



Comparative Assessment of Salinity Effects on Germination Kinetics and Early Seedling Performance in Diverse Clover Species

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ABSTRACT

Background: Soil salinity is a major abiotic constraint limiting seed germination, seedling establishment and early growth in forage legumes, particularly in arid and semi-arid regions. Early stages are highly sensitive to osmotic and ionic stresses and interspecific variation in salinity tolerance within *Trifolium* remains insufficiently characterized. Identifying tolerant species at early growth stages is therefore critical for improving pasture establishment and sustainability in salt-affected environments.

Methods: A controlled laboratory experiment evaluated salinity effects on germination kinetics and early seedling growth of five clover species (*Trifolium hybridum*, *T. michelianum*, *T. alexandrinum*, *T. incarnatum* and *T. resupinatum*). Seeds were exposed to five NaCl concentrations (0, 50, 100, 150 and 200 mM) in a completely randomized design with four replications. Germination percentage, mean germination time, germination rate index, coefficient of velocity of germination, shoot and root length, shoot-to-root ratio, seedling fresh weight, vigor index and salinity tolerance index were determined. Data were analyzed using two-way ANOVA and relationships among traits were assessed by Pearson correlation analysis.

Result: Salinity significantly reduced all traits in a concentration-dependent manner ($p < 0.05$). Species effects were significant for all measured parameters and species $\times$ salinity interactions were significant for all traits except root length. Among the species, Balansa clover (*T. michelianum*) consistently exhibited the highest germination capacity, seedling growth, vigor index and salinity tolerance index across salinity levels, indicating superior tolerance. Berseem (*T. alexandrinum*), Persian (*T. resupinatum*) and Crimson (*T. incarnatum*) clovers showed moderate tolerance, whereas Alsike clover (*T. hybridum*) was the most salt-sensitive species, characterized by reduced germination, prolonged mean germination time and poor seedling vigor. Strong correlations among germination, growth and tolerance indices highlight the importance of rapid and uniform germination for early-stage resilience under salinity.

Key words: Forage legumes, Salinity stress, Salt tolerance, Seed germination, Seedling vigor, *Trifolium*.

INTRODUCTION

Soil salinity is one of the most pervasive abiotic stresses limiting plant establishment, productivity and ecological stability in agricultural ecosystems (Sarita *et al.*, 2025). More than 20% of irrigated lands are currently affected by salinity and this proportion is expected to increase due to climate-driven alterations in evapotranspiration, unsustainable irrigation practices and secondary salinization processes (Qadir *et al.*, 2014; Cowie *et al.*, 2018). Global assessments indicate that nearly one billion hectares are naturally saline, whereas approximately 77 million ha have undergone secondary salinization, making salinity a major constraint to forage and crop production worldwide (Cherlet *et al.*, 2018). This trend necessitates the identification of plant species capable of maintaining acceptable performance under salt-affected conditions (Athar *et al.*, 2009; Jamil *et al.*, 2011).

The adverse impacts of salinity on plants arise primarily from osmotic stress and ionic stress. Elevated concentrations of soluble salts reduce soil osmotic potential, thereby limiting water uptake and inducing cellular dehydration (Acosta-Motos *et al.*, 2017). Ionic stress results from the accumulation of Na^+ and Cl^- , which

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disrupts membrane stability, impairs nutrient homeostasis, inhibits enzymatic activity and reduces photosynthetic efficiency (Munns and Tester, 2008). Na^+ toxicity is widely recognized as the predominant factor responsible for growth inhibition, whereas Ca^{2+} and K^+ play protective roles by stabilizing membranes and mitigating Na^+ -induced injury (Kinraide, 1999; Lindberg and Premkumar, 2023). The severity of injury depends on salt concentration,

exposure duration, environmental conditions and plant developmental stage (Hampson and Simpson, 1990; James *et al.*, 2011).

Seed germination and early seedling growth represent the stages most sensitive to salinity stress. Salinity delays radicle protrusion, reduces germination percentage, prolongs mean germination time, suppresses root-shoot elongation and decreases early biomass accumulation (Hubbard *et al.*, 2012; Shiade and Boelt, 2020). These effects are more pronounced during germination than at later stages, as many non-halophytic species exhibit greater sensitivity during early establishment (Rogers and Noble, 1991; Dehnavi *et al.*, 2020). Because successful stand establishment determines plant competitiveness and forage yield, evaluations at the germination stage are essential for screening salt-tolerant species and genotypes.

Legumes (Fabaceae) comprise more than 19,000 species distributed across diverse ecological zones. Their agronomic value derives from high nutritive quality and the capacity to fix atmospheric nitrogen through symbiosis with rhizobia, improving soil fertility (Frame and Laidlaw, 2007). Within this family, the genus *Trifolium* (approximately 250-300 species) is globally important for pasture-based livestock systems, particularly in Mediterranean and temperate regions (Zohary and Heller, 1984). *Trifolium* species enhance forage quality and contribute to biologically fixed nitrogen inputs in mixed swards (Ledgard, 1991; FAO, 2010).

Despite their agronomic significance, many *Trifolium* species exhibit moderate to high sensitivity to salinity relative to companion grasses or other legumes such as *Medicago* and *Melilotus* (Maas and Hoffman, 1977; Rogers *et al.*, 2008). Reductions in germination and early seedling vigor have been widely documented, often occurring at salt concentrations around or exceeding 200 mM NaCl (Ates and Tekeli, 2007; Can *et al.*, 2013; Saberi *et al.*, 2013; Ma *et al.*, 2025). Nevertheless, considerable variation exists among species and within species, reflecting divergent evolutionary histories, ecological adaptations and genetic structures. This variability underscores the importance of comparative studies to identify tolerant taxa for cultivation or breeding.

Among cultivated annual clovers, Persian clover is valued for its adaptability to poorly drained soils and high-quality forage production; however, its germination and early growth decline with increasing salinity (Heuzé *et al.*, 2015; Ghassemabadi *et al.*, 2018). Berseem clover is an important

forage crop in subtropical systems but is sensitivity to salinity during germination (Zayed, 2013; Garg *et al.*, 2016). Crimson, Alsike and Balansa clovers also exhibit variable resilience, with growth impaired at elevated NaCl levels (Gravandi, 2013; Saberi *et al.*, 2013). These responses underscore the need for comparative, germination-based evaluations. With the increasing severity of soil salinization, salt-tolerant forage legumes represent a practical strategy for sustaining productivity in marginal environments (Ashraf and Harris, 2004; Turhan and Seniz, 2010).

Given the limited number of studies comparing multiple *Trifolium* species under uniform salinity treatments, interspecific evaluations during germination and early seedling growth are needed. Assessing responses across a salinity gradient can reveal species-specific sensitivities and identify tolerant taxa. Such information is essential for forage improvement and the sustainable use of salt-affected environments.

MATERIALS AND METHODS

This research was conducted at the Seed Laboratory of the Department of Field Crops, Faculty of Agriculture, Ege University, to evaluate the effects of salinity on germination kinetics and early seedling performance in diverse clover species (*Trifolium* spp.). The plant materials are presented in Table 1.

Experimental design and salinity treatments

The experiment was conducted in a completely randomized design with four replications. Five salinity treatments were prepared using sodium chloride (NaCl) at concentrations of 0, 50, 100, 150 and 200 mM. Distilled water was used as the control solution.

Seed preparation and germination procedure

Seeds of each species were surface-sterilized with a 1.0% sodium hypochlorite (NaClO) solution for 5 min and subsequently rinsed thoroughly with distilled water. Germination tests were conducted in 15 cm glass Petri dishes lined with double-layer Whatman No. 1 filter paper. Fifty seeds were placed in each dish and 10 mL of the respective salinity solution was applied. To prevent salt accumulation, filter papers were replaced every two days. The dishes were enclosed in transparent plastic bags to minimize evaporation.

Germination tests were conducted in a growth chamber maintained at 20±1°C under dark conditions, following ISTA (2018) rules. Temperature and relative humidity (65-70%)

Table 1: Common name, scientific name and life cycle of clover species used in the study.

Common name	Scientific name	Life cycle
Alsike clover	<i>Trifolium hybridum</i>	Short-lived perennial
Balansa clover	<i>Trifolium michelianum</i>	Annual
Berseem clover	<i>Trifolium alexandrinum</i>	Annual
Crimson clover	<i>Trifolium incarnatum</i>	Annual
Persian clover	<i>Trifolium resupinatum</i>	Annual

were continuously monitored and maintained throughout the experiment. Germinated seeds were counted daily and a seed was considered germinated when the radicle reached at least 1 mm in length. Germination was expressed as a percentage.

At the end of the 10th day, ten seedlings per replication were randomly selected for measurements. Shoot and root lengths (mm) were measured using a millimeter ruler and seedling fresh weight (mg) was determined immediately to avoid moisture loss.

Evaluated germination and seedling parameters

The following germination and vigor traits were calculated:

Germination percentage (GP): Germination percentage was calculated as the ratio of germinated seeds to total seeds, multiplied by 100.

Mean germination time (MGT): Calculated according to Ellis and Roberts (1980):

$$MGT = \frac{\sum(n_i t_i)}{\sum n_i}$$

Shoot and root length (SL and RL): Average shoot and root length (mm) of seedlings measured on day 10th.

Shoot-to-root ratio (SRR): Calculated as the ratio of shoot length to root length of seedlings.

Seedling fresh weight (SFW): Mean fresh weight of seedlings, expressed in mg seedling⁻¹.

Germination rate index (GRI): Calculated as the sum of the number of seeds germinated on each day divided by the corresponding day (Kader, 2005).

$$GRI = G1/1 + G2/2 + \dots + Gx/x$$

Coefficient of velocity of germination (CVG): Defined as (Jones and Sanders, 1987):

$$CVG = \left(\frac{\sum n_i}{\sum(n_i t_i)} \right) \times 100$$

Vigour index (VI): Calculated according to Abdul-Baki and Anderson (1973) as:

$$VI = GP \times (\text{Mean shoot length} + \text{Mean root length})$$

Salinity tolerance index based on vigour (STI): Defined as the ratio of the vigor index under salinity to the vigor index under the control treatment.

Statistical analysis

All measured variables were analyzed using analysis of variance (ANOVA) with the Statistical Analysis System (SAS Institute, 2012). Prior to analysis, data were tested for normality and no transformations were required. Significant differences at $p \leq 0.05$ were evaluated by comparing treatment means using the least significant difference (LSD) test. Pearson correlation analysis was performed to evaluate relationships among germination and seedling traits under salinity treatments.

RESULTS AND DISCUSSION

The main effects of species and salinity levels, as well as their interaction, on all measured traits are presented in Table 2 and interaction effects are illustrated in Fig 1 and 2. According to the analysis of variance, both species and salinity level significantly affected all traits ($p < 0.05$). The interaction between species and salinity level was significant for all traits except root length, indicating generally trait-dependent responses, whereas relative differences in root length among species remained consistent across salinity levels.

Germination percentage differed significantly among species ($p < 0.05$), with Balansa clover showing the highest value (84.34%), followed by Berseem clover (73.00%) and Alsike clover the lowest (54.33%). Salinity exerted a strong negative effect ($p < 0.05$), decreasing germination from 94.17% in the control to 28.67% at 200 mM. Mean germination time also varied among species and salinity levels ($p < 0.05$). Alsike clover required the longest time (5.70 days), whereas Berseem clover was the fastest (4.24 days). Increasing salinity delayed germination, with values rising from 2.20 to 8.44 days.

Seedling growth was significantly influenced by both species and salinity ($p < 0.05$). Balansa clover produced the greatest shoot (35.78 mm) and root length (43.40 mm), whereas Alsike clover had the lowest values (21.70 and 31.62 mm, respectively). Increasing salinity reduced both traits ($p < 0.05$), with shoot length declining from 48.20 to 7.26 mm and root length from 60.72 to 11.08 mm. The interaction was not significant for root length ($p > 0.05$), indicating stable interspecific ranking.

Shoot-to-root ratio differed among species ($p < 0.05$), with the highest value in Balansa clover (0.83) and the lowest in Alsike clover (0.62). Salinity reduced this ratio ($p < 0.05$), with the minimum value at 200 mM (0.62), indicating greater suppression of shoot growth under stress.

Seedling fresh weight differed among species ($p < 0.05$), with Balansa clover showing the highest value (38.80 mg), followed by Berseem clover (33.30 mg) and Alsike clover the lowest (24.48 mg). Fresh weight declined sharply with salinity ($p < 0.05$), from 47.10 to 10.70 mg.

Germination rate index and coefficient of velocity of germination were significantly affected by species and salinity ($p < 0.05$). Berseem clover exhibited the highest values (24.61% day⁻¹ and 29.83%), whereas Alsike clover showed the lowest (15.33% day⁻¹ and 22.29%). Increasing salinity reduced both indices ($p < 0.05$), with germination rate index decreasing from 43.18 to 3.57% day⁻¹ and coefficient of velocity of germination from 45.86% to 11.92%.

Vigor index and salinity tolerance index were also significantly affected ($p < 0.05$). Balansa clover exhibited the highest values (711.4 and 0.60), whereas Alsike clover showed the lowest (390.0 and 0.44). Across salinity levels, vigor index decreased from 1027.0 to 63.7 and salinity tolerance index from 1.00 to 0.06, indicating a marked decline in seedling vigor and tolerance with increasing stress.

Table 2: Effects of salinity levels and clover species on germination and early seedling growth traits.

Treatments	GP (%)	MGT (day)	SL (mm)	RL (mm)	SRR	SFW (mg)	GRI (%/day)	CVG (%)	VI	STI
Clover species (C)										
Alsike	54.33 ^d	5.70 ^a	21.70 ^e	31.62 ^e	0.62 ^d	24.48 ^e	15.33 ^d	22.29 ^c	390.0 ^e	0.44 ^d
Balansa	84.34 ^a	4.46 ^c	35.78 ^a	43.40 ^a	0.83 ^a	38.80 ^a	23.01 ^b	25.93 ^b	711.4 ^a	0.60 ^a
Berseem	73.00 ^b	4.24 ^d	30.80 ^b	39.76 ^b	0.75 ^b	33.30 ^b	24.61 ^a	29.83 ^a	590.7 ^b	0.55 ^b
Crimson	68.32 ^c	4.94 ^b	25.86 ^d	35.24 ^d	0.70 ^c	29.94 ^d	20.55 ^c	26.04 ^b	493.4 ^d	0.51 ^c
Persian	68.33 ^c	4.94 ^b	28.74 ^c	37.54 ^c	0.74 ^b	31.26 ^c	21.29 ^c	26.66 ^b	534.8 ^c	0.52 ^c
Salinity levels (S)										
Control	94.17 ^a	2.20 ^e	48.20 ^a	60.72 ^a	0.80 ^a	47.10 ^a	43.18 ^a	45.86 ^a	1027.0 ^a	1.00 ^a
50 mM	89.17 ^b	3.06 ^d	40.70 ^b	51.92 ^b	0.78 ^a	42.24 ^b	29.55 ^b	33.03 ^b	830.1 ^b	0.80 ^b
100 mM	77.67 ^c	4.36 ^c	28.54 ^c	38.88 ^c	0.73 ^b	34.26 ^c	18.46 ^c	23.42 ^c	533.5 ^c	0.51 ^c
150 mM	58.66 ^d	6.22 ^b	18.18 ^d	24.96 ^d	0.72 ^b	23.48 ^d	10.04 ^d	16.52 ^d	266.0 ^d	0.25 ^d
200 mM	28.67 ^e	8.44 ^a	7.26 ^e	11.08 ^e	0.62 ^c	10.70 ^e	3.57 ^e	11.92 ^e	63.7 ^e	0.06 ^e
ANOVA										
C	**	**	**	**	**	**	**	**	**	**
S	**	**	**	**	**	**	**	**	**	**
C × S	**	**	**	ns	**	*	**	**	**	**

*p-value<0.05, **p-value<0.01, ANOVA: analysis of variance, ns: not significant.

^{a,b,c}Means followed by different letters are significantly different (p-value<0.05) according to LSD's test.

GP: Germination percentage, MG: Mean germination time, SL: Shoot length, RL: Root length, SRR: shoot-to-root ratio, SFW: Seedling fresh weight, GRI: Germination rate index,

CVG: Coefficient of velocity of germination, VI: Vigor index, STI: Salinity tolerance index.

Salinity exerted a strong, concentration-dependent inhibitory effect on germination and early seedling development across the five clover species, consistent with established models describing the combined osmotic and ionic constraints of saline environments (Munns and Tester, 2008; Acosta-Motos *et al.*, 2017). This decline was observed across germination percentage, mean germination time, shoot and root length, shoot-to-root ratio, fresh weight, germination rate index, coefficient of velocity of germination, vigor index and salinity tolerance index, indicating that salinity constrained both germination initiation and subsequent seedling growth. The interaction between species and salinity level was significant for most traits, demonstrating differences in both baseline performance and response magnitude. In contrast, the interaction was not significant for root length, suggesting that relative species ranking remained stable despite overall reductions.

The interspecific variation can be interpreted in the context of life-history strategies and ecological adaptation.

Berseem, Crimson, Balansa and Persian clovers are annual legumes associated with Mediterranean-type environments characterized by temporal variability in soil moisture and episodic stress exposure, whereas Alsike clover is a short-lived perennial typical of cool, moist temperate conditions (Frame, 2019; USDA NRCS, 2023). This divergence is relevant because annual species are often selected for rapid germination and establishment, whereas perennials may allocate more resources to persistence-related traits (Baskin and Baskin, 2014; Finch-Savage and Bassel, 2016). In this study, Balansa clover consistently exhibited superior performance for several germination and vigor attributes, whereas Alsike clover showed lower values, consistent with expectations for Mediterranean annuals.

From a mechanistic perspective, salinity-induced inhibition of germination is commonly attributed to osmotic stress restriction of water uptake and ion toxicity disrupting metabolic processes (Munns and Tester, 2008; Acosta-Motos

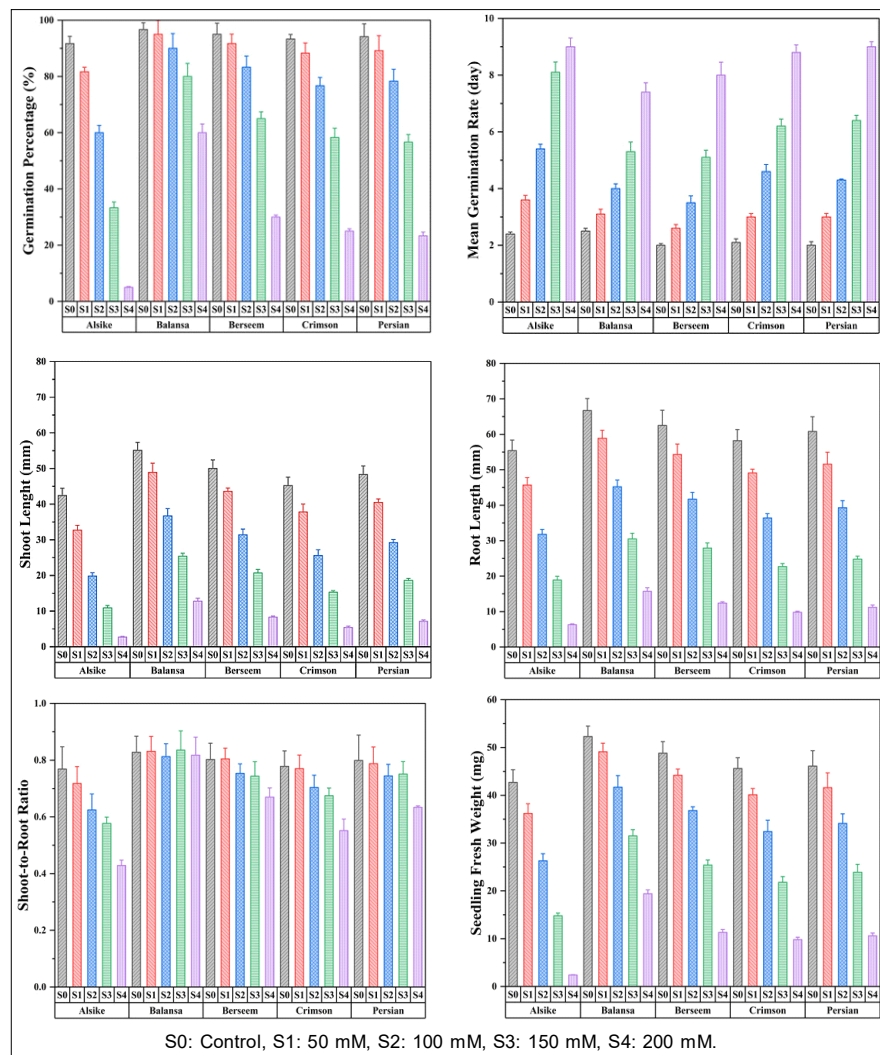


Fig 1: Interaction effects of salinity levels and clover species on germination and early seedling traits.

et al., 2017). A frequently reported regulatory axis involves enhanced abscisic acid signaling and suppressed gibberellin biosynthesis, which constrain radicle emergence and slow germination (Shu *et al.*, 2016; Singh *et al.*, 2024). The comparatively higher germination-related indices in Balansa clover may be associated with more effective osmotic adjustment and stress tolerance mechanisms, whereas the weaker performance of Alsike clover may reflect slower metabolic activation under stress condition.

Mean germination time increased with salinity, indicating delayed germination under increasing stress. Such delays are associated with reduced reserve mobilization, inhibition of hydrolytic enzymes, impaired mitochondrial function and limited energy supply during early development (Ashraf and Foolad, 2005; Farooq *et al.*, 2015). Species-level variation suggests differences in metabolic activation efficiency, with Mediterranean annual clovers potentially maintaining faster activation under transient stress. Delayed germination may narrow the establishment window and increase vulnerability to secondary stresses (Clay *et al.*, 2024).

Seedling growth responses reinforced these patterns. Shoot and root lengths declined with increasing salinity, consistent with reduced turgor, inhibition of cell expansion and disruption of cellular processes (Negrão *et al.*, 2017; Shabala and Munns, 2017; Mariyappillai and Kulanthaivel, 2024). The reduction in shoot-to-root ratio indicates greater suppression of shoot growth under stress. Root length showed a non-significant interaction, suggesting relatively

stable interspecific differences, whereas other traits were more strongly differentiated by tolerance mechanisms.

Fresh weight declined sharply with increasing salinity, reflecting reduced biomass accumulation under stress. This reduction is associated with impaired photosynthetic establishment, oxidative damage and reduced protein synthesis (Safdar *et al.*, 2019; Hasanuzzaman and Fujita, 2022). The higher fresh weight observed in Balansa clover supports its greater tolerance. Because fresh weight integrates growth and plant water status, it can effectively discriminate among species.

Germination rate index, coefficient of velocity of germination, vigor index and salinity tolerance index collectively support species resilience patterns. Declines in germination rate and velocity indicate slower and less coordinated germination, while reductions in vigor index confirm that stress effects extend beyond germination. The salinity tolerance index supports a consistent ranking, with Balansa clover exhibiting the highest tolerance and Alsike clover the lowest, reflecting adaptation to contrasting environments (Frame, 2019; USDA NRCS, 2023).

These findings indicate that increasing salinity compromises both germination and early seedling growth while revealing interspecific differences in resilience. The stronger performance of Balansa clover supports its suitability for saline-prone environments, whereas the sensitivity of Alsike clover suggests that successful establishment may require avoidance of saline conditions or management practices that minimize early-stage salinity exposure.

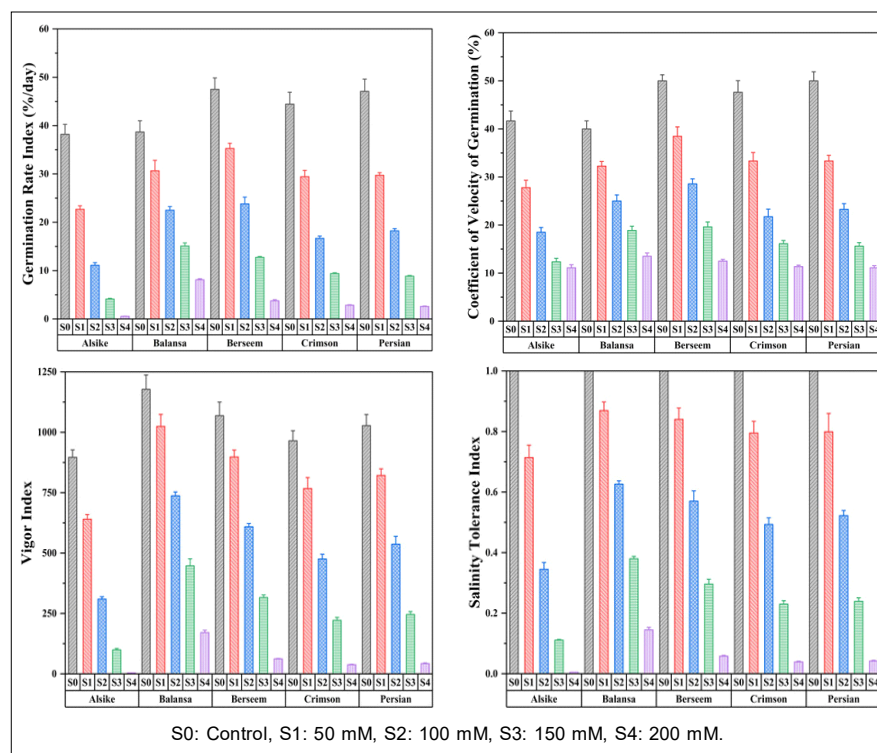


Fig 2: Interaction effects of salinity levels and clover species on germination and early seedling traits.

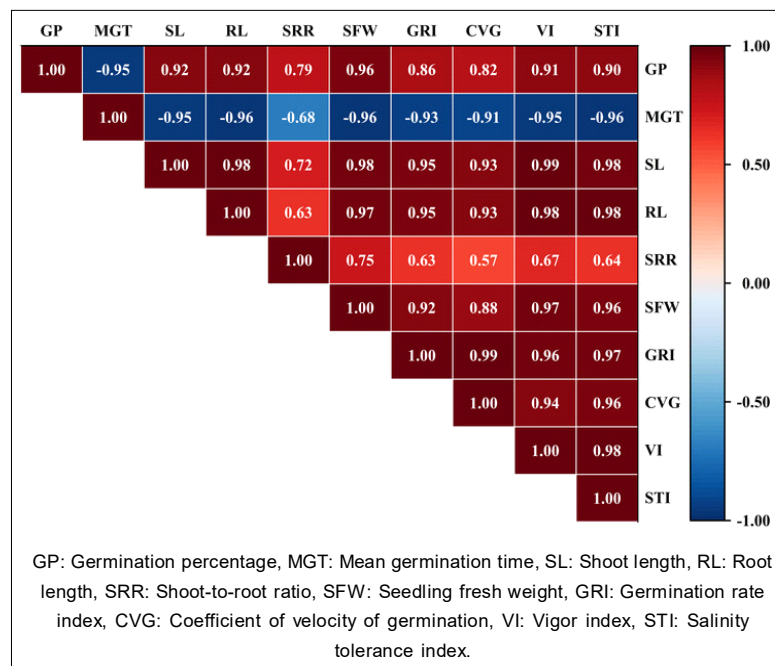


Fig 3: The correlation coefficient of the studied parameters.

Pearson correlation analysis revealed strong relationships among germination, seedling growth and salinity tolerance indices (Fig 3). Germination percentage (GP) showed a very strong negative correlation with mean germination time (MGT; $r = -0.95$), indicating that higher germination success was associated with faster germination. GP also exhibited strong positive correlations with shoot length (SL; $r = 0.92$), root length (RL; $r = 0.92$), seedling fresh weight (SFW; $r = 0.96$), vigor index (VI; $r = 0.91$) and salinity tolerance index (STI; $r = 0.90$), suggesting that improved germination translated into enhanced seedling development and tolerance.

MGT was strongly negatively correlated with growth and vigor parameters, including SL ($r = -0.95$), RL ($r = -0.96$), SFW ($r = -0.96$), germination rate index (GRI; $r = -0.93$), coefficient of velocity of germination (CVG; $r = -0.91$), VI ($r = -0.95$) and STI ($r = -0.96$), highlighting the importance of rapid germination for seedling establishment under salinity.

SL and RL were strongly correlated ($r = 0.98$) and positively associated with SFW ($r = 0.98$ and 0.97), GRI ($r = 0.95$), CVG ($r = 0.93$), VI ($r = 0.99$ and 0.98) and STI ($r = 0.98$), reflecting coordinated seedling growth and vigor. The shoot-to-root ratio (SRR) showed moderate positive correlations with SFW ($r = 0.75$), GRI ($r = 0.63$), VI ($r = 0.67$) and STI ($r = 0.64$), indicating a weaker association with overall tolerance.

GRI and CVG were highly correlated ($r = 0.99$) and strongly associated with VI ($r = 0.96$ and 0.94) and STI ($r = 0.97$ and 0.96), supporting their reliability as indicators of seed performance under stress. Overall, the correlation structure indicates that rapid and uniform germination is closely linked to improved seedling growth, vigor and

salinity tolerance, suggesting that traits such as MGT, GRI, CVG and VI may serve as effective selection criteria for identifying salt-tolerant clover species or genotypes.

CONCLUSION

This study demonstrated that Balansa clover exhibited the most robust salinity tolerance among the five clover species, maintaining higher germination capacity, seedling growth and vigor-related indices under increasing salinity. Berseem, Persian and Crimson clovers showed moderate tolerance, characterized by intermediate values across germination and seedling traits and a progressive decline as salinity intensified. In contrast, Alsike clover was the most salt-sensitive species, displaying the lowest performance for most traits and the longest mean germination time, indicating vulnerability during early establishment. The significant species $\times$ salinity interaction for most traits further confirmed that responses were trait-dependent, highlighting the importance of evaluating multiple metrics to obtain a reliable ranking of salinity tolerance in forage clovers.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

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