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Assessing *Salicornia europaea* Tolerance to Salinity at Seed Germination Stage

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Abstract: *Salicornia europaea*, a halophytic species, was investigated to assess its ability to withstand salinity during seed germination, and to identify suitable indices to interpret salt tolerance at this delicate stage. Seed germination indices (germination percentage (GP), germination energy (GE), germination value (GV), coefficient of germination velocity (CVG), germination rate index (GRI), germination peak value (GPV), mean germination time (MGT), and time to 50% germination (T_{50})) were calculated under increasing salinity (0, 100, 200, 300, 400, and 600 mM NaCl). Principal component analysis (PCA) was used to describe the relationships involving the variables that account for data variance. Two salinity thresholds were identified (100 and 600 mM NaCl) determining significant decreases in all the indices, except for T_{50} and MGT. In fact, PCA based on generated correlation circle showed significant negative correlations (r close to -1) between salt stress and GP, GE, GRI, PV, GV, and CVG, whereas no correlation was observed with T_{50} and MGT (r close to zero). Based on this, GP, GE, GRI, PV, GV, and CVG can be considered useful traits to assess salt tolerance during germination in *S. europaea*, while T_{50} and MGT, that were not affected by the range of salinity levels investigated, should not be used for this purpose.

Keywords: *Salicornia europaea*; salinity; seed germination; PCA; correlation circle

1. Introduction

Salinity in soil or water is one of the stresses most severely limiting crop production [1]. More than 20% of cultivated land worldwide is affected by salt accumulation, and this figure is feared to increase up to 50% by 2050 [2]. Salinity impairs seed germination, delays plant development, and reduces crop yield [3]. As a result, the decline in food availability, and the quest for more sustainable sources of food and forage are stirring the interest in halophyte plants. Halophytes are naturally evolved salt-tolerant plants that represent almost 2% of terrestrial species [4]. Halophytes are currently being studied for wider commercial applications, including as a source of food and forage, but also aromatic, cosmetic, and nutraceutical compounds for human uses [5]. *Salicornia* is a halophyte genus belonging to the Amaranthaceae family. It is known as pickleweed, glasswort, sea beans, sea asparagus, or crow's foot greens [6]. Besides *Salicornia europaea*, several other species of *Salicornia* are well known, such as *S. bigelovii*, *S. brachiata*, *S. virginica*, *S. maritima*, *S. ramosissima*, *S. herbacea*, and *S. persica*. These plants are commonly found at the edges of wetlands, marshes, seashores, and mudflats. They have been reported to be able to tolerate up to 500 mM salinity, as in the case of *Salicornia europea* [7], and are considered good candidates for reclamation of barren lands, salt flats, and seashores [6].

Salicornia spp. has been historically used for both edible and non-edible purposes. The aerial parts of the plant are consumed in salads or processed into pickles, beverages, or vinegar [8,9]. On the other hand, the use of this plant as a source of soda (sodium carbonate) for glass and soap making has been a common practice for several centuries [10]. Recently, additional potential uses have been proposed.

Some *Salicornia* species (e.g., *S. bigelovii*) are grown at the commercial scale to produce biofuel, livestock feeding, and for salt and oil extraction [11]. A recent study reports the suitability of some *Salicornia* species as bio-indicators of zinc and copper, also emphasizing their potential for soil phytoremediation from these metals [12]. The possibility of using *S. persica* as biofilter for the treatment of the effluent released by a recirculating maricultural system has been studied in Israel [13].

Moreover, the medical and nutraceutical properties of this genus are drawing attention, contributing to the growing interest in it [14]. The efficacy of *S. herbacea* against oxidative stress, inflammation, diabetes, asthma, hepatitis, cancer, gastroenteritis has already been reported [15]. Additionally, the powder of *S. herbacea* has been transformed into spherical granules showing the potential to be used as dietary NaCl [16]. *S. herbacea* has also proved that seed oil is stable to oxidation and eligible to be used in food processing [17]. Crude, as well as purified, polysaccharides from *S. herbacea* have demonstrated cell antiproliferation in human colon cancer [18]. Furthermore, various options to control hyperglycemia have been studied using *S. herbacea* powder on diabetic-induced rats [19].

However, despite the plentiful benefits of *Salicornia*, the consumption of these plants may also determine adverse effects. For instance, the Amaranthaceae family is known for a high oxalate content, which might be harmful to consumers [20]. A study reports *S. brachiata* as being able to accumulate heavy metals, such as cadmium, nickel, and arsenic salts [21], therefore posing a potentially serious risk to consumer health [22].

Despite the potential multiple applications, the use of halophytes as cultivated plants is still restricted due to several impediments, among which is the difficult and uneven germination. In fact, some halophytes are salt-tolerant when adults, but have a differential ecotypic response to salinity during seed germination [23,24]. Typically, germination is higher in fresh water and declines as salinity increases, albeit for some species, low salt concentrations may stimulate germination [25,26]. Many halophytes have developed mechanisms of avoidance based on seed dormancy in order to germinate when salinity is the lowest in their natural environment [27]. Often, indeed, germination occurs after a rainy periods when soil salinity is diluted, and the risk of salt stress is reduced [28].

Therefore, the domestication efforts addressing *Salicornia* should include understanding its germination behavior.

Ungar [28] observed that *S. europaea* has low germination when treated with NaCl solutions between 1% (170 mM) and 5% (860 mM), and a germination level similar to control (distilled water) when treated with solutions not exceeding 1% NaCl. In nature, *S. europaea* germinates during the winter and spring season, when the salt concentration is the lowest [28]. Additionally, Orlovsky et al. [29] studied the germination response of *S. europaea* dimorphic seeds under growing salinity and demonstrated that large seeds keep a 90% germination up to 2% NaCl concentration (342 mM), with a drastic drop to 20% at 3% NaCl (513 mM) and no germination at 5% and 7% NaCl concentration. Small seeds, instead, showed germination below 10% at 2% NaCl concentration. This explains why, in the early phase of halophyte cultivation, freshwater irrigation was recommended to ensure good germination and seedling establishment (Gallagher, 1985).

On the other hand, a growing piece of literature, as reviewed by Jisha et al. [30], demonstrates the potential of NaCl seed priming in conferring glycophyte species a higher salt tolerance, which is essentially due to the acquisition of a higher osmotic adjustment capacity. Nonetheless, negative effects with seed osmo-priming were also detected in halophytes, as reviewed by Gul et al. [27]. Therefore, in view of promoting the cultivation of *S. europaea* in Mediterranean areas affected by soil and water salinity, further investigations on germination under saline conditions are needed. In this light, this work was conducted to study the influence of salinity on *S. europaea* seed germination through different indices, with the aim of evaluating their performance and reliability, in order to detect the most suitable ones to assess salt tolerance at this delicate stage.

2. Materials and Methods

2.1. Plant Material and Germination Conditions

The experiment was set up at the Department of Agricultural and Food Sciences (DISTAL), University of Bologna, Italy. Commercial *Salicornia europaea* seeds were purchased from B & T World Seeds (Paguignan, Aigues-Vives, France). Healthy seeds were surface-sterilized with a 3% sodium hypochlorite solution for 2 min, rinsed in deionized water for 5 min, and dried at room temperature. The cold stratification method [10] was used to overcome seed dormancy. Seeds were then placed on damp filter paper in 9-cm Petri dishes that were wrapped in transparent plastics and stored in a dark refrigerator at 6 °C for 30 days. During this time, the seeds were dampened with distilled water.

Thereafter, Petri dishes were moved into an incubator at 24 °C, 70–80% relative humidity, and 16/8 h light/dark period for 18 days. At this stage, the filter paper was dampened with distilled water (0 mM NaCl—control), and five treatments at increasing salinity (100, 200, 300, 400, and 600 mM NaCl). Two replicates of 50 seeds per Petri dish were set up for each treatment. The number of seeds germinated under the given conditions was counted every other day until no more germination was observed (up to 18 days). Seeds were considered germinated when the protruding radicle was ≥ 2 mm long.

2.2. Germination Indices

The following indices were calculated: Germination percentage (GP), germination energy (GE), germination value (GV), coefficient of germination velocity (CVG), germination rate index (GRI), peak value (PV), mean germination time (MGT), and time to 50% germination (T_{50}). For each index, the formula and the source are given in Table 1.

Table 1. Description of the various germination indices used in this study.

Index	Unit	Formula	Ref.
Germination Percentage (GP)	%	$GP = \frac{\text{No. of germinated seeds}}{\text{No. of seeds}} \times 100$	[31]
Germination Energy (GE)	—	$GE = \frac{N_1}{D_1} + \frac{N_2 - N_1}{D_2} + \dots + \frac{N_j - N_{j-1}}{D_j}$ where N is the number of germinated seeds on the counting date and D the number of days	[32]
Peak Value (PV)	—	$PV = \frac{M_{ag}}{\text{No. of seeds}} \times 100$ where M_{ag} is the maximum of seeds accumulative germination	[33]
Germination Value (GV)	—	$GV = PV \times MDG^1$	[33]
Coefficient of Germination Velocity (CVG)	—	$CVG = \frac{(N_1 + N_2 + \dots + N_n) / 100 \times [(N_1 \times D_1) + (N_2 \times D_2) + \dots + (N_n \times D_n)]}{\text{where } N \text{ is the number of germinated seeds every day and } D \text{ is the number of days from seeding corresponding to } N.}$	[34]
Germination Rate Index (GRI)	% day ⁻¹	$GRI = \frac{G_1}{D_1} + \frac{G_2}{D_2} + \dots + \frac{G_n}{D_n}$ where G_1 is the germination percentage on day 1 (D_1), and so on	[31]
Time to 50% germination (T_{50})	days	$T_{50} = t_i + \frac{(N/2 - n_i) \times (t_j - t_i)}{(n_j - n_i)}$ where N is the final number of germinated seeds, and n_i and n_j are the total number of seeds that had germinated (by adjacent counts) at times t_i and t_j , when $n_i < N/2 < n_j$.	[35]
Mean Germination Time (MGT)	days	$MGT = \frac{\sum(N \times D)}{\sum N}$ where N is the number of seeds germinated on day D	[36]

¹ Mean Daily Germination (MDG): No. germinated seeds/No. of days.

2.3. Statistical Analysis

Data of the eight germination indices were subjected to a one-way ANOVA for the six salinity levels (from zero to 600 mM NaCl), using the CoStat, 6.4 statistical package (CoHort Software, Berkeley, CA, USA). Tukey's HSD test at $p \leq 0.05$ was used as a mean separation test for significant indices. Additionally, the Statistica 7 program (StatSoft, Tulsa, OK, USA) was used to perform the principal component analysis (PCA). Significant covariance between the studied parameters was defined by the correlation circle using the Pearson correlation coefficient (r) at $p \leq 0.05$ to indicate the similarity.

3. Results

3.1. Salt Effects on Seed Germination Indices

Figure 1 reports the variations determined by salt stress on germination indices. A significant decrease was detected in all indices at increasing NaCl concentration, except for MGT and T_{50} that did not exhibit any significant change. This decreasing trend could be described by combining three linear functions, whose slopes vary at the salinity levels causing significant reductions in the surveyed indices. The first significant decline was generally observed between 0 and 100 mM NaCl, but the strongest reduction occurred between 400 and 600 mM NaCl.

Optimal GP took place in distilled water (72%), and then seed germination declined to an average 45%, remaining constant between 100 and 400 mM NaCl (Figure 1A). A further decrease was evidenced at 600 mM NaCl, although germination was not totally inhibited, as the residual 28% GP demonstrates (Figure 1A).

GE showed a similar trend (Figure 1B): After the first initial drop between zero and 100 mM NaCl, seeds subjected to a salinity range between 100 and 400 mM NaCl showed a similar GE reduction (35% on average), while the strongest drop in GE was observed at 600 mM NaCl (−61%). PV, instead, decreased quite consistently across the range of salinity (Figure 1C), losing more than 50% of the initial value at 600 mM NaCl (Figure 1C).

A much stronger variation was shown in GV (i.e., the product of PV by mean daily germination) (Figure 1D). The fall in both PV (Figure 1C) and MDG (not shown) determined a multiplicative effect on GV, resulting in an almost 85% drop between 0 and 600 mM NaCl (Figure 1D).

CVG exhibited the same trend of GP, GE, and PV (Figure 1E). The highest CVG value was recorded under control conditions (77). A substantial decline was registered at the salinity level between 100 and 400 mM NaCl (−60%), and a further drop was evidenced at 600 mM NaCl (Figure 1E). GRI, which reflects the daily germination percentage, staged a similar trend, and the final drop at 600 mM NaCl was the same as CVG (−60%) (Figure 1F).

Contrarily, T_{50} was unaffected by salinity, as three to four days were needed for all the tested seeds to reach 50% germination, regardless of the salt level (Figure 1G). Likewise, MGT did not exhibit any considerable variation in response to salt concentration (Figure 1H), and the mean time seeds require to initiate and complete germination was six days either under control condition or at the highest salt level (600 mM NaCl).

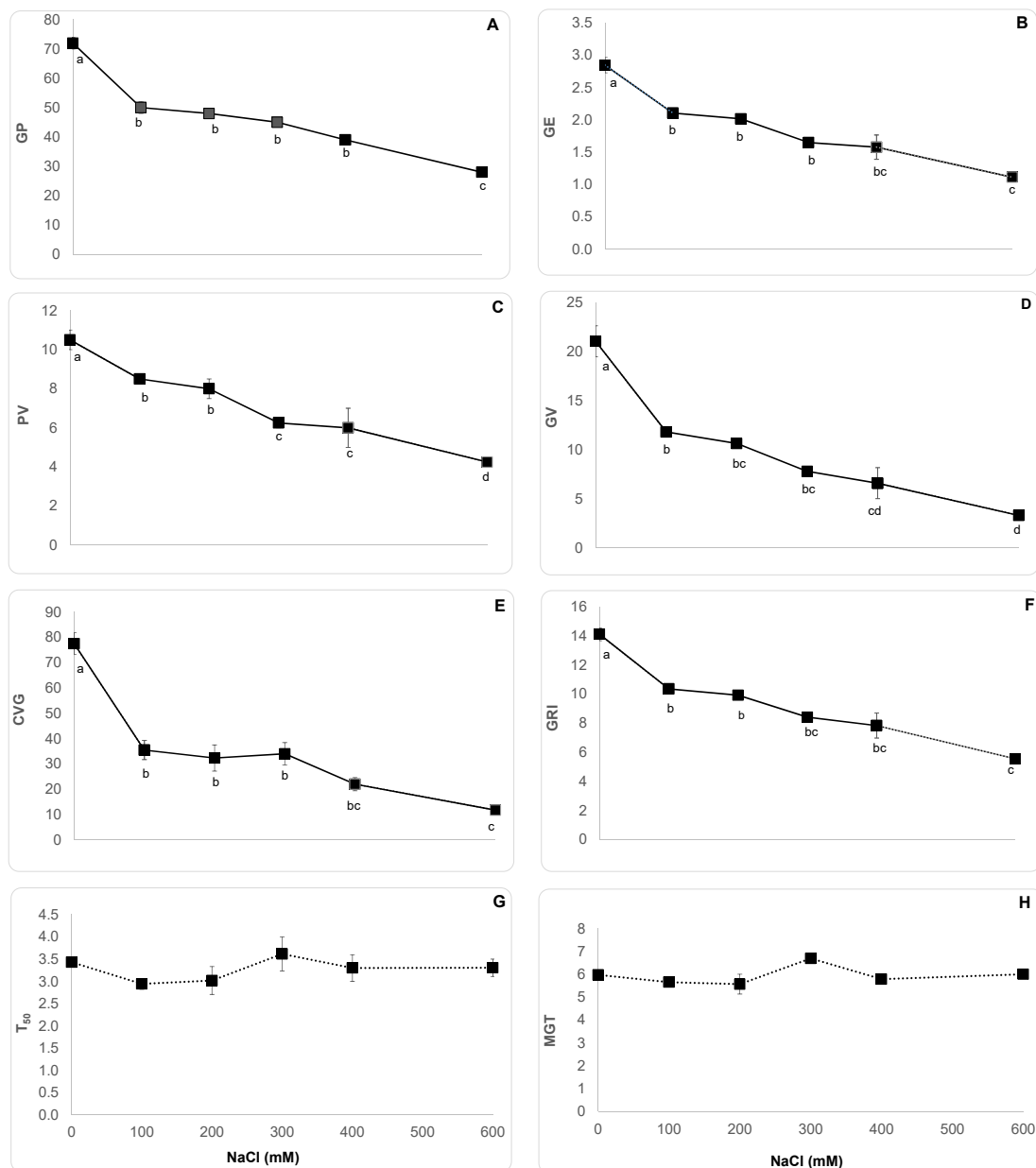


Figure 1. Effects of different salt concentrations on germination indices of *Salicornia europaea* seeds. GP, germination percentage (A), GE, germination energy (B), PV, peak value (C), GV, germination value (D), GRI, germination rate index (E), CVG, coefficient of velocity of germination (F), T₅₀, time to reach 50% of germination (G), MGT, mean germination time (H). Data presented are means ± SE.

3.2. Principal Component Analysis of Germination Indices

Principal component analysis was carried out to establish the relationship among the variables that account for the observed data variance. Eigenvalues higher than 1 were used to determine the number of principal components (Table 2). The first two principal components (PC1 and PC2) jointly explained 98% of the observed variance and were, therefore, represented in a two-dimensional space (Figure 2). PC1, plotted on the horizontal axis, explained the largest share of variance (73.8%), while PC2, plotted on the vertical axis, represented an additional 24.4% of the total variance (Table 2). Variable squared cosines were used to define variable contributions to the respective PC1 and PC2 (Table 2).

Table 2. Eigen analysis of the correlation matrix.

Principal Component Analysis		PC1	PC2
Eigenvalue		5.905139	1.954265
Total variance (%)		73.81423	24.42831
Cumulative Eigenvalue		5.905139	7.859403
Cumulative variance (%)		73.8142	98.2425
Variable Squared Cosines			
Variable		PC1	PC2
GP		0.98	1.00
GE		1.00	1.00
PV		0.97	0.99
GV		0.99	0.99
CVG		0.93	0.99
GRI		1.00	1.00
T ₅₀		0.00	0.95
MGT		0.03	0.94

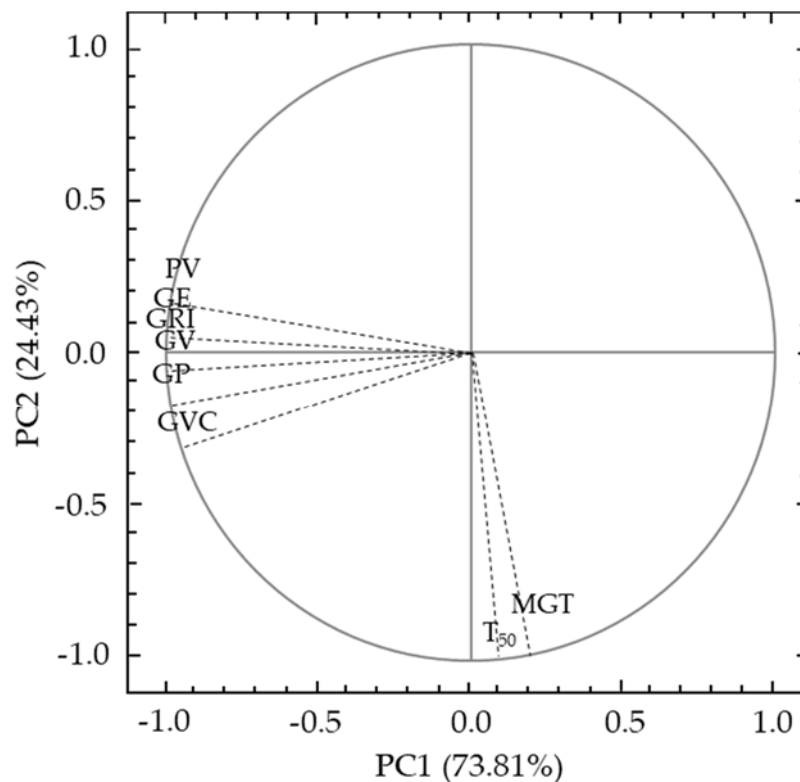


Figure 2. Site score plot of the studied variables on the first two principal components (PC1 and PC2) of *Salicornia europaea* seeds exposed to salt stress. GP, germination percentage; GE, germination energy; PV, peak value; GV, germination value; GRI, germination rate index; CVG, coefficient of velocity of germination; T₅₀, time to reach 50% of germination; MGT, mean germination time (MGT).

Figure 2 represents the site score plot of the eight indices on the two first PC of *Salicornia europaea* seeds. GP, GE, PV, GV, CVG, and GRI appeared to be negatively correlated with salt stress, being positioned on the negative side of the horizontal axis representing PC1. The highest correlations were especially observed between salt stress and GP, GE, PV, GV, and GRI (r between -0.99 and -1.00). CVG was also shown to be well correlated with salt stress ($r = -0.96$). On the other hand, T₅₀ and MGT that are located on the positive side of the horizontal axis, very close to zero, express a very low correlation with salinity stress ($r = 0.18$ and $r = 0.05$, respectively).

Figure 3 illustrates each variable's specific contribution to PC1 (total contribution = 1). The contribution of GP, GE, PV, GV, CVG, and GRI was relatively high and uniform, whereas T₅₀ and MGT contribution to PC1 was negligible.

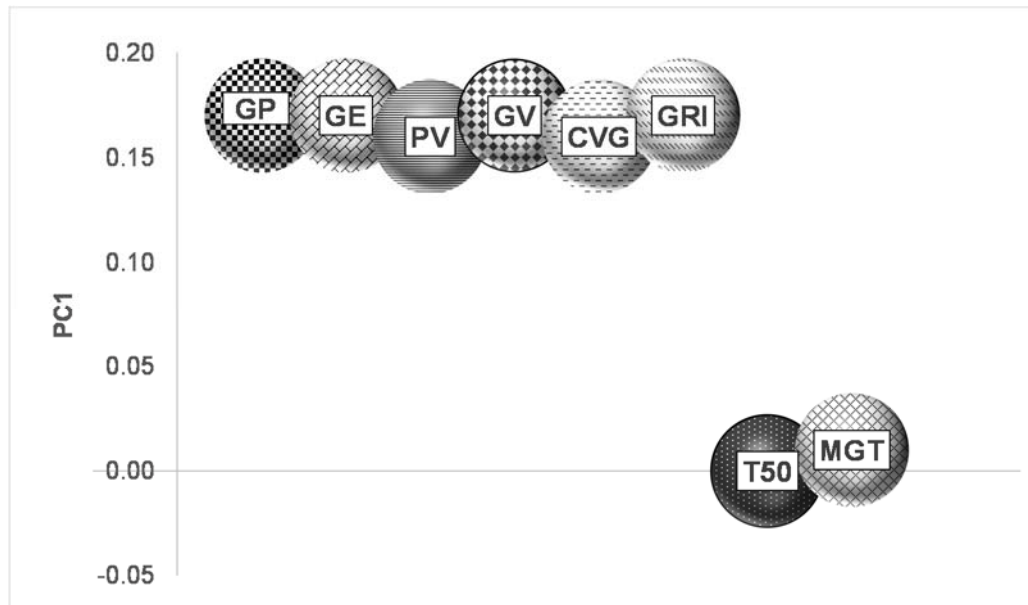


Figure 3. Contribution of the studied variables to the first principal component (PC1) of *Salicornia europaea* seeds exposed to salt stress. GP, germination percentage; GE, germination energy; PV, peak value; GV, germination value; GRI, germination rate index; CVG, coefficient of velocity of germination; T₅₀, time to reach 50% germination; MGT, mean germination time.

4. Discussion

Germination characteristics are among the most suitable criteria for assessing salt tolerance in plants [37]. Salinity is a serious constraint hindering seed germination [38], and the fact that germination indices are adversely affected by salinity is generally acknowledged [39]. In the present work, various indices were focused on assessing *Salicornia europaea* germination performance, each having a slightly different focus. Two salinity thresholds were identified: The first one was at 100 mM NaCl (low salinity threshold), where most of the indices showed the first decline, with the exception of T₅₀ and MGT (Figure 1). The second critical drop was observed at 600 mM NaCl (high salinity threshold), again with the exception of T₅₀ and MGT that remained substantially unaffected up to this level (Figure 1). Hence, most indices exhibited a consistency of the effect in a relatively wide range of salt doses (between 100 and 400 mM NaCl).

It could be argued that low-medium salt stress (up to 400 mM NaCl) might break *S. europaea* seed dormancy and promote germination. Sanoubar et al. [40] identified two thresholds of salinity response in white cabbage, respectively, at 100 mmol L⁻¹ NaCl (moderate salinity threshold) and 200 mmol L⁻¹ NaCl (high salinity threshold). Maggio et al. [41] proposed that the relationship between yield and salinity in tomato could be represented by a bilinear response function, suggesting the existence of a second physiological threshold. Such a threshold may be used to identify functional shifts between different adaptation mechanisms.

However, at a high salt concentration (600 mM NaCl), GP was severely reduced but not completely inhibited, and seeds were still able to germinate (28% vs. 72% of the control) (Table 1, Figures 1 and 2). This suggests a high tolerance of *S. europaea* towards salinity stress, proving its ability to germinate even under high salt concentration. Such tolerance was identified also in other halophytes, such as *Haloxylon ammodendron* (200 mM) [25], *Salsola affinis* (400 mM) [42], and *Bromus inermis* (200 mM) [43]. Compared to this, for *Artemisia annua*, a species that is not acknowledged to be a halophyte, Bijeh Keshavarzi [44]

reported a GP of 77.5% under no salinity, vs. 8.75% at 100 mM NaCl, and zero at only 150 mM NaCl. Seed germination of halophytes under salinity was also reported by Li et al. [45], who found that GP of *Apocynum venetum* increased with NaCl concentrations up to 150 mM, supporting the assumption that low salt stress could promote seed germination in this species. In *S. europaea* germination of small seeds was also reported to be slightly improved at 0.5 and 1% NaCl vs. no salt added [29].

Compared to this, other studies on halophytes such as *Atriplex isatidea*, *Phragmites australis*, *Sesbania cannabina*, and *Limonium bicolor* reported germination levels of 5% or lower at 300 mM NaCl [46]. Similar results were also observed in species as *Reaumuria trigyna* [47], and *Salsola vermiculata* [48]. High salinity might lead to ionic imbalance, with excess Na^+ and Cl^- ions determining irreversible damage of function and structure of cell membranes, in turn, leading to cell death [49].

Furthermore, PCA supported our quest to identify the traits contributing to explain the variable behavior in response to salinity. The first two principal components, PC1 and PC2, accounting for almost all the observed variance (Table 2), were used to plot two-dimensional scatter plots (Figure 2) [50,51]. As already mentioned, the first principal component PC1 alone expressed almost three quarters of the variability in our data set (Table 2). Consequently, PC1 could be considered the main salinity-related component. Some of the selected germination indices (GP, GE, PV, GV, CVG, and GRI) were located at the extreme left side of the horizontal axis in the loading plot, meaning that they were negatively correlated with salt stress (r close to -1) (Figure 2). This distribution of GP, GE, PV, GV, CVG, and GRI can be ascribed to the salinity factor, and the negative correlation indicates adverse effect exerted by salinity in each of these traits. Therefore, it is perceived that these indices represent a robust parameter for evaluating *Salicornia europaea* seed salinity tolerance. In contrast, T_{50} and MGT were loaded orthogonally (r close to 0) (Figure 2), indicating unsuitability to reveal salt stress. Therefore, they cannot be considered useful indices for salt tolerance screening in seed lots of this species.

5. Conclusions

The achievement of the highest germination in distilled water (control) suggests that *S. europaea* does not have a real physiological need for salt during germination. Conversely, salinity progressively affects its germination, without ever completely suppressing it. Eight seed germination indices were selected to study *S. europaea* germination under non-saline control and five levels of increasing salinity. Two salinity thresholds (100 and 600 Mm NaCl) were identified, at which all the surveyed indices were significantly reduced, with exception of T_{50} and MGT.

A principal component analysis (PCA) was carried out to identify and group the variables accounting for the largest share of data variance. PCA results showed a significant negative correlation (r close -1) between salt stress and all measured indices, with the exception of T_{50} and MGT (r close 0). Accordingly, GP, GE, PV, GV, CVG, and GRI may be considered appropriate parameters for the evaluation of salinity tolerance in *Salicornia europaea* seed lots, while T_{50} and MGT should not be addressed in salt stress assessment, as they did not reveal sufficient sensitivity to this factor.

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