

## Research Paper

## Drought stress effects on *Lathyrus* seedlings: A morpho-physiological and molecular study

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### ABSTRACT

The experiment aimed to investigate the impact of polyethylene glycol (PEG) treatments, specifically 0% (control), 15% (PEG-I), and 20% (PEG-II), on various seed quality traits, proline content, and morpho-physiological traits at both the seed (9 days) and seedling stage (18 days) in five *Lathyrus* genotypes and to relate the ISSR markers based molecular characterization of these genotypes osmotic stress. The results unveiled substantial phenotypic and molecular diversity among the accessions concerning morpho-physiological and seed quality traits. Ratan variety exhibited promise as it demonstrated a higher germination percentage (G%) at 62.78±2.42% for PEG-I and 13.33±0.10% for PEG-II, along with a lower mean germination time (MGT) of 3.80±0.071 days for PEG-I and 7.33±0.16 days for PEG-II. P-24 showcased the highest seed vigour index-I (SVI-I) at 872.06±27.02 for PEG-I, whereas Nirmal recorded the highest SVI-I at 80.03±8.53 for PEG-II. Notably, Nirmal displayed the lowest proline content, expressed as µmol per gram of fresh weight (52.45±6.27 units for PEG-I and 111.37±4.75 units for PEG-II) at the seed stage. For the seedling stage, Ratan exhibited the lowest proline content, expressed as µmol per gram of fresh weight (14.18±1.09 units for PEG-I and 10.67±0.851 units for PEG-II). Prateek demonstrated higher seedling length (42.00±1.44 cm for PEG-I and 45.88±0.30 cm for PEG-II), as well as root length (14.11±0.84 cm for PEG-I and 21.29±1.59 cm). Principal component analysis (PCA) highlighted that the most influential variables contributing to the highest variability in the PCA included SVI-I, MGT, and seedling length. Molecular characterization employing ISSR markers resulted in three clusters: CI (Ratan), CII (Pusa-24 and Mahateora), and CIII (Prateek and Nirmal), based on the genetic similarity coefficient. These genotypes exhibit the potential to be recommended and utilized in breeding programs for developing drought-tolerant *Lathyrus* germplasm.

**Key words:** *Lathyrus*, Polyethylene glycol, Primary root length, ISSR marker, Polymorphism

### INTRODUCTION

Grass pea (*Lathyrus sativus* L.), an annual legume crop with a remarkable ability to withstand drought, harks back to the Neolithic era and falls within the Fabaceae subfamily Papilionoideae. Cultivated for purposes such as animal feed (Parsa *et al.* 2023), human consumption, and bioethanol production (Tesfaw *et al.* 2021), it stands as an underutilized yet cost-effective protein source, boasting protein levels ranging from 18% to 34% in seeds and 17% in mature leaves (Lambein *et al.* 2019). These values surpass those of lupine (*Lupinus albus* L.) (32%), faba bean (*Vicia faba* L.) (24%), and field pea [*Pisum sativum* subsp. *arvense* (L.) Asch] (23%)

(Tamburino *et al.* 2012), underscoring its potential as a valuable substitute for high-protein legumes like beans and peas that thrive in more hospitable environments. This legume offers notable dietary benefits with its high digestibility, mineral, and fat content, comparable to faba beans and field peas. However, its cultivation and consumption are constrained due to the presence of β-N-oxalyl-L-α, β-diaminopropionic acid (β-ODAP or BOAA), a neurotoxin that causes neurolathyrism in humans and animals.

Abiotic stress profoundly influences and intricately connects molecular, cellular, morphological, and physiological changes in

plants. Notably, drought stress often attracts other biotic stresses, as well as ionic and salinity stress, unsettling critical processes such as cell division, plant growth, yield, and overall crop productivity (Thanmalagan *et al.* 2022). Plants experiencing drought stress tend to amass reactive oxygen species (ROS) and osmolytes, including Proline. Additionally, they undergo reduced vacuolar and cytosolic volumes, activate stress response genes, and exhibit diminished photosynthetic rates, delayed flowering, stomatal closure, low turgor pressure, and decreased transpiration rates (Zia *et al.* 2021). Prolonged exposure to drought can lead to the disruption of cell membrane integrity, nitrogen metabolism, and protein synthesis (Wasaya *et al.* 2022).

Lathyrus demonstrates unique abilities such as nitrogen fixation (Mekonen *et al.* 2022), temperature tolerance (Das *et al.* 2021), drought tolerance (Parsa *et al.* 2023), and the ability to thrive in poor soil conditions (Assefa and Bitew 2023). When incorporated into the soil, Lathyrus functions as an exceptional green manure, generating substantial quantities of nitrogen and biomass (Jeromela *et al.* 2017). In comparison to other legumes, Lathyrus exhibits specific physiological and morphological traits that contribute to its drought tolerance, including an extensive and deep root system, stems with winged margins, narrow leaves, and high proline content (Kong *et al.* 2022). These characteristics enable the plant to extract significant amounts of water from diverse soils, allowing it to withstand severe drought stress. Consequently, Lathyrus can be viewed as a robust plant species within dryland systems (Carr *et al.* 2021). However, despite these xerophytic traits, instances of drought stress during early establishment and post-flowering stages can significantly diminish Lathyrus' seed yield and overall crop growth (Gusmao *et al.*, 2012, Kong *et al.* 2015).

Germination, critically impacted by drought stress, is essential for transitioning seeds to seedlings and affects crop yields (Guo *et al.* 2022). This process involves water absorption, activation of growth pathways, and differentiation into shoot and root systems. Drought significantly lowers germination rates and seedling growth due to their dependence on adequate moisture (Fu *et al.* 2022).

In laboratory settings, conditions of drought stress can be emulated by treating seeds with a polymeric osmolyte, Polyethylene glycol-6000 (PEG-6000). PEG-6000, characterized by its high molecular

weight, inert nature, and non-toxic properties, allows for the generation of varying degrees of osmotic stress by adjusting the concentrations of PEG-6000 solutions. The high molecular weight of PEG-6000 induces water stress conditions akin to those brought about by dry soil. This method finds frequent application in the screening of genotypes for drought tolerance during the initial growth stage. PEG-6000 restricts seed growth and germination by reducing water potential. Several studies have highlighted the efficacy of *in vitro* screening using PEG as one of the most reliable methods for selecting drought-tolerant genotypes based on germination indices (Ahmad *et al.* 2022, Mas-ud *et al.* 2022). Seed treatment with PEG-6000 within a dose range of 2.5 to 52.5 mM, and a treatment duration of 4 to 168 hours post-imbibition, can stimulate enhanced tolerance and growth in crops. One plausible mechanism behind PEG treatment lies in its capacity to limit seed water uptake, thus slowing down the progression of germination-related mechanisms. This mechanism is believed to protect proteins and nucleic acids from potential damage during the transition of seeds from a dehydrated to a rehydrated state (Tabatabai *et al.* 2022). However, higher doses of PEG can significantly diminish the potential for seed germination. Moreover, PEG-treated seeds exhibit heightened antioxidant potential and the accumulation of compatible osmolytes (Binodh *et al.* 2023).

DNA markers, specifically Inter Simple Sequence Repeat (ISSR) markers, are crucial in evaluating various legumes for cultivar enhancement and characterization. ISSR markers, ranging from less than 100 bp to 300 bp, are situated between similar microsatellite regions or simple sequence repeats (SSRs) and typically include short tandem repeats (STRs) from 16 bp to 25 bp oriented oppositely (Lalrinmawii *et al.* 2023). These markers combine features of amplified fragment length polymorphism (AFLP) and microsatellites, offering the broad applicability of Random Amplified Polymorphic DNA (RAPD). They provide specific, efficient assessments without requiring primer sequence knowledge, making them highly useful in evolutionary biology, genome mapping, gene tagging, and studies of genetic diversity and phylogeny. Additionally, ISSR markers have proven valuable in identifying molecular markers for drought tolerance in lathyrus cultivars (Younis *et al.*, 2020; Das *et al.*, 2021).

Based on this background, the present study was designed to evaluate the early establishment,

screening, and assessment of five Lathyrus pea genotype seeds under varying drought stress conditions, induced by different polyethylene glycol (PEG) concentrations. Molecular characterization related to osmotic stress in these genotypes was subsequently conducted using ISSR markers. The study hypothesized that the tested genotypes would manifest variations in growth and drought tolerance traits. It was further postulated that an increase in negative osmotic potential could potentially lead to a reduction in seedling and seed germination traits. The results of this investigation will aid in the selection of the most resilient genotypes for breeding purposes, contributing to the development of drought-tolerant genotypes in the future.

## MATERIALS AND METHODS

### *Plant materials*

The present study was carried out using five Lathyrus genotypes viz., Mahateora, Pusa-24, Nirmal, Ratan, and Prateek were collected from the Department of Seed Technology, ICAR-Indian Grassland and Fodder Research Institute, Jhansi (Table 1).

### *Seedling stage experiment*

The seeds underwent surface sterilization using 1% (wt/vol) sodium hypochlorite for 15 minutes, followed by three rinses with distilled water. Subsequently, they were placed in 9 cm diameter Petri plates at a quantity of 50 seeds per plate for germination. The growth process spanned nine days, maintaining ambient room temperature, within three distinct PEG treatments, namely 0% (utilizing distilled water as the control, corresponding to 0.0 Mpa osmotic potential), 15% (equivalent to -0.30 MPa osmotic potential referred to as PEG-I), and 20% (equivalent to -0.49 MPa osmotic potential referred to as PEG-II) (Michel and Kaufmann 1973). Each Petri dish was supplemented with 5 mL of the corresponding solution, either PEG for inducing drought stress or distilled water for the control. An incubation period of 9 days was allocated for the assessment of germination percentage (G%). The count of germinated seeds was recorded daily for 9 days. Selected seedlings from all the treatments, exhibiting synchronized growth, were transplanted onto germination paper sheets immersed in distilled water for an additional nine-day period. The experimental setup followed a completely randomized design (CRD) incorporating three treatments and three

replications for each treatment across the five Lathyrus genotypes. Each plant was considered a single replicate, with traits being measured on an individual basis. The germination paper sheet with the transplanted seedlings was meticulously folded and further formed into a cylinder. This paper cylinder, containing the seedlings, was enveloped in a piece of polythene sheet secured with rubber bands. Careful attention was paid during the placement of the seedlings to ensure approximately 2.0 cm of the germination paper remained exposed to the exterior surface, with the upper end of around 1.0 cm placed within the polythene cover. These cylinders, housing the seedlings, were subsequently positioned in elongated plastic beakers, with their ends immersed in distilled water. The plants received adequate moisture through capillary action within the germination paper. The beakers containing the cylinders were maintained under controlled conditions at 25±2°C, 75% humidity, a photoperiod of 16 hours of light (with an intensity of 2000 lux), followed by 8 hours of darkness. The germination paper encircling the roots was kept moist by spraying water twice daily. After 18 days from the initiation of planting, the seedlings were carefully removed from the germination paper, ensuring the preservation of all lateral roots and root hairs.

### *Data collection*

Observations of morpho-physiological traits were recorded at regular intervals after seed sowing, following the application of PEG treatment through distinct destructive methods. Specifically, a total of three individual plants from each genotype-treatment combination were selected for observation 18 days after the application of PEG treatment, concerning the traits of interest.

### *Germination percentage (G%)*

Seed germination was recorded daily up to 7 days after the initiation of the experiment. A seed was considered germinated when the radicle emerged to ~2mm. Ten seedlings were selected randomly from the Petri dish and their mean was considered as sample data. The G % was calculated by the following formula as the cumulative number of germinated seeds with normal radicles.

$$\text{Germination \%} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds planted}} \times 100 \text{ Eq. 1}$$

### Mean germination time (MGT)

MGT in days was calculated according to Ellis and E.H (1980). It is the reciprocal of the rate of germination and indicates the germination performance of seeds.

$$MGT = \sum D(n) / \sum n \quad \text{Eq. 2}$$

Where:

n: is the number of seeds germinated on day (D)

D: is the number of days counted from the beginning of the germination test

Seed vigour index-1 (SVI)

SVI-I was calculated by the formula given by Abdul-Baki and Anderson (1973).

$$\text{Seed vigour index - 1} = \text{Germination \%} \times \text{Seedling length (cm)} \quad \text{Eq.3}$$

### Root and shoot length

The length of both the shoot and root of the seedlings was measured in centimeters (cm). For seedlings subjected to PEG treatment, root length was measured 18 days after treatment. The fresh weight of the seedlings was determined using a weighing balance and recorded in grams (gm), with an average value calculated from ten plants. To assess the dry weight, the seedlings were placed in a hot air oven set at 100°C for 24 hours and then weighed. An average value, obtained by averaging the measurements from ten plants, was recorded for each treatment. These procedures were repeated three times to ensure accuracy and reliability.

### Proline content

The proline content in both germinating seeds and seedlings was assessed using the acid ninhydrin reagent method as detailed by Bates *et al.* (1973). Proline content was quantified and expressed in  $\mu\text{mol}$  per gram of fresh weight. Initially, 250 mg samples were meticulously ground and homogenized in 5 ml of a 3% (w/v) aqueous solution of sulphosalicylic acid. Subsequently, the homogenate was transferred to 10 ml Falcon tubes and centrifuged at 16,000 g for 15 minutes. The resulting supernatant served as the enzyme extract and was then transferred to fresh test tubes. To 3 ml of this extract, precisely 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid were added. The solution was vigorously vortexed for 15-20 seconds and incubated in a water bath at 100°C for 1 hour to initiate the reaction. Afterwards, the tubes were removed from the hot water bath and placed in

an ice bucket for 5 minutes. Next, 4 ml of toluene was introduced to the mixture and vortexed for an additional 15-20 seconds. This process facilitated the separation of the chromophore-containing toluene from the aqueous phase. Subsequently, the absorbance of the toluene phase was measured at 520 nm using a spectrophotometer. Readings were obtained for the blank solution (lacking sample extract), and the blank's absorbance was subtracted from that of the sample absorbance. The proline concentration was then calculated on a fresh weight basis using a standard curve.

### DNA Extraction and PCR Amplification

Genomic DNA extraction was conducted on young green leaves employing the CTAB (cetyltrimethylammonium bromide) method with slight adjustments, as outlined by (Tiwari *et al.* 2017). Subsequently, the purified DNA was subjected to quality and quantity assessment via 0.8% agarose gel electrophoresis. To determine DNA concentration, uncut  $\lambda$  genomic DNA with known concentrations (50, 100, and 150 ng/ $\mu\text{l}$ ) was utilized to generate standard working DNA solutions in the range of 30-40 ng/ $\mu\text{l}$ . The diluted DNA was employed for amplifying ISSR markers within an Eppendorf thermocycler ([www.eppendorf.com](http://www.eppendorf.com)). The PCR reaction was set up in a 25 $\mu\text{l}$  volume, comprising 40 ng of genomic DNA, 200 pg of primer, 0.1 mM dNTPs, 1 $\times$ PCR buffer (containing 10 mM Tris, pH 8.0, 50 mM KCl, and 50 mM ammonium sulfate), 1.8 mM MgCl<sub>2</sub>, and 0.2 units of Taq DNA polymerase. The cycling conditions encompassed an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C, primer annealing at 50°C, and primer extension at 72°C, each lasting for 30 seconds. A final extension at 72°C for 10 minutes concluded the PCR process, and the PCR products were stored at 4°C. The resulting PCR products were then separated through electrophoresis in 2.0% agarose gels in 1 $\times$ TBE (Tris/Borate/EDTA) buffer, and subsequently visualized by staining with ethidium bromide. Gel images were captured using the Syngene Geldoc system ([www.syngene.com](http://www.syngene.com)). The allelic variations, depicted as banding patterns, were examined among five Lathyrus genotypes, utilizing a panel of 30 ISSR markers.

### Molecular analysis

The ISSR profiles were scored based on the size of fragments amplified across all five Lathyrus genotypes. The genetic diversity, polymorphism information content, and genetic distance-based

clustering were performed with the Unweighted Pair Group Method for the Arithmetic average (UPGMA) tree using Power Marker v3.25 (Liu and Muse 2005) and the dendrogram was constructed using MEGA 4.0 software (Kumar *et al.* 2008).

### Statistical analysis

Unless otherwise specified, all analyses were performed in triplicate to ensure data consistency. A significance level of 95% was chosen to determine statistical significance. Hierarchical cluster analysis (HCA) was conducted using the squared Euclidean distance and the Ward clustering algorithm, utilizing a trial version of IBM SPSS version 19. The correlation among the assessed parameters was assessed using the Pearson correlation coefficient. This correlation study was conducted at a 5% significance level using Jamovi version 1.2.27. To evaluate the distinctions between the assessed attributes, a one-way analysis of variance (ANOVA) was employed. Subsequently, a posthoc test, specifically Duncan's multiple range test, was executed with a trial version of IBM SPSS to assess mean differences at a significance level of  $p < 0.05$ . Principal component analysis (PCA) was applied to the data using Jamovi version 1.2.27 to elucidate observed variances and reveal potential relationships between the principal components. To enhance the loading of a parameter along the component axis and to gain insights into the pattern of a specific parameter, an orthogonal varimax rotation was applied to PCA. Furthermore, a heatmap illustrating Pearson's correlation coefficients and relative composition was generated using a web interface, MetaboAnalyst (Chong *et al.* 2019).

## RESULTS AND DISCUSSION

The exceptional agronomic potential of various Lathyrus species, particularly *Lathyrus sativus* L., is attributed to their superior tolerance to drought, salinity, and flooding, setting them apart from other leguminous plants. Considering the intricate physiological mechanisms in plants, research on legumes, such as Lathyrus, can provide insights into specific mechanisms governing resistance and tolerance to abiotic stresses (Savita *et al.* 2020). Establishing a robust early crop is one of the pivotal factors for achieving higher crop yields. Optimal crop establishment directly correlates with achieving the most suitable plant population in the field. In this context, *in vitro* studies play a crucial role in assessing the impact of changing external environments on early crop establishment

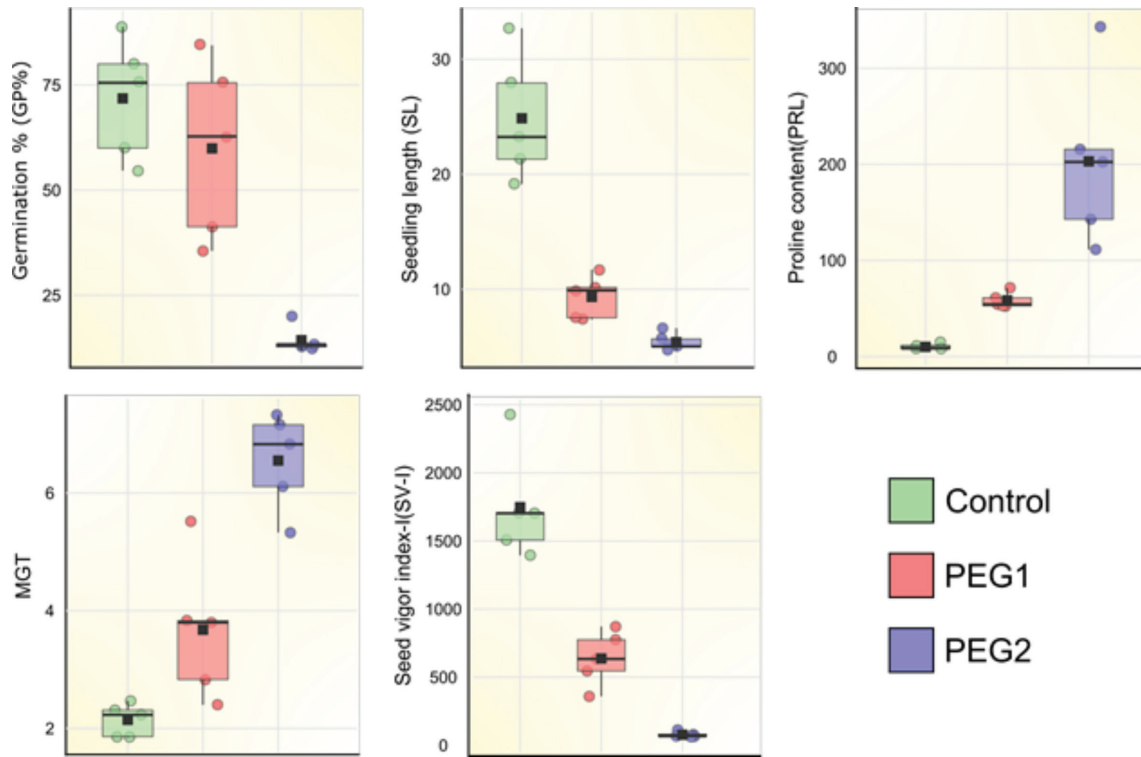
and growth. Provoking drought stress presents no challenge, as the use of high molecular weight, inert, and non-penetrating PEG effectively induces osmotic stress in plants (Zhang and Shi 2018). Despite this, *in vitro* studies focused on evaluating the effects of water stress on the early establishment of Lathyrus, particularly concerning seed quality, stress-related traits, and morpho-physiological characteristics, have received limited attention thus far.

Within the current study, all measured parameters concerning germination and seedling growth exhibited a significant reduction in response to the osmotic stress induced by the PEG treatments. It is well-recognized that seed germination and early seedling development represent the most critical stages influenced by drought stress. Our findings underscored a substantial inhibition of seed quality and seedling-related traits under osmotic stress across all assessed Lathyrus genotypes. Additionally, the proline content exhibited a significant increase under PEG treatment in all Lathyrus genotypes. The adverse impact of drought stress on these traits has been extensively demonstrated in numerous crops, including corn (Badr *et al.* 2020), wild almonds (Gholami *et al.* 2010), various grass species such as *Themeda triandra*, *Heteropogon contortus*, and *Antheophora pubescens* (van den Berg and Zeng 2006), kenaf (Tang *et al.* 2019), wheat (Rana *et al.* 2017), barnyard grass (Wu *et al.* 2019), chickpea (Ahmadpour *et al.* 2022), rice (Purbajanti *et al.* 2019), barley (Hellal *et al.* 2018) etc.

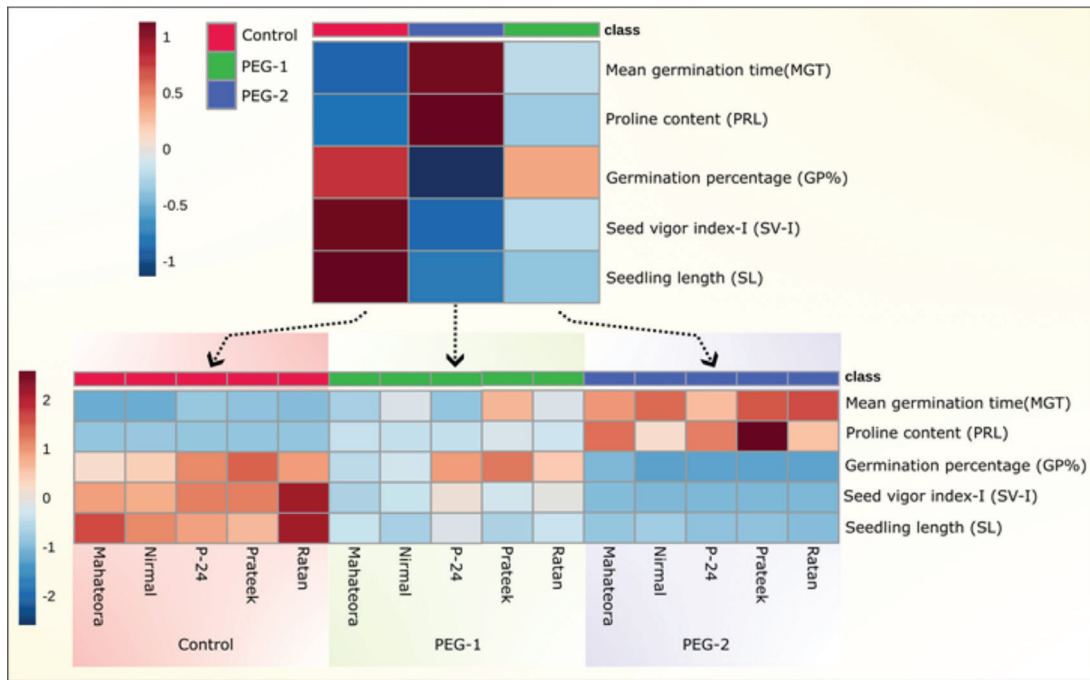
### Seed quality traits

The assessment of seed quality traits, including G%, MGT, and SVI-I, was conducted in five Lathyrus genotypes across three different PEG concentrations, as detailed in Table 2. The corresponding findings were depicted through Box-and-whisker plots in Fig 1, a heat map in Fig 2.

Regarding G% (germination percentage), notable variations were observed among the genotypes. In the absence of PEG stress, G% ranged from  $54.66 \pm 6.11\%$  (Mahateora) to  $88.88 \pm 5.87\%$  (Prateek). Conversely, under PEG 1 (15%) exposure, this range shifted from  $35.55 \pm 2.22\%$  (Mahateora) to  $84.44 \pm 2.22\%$  (Prateek). Under the influence of PEG-II (20%), the range was further constrained, fluctuating from  $12.22 \pm 0.80\%$  (Nirmal) to  $20.00 \pm 1.1\%$  (Mahateora). Notably, the most substantial reduction in G% was observed in PEG-II, followed by PEG-I, in comparison to the control. A study by



**Fig. 1.** Traits like G% (Germination %), SL (Seedling length), Proline content (PRL), MGT (Mean germination time) and SVI-I (Seed vigour index-I) in five Lathyrus genotypes 9 days under three different PEG treatments i.e. 0% (distilled water as control, equivalent to 0.0 Mpa osmotic potential), 15% (equivalent to -0.30 MPa osmotic potential as PEG-I) and 20% (equivalent to -0.49 MPa osmotic potential as PEG-II) represented through Box-and-whisker plots



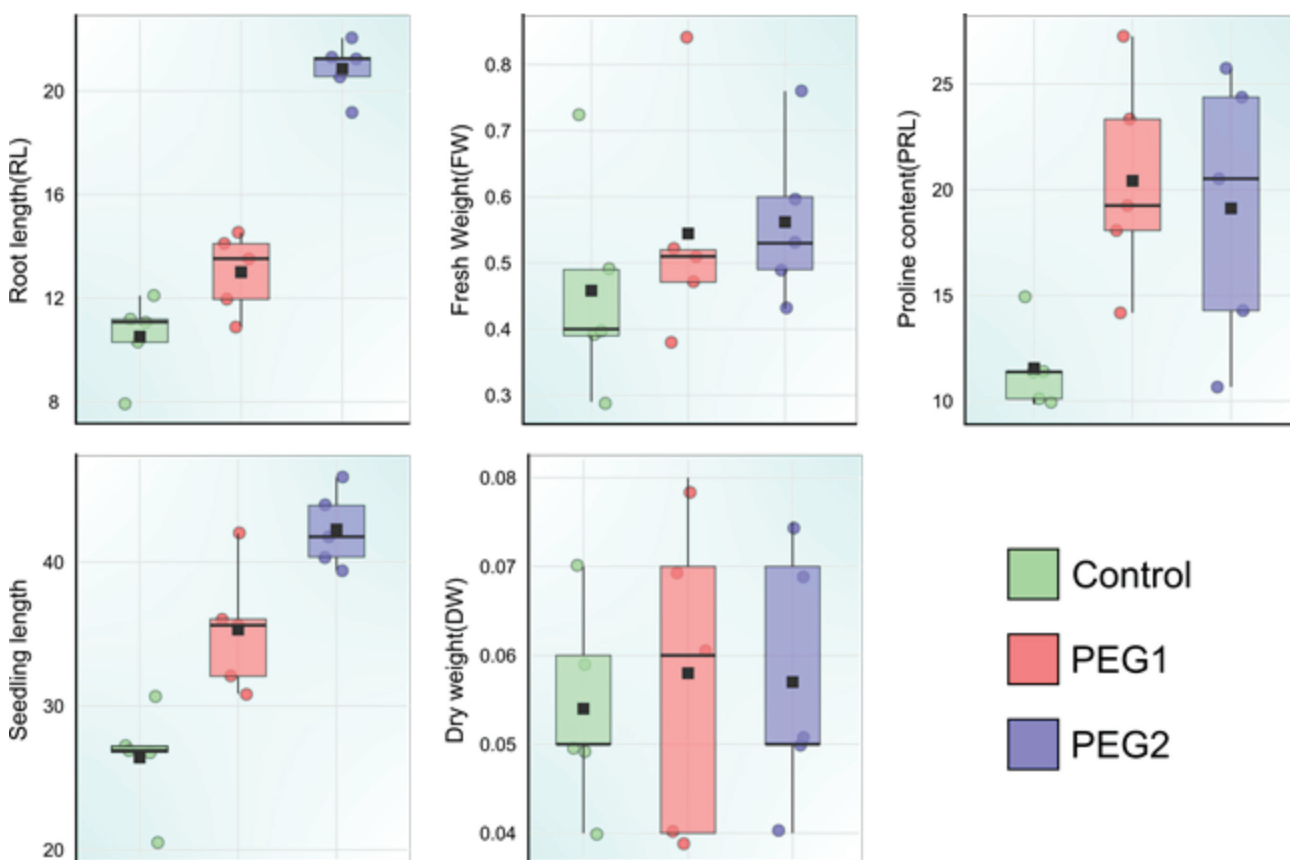
**Fig. 2.** Traits like G% (Germination %), SL (Seedling length), Proline content (PRL), MGT (Mean germination time) and SVI-I (Seed vigour index-I) in five Lathyrus genotypes 9 days under three different PEG treatments i.e. 0% (distilled water as control, equivalent to 0.0 Mpa osmotic potential), 15% (equivalent to -0.30 MPa osmotic potential as PEG-I) and 20% (equivalent to -0.49 MPa osmotic potential as PEG-II) represented through heat map

Kachout *et al.* (2019) supports these observations, reporting a decrease in osmotic potential from -0.7 to -1.0 MPa induced by PEG, leading to a significant reduction in G% from 96% to 84%. Similarly, Tokarz *et al.* (2020) demonstrated reduced seed germination under drought stress, with a decline from 100% in the control to 80% in the presence of 22 mM PEG. Osmotic stress was found to exert a detrimental effect on the seedling growth and germination indices of Lathyrus. The addition of PEG decreased the water potential of osmotic solutions, thereby limiting both water and oxygen availability for germinating seeds. Consequently, the reduced surrounding water potential induced by PEG hindered nutrient and water uptake, thereby impeding cell elongation and division under low turgor pressure. Several studies (Piwowarczyk *et al.* 2014, Tang *et al.* 2019) have highlighted the impact of dehydration stress on the elevation of reactive oxygen species (ROS) such as hydroxyl ions ( $\cdot\text{OH}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide radical ( $\text{O}_2^{\cdot-}$ ), and singlet oxygen ( $^1\text{O}_2$ ). This increase in ROS results in the oxidative

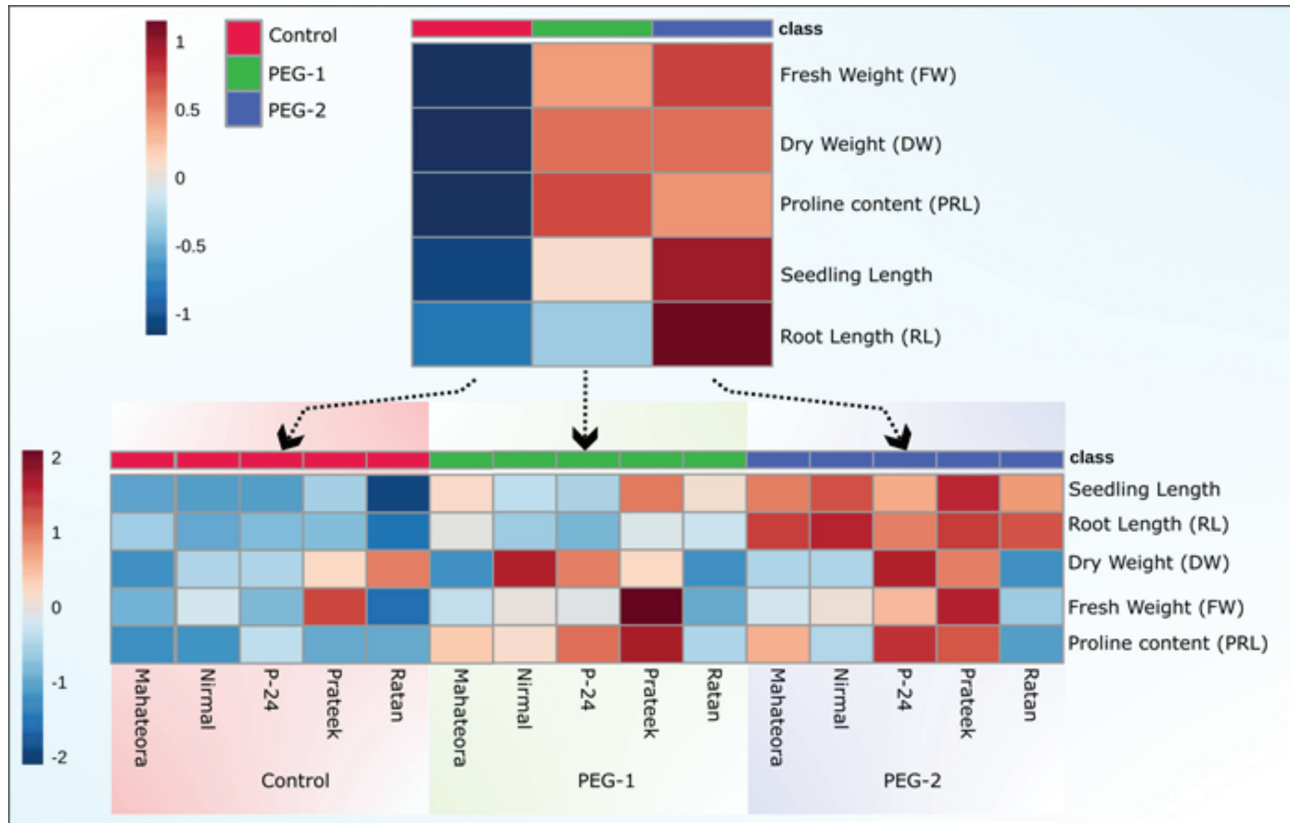
damage of multiple cellular components, including lipids, proteins, RNA, and DNA, thereby leading to reduced germination and seedling vigour.

The Mean Germination Time (MGT), expressed in days, exhibited varying trends among the different Lathyrus genotypes. In the absence of PEG-induced stress, MGT ranged from  $1.85 \pm 0.206$  (Nirmal) to  $2.47 \pm 0.545$  (P-24). Conversely, under the influence of PEG I (15%), this range shifted from  $2.40 \pm 0.187$  (P-24) to  $3.83 \pm 0.422$  (Nirmal). Under the more intense stress of PEG-II (20%), the MGT further extended, fluctuating from  $5.33 \pm 1.01$  (P-24) to  $7.33 \pm 0.16$  (Ratan).

Consistent with these findings, previous studies (Piwowarczyk *et al.* 2014, Gheidary *et al.* 2017) have demonstrated a significant increase in MGT under elevated PEG concentrations. The Mean Germination Time (MGT) serves as a metric for assessing the time required for seeds to germinate, focusing on the specific day when the maximum number of seeds have undergone



**Fig. 3.** The PRL (Proline content), RL (Root length), FW (Fresh weight), seedling length, Dry weight (DW) in five Lathyrus genotypes 18 days under three different PEG treatments i.e. 0% (distilled water as control, equivalent to 0.0 Mpa osmotic potential), 15% (equivalent to -0.30 MPa osmotic potential as PEG-I) and 20% (equivalent to -0.49 MPa osmotic potential as PEG-II) represented through Box-and-whisker plots



**Fig 4.** The PRL (Proline content), RL (Root length), FW (Fresh weight), seedling length, Dry weight (DW) in five *Lathyrus* genotypes 18 days under three different PEG treatments i.e. 0% (distilled water as control, equivalent to 0.0 MPa osmotic potential), 15% (equivalent to -0.30 MPa osmotic potential as PEG-I) and 20% (equivalent to -0.49 MPa osmotic potential as PEG-II) represented through heat map

germination. It effectively measures the rate and spread of germination by computing the day of mean germination (Talská *et al.* 2020). Notably, a lower value of MGT indicates a faster rate at which a seed population achieves germination. The observed pattern of the highest increase in MGT under PEG-II, followed by PEG-I, in comparison to the control results from the inverse relationship between MGT and germination percentage. This pattern highlights the influence of PEG-induced stress on the germination process, causing a delay in the mean germination time as the osmotic stress intensifies.

The Seed Vigour Index-I (SVI-I) exhibited diverse ranges across the *Lathyrus* genotypes under varying PEG concentrations. In the absence of PEG-induced stress, SVI-I ranged from  $1394.57 \pm 89.98$  (Nirimal) to  $2428.73 \pm 95.25$  (Ratan). Conversely, under the influence of PEG 1 (15%), this range shifted from  $359.04 \pm 80.52$  (Mahateora) to  $872.06 \pm 27.02$  (P-24). With the intensified stress of PEG-II (20%), the SVI-I range further contracted, fluctuating from  $62.35 \pm 5.90$  (Ratan) to  $113.62 \pm 3.19$

(Mahateora). Corroborating these findings, Hojjat (2020) similarly reported significant reductions in SVI-I and MGT under various concentrations of PEG (-0.00, -0.27, -0.53, and -0.80 MPa), with the most substantial decrease observed in the PEG treatment corresponding to -0.80 MPa. Seed vigour constitutes a multifaceted trait, encompassing seedling establishment, rapid germination, seed viability, dormancy, and tolerance to ageing, particularly under suboptimal conditions. SVI-I represents an integration of traits governing a seed's potential for robust performance post-sowing. It serves as a measure of the theoretical potential of seeds to exhibit their vital functions, both in unfavourable and favourable environments. Given that PEG hampers seed germination by impeding water and oxygen uptake, it concurrently exerts a negative influence on SVI.

#### Proline content

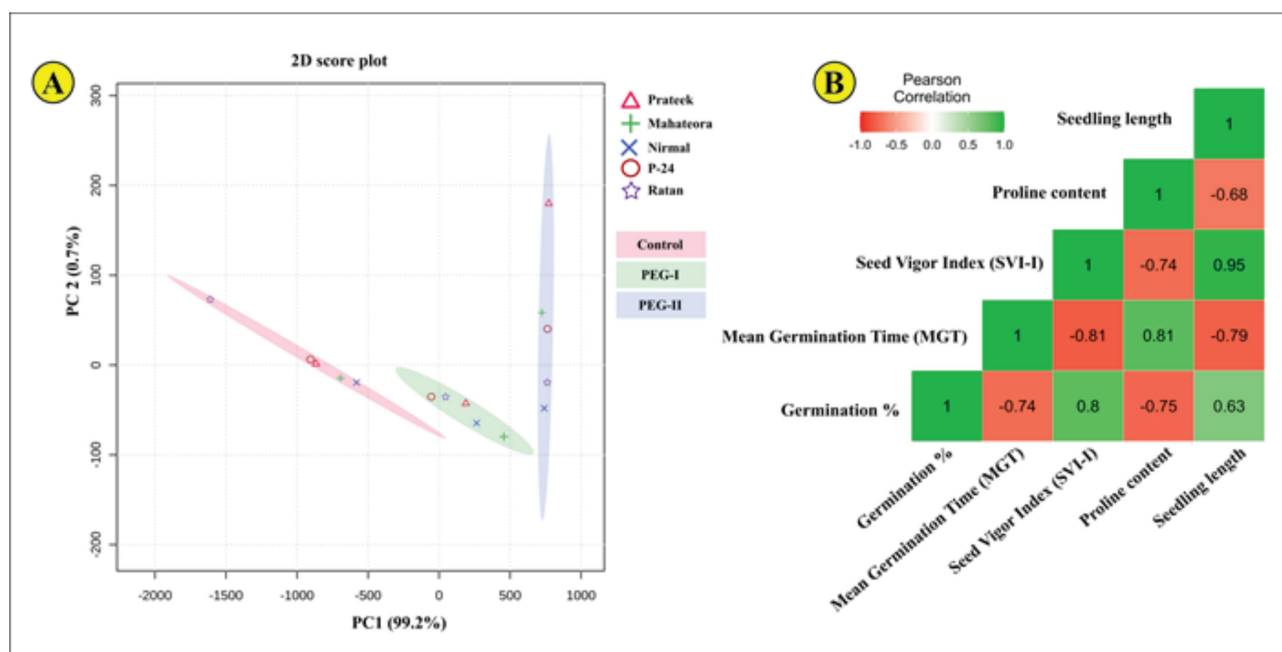
The proline content in five *Lathyrus* genotypes was assessed at three different PEG concentrations for both seed and seedling stages as indicated

in Tables 2 and 3, represented through Box-and-whisker plots in Fig. 1 and 3, heat map in Fig. 2 and 4.

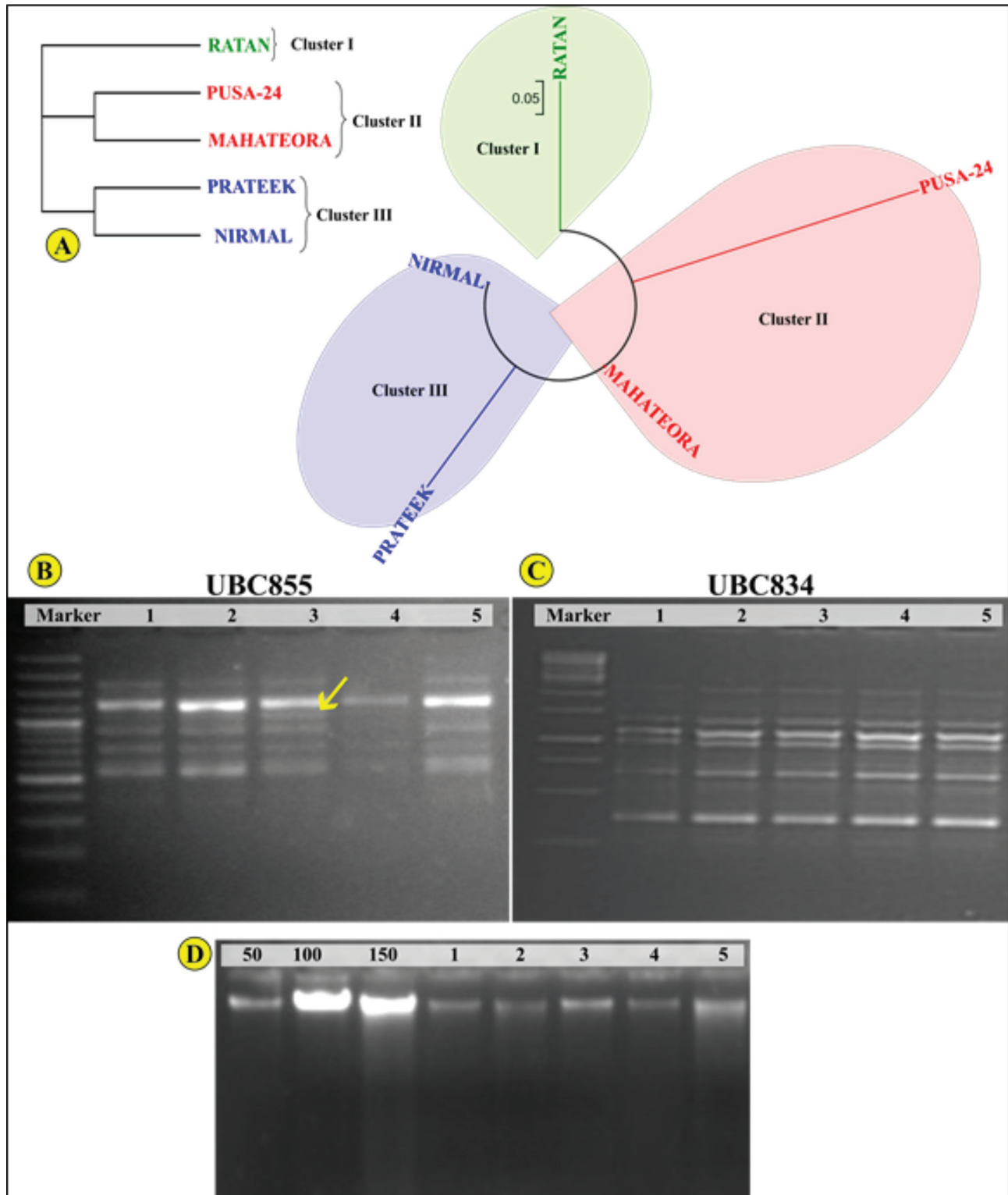
The proline content expressed as  $\mu\text{ mol g}^{-1}$  fresh weight at the seed stage varied from  $7.75 \pm 2.05$  (Ratan) to  $14.92 \pm 1.24$  (Nirmal) for control. From  $52.45 \pm 6.27$  (Nirmal) to  $71.61 \pm 1.86$  (Prateek) in PEG 1 (15%). From  $111.37 \pm 4.75$  (Nirmal) to  $343.14 \pm 46.31$  (Prateek) in PEG-II (20%). The highest increase in proline content was observed in PEG-II followed by PEG-I when compared to the control. Similarly, for the seedling stage, the proline content varied from  $9.94 \pm 1.06$  (Mahateora) to  $14.94 \pm 1.12$  (P-24) for control. From  $14.18 \pm 1.09$  (Ratan) to  $27.26 \pm 1.26$  (Prateek) in PEG 1 (15%). From  $10.67 \pm 0.851$  (Ratan) to  $25.74 \pm 1.62$  (P-24) in PEG-II (20%). The highest increase in proline content was observed in PEG-II followed by PEG-I when compared to the control. Similar results where proline increased under high PEG concentrations were also reported in *Lathyrus* by Piwowarczyk *et al.* (2014), Tokarz *et al.* (2020), Parsa *et al.* (2023), in *Thymus vulgaris* L. by Piwowarczyk *et al.* (2017), Razavizadeh *et al.* (2019).

At the seed stage, the proline content, expressed as  $\mu\text{mol g}^{-1}$  fresh weight, displayed considerable variation among the *Lathyrus* genotypes under different PEG concentrations. In the absence of PEG-induced stress, the proline content ranged from  $7.75 \pm 2.05$  (Ratan) to  $14.92 \pm$

$1.24$  (Nirmal). However, under the influence of PEG 1 (15%), this range shifted from  $52.45 \pm 6.27$  (Nirmal) to  $71.61 \pm 1.86$  (Prateek). With the intensified stress of PEG-II (20%), the proline content range expanded significantly, fluctuating from  $111.37 \pm 4.75$  (Nirmal) to  $343.14 \pm 46.31$  (Prateek). Notably, the most substantial increase in proline content was observed in PEG-II, followed by PEG-I, when compared to the control. Similar findings regarding the increase in proline content under high PEG concentrations were reported in *Lathyrus* by Piwowarczyk *et al.* (2014), Tokarz *et al.* (2020), Parsa *et al.* (2023), as well as in *Thymus vulgaris* L. by Piwowarczyk *et al.* (2017); Razavizadeh *et al.* (2019). During the seedling stage, after the removal of drought stress, the proline content persisted within a range that varied from  $9.94 \pm 1.06$  (Mahateora) to  $14.94 \pm 1.12$  (P-24) under normal conditions. However, in the presence of PEG 1 (15%), this range shifted from  $14.18 \pm 1.09$  (Ratan) to  $27.26 \pm 1.26$  (Prateek). Under the influence of PEG-II (20%), the proline content fluctuated from  $10.67 \pm 0.851$  (Ratan) to  $25.74 \pm 1.62$  (P-24). Notably, even after the removal of stress, the proline content remained higher than that of the control. Surprisingly, it was observed that both PEG 1 (15%) and PEG-II (20%) exhibited almost identical levels of proline content, indicating the persistence of elevated proline levels even after the elimination of the stress factor.



**Fig. 5.** (A) Score plots of PCA represented in two dimensions indicating the distribution of assessed traits in eight diverse lucerne genotypes, clustered statistically by the Ward clustering algorithm and based on their evaluated component loading; (B) Pearson’s *r* values are demonstrated through a heat map



**Fig. 6.** (A) A phylogenetic tree was constructed based on genetic similarity coefficients derived from ISSR markers in five distinct Lathyrus genotypes; (B) The allelic variation demonstrated by the UBC855 and (C) UBC834 ISSR markers is as follows, with M representing the 1 kb ladder marker and numbers 1 to 5 corresponding to the Lathyrus genotypes P1= P-24, 2=Nirmal, 3=Ratan, 4=Prateek, 5=Mahateora; (D) Quantification using standard Lambda DNA (50, 100, 150 ng/ $\mu$ l) of diverse Lathyrus genotypes 1= P-24, 2=Nirmal, 3=Ratan, 4=Prateek, 5=Mahateora on 2% agarose gel.

Proline, a unique proteinogenic amino acid characterized by a five-membered ring housing a protonated  $\text{NH}_2^+$  secondary amine, possesses an atypical cyclic structure that significantly influences the secondary structure of proteins, rendering them notably rigid. Beyond its role as a common constituent of proteins, proline serves diverse and distinct cellular functions. Functioning as a multifaceted osmolyte, this amino acid accumulates in significant concentrations under various abiotic stresses, particularly drought stress. It fulfills several roles, including that of a reactive oxygen species (ROS) scavenger, an osmolyte, a signaling molecule, an antioxidant defense molecule, and a stabilizer of macromolecules and metal chelator (Alvarez *et al.* 2022). Furthermore, during the alleviation of stress, the accumulated proline is catabolized to provide an energy supply for growth. Proline plays

a critical role in maintaining cell turgor and osmotic balance by upholding the integrity of the cell membrane, thereby preventing electrolyte leakage and regulating the production of ROS, effectively curbing oxidative bursts (Raza *et al.* 2023). Our findings reveal heightened proline content under PEG-II, followed by PEG-I, in comparison to the control, during both the seed and seedling stages. These findings are consistent with those of (Piwowarczyk *et al.* 2014), which demonstrate a significant increase in proline accumulation under osmotic stress. The biosynthesis of proline occurs via two distinct pathways, namely the ornithine and glutamate pathways. Under normal conditions, proline biosynthesis predominantly takes place in the cytosol; however, under conditions of drought stress, proline synthesis also occurs within the chloroplast (Ben Rejeb *et al.* 2014).

**Table 1.** Detailed information on five diverse Lathyrus genotypes.

S.No.	Genotype	Year of release	ODAP %	Adaptation area
1	Pusa 24	1974	0.20	Madhya Pradesh, Maharashtra, Chhattisgarh
2	Nirmal	1980	0.20	West Bengal
3	Ratan [Bio L212]	1997	0.06	Madhya Pradesh, Maharashtra, Bihar, Chhattisgarh, Orissa, West Bengal
4	Prateek [LS 157-14]	2006	0.08	Madhya Pradesh, Maharashtra, Chhattisgarh
5	Mahateora [RLS 4595]	2008	0.08	Madhya Pradesh, Maharashtra, Chhattisgarh

**Table 2.** Seed quality traits at 9 days under three different PEG treatments i.e. 0% (distilled water as control, equivalent to 0.0 Mpa osmotic potential), 15% (equivalent to -0.30 MPa osmotic potential as PEG-I) and 20% (equivalent to -0.49 MPa osmotic potential as PEG-II)

Genotype	Germination percentage %	MGT	SVI-I	Proline content	Seedling length
<b>Control (0.0%)</b>					
Mahateora	54.66±6.1 <sup>c</sup>	1.86±0.185 <sup>e</sup>	1507.18±150.6 <sup>b,c</sup>	8.43±0.93 <sup>e</sup>	27.93±3.07 <sup>b</sup>
Nirmal	60.00±4.01 <sup>c</sup>	1.85±0.20 <sup>e</sup>	1394.57±89.9 <sup>8c</sup>	14.92±1.24 <sup>e</sup>	23.24±1.50 <sup>b</sup>
P-24	80.00±3.84 <sup>a,b</sup>	2.47±0.54 <sup>5e</sup>	1703.65±108.1 <sup>b</sup>	11.23±1.33 <sup>e</sup>	21.31 ±1.06 <sup>c,d</sup>
Prateek	88.88±5.8 <sup>a</sup>	2.31±0.95 <sup>e</sup>	1707.62±168.0 <sup>b</sup>	8.26±0.58 <sup>e</sup>	19.14 ±0.70 <sup>d</sup>
Ratan	75.55±8.01 <sup>b</sup>	2.23±0.353 <sup>e</sup>	2428.73±95.2 <sup>5a</sup>	7.75±2.05 <sup>e</sup>	32.69 ±2.65 <sup>a</sup>
<b>PEG-I (15%)</b>					
Mahateora	35.55±2.2 <sup>d</sup>	2.83±0.491 <sup>d,e</sup>	359.04±80.5 <sup>2g</sup>	54.29±2.25 <sup>d</sup>	9.90±1.55 <sup>e,f</sup>
Nirmal	41.29±2.85 <sup>b</sup>	3.83±0.42 <sup>2d</sup>	545.96±36.97 <sup>6g</sup>	52.45±6.27 <sup>d</sup>	7.38 ±0.76 <sup>6g</sup>
P-24	75.55±5.87 <sup>b</sup>	2.40±0.18 <sup>7e</sup>	872.06±27.0 <sup>2d</sup>	52.87±4.03 <sup>d</sup>	11.71 ±1.12 <sup>e</sup>
Prateek	84.44±2.2 <sup>a,b</sup>	5.52±0.629 <sup>c</sup>	634.12±87.75 <sup>7e</sup>	71.61±1.86 <sup>d</sup>	7.52±1.04 <sup>6g</sup>
Ratan	62.78±2.42 <sup>b</sup>	3.80±0.071 <sup>d</sup>	774.72±78.25 <sup>d,e</sup>	61.14±14.37 <sup>d</sup>	10.16 ±0.88 <sup>e,f</sup>
<b>PEG-II (20%)</b>					
Mahateora	20.00±1.1 <sup>e</sup>	6.11±0.11 <sup>b,c</sup>	113.62±3.1 <sup>9h</sup>	215.68±11.05 <sup>b</sup>	5.68 ±0.16 <sup>g</sup>
Nirmal	12.22±0.8 <sup>e</sup>	6.83±0.44 <sup>a,b</sup>	80.03±8.53 <sup>h</sup>	111.37±4.75 <sup>c</sup>	6.62 ±0.87 <sup>6g</sup>
P-24	13.33±0.9 <sup>e</sup>	5.33±1.0 <sup>1c</sup>	67.04±3.04 <sup>h</sup>	202.52±13.27 <sup>b</sup>	5.03 ±0.23 <sup>g</sup>
Prateek	12.77±0.55 <sup>e</sup>	7.16±0.166 <sup>a,b</sup>	64.93±10.66 <sup>h</sup>	343.14±46.31 <sup>a</sup>	5.03 ±0.64 <sup>g</sup>
Ratan	13.33±0.10 <sup>e</sup>	7.33±0.1 <sup>6a</sup>	62.35±5.9 <sup>0h</sup>	142.84±9.37 <sup>c</sup>	4.67 ±0.44 <sup>g</sup>
<b>P Value</b>	<.0001	0.0211	<.0001	<.0001	0.0003

**Note:** Data are expressed as Mean ± Standard Deviation; Means with different superscripts within the same column for each treatment (control, PEG-I, and PEG-II) show significant difference at  $p \leq 0.05$ ; Germination percent expressed as a percentage; MGT: mean germination time, expressed in days; SVI-I: seed vigour index-I; Proline content expressed as  $\mu \text{ mol g}^{-1}$  fresh weight; Seedling length expressed in cm.

Scholarly investigations have underscored that proline primarily originates from glutamate during periods of drought stress. Within the glutamate pathway for proline biosynthesis, glutamate undergoes phosphorylation to form  $\gamma$ -glutamyl phosphate, subsequently reduced to glutamic-5-semialdehyde (GSA) through the enzymatic action of  $\Delta$ 1-pyrroline-5-carboxylate synthetase (P5CS). GSA then undergoes spontaneous cyclization, resulting in the formation of pyrroline-5-carboxylate (P5C). Finally, P5C is reduced to proline catalyzed by the enzyme  $\Delta$ 1-pyrroline-5-carboxylate reductase (P5CR) (Per *et al.*, 2017). The catabolism of proline in mitochondria is facilitated by two essential enzymes, namely proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH), ultimately yielding glutamine. Studies have indicated that the modulation of proline synthesis, evidenced by the upregulation of P5CS transcription, and the downregulation of PDH under conditions of drought stress in leguminous plants like *Lathyrus*, could contribute to an elevation in proline content within PEG-treated *Lathyrus* (both at the seed and seedling stages) (Ruszkowski *et al.* 2015, Sabbioni and Forlani 2022).

### **Morpho-physiological traits**

The morpho-physiological traits (seedling length, root length, fresh seedling weight, and dry seedling weight) of five *Lathyrus* were assessed at three different PEG concentrations as indicated in Tables 2 and 3, represented through Box-and-whisker plots in Fig 1 and 3, heat map in Fig 2 and 4.

The seedling length, expressed in centimeters, during the seed stage, exhibited a range of variability from  $19.14 \pm 0.70$  (Prateek) to  $32.69 \pm 2.65$  (Ratan) under normal conditions. With the application of PEG 1 (15%), this range shifted from  $7.38 \pm 0.76$  (Nirmal) to  $10.16 \pm 0.88$  (Ratan). Under the influence of PEG-II (20%), the seedling length range fluctuated from  $4.67 \pm 0.44$  (Ratan) to  $6.62 \pm 0.87$  (Nirmal). Notably, the highest increase in seedling length was observed in the control group, followed by PEG-I and subsequently PEG-II. Similarly, at the seedling stage after stress removal, the seedling length ranged from  $20.47 \pm 1.96$  (Ratan) to  $30.70 \pm 2.38$  (Prateek) under normal conditions. Following the application of PEG 1 (15%), this range expanded from  $30.85 \pm 0.38$  (P-24) to  $42.00 \pm 1.44$  (Prateek). With the impact of PEG-II (20%), the seedling length range extended from  $39.43 \pm 0.71$  (P-24) to  $45.88 \pm 0.30$  (Prateek). Both PEG-I and PEG-II exhibited a substantial increase in seedling length in

comparison to the control group.

Seedling growth serves as a vital indicator of drought stress susceptibility and represents a critical stage highly susceptible to water deficit. Corresponding findings have been reported by Khodarahmpour (2011), Shahzad *et al.* (2023) in maize inbred lines, OKÇU *et al.* (2005) in peas, and Muscolo *et al.* (2014) in lentils, demonstrating a decrease in seedling length with increasing PEG concentration during the seed stage while observing enhanced seedling length compared to the control during the seedling stage. Studies suggest that the decline in seedling growth under high PEG concentration during the seed stage may result from constrained cell enlargement and division, as drought stress has a suppressing effect on both cell division and elongation (Saha *et al.* 2022). Furthermore, it has been documented that drought stress impedes seedling growth through the inhibition of carbon partitioning and accumulation (Khan *et al.* 2019).

The observed reduction in seedling length under PEG-I and PEG-II treatments during the seedling stage can be attributed to the compromised stability of the plasma membrane, leading to increased electrolyte leakage when exposed to suboptimal soil moisture conditions. This electrolyte leakage results in the accumulation of reactive oxygen species (ROS), thereby causing oxidative damage during the seedling stage and subsequently leading to a decrease in seedling length. Elevated ROS levels prompt the plant to trigger the activity of antioxidant enzymes, including Ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), among others. These antioxidant enzymes play a crucial role in maintaining cell membrane integrity and preventing lipid peroxidation by effectively scavenging ROS (Zeng *et al.* 2023). Consequently, this process contributes to the observed increase in seedling length under PEG-I and PEG-II treatments after 18 days (Zhang *et al.* 2015).

The root length, measured in centimeters, during the seedling stage, exhibited a range of variability from  $7.92 \pm 0.13$  (Ratan) to  $12.10 \pm 1.13$  (Mahateora) under normal conditions. With the application of PEG 1 (15%), this range extended from  $10.90 \pm 0.66$  (P-24) to  $14.54 \pm 0.613$  (Mahateora). Under the influence of PEG-II (20%), the root length range increased from  $19.16 \pm 0.70$  (P-24) to  $22.05 \pm 1.35$  (Nirmal). Notably, the highest increase in root length was observed in PEG-II, followed by PEG-I and the control. Additionally, no significant

difference in root lengths was observed between the PEG-I treatment and the control group.

Plant roots play a pivotal role in determining water and nutrient absorption, as well as in nutrient synthesis and immobilization. The plant's response to drought stress during the seedling stage is primarily reflected in the promotion of root growth accompanied by suppressed shoot growth, ultimately leading to an increased root-to-shoot ratio (Kulkarni *et al.* 2017). Studies have suggested that seedlings develop elongated root systems, facilitating deep root penetration and proliferation in response to drought stress (Tang *et al.* 2019). Thus, in the context of the current study, the observed increase in root length in PEG-II, compared to PEG-I and the control, can be attributed to the enhanced capacity of deeper and longer root systems to facilitate drought avoidance. This is facilitated by their improved ability to access moisture from deeper soil layers, along with increased nutrient absorption, immobilization, and photosynthetic yield.

The fresh seedling weight, measured in grams, displayed a range of variability from  $0.29 \pm 0.01$  (Ratan) to  $0.721 \pm 0.08$  (Prateek) under normal conditions. Following the application of PEG 1 (15%), this range extended from  $0.381 \pm 0.040$  (Ratan) to  $0.841$  (Prateek). Under the influence of PEG-II (20%), the fresh seedling weight range expanded from  $0.43 \pm 0.006$  (Ratan) to  $0.76 \pm 0.006$  (Prateek). The results indicate that there was no significant difference in fresh seedling weight between the PEG-I, PEG-II, and control groups.

Likewise, the dry seedling weight, also measured in grams, exhibited variability ranging from  $0.04 \pm .008$  (Mahateora) to  $0.07 \pm 0.004$  (Ratan) under normal conditions. With the application of PEG 1 (15%), this range shifted from  $0.04 \pm 0.002$  (Mahateora) to  $0.08 \pm 0.005$  (Nirmal). Under the influence of PEG-II (20%), the dry seedling weight range fluctuated from  $0.05 \pm 0.002$  (Mahateora) to  $0.043 \pm 0.003$  (Ratan). The results indicate that there was no significant difference in dry seedling weight between the PEG-I, PEG-II, and control groups.

### **Principal component analysis (PCA)**

The application of the Principal Component Analysis (PCA) method enabled the meticulous scaling and comprehensive analysis of the agromorphological attributes and phytochemical composition of lucerne. This strategic utilization aimed to unravel the underlying factors governing

the observed compositional fluctuations and to illuminate potential interconnections among these traits. In the multidimensional data space, PCA algorithms swiftly discern the direction exhibiting the highest variance (Tomar *et al.* 2021). This process is predicated on the premise that greater variability (signified by increased variance) corresponds to supplementary informational content. To assess the precision of this direction, the data matrix underwent a mean-centered column-wise transformation, ensuring that the axes revolve around the centroid of the data. Consequently, the axis delineating the maximum variance designates the primary (lowest-order) principal component (PC1). Meanwhile, the second axis aligns in a direction with the highest variance, maintaining non-correlation (orthogonality) with all other directions concerning PC1. Subsequently, the second principal component (PC2) accounts for any discernible information that couldn't be elucidated through PC1, and this pattern continues for subsequent PCs. One advantageous aspect of the principal components is their mutual non-correlation, effectively capturing significant information among the parameters while validating the same information derived from the evaluated parameters (Granato *et al.* 2018). Conceptually, the principal components can be viewed as a linear amalgamation of the original variables, with each variable undergoing multiplication by a loading coefficient. From a geometrical perspective, these loadings represent the cosine values of the angle between the original variables and the principal components. These loading coefficients, spanning from +1 to -1, serve to define each individual PC. Notably, higher cosine values denote a diminished rotational angle between the variable and the PC, signifying closer alignment between the two directions and a more substantial contribution of that variable to the corresponding PC. In practice, PCA efficiently employs bi- and tridimensional graphs to effectively distil the intricate and expansive information embedded within the data (biplots, score plots, and loading plots). Each principal component delineates a distinct portion of data variation, which is indicated by the Eigenvalue (Dobriban 2017). Concomitantly, each Eigenvalue corresponds to an Eigenvector representing the variation among the respective principal components. It is essential to note that Kaiser's criterion was adhered to, emphasizing factors with Eigenvalues surpassing 1.0 (Kaiser 1960).

Before delving into the component analysis, the Kaiser-Meyer-Olkin (KMO) Measure of Sampling

Adequacy (MSA) and Bartlett's test for sphericity were enlisted as the preliminary assumption tests. Specifically, Bartlett's test was employed to compare the identity matrix with the observed correlation matrix (Tobias and Carlson 1969). This test plays a vital role in detecting potential redundancies among variables that may be explicable by a limited number of factors. Essentially, it determines whether the variables in the data exhibit significant interrelations, potentially reducible to a smaller set of variables. The test operates under the null hypothesis that the variables are orthogonal or uncorrelated. Deviation from this assumption, as indicated by a significant departure of the correlation matrix from the identity matrix, suggests a substantial correlation between the variables. In the context of our analysis, if Bartlett's test yields a significance level ( $p < 0.5$ ), it signifies that the data can be suitably condensed through PCA. Remarkably, the computed value

of Bartlett's test for all the evaluated traits was determined to be  $p < 0.001$  for both the seed stage (9 days) and the seedling stage (18 days).

An additional critical statistical test, the Kaiser-Meyer-Olkin (KMO), was employed to assess the suitability of the data for factor analysis via PCA. This test serves to gauge the extent to which the variability in the variables can be attributed to underlying causes (Lestari 2021). The suitability for PCA is heightened when the degree of variance is lower. Optimally, values between 0.9 and 1.0 are deemed appropriate for applying PCA to the data. Values falling within the range of 0.5 to 0.7 are considered adequate, while those between 0.7 and 0.9 are deemed favorable. However, when the values dip below 0.5, the PCA results may not yield sufficiently meaningful interpretations. Notably, in the current study, the KMO values ranged from

**Table 3.** Seedling traits at 18 days under three different PEG treatments i.e. 0% (distilled water as control, equivalent to 0.0 Mpa osmotic potential), 15% (equivalent to -0.30 MPa osmotic potential as PEG-I) and 20% (equivalent to -0.49 MPa osmotic potential as PEG-II)

Genotype	Seedling length	Root length after	Fresh seedling weight	Dry seedling weight	Proline content
<b>Control (0.0%)</b>					
Mahateora	27.21±3.47 <sup>f</sup>	12.10±1.13 <sup>d,e,f</sup>	0.39±0.003 <sup>f,g,h</sup>	0.04±0.00 <sup>3</sup> d	9.94±1.06 <sup>f,g,h</sup>
Nirmal	26.79±2.93 <sup>f</sup>	10.30±1.59 <sup>f</sup>	0.49±0.09 <sup>c,d,e,f,g</sup>	0.05±0.002 <sup>b</sup>	10.11±0.90 <sup>i,g</sup>
P-24	26.90±2.89 <sup>f</sup>	11.08±1.28 <sup>f</sup>	0.40±0.03 <sup>e,f,g,h</sup>	0.05±0.005 <sup>b,c</sup>	14.94±1.12 <sup>f,g,h,i</sup>
Prateek	30.70±2.38 <sup>c,f</sup>	11.19±0.54 <sup>e,f</sup>	0.721±0.08 <sup>b</sup>	0.06±0.008 <sup>b</sup>	11.38±3.79 <sup>a</sup>
Ratan	20.47±1.96 <sup>g</sup>	7.92±0.13 <sup>g</sup>	0.29±0.01 <sup>h</sup>	0.07±0.00 <sup>4</sup> a	11.40±1.97 <sup>e,f,g</sup>
<b>PEG-I (15%)</b>					
Mahateora	36.04±1.55 <sup>c,d,e</sup>	14.54±0.613 <sup>c</sup>	0.471±0.05 <sup>d,e,f,g</sup>	0.04±0.00 <sup>3</sup> c,d	19.25±2.98 <sup>d,e,f</sup>
Nirmal	32.06±1.70 <sup>e,f</sup>	11.96±0.03 <sup>d,e,f</sup>	0.52±0.021 <sup>c,d,e</sup>	0.08±0.00 <sup>5</sup> a	18.07±0.70 <sup>j</sup>
P-24	30.85±0.383 <sup>c,f</sup>	10.90±0.66 <sup>f</sup>	0.51±0.021 <sup>c,d,e,f</sup>	0.078±0.009 <sup>a</sup>	23.34±2.88 <sup>b,c,d</sup>
Prateek	42.00±1.4 <sup>a,b</sup>	14.11±0.8 <sup>c,d</sup>	0.841±0.01 <sup>4</sup> a	0.076±0.002 <sup>a</sup>	27.26±1.26 <sup>a,b</sup>
Ratan	35.61±2.49 <sup>d,e</sup>	13.53±1.70 <sup>c,d,e</sup>	0.381±0.04 <sup>0</sup> g,h	0.047±0.002 <sup>c,d</sup>	14.18±1.09 <sup>g,h,i</sup>
<b>PEG-II (20%)</b>					
Mahateora	41.76±2.93 <sup>a,b,c</sup>	21.25±0.90 <sup>a,b</sup>	0.49±0.03 <sup>c,d,e,f,g</sup>	0.05±0.00 <sup>2</sup> b,c	20.52±0.568 <sup>c,d,e</sup>
Nirmal	43.94±1.20 <sup>a,b</sup>	22.05±1.35 <sup>a</sup>	0.53±0.012 <sup>c,d</sup>	0.054±0.002 <sup>b,c</sup>	14.28±1.14 <sup>g,h,i</sup>
P-24	39.43±0.71 <sup>b,c,d</sup>	19.16±0.70 <sup>b</sup>	0.60±0.02 <sup>c</sup>	0.075±0.002 <sup>a</sup>	25.74±1.62 <sup>b</sup>
Prateek	45.88±0.3 <sup>a</sup>	21.29±1.5 <sup>9</sup> a,b	0.76±0.00 <sup>6</sup> a,b	0.074±0.002 <sup>a</sup>	24.38±1.40 <sup>b,c</sup>
Ratan	40.35±2.53 <sup>a,b,c,d</sup>	20.56±0.93 <sup>a,b</sup>	0.43±0.00 <sup>6</sup> d,e,f,g	0.043±0.00 <sup>3</sup> d	10.67±0.851 <sup>h,i,j</sup>
<b>P Value</b>	NS	0.0357	NS	<.0001	0.0001

**Note:** Data are expressed as Mean ± Standard Deviation; Means with different superscripts within the same column for each treatment (control, PEG-I, and PEG-II) show significant difference at  $p \leq 0.05$ ; Proline content expressed as  $\mu \text{ mol g}^{-1}$  fresh weight; Seedling length expressed in cm; seedling and root length expressed in cm; dry and fresh seedling weight expressed in gram.

**Table 4.** Allelic variation, genetic diversity [GD] and Polymorphic Information Content [PIC] values for microsatellite loci Inter Simple Sequence Repeats [ISSR] identified in five Lathyrus genotypes.

S.No.	Primer/ Marker	Allele No	Primer Sequence (5'-3')	G+C content (%)	No. of bands	Size range	PIC	GD
1	UBC836	3.0	AGAGAGAGAGAGAGAGYA	48.80	7	300-1400	0.31	0.34
2	UBC841	3.0	GAGAGAGAGAGAGAGA(CT)C	52.63	6	400-1600	0.31	0.34
3	UBC842	3.0	GAGAGAGAGAGAGAGAYG	52.94	6	400-1500	0.31	0.34
4	UBC855	2.0	ACACACACACACACAC (CT)T	47.6	9	400-1800	0.27	0.32
	<b>Mean</b>	<b>2.75</b>					<b>0.30</b>	<b>0.34</b>

0.602 to 0.886, indicating a significant suitability of the data for PCA analysis. Conversely, for the seed stage, the values spanned from 0.259 to 0.492, implying the inadequacy of the data for conducting PCA analysis. This observation underscores the limitations in utilizing the PCA approach for the data derived from the seed stage.

Following the discerning selection of representative principal components (PCs) based on the explained variance and the grouping/differentiation of samples, the loadings were meticulously examined to elucidate the fundamental relationship embedded within the data structure. In essence, the loadings can be interpreted as linear combination coefficients of the original variables from which the principal components were derived. To compute the factor loadings (FLs), the variable coordinates were divided by the square root of the eigenvalues corresponding to the specific component. Notably, positive factor loading values suggest that the factor will be positioned higher along the positive axis of the respective principal component. In the context of the present study, SVI-I (FL1: 0.953), Seedling length (FL: 0.900), and G% (FL1: 0.865) demonstrated positive factor loadings, whereas MGT (FL1: -0.920) and Proline content (FL1: -0.881) displayed negative values.

Remarkably, PC1 accounted for 99.2% of the data variability, whereas PC2 explained 0.7% of the variability. Notably, the pivotal variables contributing significantly to the highest variability in the PCA encompass SVI-I, MGT, and seedling length. Additionally, uniqueness denotes the portion of variance that is distinct to a particular variable and cannot be explicated by individual components. As a rule, higher values of 'uniqueness' for a variable correspond to a diminished share of the variable in the component model. In the present study, the G% trait exhibited the highest uniqueness value (0.2521), while SVI-I displayed the lowest value (0.0917). To visually represent the distribution of the assessed traits across eight distinct lucerne genotypes, the score plots of the PCA, depicted in two dimensions, were employed. These score plots were generated to delineate the statistical clustering of the genotypes through the application of the Ward clustering algorithm, leveraging their respective evaluated component loading values, as depicted in Fig 5A.

### Correlation analysis

For the seed stage (9 days), a comprehensive

evaluation of the correlation coefficients was conducted individually among Germination %, Mean Germination Time (MGT), SVI-I, Proline content, and Seedling length. Pearson's correlation test was employed to ascertain significant correlations between these traits. With a threshold of  $p < 0.05$ , a Pearson's  $r$  value  $> 0$  indicates a significant positive correlation, while  $r < 0$  indicates a significant negative correlation. The detailed results of the Pearson's  $r$  values are visually presented through a heat map in Figure 5B. In the context of the seed stage, the Germination % exhibited negative correlations with MGT ( $r: -0.739$ ,  $p: 0.002$ ) and Proline content ( $r: -0.754$ ,  $p: 0.001$ ). Notably, the negative correlation between Germination % and MGT can be attributed to the reciprocal relationship between MGT and the rate of germination. Studies suggest that proline triggers the generation of reactive oxygen species (ROS) in mitochondria via the electron transport system, leading to the activation of hypersensitive response and fluctuations in cellular signaling and germination processes (Munir *et al.*, 2021). Consequently, this contributes to the negative correlation observed between proline and Germination %. Furthermore, Germination % demonstrated positive correlations with SVI-I ( $r: 0.800$ ,  $p: < 0.001$ ) and Seedling length ( $r: 0.627$ ,  $p: 0.012$ ). This positive relationship can be attributed to the concept that SVI-I serves as a complex seed trait determining the potential for uniform germination, emergence, and growth across diverse environmental conditions (Rajjou *et al.*, 2012). Conversely, MGT exhibited negative correlations with SVI-I ( $r: -0.812$ ,  $p: < 0.001$ ) and Seedling length ( $r: -0.794$ ,  $p: < 0.001$ ). This phenomenon can be explained by the fact that seeds with higher SVI-I tend to germinate more rapidly, leading to reduced MGT and increased seedling length. Notably, MGT displayed a positive correlation with Proline content ( $r: 0.813$ ,  $p: < 0.001$ ), in line with the previously mentioned inhibitory effect of proline on seed germination, thereby extending the time taken for seedlings to germinate (resulting in higher MGT). Additionally, SVI-I exhibited a negative correlation with Proline ( $r: -0.739$ ,  $p: 0.002$ ) and a positive correlation with Seedling length ( $r: 0.948$ ,  $p: < 0.001$ ). Furthermore, Proline was found to have a negative correlation with Seedling length ( $r: -0.685$ ,  $p: 0.005$ ).

### Molecular Characterization Using ISSR Markers

The study encompassed the application of 30 ISSR (Inter Simple Sequence Repeat) primers to five

Lathyrus genotypes, namely Pusa-24, Nirmal, Ratan, Prateek, and Mahateora. Notably, only 20 primers yielded amplification, and among them, four were found to be polymorphic, namely UBC855, UBC836, UBC842, and UBC841 (Fig. 6; Table 4). The resultant analysis led to the categorization of the genotypes into three distinct clusters, denoted as CI (Ratan), CII (Pusa-24 and Mahateora), and CIII (Prateek and Nirmal), based on the similarity coefficient (Fig 3). The discernment of high Polymorphic Information Content (PIC) and genetic diversity values facilitated the identification of the most informative markers, namely UBC836, UBC842, and UBC841, with PIC values of 0.30 and 0.34, respectively (Table 4). Notably, the emergence of unique bands further facilitated the identification of diverse germplasm lines, a process crucial for cultivar barcoding. Leveraging molecular markers, the identification of suitable genotypes for commercial cultivation within the region becomes feasible, predicated on discrimination and resolving power. The ISSR markers demonstrated a substantial degree of genetic diversity, amplifying a total of 33 alleles, ranging from 3 to 5, with a mean of 2.75 alleles per locus. However, the low PIC value derived from ISSR genotyping indicated the necessity for the inclusion of a more substantial number of highly polymorphic markers in the study to avert biased distribution.

## CONCLUSION

The current investigation has provided significant insights into the impact of PEG treatment on both seed quality and morphophysiological characteristics across five distinct Lathyrus genotypes, namely Ratan, Prateek, Mahateora, Pusa-24 (P-24), and Nirmal. Comparative analysis with the control group revealed that the most notable reductions in both germination percentage (G%) and seedling vigour index-I (SVI-I) were evident in the PEG-II treatment, followed closely by the PEG-I treatment. Furthermore, heightened concentrations of PEG were found to correlate with a marked increase in mean germination time (MGT), with the most significant elevation observed in the PEG-II treatment, closely trailed by the PEG-I treatment. Concomitantly, an increase in proline content was recorded, notably accentuated in the PEG-II treatment, followed by the PEG-I treatment, as compared to the control group. Both PEG-I and PEG-II treatments exhibited enhanced seedling length compared to the control group. Similarly, the most substantial rise in root length was observed in the PEG-II treatment, followed

by the PEG-I treatment and the control group. Notably, no significant disparity in root lengths was detected between the PEG-I treatment and the control group. However, the investigation did not reveal any significant difference in fresh and dry seedling weight among the PEG-I, PEG-II, and control groups. The integration of high polymorphic information content (PIC) and genetic diversity values played a crucial role in identifying the most informative markers, namely UBC836, UBC842, and UBC841, each exhibiting PIC values of 0.30 and 0.34. The discernible presence of unique bands aided in the distinction of diverse germplasm lines, effectively functioning as a cultivar barcoding system. The utilization of ISSR markers uncovered a notable level of genetic diversity, amplifying a total of 33 alleles across the loci, ranging from 3 to 5 alleles per locus, with a mean of 2.75 alleles per locus. Consequently, these discernible genotypes hold promising potential and can be strongly recommended for the purpose of breeding superior Lathyrus genotypes, particularly well-adapted to conditions characterized by moisture stress. The development of these superior Lathyrus genotypes may prove pivotal in fostering varieties that demonstrate heightened tolerance to drought stress, consequently leading to elevated yields.

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## DISCLOSURE STATEMENT

The authors declare that they have no conflicts of interest.

## REFERENCES

- Abdul-Baki AA and Anderson JD. 1973. Vigor Determination in Soybean Seed by Multiple Criteria 1. *Crop Science* **13**(6): 630-633.
- Ahmad NS, Abid MA and Al-Assie AA. 2022. Profiling of foxtail millet (*Setaria italica* L.) germplasm using in vitro drought screening and SSR markers. *Plant Genetic Resources* **20**(4): 277-289.
- Ahmadpour R, Bachari Y and Hosseinzadeh SR 2022. The role of compost tea in mitigating the negative effects of drought stress caused by polyethyleneglycol in chickpea seeds (Adel cultivar) by evaluating germination indices. *Iranian Journal Pulses Research* **13**(2): 37-49.

- Alvarez ME, Savouré A and Szabados L. 2022. Proline metabolism as regulatory hub. *Trends in Plant Science* **27**(1): 39-55.
- Assefa E and Bitew Y. 2023. Enhancing the land use efficiency of low-land rice (*Oryza sativa* L.)-Grass pea (*Lathyrus sativus* L.) additive series relay intercropping in North-Western Ethiopia: A farmer's indigenous knowledge. *Plos one* **18**(7): p. e0281410.
- Badr A, El-Shazly HH, Tarawneh RA and Börner A 2020. Screening for Drought Tolerance in Maize (*Zea mays* L.) Germplasm Using Germination and Seedling Traits under Simulated Drought Conditions. *Plants* **9**(5): 565.
- Bates LS, Waldren RP and Teare ID. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**(1): 205-207.
- Ben Rejeb K, Abdelly C and Savouré A. 2014. How reactive oxygen species and proline face stress together. *Plant Physiology and Biochemistry* **80**: 278-284.
- Van den Berg L and Zeng YJ. 2006. Response of South African indigenous grass species to drought stress induced by polyethylene glycol (PEG) 6000. *South African Journal of Botany* **72**(2): 284-286.
- Binodh AK, Kathiresan PK, Thankappan S and Senthil A. 2023. Acclimatization of non-cultivated rice landraces to early moisture stress mediated by enzymatic antioxidants and osmolyte accumulation. *Biocatalysis and Agricultural Biotechnology* **47**: p. 102623.
- Carr PM *et al.* 2021. Annual forage impacts on dryland wheat farming in the Great Plains. *Agronomy Journal* **113**(1): 1-25.
- Chong J, Yamamoto M and Xia J. 2019. MetaboAnalystR 2.0: From Raw Spectra to Biological Insights. *Metabolites* **9**(3): 57.
- Das A, Parihar AK, Barpete S, Kumar S and Gupta S. 2021. Current Perspectives on Reducing the  $\beta$ -ODAP Content and Improving Potential Agronomic Traits in Grass Pea (*Lathyrus sativus* L.). *Frontiers in Plant Science* **12**: 703275.
- Dobriban E. 2017. Sharp detection in PCA under correlations: All eigenvalues matter. *The Annals of Statistics* **45**(4): 1810-1833.
- Ellis RH and EH R. 1980. Towards a rational basis for testing seed quality. In: *Seed Production*. Butterworth-Heinemann, London: 605-635.
- Fu X, Wang J, Shanguan T, Wu R, Li S, Chen G and Xu S. 2022. SMXLs regulate seed germination under salinity and drought stress in soybean. *Plant Growth Regulation* **96**(3): 397-408.
- Gheidary S, Akhzari D and Pessaraki M. 2017. Effects of salinity, drought, and priming treatments on seed germination and growth parameters of *Lathyrus sativus* L. *Journal of Plant Nutrition* **40**(10): 1507-1514.
- Gholami M, Rahemi M and Kholdebarin B. 2010. Effect of drought stress induced by polyethylene glycol on seed germination of four wild almond species. *Australian Journal of Basic and Applied Sciences* **4**(5): 785-791.
- Granato D, Santos JS, Escher GB, Ferreira BL and Maggio RM. 2018. Use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate association between bioactive compounds and functional properties in foods: A critical perspective. *Trends in Food Science & Technology* **72**: 83-90.
- Guo Y *et al.* 2022. Seed Priming With Melatonin Promotes Seed Germination and Seedling Growth of Triticale hexaploide L. Under PEG-6000 Induced Drought Stress. *Frontiers in Plant Science* **13**: 932912.
- Gusmao M, Siddique KHM, Flower K, Nesbitt H and Veneklaas EJ. 2012. Water Deficit during the Reproductive Period of Grass Pea (*Lathyrus sativus* L.) Reduced Grain Yield but Maintained Seed Size. *Journal of Agronomy and Crop Science* **198**(6): 430-441.
- Hellal FA, El-Shabrawi HM, Abd El-Hady M, Khatab IA, El-Sayed SAA and Abdelly C. 2018. Influence of PEG induced drought stress on molecular and biochemical constituents and seedling growth of Egyptian barley cultivars. *Journal of Genetic Engineering and Biotechnology* **16**(1): 203-212.
- Hojjat SS. 2020. Effects of TiO<sub>2</sub> Nanoparticles on Germination and Growth Characteristics of Grass Pea (*Lathyrus sativus* L.) Seed under Drought Stress. *Nanotechnologies in Russia* **15**(2): 204-211.
- Jeromela, Ana M, Aleksandar M Mikić, Svetlana Vujić, Branko Čupina, Đorđe Krstić, Aleksandra Dimitrijević, Sanja Vasiljević, Vojislav Mihailović, Sandra Cvejić, and Dragana Miladinović. 2017. Potential of Legume-Brassica Intercrops for Forage Production and Green Manure: Encouragements from a Temperate Southeast European Environment. *Frontiers in Plant Science* **8**: 245916.
- Kachout SS, Benyoussef S, Zoghalmi A and Chakroun M. 2019. Effect of water deficit during germination and flowering period of grass pea (*Lathyrus sativus* L.). *International Journal of Plant Breeding and Genetics* **13**(1): 12-18.
- Kaiser HF. 1960. The Application of Electronic Computers to Factor Analysis. *Educational and Psychological Measurement* **20**(1): 141-151.
- Khan MN *et al.* 2019. Morpho-physiological and biochemical responses of tolerant and sensitive rapeseed cultivars to drought stress during early seedling growth stage. *Acta Physiologiae Plantarum* **41**(2): 25.
- Khodarahmpour Z. 2011. Effect of drought stress induced by polyethylene glycol (PEG) on germination indices in corn (*Zea mays* L.) hybrids. *African Journal of Biotechnology* **10**(79): 18222-18227.
- Kong H, Palta JA, Siddique KHM, Stefanova K, Xiong

- YC and Turner NC. 2015. Photosynthesis is Reduced, and Seeds Fail to Set and Fill at Similar Soil Water Contents in Grass Pea (*Lathyrus sativus* L.) Subjected to Terminal Drought. *Journal of Agronomy and Crop Science* **201**(4): 241-252.
- Kong HY, Zhu H, Zhou R, Akram NA, Wang YB, Jiao CJ and Xiong YC. 2022. Role of abscisic acid in modulating drought acclimation, agronomic characteristics and  $\beta$ -N-oxalyl-L- $\alpha,\beta$ -diaminopropionic acid ( $\beta$ -ODAP) accumulation in grass pea (*Lathyrus sativus* L.). *Journal of the Science of Food and Agriculture* **102**(6): 2553-2562.
- Kulkarni M, Soolanayakanahally R, Ogawa S, Uga Y, Selvaraj MG and Kagale S. 2017. Drought Response in Wheat: Key Genes and Regulatory Mechanisms Controlling Root System Architecture and Transpiration Efficiency. *Frontiers in Chemistry* **5**: 106.
- Kumar S, Nei M, Dudley J and Tamura K. 2008. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* **9**(4): 299-306.
- Lalrinmawii Mir H and Perveen N. 2023. Recent Advances in the Use of Molecular Markers for Fruit Crop Improvement. In: Kumar, N. ed. *Molecular Marker Techniques: A Potential Approach of Crop Improvement*. Singapore: Springer Nature: 339-355.
- Lambein F, Travella S, Kuo YH, Van Montagu M and Heijde M. 2019. Grass pea (*Lathyrus sativus* L.): orphan crop, nutraceutical or just plain food? *Planta* **250**(3): 821-838.
- Lestari MW. 2021. Yield Determinants of Some Sweet Potato (*Ipomoea batatas* L) Clones Using Principal Component Analysis. *IOP Conference Series: Earth and Environmental Science* **828**(1): p. 012006.
- Liu K and Muse SV. 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics* **21**(9): 2128-2129.
- Mas-ud MA et al. 2022. Screening for drought tolerance and diversity analysis of Bangladeshi rice germplasms using morphophysiology and molecular markers. *Biologia* **77**(1): 21-37.
- Mekonen DA, Abraham A, Oselebe H, Afiukwa C, Ilesanmi O and Abebe TD. 2022. Genetic diversity and population structure analysis of Grass pea (*Lathyrus sativus* L.) accessions collected from North-Western Ethiopia using SSR markers. *Genetic Resources and Crop Evolution* **69**(3): 1247-1260.
- Michel BE and Kaufmann MR. 1973. The Osmotic Potential of Polyethylene Glycol 6000 1. *Plant Physiology* **51**(5): 914-916.
- Munir O, Bengu TU, Pedro GC, Anum K, Alvina G and Mirza H. 2021. Osmoregulation and its actions during the drought stress in plants. *Physiologia plantarum* **172**(2): 1321-1335.
- Muscolo A, Sidari M, Anastasi U, Santonoceto C and Maggio A. 2014. Effect of PEG-induced drought stress on seed germination of four lentil genotypes. *Journal of Plant Interactions* **9**(1): 354-363.
- Okcu G, Kaya M and ATAK M. 2005. Effects of Salt and Drought Stresses on Germination and Seedling Growth of Pea (*Pisum sativum* L.). *Turkish Journal of Agriculture and Forestry* **29**(4): 237-242.
- Parsa M, Kashanchi M, Zeinali A and Pourfakhraei E. 2023. Overexpression of zinc finger (GpZF) transcription factor promotes drought tolerance in grass pea (*Lathyrus sativus* L.). *South African Journal of Botany* **153**: 178-187.
- Per TS, Khan NA, Reddy PS, Masood A, Hasanuzzaman M, Khan MIR and Anjum NA. 2017. Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. *Plant Physiology and Biochemistry* **115**: 126-140.
- Piwowarczyk B, Kamińska I and Rybiński W. 2014. Influence of PEG generated osmotic stress on shoot regeneration and some biochemical parameters in *Lathyrus* culture. *Czech Journal of Genetics and Plant Breeding* **50**(2): 77-83.
- Piwowarczyk B, Tokarz K, Makowski W and Łukasiewicz A. 2017. Different acclimatization mechanisms of two grass pea cultivars to osmotic stress in in vitro culture. *Acta Physiologiae Plantarum* **39**(4): 96.
- Purbajanti ED, Kusmiyati F, Fuskhah E, Rosyida R, Adinurani PG and Vincēviča-Gaile Z. 2019. Selection for drought-resistant rice (*Oryza sativa* L.) using polyethylene glycol. *IOP Conference Series: Earth and Environmental Science* **293**(1): p. 012014.
- Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C and Job D. 2012. Seed Germination and Vigor. *Annual Review of Plant Biology* **63**(1): 507-533.
- Rana MS, Hasan MA, Bahadur MM and Islam MR. 2017. Effect of Polyethylene Glycol induced water stress on germination and seedling growth of wheat (*Triticum aestivum*). *The Agriculturists* **15**(1): 81-91.
- Raza A, Charagh S, Abbas S, Hassan MU, Saeed F, Haider S, Sharif R, Anand A, Corpas FJ, Jin W and Varshney RK. 2023. Assessment of proline function in higher plants under extreme temperatures. *Plant Biology* **25**(3): 379-395.
- Razavizadeh R, Farahzadianpoor F, Adabavazeh F and Komatsu S. 2019. Physiological and morphological analyses of *Thymus vulgaris* L. in vitro cultures under polyethylene glycol (PEG)-induced osmotic stress. *In Vitro Cellular & Developmental Biology Plant* **55**(3): 342-357.
- Ruszkowski M, Nocek B, Forlani G and Dauter Z. 2015. The structure of *Medicago truncatula*  $\delta$ 1-pyrroline-5-carboxylate reductase provides new insights into regulation of proline biosynthesis in plants. *Frontiers*

- in Plant Science **6**: 167226.
- Sabbioni G and Forlani G. 2022. The Emerging Role of Proline in the Establishment and Functioning of Legume-Rhizobium Symbiosis. *Frontiers in Plant Science* **13**: 888769.
- Saha D et al. 2022. Drought stress responses and inducing tolerance by seed priming approach in plants. *Plant Stress* **4**: 100066.
- Savita, Tomer A and Singh SK. 2020. Drought Stress Tolerance in Legume Crops. In: Hasanuzzaman, M. ed. *Agronomic Crops* **3**: 149-155.
- Shahzad A, Gul H, Ahsan M, Wang D and Fahad S. 2023. Comparative Genetic Evaluation of Maize Inbred Lines at Seedling and Maturity Stages Under Drought Stress. *Journal of Plant Growth Regulation* **42**(2): 989-1005.
- Tabatabai SMT, Goshasbi F and Bakhshi B. 2022. Evaluation of the effect of polyethylene glycol (PEG) on germination and morphological characteristics of bread wheat. *Cereal Research Communications* **50**(4): 1009-1015.
- Talská R, Machalová J, Smýkal P and Hron K. 2020. A comparison of seed germination coefficients using functional regression. *Applications in Plant Sciences* **8**(8): e11366.
- Tamburino R, Guida V, Pacifico S, Rocco M, Zarelli A, Parente A and Di MA. 2012. Nutritional values and radical scavenging capacities of grass pea (*Lathyrus sativus* L.) seeds in Valle Agricola district, Italy. *Australian Journal of Crop Science* **6**(1): 149-156.
- Tang D, Wei F, Qin S, Khan A, Kashif MH and Zhou R. 2019. Polyethylene glycol induced drought stress strongly influences seed germination, root morphology and cytoplasm of different kenaf genotypes. *Industrial Crops and Products* **137**: 180-186.
- Tesfaw A, Kosebent D, Oner ET and Assefa F. 2021. Bioethanol production from grass pea and wild oat hydrolysates using *S. cerevisiae* ETP53, *K. marxianus* ETP87, and *P. fermentans* ETP22. *Discover Energy* **1**(1): 1-12.
- Thanmalagan RR, Jayaprakash A, Roy A, Arunachalam A and Lakshmi PTV. 2022. A review on applications of plant network biology to understand the drought stress response in economically important cereal crops. *Plant Gene* **29**: 100345.
- Tiwari S, Tomar RS, Tripathi MK and Ahuja A. 2017. Modified Protocol for Plant Genomic DNA Isolation. *Indian Research Journal of Genetics and Biotechnology* **9**(4): 478-485.
- Tobias S and Carlson JE. 1969. Brief Report: Bartlett's Test of Sphericity and Chance Findings in Factor Analysis. *Multivariate Behavioral Research* **4**(3): 375-377.
- Tokarz B, Wójtowicz T, Makowski W, Jędrzejczyk RJ and Tokarz KM. 2020. What is the Difference between the Response of Grass Pea (*Lathyrus sativus* L.) to Salinity and Drought Stress? – A Physiological Study. *Agronomy* **10**(6): 833.
- Tomar M et al. 2021. Nutritional composition patterns and application of multivariate analysis to evaluate indigenous Pearl millet (*Pennisetum glaucum* (L.) R. Br.) germplasm. *Journal of Food Composition and Analysis* **103**: 104086.
- Wasaya A, Hassan J, Yasir TA, Ateeq M and Raza MA. 2022. Foliar Application of Silicon Improved Physiological Indicators, Yield Attributes, and Yield of Pearl Millet (*Pennisetum glaucum* L.) Under Terminal Drought Stress. *Journal of Soil Science and Plant Nutrition* **22**(4): 4458-4472.
- Wu LM, Fang Y, Yang HN and Bai LY. 2019. Effects of drought-stress on seed germination and growth physiology of quinclorac-resistant *Echinochloa crusgalli*. *Plos one* **14**(4): e0214480.
- Younis A, Ramzan F, Ramzan Y, Zulfiqar F, Ahsan M and Lim KB. 2020. Molecular Markers Improve Abiotic Stress Tolerance in Crops: A Review. *Plants* **9**(10): 1374.
- Zeng FS, Yao YF, Wang LF and Li WJ. 2023. Polysaccharides as antioxidants and prooxidants in managing the double-edged sword of reactive oxygen species. *Biomedicine & Pharmacotherapy* **159**: 114221.
- Zhang C and Shi S. 2018. Physiological and Proteomic Responses of Contrasting Alfalfa (*Medicago sativa* L.) Varieties to PEG-Induced Osmotic Stress. *Frontiers in Plant Science* **9**: 333359.
- Zhang F et al. 2015. Seed Priming with Polyethylene Glycol Induces Physiological Changes in Sorghum (*Sorghum bicolor* L. Moench) Seedlings under Suboptimal Soil Moisture Environments. *Plos one* **10**(10): e0140620.
- Zia R, Nawaz MS, Siddique MJ, Hakim S and Imran A. 2021. Plant survival under drought stress: Implications, adaptive responses, and integrated rhizosphere management strategy for stress mitigation. *Microbiological Research* **242**: 126626.