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# Effect of polyethylene glycol, temperature, and stratification in thirty plant species: Implications for conservation and management

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**Abstract** Seed ecology is a fundamental view of a population's regenerative potential and under climate change needs investigation for proper management and conservation of plant populations. In the Mediterranean basin extreme rainfall variability is expected to prevail during the next decades. The aim of the study is to investigate the germination potential of thirty plant species by placing their seeds under gradients of water potentials with the use of polyethylene glycol, a polymer used to mimic water stress in seeds to assess their adaptive potential. We applied three different osmotic potentials (-0.03 MPa , -0.50 MPa, -1.56 MPa) using PEG6000 in seeds from thirty different plant species. Prior PEG treatment seeds grouped to non-stratified and stratified ones at +2°C for 1 month. Seeds were then germinated under either constant +21°C or alternate +25°C/+15°C temperature with 12-hours photoperiod. Germination was affected by PEG level, stratification and temperature which acted either independently or in combination, and the response to those factors was species specific. Most species that PEG had affected their germination, responded positively in water stress at -0.5 MPa and some of them further increased their seed germination under -1.56 MPa. However, in species where PEG had no effect, their germination response was driven by the other two factors either independently or in combination. Finally, there were also species that presented low germination, unaffected by the studied factors. The present study demonstrates the ability of several Mediterranean species to germinate under various levels of water stress and can be applied to species management and conservation strategies.

**Keywords:** PEG6000, germination, water stress, climate change, cold stratification.

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## Introduction

The incident and the intensity of drought stress across the Mediterranean basin is gradually increasing as a consequence of climate change which can affect species population dynamics. Successful germination is complex process affected by many factors, with water availability and temperature being the most significant. Temperature changes may affect vital processes controlling seed germination such as membrane permeability, cytosolic enzyme activity or influencing water uptake (Bewley & Black 1994, Taiz & Zeiger 2010). The environmental signal triggering germination is the increase in temperature during the favored period of the year. When the seed receives the signal, the soil moisture availability becomes the fundamental factor for germination to begin since water absorption is the first stage for metabolism to start. Thus, soil water availability plays a crucial role in germination onset and subsequent seedling growth (Ansari et al. 2013, Bhatt et al. 2020). Drought stress, due to limited water availability, may negatively impact plant regeneration, seedling growth, and survival (Toscano et al. 2017, Du et al. 2019, Ding et al. 2020) because the reduced osmotic potential of dehydrated seeds inhibits metabolism (Bradford et al. 2013). When water levels are low, seeds do not germinate until water potential is re-established, even if they are not in a dormant state (Hadas & Stibbe 1973). Additionally, water potential and temperature often interact in regulating seed germination rate and speed (El-Sharkawi et al. 1989, Mesgaran et al. 2017, Ostadian Bidgoly et al. 2018).

Drought stress response may vary with species since taxa from different climate provenances or with different ecological traits (e.g., annual vs perennial) may display different responses towards water limitation (Hegarty 1977, Dirik 2000, Volis & Bohrer 2013, Baskin & Baskin 2014, Miranda et al. 2014, Bhatt et al. 2019, Fan et al. 2021). A reduction in seed germination under low water potential is the trend most often reported for different taxa (Wan-li et al. 2004, Barrios et al. 2021, Bhatt et al. 2022). Evaluating species drought tolerance during the seed germination stage could allow for the understanding of regeneration ecology, species persistence and how they might be affected in a changing climate (Zhang et al. 2017, Dantas et al. 2020). It can also help to design successful management and conservation strategies when most needed (Zhu et al. 2006).

The effect of limited water potential (i.e., water stress) in seed germination can be studied by adding either limited water or an osmotica solution, such as sodium chloride or polyethylene glycol (PEG), in a germination substrate (e.g., soil) (Larson & Schubert 1969, Kaufmann & Eckard 1977, Falusi et al. 1983, Maraghni et al. 2010). Osmotic solutions, such as PEG 6000, lower the water potential, simulating drought stress conditions in seeds, thus allowing for assessing their germination ability and speed under potential unfavorable environmental conditions (Michel & Kaufmann 1973). The response of seeds under imposed drought stress with use of PEG 6000 has been used to infer how plant species will be affected by climate change scenarios (Krichen et al. 2014).

Polyethylene glycol (PEG) is a non-toxic, non-ionic polymer with high solubility in water and is widely used to mimic and induce drought stress in plants by decreasing the water potential in the medium. It is not expected to penetrate into plant cells because of its high molecular weight (Carpita et al. 1979). It has been traditionally used to estimate drought resistance in crop or forest plant species at the seed, seedling, or adult plant stages. It is generally reported that seed germination reduces the amount of water absorption necessary for overall seed metabolic processes (e.g., amylases activity) when PEG levels are increased.

Successful germination of seeds under water stress is a process which also depends on germination temperature and seed priming (Dawood 2018). Temperature of germination promotes cellular activity and respiration necessary for the activation of various seed metabolic pathways. Cold stratification acts as a priming method for seeds, resulting in an increase in the levels of germination hormones, such as gibberellin, and a reduction of the inhibitor hormones, such as abscisic acid (Nautiyal et al. 2023). However, despite that polyethylene glycol is used as a method for assessing water stress tolerance, it also acts as an osmopriming factor that suppresses water uptake triggering the seed to initiate pre-germination metabolic activities (Abid et al. 2018, Lei et al. 2021, Busaidi et al. 2023).

In the Mediterranean ecosystems, seasonal water fluctuation is the main factor controlling seedling emergence and establishment. Climate modeling generally predicts lower and more variable precipitation heights and an increase in mean temperature for the Mediterranean Basin (Calbó 2009). Climate change and global warming greatly threaten native plant species by increasing mean temperature and reducing mean precipitation. Regeneration success is a vital stage of a population's dynamics under climate change since early germination stages are among the most at risk under climate change (Walck et al. 2011). Thus, determination of species seed drought tolerance under different osmotic potentials is critical for inferring how seed germination will be affected under climate change caused by limited water and temperature fluctuation (van den Berg & Zeng 2006, Yi et al. 2019, Dantas et al. 2020).

The aim of the study is to investigate the germination potential of thirty plant species under water stress conditions by placing their seeds under three different gradients of water potentials with the use of the osmotica PEG 6000 and germinate them under constant or alternate temperature. We also investigated if cold stratification, which mimics the winter season and acts as a "priming treatment", can ameliorate the effect of the applied water potentials on seed germination. All different treatments were also evaluated as promising methods for the development of reproduction protocols, which for some of the species, such as *Silene frivaldszkyana*, are generally absent from the published literature.

Additionally, the study can help us to reach conclusions about the effect of climate change on the regeneration capacity of the above Mediterranean species and to design measures and strategies for proper conservation and management.

## Materials and Methods

### Studied species

Infructescences from thirty different plant species were collected during September 2021 and they were stored for 1 week at room conditions inside nylon mesh to dry. Afterwards, the seeds were extracted from the fruits and placed inside petri dishes until the experiment initiation.

The collected species and their coordinates are reported in Table 1 and their ecological traits based on reports by Lafranchis and Sfikas (2009) and Dimopoulos et al. (2013) in Supplementary table S2.

### PEG osmotic potentials preparation

Water stress conditions were imposed in seeds by placing them at one of three PEG concentration treatments simulating three different levels of osmotic potential at 21°C: low (-0.03 MPa, 36.79 g/L), medium (-0.50 MPa, 193.46 g/L), and high (-1.56 MPa, 347.20 g/L). The PEG 6000 levels were selected based on literature references for various plant taxa (Wan-li et al. 2004, Kaya et al. 2006, Zhu et al. 2006, Krichen et al. 2014, Toscano et al. 2017, Yousefi et al. 2020, Krichen et al. 2023). Untreated (i.e., control) seeds that were not subjected to water stress were also used. The PEG levels were prepared having their osmotic potential calculated according to Michel & Kaufmann (1973). Seeds in each PEG level treatment was placed between Whatman filter papers regularly moistened to maintain constant attachment of seeds to the osmotica solutions.

**Table 1** List of the different plant species used in the study.

| Species | Common name | Coordinates in WGSA 84 (Longitude, Latitude) | Site Toponymy |
|---|---|---|---|
| *Achillea clypeolata* Sibth. & Sm. | Milfoil | 41.472139N, 24.143282E | Simidas forest, Kato nevrokopi |
| *Achillea millefolium* L. | Common Yarrow | 41.470730N, 24.142215E | Simidas forest, Kato nevrokopi |
| *Arabis turrita* L. | Tower rock cress | 41.472422N, 24.142038E | Simidas forest, Kato nevrokopi |
| *Asphodeline lutea* L. | Yellow asphodel | 41.200179N, 24.029665E | Petrousa, Drama |
| *Briza media* L. | Common quaking grass | 41.469229N, 24.150186E | Simidas forest, Kato nevrokopi |
| *Campanula trachelium* (Boiss. & Heldr.) Hayek. | Nettle-leaved Bellflower | 41.474671N, 24.140980E | Simidas forest, Kato nevrokopi |
| *Centaurea nervosa* Willd. | Plume Knapweed | 41.471144N, 24.131745E | Simidas forest, Kato nevrokopi |
| *Cistus creticus* L. | Pink rock-rose | 41.482067N, 24.148987E | Kariotou Kalyvia, Kato Nevrokopi |
| *Clematis flammula* L. | Fragrant clematis | 41.474212N, 24.141738E | Simidas forest, Kato nevrokopi |
| *Clematis vitalba* L. | Traveller's Joy | 40.758553N, 21.164581E | Prespes, Kastoria |
| *Dianthus cruentus* Griseb. | Blood carnation | 41.477636N, 24.144403E | Simidas forest, Kato nevrokopi |
| *Digitalis viridiflora* Lindl. | Large yellow foxglove | 41.476732N, 24.146727E | Simidas forest, Kato nevrokopi |
| *Epilobium hirsutum* L. | Great willow herb | 41.410200N, 24.106493E | Despatis Potamon, Kato Nevrokopi |
| *Eupatorium cannabinum* L. | Hemp agrimony | 41.485519N, 24.158716E | Kariotou Kalyvia, Kato Nevrokopi |
| *Humulus lupulus* L. | Common hop | 41.269690N, 24.487115E | Mesochori, Paranesti |
| *Lagurus ovatus* L. | Bunny tail grass | 40.592994N, 23.762642E | Olympiada, Chalkidiki |
| *Lilium martagon* L. | Martagon lily | 40.918048N, 24.039156E | Pangaion, Serres |
| *Linaria pelisseriana* (L.) Mill. | Jersey toadflax | 41.476093N, 24.142466E | Simidas forest, Kato nevrokopi |
| *Lychnis coronaria* (L.) Desr. | Rose campion | 41.466203N, 24.145467E | Simidas forest, Kato nevrokopi |
| *Lythrum salicaria* L. | Purple loosestrife | 41.395472N, 24.112733E | Potamoi, Kato Nevrokopi |
| *Physalis alkekengi* L. | Bladder cherry | 41.393301N, 24.118536E | Potamoi, Kato Nevrokopi |
| *Potentilla detommasii* Ten. | Wild cinquefoil | 41.467475N, 24.147844E | Simidas forest, Kato nevrokopi |
| *Saponaria officinalis* L. | Soapwort | 41.468738N, 24.142264E | Simidas forest, Kato nevrokopi |
| *Silene frivaldszkyana* Hampe | No common name | 41.472341N, 24.136606E | Simidas forest, Kato nevrokopi |
| *Silene viridiflora* L. | Bold green flower | 41.471282N, 24.134091E | Simidas forest, Kato nevrokopi |
| *Tanacetum corymbosum* (L.) Sch. Bip. | Corymbflower tansy | 41.481887N, 24.139563E | Simidas forest, Kato nevrokopi |
| *Thymus thracicus* Velen. | Thyme | 41.469975N, 24.141587E | Simidas forest, Kato nevrokopi |
| *Trifolium pratense* L. | Red clover | 41.462709N, 24.144275E | Simidas forest, Kato nevrokopi |
| *Veratrum lobelianum* Bernh. | European white hellebore | 41.394773N, 24.631054E | Lepidas Forest, Paranesti |
| *Verbascum humile* Janka | Mullein | 41.471340N, 24.140957E | Simidas forest, Kato nevrokopi |

## Germination experiment

Species seeds used in the current study were grouped at two categories, non-stratified and cold stratified, prior to the application of the PEG treatment. The germination experiment aimed to test the effect of water stress imposed by the application of PEG in seeds and whether it can be ameliorated under low temperatures during cold stratification for 2 months at +0°C. Seeds both untreated and treated with PEG were germinated under constant temperature of +21°C or diurnal fluctuating temperatures of +25/+15°C under 12-hours photoperiod with the higher temperature to coincide with the light cycle (Zhu et al. 2006, Veiga-Barbosa and Pérez-García 2014, Bhatt et al. 2022). Four repetitions of 100 seeds per treatment combination were used. Seeds in each treatment were placed between filter papers moistened with distilled water or PEG.

Seeds were continuously exposed to water stress by adding daily fresh PEG solution, when necessary, throughout the duration of the experiment.

At the end of the germination experiment, ungerminated seeds in each treatment were checked if being filled or empty through seed-cut test and final germination percentages were corrected on the base of filled seeds that contained firmly developed embryos (Thomas et al. 1994). Thus, corrected germination percentages (CGP) were calculated based on the formula by Weller et al. (2016), modified by replacing the number of viable embryos with the number of filled seeds:

CGP(%)=Ngerm/(Ngerm+Nfilled)×100 (Eq. 1)

where: Ngerm is the number of germinated seeds and Nfilled is the number of ungerminated seeds with firm embryos.

Finally, mean germination values for all the three PEG levels (namely species means) were calculated for each species, according to van den Berg & Zeng (2006) (Supplementary Table 1).

## Statistical analysis and experimental design

A factorial experimental design with three factors (2 x 2 x 4) arranged in a completely randomized block design was applied in the study. Germination temperature was the first factor with two levels (+21°C, +25/+15°C), cold stratification was the second one with two levels (stratified, non-stratified) while the third factor was PEG treatment with four levels (Untreated, -0.03 MPa, -0.5 MPa, -1.5 MPa). Germination counts were taken every 3 days for a total of 15 weeks and seeds were considered germinated if the radicle protruded 2 mm from the seed coat. Each treatment was replicated four times and mean germination values were calculated. Percentage germination data was arcsine square root transformed to meet normality, checked through Shapiro–Wilk test, and homogeneity, checked through Levene's test (Zhu et al. 2006, Yi et al. 2019). Differences in mean germination values were checked through ANOVA analysis while the significance of temperature, PEG level or stratification as germination factors was checked through generalized linear modeling. Additionally, cluster analysis was performed via K-means Clustering (Bhatt et al. 2022). All the statistical analyses were performed in SPSS v.19 (Chamorro et al. 2013).

## Results

Water stress imposed by PEG application affected final germination in most of the studied species. In non-stratified seeds PEG application significantly increased germination under alternate temperature in *S. frivaldszkyana*, *Thymus thracicus*, *Lycnhis coronaria*, *Eupatorium cannabinum*, *Potentilla detommasii*, *Silene viridiflora*, *Verbascum humile*, *Centaurea nervosa*, *Lagurus ovatus*, and *Briza media*, while PEG generally enhanced seed germination in *Tanacetum corymbosum*, *Arabis turrita*, *Lythrum salicaria*, *Campanula trachelium* and *Dianthus cruentus*, irrespective of temperature regim (Table 2). With reference to the PEG level effect most of the above species responded positively to low (-0.03 MPa) or medium stress level (-0.5 MPa), except *S. frivaldszkyana*, *L. ovatus*, *A. turrita*, *C. trachelium* and *L. salicaria*, where high germination of non-stratified seeds was also retained under the high-level (-1.56 MPa) stress treatment. Regarding the rest of the species with non-stratified seeds, PEG application did not generally affect final germination, except *Epilobium hirsutum* and *Asphodeline lutea* where no germination was recorded under alternate temperatures at medium or high PEG level (Table 2).

Germination increase under PEG application was also observed in stratified

**Table 2** Germination percentages of non-stratified seeds under different water stress level and germination temperature (mean values ± standard error).

| Species | PEG concentration (MPa) / Germination Temperature (ºC) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | no PEG/21 | -0.03/21 | -0.5/21 | -1/21 | no PEG/25-15 | -0.03/25-15 | -0.5/25-15 | -1/25-15 |
| *Achillea clypeolata* | 21.06±2.13c | 15.10±3.06bc | 12.08±2.79ab | 6.00±2.00a | 10.00±1.35ab | 8.00±1.63ab | 8.10±2.25ab | 7.96±1.63ab |
| *Achillea millefolium* | 15.09±1.36a | 12.06±1.63a | 9.08±3.06a | 11.16±1.95a | 20.13±2.00b | 20.01±0.91b | 18.30±0.89b | 9.96±2.01a |
| *Arabis turrita* | 6.00±1.08e | 14.00±2.58c | 89.00±4.12a | 41.75±0.85d | 48.00±1.82b | 85.00±6.40a | 85.00±6.81a | 96.00±2.83a |
| *Asphodeline lutea* | 8.00±0.91a | 14.00±2.58ab | 10.00±2.00ab | 11.001.00ab | 16.00±1.08b | 14.00±1.15ab | 0c | 0c |
| *Briza media* | 8.25±2.06a | 15.00±4.12a | 12.00±2.31a | 10.00±3.46a | 6.00±1.82a | 11.00±3.00a | 54.00±5.94b | 69.75±3.84b |
| *Campanula trachelium* | 13.00±0.91b | 30.00±0.41c | 63.00±1.29e | 78.00±0.58a | 79.00±1.08a | 88.00±6.93ad | 98.00±2.00d | 82.75±6.18ad |
| *Centaurea nervosa* | 20.00±1.83a | 88.75±1.49c | 16.17±1.69a | 16.04±1.50a | 42.21±1.49b | 48.39±10.55b | 98±1.15c | 48.11±7.46b |
| *Cistus creticus* | 8.03±0.73a | 10.02±3.45a | 11.03±1.01a | 12.00±1.63a | 8.00±0.71a | 10.08±1.11a | 12.12±3.64a | 10.00±1.15ab |
| *Clematis flammula* | 18.00±1.00a | 18.00±1.15a | 19.00±3.42a | 12.00±2.83a | 14.00±0.71a | 11.00±1.91a | 13.00±2.52a | 16.00±4.90a |
| *Clematis vitalba* | 16.00±1.63a | 15.00±3.00a | 15.00±4.43a | 13.00±3.79a | 19.00±1.08a | 21.00±4.73a | 14.00±3.46a | 27.00±8.54a |
| *Dianthus cruentus* | 56.56±1.55d | 64.32±1.61b | 24.32±1.74e | 11.13±3.36f | 59.00±1.96d | 80.89±6.31a | 80.17±7.02a | 45.22±1.89c |
| *Digitalis viridiflora* | 21.00±0.71c | 30.00±10.00bc | 20.00±2.83c | 21.00±5.74c | 72.00±2.45a | 69.00±11.12a | 36.00±2.83b | 33±6.40bc |
| *Epilobium hirsutum* | 4.00±0.71a | 6.00±1.15a | 7.00±1.00a | 13.00±5.26a | 9.00±1.47a | 0b | 0b | 0b |
| *Eupatorium cannabinum* | 16.00±1.22b | 33.50±1.55d | 36.09±0.63d | 25.10±1.83b | 9.04±1.57c | 13.17±1.96b | 76.22±5.08a | 6.01±2.00e |
| *Humulus lupulus* | 14.00±1.22a | 13.00±2.35a | 16.00±5.89a | 16.00±0.91a | 14.00±1.78a | 21.00±1.00a | 14.00±2.00a | 13.00±1.91a |
| *Lagurus ovatus* | 18.00±2.27a | 23.00±6.81a | 49.00±9.00ab | 26.00±3.83a | 71.00±3.81bc | 90.00±6.22cd | 94.75±2.78d | 94.00±4.76d |
| *Lillium martagon* | 23.00±1.08a | 20.00±2.31a | 21.00±5.74a | 18.00±3.46a | 21.00±2.80a | 12.00±4.00a | 13.00±3.42a | 16.00±3.27a |
| *Linaria pelisseriana* | 13.00±0.91a | 12.00±2.31a | 16.00±4.62a | 14.00±2.00a | 11.00±1.47a | 10.00±2.58a | 18.00±1.15a | 12.00±1.63a |
| *Lychnis coronaria* | 71.00±3.03b | 64.00±8.49b | 23.00±1.91a | 33.00±4.36a | 83.00±3.03b | 86.00±6.00b | 98.00±1.15c | 81.00±9.00b |
| *Lythrum salicaria* | 12.00±1.29d | 66.00±8.65ab | 65.00±5.43ab | 70.00±8.73ab | 19.00±0.91c | 75.00±2.08a | 74.00±0.91a | 56.00±5.76b |
| *Physalis alkekengi* | 8.00±1.47a | 8.00±8.00a | 7.00±7.00a | 10.00±10.00a | 9.00±0.41a | 9.00±1.00a | 7.00±1.00a | 6.00±1.16a |
| *Potentilla detommasii* | 20.00±1.58b | 10.00±3.46 bc | 8.00±0.41c | 10.00±4.16 bc | 20.00±2.48b | 20.00±2.83b | 82.00±8.41a | 17.00±3.00b |
| *Saponaria officinalis* | 14.00±2.20a | 11.00±4.43a | 11.00±2.52a | 10.00±1.15a | 9.00±2.04a | 9.00±1.00a | 9.00±1.91a | 9.00±3.42a |
| *Silene frivaldszkyana* | 36.00±1.58a | 39.00±5.51a | 13±1.00b | 14±2.58b | 31.00±1.78a | 65.00±4.26c | 100.00±0.00d | 100±0.00d |
| *Silene viridiflora* | 52.00±3.39c | 46.00±2.58c | 48.00±7.12c | 16.00±6.73d | 87.00±2.27b | 100.00±0.00a | 94.00±2.58b | 81.00±3.79b |
| *Tanacetum corymbosum* | 16.12±1.06b | 22.12±1.87b | 72.51±3.00c | 14.17±2.63b | 44±2.35a | 59.47±10.02ac | 98.49±0.96d | 68.61±6.64c |
| *Thymus thracicus* | 15.00±1.35b | 25.00±2.12a | 27.00±2.89a | 16.00±2.12b | 27.00±2.35a | 35.00±5.74a | 90.00±3.46c | 33.00±5.26a |
| *Trifolium pratense* | 13.00±1.22a | 15.00±4.72ab | 10.00±2.00a | 10.00±1.68a | 19.00±1.47b | 19.00±1.58b | 20.00±2.83b | 12.00±1.78a |
| *Veratrum lobelianum* | 13.00±1.47a | 13.00±1.00a | 13.00±2.52a | 16.00±4.32a | 14.00±0.91a | 13.00±1.91a | 12.00±2.83a | 15.00±4.12a |
| *Verbascum humile* | 68.18±1.70a | 66.17±0.44a | 52.40±0.64c | 22.06±0.06d | 33.00±0.41b | 32.32±4.82b | 100.00±.000e | 66.74±3.95a |

Note: Values within rows followed by different small letter represent difference at 0.05 level of significance seeds of *Trifolium pratense*, *T. thracicus*, *E. cannabinum*, *P. detommasii*, *L. coronaria*, *T. corymbosum*, *Cistus creticus*, *V. humile*, *E. hirsutum*, and *C. trachelium* seeds when germinated at constant rather than alternate temperature (Table 3).

**Table 3** Germination percentages of cold stratified seeds under different water stress level and germination temperature (mean values ± standard error).

| Species | PEG concentration (MPa) / Germination Temperature (°C) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | no PEG/21 | -0.03/21 | -0.5/21 | -1/21 | no PEG/25-15 | -0.03/25-15 | -0.5/25-15 | -1/25-15 |
| *Achillea clypeolata* | 18.00±1.68b | 7.05±2.98a | 7±2.52a | 12.00±4.32ab | 11.00±1.22ab | 9.00±2.52a | 8.90±3.34a | 6.08±1.20a |
| *Achillea millefolium* | 16.19±1.23a | 14.00±2.58a | 20±2.31a | 22.10±6.72a | 26.94±5.82a | 0b | 0b | 0b |
| *Arabis turrita* | 67.00±2.74a | 44.00±0.00c | 40.00±4.89c | 20.00±4.61d | 20.00±1.96d | 23.00±3.00d | 86.00±6.00b | 87.00±6.60b |
| *Asphodeline lutea* | 10.00±1.77a | 8.00±1.63a | 11.00±1.91a | 9.00±1.00a | 9.00±1.40a | 0b | 0b | 0b |
| *Briza media* | 9.00±1.22bc | 9.00±1.91bc | 10.00±2.00bc | 6.00±2.58bc | 14.00±2.85ab | 10.00±3.46bc | 6.00±1.15c | 23.00±3.41a |
| *Campanula trachelium* | 18.00±2.74b | 21.00±3.79bc | 27.00±0.41c | 28.00±0.41c | 68.00±3.37a | 70.00±6.22a | 44.00±4.90d | 36.00±5.16cd |
| *Centaurea nervosa* | 31.09±2.56bc | 39±13.00bc | 54±11.37c | 34.91±4.99bc | 25.00±2.97b | 24±4.00b | 41±9.75bc | 9±1.00a |
| *Cistus creticus* | 9.05±2.97a | 12.13±1.68ab | 36.10±6.35c | 32.00±4.90c | 29.00±1.96c | 24.17±16.30abc | 24.00±4.00bc | 9.05±2.97a |
| *Clematis flammula* | 15.00±1.47a | 13.00±3.00a | 14.00±3.46a | 11.00±1.91a | 12.00±1.68a | 15.00±1.91a | 11.00±1.00a | 10.00±3.46a |
| *Clematis vitalba* | 21.00±1.08a | 20.00±5.16a | 22.00±1.15a | 18.00±2.58a | 18.00±2.27a | 16.00±1.63a | 21.00±2.52a | 16.00±3.65a |
| *Dianthus cruentus* | 35.07±2.69a | 42.00±2.27ab | 68.00±5.66c | 78.19±1.06cd | 52.00±2.86b | 50.00±1.96b | 71.35±5.75cd | 82.00±6.06d |
| *Digitalis viridiflora* | 29.00±0.71b | 37.00±5.00b | 35±5.51b | 32±9.09b | 12.00±1.35a | 10.00±4.16a | 12.00±4.32a | 13.00±3.42a |
| *Epilobium hirsutum* | 12.00±1.58d | 26.00±6.22b | 29.00±9.29b | 73.00±9.15a | 36.00±1.29b | 15.00±1.00d | 32.00±4.32b | 22.00±6.22d |
| *Eupatorium cannabinum* | 22.00±2.74ab | 56.37±7.18c | 28.26±2.75b | 12.00±1.63ab | 29.43±2.59b | 26.21±3.53b | 21.06±5.23ab | 9.05±4.98a |
| *Humulus lupulus* | 15.00±2.16ab | 16.00±1.63ab | 17.00±2.52b | 10.00±0.41a | 40.00±3.16c | 36.00±3.65c | 20.00±3.27b | 21.00±2.52b |
| *Lagurus ovatus* | 91.00±4.43a | 92.00±4.32a | 89.00±4.43a | 84.00±2.16a | 75.00±4.80b | 73.00±3.42b | 91.00±4.43a | 92.00±1.63a |
| *Lillium martagon* | 14.00±1.08a | 11.00±1.91a | 13.00±1.91a | 18.00±3.83a | 13.00±1.68a | 15.00±5.26a | 14.00±3.46a | 14.00±3.83a |
| *Linaria pelisseriana* | 13.00±0.91a | 13.00±3.42a | 12.00±4.00a | 17.00±4.43a | 10.00±2.38a | 9.00±1.91a | 12.00±0.00a | 11.00±2.52a |
| *Lychnis coronaria* | 14.00±1.96a | 12.00±5.16a | 16.00±3.27a | 62.00±10.39c | 61.00±3.11c | 52.00±7.48c | 42.00±4.76b | 35.00±1.91b |
| *Lythrum salicaria* | 14.00±1.22e | 64.00±2.48a | 78.00±1.58b | 100.00±0.00c | 52.00±4.14a | 87.00±5.12b | 43.00±1.91d | 16.00±1.63e |
| *Physalis alkekengi* | 9.00±1.35a | 5.00±1.00a | 7.00±2.52a | 6.00±2.00a | 9.00±1.91a | 5.00±1.91a | 8.00±1.63a | 8.50±1.85a |
| *Potentilla detommasii* | 14.00±1.96b | 14.00±2.58b | 67.00±6.61a | 75±15.52a | 12.00±1.08b | 8.00±2.31b | 13.00±5.00b | 10.00±2.59b |
| *Saponaria officinalis* | 12.00±2.04a | 10.00±3.46a | 13.00±2.52a | 11.00±4.43a | 13.00±1.78a | 10.00±2.58a | 15.00±1.91a | 12.00±2.31a |
| *Silene frivaldszkyana* | 17.00±1.47a | 18.00±2.58a | 49.00±5.45b | 42.00±9.10b | 61.00±1.96b | 64.00±12.00b | 100±0.00c | 49.00±12.26b |
| *Silene viridiflora* | 53.00±1.08b | 52.00±6.06b | 62.00±4.76b | 78.00±2.00a | 55.00±2.04b | 62.00±6.26bc | 76.00±1.63ac | 84.00±2.83a |
| *Tanacetum corymbosum* | 20.00±1.08b | 22.00±3.83bc | 38.00±4.83c | 74.00±10.89a | 61.92±5.37a | 61.13±6.55a | 27.00±9.15bc | 22.08±4.73b |
| *Thymus thracicus* | 11.00±2.52a | 12.00±1.63a | 58.00±7.39b | 49.00±10.25b | 13.00±1.08a | 12.00±2.83a | 10.00±3.46a | 19.00±2.52a |
| *Trifolium pratense* | 33.00±2.80a | 32.00±5.66a | 58.00±2.58bc | 64.00±4.00b | 41.00±2.38a | 40.00±7.08ac | 36.00±6.32a | 36.00±8.16a |
| *Veratrum lobelianum* | 14.00±2.35a | 14.00±4.76a | 18.00±3.83a | 17.00±6.40a | 17.00±1.87a | 17.00±1.91a | 16.00±3.27a | 18.00±4.76a |
| *Verbascum humile* | 38.18±1.90b | 44.22±0.22c | 42.13±4.85bc | 73.00±0.41d | 86.00±1.83a | 67.00±5.97d | 4.01±1.63e | 1.25±0.25e |

Note: Values within rows followed by different small letter represent difference at 0.05 level of significance

In *L. ovatus* and *A. turrita* increase in germination of PEG treated seeds was observed under alternate temperature. Increased germination under water stress was observed in cold stratified seeds of *S. frivaldszkyana*, *S. viridiflora*, *L. salicaria* and *D. cruentus* under both constant and alternate temperature. Regarding water stress level and incubation temperature most of the positively responded species in PEG treatment application generally increased their stratified seeds germination at low or medium but not high stress level except for *S. frivaldszkyana*, *T. thracicus*, *P. detommasii*, *L. coronaria*, *T. corymbosum*, *Cistus creticus*, *S. viridiflora*, *V. humile*, *E. hirsutum*, *L. ovatus*, *L. salicaria*, *A. turrita* and *D. cruentus*. In the rest of the species, PEG application did not affect germination except for *A. lutea* and *Achillea millefolium* where no germination recorded under alternate temperatures at any PEG level.

Generalized linear model analysis revealed significant interaction of PEG level with seed pretreatment (i.e. cold stratification) in 18 out of the 30 species of the study and with germination temperature in 17 out of the 30 species (Table 4) showing species specific response to water stress. In general,

**Table 4** Significance of factor effect on germination through Generalized linear model analysis.

| Species | Factor | | | | | |
|---|---|---|---|---|---|---|
| | Seed pretreatment | Germination temperature | PEG level | Seed pretreatment x PEG level | Germination temperature x PEG level | Germination temperature x Seed pretreatment |
| *Achillea clypeolata* | .212 | **.016** | **.000** | .333 | .237 | .318 |
| *Achillea millefolium* | **.001** | **.002** | .660 | .093 | .198 | .278 |
| *Arabis turrita* | .398 | **.000** | **.000** | **.004** | .016 | **.000** |
| *Asphodeline lutea* | .984 | .053 | .064 | **.000** | **.004** | .315 |
| *Briza media* | **.000** | **.000** | **.000** | **.000** | **.000** | **.021** |
| *Campanula trachelium* | **.000** | **.000** | **.000** | **.000** | **.000** | .000 |
| *Centaurea nervosa* | **.000** | .247 | **.000** | **.011** | **.000** | **.000** |
| *Cistus creticus* | **.000** | .907 | .040 | .246 | **.000** | .920 |
| *Clematis flammula* | **.032** | **.048** | .258 | .775 | .415 | .395 |
| *Clematis vitalba* | .222 | .445 | .969 | .245 | .648 | **.032** |
| *Dianthus cruentus* | **.001** | **.000** | **.006** | **.000** | **.011** | **.000** |
| *Digitalis viridiflora* | **.000** | .488 | **.016** | **.003** | .057 | **.000** |
| *Epilobium hirsutum* | **.000** | **.003** | **.000** | **.000** | **.000** | .451 |
| *Eupatorium cannabinum* | .643 | **.004** | **.000** | **.000** | **.000** | .387 |
| *Humulus lupulus* | **.000** | **.000** | .007 | .026 | .005 | .000 |
| *Lagurus ovatus* | **.000** | **.000** | **.000** | .089 | **.010** | **.000** |
| *Lillium martagon* | .035 | .074 | .303 | .475 | .960 | .113 |
| *Linaria pelisseriana* | .263 | .100 | .155 | .437 | .372 | .411 |
| *Lychnis coronaria* | **.000** | **.000** | .580 | **.006** | **.001** | **.010** |
| *Lythrum salicaria* | .118 | **.044** | **.000** | .108 | **.000** | **.006** |
| Physalis alkekengi | .543 | .914 | .835 | .824 | .977 | .716 |
| *Potentilla detommasii* | .917 | .477 | **.000** | **.025** | **.001** | **.000** |
| *Saponaria officinalis* | .115 | .494 | .382 | .665 | .947 | .098 |
| *Silene frivaldszkyana* | .576 | **.000** | **.000** | **.006** | **.000** | **.043** |
| *Silene viridiflora* | **.035** | **.000** | **.003** | **.000** | **.002** | **.000** |
| *Tanacetum corymbosum* | **.023** | **.000** | **.000** | **.000** | **.003** | .000 |
| *Thymus thracicus* | **.000** | .640 | **.000** | **.001** | .238 | **.000** |
| *Trifolium pratense* | **.000** | .873 | .497 | **.003** | **.005** | **.001** |
| *Veratrum lobelianum* | .176 | .537 | .814 | .872 | .760 | .392 |
| *Verbascum humile* | **.009** | .760 | .109 | **.000** | .247 | **.024** |

Note: Table cells with grey fonts represent significant p-value at 0.05 level.

non-stratified seeds performed better when water stressed under alternate temperature while the reverse trend was observed for stratified seeds. For example, the PEG effect interacted with both cold stratification or incubation temperature in *S. frivaldszkyana*, *C. trachelium*, *D. cruentus*, *P. detommasii*, *S. viridiflora*, and *C. nervosa*. However, in *T. thracicus* and *V. humile* PEG interacted with the cold stratification but not with incubation temperature, while the opposite was observed in *C. creticus*, *L. ovatus*, and *L. salicaria*.

K-means clustering analysis of whole germination treatments determined three groups (clusters) (Figure 1).

Species assigned to cluster 1 had the lowest germination percentages in total. In the species assigned to cluster 2, the germination percentages of water stressed seeds were unaffected by cold stratification when placed at constant rather than alternate temperature, where non-stratified generally performed better. Finally, species assigned to cluster 3 germinated better under alternate temperature when cold stratified contrary to non-stratified ones, when germination took place under constant temperature.

## Discussion

The germination response of native plant species under water stress is a subject of importance under the ongoing climate change to the view of species persistence. PEG is a polymer traditionally used to infer water stress through osmotic adjustment in plant tissues. In general, a reduction in seed germination under PEG application has been reported for various plant taxa (Kaur et al. 1998, Maraghni

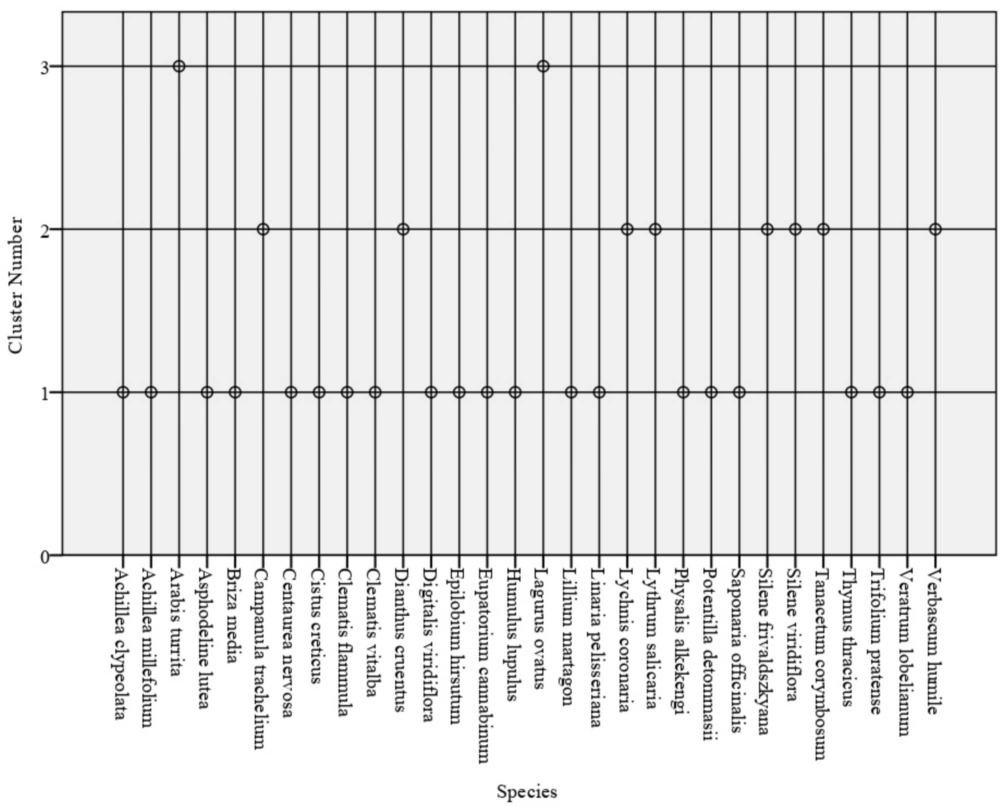


**Figure 1** Species classification based on K-means cluster analysis of germination data.

et al. 2010, Cochrane et al. 2014, Krichen et al. 2017, Bhatt et al. 2022, Müller et al. 2022). In our study, the PEG effect on seed germination response varied according to the species considered and was dependent on incubation temperature, cold stratification and level of water stress.

## Cold stratification

Seed cold stratification is a laboratory method to alleviate certain types of dormancy such as physiological or morphophysiological ones. Its ecological meaning is a seed dormant period imposed by inhibitory hormones to prevent seed germination during the winter period (Finch-Savage & Leubner-Metzger 2006). In our study cold stratification proved to be significant according to generalized linear model analysis in almost half of the studied species (e.g., *T. thracicus*, *T. pratense*, *C. nervosa*, *D. cruentus*). Most of the species expressing this type of dormancy that required cold stratification are commonly late flowering species (i.e., late germinating) that usually germinate in the end of spring and early summer (Supplementary Table 2). Thus, cold stratification is an adaptive trait in species that usually grow in relatively warmer sites with high mean annual temperature, but intense winter period through out of which become physiologically dormant to overcome unfavorable winter events (e.g., drought-freezing periods) (Klupczyńska & Pawłowski 2021).

## Incubation temperature

Temperature is the environmental signal that triggers germination when dormancy has been alleviated. The determination of the optimal germination temperature is of critical importance for the development of reproduction protocol assays from seeds (Murdoch & Kebreab 2013). Usually, alternate rather than constant temperature regimes stimulate greater seed germination, but this is species specific, related to the depth of seed burial in the soil as well as the community structure (Thompson & Grime 1983, Liu et al. 2013). Similarly, in our study, the effect of temperature (irrespective of cold stratification or PEG level) was species specific. However, most of the species had their germination favored under alternate than constant temperature.

The authors attributed this to the fact that the studied species, except *T. pratense* (typical legume with long seed longevity), *C. creticus* (species with long seed longevity) (Scuderi et al. 2010), and *Physalis alkekengi* (species with long seed longevity) (Walters et al. 2005) do not usually form permanent seed banks but are rather scattered on the top soil layers below leaf cover, experiencing highly fluctuating temperatures (day-night) and lurking for suitable germination timing. However, some of the studied species can form transient but not permanent seed banks, such as *A. turrita*, *T. corymbosum* and *Lilium martagon* (Cerabolini et al. 2002), *L. salicaria* (Roovers et al. 2006, Schellenberger et al. 2022), *C. trachelium* and *E. cannabinum* (Kjellsson 1992), *B. media* (Jensen 2004) and *H. lupulus* (Touzard et al. 2002). The species that usually form transient seed banks possess shallow physiological dormancy and germination easily occurs after a winter passage in the ground, which could explain the demand for cold stratification (Honda 2008, Escobar & Cardoso 2015).

## Effect of Water stress and interaction with pretreatment and temperature

The magnitude of PEG effect was level specific and their effect on germination response was species specific. In almost all the studied species the germination percentage of the control seeds did not significantly differ from those treated with low PEG level (-0.03 MPa), indicating that the specific PEG level inferred very low degree of water stress in seeds and all species are able to withstand it. Intermediate stress level (-0.50 MPa) was in general the most successful in promoting germination (e.g., *V. humile*, *T. corymbosum*, *T. thracicus*, *C. nervosa*). On the other hand, for example in *L. ovatus*, high level water stress (-1.56 MPa) increased seed germination, while the opposite was observed for *V. humile*. Thus, it can be stated that germination response to PEG application is not only level dependent, but it is also species specific. This can be attributed to either species ontogeny or adaptation to its ecological site conditions (i.e., germination niche) (Wagner et al. 2011, Bhatt et al. 2022, Müller et al. 2022).

Water stress applied via PEG solutions promoted the germination of non-stratified seeds in some of the studied species, such as *L. salicaria*, *A. turrita* and *C. trachelium*, irrespective of incubation temperature, indicating that they are tolerant to moisture stress and thus able to germinate under low water potential in areas with seasonally low precipitation heights (Dantas et al. 2020).

However, subjecting seeds of the same species to cold stratification differentiated the germination response to PEG according to species and germination temperature. For A. turrita germination response to PEG was generally reduced under constant than alternate temperature while the opposite was observed for *L. salicaria* and *C. trachelium*. Thus, germination response of seeds under water stress was dependent upon cold stratification treatment and incubation temperature (El-Sharkawi et al. 1989, Kebreab and Murdoch 1999, Zhou et al. 2009, Krichen et al. 2017). For example, in non-stratified seeds of *T. corymbosum* the increase of PEG concentration (i.e., higher water stress) initially promoted germination at low and medium levels (-0.03 and -0.5 MPa), but reduced it at the high level (-1.56 MPa). In the same species, germination response increased in general at the high PEG concentration level after seed cold stratification. Thus, the cold stratification of seeds might have acted as priming treatment, improving its response to water stress and enhancing germination promoter hormones, such as $GA_3$, in seeds, which ameliorated possible negative PEG effect at the high concentration level (Kaur et al. 1998, Wagner et al. 2011).

On the other hand, the application of PEG reduced or inhibited the germination response towards control (no PEG addition) in non-stratified seeds of species such as *D. cruentus*, *S. viridiflora* and *S. frivaldszkyana*, indicating at first site low tolerance to water stress. However, this negative effect was temperature dependent since germinability increased under alternate temperature rather than constant temperature. Thus, seeds are possibly non dormant and alternate temperature triggers germination under medium or high PEG levels, indicating tolerance to water stress conditions. However, cold stratification in the seeds of the above species promoted germination under alternate rather than constant incubation temperature, indicating a significant interaction between incubation temperature and cold stratification when the seeds are placed under PEG water stress and furthermore it is species dependent and could be attributed either to species ontogeny or site microenvironmental factors (van den Berg & Zeng 2006, Bhatt et al. 2022).

However, not all the species had their germination response affected by PEG application. For example, *P. alkehengi*, *Saponaria officinalis*, *Veratrum lobelianum*, *Clematis vitalba*, L. *martagon*, *Clematis flammula* did not differ in germination capacity under water stress irrespective of cold stratification or incubation temperature. Thus, PEG as a stress osmopriming factor or cold stratification as a dormancy alleviation treatment were not enough to trigger germination. Thus, we speculate the existence of deeper dormancy in seeds, which needs different treatments, such as exogenous germination hormones, to alleviate possible dormancy (Wagner et al. 2011).

## Implications for management and conservation under climate change

Understory species such most of the current study constitute important components of many Mediterranean forest understories and their growth and survival under the climate change, contributes to the overall ecosystem carbon fixation and use (Gonzalez et al. 2013, McIntosh et al. 2016). Additionally, it is generally known that many understory species are connected to specific habitat types and their regeneration is an indicator of forest regeneration dynamics (McLachlan and Bazely 2008, Burrascano et al. 2009). The knowledge of seed ecology is a prerequisite for the development of proper management practices to maintain viable populations, with increasing significance especially for endemic species. This study showed that cold stratification and type of germination temperature had a great effect on seed germination for most of the studied species. These treatments are easy to apply and can be introduced in the development of reproduction protocols for the restoration and maintenance of genetic variability through management practices, such as micro-reserves or botanical gardens for ex-situ protection (Zheng et al. 2005, Al Hassan et al. 2021).

Changes in temperature and precipitation, with increasing drought periods due to climate change, will affect many aspects of seed ecology, such as germination, and dormancy release (Walck et al. 2011). Thus, seed performance in terms of germination, the earliest phase for plant development, under water stress is a crucial step that needs to be investigated for selection properties of adaptive populations and the application of management, conservation, and restoration purposes (van den Berg & Zeng 2006, Cochrane et al. 2014, Zerpa-Catanho et al. 2019, Bhatt et al. 2021, Zagoub et al. 2022). In our study, PEG improved germination rates in many of the studied species, suggesting that it could be easily used as a priming method (osmopriming) for developing reproduction protocols for successful species restoration (Wagner et al. 2011, Lin et al. 2017). Since PEG mimics water stress in seeds, it could also be used to assess the performance of species populations in terms of seed vigor under periods of low rainfall and high temperature.

## Conclusions

In our study, PEG generally enhanced seed germination, especially at the intermediate level of -0.50 MPa which shows that most of the studied species, extensively distributed in the Balkans and Southern Europe, can withstand at least mild water stress. However, many of the studied species maximized their germinability under even higher PEG levels (-1.56 MPa), showing better stress adaptive potential under climate change in the Mediterranean area where extreme rainfall variability events are predicted.

### Conflict of interest

The authors declare that they have no conflict of interest.

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