016 ^{a}	0.375 ± 0.011 ^{a}

[1] The different letters indicate a significant ($p < 0.05$) difference. Data in columns with no letters do not differ statistically. [2] Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol.

Chitosan (2%) with gibberellic acid produced the highest shoot dry weight, followed by 2% chitosan–proline-betaine−glycerol and gibberellic acid alone, significantly surpassing the water-treated seeds. Most other treatments were either comparable to or below the performance of the water-treated seeds. The lowest shoot biomass was recorded for 2% chitosan–gibberellic acid−glycerol and 2% chitosan–proline-betaine, both less than half of the water-treated seeds' value, confirming their inhibitory effect on above-ground growth.

The root-to-shoot dry weight ratio reflects the carbon partitioning strategy between below- and above-ground tissues. The highest ratios were observed in 1.5% chitosan–proline-betaine, 2% chitosan–gibberellic acid-glycerol, and 1% chitosan–gibberellic acid−glycerol, indicating a pronounced shift toward root investment. Notably, gibberellic acid alone and 1% chitosan–gibberellic acid yielded the lowest ratios, significantly below those of the water-treated seeds. This reveals an important dual perspective: gibberellic acid alone favors shoot allocation, whereas gibberellic acid combined with glycerol shifts allocation heavily toward roots, suggesting that glycerol may modulate the hormonal signal governing biomass partitioning.

Plant fresh weight was greatest in 2% chitosan with gibberellic acid, the only treatment to exceed 3 g and significantly above all others. The 2% chitosan combined with amino acids and glycerol, as well as gibberellic acid applied alone, performed comparably to the water-treated seeds. Below this level, the 2% chitosan with gibberellic acid and glycerol and the 2% chitosan with proline-betaine treatments consistently showed the poorest performance.

Total dry weight closely mirrored fresh weight trends. Chitosan (2%) with gibberellic acid, followed by 2% chitosan with proline-betaine and glycerol, achieved the highest values, approximately 23% above the water-treated lines. Chitosan (1.5%)–proline-betaine−glycerol slightly tended to exceed the water-treated seeds. The lowest dry weights were observed in 2% chitosan with gibberellic acid and glycerol and 2% chitosan with proline and betaine, representing 51% lower plant dry weight than water-treated seeds.

3.3. Comparison of Treatments

3.3.1. Petri Dish Experiment

Analysis revealed two major patterns in the germination and growth data. The first pattern is linked to overall germination vigour and shoot performance, where gibberellic acid-based treatments showed a tendency towards improved shoot performance. Gibberellic acid alone and chitosan−gibberellic acid combinations followed a similar response pattern. At the opposite extreme, the 1% chitosan treatments, both alone and combined with proline-betaine supplementation, germinated more slowly and produced the shortest shoots, even relative to the water-treated seeds. The second pattern is focused on root growth. Root-related traits showed coordinated variation in the heatmap analysis, with mean root growth rate representing the parameter with statistically significant treatment differentiation. Biostimulant combinations were associated with a more favorable root-related response profile, particularly due to higher mean root growth rate values. Proline-betaine applied alone clustered with the highest root-related response values, followed by 2% chitosan with proline-betaine, 1% chitosan with proline-betaine and glycerol, and 1.5% chitosan with proline-betaine. A similar pattern was observed across treatments containing the amino acid biostimulant mixture. In contrast, the GA_3 treatments, despite their favorable shoot growth profile, tended to cluster with lower root-related trait values; 1.5% chitosan with gibberellic acid and 2% chitosan showed lower root response profiles in the heatmap analysis (Figure 4). This trade-off was also reflected in the root-to-shoot weight ratio. Chitosan applied alone, particularly at 1%, clustered with lower-performance profiles relative to several other treatments. Water-treated seeds serve as a useful baseline, allowing for a clear distinction between the active treatments. Glycerol appears to mod-

ulate but not dominate, these effects; its presence slightly shifts the treatments without changing the profile of the mixtures, which justifies its role as a "plasticizer" in combination with chitosan.

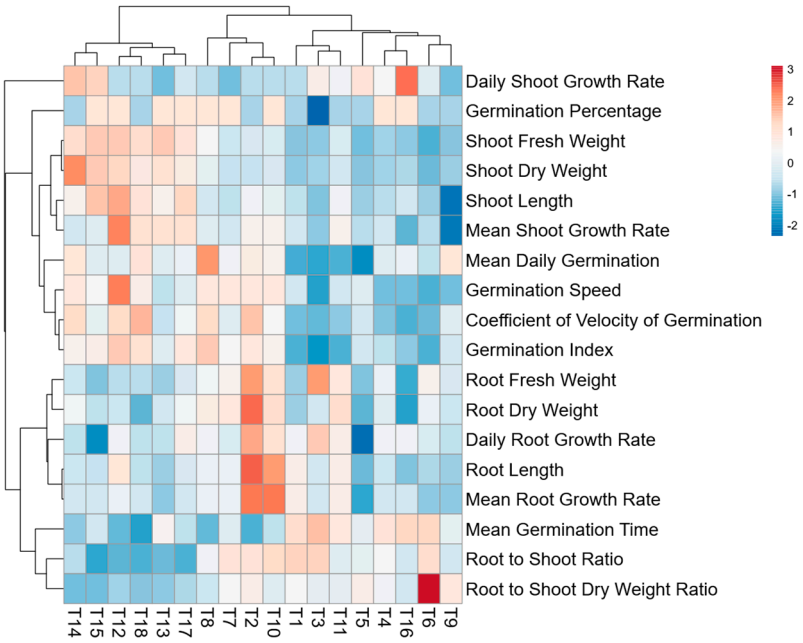


Figure 4. Heatmap analysis of *P. trifoliata* seed growth parameters per treatment in Petri dish conditions. Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol. Certain parameters shape the visual pattern while remaining statistically non-significant.

3.3.2. Soil-Based Experiment

The addition of glycerol to the chitosan, proline-betaine mixtures (particularly in 1.5% chitosan–proline-betaine−glycerol and 2% chitosan–proline-betaine−glycerol) substantially improved overall seedling quality compared to the same formulations without glycerol. Chitosan (2%) with gibberellic acid produced the best combination of rapid germination and high biomass, making it the most promising treatment for both early establishment and seedling robustness. Higher chitosan concentrations (2%) were associated with greater variation among formulations, with responses depending on the accompanying components; formulations containing 2% chitosan showed either enhanced biomass-related performance or reduced seedling growth responses, depending on the co-formulated compounds. Water-treated and amino acids-only-treated seeds were outperformed by the best-performing combination treatments but remained above the least favorable formulations.

The heatmap clustering reveals an asymmetry of effectiveness between the tested mixtures. Proline-betaine-based pairings were split across three clusters depending on the chitosan concentration and glycerol adjuvant, suggesting that they respond sensitively to the formulation context. Gibberellic acid-based pairings clustered tightly together regardless of concentration, suggesting that it dominates whatever it is paired with. Pure chitosan applications exhibit different attributes: 1% chitosan behaves like a germination promoter and was positioned in the small intermediate cluster, while 1.5% chitosan and 2% chitosan behave as suppressants and were positioned in the top cluster. However, the impact of soil conditions can lead to an interaction between chitosan concentration and the

choice of partner compound, resulting in either the best or the worst outcome depending on what is added to the overall synthesis (Figure 5).

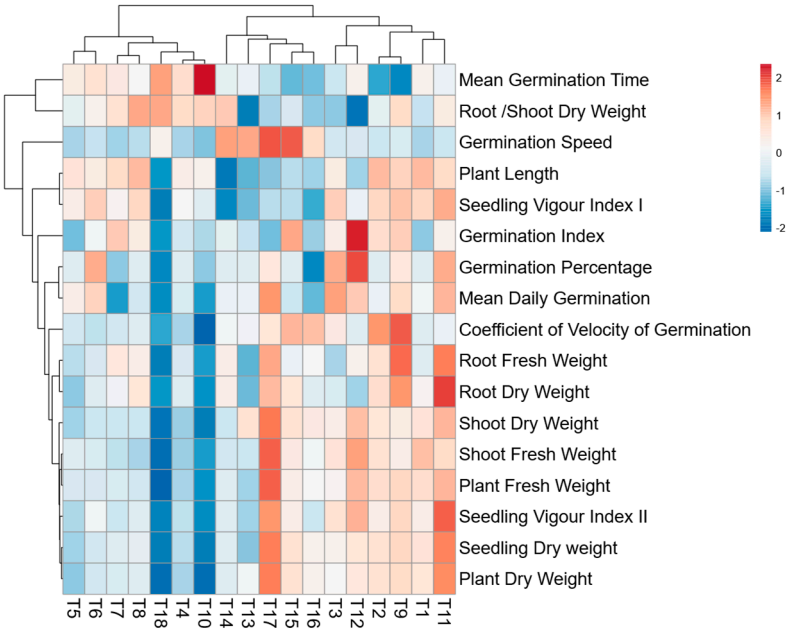


Figure 5. Heatmap analysis of *P. trifoliata* seed growth parameters per treatment in soil conditions. Treatments: T1, water-treated control; T2, biostimulant amino acids alone; T3–T5, chitosan at 1%, 1.5%, and 2%, respectively; T6, T8, and T10, chitosan (1%, 1.5%, and 2%) combined with biostimulant amino acids, respectively; T7, T9, and T11, the same combinations supplemented with glycerol; T12, GA_3 alone; T13, T15, and T17, chitosan (1%, 1.5%, and 2%) combined with GA_3, respectively; T14, T16, and T18, the same combinations supplemented with glycerol. Certain parameters shape the visual pattern while remaining statistically non-significant.

4. Discussion

Trifoliate orange (*Poncirus trifoliata*) produces large, polyembryonic, desiccation-sensitive seeds that germinate best at moderate-to-warm soil temperatures (20–40 °C with an optimum value of 25 °C) [55]. Seeds contain a mean of 1.4 embryos per seed, with 31.5% being polyembryonic, following Swingle citrumelo (*C. paradisi* × *P. trifoliata*) with higher rates of 48.1% polyembryonic seeds and 1.6 embryos per seed [56]. Larger fruits yield more viable seeds, and fruit weight is positively correlated with fruit length, fruit diameter, viable seed number, seed weight, and seed germination. Seeds of *P. trifoliata*, extracted from the fruit, water-rinsed, surface-sterilized in hot water (51.7 °C, 10 min), coated with fungicide, surface-dried, and with their seed coats removed, germinated across a broad range of 9–38 °C over 9–84 days, achieving 100.00% emergence. Retaining the seed coats narrowed this range to 11–36.5 °C, delayed and prolonged germination to 17–101 days, and reduced emergence to 81.25%, while also shifting the optimum germination temperature by 1 °C [9].

Herein, the examined trifoliate orange seeds were extracted from mature fruits, reflecting an important experimental standardization parameter. Seed maturity, determined by the developmental stage of the harvested fruit, shapes both dry weight and water content. Across four fruit ripening stages [solid-green, yellow-green, ripe, and overripe (4 weeks post-ripening)], seeds extracted from the fruits steadily accumulated dry matter, with the mean dry weight rising from 93.4 to 144.5 mg. The water content moved inversely, falling from 129.3% of the dry weight in seeds from solid-green fruits to 79.5% (yellow-green) and 64.7% (ripe), before rising slightly to 70.6% in seeds from overripe fruits. Germination at

25 °C (radicle: ≥2 mm) reached 96–100% across all maturity levels, although germination times differed [57].

Under Petri dish growth conditions, seeds are subjected to highly controlled conditions, including uniform temperature, light, humidity, and moisture. Moisture is consistently maintained through capillary action, eliminating drought stress and minimizing mechanobiological responses due to the absence of soil particle resistance, allowing the radicle to emerge freely. However, these artificial conditions do not accurately reflect seed emergence responses in soil environments. In contrast, seed trials conducted in soil are exposed to spatial geometries with physicochemical fluctuations, complicating observation without disturbing the seedlings. Root development remains hidden, and the simulation of natural soil depth and conditions more closely approximates field conditions. Consequently, the parameters for assessing seed germination in soil differ from those in Petri dish experiments. The minimal variability that yielded non-significant differences under Petri dish conditions, particularly for the coefficient of velocity of germination (CVG), mean germination time (MGT), and germination index (GI), may reflect the inherent biological heterogeneity of the seeds, as citrus polyembryony allows a zygotic embryo to coexist with multiple nucellar (asexual) embryos within a single seed.

There are many cases where chitosan has been reported to enhance seed germination in large crop plant species via the following: (a) increased water absorption, permeability, and enzyme activity, (b) stimulation of gibberellic acid and regulation of abscisic acid, and (c) increasing superoxide dismutase, catalase, and peroxidase activities, reducing oxidative stress [58]. However, apart from this generalized inducible scheme, the seed chemical composition, which is a species-related characteristic, and the type of applicable chitosan can differentiate the intensity and spectrum of the responses [59]. Soil incorporation offers an alternative delivery route for chitosan, conveying the polymer directly into the seed microenvironment without requiring seed-coating technologies. A granular composite amendment containing chitosan was mixed uniformly through the 0–20 cm tillage layer at 0.2–0.5% of soil dry weight to alleviate salinity stress and improve crop establishment on moderate-to-severe saline-alkali land (pH: 8.5–10.5; electrical conductivity: 5–12 mS/cm) [60]. This practice is not transferable to *P. trifoliata*, which favors slightly acidic to neutral soils: above pH 7.5, reduced availability of micronutrients such as iron induces lime-induced chlorosis and depressed yields [61].

The pairing of chitosan with glycerol has become one of the most frequently recurring formulation motifs in biodegradable seed-treatment technology. In degradable plastic films, glycerol was incorporated into chitosan–acetic acid systems in small proportions (1–2 mL of a 1% solution per 1–5 g of chitosan), conferring the mechanical flexibility that chitosan alone cannot provide. By positioning itself between the chitosan polymer chains, glycerol reduces intermolecular hydrogen bonding and lowers the glass transition temperature, rendering the dried film flexible [62]. Furthermore, plasticized chitosan-based liquid mulch exhibited a tensile strength of approximately 17 MPa and an elongation at break exceeding 130%, while retaining water solubility and biodegradability; upon soil application, it enhanced cabbage seed germination to 74.6% [63]. Chili seeds coated at a lignin–chitosan–glycerol ratio of 0.25:1.0:0.2 reached 100% germination after one week of storage and retained 40–80% after five weeks [64].

The literature on the effects of glycerol on plant growth is mixed, with the direction of the response contingent upon concentration, mode of application, and the tissue or species examined. At the stimulatory end, diluted glycerol can act as a short-term growth promoter. Foliar sprays at approximately 5–50 mM enhanced fresh and dry weight and shoot length by 46–158% in corn, carrot, and spearmint. Glycerol also shows promise as a plasticizer in hydrogel soil formulations, since glycerol-plasticized cellulose hydrogel

enhanced germination under moisture stress, raising wheat germination by 21.88% and increasing leaf number after 21 days [65]. In contrast, inhibitory findings demonstrated that crude glycerin can be applied as a weed-seed suppressant, with the effect scaling in proportion to the glycerol concentration [31]. Neutral responses have also been documented between these extremes. Glycerol concentrations below approximately 100 μM do not significantly alter *Arabidopsis* root length [30], and dark-grown hypocotyl elongation is not inhibited by glycerol, indicating a tissue-specific dimension to the response [66].

Glycerol's role exhibits a dual set of properties. As a plasticizer, it governs whether the coating physically permits germination; but as a small, hygroscopic, water-miscible polyol, it also influences the moisture environment immediately around the seed [28]. In this work, under soil conditions, glycerol plays a dual and context-dependent role. In proline-betaine formulations, glycerol is essential for efficacy (2% chitosan–proline-betaine−glycerol vs. 2% chitosan–proline-betaine). In gibberellic acid formulations, however, glycerol addition to 2% chitosan is strongly detrimental (2% chitosan–gibberellic acid vs. 2% chitosan–gibberellic acid−glycerol). This asymmetry suggests that glycerol interacts differently with the two bioactive agents, possibly affecting release kinetics or phytohormone balance. The divergent responses observed in soil and Petri dish environments likely reflect a physical–mechanobiological trade-off. Exogenous GA_3 promotes rapid cell elongation, often resulting in longer hypocotyls accompanied by tissue spindling and reduced structural integrity. Although such elongation is advantageous under the unconstrained conditions of a Petri dish, slender tissues may lack the mechanical rigidity required for soil penetration and emergence from compacted substrates. Therefore, elongation that is not matched by proportional reinforcement of the cell wall may heighten susceptibility to mechanical impedance, increasing both biomechanical stress and the energetic cost of emergence. This imbalance between growth stimulation and structural support may ultimately constrain seedling elongation, establishment, and early developmental performance in the soil.

The oligomeric chitosan−polyvinyl alcohol−glycerol formulation was reported to enhance the soybean germination rate by over 25% [67]. Several seed-coating patents employ chitosan in combination with glycerol and other components, demonstrating enhanced germination and growth rates in coated broccoli and rice seeds [68,69]. Pretreating long-term stored seeds of *Avicennia marina* with 15 g/L chitosan in 1% acetic acid with 1% glycerol, followed by storage in moist sand at low temperature, delayed viability loss and preserved a 40% germination capacity after four months [70].

For corn seeds, a 2% chitosan coating plasticized with 0.30 g glycerol per g of chitosan yielded favorable physical properties, supporting a germination capacity above 90%. In contrast, the germination speed index (GSI) was sensitive to coating composition, declining at higher chitosan concentrations, likely because the resulting thicker, more continuous film prolonged water uptake and delayed radicle protrusion [26].

Among the parameters studied, the vigour index showed the greatest improvement in seeds under exogenous supplementation with chitosan and glycerol. Similarly, *Ricinus communis* seeds treated with a formulation combining chitosan (1.5%), polyethylene glycol 6000 (0.5%), glycerol (1%), and genus *Trichoderma* fungi (1%, 10^8–10^{10} cfu) yielded a vigour index of 3745, corresponding to 100.0% germination, a shoot length of 9.6 cm, and a root length of 27.1 cm [28]. It should be noted that, when comparing vigour index data across studies or conditions, the timing of measurement emerges as a critical variable.

Gibberellic acid-encapsulated chitosan nanoparticles enhance Fabaceae seed germination through both direct effects and indirect interactions mediated by nitrogen-fixing microorganisms, although the cross-kingdom cellular signaling underlying this process remains to be clarified [71,72]. Exogenous gibberellic acid elicits a more immediate response, whereas chitosan appears to indirectly raise endogenous gibberellic acid via a

separate or complementary biochemical route. To fully assess the role of gibberellic acid in *Poncirus trifoliata* seed germination, three parameters should be quantified in the next step: a) endogenous gibberellic acid content, b) the delivery kinetics of gibberellic acid applied alone versus in chitosan-based formulations, and c) gibberellic acid half-life under both Petri dish and soil conditions.

Gibberellic acid was a major contributor to shoot growth in *P. trifoliata* seed lines grown in Petri dishes. Across the evaluated parameters, gibberellic acid-containing treatments showed the strongest responses for shoot elongation and mean shoot growth rate while being associated with lower root-to-shoot ratios. The exogenous presence of proline-betaine was associated with a more favorable root-related response profile, particularly reflected in mean root growth rate, which showed significant treatment differentiation. Chitosan treatments at all concentrations, whether alone or combined with proline and betaine or gibberellic acid, generally produced intermediate responses that did not differ significantly from the control in most parameters. The addition of glycerol did not confer a consistent additional benefit across the tested combinations, possibly due to physicochemical interactions in the seed coating mixture [73].

Under soil experimental conditions, the application of a 1.5% chitosan mixture combined with proline-betaine and glycerol consistently enhanced CVG while reducing MGT, whereas the 2% chitosan–proline-betaine–glycerol combination proved most effective in improving SVI, SVII, root fresh weight, and seedling dry weight. Under these conditions, the glycerol–chitosan matrix supplemented the proline-betaine-mediated microenvironmental growth-promoting activities at the seed surface. A possible explanation is that root penetration among soil particles requires osmoregulation, membrane integrity, and redox balance, which can be supported by exogenous proline-betaine administration. In contrast, 2% chitosan with proline and betaine exhibited the slowest germination CVG and MGT and the poorest seedling growth, indicating a potential inhibitory effect without glycerol supplementation.

In all other cases, where gibberellic acid predominates over amino acid-mediated biostimulation, germinating seeds and seedlings preferentially activate specialized pathways in cell elongation and reserve mobilization [74,75]. The hormonal advantages of gibberellic acid, namely antagonizing abscisic acid signaling, activating gibberellin-responsive genes, and inducing α-amylase and other hydrolytic enzymes, frame a response that could not be readily achieved through amino acid-based stimulation. The overall germination capacity (%) remained unaffected by the treatments, indicating that the coatings modulate germination kinetics rather than seed viability.

5. Conclusions

Pre-sowing chitosan-based seed coating offers an effective strategy for improving the propagation of *Poncirus trifoliata*. Critically, applied coating treatments influenced germination timing and seedling vigour-related responses rather than altering intrinsic viability, which remained uniformly high in both exposed conditions (86.67–100% in Petri dishes and 60–93.33% in soil).

The hormonal and amino acid additives influenced seedling development along two complementary axes. Gibberellic acid-containing treatments were associated with increased shoot elongation and lower root-to-shoot ratios in some formulations. In contrast, proline-betaine-containing treatments were associated with more favorable root-related responses, particularly mean root growth rate, indicating a functional trade-off in resource allocation. In soil, coatings modulated germination kinetics, with the fastest emergence from gibberellic acid in 1.5–2% chitosan (~3 times the control) and the most uniform, rapid germination from 1.5% chitosan−proline-betaine−glycerol (MGT: ~25 days).

Seedling establishment proved highly dependent on formulation. Although gibberellic acid accelerated emergence, several GA_3 treatments reduced seedling length in soil (up to ~57%), suggesting a possible absence of GA_3–coating interaction in the Petri dish. Two formulations stand out as recommended compositions: 2% chitosan with gibberellic acid (without glycerol), which united rapid emergence with maximum biomass (fresh weight: >3 g), and 2% or 1.5% chitosan−proline-betaine−glycerol, which consistently increased root biomass (~28–41% above water-treated lines) and overall seedling quality. Glycerol played a notably dual role, being essential to the amino acid schemes but detrimental within gibberellic acid mixtures, particularly at 2% chitosan, likely through its influence on coating properties and hormone-release kinetics. Plain chitosan without an appropriate co-factor conferred no comparable benefit, indicating that the co-formulated components strongly influenced the resulting treatment performance.

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