EGYPTIAN JOURNAL OF BOTANY (EJBO)

Seed priming strategies of *Hordeum vulgare* L. as a sustainable approach to alleviate drought stress

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Hordeum vulgare L. (Barley) is an essential economic cereal crop used mainly in breads and health products. This study focuses on applying different priming strategies to barley seeds to alleviate drought stress, which poses a serious threat to crop production. Seed priming techniques in the current experiments include hydropriming with water, phytopriming using *Asphodelus microcarpus* L. aqueous extract (5 and 10%), halopriming using zinc chloride (1, 0.25, and 0.05gm/L), and nanopriming using zinc oxide nanoparticles (1, 0.25, and 0.05gm/L). These strategies were applied under different field capacity levels (80, 60, and 40%). Percentage of germination, germination energy, germination velocity, shoot and root length, vigor index, and leaf area, as well as protein patterns, were estimated at vegetative stage. The study's findings revealed that several priming strategies considerably increased *Hordeum vulgare* L. drought resistance by improving germination parameters, plant establishment, and overall plant development under drought conditions. Furthermore, the study evaluated whether changes in protein patterns could induce drought tolerance through different treatments. The seed priming strategies examined in this work have shown significant potential in relieving drought stress in barley. More research is needed to determine the best priming techniques for different barley varieties and to evaluate their long-term impact on crop performance.

Keywords: *Asphodelus microcarpus,* Barley, Drought tolerance, Priming techniques, ZnO-nanoparticles

ARTICLE HISTORY

Submitted: April 15, 2025

Accepted: July 3, 2025

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DOI: 10.21608/ejbo.2025.375568.3264

EDITED BY: Fawzy M. Salama

INTRODUCTION

One ubiquitous environmental problem that severely reduces crop productivity globally is drought stress, which has an adverse impact on the growing, development, and yield of various plant species (Seleiman et al., 2021). It has varying impact on seed germination, vegetative and reproductive development, and maturation phases, based on how frequently and severely drought stress occurs (Anjum et al., 2017). The primary effect of drought stress is on seed germination and the vegetative stage, impacting the molecular, physiological, biochemical, and morphological characteristics, and causing a reduction in overall seedling growth (Yigit et al., 2016). Among these, *Hordeum vulgare* L., or barley, is one of the world's most significant cereal crops in terms of nutrition and economics. Due to its drought stress tolerance, barley requires creative and sustainable ways to increase its productivity and resilience in water-constrained environments (Hussain et al., 2022).

Pre-sowing treatments, such as seed priming, can be applied in a variety of ways to improve seed vigor and early germination (Evenari, 1984). Priming seeds is a pre-germinative improvement strategy that stimulates the early emergence of seedlings by modulating metabolic processes in the early stages of germination. It has been stated to as the most essential short-term technique to alleviate the adverse impact of drought on plants (Khan et al., 2019). The main objective of this pre-sowing procedure is to activate the process of germination in the seed's metabolic machinery and prepare the seeds for radicle protrusion while preventing radicle emergence (Nawaz et al., 2013). Prime seeds have a more effective germination process, resulting in higher germination rates and uniformity than non-primed ones (Moosavi et al., 2009). It provides improved and uniform germination by lowering imbibition time (Brocklehurst & Dearman, 1983), enhancing pre-germinative activation of enzymes and compound synthesis (Hussain et al., 2016), and restoring damaged DNA (Macovei et al., 2017). It has recently been used in numerous crops such as wheat and maize to alleviate the negative effects of drought stress (Hussain et al., 2022; Elnajar et al., 2024; Manavalagan et al., 2024).

Seed priming methods like hydropriming (HyP), phytopriming (PhP), halopriming (HaP), and nanopriming (NP) apply moderate stress to plants, promoting genes and proteins that contribute to tolerance, such as abundant late embryogenesis, which may enhance drought stress resistance (Thomas et al., 2020; Sen et al., 2021). HyP has demonstrated faster germination and greater root growth (Ibrahim, 2016; Ermiş et al., 2021). It reduces the detrimental effects of a lower water potential gradient caused by drought stress, which can inhibit primary root protrusion (Ibrahim, 2016). PhP refers to the use of an aqueous plant extract for seed priming. Extracts from several agricultural and tree residues have been observed to have growth-promoting properties (Imran et al., 2014; Rafi et al., 2015). Numerous studies have proven that diverse botanicals can improve seed germinability potential under a variety of environmental circumstances (Imran et al., 2014; Rafi et al., 2015). Priming with *Asphodelus microcarpus* L. extract is a promising way to improve the drought tolerance of *Hordeum vulgare* L. This extract may activate certain biochemical and molecular pathways within the seeds, supporting improved stress response mechanisms (Ghoneim et al., 2013). HaP is a typical procedure in which seeds are soaked in an inorganic salt solution, such as NaCl, $CaCl_2$, KNO3, etc. (Kumari et al., 2017). Inorganic salts decrease the seeds' water potential, resembling the effects of drought stress (Patade et al., 2009). HaP causes a physiological reaction in seeds that affects plant stress memory, leading plants to react strongly to imminent abiotic stress (Jisha et al., 2013). Plant stress memory is maintained following seed HaP and being subjected to osmotic stress (Llorens et al., 2020). Consequently, seed HaP is extremely effective in strengthening plant resistance to adverse environmental conditions and in improving production (Khaing et al., 2020; Hussain et al., 2022). NP, or the pre-treatment of seeds with nanoparticles, is one method for overcoming these restrictions and successfully increasing plant resistance to future stress conditions. NP can help to improve abiotic stress tolerance by inducing a diversity of metabolic and physiological mechanisms (Yavari et al., 2023). Zinc is one of the most essential micronutrients, playing a role as a regulatory cofactor for numerous enzymes and in chlorophyll formation, fertilization, pollen function, and seed germination, resulting in increased plant growth and yield (Cakmak, 2000). ZnO nanoprimed seeds have demonstrated resilience to drought stress (Rai-Kalal et al., 2021).

The objective of this research is to investigate different seed priming procedures (HyP, PhP, HaP, and NP) for *Hordeum vulgare* L., with the specific goal of mitigating drought stress. The study aims to better understand how pre-sowing treatments affect germination, plant vigor, and overall performance of *Hordeum vulgare* L. seeds under water shortage situations, offering useful insights for the development of sustainable agriculture methods in drought-prone countries. Finally, the purpose is to improve barley cultivation resilience and promote food security in water-limited environments.

MATERIALS AND METHODS

The seeds of barley (*Hordeum vulgare* L.; Giza 126 cultivar) were obtained from the Agriculture Research Institute (Giza, Egypt).

Collection and preparation of *Asphodelus microcarpus* L. for PhP

Tuberous roots of *Asphodelus microcarpus* L. were collected from different habitats in Borg El-Arab, Egypt's western Mediterranean region, from April to May 2022. The plant materials were cleaned using tap and bi-distilled water, then allowed to air dry in a shaded area. Subsequently, they were cut into small pieces and ground in a Wiley Mill to a fine, uniform texture. The powder was stored in dried glass jars at room temperature until use. Stock aqueous extracts and subsequent dilutions were prepared by combining a constant weight (100gm) of dried powder and 1000mL of distilled water. The extraction was carried out in the dark for 48h at 25^0C. The mixture was then filtered using Whatman No. 41 filter paper. The extract was kept in a refrigerator at 5^0C until used. The stock solution was then used to prepare dilutions (5, 10, 20, and 40%, in addition to a control) for a preliminary test to select the most effective concentration and seed soaking period.

Preparation of zinc chloride ($ZnCl_2$) for HaP and ZnO nanoparticles (ZnONPs) for NP and zinc chloride ($ZnCl_2$) for HaP

Aqueous solution of zinc chloride ($ZnCl_2$) with different concentrations (1, 0.25, and 0.05g/L) were prepared for HaP. ZnO nanoparticles for photoelectric applications were purchased from Nanotech. According to Pacholski et al. (2002), these nanoparticles were prepared when potassium hydrolyses and condenses zinc acetate dihydrate hydroxide in a low-temperature alcohol solution. The excess mother liquor was eliminated from the ZnO nanoparticles, the precipitate accumulated at the bottom, and it was cleaned using methanol. After that, the precipitate was spread throughout a mixture of methanol and chloroform. The physical characteristics of the utilized ZnO nanoparticles were derived from the attached pamphlet, while their shape was define using a JEOL JSM-1400 PLUS transmission electron microscope from Japan. We measured X-ray diffraction (XRD). Different diluted solutions were prepared (1, 0.25, and 0.05g/L) for NP.

Preparation and sterilization of seeds for lab experiments

Before germination, the uniform *Hordeum vulgare* L. seeds were sterilized for 2 to 3min in a diluted sodium hypochlorite solution (3%). They were then washed thoroughly with running tap water, followed by rinsing more than three times with distilled water.

Preliminary test

Before sowing, sterilized *Hordeum vulgare* seeds were soaked in a previously prepared aqueous extract of *Asphodelus microcarpus* L. at different concentrations (0, 5, 10, 20, and 40%). Each concentration had a different soaking period (6, 8, and 10h) resulting in 15 treatments with 5 replicates (15×5) to determine the preferable concentration and time of priming (Figure 1a).

After soaking in distilled water, the seeds were allowed to air dry for 12 hours between two filter sheets, returning them to their initial moisture content. Nine cm petri dishes containing primed and non-primed seeds were covered with Whatman No. 41 filter paper. Each Petri dish contained twenty seeds. Untreated seeds were used as control. From preliminary tests, 5% and 10% aqueous extract concentrations for 12h were selected for priming seeds.

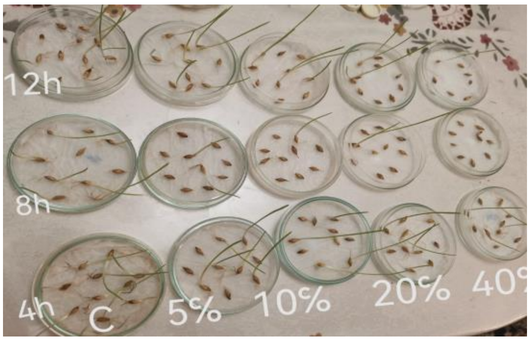
Growth experiment

Sterilized uniform barley seeds were hydroprimed by soaking in distilled water for 12 h, phytoprimed by soaking seeds in 5 and 10% of *Asphodelus microcarpus* L. aqueous extract for 12h, Nanoprimed by soaking in nano particles of zinc oxide (1, 0.25, and 0.05g/L) and Haloprimed using zinc chloride (1, 0.25, and 0.05g/L) for 12h. The experiment was established in April 2023. Ten prepared, primed seeds were individually planted in each plastic pot (15cm diameter, 20cm length, with bottom holes) 0.5cm below the soil surface. Each pot contained 1kg of soil (Figure 1b). The pots were arranged in a completely randomized block design with five replicates in a greenhouse at Alexandria University's Faculty of Education. Three varying soil field capacity (FC) levels were maintained (80, 60, and 40% FC). Water loss was measured at at 24-hour intervals to calculate the average soil-plant evapotranspiration. The greenhouse conditions were maintained at a temperature of 20±2ºC, a relative humidity of 75±2%, and a light/dark photoperiod of 14/10. After 21 days, once the plants were well-established, homogeneous plants from each treatment were carefully removed. They were cleaned with tap water to remove adhering soil particles and then gently wiped with filter paper moistened with distilled water. Samples were subsequently divided into shoots and roots for determination of growth parameters.

Growth parameters

Fifteen individuals were selected from each treatment, and several germination parameters were recorded. Germination percentage (GP) was calculated by dividing the number of germinated seeds by the total number of seeds and multiplying by 100. Germination energy (GE) was the number of seeds that germinated after four days divided by total number of seeds tested and multiplying by 100. The formula for germination velocity (GVe) is $\sum GP/t$, where t is the total germination time and GP is the germination percentage. To estimate shoot length (SL) and root length (RL), five plant individuals per treatment were measured using measuring tape. The shoot/root ratio was calculated for each treatment. Finally, the vigor index (SVI) is estimated by multiplying the total length (cm) by the germination percentage, as described by Hossain et al. (2005).

a:



b:

Figure 1. a. Petri dish experiment sowing a sterilized seed soaked in aqueous extract of *Asphodelus microcarpus* L. with different concentrations (0, 5, 10, 20 and 40%) in different soaking period (4, 8 and 12h) to determine the preferable concentration and time of priming. **b.** Pot experiment for priming seeds with hydropriming, phytopriming by using *Asphodelus microcarpus* L. aqueous extract (5 and 10%), Nanopriming using nano particles of zinc oxide (1, 0.25 and 0.05gm/L) and Halopriming using zinc chloride (1, 0.25 and 0.05gm/L) for 12h and sowed under conditions of normal irrigation 80% and drought stress 60 and 40% field capacity in barley seeds

Leaf area (A) was calculated by measuring leaf width and length (cm) and estimated using the equation A= 0.667*L*W (Cain & Castro, 1960), where W is the leaf width, L is the leaf length, and 0.667 is a correction factor used to convert the leaf's area from the rectangular product of its width and length.

Protein electrophoresis

0.5gm seedling leaves were homogenized 3x in 500µL phosphate buffer (0.6M, pH 7) with the help of glass beads and liquid nitrogen using a Fast Prep®-24 homogenizer. Cell debris was removed by centrifugation for 10min at 10,000 rpm. Supernatants containing protein were collected in Eppendorf tubes and stored at -20°C till loading. Sodium Dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the standard method for protein separation and differentiation by MW (Laemmli, 1970).

Polymorphism % = (Σ bands for each sample –common bands for all samples)/Σ bands of all samples*100

Statistical analysis

Results were reported as the mean ± SE of the mean. The traditional one-way analysis of variance (ANOVA) was applied to the data from the current study using The Cohort Software Company's COSTAT 2.00 statistical analysis application (Zar, 1984). Duncan's Multiple Range Test was used to distinguish differences in treatment means at a 5% level of significance. Protein molecular weights and molecular banding patterns were examined using Image Lab software and Gel Doc XR+ (BioRad).

RESULTS

Characteristics of ZNPs

The size and form of ZNPs were examined using a transmission electron microscope, (TEM). As indicated in Table 1 and Figure 2, the ZNPs used in this investigation are white powders with spherical shapes and average sizes between 20 and 30nm.

Table 1. Characterization of ZNPs

Appearance (Color)	White
Appearance (Form)	Powder
Solubility	Stable colloid in mixture of Methanol and chloroform
Optical Prop. (Abs.)	λmax = 301nm, and 380nm
Avg. Size (TEM)	20 ± 3nm
Shape (TEM)	Spherical like shape

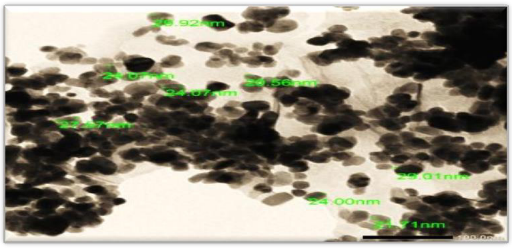


Figure 2. Shape and size of ZNPs by Transmission Electron Microscope

X-ray diffraction (XRD)

It was employed to verify that the synthesized ZnO nanomaterial was crystalline (Figure 3). The peaks at 2ϴ values of 31.8, 34.44, 36.27, 39.32, 47.57, 49.92, 56.63, 62.88, 65.89, 67.9, and 69.11 represented the crystal planes. The strong, narrow peaks indicate the product's well-crystalline particle nature. Similar outcomes were reported by Surbhi et al. (2013).

Growth parameters

The effect of different priming treatments (HyP, PyP, HaP, and NP), in addition to un-priming (UP), under different levels of drought stress was illustrated in Figure 4. The germination percentage (GP) was inhibited in response to drought stress, but the inhibition was more obvious for un-priming than for priming treatments (Figure 5a). The improvements in GP against drought stress were more obvious in response to 5% PyP treatment at 40% FC relative to UP seeds. Germination energy (GE) varied in response to drought stress (Figure 5b), it decreased in the UP treatment, but the improvement was obvious in response to priming treatments. The 5% PyP represented the most significant improvement in GE, especially at 60 % FC. Germination velocity (Gve) was significantly affected by drought stress (Figure 5c). The priming treatments improved Gve under drought stress when compared with UP. The most obvious improvement was represented in PyP at 60% FC.

The shoot and root lengths of barley in all treatments were significantly affected by drought stress (Figure 6 a & b). The shoot length at 60% FC exhibited an increase in response to most priming treatments as compared to UP. However, the data show a remarkable increase in root length in the UP treatment at 60% FC, while it increased only slightly in response to most HaP and NP treatments relative to their controls.

Data presented in Figure 6a and 6b showed that shoot and root length were significantly reduced by 40 % FC in all studied treatments relative to their control (80%, well watered condition). Priming treatments reduced this inhibition, but with different levels. The most significant improvement in shoot length under drought stress (40%) was recorded in response to PyP (10%) and both low and moderate concentration for HaP. Conversely, the most significant improvement in root length was in response to 1g NP. Seed vigor index (SVI) has been shown to be greatly affected by drought stress, and it tended to increase with priming treatments (Figure 6c). The effects of different priming treatments on the leaf area of barely were shown in Figure 6d. The study noted a significant reduction in leaf area under the highest drought stress, while at 60% FC, there was an increase in leaf area in response to most treatments. The greatest increase in leaf area was recorded in response to the 1g NP treatment under 60% FC.

The electrophoretic patterns (SDS-PAGE) for the total protein in barley, after different priming techniques under well-watered condition (80% FC), indicated that the protein bands ranged from a minimum of 15KDa to a maximum of 180KDa (Figure 7a). Nine common bands were observed. Two specific bands, at molecular weights of 75 and 100KDa, were present only in the control and disappeared in all priming treatments. The percentage of polymorphism varied from 27% for UP to 30% in most priming treatments. Conversely, the electrophoretic patterns (SDS-PAGE) for the total protein in barley after different priming techniques with 60 % FC indicated the protein bands ranging from a minimum of 22KDa to a maximum of 100KDa (Figure 7b). Eight common bands were observed. The percentage of polymorphism varied from 20% to 42%. No specific bands were found in any of these treatments. Under drought stress (40%FC), the electrophoretic patterns (SDS-PAGE) for the total protein in barley, after different priming techniques, indicated protein bands ranging from a minimum of 17KDa to a maximum of 100KDa (Figure 7c). Fifteen common bands were observed. Bands at molecular weights of 18, 19KDa were present in all treatments except UP. A specific band with a molecular weight of 100KDa was present in UP. There were three polymorphic bands and fourteen monomorphic bands. The total number of bands under moderate drought stress 40% FC was 19, which is greater than 15 bands observed at 80% FC.

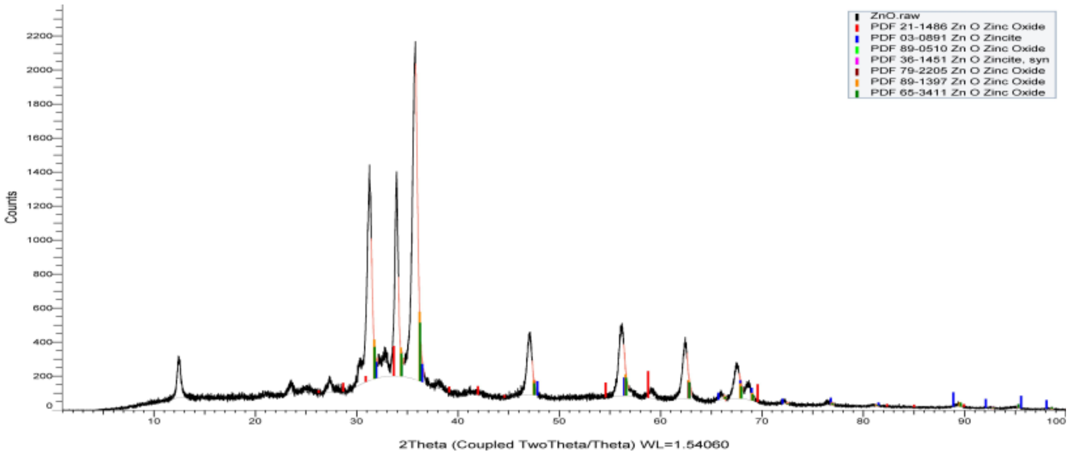


Figure 3. Demonstrate the different peaks through crystalline nature of synthesized ZnO nanomaterial

Figure 4. Effect of priming barley seeds with **a)** Hydropriming, phytopriming by using *Asphodelus microcarpus* L. aqueous extract (5 and 10%), **b)** Halopriming using zinc chloride (1, 0.25 and 0.05gm/L) and **c)** Nanopriming using nano particles of zinc oxide (1, 0.25 and 0.05gm/L) under conditions of normal irrigation 80% and drought stress 60 and 40% field capacity on growth

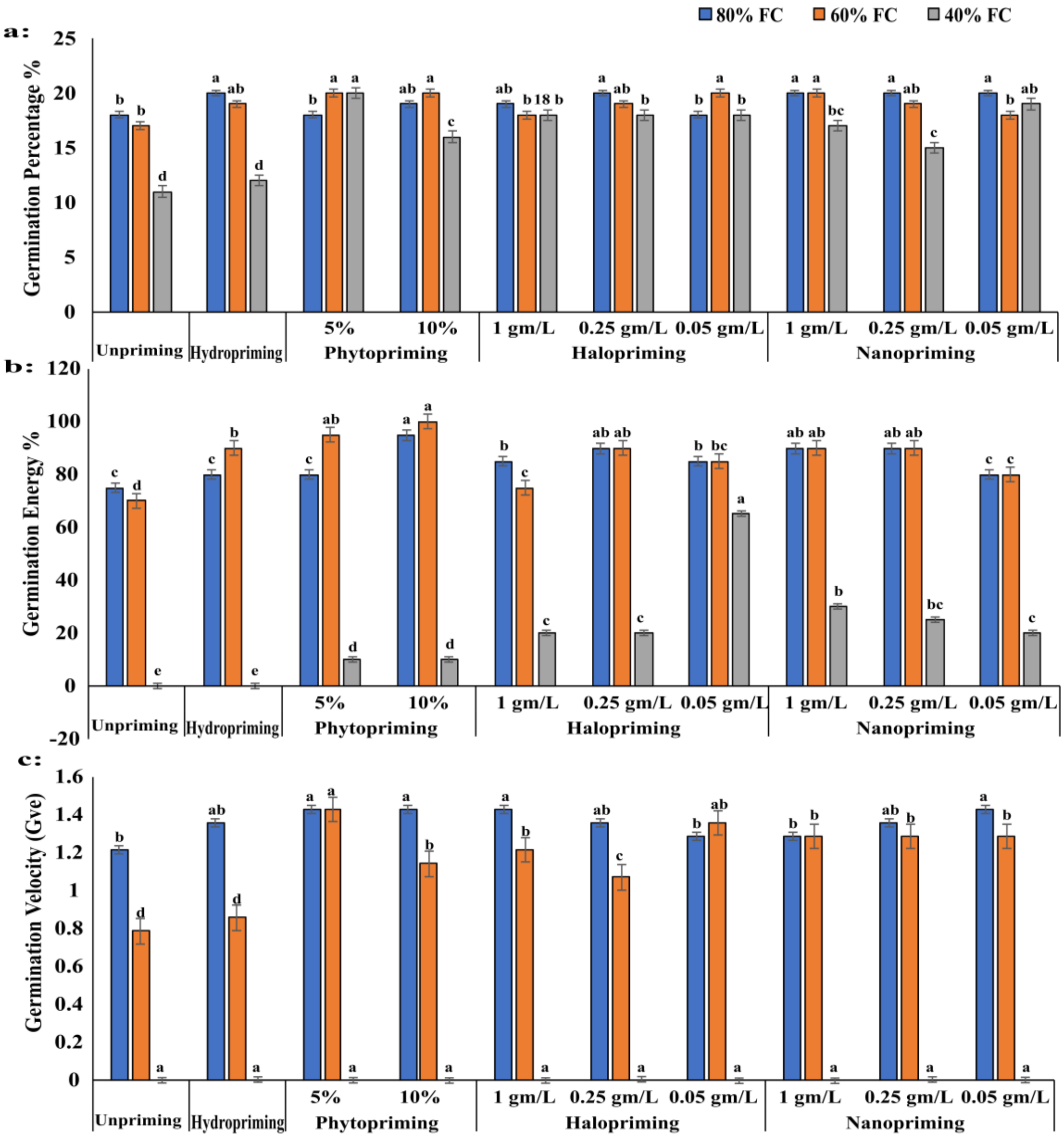


Figure 5. Effect of priming barley seeds with hydropriming, phytopriming by using *Asphodelus microcarpus* L. aqueous extract (5 and 10%), Halopriming using zinc chloride (1, 0.25 and 0.05gm/L) and Nanopriming using nano particles of zinc oxide (1, 0.25 and 0.05gm/L) under conditions of normal irrigation 80% and drought stress 60 and 40% field capacity on germination percentage (GP), germination energy (GE) and germination velocity (Gve) using a Tukey's test at 5% probability [Columns with the same letters did not differ between treatments; the results are shown as mean±standard error]

DISCUSSION

Drought stress is one of significant abiotic element that suppress seed germination and plant growth. (Hatzig et al., 2018). In our research we found that drought stress can cause severe damage in barley seeds germination and other growth parameters. Previous research have demonstrated that when the level of drought stress exceeds a particular threshold, normal physiological metabolic activities are interrupted, resulting in a variety of physiological effects and reduced growth (Yang et al., 2020). Drought stress reduces overall growth by affecting morphological, physiological, biochemical, and molecular traits during the germination and plant stages (Yigit et al., 2016).

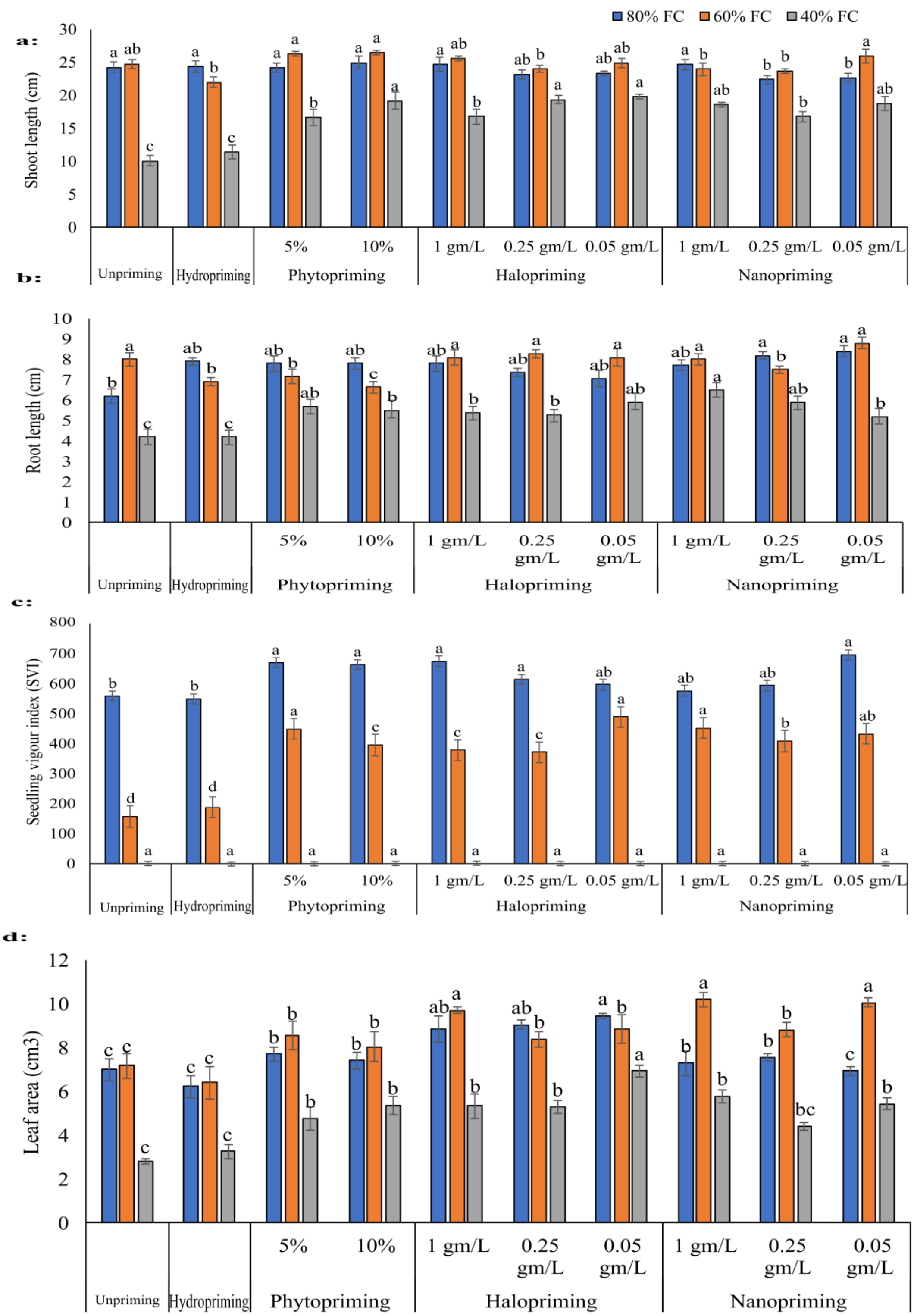


Figure 6. Effect of priming barley seeds with hydropriming, phytopriming by using *Asphodelus microcarpus* L. aqueous extract (5 and 10%), Halopriming using zinc chloride (1, 0.25 and 0.05gm/L) and Nanopriming using nano particles of zinc oxide (1, 0.25 and 0.05gm/L) under conditions of normal irrigation 80% and drought stress 60 and 40% field capacity on germination shoot length (cm), root length (cm), seedling vigour index (SVI) and leaf area (cm^3) using a Tukey's test at 5% probability [Columns with the same letters did not differ between treatments; the results are shown as mean±standard error]

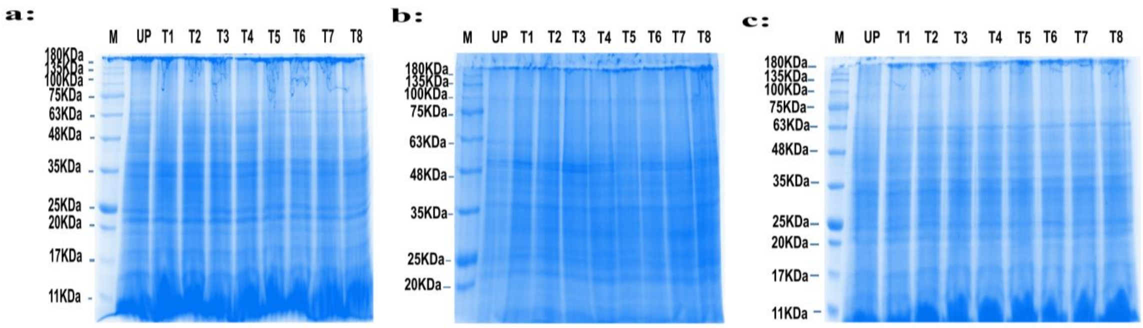


Figure 7. Vegetative protein electrophoresis of barley according to different priming treatments for 8h. (M, UP; T1 and T2 phytopriming with 5% and 10% *Asphodelus microcarpus* L. extract; T3, T4, T5 halopriming with different Zn concentration (1g/L, 0.25g/L, 0.05g/L, respectively); T6, T7, nanopriming with different concentrations of ZnONPs (1g/L, 0.25g/L, 0.05g/L, respectively) at different field capabilities **a**: 80%, **b**: 60% and **c**: 40% FC

Plants may benefit from seed priming as a pre-germinative treatment by receiving a moderate stress signal that creates a priming memory, which increases their ability to withstand future stress and diminishes the negative effects of drought stress (Gour et al., 2023). Seed priming increased germination and early seedling formation during drought stress (Li & Liu, 2023). Seed priming treatments increased germination percentage, normality percentage, and plant vigor index, while decreasing mean time to germination, germination uniformity, and coefficient of velocity of germination in *Secale montanum* under stressed conditions (Tabatabaei & Ansari, 2020). In this study, we determined the positive effect of different priming strategies in barley seeds to alleviate drought stress. The increase in germination percentage, seedling percentage, vigor index, and germination index may be the result of mobilized food material reserves, activation and resynthesis of some enzymes, improved ATPase activity, RNA and acid phosphatase synthesis, as well as improved priming of amylases, lipases, and proteases (Ashraf & Foolad, 2005). Our results showed that HyP significantly enhanced plant vigor, germination, seedling emergence, and crop growth drought-stressed conditions. Numerous studies have demonstrated that HyP, as opposed to non-priming, promotes plant development and germination by a factor of three to four amid drought stress conditions (Kaur et al., 2002).

Our results indicate that the highest germination percentage under drought stress was achieved with a 5% concentration of PhP treatment. Priming with *Aloe vera*, *Moringa olifera,* or sugar beet aqueous extracts can be recommended to farmers for achieving higher yields under stress conditions (Imran et al., 2014). Under both ideal and stressful circumstances, HaP increases the production, germination metabolism, and stand establishment of numerous crops (Sher et al., 2019). The greatest increase in shoot length under drought stress was observed when 0.25 and 0.05gm/L $ZnCl_2$ were used as treatments.

When seeds are treated with a halopriming solution, their water intake is reduced. This delays the start of the growth phase and prolongs the activation phase, during which protein synthesis, early DNA replication, and chromosomal and DNA damage repair occur (Paul & Roychoudhury, 2017). The essentials of the priming treatment, including the treatment composition, concentration, and duration are important factors that help determine germination achievement and plant establishment. According to Ghafari & Razmjoo (2013), ZnO nanoparticle seed priming improved plant growth and germination. To encourage seed germination and vigor, effective priming compounds, such as ZnO, can be transformed into nanoparticles and used in seed priming therapy (Dutta, 2018). Our study found that seed priming with ZnO nanoparticles had a substantial impact on shoot length, root length and SVI. By preventing the breakdown of chlorophylls due to oxidative stress damage, wheat plants cultivated from ZnO nanoprimed seeds demonstrated resistance to drought stress. Physiologically, the increased photosynthetic efficiency, which is closely linked to increased biomass, may be the cause of NP's positive benefits during drought (Rai-Kalal et al., 2021).

The presence and disappearance of certain protein bands indicated that the drought stress caused some proteins to rise and others to decrease (Amini et al., 2007). The occurrence of novel protein bands in response to drought stress levels raised the possibility that these proteins are responsible for inducing drought resistance in various barley genotypes (Bi et al., 1997). One explanation for the loss of some protein bands during drought stress is that the genes responsible for protein synthesis may have been entirely suppressed due to the stress. As a result, the developing tissue may have lost its capacity to produce these proteins in response to stress. Another possibility is that the stress-induced inhibition of the genes led to partial suppression, rather than complete reversal of the inhibition, which was not achieved (Mohamed, 2005). Rashad et al. (2023) indicated that Giza 126 of barley is among the most stable genotypes, appearing to express or inhibit bands the least. In Giza 126, only a small number of genes were directing protein expression, or gene expression was more stable under drought conditions (Amini et al., 2007). There has been a significant accumulation of processes by which drought stress may cause the development of certain polypeptides in drought-stressed plants. These polypeptides, known as osmotin, were specific to tobacco cells because they were synthesized and accumulated while being gradually osmotically adjusted to desiccation stress (Mohamed, 2005).

CONCLUSION

Climate change presents significant obstacles for food production, and seed priming may be an effective method to improve plant development and increase drought tolerance in unfavourable conditions. The current study exhibited that the barley growth improvement in response to priming against drought stress was as follows: phytopriming> nanopriming> halopriming> hydropriming relative to unpriming. Further studies under natural field conditions are needed to gain more insights into the effects of different seed priming techniques on barley yield management.

Acknowledgements authors : The authors would like to thank members of the Biology and Geology department Faculty of Education, Alexandria University for their cooperation and give them the opportunity to use the department's labs

Funding: No funding was received for conducting this study.

Availability of data and materials: The authors declare that the data supporting the findings of this study are available within the paper, and its supplementary information files.

Competing interests: The authors declare no competing interests.

Authors' contributions: The experiments were designed and planned by M. M. The experiments were conducted by R. M. and S. M., who also helped analyse the findings and led the manuscript's writing. Each author offered insightful criticism and contributed to the development of the study, analysis, and manuscript.

Ethics approval and consent to participate : not applicable

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