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In vitro Responses of the *Lotus corniculatus* cv. 'AC Langille' to NaCl-induced Salt Stress

Lotus corniculatus cv. 'AC Langille'nin NaCl indüklü Tuz Stresine Karşı *In vitro* Tepkisi

Abstract

Lotus corniculatus L. is a legume plant that has a very important economic value for ecology and agriculture and is also used as a forage plant worldwide due to its high nutritional value. The tolerance of *L. corniculatus* to salt stress is lower than of other *Lotus* species (such as *Lotus tenuis*). Since breeding studies focused on high nutritional value and feed efficiency while developing new commercial varieties, tolerance to limiting environmental factors such as salt stress remained low in these varieties. However, studies on determining the tolerance of these commercial varieties to salt stress have been limited to date. This study was conducted under *in vitro* conditions with 4 replications, according to a completely randomized trial design, in order to examine the effect of different salt stress levels on germination and initial seedling growth period in 'AC Langille', a commercial variety of *L. corniculatus*. For this purpose, the seeds of *L. corniculatus* were planted in Murashige and Skoog/Gamborg (MG) medium containing NaCl at three different concentrations (0, 40, and 80 mM). Germination and growth parameters were calculated. According to the research results, it was determined that the applied NaCl concentrations had a statistically significant effect on germination (except mean germination time) and growth parameters, and negatively affected both germination (except mean germination time) and growth. It has been observed that salt stress affects the root more

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negatively than the shoot. It was observed that 80 mM NaCl concentration reduced the seedling vigor index by 41.27%.

Keywords: *Lotus corniculatus* L., salt stress, *in vitro*, germination, initial seedling growth

Özet

Lotus corniculatus L. ekoloji ve tarım için oldukça önemli ekonomik değeri olan, dünya genelinde yüksek besin değeri nedeniyle yem bitkisi olarakta kullanılan bir baklagil bitkisidir. *L. corniculatus*'un tuz stresine olan toleransı diğer *Lotus* türlerine göre (*Lotus tenuis* gibi) düşüktür. Islah çalışmalarında yeni ticari çeşitler geliştirilirken yüksek besin değeri ve yem verimine odaklanıldığı için bu çeşitlerde tuz stresi gibi sınırlayıcı çevresel faktörlere karşı tolerans düşük kalmıştır. Bununla birlikte, günümüze kadar üretilen bu ticari çeşitlerin tuz stresine karşı toleransının belirlenmesi çalışmaları sınırlı kalmıştır. Bu çalışma farklı tuz stresi seviyelerinin *L. corniculatus*'un bir ticari çeşidi olan 'AC Langille"de çimlenme ve erken fide gelişim dönemi üzerine olan etkisini incelemek amacıyla tamamen tesadüfü deneme desenine göre 4 tekerrürlü olarak *in vitro* şartlarda yürütülmüştür. Bu amaçla *L. corniculatus*'un tohumları üç farklı konsantrasyonda (0, 40 ve 80 mM) NaCl içeren Murashige ve Skoog/Gamborg (MG) ortamına ekilmiştir. Çimlenme ve büyüme parametreleri hesaplanmıştır. Araştırma sonuçlarına göre, uygulanan NaCl konsantrasyonlarının istatistiki açıdan çimlenme (ortalama çimlenme süresi hariç) ve büyüme parametreleri üzerine önemli derecede etkisinin olduğu, ve hem çimlenmeyi (ortalama çimlenme süresi hariç) hemde büyümeyi olumsuz etkilediği tespit edilmiştir. Tuz stresinin kökü sürgüne göre daha fazla olumsuz etkilediği gözlemlenmiştir. 80 mM NaCl konsantarasyonun fide canlılık indeksini %41.27 oranında azalttığı görülmüştür.

Anahtar Kelimeler: *Lotus corniculatus* L., tuz stresi, *in vitro*, çimlenme, erken fide gelişimi

Introduction

Today, approximately one billion hectares of land area worldwide are affected by salt; this accounts for approximately 7% of the Earth's total land surface (Hopmans et al., 2021). While the majority of salt-affected irrigated lands can be attributed to natural geochemical processes, around 30% of such lands worldwide are damaged by secondary salinization caused by human activities (Hopmans et al., 2021). Exposure to salinity stress can induce several forms of stress in plants, including ionic, oxidative, and osmotic stress, besides disrupting hormonal balance (Sári et al., 2023). Soil with water-soluble salts over 4 dS m^{-1} (~ 40 mM) is classified as saline (Shokat and Großkinsky 2019). The essential phase for crop plants' susceptibility to salinity often occurs during the stages of germination and initial seedling growth. The temporal relationship between seed germination and seedling emergence in plants has a significant impact on their level of tolerance. Therefore, the species or cultivars that exhibit rapid germination or emergence acquire advantages in terms of their tolerance to NaCl (Beyaz et al., 2011). The impact of this particular stressor on agricultural output was examined across various levels, commencing with *in vitro* experimentation with plant tissue culture, followed by hydroponics, pots, and ultimately field conditions (Sári et al., 2023).

Tissue culture has been identified as a highly effective approach for investigating the responses of plants to stressful conditions (Dogan, 2020). In plant tissue culture, the manipulation of factors such as light, temperature, humidity, nutrient composition, and quantity can be precisely regulated. Consequently, any impact on plant growth resulting from the application of stress factors solely within the culture medium can be readily, expeditiously, cost-effectively, and dependably detected.

Lotus corniculatus L. (allotetraploid) is recognized for its significant value as animal feed (Büyükyıldız et al., 2023). It exhibits promising potential for replacing white clover and alfalfa in temperate regions worldwide (Diaz et al., 2005; Hunt et al., 2015; Savic et al., 2019). Furthermore, research has indicated that its utilization as a forage can lead to enhanced dairy production in comparison to alternative forage options (Diaz et al., 2005; Hunt et al., 2015; Savic et al., 2019). This particular species belongs to the outbreeding *Lotus* types that are extensively utilized for commercial purposes due to their ability to produce abundant aboveground biomass with excellent nutritional content (Sanchez et al., 2011; Savic et al., 2019). Nevertheless, due to the advantageous selection of these traits, this particular forage crop demonstrates a lower capacity to tolerate salinity (Sanchez et al., 2011; Bao et al., 2014; Savic et al., 2019).

Since the breeding studies of commercially bred *Lotus* cultivars focus mostly on yield and productivity, their tolerance to other abiotic stress factors, especially salinity, is very low (Escaray et al., 2014; Escaray et al., 2019). Although the responses to salinity in some commercially bred lotus cultivars have been investigated in studies at the physiological, biochemical, and molecular levels (Antonelli et al., 2021; Azarafshan and Abbaspour, 2014; Bao et al., 2014; Teakle et al., 2006; Teakle et al., 2007; Uchiya et al., 2016), studies on determining the responses in the germination and early seedling development period, which are the most sensitive stages in tolerance to salinity, as in all plants, have remained quite limited. So far, only Büyükyıldız et al. (2023) examined the responses of *Lotus corniculatus* L. var. 'Sarıyıldız' to salinity during the germination and initial seedling growth period under *in vivo* conditions. However, it is well known that the responses of plants to salt stress may vary depending on plant species, and that even different varieties/cultivars within the same species show very different responses. In addition, Beyaz et al. (2011) reported that the responses of two different sainfoin species to the same doses of salt stress may differ even under *in vivo* and *in vitro* conditions. Therefore, this study was carried out to determine the responses of *Lotus corniculatus* L. cultivar 'AC Langille', which was bred for commercial purposes, to different salinity levels during germination and early seedling development periods under *in vitro* conditions.

Materials and Methods

Plant Material

The plant material used in this study consisted of the seeds of the *Lotus corniculatus* L. cultivar 'AC Langille', which were gathered in 2012 and provided by the Plants, Soils and Climate Department at Utah State University. The entity known as 'AC Langille' was established by the Nappan Research Farm, which is a part of Agriculture and Agri-Food Canada. It was generated using two cycles of mass selection for winter hardiness and one cycle of mass selection for seedling vigor. The original material consisted of six distinct germplasms selected from the cultivar 'Leo' and provided by the Crop Science Department of the University of Guelph (Papadopoulos et al., 1998).

Plant Tissue Culture and Salt Treatments

The basal growth media used was the standard Murashige and Skoog/Gamborg medium obtained from Plant Media, USA. It consisted of 3% sucrose from Research Product International, USA, and 0.7% agar from Plant Media, USA. Prior to subjecting to autoclaving, the sample should be exposed to a temperature of 121 degrees Celsius and a pressure of 7.25 pounds per square inch absolute for a duration of 20 min. The acidity level of the medium was modified to a pH of 5.7. The seeds of *L. corniculatus* were subjected to surface sterilization using a 50% solution of commercial bleach (Clorox-USA) containing 8.25% sodium hypochlorite. Additionally, one drop of Tween-20 (Acros Organics) was added to the solution. The sterilization process lasted for 20 min, after which the seeds were rinsed three times with distilled water. The seeds were planted on a layer of material that was 2.5 cm thick in magenta boxes measuring 7.62 cm in length, 7.62 cm in width, and 10.16 cm in height. These boxes were obtained from BioWorld, a company based in the USA. Seeds were planted on a basic medium with varying amounts of NaCl (0-control-, 40, and 80 mM) added. The seeds were germinated and the seedlings were grown at a temperature of 25±1 °C. White fluorescent lamps were used to provide light at an intensity of 30 μmol m^{-2} s^{-1} (PAR) for 16 h, followed by 8 h of darkness.

Germination assay and Morphological Observations

Germination assays and morphological observations were made considering Beyaz (2023). Once the radicle reached a length of 2 mm, the seed was said to have germinated. Commencing on the 9th day, the frequency of germinations was recorded at 24 h intervals (ISTA, 2003).

The mean germination time (MGT) was determined using the method described by Ellis and Roberts (1980). The formula (∑Dn/∑D -Eq. 1-) MGT is defined as the sum of the number of freshly germinated seeds on each day D, divided by the sum of the total number of days since the start of the experiment. The germination rate of seeds under salt stress conditions was determined using the following equation:

Germination percentage (GP) = (Number of germinating seeds/Total number of seeds) × 100 (Al-Enezi et al., 2012) (Eq. 2)

Measurements were taken on the seedlings that grew 14 days after the experiment began, including observations of shoot and root length (in centimeters) as well as fresh and dry weight (in grams). The dry weights of the samples were determined by drying them in an oven (VWR Scientific Inc., USA) at a temperature of 70°C for a duration of 48 h (Beyaz et al., 2011). The formulas used to compute water content (WC), dry matter (%) (DM), and vigor index (VI) are as follows:

Water content (WC) = (fresh weight – dry weight)/ fresh weight ×100 (Zheng et al., 2008) (Eq. 3)

Dry matter (DM) = (dry weight/fresh weight) × 100 (Bres et al., 2022) (Eq. 4)

Seedling Vigor index (SVI) = (average root length + average hypocotyl length) x germination percentage (GP) (Abdul-Baki & Anderson, 1973) (Eq. 5)

The germination rate index (speed of germination) was calculated using the formula GRI = Σ No of Germinated Seeds/ Σ No of Days according to Maguire (1962). (Eq. 6)

Statistical Data Analysis

The experiment was conducted using a completely randomized block design, with four replications. The gathered data were analyzed using a one-way Analysis of Variance (ANOVA) in the statistical software package SPSS 22. The statistical significance of the means was evaluated using the Duncan Multiple Range Test (DMRT) at a significance threshold of $P \leq 0.05$. Prior to doing statistical analysis, the data expressed as percentages underwent an Arcsine transformation, as recommended by Snedecor and Cochran (1967).

Results and Discussion

In this study, the effect of different NaCl concentrations on germination and morphological changes in 14-day-old initial seedlings (Figure 1) of *Lotus corniculatus* L. cultivar 'AC Langille' was examined under *in vitro* conditions. The impact of increasing NaCl concentrations on the germination percentage (GP), mean germination time (MGT), and germination rate index (GRI) were presented in Figure 2A-2B-2C. It was observed that there existed a statistically significant ($P \leq 0.01$) disparity in GP and GRI across different NaCl concentrations. On the other hand, there was no statistical difference between NaCl concentrations in terms of MGT. Due to increasing NaCl concentrations, GP and GRI decreased. This decrease was calculated as 50% for GP and 66.21% for GRI, respectively, when comparing the control with the highest NaCl concentration (80 mM). However, due to increasing NaCl concentrations, there was a negligible decrease in MGT. This decrease was recorded as 0.14% (Figure 2B). Seed germination is often negatively affected in many plants due to the osmotic effect, ion toxicity, and oxidative stress caused by high doses of NaCl. Büyükyıldız et al. (2023) reported that increased NaCl concentrations (200 ppm to 10000 ppm) negatively affected ($P \leq 0.01$) germination rate and germination index of *L. corniculatus* variety 'Sarıyıldız'. Teakle et al. (2010) stated that increased NaCl-induced salinity negatively effected seed germination of commercial cultivars and accessions of *L. tenuis*. The fact that the averages of the groups are not statistically significant at increasing NaCl concentrations in terms of MGT means that the NaCl concentrations applied in the research are not at a level that would affect the germination period of *L. corniculatus* cv. 'AC Langille'. The application of salt stress frequently resulted in a higher mean germination time in comparison to the control group. However, it should be noted that the increase in salt concentration did not consistently lead to a proportional increase in the mean germination time (Büyükyıldız et al., 2023). Parallel with these results, Beyaz and Kazankaya (2024) reported that GP and GRI in *L. corniculatus* cultivar 'Leo' decreased under 40 and 80 mM NaCl treatments. Cokkızgın (2012) noted that all the examined parameters such as germination index (GI), coefficient of velocity of germination (CVG), germination percentage (GP), and seed vigor index (SVI) decreased with increasing osmotic potential of NaCl (0.3 mPa to 1.5 MPa NaCl) concentration, except MGT in *Phaseolus vulgaris* L. Increased NaCl-induced salinity decreased the seed germination of many other legume plants were found with the findings of various researchers (Beyaz et al., 2018; Bhattarai et al., 2020). The process of seed germination is a complex developmental phenomenon that is impacted by a combination of intrinsic and extrinsic variables. The influence of salinity stress on seed germination can be linked to the delayed uptake of water and a reduction in the activity of α-amylase, an enzyme responsible for the breakdown of starch (Atta et al., 2023).

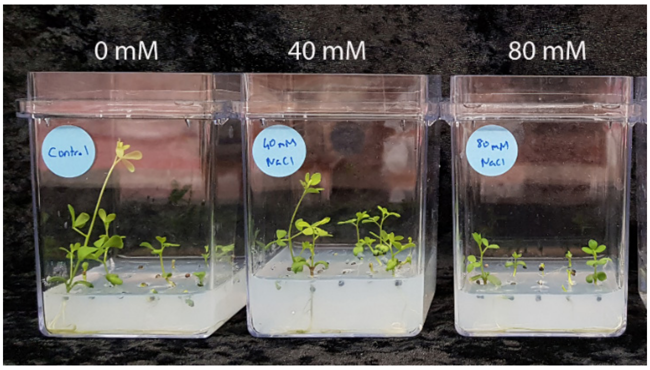
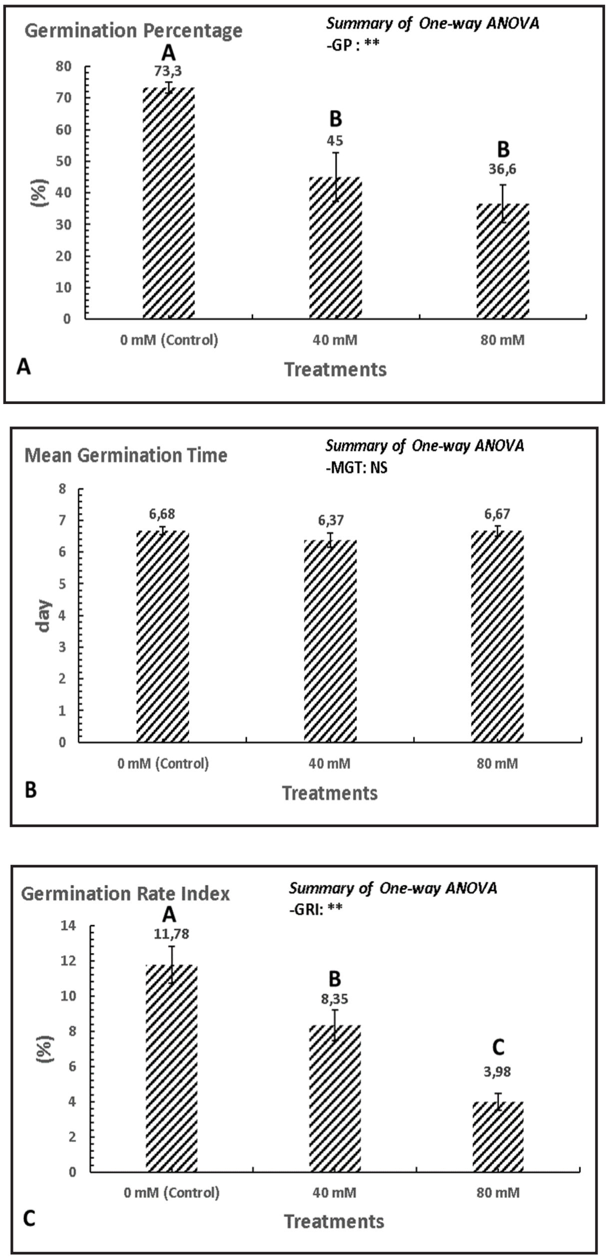
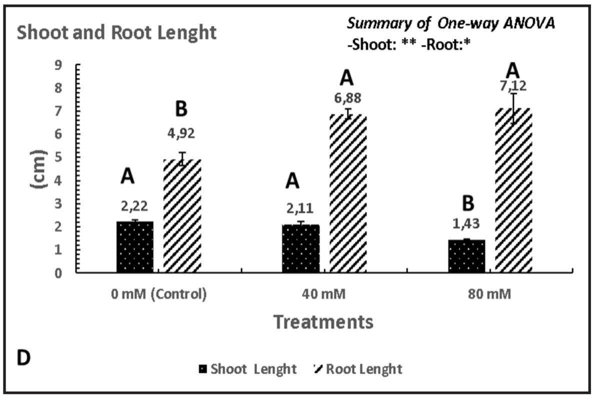
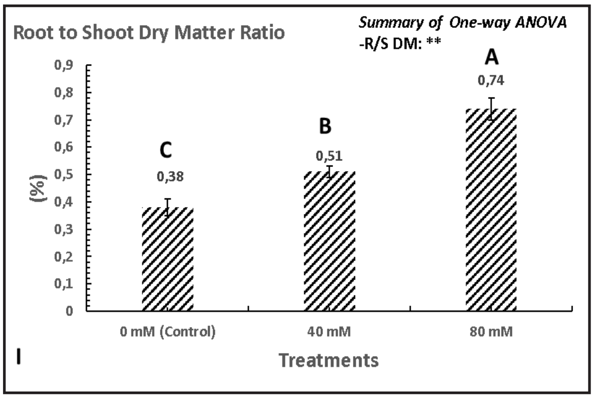
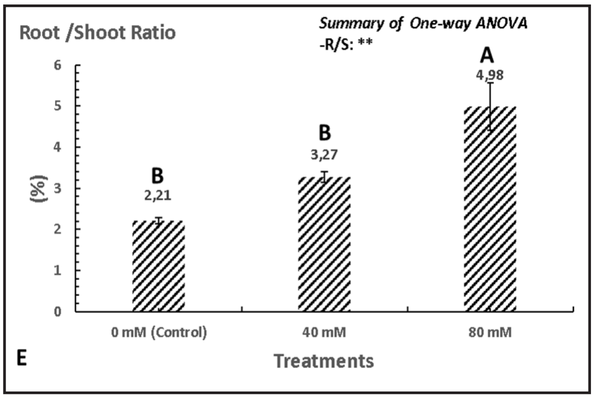


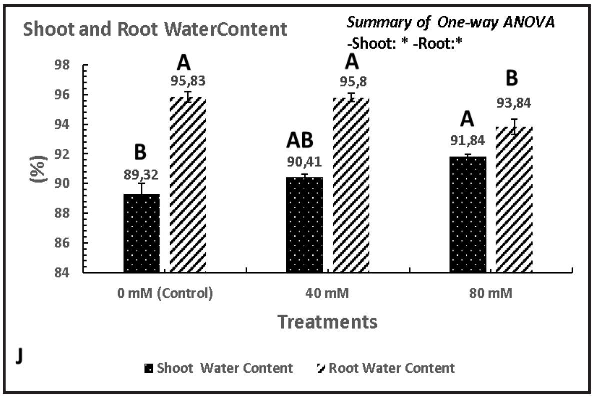
Figure 1. The morphology of *L. corniculatus* cv. 'AC Langille' seedlings after NaCl-induced salt stress treatments (after 14 days of germination)

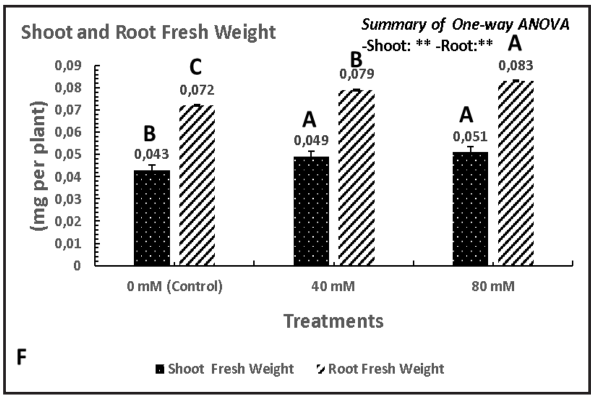


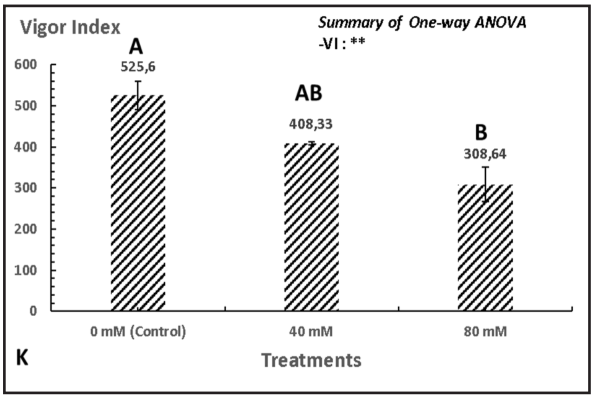












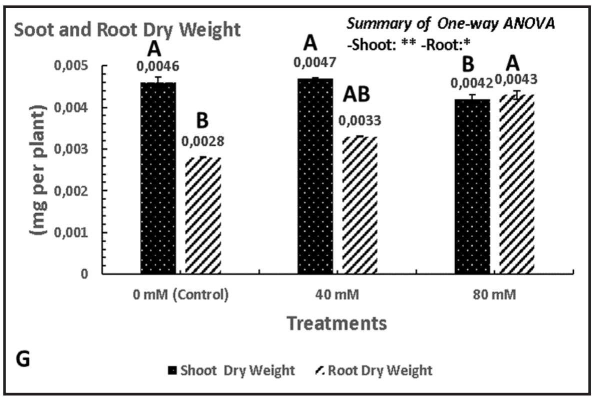


Figure 2. Effects of NaCl-induced salt stress on germination percentage, mean germination time, germination rate index, shoot lenght, root lenght, root to shoot ratio, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, shoot dry matter, root dry matter, root to shoot dry matter, shoot water content, root water cantent, seedling vigor index. ** $P \leq 0.01$; * $P \leq 0.05$; NS: non-significant.

In the present study, shoot length (SL), root length (RL), root to shoot ratio (R/S), shoot fresh weight (SFW), root fresh weight (RWF), shoot dry weight (SDW), root dry weight (RDW), shoot dry matter (SDM), root dry matter (RDM), root to shoot dry matter (R/S DM), shoot water content (SWC), root water content (RWC), and seedling vigor index (SVI) parameters were examined to determine the effects of different NaCl concentrations on initial seedling growth. The effects of NaCl concentrations on SL, RL, and R/S were given in Figure 2D-2E. It was determined that there was a statistical difference between NaCl concentrations in terms of these parameters ($P \leq 0.01$). According to the findings, a decrease in SL and an increase in RL and R/S were detected due to increasing NaCl concentrations. When the control and 80 mM NaCl concentrations were compared, the decrease in SL was found to be 35.58%, and the increases in RL and R/S were 44.71% and 125.33%, respectively. The shoot and root length are the most essential characteristics for salinity because roots are in direct contact with soil and receive water from soil and shoots supply it to the rest of the plant. For this reason, root length and shoot length provide a crucial indication of the response of plants to salt stress (Aydinşakir et al., 2015). From the research findings, different scenarios can be put forward to understand the mechanism underlying the decrease in shoot length and increase in root length in *L. corniculatus* cv. 'AC Langille' seedlings due to increasing NaCl concentrations. i) Perhaps the increase in root length requires increased surface for uptake of water and other macro/micro (including Na and Cl) elements. ii) Perhaps removal of toxic ions from the root system. iii) Perhaps activation of another adaptation mechanism. However, more detailed studies such as physiological, biochemical, and molecular analyzes are needed to elucidate this mechanism. Antonelli et al. (2021) reported that the root to shoot ratio of different *L. corniculatus* cultivars increased depending on the increasing salt level at greenhouse conditions. In addition, Beyaz and Kazankaya (2024) stated that found that while SL decreased, RL and R/S ratio increased in *L. corniculatus* cultivar 'Leo' under 40 and 80 mM NaCl treatments. On the other hand, several studies exploring the effects of salt stress on the *L. corniculatus* also demonstrate that salt stress has a negative influence on shoot, root, and seedling growth (Azarafshan and Abbaspour, 2014; Büyükyıldız et al., 2023; Galloway et al., 2010; Teakle et al., 2006). The combination of osmotic and drought stress, resulting from salt accumulation in plants, leads to a reduction in water uptake by plant tissues. Consequently, there is a reduction in cellular growth and development. Hence, the suppression of water absorption and its consequential impact on cellular growth and development represents a significant factor contributing to the reduction in shoot and root growth (Emek, 2018).

Different NaCl concentrations significantly affected the SDM, RDM, and R/S DM of *L. corniculatus* seedlings (Figure 2H-2I). Due to increasing NaCl concentrations, SDM decreased and RDM increased. The highest SDM (10.67%) was obtained in control, while the lowest SDM (8.15%) was measured under 80 mM NaCl treatment. On the other hand, the highest RDM (6.15%) was obtained in 80 mM NaCl treatment, whereas the lowest RDM (4.16%) was measured under control treatment. However, the maximum value of R/S DM (0.74%) was recorded for the 80 mM NaCl concentration, whereas the minimum value of R/S DM (0.38%) was observed in control. Therefore, research findings have shown that, depending on increasing NaCl concentrations, SDM decreases, and on the contrary, RDM and R/S DM increase. When the control and 80 mM NaCl concentrations were compared, there was a decrease of 23.61% in SDM and an increase of 47.83% and 94.73% in RDM and R/S DM, respectively. This can perhaps be interpreted as the root organ being less affected by the applied concentrations of NaCl than the shoot. Similar to these findings, Beyaz and Kazankaya (2024) found that RDM and R/S DM parameters increased in 40 and 80 mM NaCl treatments in another commercial *Lotus* variety, 'Leo', and contrary to our findings, SDM also increased in 40 and 80 mM NaCl treatments. In the light of these data, it can be speculated that cv. 'Leo' is less negatively affected by the possible negative effects of salt in terms of RDM, R/S DM, and SDM parameters that cv. 'Langille'. On the other hand, several studies have been conducted to examine the impact of salt stress on the *L. corniculatus* plant, and these studies consistently demonstrate that salt stress adversely affects the dry matter of both shoots and roots (Teakle et al., 2006; Antonelli et al., 2021).

SWC and RWC of *L. corniculatus* seedlings were significantly ($P \leq 0.05$) impacted by different NaCl concentrations (Figure 2J). While the maximum SWC (91.84%) was recorded at 80 mM NaCl concentration, the lowest SWC (89.32%) was recorded in the control. On the contrary, while the maximum RWC (95.83%) was recorded at control, the lowest RWC (93.84%) was recorded in 80 mM NaCl concentration. Therefore, the research results showed that with increasing NaCl concentrations, there was an increase in water content in the shoot and a decrease in the root. When comparing the control group with the highest salt level applied, 80 mM NaCl concentration, this increase was 2.82% for SWC, and on the other hand, this decrease was 2.07% for RWC. The root-water-uptake (RWU) of plants is constrained by salinity stress, which also affects plant growth (Wang et al., 2012). Therefore, it can be speculated that this decrease in water in the root is due to the osmotic potential created by the salt. Similarly, Beyaz and Kazankaya (2024) reported that SWC decreased at 40 and 80 mM NaCl concentrations, while RWC increased in *L. corniculatus* cultivar 'Leo'. However, Wouyou et al. (2019) stated that NaCl-induced salt stress causes a slight increase in shoot water content of *Amaranthus cruentus* L. cultivar seedlings at 30 mM NaCl and no effect at 90 mM NaCl.

The effect of different NaCl concentrations on SVI in *L. corniculatus* is seen in Figure 2K. SVI values of *L. corniculatus* against the applied NaCl concentrations varied between 308.64 and 525.60, and SVI decreased as a result of the increased NaCl concentrations and this decrease was found to be statistically significant ($P \leq 0.01$). There was a 41.27% decrease in SVI at 80 mM NaCl concentration. Since this decrease is based on the seed germination percentage and seedling development (shoot and root length) of the vigor index, it can be said that the negative effect of salt on these parameters is observed. In general, it has been reported in the literature (Büyükyıldız et al., 2023; Cokkızgın, 2012; Dehnavi et al., 2020; Khajeh-Hosseini et al., 2003) that the seedling vigor index decreased in other crops due to increased salinity levels, similar to the results of this study. Parallel to these results, Beyaz and Kazankaya (2024) reported that SVI for *L. corniculatus* cultivar 'Leo' decreased at 80 mM NaCl concentration.

Overall, the data obtained showed that germination and initial seedling growth parameters of *L. corniculatus* cv. 'AC Langille' responded differently under salt stress. NaCl concentrations applied in the study had a significant effect on germination parameters (except MGT). While GP and GRI decreased due to increasing NaCl concentrations, there was no change in MGT values. However, the NaCl concentrations applied in the study significantly affected all measured growth parameters statistically. Depending on increasing NaCl concentrations, an increase in root growth and development parameters (RL, R/S, RFW, RDW, RDM, and R/S DM) was observed. However, a decrease in root growth and development parameters (SL, SFW, SDW, SDM) and SVI was observed. In addition, RWC decreased while SWC increased with increasing NaCl concentrations.

Conclusion

As a result of the research, it was seen that the NaCl-induced salt stress levels (40 and 80 mM NaCl) applied in the research retarded the germination of commercial *L. corniculatus* cv. 'AC Langille'; however, it encouraged root development and inhibited shoot development, and SVI decreased significantly. Commercial *Lotus corniculatus* cultivar(s) with high forage yield and seedling vigor are said to have low tolerance to abiotic stress factors, including salt. Therefore, in future *L. corniculatus* breeding studies, it is important to increase not only agronomic characters such as high forage yield and seedling vigor, but also tolerance to abiotic stress factors such as salinity. Otherwise, agricultural benefit from this plant will be limited in marginal agricultural areas with salinity.

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