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Interactive Effects of the Temperature and Salinity on Germination of Alfalfa (*Medicago sativa* L.)

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ABSTRACT

Background: Temperature and salinity are crucial environmental factors that significantly affect seed germination. The current study aimed to determine optimal temperature (18°C, 25°C and 32°C) and seed germination traits of alfalfa genotypes (genotype 3, genotype 9, genotype 16) and CUF-101 (control variety) for comparison purposes at different salinity levels.

Methods: Germination energy (GE), germination percentage (GP), germination index (GI), mean germination days (MGD), root length (RL), shoot length (SL), fresh weight (FW), dry weight (DW), seedling vigour (SV) were analyzed using JMP v17.0.

Result: The seeds of CUF-101 were greater for all germination traits than those of the other alfalfa genotypes, excluding MGD. The results showed that the highest germination traits were achieved at 25°C (T_{25}) for all treatments, which was determined to be the optimal temperature. All germination traits of both CUF-101 and alfalfa genotypes decreased drastically due to the interactive effects of the highest temperature and increasing salt levels. Genotype 16 was found to be the alfalfa genotype closest to CUF-101, while genotype 9 was indicated the least similar genotype to CUF-101.

Key words: Abiotic stress, $CaCl_2$, NaCl, Salinity, Synthetic variety, Temperature.

INTRODUCTION

Salinization is one of the most harmful abiotic stressors affecting plant growth and agricultural efficiency worldwide (Malik *et al.,* 2022). This phenomenon poses a considerable threat to agricultural sustainability as it causes damage to plants, reduces seedling growth, impedes root and shoot length and decreases establishment success (Shiade and Boelt, 2020). Salinization is the primary cause of soil degradation and its lethal effects on ecological restoration (Zhang *et al.,* 2015). Approximately 20% of irrigated land in arid and semi-arid regions is salt-affected, posing a significant challenge to agricultural management (Hutchinson *et al.,* 2018). It has been estimated that over 50% of arable land will be salinized by 2050, highlighting the urgent need for effective mitigation and remediation strategies.

Successful seed germination and strong root system is essential for the establishment of healthy plants and can significantly affect crop yield and quality (Hubbard *et al.,* 2012; Gao *et al.,* 2023; Wang *et al.,* 2024). Several factors can affect seed germination, including environmental conditions such as temperature, moisture, light, water and soil salinity (Weber, 2009; Farooq *et al.,* 2021). The effect of salinity on plants depends on the number and total amount of salts [sodium chloride (NaCl), sodium sulfate (Na_2SO_4), calcium chloride ($CaCl_2$), calcium sulfate ($CaSO_4$) and potassium chloride (KCl)] in the soil and irrigation water (Wali *et al.,* 2021). Excessive concentrations of these soluble salts in the root zone can pose a significant threat to physiological and metabolic disorders during plant growth and development (Szabolcs, 1989). Alfalfa is a moderately salt-tolerant legume and its seed germination is highly sensitive to salinization (Diaz *et al.,* 2018), which has been widely studied, utilizing NaCl to induce salinity stress. Limited research has been conducted on the effects of other types of salts on alfalfa (Bhattarai *et al.,* 2020).

One of the soluble salts, NaCl, is the predominant salt type that can accumulate in soils through irrigation with saline water. High concentrations of NaCl can lead to the displacement of essential plant nutrients, such as potassium and calcium and negatively affect plant growth and yield. Calcium (Ca) is the primary regulator of plant metabolism and development and is used in agriculture for several purposes, such as improving soil structure, reducing soil alkalinity and providing calcium and chloride ions as essential plant nutrients (Wang *et al.,* 2022). However, excessive application of $CaCl_2$ can have negative effects on the seed germination of forage crops, including reduced germination rates, root and shoot lengths, decreased seedling vigour and plant injury (Zhang *et al.,* 2019). Alfalfa seed germination exhibits higher sensitivity to $CaCl_2$ and NaCl salts and greater tolerance to KCl (Azhdari *et al.,* 2010).

Salinity and temperature are important environmental factors that significantly affect seed germination. When these factors were combined at higher levels in the natural

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habitat, all stages of growth and development, including seed germination, seedling growth, chlorophyll content and proline accumulation, were affected negatively (Ashraf and Harris, 2013; Durr *et al.,* 2015; Bidgoly *et al.,* 2018; Prerna *et al.,* 2021; Khaeim *et al.,* 2022). It is widely known that extreme warming may become a common threat in the future, negatively affecting seed germination and influencing the adaptability and germination of forage legumes (Brar *et al.,* 1991). Therefore, it is essential to investigate the interactive effects of salinity and temperature on seed germination to understand how plants respond to environmental stressors.

Adequate information is not available to elucidate the interactive effect of temperature and soluble salts (NaCl, $CaCl_2$ and KCl) on the early stage of germination traits on alfalfa. This knowledge will provide an important framework for restoring salinity inflicted through the cultivation of alfalfa and its breeding prospects. The current study aimed to determine; (1) suitable temperatures for optimal seed germination compared to CUF-101 (control variety), (2) the best genotype with equivalent or higher germination traits to CUF-101.

MATERIALS AND METHODS

The cv. "CUF-101", a non-dormant alfalfa variety, was used as a control and the non-dormant genotypes 'Genotype 3', 'Genotype 9' and 'Genotype 16' were used as experimental genotypes (n=4) for comparison. CUF-101 was selected for its non-dormant growth characteristics (9-dormancy level), similar to the alfalfa genotypes in the current study and is classified by the Certified Alfalfa Seed Council as the top 11 alfalfa in the Fall Dormancy Class (FDC). The alfalfa genotypes were synthetic genotypes obtained from 192 clones from Peru and cultivated in the Urfa Province of Türkiye. They were selected based on dormancy level, seedling growth, seed yield, cutting yield and mature plant growth.

Temperature and salinity treatments

A laboratory experiment was conducted over a gradient of three stable temperatures (18°C, 25°C, 32°C) (n=3) under six salinity levels (100 and 200 mM NaCl, $CaCl_2$ and KCl for each) (n=6). Temperatures were provided using an adjustable incubator at the Field Crops Department Labs, Faculty of Agriculture, Ankara University. CUF-101 was used as a control variety to compare the other three alfalfa genotypes under different salt types, salinity levels and temperatures. Temperatures (18°C, 25°C, 32°C) and salt-treated groups [(100 mM NaCl (156.25 µS/cm), 100 mM $CaCl_2$ (181.81 µS/cm), 100 mM KCl (142.85 µS/cm), 200 mM NaCl (312.50 µS/cm), 200 mM $CaCl_2$ (363.62 µS/cm), 200 mM KCl (285.70 µS/cm)] were labelled and mentioned as T_{18}, T_{25}, T_{32} and S_1, S_2, S_3, S_4, S_5, S_6, in the same order.

Germination tests

The germination test was performed according to rules, with 50 seeds per plot of CUF-101 and alfalfa genotypes for each treatment (ISTA, 2017). Fifty seeds were placed on three-layers of filter paper and irrigated with 5 mL of the respective solutions for each filter paper. After rolling the filter papers with the seeds, they were placed in sealed plastic bags to prevent moisture loss. Each rolled paper was replaced every 2 days after incubation to avoid salt accumulation. Seeds were germinated at three different temperatures with a 12-h light photoperiod (cool white fluorescent lamps, 200 µmol m^{-2} s^{-1}, 400-700 nm) to screen and evaluate the effects of the soluble salts (NaCl, $CaCl_2$, KCl) (Merck, Germany) to determine the optimal temperatures for seed germination of CUF-101 (control variety) and alfalfa genotypes. Seeds were considered germinated with the emergence of roots (≥ 2 mm) (Bhattarai *et al.,* 2020). Germination energy (GE), germination percentage (GP), germination index (GI), mean germination days (MGD), root length (RL), shoot length (SL), fresh weight (FW), dry weight (DW) and seedling vigour (SV) were calculated using formulas (Maguire, 1962; Abdulbaki and Anderson, 1973).

$$\text{Germination Index (GI)} = \frac{\sum \text{(Number of germinated seeds)}}{\text{Day of count}}$$

$$\text{Mean germination day (MGD)} = \sum (Dn) / \sum (n)$$

Where,

n= Number of seeds germinated on day D.

D= Number of days from the beginning of the germination test.

$$\text{Seedling vigour (SV)} = \frac{\text{Germination percentage} \times \text{Seedling length}}{100}$$

Germination energy and germination percentage were recorded on the 7th and 10th days, as described by the International Seed Testing Association (ISTA, 2017). Seedling vigor was measured in 10th day. Root length (cm), shoot length (cm), fresh weight (g) and dry weight (g) were measured on the 10th day by randomly selecting 20 seeds per replication. Remains of the alfalfa seeds were included in fresh weight and dry weight. The fresh parts of the alfalfa seeds were dried for 72 hours at 60°C to calculate their dry weights.

Statistical analysis

For all variables, JMP v17.0 was used to analyze the variance in the current study (SAS, 2017). The balanced completely randomized design (CRD) in factorial scheme was conducted using with three replications. The treatments were three stable temperatures [18°C (T_{18}), 25°C (T_{25}), 32°C (T_{32})] (n=3), six salinity levels from three types of salts with two levels [100 mM NaCl (S_1), 100 mM $CaCl_2$ (S_2), 100 mM KCl (S_3), 200 mM NaCl (S_4), 200 mM $CaCl_2$ (S_5) and 200 mM KCl (S_6)] (n=6) and four alfalfa plants (a control variety and three genotypes) (n=4). The Shapiro-wilk and Levene's tests were used to check the normal distribution of the variables and the ANOVA assumptions for equality of the

error variances and residual normality, respectively. A three-way ANOVA ($p \leq 0.05$) was then performed using the F-test to distinguish the variance among CUF-101 and alfalfa genotypes. The differences among means were assessed utilizing Duncan's multiple range test. Principal component analysis (PCA) was performed using JMP v17.0 to determine the multivariate ordination of nine germination traits of CUF-101 and three alfalfa genotypes, salt levels and three stable temperatures.

RESULTS AND DISCUSSION

Germination energy and germination percentage

The results showed that germination energy was significantly affected by genotype (F=57.65; df=3; p<0.001), temperature (F=791.64; df=2; p<0.001), salinity level (F=81.06; df=5; p<0.001) and interaction of temperature × genotype (F=19.91; df=6; p<0.001), temperature × salinity level (F=7.51; df=10; p<0.001), genotype × salinity level (F=9.40; df=15; p<0.001), temperature × genotype × salinity level (F=1.95; df=30; p=0.0058).

The highest germination energy of CUF-101 and alfalfa genotypes was observed at 25°C (T_{25}), while the lowest germination energy was observed at 32°C (T_{32}). CUF-101 had the highest germination energy (85.61%) followed by genotype 16 (71.39%) (Table 1). The highest germination energy was also obtained at T_{25} and 100 mM of NaCl (95.00%) and KCl (91.67%) in CUF-101, followed by genotype 16. The germination energy of genotype 16 under 100 mM of $CaCl_2$ at T_{25} (91.33%) was higher than CUF-101 (87.67%) (Table 2). The lowest germination energy was recorded at T_{32}. Genotype 16 had the lowest germination energy, with 200 mM NaCl and $CaCl_2$ (9.33%). T_{25} had a positive effect on germination energy for CUF-101 and alfalfa genotypes, although T_{32} had a lethal effect on germination energy. According to the temperature, the germination energy of CUF-101 and alfalfa genotypes was lined T_{25}, T_{18} and T_{32} (Fig 1).

The ANOVA showed that germination percentage was significantly affected by genotype (F=70.09; df=3; p<0.001), temperature (F=1733.83; df=2; p<0.001), salinity level (F=92.41; df=5; p<0.001) and interaction of temperature × genotype (F=31.48; df=6; p<0.001), temperature × salinity level (F=9.13; df=10; p<0.001), genotype × salinity level (F=8.30; df=15; p<0.001) and temperature × genotype × salinity level (F=2.58; df=30; p=0.001).

The germination percentage of the alfalfa plants was the highest at T_{25} with 91.06% in CUF-101, followed by genotype 16 with 78.11%. Similar to the germination energy, T_{32} had a negative effect on germination percentage, with the lowest germination percentage values (Table 1). The highest germination percentage was obtained at T_{25} and 100 mM of KCl (97.33%) and NaCl (96.33%) in CUF-101, followed by genotype 16. The highest germination per centage at T_{25} under 100 mM $CaCl_2$ was recorded in genotype 16 (95.33%) (Table 2). The lowest germination percentage was observed at T_{32}, similar to the germination energy. Genotype 16 had the lowest germination percentage, with 200 mM NaCl (12.00%) and $CaCl_2$ (12.66%). In particular, the germination percentage followed a decreasing trend from T_{25} to T_{32}, due to the interactive effects of temperature and increasing salt levels ($T_{25} > T_{18} > T_{32}$) (Fig 1).

Germination indexes and mean germination days

The ANOVA showed that germination index was significantly affected by genotype (F=100.66; df=3; p<0.001), temperature (F=833.36; df=3; p<0.001), salinity level (F=259.47; df=5; p<0.001) and interaction of temperature × genotype

Table 1: Temperature × genotype interaction mean values.

	GE (%)	GP (%)	GI (%)	MGD (days)	RL (cm)	SL (cm)	FW (g)	DW (g)	SV
					(T_{18})				
CUF-101	69.44^{b}	75.00^{bc}	58.11^{bc}	7.50^{bc}	1.96^{b}	2.40^{bc}	0.222^{cd}	0.165^{a}	337.16^{b}
G-3	65.28^{bc}	71.44^{cd}	51.45^{c}	7.14^{bc}	1.79^{bc}	2.07^{d}	0.197^{d}	0.074^{b}	276.59^{c}
G-9	59.17^{cd}	68.67^{d}	43.51^{d}	6.87^{c}	1.36^{d}	1.93^{d}	0.166^{e}	0.066^{b}	229.30^{d}
G-16	66.67^{bc}	72.39^{bcd}	54.47^{bc}	7.24^{bc}	1.80^{bc}	2.17^{cd}	0.200^{d}	0.091^{ab}	293.99^{c}
					(T_{25})				
CUF-101	85.61^{a}	91.06^{a}	73.49^{a}	3.47^{e}	2.36^{a}	2.91^{a}	0.340^{a}	0.115^{ab}	488.37^{a}
G-3	70.11^{b}	76.44^{bc}	58.60^{b}	3.71^{e}	1.84^{bc}	2.38^{bc}	0.232^{c}	0.066^{b}	339.84^{b}
G-9	53.00^{d}	58.9^{4e}	37.72^{d}	3.88^{e}	1.65^{c}	2.14^{cd}	0.208^{cd}	0.058^{b}	228.58^{d}
G-16	71.39^{b}	78.11^{b}	60.89^{b}	3.21^{e}	1.89^{bc}	2.47^{b}	0.271^{b}	0.069^{b}	352.27^{b}
					(T_{32})				
CUF-101	30.72^{e}	34.67^{fg}	26.42^{e}	9.11^{a}	0.79^{e}	1.23^{e}	0.204^{d}	0.074^{b}	74.13^{ef}
G-3	32.89^{e}	37.11^{fg}	24.74^{e}	7.61^{b}	0.97^{e}	1.12^{ef}	0.145^{e}	0.048^{b}	93.20^{ef}
G-9	28.33^{e}	32.11^{g}	23.83^{e}	5.89^{d}	0.76^{e}	0.93^{f}	0.143^{e}	0.046^{b}	59.49^{f}
G-16	34.44$_{e}$	38.88^{f}	26.56^{e}	7.64^{b}	1.04^{e}	1.15e^{f}	0.154^{e}	0.094^{ab}	99.9^{e}

GE: Germination energy, GP: Germination percentage, GI: Germination index, MGD: Mean germination days, RL: Root length, SL: Shoot length, FW: Fresh weight, DW: Dry weight, SV: Seedling vigour, G-3: Genotype 3, G-9: Genotype 9, G-16: Genotype 16. All values shown by different letters in a row are significantly different.

Table 2: Comparison of CUF-101 (Control variety) and genotype 16 under two stable temperatures and salinity levels.

Temperature	25°C (T_{25})						32°C (T_{32})					
Salinity	S_1	S_2	S_3	S_4	S_5	S_6	S_1	S_2	S_3	S_4	S_5	S_6
Germination energy												
CUF-101	95.00^{a}	87.67^{a}	91.67^{a}	80.00^{a}	79.00^{a}	80.33^{a}	40.00^{c-f}	33.00^{def}	34.67^{def}	29.67^{ef}	23.00^{fg}	24.00^{fg}
G-16	90.00^{a}	91.33^{a}	86.67^{a}	44.00^{b-e}	58.67^{b}	57.67^{bc}	50.67^{bcd}	54.67^{bc}	47.33^{b-e}	9.33^{g}	9.33^{g}	35.33^{def}
Germination percentage												
CUF-101	96.33^{ab}	95.00^{ab}	97.33^{a}	86.00^{ab}	83.67^{ab}	88.00^{ab}	41.33^{e-h}	38.67^{fgh}	41.33^{e-h}	30.00^{ghi}	27.33^{hi}	29.33^{ghi}
G-16	92.00^{ab}	95.33^{ab}	92.67^{ab}	46.00^{d-g}	64.67^{cd}	78.00^{bc}	52.67^{def}	58.00^{de}	51.33^{def}	12.00^{i}	12.67^{i}	46.67^{efg}
Germination index												
CUF-101	84.84^{ab}	81.68^{abc}	94.22^{a}	54.60^{de}	58.69^{de}	66.89^{cd}	33.26^{g}	30.34^{g}	52.63^{ef}	5.99^{h}	7.41^{h}	28.90^{g}
G-16	77.34^{bc}	88.67^{ab}	78.59^{bc}	25.76^{g}	36.39^{g}	58.61^{de}	34.16^{g}	39.08^{fg}	36.56^{g}	5.16^{h}	6.31^{h}	27.15^{g}
Mean germination days												
CUF-101	3.00^{fgh}	2.93^{fgh}	2.73^{gh}	3.87^{efg}	4.13^{efg}	4.13^{efg}	8.60^{ab}	9.63^{a}	8.37^{ab}	9.50^{a}	8.80^{ab}	9.73^{a}
G-16	1.20^{h}	4.67^{d-g}	1.26h	5.80^{cde}	5.26^{def}	5.13^{def}	4.60^{d-g}	9.20^{a-e}	6.47^{bcd}	9.53^{a}	7.80^{abc}	8.07^{abc}
Root length												
CUF-101	3.39^{a}	2.18^{de}	3.21^{ab}	2.10^{e}	1.12^{ijk}	2.14^{de}	1.39^{f-i}	0.40^{lm}	1.21^{g-j}	0.29^{lm}	0.47^{lm}	1.00^{ijk}
G-16	2.76^{bc}	1.68^{e-h}	2.64^{cd}	1.50^{f-i}	0.61^{kl}	1.87^{ef}	1.49^{f-i}	0.76^{jkl}	1.71^{efg}	0.66^{kl}	-	1.21^{hij}
Shoot length												
CUF-101	3.40^{bc}	3.12^{cd}	4.37^{a}	1.95^{gh}	1.43^{ijk}	3.18^{cd}	1.40^{i-l}	1.12^{j-m}	2.37^{fg}	0.33^{no}	0.82^{m}	1.36^{i-l}
G-16	2.90^{cde}	2.54^{ef}	3.85^{ab}	1.32^{j-m}	0.91^{klm}	2.75^{def}	1.69^{hij}	1.09^{klm}	1.89^{ghi}	0.83^{lmn}	-	1.41^{ijk}
Fresh weight												
CUF-101	0.392^{ab}	0.347^{b}	0.429^{a}	0.283^{b}	0.235^{cd}	0.355^{b}	0.206^{de}	0.169^{ef}	0.376^{ab}	0.140^{fg}	0.121^{fg}	0.215^{de}
G-16	0.253^{cd}	0.240^{cd}	0.247^{cd}	0.163^{efg}	0.127^{fg}	0.172^{ef}	0.203^{de}	0.160^{efg}	0.170^{ef}	0.137^{fg}	0.111^{fg}	0.147^{fg}
Dry weight												
CUF-101	0.147	0.109	0.135	0.105	0.074	0.121	0.079	0.066	0.135	0.045	0.042	0.077
G-16	0.090	0.082	0.083	0.053	0.048	0.063	0.076	0.059	0.063	0.086	0.034	0.053
Seed vigor												
CUF-101	656.13	502.23	737.36	351.45	214.72	468.34	117.24	58.33	148.26	17.15	34.79	69.01
G-16	520.21	403.41	601.14	128.56	100.27	360.01	167.66	106.97	184.83	17.73	-	122.22

GE: Germination energy, GP: Germination percentage, GI: Germination index, MGD: Mean germination days, RL: Root length, SL: Shoot length, FW: Fresh weight, DW: Dry weight, SV: Seedling vigour, T_{25}: 25 C°, T_{32}: 32 C°, S_1: 100 mM NaCl, S_2: 100 mM $CaCl_2$, S_3: 100 mM KCl, S_4: 200 mM NaCl, S_5: 200 mM $CaCl_2$, S_6: 200 mM KCl, G-16: Genotype 16. All values shown by different letters in a column are significantly different.

(F=30.66; df=6; p<0.001), temperature × salinity level (F=8.14; df=10; p<0.001), genotype × salinity (F=8.93; df=15; p<0.001) and temperature × genotype × salinity level (F=2.87; df=30; p<0.001).

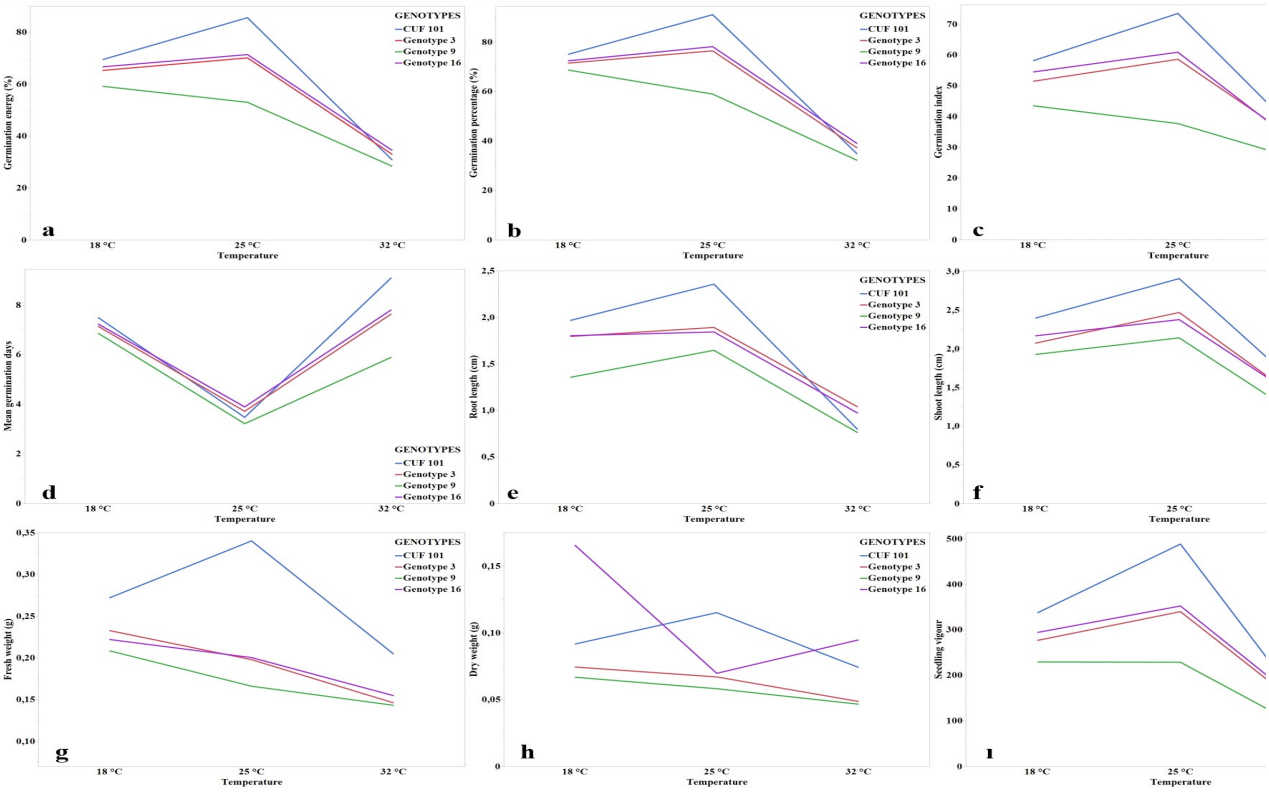
The germination index was the highest at T_{25} in CUF-101 (73.49%), followed by genotype 16 (60.89%) (Table 1). The genotype 16 had a higher germination index at T_{32}, compared to CUF-101, similar to germination energy and germination percentage. The negative effect of T_{32}, on the germination index was significantly higher compared to T_{18} and T_{25} (Fig 1). The highest germination index was obtained at T_{25} and 100 mM of KCl in CUF-101 (94.22%), followed by genotype 16 under 100 mM of $CaCl_2$ (88.67%) (Table 2). The lowest germination index was found at T_{32}, similar to the germination energy and germination percentage. CUF-101 and genotype 16 had the lowest germination index values with 200 mM NaCl (5.16% and 5.99%), respectively. KCl had the highest germination index at all three stable temperatures (Fig 4). The most lethal effect of salt type on the germination index was observed with NaCl, followed by $CaCl_2$ and KCl in the same order.

The ANOVA showed that mean germination days were significantly affected by genotype (F=56.64; df=3; p<0.001), temperature (F=933.83; df=2; p<0.001), salinity level (F=76.91; df=5; p<0.001) and interaction of temperature × genotype (F=26.18; df=18; p<0.001), temperature × salinity level (F=7.86; df=10; p<0.001), genotype × salinity (F=6.95; df=15; p<0.001) and temperature × genotype × salinity level (F=2.18; df=30; p=0.0016).

T_{25} gave the earliest mean germination days compared to T_{18} and T_{32} (T_{25} > T_{18} > T_{32}) (Fig 1). CUF-101 and genotype 16 were the two earliest alfalfa plants at T_{25} (3.47 and 3.21 days), respectively (Table 1). Genotype 16 had the earliest mean germination days under 100 mM of NaCl (1.20 days) and KCl (1.26 days) at T_{25}, which were earlier than CUF-101. The latest mean germination days were obtained at T_{32} and 200 mM of KCl in CUF-101 (9.73 days), followed by genotype 16 under 200 mM $CaCl_2$ (9.53 days) (Table 2).

Root length and shoot length

The ANOVA showed that root length was significantly affected by genotype (F=36.68; df=3; p<0.001), temperature (F=662.33; df=2; p<0.001), salinity level (F=359.27; df=5; p<0.001) and interaction of temperature × genotype (F=11.60; df=6; p<0.001), temperature × salinity level (F=11.44; df=10; p<0.001), genotype × salinity level (F=4.23; df=15; p<0.001), except temperature × genotype × salinity level (F=2.49; df=30; p=0.0003) (p≤0.01).



Note: a: Germination energy, b: Germination percentage, c: Germination index, d: Mean germination days, e: Root length, f: Shoot length, g: Fresh weight, h: Dry weight, ı: Seedling vigour, Blue line: CUF-101, Red line: Genotype 3, Green line: Genotype 9, Purple line: Genotype 16.

Fig 1: The effect of temperature × genotype interactions to the germination traits.

The root length of CUF-101 at T_{25} (2.36 cm) and T_{18} (1.96 cm) was higher compared to T_{32} (0.79 cm). Genotype 16 was the closest genotype to CUF-101 in terms of root length at T_{25} (1.89 cm) and T_{18} (1.80 cm) (Table 1). The highest root length was obtained at T_{25} and 100 mM of NaCl (3.39 cm) and KCl (3.21 cm) in CUF-101, followed by genotype 3. The root length of genotype 3 was recorded at T_{18} and 100 mM NaCl (3.33 cm). Genotype 3, genotype 9 and genotype 16 did not survive the interactive effects of T_{32} and 200 mM $CaCl_2$. These genotypes germinated but were not sufficiently developed to progress the next stage (vegetative stage) of the plant life cycle, except for CUF-101. Both temperature from T_{25} to T_{32} and increasing salinity levels' resulted in reduced root length. $CaCl_2$ was the salt type that had the greatest negative effect on root length, followed by NaCl and KCl, especially at T_{32}.

The ANOVA showed that shoot length was significantly affected by genotype ($F=42.27$; $df=3$; $p<0.001$), temperature ($F=1961.38$; $df=3$; $p<0.001$), salinity level ($F=531.40$; $df=5$; $p<0.001$) and interaction of temperature × salinity level ($F=21.08$; $df=10$; $p<0.001$), genotype × salinity level ($F=11.26$; $df=15$; $p<0.001$), temperature × genotype × salinity level ($F=4.17$; $df=30$; $p<0.001$), except temperature × genotype ($F=4.06$; $df=6$; $p=0.0095$) ($p\leq0.01$).

The highest shoot length of CUF-101 and alfalfa genotypes was obtained at T_{25}, followed by T_{18} and T_{32} (Fig 1). The highest shoot lengths at T_{25} were found for CUF-101 (2.91 cm) and genotype 16 (2.47 cm), (Table 1). CUF-101 (4.37 cm) and genotype 16 (3.85 cm) had the highest shoot lengths under the interactive effects of 100 mM KCl at T_{25} and T_{18}, respectively (Table 2). Genotype 3, genotype 9 and genotype 16 did not survive at the T_{32} under 200 mM $CaCl_2$.

Fresh weight, dry weight and seed vigor

The ANOVA showed that fresh weight was significantly affected by genotype ($F=274.97$; $df=3$; $p<0.001$), temperature ($F=235.63$; $df=2$; $p<0.001$), salinity level ($F=258.91$; $df=5$; $p<0.001$) and interaction of temperature × genotype ($F=40.40$; $df=6$; $p<0.001$), genotype × salinity level ($F=14.03$; $df=15$; $p<0.001$), temperature × genotype × salinity level ($F=7.31$; $df=30$; $p<0.001$), except temperature× salinity level ($F=3.67$; $df=10$; $p=0.0003$) ($p\leq0.01$).

The temperature effects on the fresh weight were T_{25} > T_{18} > T_{32} (Fig 1). CUF-101 had the heaviest fresh weight (0.340 g), followed by genotype 16 (0.271 g) under all three stable temperatures (Table 1). The fresh weight was also the heaviest for CUF-101 under the interactive effects of T_{25} and 100 mM KCl (0.429 g) and NaCl (0.392 g). The lightest fresh weight was obtained at T_{32} with 200 mM $CaCl_2$ for genotype 9 (0.087 g). This was followed by genotype 3 at T_{32} with 200 mM $CaCl_2$ (0.092 g). Compared to the different salt types in comparison to each other, CUF-101 and alfalfa genotypes were more resistant to KCl, although they were more sensitive to $CaCl_2$ (Fig 4).

The ANOVA showed that dry weight was significantly affected by genotype ($F=3.70$; $df=3$; $p=0.001$), salinity level ($F=2.91$; $df=5$; $p=0.0162$), temperatures × genotype ($F=3.47$; $df=6$; $p=0.0187$) ($p\leq0.05$), except temperature ($F=4.11$; $df=2$; $p=0.106$), temperature × salinity level ($F=1.07$; $df=10$; $p=0.387$), genotype × salinity level ($F=0.72$; $df=15$; $p=0.757$) and temperatures × genotype × salinity level ($F=1.27$; $df=30$; $p=0.182$).

The dry weight of CUF-101 and alfalfa genotypes showed variability (0.057-0.147 g) (Table 1). CUF-101 had the heaviest dry weight, followed by genotypes 16, 3 and 9. The effect of the salinity levels on dry weight ranged (0.043-0.114 g). The heaviest dry weight was obtained with 100 mM NaCl, whereas the lightest dry weight was obtained with 200 mM $CaCl_2$ (Table 2). The most damaging salt type for dry weight was $CaCl_2$ (0.057 g), followed by KCl (0.087 g) and NaCl (0.097 g).

The ANOVA showed that seedling vigour was significantly affected by genotype ($F=189.77$; $df=3$; $p<0.001$), temperature ($F=981.83$; $df=3$; $p<0.001$), salinity level ($F=371.21$; $df=5$; $p<0.001$) and interaction of temperature × genotype ($F=62.44$; $df=6$; $p<0.001$), temperature × salinity level ($F=32.12$; $df=10$; $p<0.001$), genotype × salinity level ($F=9.67$; $df=15$; $p<0.001$), temperature × genotype × salinity level ($F=3.05$; $df=30$; $p<0.001$).

The maximum seedling vigour values were obtained at T_{25}, followed by T_{18} and T_{32}. The maximum seedling vigour was observed for CUF-101 at T_{25} with 100 mM KCl (737.36) and NaCl (656.13) (Table 2). Genotype 16 was incubated at the same temperature with 100 mM KCl (601.14). Except for CUF-101, genotypes 3, 9 and 16 did not survive the interactive effects at T_{32} and 200 mM $CaCl_2$. The lowest seedling vigour was observed in CUF-101 (17.15), genotype 16 (17.73) and genotype 9 (26.54) at T_{32} with 200 mM NaCl, in the same order.

Principal component analysis (PCA) of CUF-101 and alfalfa genotypes

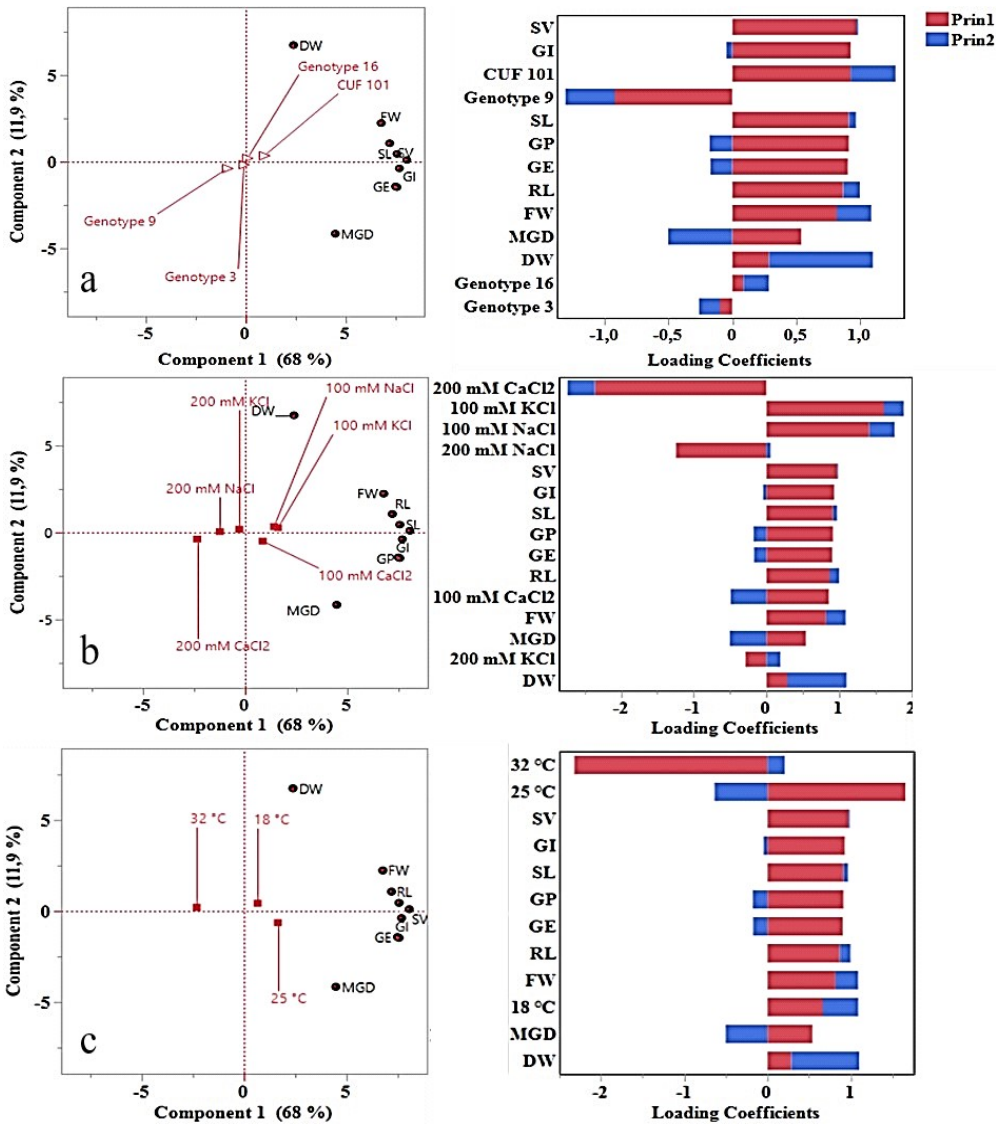
PCA revealed a high level of variation in the genotypes. The variation examined by PCA showed that two principal components with more than two eigenvalues accounted for 79.90% of the total variance among the nine germination traits, with germination energy (0.366) and germination percentage (0.369) being the main contributors (Fig 2). All PC1 contributors had positive associations, whereas PC2 contributors had negative associations. CUF-101 and genotype 16 are positively associated with components 1, 2 and the loading matrix (Fig 2). PCA plots about temperatures and salinity levels showed that all salt types with 100 mM and T_{25} were optimal conditions that had positive associations with the PCA plots (Fig 2).

Pearson's correlation coefficients of the quantitative germination traits were tested to determine the relationship between germination percentage and other germination traits (Fig 3). Germination percentage had very strong and positive relationship with germination energy (+0.98) and germination index (+0.89). Secondly; shoot length (+0.72), root length (+0.66) and fresh weight (+0.64) may be

considered to have strong relationship with germination percentage (Fig 3).

The current study aimed to investigate the interactive effects of temperature and different salt levels on CUF-101 and alfalfa genotypes during the germination phase. Temperature and salinity have a statistically significant effect on seed germination (Weber, 2009; Farooq *et al.,* 2021). The combination between them can be complex, with different temperature and salinity levels leading to various effects on seed germination and seedling growth (Munns and Tester, 2008).

The interactive effects of increasing temperature and salinity progressively reduced germination energy and percentage in CUF-101 and the three alfalfa genotypes. Li *et al.* (2010) and Soltani *et al.* (2012) emphasized that salinization reduces the germination percentage of alfalfa. Malik *et al.* (2022) also stated that increasing temperature and salinity levels significantly reduced the germination energy and germination percentage at 30-35°C in their study, which had a similar temperature (T_{32}) with the current study. When combined with high temperatures, excessive concentrations of salt ions can impede water uptake by



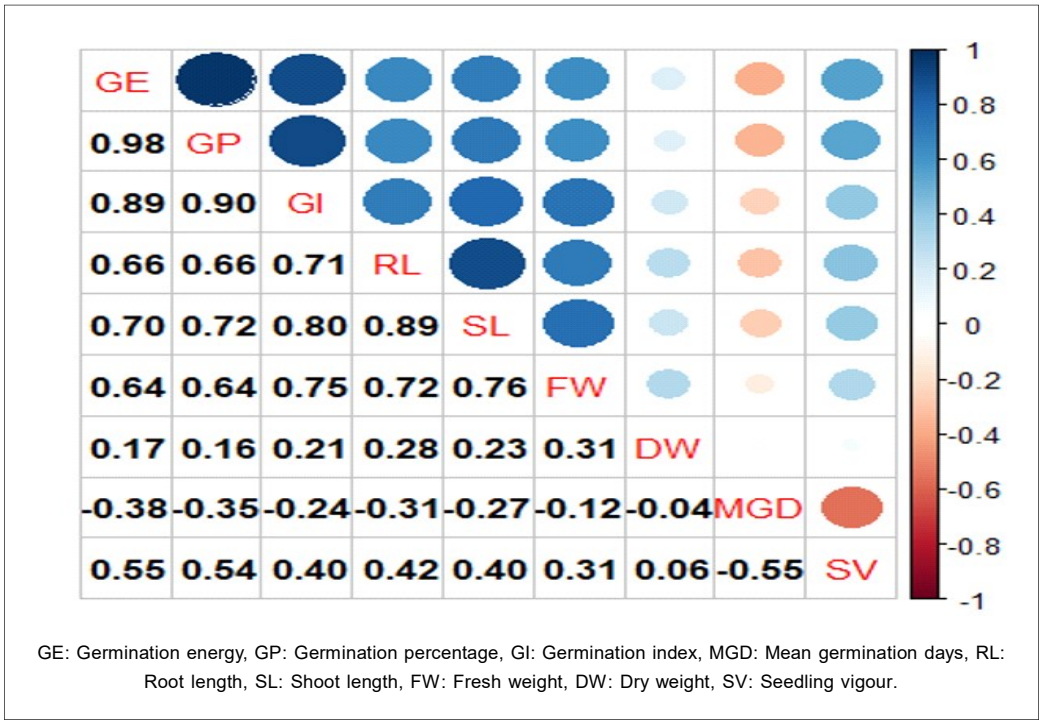
a: CUF-101 and genotypes, b: Salinity levels, c: Temperatures. Note: GE: Germination energy, GP: Germination percentage, GI: Germination index, MGD: Mean germination days, RL: Root length, SL: Shoot length, FW: Fresh weight, DW: Dry weight, SV: Seedling vigour.

Fig 2: Biplot analysis of CUF-101 and alfalfa genotypes and their loading coefficients.

germinating seeds (Mangwane *et al.,* 2021). The results indicated that the optimal temperature (T_{25}) to the highest temperature (T_{32}) and higher salinity delayed germination energy and percentage very sharply, which was in agreement with Sharavdorj *et al.* (2021) (Fig 4). Genotype 16 was the alfalfa genotype, which had similar features to CUF-101 in terms of germination energy and percentage. In addition, genotype 16 was more resistant to the interactive effects of T_{32} and had lower salinity levels than CUF-101. Alfalfa genotypes showed a greater alleviating effect on germination percentage at T_{25} and T_{32} under 100 mM $CaCl_2$ compared to 100 mM NaCl and KCl. At the higher salinity level of the salts (200 mM) on CUF-101 and alfalfa genotypes, KCl had the highest germination percentage over NaCl and $CaCl_2$. Steppuhn *et al.* (2012) determined that $CaCl_2$, NaCl and their mixture reduced the alfalfa emergence (3.00-30.00%).

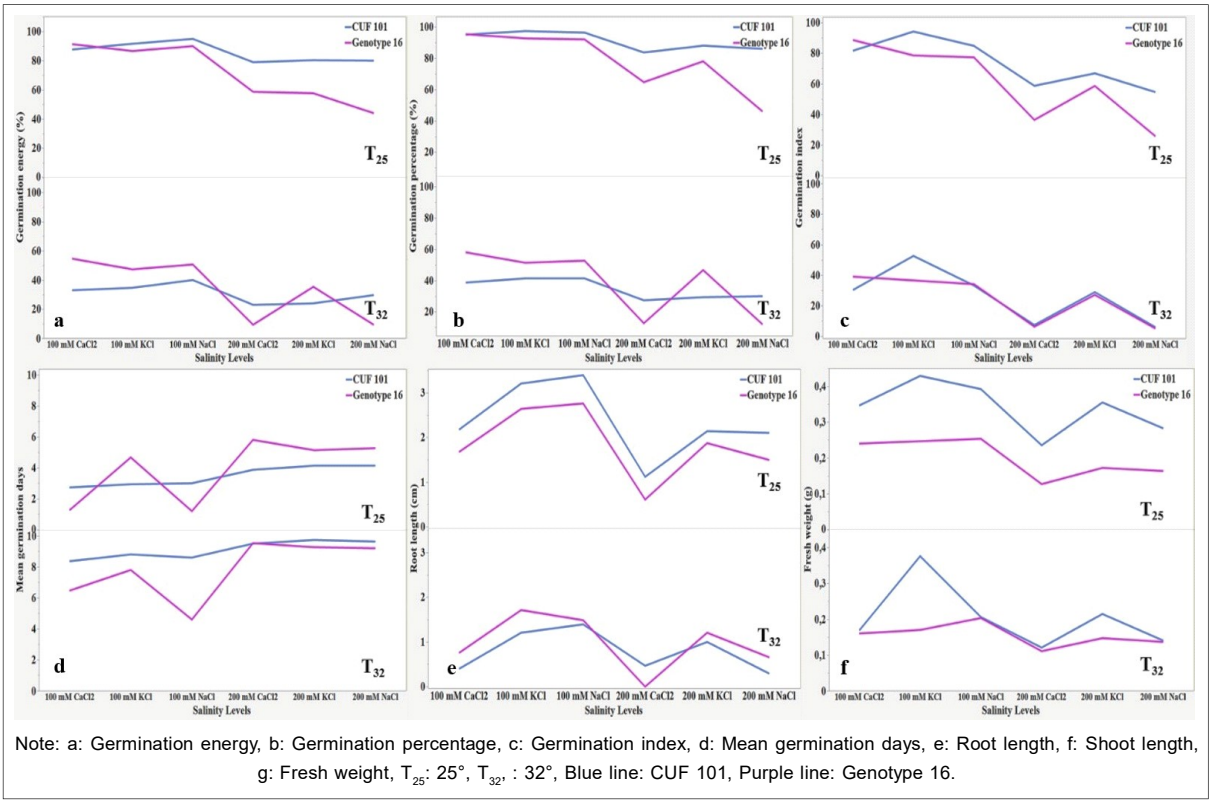
Germination index is a quantitative measure of seed viability and germination potential. It is also used as an indicator of seed quality and vigour. The germination index increased from T_{18} to T_{25} in CUF-101 and alfalfa genotypes except for genotype 9, but it decreased dramatically from T_{25} to T_{32} (Fig 1). These results showed that the T_{25} can mitigate the inhibitory effect of salt stress on the germination index of CUF-101 and alfalfa genotypes, except genotype 9. NaCl had a significantly higher lethal effect on the germination index than $CaCl_2$ and KCl in CUF-101 and alfalfa genotypes at T_{25}. The germination index drastically decreased with increasing temperature and salinity, which was consistent with the results of Wang *et al.* (2022). Higher salinity level (200 mM) of $CaCl_2$ and KCL at T_{25} showed a superior germination index compared to NaCl for CUF-101 and alfalfa genotypes (Fig 4). It is also well known that salinity negatively correlates with the germination index (Saddiq *et al.,* 2021). Salinization, combined with temperature, increased the mean germination days. Sepehri *et al.* (2015) stated that mean germination days varied for alfalfa genotypes (1.00-3.60 days), which was parallel to results at T_{25} under 100 mM NaCl and KCl in the current study. In all three stable temperatures, T_{32} was negatively affected by mean germination days ($T_{32} > T_{18} > T_{25}$), especially when it is combined with 200 mM NaCL and $CaCl_2$ and KCl. The 200 mM NaCl and T_{32} combination delayed mean germination days longer than the other salt types. It was observed that alfalfa plants were more sensitive to NaCl than $CaCl_2$ and KCl when the temperature increased.

Among the various parameters used to assess salinity tolerance, root and shoot lengths were considered the most important. This is because roots are the primary organs responsible for water and nutrient uptake from the soil and their growth and function are directly affected by salt stress (Saddiq *et al.,* 2021; Yu *et al.,* 2021). A similar pattern was observed for germination traits such as seedling growth; root length, shoot length, fresh weight and dry weight all decreased with increasing salinity in both CUF-101 and alfalfa genotypes. T_{25} provided an available seedling establishment, which could minimize competition in the seedling stage as a suitable thermo-period. The interactive effects of salinity and temperature, through osmotic and specific ion toxic effects, ultimately limited root length and



GE: Germination energy, GP: Germination percentage, GI: Germination index, MGD: Mean germination days, RL: Root length, SL: Shoot length, FW: Fresh weight, DW: Dry weight, SV: Seedling vigour.

Fig 3: Correlation coefficients of the alfalfa plants' germination traits.



Note: a: Germination energy, b: Germination percentage, c: Germination index, d: Mean germination days, e: Root length, f: Shoot length, g: Fresh weight, T_{25}: 25°, T_{32}, : 32°, Blue line: CUF 101, Purple line: Genotype 16.

Fig 4: Temperature × genotype × salinity level interactions of some germination traits for CUF-101 and genotype 16.

shoot length in the current study, which is in agreement with Niste *et al.* (2015) and Sepehri *et al.* (2015). In particular, when the temperature reached T_{25} with 200 mM $CaCl_2$, there was no development in root length or shoot length for the alfalfa genotypes in the current study. The lethal effect of $CaCl_2$ on alfalfa plants was more evident at high temperatures than that of NaCl and KCl. The closest genotype root length to CUF-101 (3.39 cm) was genotype 3 (3.33 cm) at T_{18} under 100 mM NaCl in the current study. The shoot length was the highest for CUF-101 at T_{25} (4.36 cm). Genotype 16 was the closest genotype to CUF-101 under a similar salinity level of salt type at the T_{25}. The reduction of fresh and dry weights is a common phenomenon in plants under salinity stress. Similar trends were observed for fresh weight, dry weight and root and shoot lengths. The reduction of fresh weight and dry weight was observed from T_{18} to T_{32}. The fresh weight of the alfalfa genotypes decreased from T_{18} to T_{32}, except for CUF-101. The genotype with the lowest reduction and the closest genotype to CUF-101 was genotype 16. This genotype's fresh weight decreased (10.00%) from T_{18} to T_{25} compared to other alfalfa genotypes. Seedling vigour was dramatically reduced by increasing temperature and salinity level for CUF-101 and all alfalfa genotypes, similar to the findings of Baha (2022). Seedling vigour was the maximum at T_{25} compared to the other two stable temperatures ($T_{25} > T_{18} > T_{32}$). The combination of 100 mM KCl and T_{25} showed the maximum seedling vigour. Similar to the purposes of Benlioglu *et al.* (2022), Ozkan *et al.* (2022) and Guo and Shi (2024), PCA revealed the similar links between the some parameters which were germination traits for CUF-101 and genotype 16 in the current study (Fig 2). Especially, PC1 separated the salinity tolerance genotype with higher potential performance from genotypes sensitive to salinity with low potential performance. PCA, a multivariate analysis, highlighted the relationships between CUF-101 and genotype 16 in contrast to genotype 3 and genotype 9. CUF-101 and genotype 16 were considered salt-tolerant alfalfa plants, while genotype 3 and genotype 9 were salt-sensitive.

CONCLUSION

Selecting the most tolerant and sensitive plants under different environmental stresses and determining their germination traits are crucial for agriculture and sustainability. The current study observed significant variations in alfalfa plants under the interactive effects of temperature, salt type and salt level. Temperature was found to play an essential role in modulating the tolerance of seeds to salinity stress during the germination phase of alfalfa, with the maximum level of tolerance observed at 25°C (T_{25}) in the current study. T_{25} mitigated the harmful effects of temperature and salinity in all alfalfa plants. The germination traits of alfalfa plants

showed higher sensitivity to NaCl and $CaCl_2$ salts but displayed greater tolerance to KCl. As the temperature increased towards 32°C (T_{32}), all germination traits of both CUF-101 and alfalfa genotypes decreased dramatically owing to the interactive effects of temperature and increasing salt levels. In particular, alfalfa genotypes did not develop roots and shoots at 200 mm $CaCl_2$ and T_{32}. The findings of this study indicated that genotypes 16 and 9 are salt-tolerant and salt-sensitive, respectively. Germination per centage prediction is one of the most significant traits for decision-making regarding plant breeding prospects.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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