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Research article

Advanced seed invigoration techniques enhance germination performance of *Trifolium* species under salinity and drought stress

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Abstract

An experiment was carried out to evaluate the response of primed and unprimed seeds of red clover (*Trifolium pratense*) and white clover (*T. repens*) to drought and salinity stress during early seedling development. The study demonstrated that both stress conditions delayed seed germination in all treatments; however, primed seeds exhibited a comparatively enhanced germination response under both drought and salt stress compared to unprimed seeds. Key germination parameters, including germination percentage (G%), speed of germination (SG), seedling length, and vigour index, were notably higher under salt stress (NaCl) than under drought conditions simulated with polyethylene glycol (PEG), in both clover species. This indicates that salinity stress had a less severe impact on early seedling growth than drought stress. Hydropriming significantly reduced the mean germination time (MGT) and enhanced the SG, G%, and vigour index across all stress treatments relative to the control, suggesting its potential as an effective strategy to enhance stress tolerance in clover species during the germination stage.

Keywords: Clover, Drought, Fodder, Germination, Pasture species, Salinity, Seed priming

Introduction

Red clover (*Trifolium pratense* L.) and white clover (*T. repens* L.) are among the most significant forage legumes used in temperate pastures grazed by ruminants such as cattle and sheep (Laidlaw and Teuber, 2001; Frame and Newbould, 1986). The increasing demand for high-quality forage, coupled with the reduction in arable land, has necessitated the cultivation of clover on marginal lands often characterized by poor soil fertility, salinity, and water scarcity. In the context of global climate change, agricultural productivity is increasingly challenged by a multitude of abiotic and biotic stresses (Bartels and Sunkar, 2005). Among these, abiotic stresses, particularly drought and salinity, are recognized as predominant constraints, contributing to over 50% yield reductions across various crops (Buchanan *et al.*, 2000; Bartels and Sunkar, 2005).

Seeds form the foundation of crop production, serving as carriers of genetic potential and a conduit for technological innovations in agriculture (Sanjay *et al.*, 2016b). However, environmental stresses, especially those associated with climate variability, can significantly impair seed germination and seedling establishment, primarily through physiological and biochemical disruptions. In particular, water stress leads to irregular germination, delayed seedling emergence, and weakened early plant development (Mwale *et al.*, 2003; Okcu *et al.*, 2005). Salinity further compounds these challenges by reducing water uptake and causing ionic toxicity due to the accumulation of sodium (Na^+) and chloride (Cl^-) ions, thereby disrupting nutrient balance and physiological functions in germinating seeds (Mishra *et al.*, 2023).

Range legumes play a crucial role in improving soil conditions under drought and salinity stress by enhancing soil structure and fertility. Their deep root systems help in soil aeration, water retention, and nutrient cycling, while nitrogen-fixing abilities improve soil nutrient content, particularly nitrogen. Additionally, these legumes can tolerate harsh conditions, making them ideal for restoring and maintaining soil health in stressed environments. Legume crops, particularly range legumes, play a vital role in improving soil conditions under drought and salinity stress. These crops are capable of fixing atmospheric nitrogen, enhancing soil fertility, and improving its structure, which helps in mitigating the adverse effects of water scarcity and high salinity (Kumar *et al.*, 2024). The root systems of legumes also contribute to organic matter accumulation, fostering soil microbial activity, which further enhances soil health.

However, establishing legumes in such challenging environments requires specific management practices. Seed priming, a technique that involves pre-soaking seeds in water or nutrient solutions, has been shown to improve seed germination, seedling vigor, and overall crop establishment under drought and salinity conditions. This practice helps by enhancing water absorption, activating metabolic processes, and increasing the tolerance of seedlings to abiotic stresses, making it a critical step for the successful cultivation of range legumes in marginal lands (Heydecker and Coolbaer, 1977; Bradford, 1986). The application of growth regulators during priming has also been shown to enhance the antioxidant capacity of seedlings, thereby improving stress resilience (Sanjay *et al.*, 2016a). The positive impact of seed priming has been documented in several fodder crops viz., fodder maize (Tondey *et al.*, 2021 ; Sharifi *et al.*, 2016), fodder sorghum (Chen *et al.*, 2021) *Lectuca sativa* (Adhikari *et al.*, 2022), Mombasa grass (Saleem *et al.*, 2025) and forage cowpea (Ramya *et al.*, 2021). The present study aims to evaluate the influence of drought and salinity stress on seed germination parameters of clover and to identify effective priming strategies that enhance seed performance and early seedling vigor under degraded soil conditions. The ultimate goal is to facilitate the establishment of clover as a pioneering species for pasture development in marginal lands.

Materials and Methods

This study was carried out at the Indian Institute of Seed Science, Maunath Bhajan (Uttar Pradesh), India. The fresh seed lots of red and white clover obtained from the Regional Research Station, Indian Grassland and Fodder Research Institute, Srinagar (Jammu and Kashmir), India, were used for the study.

Assessment of seed viability and dormancy: Seed viability is a fundamental attribute that indicates the physiological "aliveness" or potential of a seed to germinate under favorable conditions. To evaluate seed viability, a tetrazolium chloride (TZ) test was performed following standard procedures. Seeds were first soaked in distilled water overnight to facilitate hydration. Subsequently, the seeds were incubated in a 1% (w/v) tetrazolium solution for 18 hours at 30 ± 1°C. After incubation, the seed coats were carefully removed, and viability was assessed based on the presence and intensity of red staining in the embryonic tissues, indicative of formazan formation as a result of dehydrogenase enzyme activity (ISTA, 2015). Seed dormancy, a biological mechanism that prevents germination under otherwise favorable conditions, is particularly prevalent in temperate species. In legumes, physical dormancy is the dominant form, typically caused by impermeable seed coats that obstruct water uptake during the initial stages of germination. To assess the presence of physical dormancy in clover seeds, a seed imbibition test was conducted. For the imbibition assay, three replicates of 1 g intact seeds were immersed in distilled water at 25 ± 1°C. At 2-hour intervals, seeds were removed, gently surface-dried using blotting paper, and weighed. This process continued until a stable (constant) seed weight was recorded, indicating the cessation of water uptake. The percentage increase in weight due to water imbibition was calculated using the formula: Water uptake (%) = $[(W' - W_0)/ W_0] \times 100$; where W_t was the weight at time t, and W_0 was the initial seed weight prior to imbibition.

Seed priming: Following the assessment of seed viability and dormancy, seed moisture content was determined in accordance with the guidelines of the International Seed Testing Association (ISTA, 2015). Subsequent to this, seed priming treatments were conducted. For each priming treatment, seeds were immersed in the respective priming solutions and incubated at a controlled temperature of 20°C for a duration of 16 hours. After incubation, the seeds were withdrawn from the solutions and subjected to shade drying to restore their initial moisture content prior to sowing. Three distinct priming strategies were employed in this study across both clover species-

- Hydropriming: Seeds were soaked in distilled water at a seed-to-solution ratio of 1:5 (v/v);
- Halopriming: Seeds were treated with 1% solutions of calcium chloride ($CaCl_2$), zinc sulfate ($ZnSO_4$), and potassium nitrate (KNO_3);
- Hormonal priming: Seeds were soaked in a 100-ppm solution of salicylic acid.

Germination test: Primed seeds were first equilibrated to their original moisture content prior to the initiation of germination assays. Germination tests were conducted under three distinct environmental conditions: control (non-stress), saline stress, and drought stress. Salinity stress was simulated by preparing sodium chloride (NaCl) solutions at concentrations of 50 and 100 mM, corresponding to electrical conductivity (EC) values of 4.82 and 9.91 dS m^{-1}, and solute potentials (ψs) of −2.49 and −4.98 bar, respectively. Osmotic potential of NaCl solution was calculated by using the van't Hoff equation ($\psi_s = -iCRT$) where C is the concentration in molarity of the solute, i is the van't Hoff factor, R is the ideal gas constant, and T is the absolute temperature (Coons *et al.*,1990). Drought stress was imposed using polyethylene glycol (PEG 6000) solutions at concentrations of 5% and 15% (w/v), which generated osmotic potentials of −0.47

and −3.02 bar, respectively, as described by Michel (1983). For each treatment, germination was evaluated using the 'top of paper' method with three replicates of 50 primed seeds. Seeds were placed on two layers of filter paper moistened with either deionized water (control), PEG solution (5 or 15%), or NaCl solution (50 or 100 mM), depending on the stress condition. The petri dishes were then incubated in the dark at a constant temperature of 20 ± 0.5°C and a relative humidity of 90 ± 5%, following the standard protocol recommended by the International Seed Testing Association (ISTA, 2015). To mitigate solute accumulation, the filter papers were replaced every two days in both the saline and drought treatments (Rehman *et al.*, 1996). Germination parameters were recorded for both *T. pratense* (red clover) and *T. repens* (white clover) throughout the experimental period and calculated as mentioned below.

Time to 50% germination (t_{50}): Time taken to 50% germination was calculated according to the formula of Coolbear *et. al.* (1984).

$T_{50\%} = t_i + \left[\frac{\frac{N}{2}-n_i}{n_j-n_i}\right](t_j - t_i)$ Where N is the number of final emergence count and ni, cumulative number of seeds emerged at adjacent days ti and tj when ni < (N+1)/2 < nj.

Mean germination time (MGT): MGT was calculated according to the following equation of Ellis and Roberts (1981). MGT is reciprocal of germination rate and shown to be highly indicative of the emergence performance of different seed lots.

$MET = \frac{\sum Dn}{\sum n}$ Where n is the number of seeds newly germinated on day D and D is the number of days calculated from the beginning of the germination test.

Speed of germination (SG) or germination energy (GE)*:* Speed of germination was calculated using the following formula of the Association of Official Seed Analysts (AOSA, 1983).

$$\text{Speed of Germination} = \frac{\text{No. of emerged seeds}}{\text{Days of first count}} + ----+ \frac{\text{No. of seed emerged}}{\text{days of final count}}$$

Germination percentage (%): Germination percentage was calculated as the proportion of seeds that produced normal seedlings under standard germination conditions as described by ISTA.

Seedling length (cm): Seedling length was measured as the combined length of the root and shoot of normal seedlings. Measurements were taken from a representative sample at the final germination count and expressed in centimeters.

Seedling vigour index-I*:* Seedling vigour index-I was calculated by multiplying germination percentage by the mean seedling length (cm).

Statistical analysis*:* All the treatments, including the control, were replicated three times. The recorded data were subjected to statistical analysis with ANOVA to test the Tukey Honest Significant Difference of treatment means at 5% probability level ($p \leq 0.05$) by using the statistical analysis software (SAS) version 9.3. The percentage data were transformed into arcsine values before the analysis.

Results and Discussion

Seed viability and water imbibition behaviour: Tetrazolium staining confirmed that the seed lots used in the present study were of high physiological quality, with viability values of 98.33% in red clover and 97.67% in white clover (Figs 1a-b). Such high viability ensured that subsequent differences in germination behaviour were attributable to imposed treatments rather than inherent seed quality variation. The imbibition pattern (Fig 1c) showed a rapid initial uptake of water followed by a

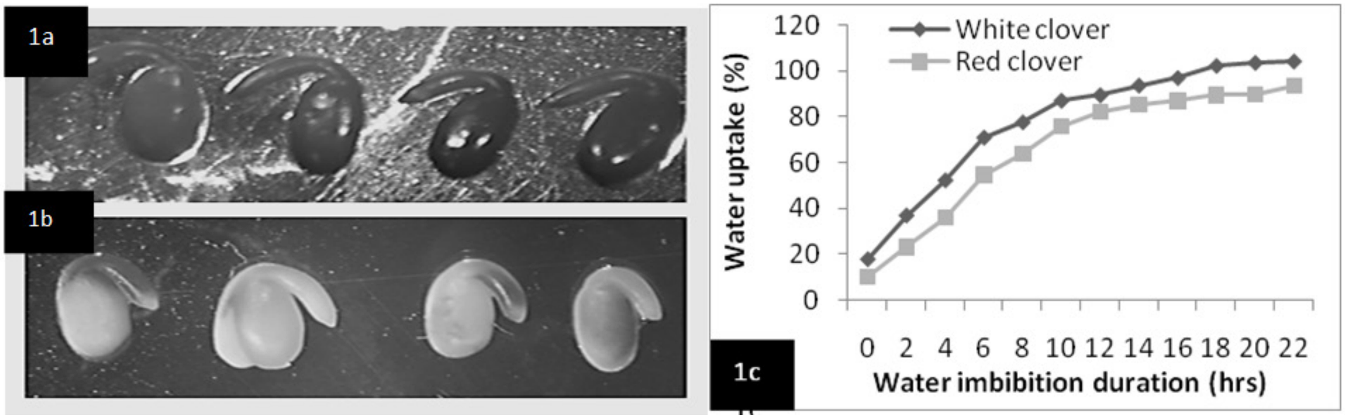

Fig 1. Viable (1a) and non-viable (1b) seed of red clover; water imbibition curve (1c) of red and white clover

gradual approach to a plateau, with both species attaining approximately 80 to 90% of their final hydrated weight within 15 to 16 hours. The absence of delayed water entry indicates that neither red clover nor white clover seeds possessed physical dormancy. The uniform hydration pattern further supports the high viability observed in Fig 1a and confirms that the seed coats were permeable, allowing normal progression to metabolic activation**.**

Effect of priming on germination and seedling growth: A statistically significant three-way interaction (p <0.05) among priming treatments, stress type, and stress intensity was observed for all parameters, indicating that priming efficacy was strongly influenced by both the nature and severity of stress. Both PEG-induced drought and NaCl-induced salinity significantly reduced germination percentage in red and white clover; however, PEG caused greater inhibition than NaCl at comparable osmotic potentials, suggesting a stronger osmotic constraint on water uptake and radicle emergence. Hydropriming consistently produced the shortest time to germination and the highest germination percentage under both optimal and stress conditions (Fig 2). Under non-stress conditions, hydroprimed seeds recorded maximum germination in red clover (86.51%) and white clover (84.64%), markedly higher than stressed treatments. The superior performance of hydropriming under stress reflects improved physiological preparedness of seeds prior to stress exposure (Table 1).

Recovery analysis revealed that non-germinated seeds from PEG treatments achieved 100% germination after transfer to distilled water, confirming that PEG-induced inhibition was reversible and primarily osmotic. In contrast, NaCl-exposed seeds showed reduced recovery, indicating additional ionic toxicity that likely impaired membrane stability and metabolic processes.

Table 1. CD value (p ≤0.05 level) from analysis of variance for different traits

Species	MGT (hrs)	SG	GP (%)	SL (cm)	SV-I
Red clover	0.694*	4.572*	2.724*	0.593*	135.811*
White clover	0.782*	4.394*	2.551*	0.572*	128.054*

*(p <0.05); (MGT: Mean germination time; SG: Speed of germination; GP: Germination percent; SL: Seedling length; SV-I: Seed vigour index-I

Seedling length followed trends similar to germination (Fig 3). Hydroprimed seeds produced the longest seedlings across environments, followed by salicylic acid and haloprimed treatments, while unprimed seeds performed the poorest. Differences between hormonal and haloprimed treatments were not significant (p < 0.05). Increasing stress intensity from −2.49 to −4.98 bars (NaCl) and −0.47 to −3.02 bars (PEG) significantly reduced seedling length, with PEG exerting the stronger inhibitory effect (Table 1). Enhanced performance of primed seeds is attributable to pre-germinative metabolic repair and activation, including membrane stabilization, RNA and protein synthesis, and efficient reserve mobilization (Varier *et al*., 2010; Xing *et al*., 2025; Ntshalintshali *et al*., 2025; Gao *et al*., 2024). Similar improvements in growth and reduced mean germination time have been reported by Kale and Takawale (2019) and Ali *et al*. (2016)

Germination speed response under salinity and drought stress: As osmotic stress intensified, mean germination time (MGT) increased markedly in both species (Fig 4), indicating delayed radicle protrusion under reduced water availability. Similar stress-induced increases in MGT have been attributed to limited water uptake and impaired mobilization of stored reserves under low osmotic potential (Pace *et al*., 2012; Nowicki *et al*., 2025). Germination speed was highest under moderate stress levels, PEG (5%) and NaCl (50 mM), but declined sharply at higher intensities, particularly under PEG (15%, ψs = -3.02 bar), as compared with NaCl (100 mM, ψs = -4.98 bar).

Hydroprimed seeds consistently exhibited superior germination speed under all stress treatments (44.45 and 43.41) at PEG 5%, followed by salicylic acid-primed

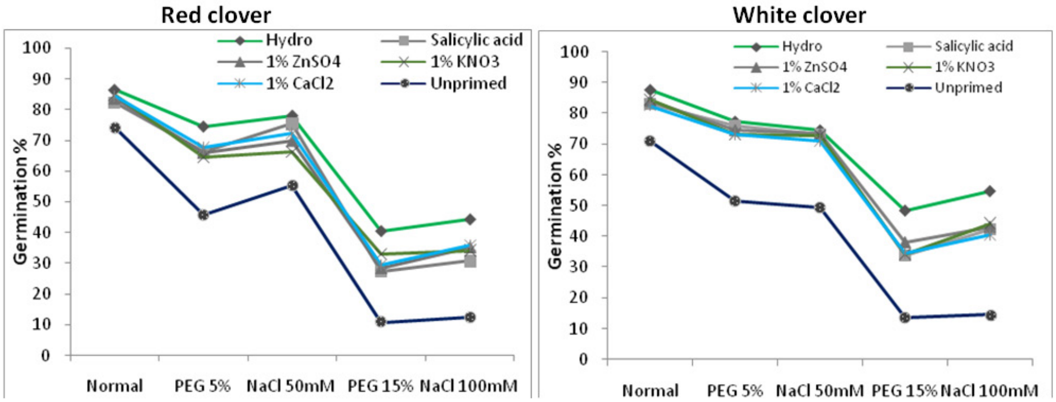

Fig 2. Effect of seed priming on germination (%) under different stress conditions in red and white clover

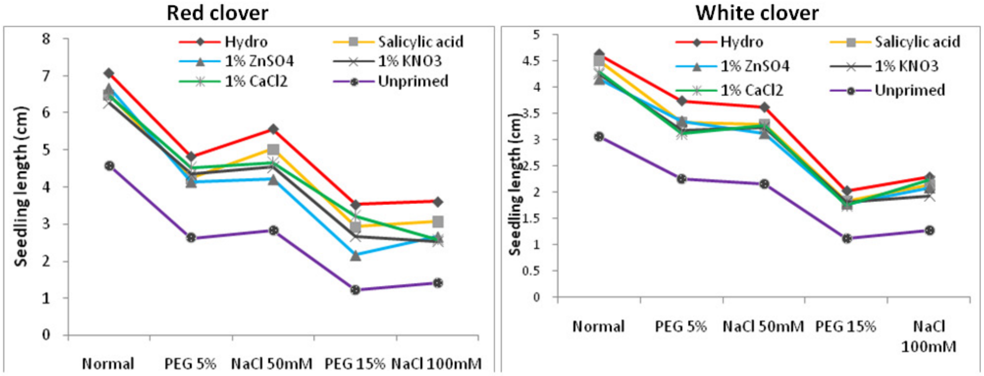

Fig 3. Effect of seed priming on seedling length under different stress conditions in red and white clover

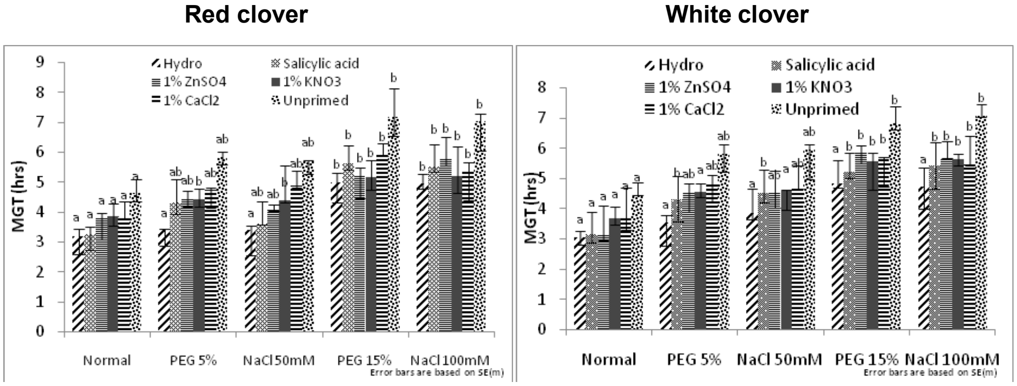

Fig 4. Effect of seed priming on mean germination time under different stress conditions in red and white clover species

seeds (37.07 and 41.28 at PEG 5%) in red and white clover, respectively (Fig 5). The comparatively greater inhibition of germination speed under PEG suggests that osmotic constraint, rather than ionic toxicity, was the dominant limiting factor during early germination. PEG-8000, owing to its high molecular weight, remains external to the seed coat and restricts water availability without penetrating tissues, thereby maintaining a strong external osmotic barrier. In contrast, Na^+ and Cl^- ions can enter seeds, lowering internal osmotic potential and facilitating partial water uptake, although prolonged exposure may induce ionic toxicity (Verma *et al*., 2014; Gour *et al*., 2023). Comparable observations have been reported in *Medicago sativa* (Wu *et al*., 2011) and in *Lolium* (Craciun *et al*., 2025), where PEG exerted stronger inhibitory effects than iso-osmotic NaCl solutions (Sayar *et al*., 2010; Sousa *et al*., 2008; Soni *et al*., 2023).

Seed vigour index and stress tolerance: Seed vigour index-I (SVI-I) varied significantly among priming treatments and stress levels (Fig 6). Hydroprimed seeds consistently achieved the highest SVI-I values under both

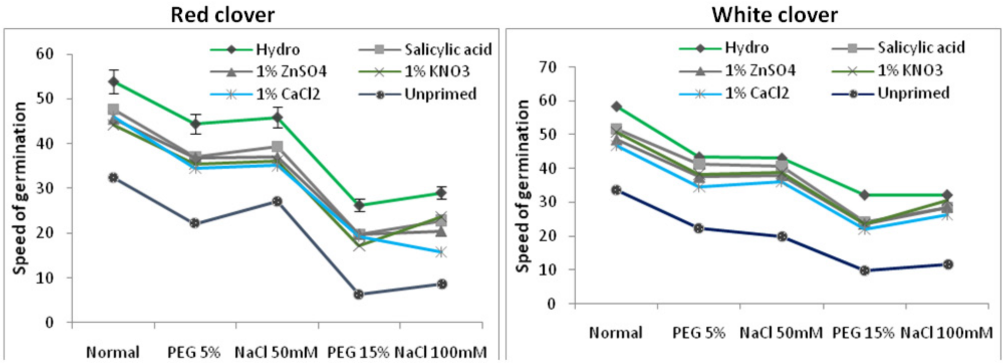

Fig 5. Effect of seed priming on germination speed under different stress conditions in red and white clover

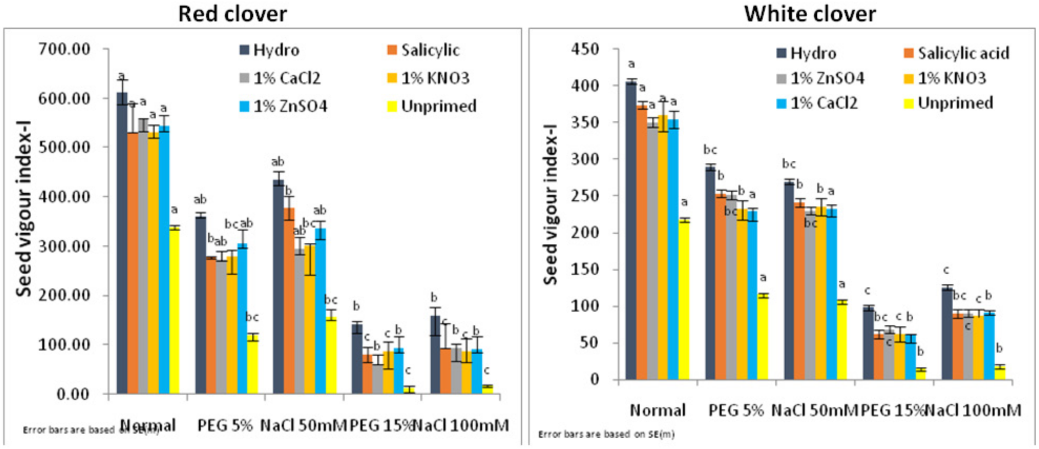

Fig 6. Effect of seed priming on seed vigour index-I under different stress conditions in red and white clover

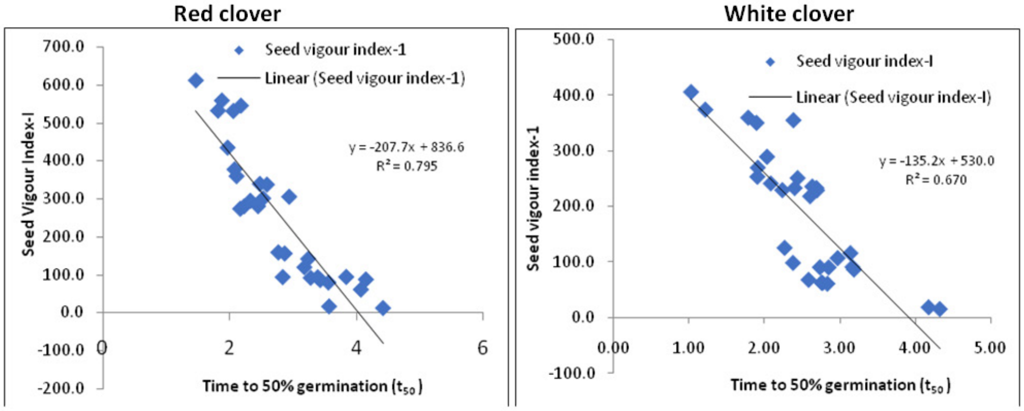

Fig 7. Correlation between t_{50} germination and seed vigour index-I

Table 2a. Coefficient of correlation (r) between different seed quality attributes in red clover

Attributes	MGT (hrs)	SG	GP	SL (cm)	SV-I
MGT	1	-0.990**	-0.960*	-0.959*	-0.962*
SG		1	0.970**	0.968*	0.979**
GP			1	0.986*	0.993**
SL				1	0.995*
SV-I					1

Table 2b. Coefficient of correlation (r) between different seed quality attributes in white clover

Attributes	MGT (hrs)	SG	GP	SL (cm)	SV-I
MGT	1	-0.986**	-0.968*	-0.980*	-0.986*
SG		1	0.987**	0.984*	0.991**
GP			1	0.989*	0.995**
SL				1	0.997*
SV-I					1

*(p <0.05); **(p <0.01); MGT: Mean germination time; SG: Speed of germination; % GP: Germination percent; SL: Seedling length; SVI-I : Seed vigour index-I

control and stress conditions, including PEG (5 and 15%) and NaCl (50 and 100 mM), with a peak value of 118.91. This demonstrates the effectiveness of hydropriming in enhancing early seedling establishment and resilience to osmotic stress (Al-Farsi *et al*., 2025; Fu *et al*., 2025; Singhal *et al*., 2023).

Correlation analysis revealed strong positive linear relationships between germination percentage and SVI-I, with coefficients of determination (R^2) of 0.79 for red clover (Table 2a) and 0.67 for white clover (Table 2b), as illustrated in Fig.7. These findings indicate that a substantial proportion of variation in seed vigour was explained by germination performance. Thus, germination percentage may serve as a reliable predictor of early seedling vigour in both species (Basu and Groot, 2023; Powell, 2022). Although not presented, the parameter t_{50} (time to 50% germination) further supported the superior performance of primed seeds, which reached 50% germination earlier and produced more vigorous seedlings. This parameter may provide a rapid and practical indicator of seed lot vigour without requiring complete germination assays.

Conclusion

The collective results demonstrate that seed priming, particularly hydropriming (16 hrs), significantly mitigates the adverse effects of drought and salinity stress on red and white clover. By enhancing germination speed, seedling growth, and vigour index, hydropriming offers a simple and cost-effective strategy suitable for large-scale and farmer-level adoption. Improved establishment under stress-prone environments characterized by irregular rainfall or saline soils can ultimately contribute to more stable forage production.

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