FORESTRY IDEAS, 2019, vol. 25, No 1 (57): 160–170

ASSESSING OF THE TOLERANCE OF *PINUS HALEPENSIS* MILL. SEEDS TO WATER AND SALINE STRESS AT THE GERMINATION STAGE

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Received: 04 May 2018 Accepted: 12 June 2019

Abstract

Arid and semi-arid ecosystems cover a large part of the southern fringe countries of the Mediterranean basin. Drought and salinization are the major processes of steppe land degradation. The objective of this work was to investigate the tolerance to water and saline stress of Aleppo pine (*Pinus halepensis* Mill.) seeds at the germinating stage. The adopted methodology used increasing concentrations under a controlled temperature of 20 °C for 30 germination days of seeds. Germination responses of seeds to different degrees of salt stress caused by NaCl (1, 2, 3, 4, and 5 $g \cdot L^{-1}$) and the water stress caused by polyethylene glycol (–0.05, –0.25, –0.5, –1, and –2 bars) under a temperature of 20 °C showed that salt and water stress have decreased the percentage of seed germination of Aleppo pine seeds for a period of 30 days. In the control treatment (distilled water) with no stress the seeds showed a germination rate of 89 % for salt stress and 90 % for water stress. The results of the study showed that salt and drought have a depressive effect on the germination rate of Aleppo pine with a tolerance of 4 $g \cdot L^{-1}$ and –1 bars for the stresses used.

Key words: Aleppo pine, drought, germination tests, NaCl, Polyethylene glycol (PEG), semi-arid.

Introduction

Algeria is one of the most water-deficient regions in the world, and the water resources are subjected to strong natural, climatic and anthropogenic pressures (Bellal 2011). The water deficit is more than 20 % for the western region, 13 % for the central and 12 % for the eastern part of the country (Ould Amara 2000, Bouguerra 2001). Arid and semi-arid area cover very large surface and are characterized by a high rainfall irregularity. These areas receive an annual average rainfall between 100 and 300 mm for the arid and between 300 and 600 mm for the semi-arid one (Le Houerou 1995, Nedjraoui 2003, Ramade 2003). Besides, they are characterized by very restrictive edapho-climatic conditions, thus posing challenges to survival of some indigenous plant species, which are subjected to constant stress by the severe environment.

In these regions, the availability of water, salinity and some other soil characteristics are among the main factors limiting plant productivity (Zid and Grignon 1991). In Algeria, the long-observed drought has clearly led to the process of salinization of soils on 3.2 million hectares (Benmahioul et al. 2009). This natural constraint has had a huge impact on the stability of ecosystems and caused a large part of desertification and soil salinization (Sadio 1989, Szabolcs 1992).

Aleppo pine is a major forest tree species in the Mediterranean basin, covering more than 250,000 km^2 (Quezel 2000). In Algeria it occupies 850,000 ha (Mezali 2003). The pine forests have been experiencing intense human pressure for centuries (clearing, illegal logging, fire, grazing), causing deforestation and decline of native plants. Aleppo pine is the most widely used tree in the reforestation in arid and semi-arid degraded areas. It is characterized by relatively shorter life span, usually not exceeding 150 years. Its growth can reach 10 m in 20 years. It is a tree that has a pivoting rooting depending on the nature of the soil and its fertility. Like other pines, it is a monoecious tree characterized by winged seeds between 5 and 7 mm in length (Bentouati 2006). Old Aleppo pine stands do not always regenerate easily, except in the case of fire. Foresters encounter difficulties in regenerating these mature stands, where the regeneration is often absent. In this context, managers seek to understand and know the techniques and treatments that facilitate the natural regeneration of these pine forests (Prévosto et al. 2009).

Seed dormancy is one of the tolerance mechanisms developed by plants found under conditions of high aridity, temperature, and salinity (Fenner 1985). The maintenance of plants under limiting environmental conditions is primarily dependent on the success of germination (Ungar 1982, 1991). Several factors interact in the regulation of germination (water, temperature, light and salinity). Also, germination is regulated by genotypic characteristics (Gutterman 1994). Thus, seed germination is the most sensitive stage of plants exposed to salt and water stress (Boulghalagh et al. 2006). Most plants may be considered to be more sensitive to salinity during their germination and emergence phases (Maillard 2001). Among the causes of inhibition of germination in the presence of salt, the change in hormonal equilibrium was evoked (Kabar 1987). A delay in the germination caused by salinity was shown in several species (Ndour and Danthu 1998, Benata et al. 2006, Boulghalagh et al. 2006). Work carried out on Halophytes showed that the inhibitory effect of NaCl on germination would be essentially osmotic in nature, with salt preventing the implantation of the seed (Katembe et al. 1998).

In relation to the above statements, the aim of this work was to study the effect of the main environmental constraints affecting seed germination of Aleppo pine, including drought and salinity, modelled in laboratory conditions. Aleppo pine was selected as a subject of study because of the problems existing with its regeneration in arid and semi-arid areas, which are the last ramparts against desertification.

Materials and Methods

Collection of Aleppo pine seeds

Mature three-year-old cones were selected for our study. They were harvested in early August 2016. Further on, they were put in plastic bags and exposed to the sun for one or two months to allow the cones to burst and release the seeds (General Directorate of Forests nursery Technique).

Cones from older trees were harvested in the pinewood of Fenouane (geographic coordinates 34°30’13.08’’ N; 3°28’17.16’’ E) (Fig. 1), located in the territory of the wilaya of Saida, some 550 km southwest of Algiers and about thirty kilometres to the west of Saida (BNEF 1990). It is managed by the Conservation service of the forests of Saida and is part of the district of Ain El-Hajar wilaya of Saida (Algeria).

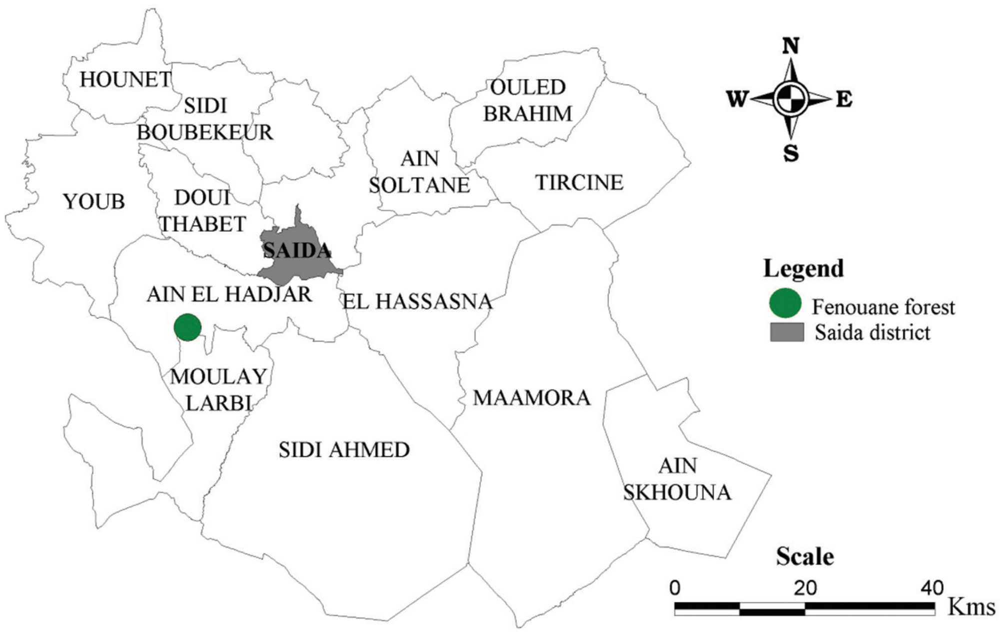

Fig. 1. Location of the harvest area of the *Pinus halepensis* grains (BNEDER 1992, modified by Zouidi).

Effect of osmotic stress on germination

Integumentary inhibition was lifted following a protocol that recommends disinfection of seeds for 10 minutes in an 8 % sodium hypochlorite solution and then rinsing the seeds by water several times followed by soaking the seeds in distilled water for 24 hours (Nedjimi et al. 2014). Germination tests were carried out under different levels of water potentials through the use of Polyethylene glycol (PEG), which forms a non-permeable, hydrosoluble non-ionic polymer for the cells. It is used to induce a water deficiency by reducing water availability without causing physical damage to plants (Romo et al. 2001), having a molar mass of 6000. Germination tests were carried out with 20 seeds per petri dish (diameter 9 cm).

Each petri dish was coated with two layers of filter paper at optimum germination temperature (20 °C). Polyethylene glycol (PEG) 6000 solutions of increasing concentrations and inducing also increasing water potentials (by the equation established by Michel and Kaufman (1973)) were used to induce the different levels of osmotic stress tested. The water potential values tested were 0, -0.05, -0.25, -0.5, -1, and -2 bars. The duration of the test was set at the germination period, which spread over 30 days, and the counting of the germinated seeds was done daily. According to Michel and Kaufman (1973), the equation (1) linking the various parameters was as follows:

$$\Psi_h = -(1.18 \cdot 10^{-2})\,C - (1.118 \cdot 10^{-4})\,C^2 + \\ + (2.67 \cdot 10^{-4})\,C \cdot T + (8.39 \cdot 10^{-7}) \cdot C^2 \cdot T, \quad (1)$$

where: Ψ_h – is water potential in bar; T– incubation temperature in °C; C– concentration of PEG 6000 in g·L^{-1}.

Effect of salinity on germination

We conducted the germination tests under saline stress using the optimum germination conditions determined by previous experiment. Seeds were germinated in petri dishes on filter paper, watered daily with distilled water containing different concentrations of NaCl (0, 1, 2, 3, 4, and 5 g·L^{-1}) and arranged at optimum germination temperature (20 °C) (Thanos and Skordilis 1987). The test duration was determined by the 30-day germination period. Germination was monitored every 24 hours, and germinated seeds were counted and removed from the batch. The time taken for the germination percentages of all replicas to reach 50 % was recorded as TG50. The counting of germinated seeds was carried out on a daily basis.

Parameters measured

The germination rate for each batch (TG) is the best indicator for identifying the concentration of PEG and NaCl that present the physiological limit of germination of the seeds. It is expressed by the ratio of the number of seeds germinated to the total number of seeds. On the germination test the definitive percentage of germination was determined (Agrobio 2013). The rate of germination may be expressed by the median germination time (Scott et al. 1984) or by the mean germination times (the time at which 50 % of the seed germination is reached) (Côme 1970, Lachiheb et al. 2004). It is expressed in equation (2):

$$V = T_1 + [(0.5 - G_1/G_2 - G_1)] \cdot (T_2 - T_1), \quad (2)$$

where: G_1 (%) is the cumulative seed germination with a value closer to 50 % (lower) and G_2 (%) cumulative seed germination with a value closer to 50 % (higher) (Lachiheb et al. 2004); T_1 – number of days is the cumulative percentage of sprouted seeds whose value is closer to 50 % (lower); T_2– number of days is the cumulative percentage of sprouted seeds whose value is closer to 50 % (higher).

Statistical analysis

The results were subjected to analysis of variance (ANOVA) with a single factor of variation at 5 % probability level ($P \leq 0.05$) after controlling normality distribution for comparing the averages of germination rates with stress. The post-hoc Fisher (LSD) test was applied for multiple comparisons of means. We used Statistica software package version 7.0.

Results

Effect of osmotic stress on the germination

Our results showed the effect of increasing PEG concentrations on Aleppo pine seeds subjected to different osmotic stresses. Thus for untreated seeds (controls) the germination rate was highest – 90 % with a short latent phase that lasts only six days. It was also noted that the application of osmotic stress of (-1 bar) resulted in a low germination rate not exceeding 21 %, while there was no germination at higher stress (-2 bar).

The analysis of the variance (ANOVA single factor) showed a highly significant effect (P<0.001) of the different concentrations of Polyethylene glycol (PEG 6000) on the rate and velocity of germination of the seeds of Aleppo pine (Fig. 2).

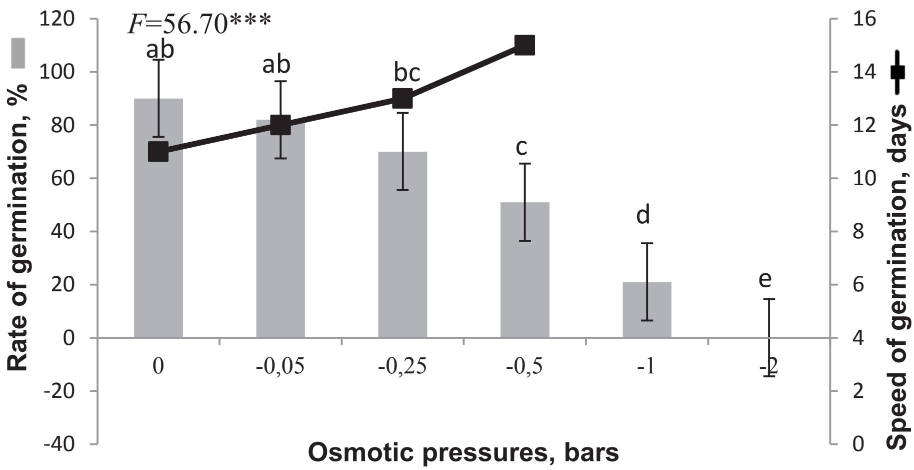

Fig. 2. Effect of different osmotic pressures on the rate and velocity of germination of Aleppo pine seeds.

Note: The bars and whiskers represent the mean ± standard deviation (n=5 repetitions). Significance level: * – P<0.05, ** – P<0.01, *** – P<0.001, NS – not significant.

Effect of saline stress on the germination

The results of the effect of NaCl on the germination of Aleppo pine seeds are presented in Fig. 3. The first germination of seeds was observed in petri dishes containing seeds soaked in distilled water after 7 days of experiment initiation. The results also showed that seed germination rates decreased with increasing of NaCl dose from the control to 4 $g \cdot L^{-1}$. It was observed that the germination rate of Aleppo pine seeds differed from one concentration to another. Based on these results, a maximum value of 89 % for unstressed NaCl seeds was observed, and a gradual decrease was registered with the increase in NaCl concentration to 21 % for the highest saline stress (4 $g \cdot L^{-1}$). Our results clearly show that the seeds of *Pinus halepensis* germinate better in the absence of salt or in a medium enriched with NaCl at low concentration 1, 2 and 3 $g \cdot L^{-1}$. When the salt concentration increased to 5 $g \cdot L^{-1}$ NaCl, seeds did not germinate. In general, the rate of germination decreases considerably with increasing saline stress of the substrate.

The results of ANOVA showed that there is a significant difference in the germination rate of Aleppo pine (P<0.001) seed soaked by different salt-concentrated solutions (Fig. 3).

Salt affects the rate of germination, and a slowdown was observed with the increase in salt in the water. In the absence of salt, the seeds required 11 days

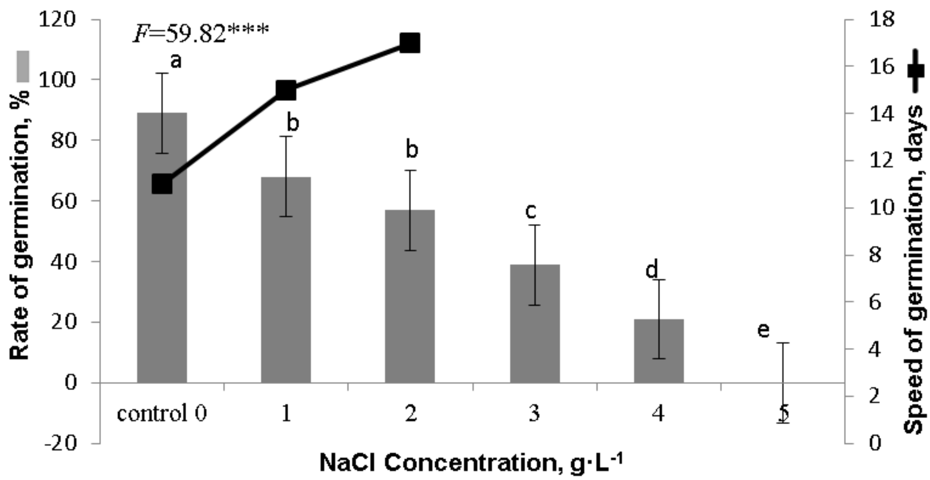

Fig. 3. Effect of different saline stress on the rate and speed of germination of Aleppo pine seeds.

Note: The bars and whiskers represent the mean ± standard deviation (n=5 repetitions). Significance levels: * – P<0.05, ** – P<0.01, *** – P<0.001, NS – not significant.

to reach a rate of 50 %, while in the presence of salt (1 and 2 g·L^{-1}) they needed 15 and 17 days, respectively, to reach 50 % germination in comparison with the controls.

Discussion

Effect of water stress on germination

Aleppo pine is an important forest tree species in the Mediterranean area. In Algeria, it is used as a major species in reforestation and repopulation to ensure the equilibrium and maintenance of many semi-arid and arid ecosystems (Bentouati et al. 2005, Kadik 1986). Indeed, some authors have shown that due to its plasticity and its low requirements, Aleppo pine remains the most used species in these degraded areas. It adapts to all soil types, when the climatic conditions are favourable (Bentouati et al. 2005, Kadik 1986).

The use of this species in afforestation programmes offers a sustainable reforestation solution in arid and semi-arid areas characterized by warm and dry climate and would, therefore, provide opportunities for diversified farms. Nevertheless, the success of the germination and growth phases of this species inevitably results in a good knowledge of its germination and developmental characteristics, as well as its behaviour towards environmental conditions. Our results show that the average germination rate of control treatment (0 bars) is 90 %. According to Hamrouni et al. (2011), the germination rates in Tunisia exceed 80 % irrespective of the provenance and the place of cultivation.

The same authors showed that the germination rate can reach 70 % for good quality lots (-0.05 bars) and (-0.25 bars). These results indicate that the germination rate decreases considerably with increasing substrate water stress. Usually most of the studies on the impact of osmotic stress on germination show that the germination rate decreases considerably with increasing the stress. Similar results were obtained in various studies on *Pinus ponderosa* Douglas ex C.Lawson and *P. eldarica* Medw. (Djavanshir and Reid 1975), *P. brutia* Ten and *P. eldarica* (Calamassi et al. 1980), *P. taeda* L. (Dunlap and Barnett 1984), and *P. halepensis* and *P. brutia* (Thanos and Skordilis 1987).

In the absence of sufficient moisture, even if the seed is properly placed in the soil, it does not evolve, and in case of drought persistence the situation can result in an absence of emergence (Feliachi et al. 2001).

Drought is one of the major environmental factors that greatly affects the germination of cultivated species and reduces their survival in early stages of development. Our results also show that the rate of germination becomes slow with increasing of stress. According to Bliss et al. (1986b) the delayed germination of the seeds, as well as the decrease in the average daily germination of all genotypes with the increase in water stress is explained by the time necessary for the seed to put in place mechanisms allowing it to adjust its internal osmotic pressure. When the seeds are subjected to osmotic pressure, this velocity becomes even lower, while the final percentage of germination is affected only when the osmotic potential is low. This is observed in most *Pinus* species (ISTA 1976 in FAO 1992) and the delay can be attributed to the seed layer acting as a barrier to water penetration into the embryo (Thalouarn 1975).

Falusi et al. (1983) studied the effects of substrate water potential on germination and on the first stage of root growth of 4 provenances of Aleppo pine (Israel, Morocco, Greece, and Italy) and showed a significant reduction in germination in % at -2 bars, while root growth was assigned to -4 bars. Different sources demonstrated significantly different responses to water stress. Our results showed that seeds harvested in the semi-arid zone exhibited significantly different responses to water stress with great germination power under ideal conditions (control). On the other hand, those who have undergone stress presented a low rate of germination and therefore are less resistant to the extreme climatic conditions of the zone.

Effect of saline stress on germination

The results of the variance analysis revealed significant differences in the germination rate of Aleppo pine ($P<0.001$) seeds soaked by different salt-concentrated solutions. Indeed, the higher salt concentrations of the environment caused decreasing of germ power. Most plants are more susceptible to salinity during their germination and emergence phases (Maillard 2001). Of the causes of inhibition of germination in the presence of salt, the change in hormonal equilibrium has been outlined (Ungar 1978, Kabar 1987). Although halophytes have very high salt content in their adult tissues, their seeds are not as salt tolerant at the germination stage (Belkhodja and Bidai 2004). This stage is often limited by soil salinity and is more sensitive than other stages (Bouda and Haddioui 2011). Salinity may result in a decrease in the final germination rate and may in turn lead to the establishment of irregular stands and reduce harvest yields (Yildirim and Güvenç 2006).

The excessive presence of soluble salts can cause high osmotic pressure in plants and inhibition of seed germination as well as the development of the whole plant by reducing its ability to retain water with consequences on the level of growth and metabolic activity (Belkhodja and Bidai 2004). The inhibitory effects of salinity on the germination process can also be explained by altered enzymatic activity, which is indispensable for cell reactivation during this phase. Thus, salinity inhibits the activity of several enzymes (Larcher 1995). Our results clearly show that the seeds of Aleppo pine germinate better in the absence of salt with a good concentration rate and at a speed accelerate and even in a medium enriched with NaCl at low concentration 1, 2, and 3 $g \cdot L^{-1}$.

Increasing of salt concentration to 5 $g \cdot L^{-1}$ NaCl alters the seed germination. Salt stress tolerance in pines is moderately low as shown by Rocco et al. (2013). In the presence of salt stress the proteins in the Aleppo pine needles affected by stress include the proteins involved in photosynthesis. Aleppo pine is affected by high concentrations, which was reported also for other species. For example, Bouda and Haddioui (2011) applied the same treatment on *Atriplex* seeds, and found that the rate of germination inhibition of seeds stressed by NaCl stops at 5 $g \cdot L^{-1}$. The decrease in germination rate and the slowing of germination velocity of seeds exposed to salinity is explained by an increase in external osmotic pressure, which affects the absorption of water by seeds due to accumulation of Na^+ and Cl^- ions in the embryo (Groome et al. 1991). This toxic effect can lead to the alteration of the metabolic processes of germination and in extreme cases to death of the embryo by excess of ions. Thus the emergence of radicle would be controlled by the osmolality of the medium (Bruggeman et al. 2002).

According to Bliss et al. (1986a) the delay in germination following the increase in salt stress is due to the fact that the salt seems to prevent the hydration of the seeds to reach the threshold when germination becomes visible. This is also due to the time required for the seed to put in place mechanisms allowing it to adjust its internal osmotic pressure and thus allowing for a germination.

Sidari et al. (2008) showed that seed germination and the activities of the main enzymes involved in *Pinus* seed reserve utilization (glyoxylate cycle enzymes) decreased with increasing PEG, NaCl and seawater concentrations.

Conclusions

In the arid and semi-arid zones of Algeria, the long-observed drought has clearly led to salinization of the soils. The combination of these two natural stresses becomes increasingly stressful for the germination and growth of plants in their natural environment. The study of the germination of Aleppo pine is very important since it is considered very important species for reforestation. It is introduced to restore the degraded areas and occupied bare land in steppe areas, in arid and semi-arid zones. The main peculiarities of the germ behaviour of Aleppo pine seeds in the face of abiotic stresses can be summarized as follows: For water stress, the germination rate and the average daily germination are strongly affected and decrease with increasing the concentration of added PEG. In the absence of stress, the maximum germination rate in the presence of sufficient moisture is about 90 %. Stressed seeds did not withstand more than -1 bar PEG with low germination rates (21 %). For saline stress the species germinated better in a salt-free treatment (control) with a germination rate of 89 % and an average daily germination of 2.96. On the other hand, the seeds with a stress in the order of 4 g·L^{-1} NaCl have a low germination rate of the order of 21 %. The rate of germination could be considered an early criterion for selection of plant species tolerant to salt stress and water stress. According to the results obtained, it can be concluded that Aleppo pine is a fragile species at the time of seed in dry media and media rich in NaCl and does not tolerate large concentration of NaCl. The estimated degree of tolerance to the stress salts is 4 g·L^{-1} and to water stress is -1 bar.

The rate of germination decreases considerably with the increase of abiotic stress, whether water or saline. The natural regeneration of pine is difficult to achieve, because it is subjected to multiple abiotic and biotic constraints and their interactions, which remain only partially known (Adili 2012). A better understanding of these factors is necessary for the development of silvicultural recommendations to increase natural regeneration.

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