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RESEARCH ARTICLES

In vitro responses of *Lotus corniculatus* L. cultivar "Leo" to PEG-induced drought stress

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Abstract

A perennial species of high-quality legume fodder, *Lotus corniculatus* L., is susceptible to drought, and productivity is decreased by water scarcity. However, studies on the drought responses of *L. corniculatus* cultivar(s) are very scarce. The in vitro culture technique is a useful method for examining how various plants react to water stress. In this study, in vitro water stress conditions induced by polyethylene glycol (PEG_{6000}) were used to evaluate the germination and early seedling growth of *L. corniculatus* cultivar "Leo". For this purpose, *L. corniculatus* seeds were cultured in MS (Murashige and Skoog/Gamborg) medium containing 0%, 4%, and 8% w/v PEG_{6000} for 14 days. Seed germination and seedling growth parameters were tested. Low concentrations of PEG (4%, − 0.03 MPa) promoted the seed germination and germination rate index (speed of germination) and shortened the mean germination time of "Leo", whereas high concentrations (8%, − 0.1 MPa) inhibited the germination parameters. Increasing PEG concentration caused a decrease in the length, fresh weight, dry weight and water content of shoots and roots, and an increase in the dry matter ratio of shoots and roots. However, the seedling vigor index decreased significantly with increasing PEG concentration. In addition, it has been determined that root is more affected by drought stress than shoot. The results of this study showed that the yield of *L. corniculatus* cultivar "Leo" will decrease significantly under drought stress in the osmotic potential of 8% (− 0.1 MPa) PEG concentration.

Keywords *Lotus corniculatus* L. · Abiotic stress · Polyethylene glycol (PEG) · Drought · Germination · Seedling traits · In vitro

Introduction

Lotus corniculatus L. (birds foot trefoil-BFT-) is an important tannin-containing legume forage with high protein content and low saponin level, and also it is a model plant among forage legumes, with best reproducibility (Bao et al. 2014; Hunt et al. 2014). Due to its main agricultural benefits, which include its ability to grow in soils with low fertility, acidity, and salinity, as well as its widespread use to stop roadside erosion, it is regarded as the most important forage plant in terms of agriculture. It also has anti-bloating properties because of its tannin content (Sun et al. 2014). For hay production, it is planted as a single crop, or when used for grazing, it is combined with grasses such as bluegrass (*Poa pratensis* L.), bromegrass (*Bromus inermis* Leyss.), cock's-foot (*Dactylis glomerata*), timothy (*Phleum pratense* L.). It outperforms alfalfa (*Medicago sativa* L.) and white clover (*Trifolium repens* L.), the two most common temperate zone fodder legumes, in marginal soils and vast grassland management regimes (Inostroza et al. 2015). *L. corniculatus* displays typical Mediterranean plant characteristics and is acknowledged to be moderately drought tolerant, however there are genotypic variances in its tolerance to irradiation, temperature, CO_2 concentrations, and drought stress (Ünlüsoy et al. 2022). On a worldwide scale, drought stress is one of the most significant abiotic stresses and results in significant crop losses (Luo et al. 2020). Around 80% of the world's agriculture is rain-fed, and the majority of it is vulnerable to drought, which lowers agricultural output. Climate change will cause droughts to occur more frequently and with greater severity, endangering global food security (Mphande et al. 2020). Due to lower water availability and higher water losses (via evapotranspiration) as a result of a warmer and drier climate, drought stress

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negatively impacts plants (Ahmed et al. 2022). Cells' equilibrium is impacted by water stress since there is less water available in their cytoplasm. Osmotic and oxidative stress are brought on by a cell's lack of water and harm physiological, biochemical, and molecular functions (Martínez-Santos et al. 2021). The success of seed germination determines the establishment of seedlings and subsequent growth in plants. Plants are very vulnerable to drought stress at this stage (Yousefi et al. 2020). Research on how plants respond to abiotic stress conditions can be conducted in vitro. For stress factors like drought, this procedure is quick, simple, and efficient (Adak et al. 2018). One of the most popular osmotic agents used to study the effects of in vitro water stress in plants is high molecular weight polyethylene glycol (PEG), which is highly soluble in water and non-penetrable in cells and produces a negative osmotic potential in the culture medium without producing toxicity (Zivcak et al. 2016). PEG molecules with a molecular weight larger than 3000 are not absorbed, and PEG does not enter the cell wall space. The vast majority of cultivars of *L. corniculatus* are drought-sensitive (Bao et al. 2014). To the best of our knowledge, it is unclear how much the osmotic effects of PEG_{6000} have on the germination and seedling growth of *L. corniculatus* cultivar(s). This investigation was done to find out how *L. corniculatus* cultivar "Leo" germination and seedling growth were affected by PEG_{6000} simulated drought stress under in vitro condititons.

Materials and methods

Plant material

In this study, the seeds of the *L. corniculatus* L. cultivar "Leo", harvested in 2022 and supplied by Utah State University, Plants, Soils and Climate Department, were used as plant material.

Plant tissue culture and drought treatments

The growth medium, standard Murashige and Skoog/Gamborg (Plant Media, USA) (Gamborg et al. 1968), contained 3% sucrose (Research Product International, USA) and 0.25% phytagel (Plant Media, USA). For drought stress, seeds were sown on standard MS medium with 4% and 8% w/v PEG_{6000}. 4% and 8% w/v PEG_{6000} correspond to osmotic potential of − 0.3 bar (− 0.03 MPa) and − 1 bar (− 0.1 MPa), respectively (Michel and Kaufmann 1973). Before autoclaving at 121 °C, 7.25 psi for 20 min, the pH of the medium was corrected to 5.7 with 1 M NaOH or HCl. *L. corniculatus* seeds were surface sterilized in 50% commercial bleach (Clorox-USA, containing 8.25% sodium hypochlorite) in which 1 drop of Tween-20 (Acros Organics) was added for 20 min and then rinsed 3 times with distilled water. Germination of seeds and subsequent seedling development were carried out at 25 ± 1 °C under white fluorescent lamps at an intensity of 30 μmol m^{-2} s^{-1} (PAR) in a photoperiod of 16 h light and 8 h dark.

Germination test and morphological observations

When the growing radicle lengthened to 2 mm, the seed was considered germinated. For 14 days, the proportion of seeds that germinated was recorded every 24 h (ISTA 2003). Mean germination time (MGT) was calculated according to Ellis and Roberts (1980). $MGT = \sum Dn/\sum D$, where n is the number of freshly germinated seeds on day D, and D is the number of days since the start of the experiment. The percentage of seeds that germinated after being exposed to drought stress was estimated using the equation:

$$\text{Germination percentage (GP)} = (\text{number of germinating seeds}/\text{total number of seeds}) \times 100\ (\text{Al-Enezi et al. 2012})$$

Morphological observations (shoot and root length (cm); fresh and dry weight (g) were made on the seedlings that developed 14 days after the start of the experiment (Fig. 1). Dry weights were calculated after samples were dried in an oven (VWR Scientific Inc., USA) at 70 °C for 48 h (Beyaz et al. 2011). Water content (WC), dry matter (%) (DM) and vigor index (VI) were calculated according to the following formulas, respectively:

$$\text{Water content (WC)} = (\text{fresh weight} - \text{dryweight})/\text{freshweight} \times 100\ (\text{Zheng et al. 2008})$$

$$\text{Dry matter (DM)} = (\text{dry weight}/\text{fresh weight}) \times 100\ (\text{Bres et al.2022})$$

$$\text{Seedling vigor index (SVI)} = (\text{average root length} + \text{average hypocotyl length}) \times \text{germination percentage (GP)}\ (\text{Abdul - Baki and Anderson 1973})$$

Germination rate was expressed as the germination rate index (GRI) according to Maguire (1962):

Fig. 1 14-Day-old *Lotus corniculatus* cv. "Leo" seedlings at different levels of PEG_{6000}-induced drought under in vitro conditions. **A** 0% w/v; **B** 4% w/v; **C** 8% w/v PEG_{6000}

$$GRI = \frac{\Sigma \text{no of germinated seeds}}{\Sigma \text{no of days}}$$

Statistical analysis

The study was carried out as a completely randomized design with 3 replications where each magenta box was considered a replication and 20 seeds were planted in each magenta box. For each treatment, a one-way ANOVA was done using the SPSS statistical program (Version 22). The means were compared using the Duncan multiple range test at $P < 0.01$. Before statistical analysis, the data in percentages were transformed using Arcsine transformation (Snedecor and Cochran 1967). Each treatment's standard deviation was also calculated.

Results

The percentage of seeds that germinated ($P < 0.01$), the mean germination time ($P < 0.05$), and the germination rate index ($P < 0.01$) of *L. corniculatus* were all significantly impacted by PEG-induced drought stress (Fig. 2A–C). The 4% PEG treatment saw the highest percentage of seeds that germinated (100%) whereas the 8% PEG treatment saw the lowest percentage of seeds that germinated (92.9%) (Fig. 2A). Germination percentage decreased by 3.83% following 8% PEG treatment compared to control. In the control, 4% PEG, and 8% PEG treatments, the mean germination time was observed as 3.29 days, 3.22 days, and 3.26 days, respectively (Fig. 2B). Compared to the control, the mean germination time was shortened by 2.12% under 4% PEG treatment and 0.91% with 8% PEG treatment. The highest germination rate index was obtained from 4% PEG treatment with 35.47%, then in the control with 31.33%, and with 8% PEG treatment with 29.46%, respectively. The germination rate index (speed of germination) was reduced by 5.96% under 8% PEG treatment. The drought stress brought on by the PEG treatment had an impact on the *L. corniculatus* seedling characteristics. The maximum shoot length (3.22 cm) was recorded in the control, 2.04 cm in the 4% PEG treatment, and 1.35 cm in the 8% PEG treatment. Similarly, the longest root measured in the control was 6.47 cm, whereas the shortest measured in the 8% PEG treatment was 1.79 cm. Overall, under 4% PEG and 8% PEG treatments, drought stress decreased the shoot length by 36.64% and 58.07% while the root length decreased by 34.31% and 72.33%. Furthermore, compared with control, the root shoot ratio was also increased by 13.82% under 4% PEG treatment and reduced 29.25% under 8% PEG treatment (Fig. 2E). Under conditions of drought brought on by the PEG treatments, the fresh and the dry weight of the shoot and root of *L. corniculatus* seedlings were considerably ($P < 0.01$) impacted (Fig. 2F, G). The maximum fresh weights of the shoots and roots (0.05 mg per plant and 0.077 mg per plant, respectively) were measured in the control, whereas the lowest fresh weights of the shoots and roots (0.018 mg per plant and 0.009 mg per plant, respectively) were determined following 8% PEG treatment. Similar to this, the control group had the highest dry weight of the shoot (0.0047 mg per plant) and root (0.0042 mg per plant), whereas the 8% PEG treatment had the lowest dry weights of the shoot (0.0036 mg per plant) and root (0.0015 mg per plant). The highest decrease in fresh weight of shoot and root by 67.85% and 88.31%, as well as dry weight of shoot and root by 23.40% and 14.28%, was recorded under 8% PEG treatment, respectively, compared to control. The PEG-induced drought conditions also had a significant ($P < 0.01$) impact on dry matter ratio of the shoot and root (Fig. 2H). Dry matter ratio was recorded at 8.39%, 15.21% and 20.51% under control, 4% PEG and 8% PEG treatments, respectively for shoot. For root, dry matter ratio was recorded at 5.44%, 9.68% and 16.66% under control, 4% PEG and 8% PEG treatments, respectively. However, the same values (0.64%) were obtained for the root shoot dry matter ratio from the control and 4% PEG, however, the 0.81 value was obtained for 8% PEG (Fig. 2İ). In terms of water content, there was a significant decrease in both shoot and root (Fig. 2I). Shoot water content was recorded at 91.6%, 84.78% and 79.48% under control, 4%

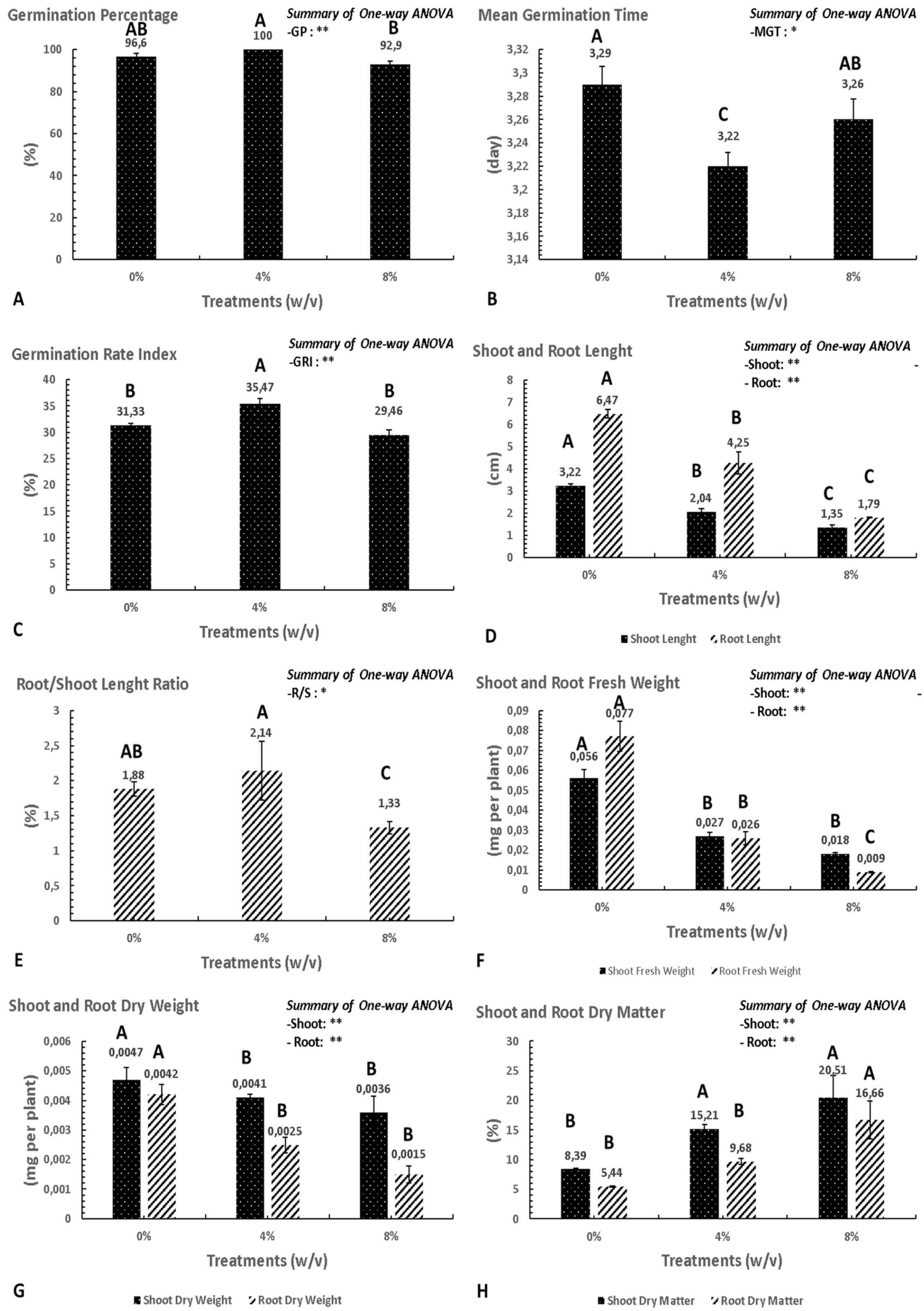

Fig. 2 HYPERLINK "sps:id::fig1||locator::gr1||MediaObject::0"The effect of different levels (0%, 4%, and 8% w/v PEG_{6000}) of the PEG-induced drought stress on germination percentage, mean germination time, germination rate index, shoot and root length, root/shoot ratio, shoot and root fresh weight, shoot and root dry matter, root to shoot dry matter ratio, shoot and root water content, and seedling vigor index of 14-days-old BFT seedling. $^{**}P<0.01$; $^{*}P<0.05$. Each treatment's standard deviation was also calculated

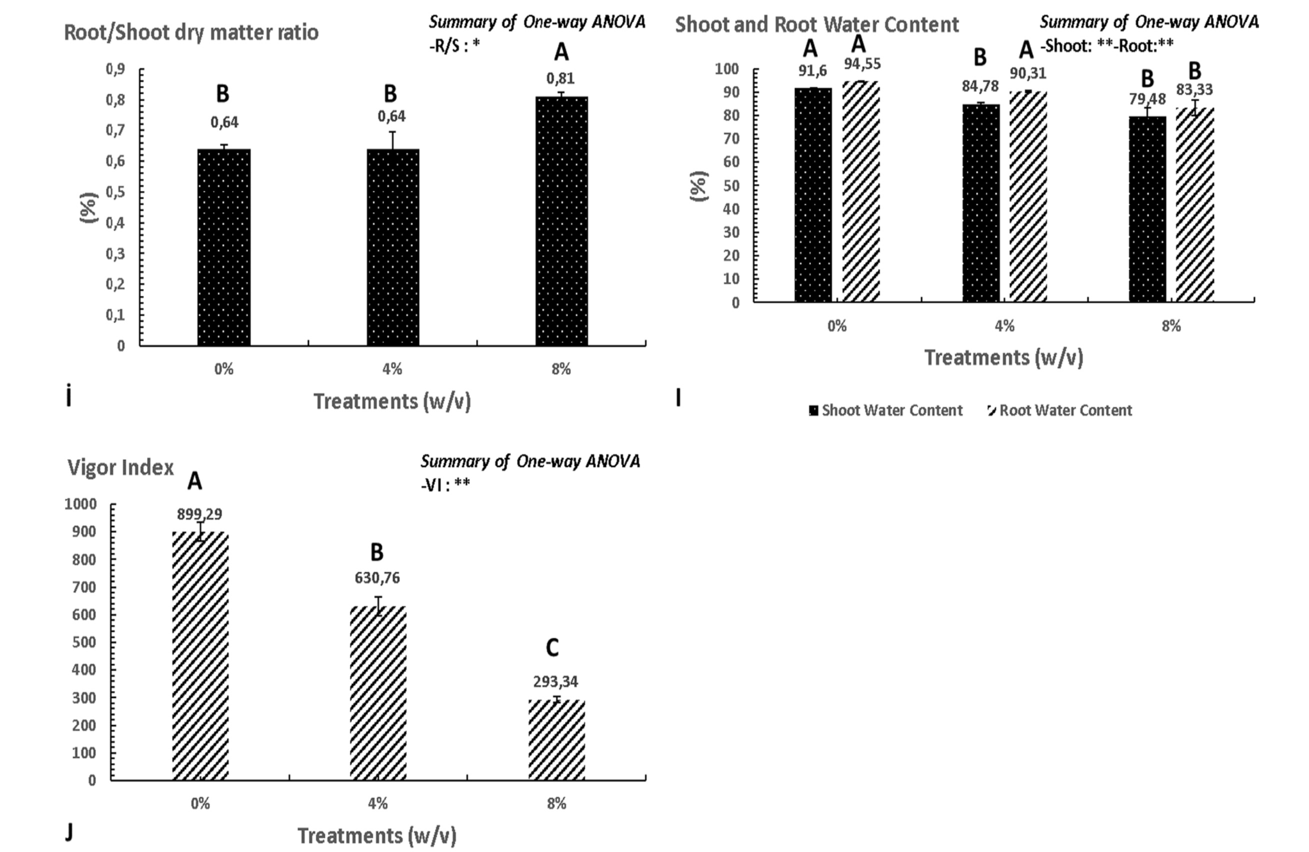

Fig. 2 (continued)

PEG and 8% PEG treatments, respectively. For root water content, 94.55%, 90.31%, and 83.33% values are obtained from control, 4% PEG and 8% PEG treatments, respectively. The PEG treatments had a negative impact ($P < 0.01$) on the *L. corniculatus* seedlings' seedling vigor indices (Fig. 2J). Seedling vigor index decreased due to increasing PEG treatments. The seedling vigor index decreased by 29.86% and 67.38% under 4% PEG and 8% PEG treatments, respectively.

Discussion

The findings of this study show that varying PEG concentrations (0%, 4%, and 8%) have an impact on *L. corniculatus* seed germination and growth when osmotic stress conditions are present (Fig. 2). According to research (Jacome-Blasquez et al. 2016), the PEG has been widely utilized to imitate the effects of drought, particularly on seed germination. The essential stage of a plant's life cycle is germination, which is influenced by a number of elements, particularly hormones, light, temperature, and moisture availability (Ahmed et al. 2022). In this study, it was observed that the germination parameters (germination percentage, mean germination time, and germination rate index) gave different responses to the applied PEG treatments (Fig. 2A–C). It was observed that the germination percentage and germination rate index increased and the mean germination time was shortened in 4% of PEG treatment. On the other hand, in the 8% of PEG treatment, it was observed that the germination percentage and germination rate index decreased, and the mean germination time was shortened a little. According to these results, it was observed that 4% of PEG treatment had a positive effect on germination parameters, while 8% of PEG treatment had a negative effect on germination parameters. PEG (especially, 6000 is more preferred for seeds due to its non-toxicity, large molecule, and not penetrating the seed while reducing the water potential) is an osmotic substance and/or agent such as mannitol, glycerol, and sucrose used in osmopriming studies, and it is reported that it can have positive effects on germination at low doses (Li et al. 2013; Aydınoglu 2019), while it can have negative effects at high doses (Wang et al. 2009; Boureima et al. 2011; Yousefi et al. 2020; Ahmed et al. 2022). A low concentration PEG solution can be employed as an osmotic regulator to control the level and status of water absorption in cells. This can stabilize water absorption in seeds, which will increase their germination and tidy rate

(Jia et al. 2020; Huo and Ma 2022). Therefore, according to the results of this study, 4% PEG treatment may be considered as low dose PEG for *L. corniculatus* and it can be speculated that the positive effect on germination is due to this reason. However, the osmotic potential created by 8% of PEG treatment can be considered as the threshold value at which the positive effect on the germination of *L. corniculatus* begins. Similarly, Aydınoglu (2019) reported that osmopriming application with PEG_{6000} eliminated the negative effects of abiotic stress factors such as salt and drought on germination in different forage crops such as *Vicia sativa, Vicia narbonensis*, *Vicia pannonica, Pisum sativum sp. arvense*. In addition, parallel to the results of this study, Li et al. (2013) noted that low-dose (5%; − 0.3 MPa) PEG_{6000} had a positive effect on germination characteristics of endemic desert plants (*Eremosparton songoricum*), while high-dose (15% PEG; − 0.9 MPa and 30% PEG; − 1.8 MPa) PEG_{6000} had a negative effect on germination characteristics. Hou and Ma (2022) reported that medicinal liquorice (*Glycyrrhiza*) species' seed germination was encouraged by low PEG concentrations (5%), while it was prevented by high quantities (25% and 30%) in all three species of medicinal liquorice.

The findings of this study indicate that the in vitro growth of *L. corniculatus* is affected by osmotic stress conditions. It was observed that seedling growth parameters such as shoot and root lengths, root shoot ratio (except, 4% PEG), and fresh and dry weights of shoot and root decreased significantly ($P < 0.01$) with increasing PEG concentrations. (Fig. 2D–G). Similar findings were made in earlier studies by Ahmed et al. (2022), who found that lower osmotic potential (10% and 20% PEG_{6000}) during drought conditions had a negative impact on sesame seedling characteristics. When the highest PEG concentration (8%) was compared with the control group, in terms of length, fresh weight and dry weight, it was observed that there was a greater decrease in the root (72.33%, 88.31% and 64.28%, respectively) than shoot (58.07%, 67.85% and 23.40%, respectively). The root is one of the organs that first encounters the stress factor and is most affected by the negative effects of the stress factor. Therefore, this situation seen in the root is inevitable. On the other hand, under PEG_{6000}-induced drought stress, it has been reported that the shoots of the seedlings decreased more than the roots in terms of length, fresh and dry weight in some other crops (Kafi et al. 2005; Badr et al. 2020; Ahmed et al. 2022). For the selection of species that can withstand droughts, the root system is regarded as a crucial factor (Yousefi et al. 2020). *L. corniculatus* is known as a moderately drought-tolerant forage crop (Bao et al. 2014; Ünlüsoy et al.2022). The results of the research showed that the root system of *L. corniculatus* cultivar "Leo" was negatively affected (by about 50%) by 4% PEG concentration, which can be considered as a low dose. However, there is a loss of up to 75% in the growth parameters of the root (length, wet weight and dry weight), although it is counted as low at a rate of 8% in the drought studies created with PEG. Therefore, in general, in light of the above data, it has been observed that the root system of *L. corniculatus* cultivar "Leo" is not strong enough to protect the shoot and its own system from the negative effects of drought, even at low concentrations of osmotic potential. In the results of the study, an increase in length was observed at 4% PEG concentration (Fig. 2E) in root shoot ratio, but this was interpreted as due to the low-dose inducing effect of PEG. However, similar to the above results, decreases in both shoot and root growth parameters were reported in various plants due to increased PEG concentrations (Wang et al. 2009; Yousefi et al. 2020; Tong et al. 2021; Ahmed et al. 2022).

Under stress conditions such as drought, biomass distribution between the organs (shoot and root) can be different. According to Kage et al. (2004), plant productivity under drought stress conditions is significantly correlated with dry matter partitioning and biomass disposal. To maximize root soil penetration, assimilate distribution is changed, increasing the root-shoot dry matter ratio (R/S) under drought stress (Luo et al. 2020). The results of this study showed an increase in both shoot and root dry matter with increasing PEG concentration (Fig. 2H), however, there was no difference in dry matter ratio between control and 4% PEG, but a significant increase between 8% PEG (Fig. 2İ). Similarly, Piwowarczyk et al. (2014) reported that the dry matter ratio in shoots of *Lathyrus* increased due to increasing PEG_{6000} concentrations (50, 100 and 150 g/l) in MS medium. Dehydration and the synthesis of new materials such as lipoxygenases (Tyagi et al. 1995), free polyamines (Xiong et al. 2006) and proline (Tyagi et al. 1999) needed to maintain higher osmoticum levels necessary to sustain water absorption are two processes that are associated with the increasing buildup of dry biomass (Soni et al. 2011). In our results, it was observed that the water content of both shoots and roots decreased significantly ($P < 0.01$) due to increased PEG levels. (Fig. 2I). Therefore, the decrease in water content in both organs can explain the increase in both shoot and root dry matter ratios. However, the increase in root shoot dry matter ratio at 8% PEG can be interpreted as the assimilates formed as a result of photosynthesis are shifted to the root in greater amounts in order to strengthen the root under drought stress and the tolerance against the stress factor is tried to be increased. Similar to the data presented above, Inostroza et al. (2015) reported that drought stress decreased the dry matter content of *L. corniculatus* cultivars in their greenhouse study, however, Luo et al. (2020) noted that water restriction decreased the dry matter ratio in alfalfa. The main effects of stress are a decrease in water content and damage to cellular membranes, which help the body deal with drought stress (Zhou et al. 2012). According to Al-Khayri and Al-Bahrany (2004), water stress caused by increased PEG concentrations is inversely proportional to the water content in plant tissues. Similar to this comment, the results of this study showed that the water content of both shoots and roots decreased under increased PEG concentrations (Fig. 2I). It is seen that this decrease (13.23%) is more in shoot when compared to control with high PEG level (8%). The seedling vigor index is one of the important morphological parameters that provide important information such as drought resistance and plant growth in plants under drought stress and needs to be calculated. According to the results of the current study, the seedling vigor index of *L. corniculatus* cultivar "Leo" decreased significantly ($P < 0.01$) in increased PEG concentrations (Fig. 2J). It was observed that this decrease was very high in 4% and 8% PEG (29.86% and 67.38%, respectively), which were considered very low concentrations in the creation of drought stress. Germination percentage (Fig. 2A), the shoot and root length (Fig. 2D) are used to calculate the seedling vigor index. While germination percentage is not affected much by PEG concentrations, it has been determined that shoot length and root length are affected very seriously. Therefore, the decrease in seedling vigor index in *L. corniculatus* cultivar "Leo" can be explained in this way. Similarly, Tong et al. (2021) reported that the seedling vigor index of white clover (*T. repens* L.), another important legume forage crop, was seriously affected and decreased even under light drought stress induced by low dose PEG (10%). In addition, Özkurt et al. (2019) reported that drought stress caused a decrease in seedling vigor index in different alfalfa (*M. sativa* L.) cultivars.

Conclusions

In vitro osmotic stress caused by polyethylene glycol had an impact on seed germination and early seedling growth in the *L. corniculatus* L. cultivar "Leo". Low PEG (4%) had a positive effect on germination. On the other hand, applied PEG concentrations (especially 8% PEG) had a very strong negative effect on growth. Above-ground biomass (calculated by averaging the decreases in shoot length, shoot fresh and shoot dry weight) decreased by 33.72% under 4% PEG treatment and by 49.77% under 8% PEG treatment. However, under-ground biomass (calculated by averaging the decreases in root length, root fresh and root dry weight) decreased by 47.00% under 4% PEG treatment and 64.97% under 8% PEG treatment. Therefore, when the underground and above-ground biomass is evaluated cumulatively, the total biomass of *L. corniculatus* cultivar "Leo" decreases by 57.37% at 8% PEG (-0.1 MPa). Changes in physio-biochemical properties and molecular studies can be evaluated in a future study to better understand the mechanism of water stress tolerance of *L. corniculatus*.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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