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The Effects of Several Types of Induced Abiotic Stress on *Cephalaria joppensis* Germination under Controlled Conditions

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Abstract

The recently domesticated species, *Cephalaria joppensis* (CJ), is emerging as a new alternative forage crop in Israel. It has high biomass potential and nutritional values that are comparable to forage wheat. However, CJ emerges slowly under cold conditions, which hinders its development as a major winter crop. Additional tolerance for abiotic stress would improve its performance as a forage crop. We examined the effects of several abiotic factors (*i.e.*, cold, salinity, drought and pH) on CJ germination under controlled conditions. The effect of temperature was studied by incubating seeds at different temperatures between 7˚C and 35˚C. The effects of salinity, osmotic potential and pH were tested by incubating seeds at different NaCl, PEG and pH levels, respectively. Temperature, salinity and osmotic potential significantly affected germination; whereas pH did not. Temperature did not affect the final proportion of germinated seeds, but did affect other germination-rate variables, indicating that germination rate might be the limiting factor under field conditions. Salinity also affected germination-rate variables, but not the proportion of seeds that germinated. Notably, CJ was found to be relatively resistant to high salt concentrations, with a 273 mM NaCl threshold for germination, indicating its potential as a relatively salt-tolerant forage crop. Both the proportion of germinated seeds and the germination rate were highly sensitive to the osmotic-potential treatments, indicating that drought resistance will remain the biggest challenge for CJ. This study provides baseline data for a rapid and efficient system for further screening for abiotic-stress tolerance among wild and cultivated lines of CJ.

Keywords

Cephalaria joppensis, Abiotic Stress, Seed Germination

1. Introduction

Wheat constitutes a significant component of the silage and forage grown in Mediterranean regions. In Israel, for example, wheat accounts for about 50% of forage production [1]. Yet, grown as a winter crop without additional irrigation, wheat forage has several disadvantages, including relatively low yields (10 - 14 dry tons/ha) [1] [2] [3], farmers' preference for growing wheat for grain instead of forage and an acute shortage of lands for crop rotation. As a result, in recent years, there has been a shortage of local rough forage for livestock. One possible solution is the development of alternative high-yielding winter crops that would improve the profitability of forage-oriented agricultural production and could be incorporated into better crop-rotation systems.

Cephalaria joppensis (CJ), whose domestication was recently initiated [4], is a promising complementary crop for wheat silage. CJ has many advantages relative to other broad leaf rotation crops (e.g., forage legumes). It is very easy and inexpensive to grow, naturally resistant to many pests and has high yield potential. In addition, its nutritional quality is equivalent to that of wheat and there is little accumulation of nitrites in the silage [4] [5]. This crop is harvested relatively late in the spring season and does not need to dry out in the field before ensiling. Initial observations have identified CJ as a very good fallow crop (Galili, personal observation).

Nevertheless, several factors limit the promotion of this species. CJ is sensitive to most existing commercial herbicides, and it grows relatively slowly in the colder weather of the winter; it begins to grow rapidly only in the early spring. These two characteristics make weed management in this crop particularly challenging, since the competing weeds grow faster than the crop under cold conditions. Drought is another important factor that hinders the expansion of CJ cultivation. The only existing commercial variety, "Rishon", performs poorly under drought conditions and, therefore, is not grown in areas with less than 350 mm of annual precipitation. Indeed, the need for more drought-tolerant varieties is becoming increasingly critical in light of the increased desertification around the world [6].

In many dry and semi-arid regions, slightly saline water from deep wells is being used for crop irrigation. This can lead to reductions in crop fitness and production due to salinity, as well as to severe accumulation of salts in the soil. One possible solution is the development of new salt-resistant cultivars. Wild CJ maybe considered naturally resistance to salt stress, since several accessions have been collected from very salty soils and seaside sites (Galili, personal observation). Yet, the direct effects of salinity on CJ germination and growth have not been previously studied.

Genetic and agronomic improvements that include the introduction of herbicide resistance and increased plant vigor and crop uniformity under stressful conditions could help CJ to become a leading forage crop in rotation systems. In this study, we examined the effects of temperature, salt, osmotic potential and pH on CJ seed germination under controlled conditions. This work is part of an effort to develop new tools for screening for abiotic-stress resistance among wild and developed lines of CJ. Although they concern only one aspect of plant development (*i.e.*, germination), our results serve as the first indications of CJ's responses to abiotic-stress conditions and may serve as a baseline for future breeding/selection work.

2. Materials and Methods

2.1. Plant Material

CJ seeds, cv. Rishon, were used for this study. We sterilized the seeds by immersing them in 1% bleach for 30 min and then rinsing them five times in distilled water. The seeds were dried in a sterile laminar hood and then kept at room temperature until they were used.

2.2. Stress Experiments

Each experiment was performed in six replications with 25 seeds each. To that end, 50 sterilized seeds were placed on two-layer germination paper using a vacuum plate. The paper's surface was divided into two areas, each containing 25 seeds. The seeds were covered with additional wet germination paper and the papers were rolled into cylinders, placed inside polyethylene bags (three cylinders per bag) and covered with aluminum foil to prevent light penetration. For the temperature experiment, the cylinders were incubated for 2 weeks at 7˚C, 10˚C, 12˚C, 15˚C, 20˚C, 25˚C, 30˚C and 35˚C (three cylinders at each temperature). During this period, the germination rate was determined every day by counting the number of seeds with >2 mm radicles. At the end of the 2 weeks, the length, fresh weight and dry weight (after 3 days at 65˚C) were determined for each seedling in each replication.

The salinity experiment used the same design described above, with six replications, but salt solutions were used instead of regular water. Five salt concentrations were used with concentrations of: 0 mM (distilled water), 40 mM, 80 mM, 120 mM and 160 mM NaCl. For the osmotic-potential experiment, the same design was used, but the cylinders were placed in polyethylene glycol (PEG) solutions at 0 Mpa (control), 0.1 Mpa, 0.2 Mpa, 0.4 Mpa 0.8 Mpa and 1Mpa.PEG 6000 (Duchefa; Netherlands) was used with a specific formula, as described by [7]. For the pH experiment, nine pH levels (*i.e.*, 4 - 11) were tested and tap water (pH 6.8) was used as a control. The pH solutions were prepared as described by [8]. In all three experiments, the paper cylinders were incubated in the dark at 25˚C for 10 days. At the end of the incubation period, we measured the length, fresh weight and dry weight of each seedling.

2.3. Calculation of Germination Variables

The percentage of seeds that germinated (%G) was determined using the following formula:

$$\%G = \frac{100 * \text{number of germinated seeds}}{\text{Total number of seeds}} \tag{1}$$

Germination index (GI), the estimated germination rate of the seeds, was calculated using this formula:

$$GI\left(\text{seed/day}\right) = \sum\nolimits_{d=1}^{n} \frac{\text{number of germinated seeds at Day } d}{d} \tag{2}$$

In that formula, d is the number of days from the initiation of the experiment.

The mean germination time (MGT) was calculated using the following formula:

$$MGT\left(\text{days}\right) = \frac{\left(\sum\nolimits_{d=1}^{n} \text{number of germinated seeds at Day } d\right) \times d}{\text{Total number of germinated seeds}} \tag{3}$$

The seed vigor index (SV) was calculated as follows:

$$SV = \text{mean seed length} \times \%G \tag{4}$$

To calculate the point at which 50% of the seeds had germinated, the following formulas were used:

$$\left(\text{Hours}\right) D_{50}HR = \left(24 \times D_{n-1}\right) + \left(24/GSD_n\right) \times \left(TGS/2 - GSD_{n-1}\right) \tag{5}$$

$$\left(\text{Days}\right) D_{50} = D_{50}HR/24 \tag{6}$$

In those formulas, D_{50} is the number of hours or days until 50% of the seeds had germinated, D_n is the Day n from the initiation of the experiment until the total number of germinated seeds exceeded 50% of the total number of seeds that had germinated by the end of the experiment. GSD is the number of germinated seeds on D_n and TGS is the total number of germinated seeds.

The threshold temperature, osmotic potential and salt concentration for germination were calculated as previously described [9]. Statistical analyses were done using the JMP 5.0 software package (SAS Institute Inc., Cary, NC).

3. Results

3.1. The Effect of Ambient Temperature on Seed-Germination Variables

The effect of ambient temperature on the germination of CJ seeds is shown in **Figure 1**. As shown in that figure, almost 100% of the seeds germinated at all of the examined temperatures, with the exception of the 35˚C treatment, in which the germination rate declined significantly to 40% (**Figure 1(a)**). In contrast, the germination index (GI), which represents the change in germination rate, increased significantly from 3.6 seeds/day at 7˚C to 20.7 seeds/day at 30˚C (**Figure 1(b)**). No significant difference in GI was noted between 12˚C and 15˚C. There

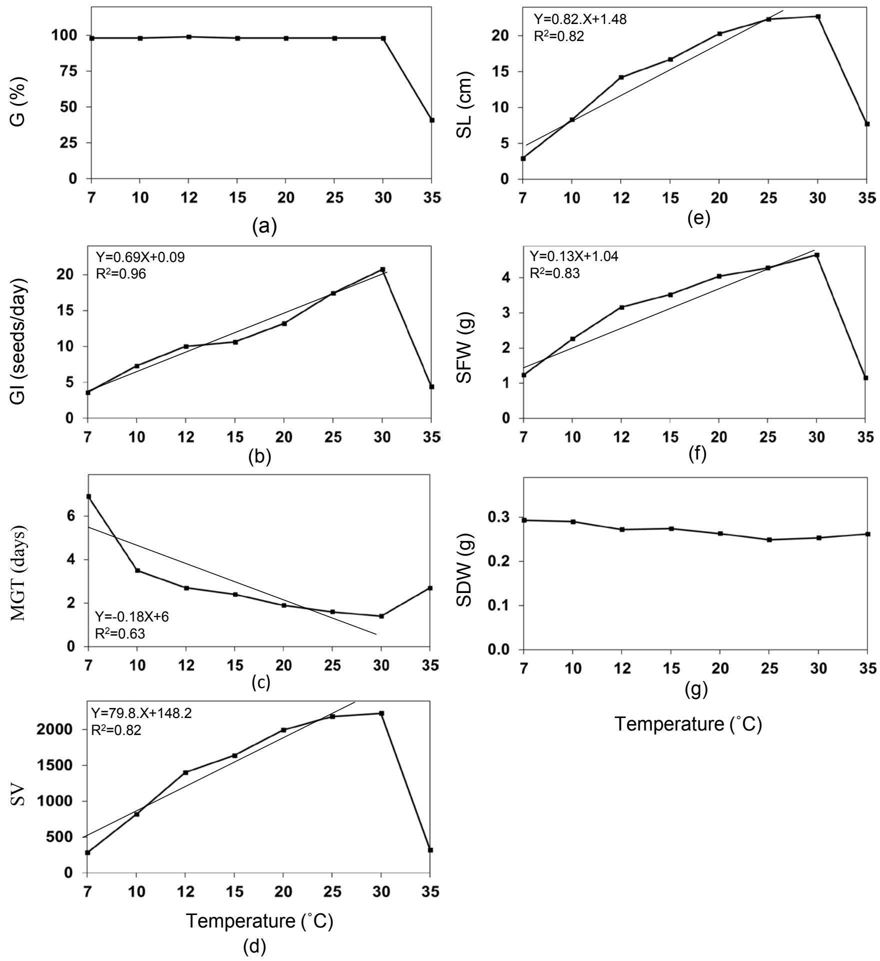

Figure 1. The effects of temperature on (a) the percentage of seeds that germinated (G); (b) the germination index (GI); (c) mean germination time (MGT); (d) seed vigor (SV); (e) seedling length (SL); (f) seedling fresh weight (SFW) and (g) seedling dry weight (SDW). The graphs show the average values and standard errors for 6 replicates of 25 seeds each. The straight line represents the linear correlation among the temperature treatments.

was a positive linear correlation between GI and temperature over the 7˚C - 30˚C range (R^2 = 0.96). However, increasing the ambient temperature to 35˚C resulted in a sharp decrease in GI that was significantly different from that observed in all of the other temperature treatments, even the 7˚C treatment. In contrast, mean germination time (MGT) significantly decreased with the increase in ambient temperature, from 6.9 days at 7˚C to 1.4 days at 30˚C, with the largest increase seen between 7˚C and 10˚C (**Figure 1(c)**). There was a negative linear correlation between MGT and temperature over the 7˚C - 30˚C range (R^2 = 0.63). Increasing the temperature to 35˚C resulted in a significant increase in MGT, although the MGT at 35˚C was not significantly different from that observed for the 10˚C and 12˚C treatments. Similarly, the germination time for 50% of the seeds decreased significantly from 4.5 days at 7˚C to 0.8 days at 30˚C (data not shown). The germination-threshold temperature was calculated from this value and found to be 2.4˚C.

Seed vigor, seedling length and seedling fresh weight all corresponded to GI and were found in positively correlated with temperature (R^2 between 0.82 and 0.83). Seedling vigor increased significantly from 284 at 7˚C to 2266 at 30˚C (**Figure 1(d)**). Seedling length increased significantly from 2.9 cm at 7˚C to 22.7 at 30˚C (**Figure 1(e)**) and seedling fresh weight increased significantly from 1.23 g at 7˚C to 4.65 g at 30˚C (**Figure 1(f)**). For all of these variables, no significant differences were found between the 25˚C treatment and the 30˚C treatment, which both yielded results significantly better than those observed for the 35˚C treatment. In contrast to seedling fresh weight, seedling dry weight did not vary much between the different temperature treatments (**Figure 1(g)**). Since no differences were noted in any of the examined parameters between 25˚C and 30˚C (aside from GI), we concluded that this range is the optimum temperature range for the germination of cv. Rishon. Based on that finding, the effects of all of the other types of stress (*i.e.*, salt, PEG, pH) were examined at 25˚C.

3.2. The Effect of Salt Concentration on Seed-Germination Variables

The effect of NaCl concentration on the germination of CJ seeds is described in **Figure 2.** Similar to the temperature experiments, no difference was found between the salt treatments in terms of %G and almost 100% germination was observed for all of the NaCl treatments (**Figure 2(a)**). Unlike %G, GI was influenced by the salt concertation and significantly decreased from 15.7 seeds/day in distilled water to 6.6 seeds/day in 160 mM NaCl (**Figure 2(b)**). There was a significant negative linear correlation between GI and that range of salt concentrations (R^2 = 0.94; **Figure 2(b)**). In accordance with that decrease in GI, MGT increased significantly between 1.7 days to 3.7 days, from 0 mM to 160 mM, respectively (**Figure 2(c)**). In addition, the amount of time required for 50% of the seeds to germinate increased significantly from 1.0 days at 0 mM NaCl to 3.8 days at 160 mM NaCl (data not shown). The threshold for osmotic potential was extrapolated from those figures and found to be 273 mM NaCl. Seed vigor, seedling length and seed fresh weight all corresponded to GI and were found to be negatively correlated with NaCl concentration (R^2 between 0.74 and 0.80). Seedling vigor significantly decreased from 1459 at 0 mM NaCl to 501 at 160 nm NaCl, but no significant differences in seedling vigor were noted between 0mMNaCland 40 mM NaCl (**Figure 2(d)**). Seedling length decreased significantly from 15.2 cm at 0 mM to 5.3 at 160 nm NaCl, but no significant differences in seedling length were noted between 0 mM and 40 mM (**Figure 2(e)**). Seedling fresh weight decreased significantly from 1.59 g at 0 mM NaCl to 0.81 g at 160 mM NaCl, but no significant differences in seedling fresh weight were noted between 0 mM and 40 mM or 80 mM (**Figure 2(f)**). As in the temperature experiment, the dry-weight values from the salt experiment did not show any significant change in either direction (**Figure 2(g)**).

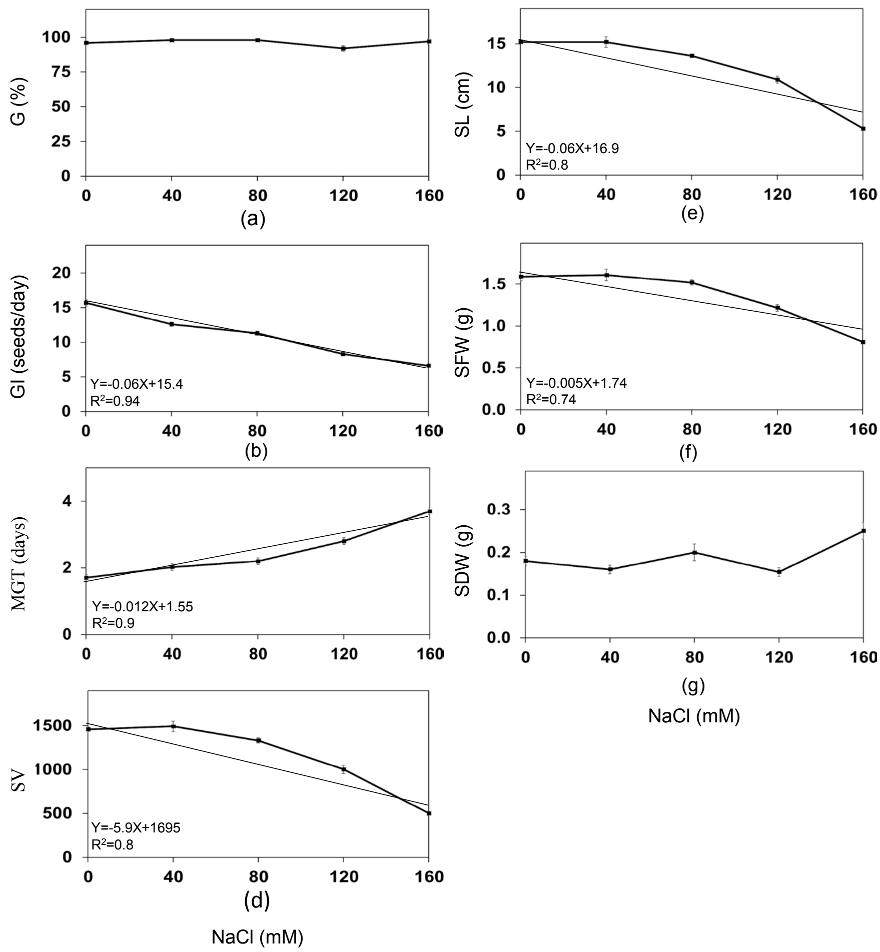

Figure 2. The effects of different NaCl concentrations on (a) the percentage of seeds that germinated (G); (b) the germination index (GI); (c) mean germination time (MGT); (d) seed vigor (SV); (e) seedling length (SL); (f) seedling fresh weight (SFW) and (g) seedling dry weight (SDW). The graphs show the average values and standard errors for 6 replicates of 25 seeds each. The straight line represents the linear correlation among the salt treatments.

3.3. The Effect of Osmotic Potential on Seed-Germination Variables

The effect of osmotic potential on the germination of CJ seeds was tested as an indicator of drought tolerance. Different levels of osmotic potential were established through the use of different concentrations of PEG. As shown in **Figure 3**, the effect of osmotic potential was different from that of salinity, with the exception of %G. Almost 100% germination was observed only up to −0.4 MPa (**Figure 3(a)**). Beyond that point, %G decreased significantly, reaching 79% at −0.6 MPa, 21% at −0.8 MPa and 5.6% at −1 MPa. GI also decreased significantly as the osmotic potential increased, from 15.7 seedlings/day at 0 MPa to 0.2 seedlings/day at −0.8 MPa (**Figure 3(b)**). The negative linear correlation between GI and osmotic potential had an R^2 value of 0.94.As expected, a very different trend was observed for MGT. MGT increased significantly as osmotic potential increased, from 1.7 days at 0 MPa to 7.5 days at −0.8 MPa (**Figure 3(c)**).

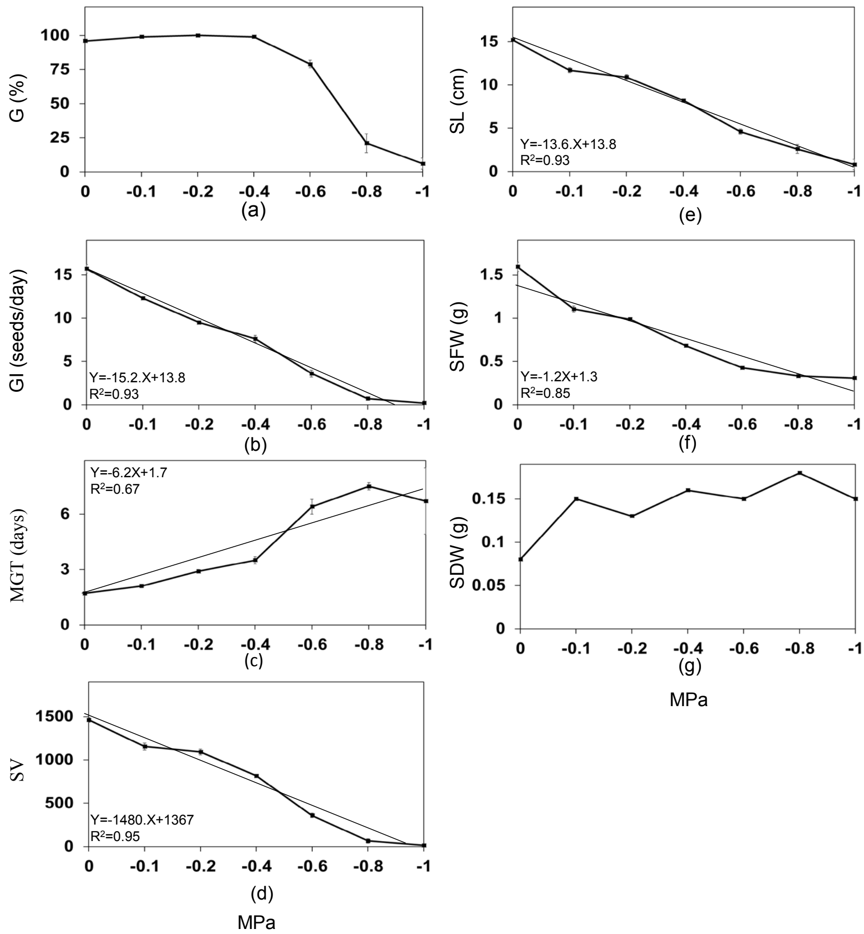

Figure 3. The effect of osmotic potential on (a) the percentage of seeds that germinated (G); (b) the germination index (GI); (c) mean germination time (MGT); (d) seed vigor (SV); (e) seedling length (SL); (f) seedling fresh weight (SFW) and (g) seedling dry weight (SDW). The graphs show the average values and standard errors for 6 replicates of 25 seeds each. The straight line represents the linear correlation among the osmotic-potential treatments.

In addition, the amount of time required for 50% of the seeds to germinate increased significantly from 1.3 days at 0 MPa to almost 5 days at −0.8 MPa (data not shown). The threshold for osmotic potential was extrapolated from those figures and found to be around −0.95 MPa. Significant negative correlations were observed between seed vigor, seedling length and seedling fresh weight, on the one hand, and osmotic potential, on the other, with R^2 values of 0.95, 0.96 and 0.85, respectively. Seedling vigor decreased from 1459 at 0 MPa to 13 at −1 MPa (**Figure 3(d)**). Seedling length decreased from 15.2 cm at 0 MPa to 0.8 cm at −1 MPa (**Figure 3(e)**) and seedling fresh weight decreased from 1.59 g at 0 MPa to 0.3 g at −1. MPa (**Figure 3(f)**). As osmotic potential increased, a slight increase in seedling dry weight was noted (**Figure 3(g)**).

3.4. The Effect of pH on Seed-Germination Variables

These data are presented in **Figure 4**. As shown, the examined pH levels in this

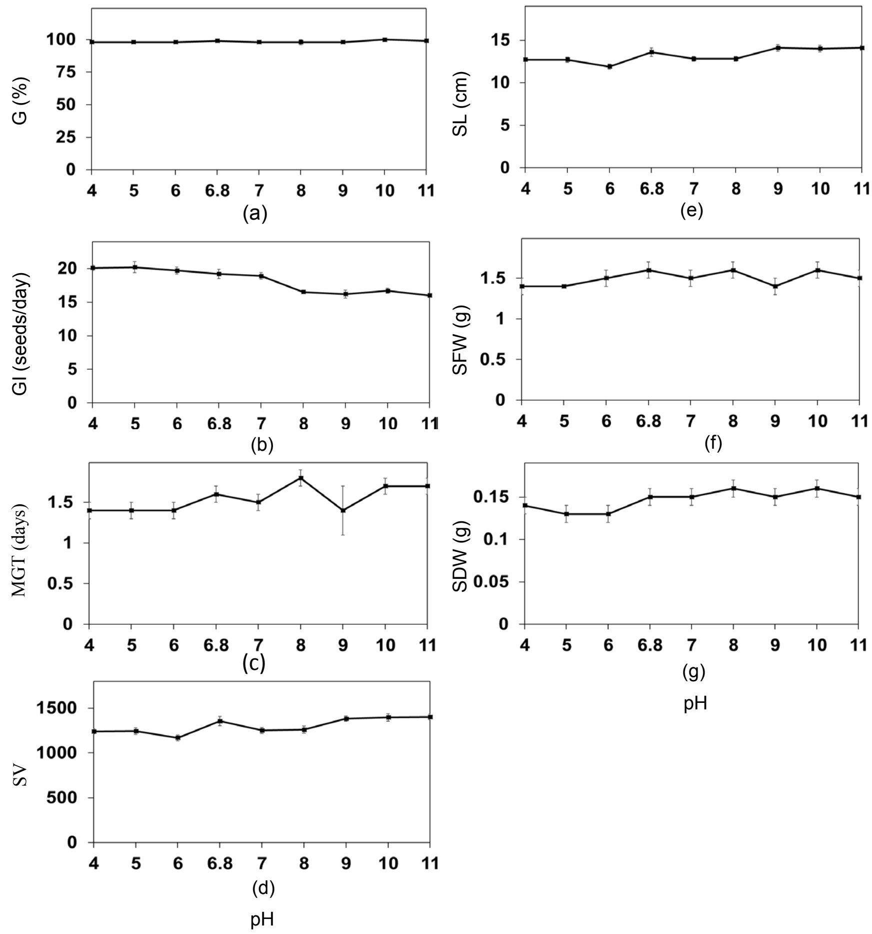

Figure 4. The effect of pH on (a) the percentage of seeds that germinated (G); (b) the germination index (GI); (c) mean germination time (MGT); (d) seed vigor (SV); (e) seedling length (SL); (f) seedling fresh weight (SFW) and (g) seedling dry weight (SDW). The graphs show the average values and standard errors for 6 replicates of 25 seeds each. The straight line represents the linear correlation among the pH treatments.

experiment did not influence on the germination of CJ seedlings. Almost 100% germination occurred in all pH treatments between pH 4and pH11 (**Figure 4(a)**). Only minor differences in the other germination variables were noted between the pH treatments (**Figures 4(b)-(g)**).

4. Discussion

Abiotic stresses such as temperature, drought and salinity decrease agricultural production and are becoming an increasingly important problem [10]. These stresses can delay, reduce or prevent germination [11]. Seed germination could be an efficient stage at which to select abiotic stress-tolerant agricultural crops, since this stage influences the final stand in the field, which strongly affects later stages of crop production [12]. In this study, the effects of several types of abiotic stress on CJ germination were examined for the first time. In general, it was found that the germination of the seedlings of cv. Rishon was affected by temperature, salinity and osmotic potential, but not pH.

Notably, ambient temperature was not found to affect the final percentage of germinated seeds (almost 100% of the seeds germinated at all of the examined temperatures). Yet, sharp decrease in all parameters tested except dried weight was observed between 30˚C and 35˚C. This could be due to that the 35˚C is above the optimum temperature (25˚C - 30˚C) for CJ germination. High temperature might also avoid wild CJ germination in unexpected early season rainfalls, when temperatures are still high, followed by a long term of dry conditions. Ambient temperature, though, did affect the other germination variables (*i.e*., GI, seedling vigor, MGT, seedling length and seedling fresh weight). This result indicates that the main problem of cv. Rishon under field conditions is not its germination potential at lower temperatures, but other germination variables. This is also reflected in its relatively low germination-threshold temperature (2.4˚C), which is much lower than the temperatures typical of the Mediterranean autumn (when CJ seeds are sown). It is reasonable to conclude that the reduction in CJ growth during the autumn is due to other variables, which are connected to seedling development as opposed to the initiation of germination. This characteristic of the species can serve as a defense mechanism in the wild, in which the plant is sheltered under the weed canopy during the winter, where it is protected from foraging, and only begins to grow rapidly in the early spring. Interestingly, unlike seedling fresh weight, seedling dry weight was not affected by most of the temperature treatments. This could be due to the fact that the seedlings were germinated in the dark and, therefore, the observed increases in seedling length and seedling fresh weight reflect water absorption as opposed to the accumulation of dry matter. The decrease in seedling dry weight at higher temperatures could be a result of higher respiration rates at those temperatures.

Similar to the results observed from the temperature experiments, no difference was found between salt treatments in terms of %G; almost 100% germination was observed at all of the examined NaCl concentrations. Like temperature, salinity was found to have some effect on germination-rate variables. It is important to note that salt-stress resistance is a trait that is closely associated with development and salinity resistance at a particular phenological stage does not necessarily imply complete resistance throughout the life cycle [13] [14] [15]. However, several studies have indicated that germination and seedling establishment are very crucial stages in the development of salt-sensitive species [16] [17] [18] [19], which affect the total proportion of germinated seeds, the speed at which those seeds germinate and initial plant growth. In Israel, some wild CJ lines have been found very close to the seashore, indicating that there may be some natural variation in salt resistance in this species. Therefore, it is not surprising that the threshold salt concentration was estimated in this study to be 273 mM. That value is similar to those found for other forage cereal plants like wheat [20], sorghum [12] [21] and other forage grasses [22] and higher than the values observed for forage legumes such as white clover [23] and alfalfa [24]. As in the temperature experiment, in the salt experiment, there were no significant trends in terms of dry weight (**Figure 2(g)**), indicating once again that the differences observed in seedling length and seedling fresh weight were due to the absorption of water by the seedlings grown in the dark.

Drought tolerance is one of the most challenging traits to select in field crops. The biggest difficulty is how to control and repeat field trials [25]. Richards, [26] suggested utilizing the germination stage of seedlings under controlled conditions for selecting for drought tolerance. Khakwani *et al.* [27], used different methods to evaluate the drought tolerance of six wheat cultivars and identified lines in which there was a strong correlation between the results seen under controlled conditions and the drought resistance observed in the field. Similar results were found in studies involving sweet potato [28]. One way to screen for drought-tolerance under controlled conditions is to use PEG to introduce drought-like stress conditions. Exposure of the germinating seeds to PEG mimics drought conditions, usually without other side effects [29], and this technique has been successfully used for drought-resistance screening in many crops [30].

In this study, the effect of osmotic potential on %G was relatively high and significantly decreased %G was observed even at −0.6 MPa, with a sharp decline between −0.6 MPa and −0.8 MPa. Similar results have been reported for other field crops such as corn, canola, barley [31] [32], and chickpea [33]. In addition, the use of an osmotic potential between −0.4 and −0.8 MPa was suggested in screens for drought stress [34]. The sharp decline in the %G below −0.6 MPa demonstrates that the absence of drought resistance will remain the biggest challenge for the development of abiotic-stress resistance in CJ. Interestingly, pH was not found to have any effect on the development of CJ seedlings, indicating that CJ could be growing in both alkaline soils (pH ranging from 7 to 9) and acidic soils (pH ranging from 5.5 to 7). Therefore, pH is not expected to limit the expansion of CJ cultivation.

5. Conclusion

In summary, the current study serves as an excellent baseline for the development of systems for the selection of abiotic-stress tolerance in CJ. For example, according to the results of this experiment, our suggestion is to screen new germplasm for temperature, salt and osmotic stress at 7˚C, 160 mM and −0.8 MPa levels, respectively. Recent field screenings of ~40 wild lines of CJ have revealed significant variation in plant morphology and maturity (data not presented here). A rapid and efficient selection system is now available for the evaluation of natural and cultivated variation in CJ.

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