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Seed diversity and germination behavior in *Nerium oleander* L.

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Abstract

Nerium oleander var. 'Villa Romaine', commonly called oleander, is an ornamental shrub that exists in Tunisia in the wild and cultivated form. This plant is frequently propagated and produced in nurseries and used in urban plantings and on roadsides. The effects of nature of the plants (spontaneous or cultivated), climatic conditions (localities), and altitude (from 6 to 447 m) on seed shape, size, and germination of Tunisian populations, were investigated. Seeds were harvested from 23 locations belonging to six bioclimatic stages and distributed from North to South of Tunisia. The seed parameters studied were area, roundness, J index, and germination rate. A significant variation was obtained between populations, categories (cultivated and spontaneous), bioclimatic zones and altitudes. Seeds of cultivated plants were the smaller and showed the higher germination rates, compared with spontaneous populations, which had large seeds and a low germination rate. Climatic conditions and geographical location had an effect on seed morphology; seeds in lower humid were small for both cultivated and spontaneous types, they also showed a low J index value and a high germination rate. On the other hand, in upper Saharan, seeds were large and roundness and germination rate were the lowest. Also, seeds from plants in higher altitudes were the smaller and presented the lower values of the J index compared with those collected from low-altitude sites.

Keywords: diversity; germination*; Nerium oleander* L.; seed; shape

Introduction

Nerium oleander L., an evergreen shrub or small tree, is a unique species of the genus *Nerium* from the Apocynaceae family. Even though being toxic in all its parts, this species is widely cultivated, resulting in one of the most poisonous among cultivated plants. It belongs to the tropical element of the Mediterranean scrub flora, that is, a set of taxa of tropical origin that probably evolved before the onset of the Mediterranean climate but now coexist with more recent, purely Mediterranean elements (Quezel, 1985). In Tunisia*, N. oleander* grows both spontaneously and cultivated. Its fruit (5-15 cm in length and 6-10 mm in diameter) consists of two follicles. The young unripe small fruit is green but it turns red when fully grown but still unripe. Upon maturation, the fruit dries, turns brown, and its follicles twist to liberate the many small, plumed, wind-dispersed seeds (Lev-Yadun*et al.*, 2009). The seed of *N. oleander* has an oblong shape and excluding

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ornamentation, the 100 seed weight is 0.3 g. The length, width and thickness of the seed are 4.7, 1.5 and 0.7 mm respectively (Demonty *et al.*, 2014; Figure 1). Molecular analyses of this species by RAPD and AFLP show its high genetic variability (Zibbu and Batra, 2015). *Nerium oleander* var. 'Villa Romaine' investigated in this work is characterized by its simple flowers, light pink and slightly fragrant. Its presence in the arid regions of Tunisia reflects its drought tolerance. The plant is propagated by seeds or cuttings. A germination rate above 82% and a rooting percentage of 100% were obtained for sowing and cuttings respectively (Simion and Anton, 2009).

Figure 1. *Nerium oleander*. Plants growing in Bou Hedma (site number 19). Flower, leaves, fruit, seeds. The hairs were removed from the seed for the morphological study

Seed morphology may give information useful in the phenotypic characterization and phylogenetic relationships between varieties and cultivars. An accurate description of seed shape may be done by comparison with geometric figures that resemble seed images. This method has been developed in the model plant (*Arabidopsis thaliana* L., Cervantes *et al.* (2010) as well as in the model legumes, *Lotus japonicus* L. and *Medicago truncatula* Gaertn (Cervantes *et al.*, 2012). Also, several studies have analyzed the diversity of *Silene* species and populations with seed shape based on the comparison with geometric models, by comparing the seeds to the ellipse and cardioids shapes (Martín-Gómez*et al.*, 2020, 2022a, 2022b; Juan *et al.*, 2022; Rodríguez-Lorenzo *et al.*, 2022) studied the diversity. In *Capparis spinosa* L., the comparison of seed images with the cardioid curve was helpful to describe differences between two subspecies (Saadaoui *et al.*, 2013). In the Euphorbiaceae *Jatropha curcas* L. and *Ricinus communis* L., seed shape was quantified by comparison with an ellipse; the form/production relationship has been verified (Saadaoui *et al.,* 2015, 2017; Martín-Gómez *et al.*, 2016).

We have established a protocol to analyze seed shape in *N. oleander*. The model applied for the quantitative geometric analysis has been an ellipse. Quantification with a model allows the comparison of seed shape in populations grown in different climatic regions as well as between seeds proceeding from plants grown spontaneously or cultivated in 23 locations in Tunisia. The main objectives of this work are to analyze the seed variability between populations, sites, and states (spontaneously or cultivated) and the effect of these characteristics on seed germination. We present in the study the variations in size, shape, and germination rate of seeds in plants of *Nerium oleander*.

Materials and Methods

Plant material

Mature seeds were obtained in the spring of 2019 from developed plants growing in 23 locations in Tunisia (Table 1). Seeds were collected from five plants per location. Ten fruits were harvested from each individual, the seeds of each population were pooled and 30 seeds per location were used in the analysis.

A total of 708 seeds from 23 populations of *Nerium oleander* var. 'Villa Romaine' grown in six different climatic regions throughout Tunisia was analyzed (Table 1). From the total, 570 seeds proceed from cultivated plants, growing on roadsides and 138 belong to spontaneous populations. Cultivated plants were obtained by cuttings, from spontaneous plants, and after several generations. In nature, *N. oleander* is mainly propagated by seeds.

Table 1. Geographic localization of studied populations

<table>
<tr><th>Population code</th><th>Population</th><th>Latitude (N)</th><th>Longitude (E)</th><th>Altitude (m)</th><th>Type</th><th>Bioclimate*</th></tr>
<tr><td>1</td><td>Rimel</td><td>37°03' 41.31"</td><td>9° 30' 34.51"</td><td>149</td><td>Cultivated</td><td rowspan="5">Lower Humid</td></tr>
<tr><td>2</td><td>Teskraya</td><td>37° 15' 14.55"</td><td>9° 54' 36.17"</td><td>6</td><td rowspan="3">Spontaneous</td></tr>
<tr><td>3</td><td>Bellif</td><td>37° 04' 05.53"</td><td>9° 01' 33.80"</td><td>77</td></tr>
<tr><td>4</td><td>Ain Snoussi 1</td><td>36° 49' 45.95"</td><td>8° 55' 50.29"</td><td>386</td></tr>
<tr><td>4'</td><td>Ain Snoussi 2</td><td>36° 49' 12.72"</td><td>8° 55' 27.49"</td><td>447</td><td rowspan="10">Cultivated</td></tr>
<tr><td>5</td><td>Sidi Bou Said</td><td>36° 51' 10.47"</td><td>10° 19' 08.57"</td><td>10</td><td rowspan="3">Upper Semiarid</td></tr>
<tr><td>6</td><td>Grombalia</td><td>36° 35' 26.66"</td><td>10° 28' 24.49"</td><td>70</td></tr>
<tr><td>7</td><td>Sidi Khelifa</td><td>36° 14' 49.02"</td><td>10° 25' 29.13"</td><td>30</td></tr>
<tr><td>8</td><td>Sousse</td><td>35° 46' 52.35"</td><td>10° 36' 52.35"</td><td>41</td><td rowspan="3">Lower Semiarid</td></tr>
<tr><td>9</td><td>Msaken</td><td>35° 41' 49.74"</td><td>10° 33' 54.10"</td><td>46</td></tr>
<tr><td>10</td><td>Monastir</td><td>35° 45' 49.16"</td><td>10° 48' 22.80"</td><td>33</td></tr>
<tr><td>11</td><td>Kairouan</td><td>35° 40' 30.0246"</td><td>10° 06' 44.48"</td><td>54</td><td rowspan="4">Upper Arid</td></tr>
<tr><td>12</td><td>Hbira</td><td>35° 10' 55.48"</td><td>10° 17' 05.80"</td><td>140</td></tr>
<tr><td>13</td><td>Sfax</td><td>34° 49' 07.24"</td><td>10° 42' 17.21"</td><td>54</td></tr>
<tr><td>14</td><td>Bou Hedma</td><td>34° 29' 20.41"</td><td>9° 26' 41.90"</td><td>346</td><td>Spontaneous</td></tr>
<tr><td>15</td><td>Bir Ali Ben Khelifa</td><td>34° 44' 17.55"</td><td>10° 05' 23.76"</td><td>171</td><td rowspan="5">Cultivated</td><td rowspan="8">Lower Arid</td></tr>
<tr><td>16</td><td>Gafsa</td><td>34°26' 06.27"</td><td>8° 46' 50.95"</td><td>317</td></tr>
<tr><td>17</td><td>Skhira</td><td>34°17'36.57"</td><td>10°03'56.27"</td><td>32</td></tr>
<tr><td>18</td><td>Nahel</td><td>33° 52' 28.07"</td><td>10° 03' 51.07"</td><td>27</td></tr>
<tr><td>19</td><td>Hamma</td><td>33° 52' 50.56"</td><td>9° 46' 24.98"</td><td>60</td></tr>
<tr><td>20</td><td>Ghandri</td><td>33° 43' 36.18"</td><td>10° 09' 07.88"</td><td>43</td><td>Spontaneous</td></tr>
<tr><td>21</td><td>Zerkine</td><td>33° 41' 00.70"</td><td>10° 15' 29.94"</td><td>25</td><td rowspan="3">Cultivated</td></tr>
<tr><td>22</td><td>Medenine</td><td>33° 21' 40.85"</td><td>10° 28' 14.03"</td><td>112</td></tr>
<tr><td>23</td><td>Kebili</td><td>33° 41' 53.23"</td><td>8° 57' 56.06"</td><td>33</td><td>Upper Saharan</td></tr>
</table>

* Bioclimates according to the classification of Emberger (1955)

Image capture

Photographs of longitudinal views of seeds were taken with a digital camera Nikon DS-Fi1 adapted to a Nikon 'SMZ-1500' stereo-microscope. Composed images containing the model (ellipse; see later) and each seed were elaborated with the software image Corel PHOTO-PAINT X7. Quantification of areas was done with Image J (Java Image Processing Program). In this process, a microscopy photograph of graph paper was used to convert pixels into mm.

Quantitative morphology

Two magnitudes were used for the quantitative morphological analysis: Roundness and J index. Roundness, obtained with Image J, is defined by Ferreira and Wayne (2010) as:

$$R = \frac{4 \text{ x area}}{\pi[\text{Major axis}]^2}$$

J index reflects the percent similarity of the seed image with a geometric figure used as a model. Based on seed measurements and image comparisons, the model used for shape quantification is an ellipse with a relation between the major and minor axis of 3.7. The model was obtained by superimposing the profiles of all the seeds and selecting color mask (black) of higher intensity with Corel PHOTO-PAINT X7 (Figure 2).

The ratio between major and minor axis of 3.7 is also obtained as the mean value in all the seeds. Quantification of the adjustment as done in each seed by superimposing the seed image with the model (ellipse), and the ratio of the areas in two regions: The common region in the ellipse and the seed image (C), and the total area occupied by the ellipse and the seed image (Figure 2). The index of adjustment (J) is defined by:

$$J = \frac{\text{area}(C)}{\text{area}(C) + \text{area}(D)} \times 100$$

Where C represents the common region and D the regions not shared (total area is the sum of shared and non-shared). Note that J ranges between 0 and 100, and decreases when the size of the non-shared region grows. It equals 100 when cardioid and seed image areas coincide, i.e., area (D) is zero.

J Index was calculated for a total of 708 seeds (30 seeds per population; except in the Bellif population 3 of only 18 seeds).

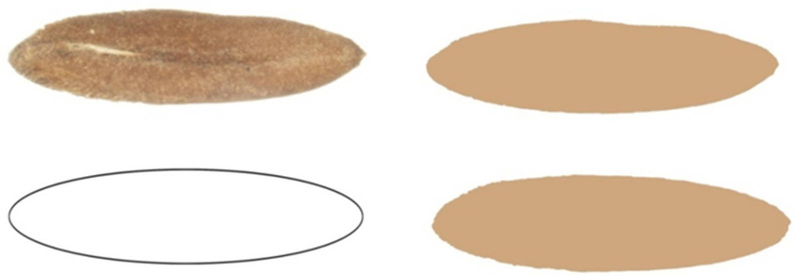

Figure 2. Left: Image of a seed (top) and model ellipse (bottom). Right: The common region in the ellipse and the seed image (top), and the total area occupied by the ellipse and the seed image (bottom). J index is the ratio between both magnitudes

Germination tests

Thirty seeds from each location were deposited in water agar 1% in Petri dishes. A total of 708 seeds were analyzed after 7 days of germination at 18 °C. Twenty-five seeds per Petri dish were tested, with four repetitions

Statistical analysis

Analysis of variance (ANOVA) was applied to the comparison of magnitudes between populations and groups of populations (climates) as well as seed types (collected from spontaneous and cultivated plants) between the diverse populations. The magnitudes analyzed were seed area, roundness, J index, and germination rate. Post-hoc analysis was carried out using the Tukey test for samples of similar sizes such as, for example, comparisons between seed types in a population, the Scheffé test for groups of populations (altitude, climatic regions, or comparison between spontaneous and cultivated populations; in general, samples are of different sizes).

Seed germination is a dynamic process. The analysis of seed germination in time was done with a GLM (General Linear Model, Christensen, 6; McCullagh and Nelder, 1989). This method allows to work with qualitative variables (plant types or varieties; here the comparison between spontaneous and cultivated populations) and combines regression analysis throughout successive moments with ANOVA involving values in all time points. GLM was done with two fixed factors: plant source, with two plant types (spontaneous and cultivated), with 138 levels for spontaneous plant type, 570 levels for cultivated, and one random factor, seed.

Statistical treatment and graphics were done with SPSS® v. 23. For the analysis, the significance level p=0.05 was established; in addition, six digits of precision were used throughout the calculations.

Results

Effect of population

For all the morphological parameters analyzed (area, roundness and J Index), the variation is significant between populations (P <0.05). Ain Snoussi 2, cultivated plants, showed the lowest area (4.44 mm^2) and Teskraya, spontaneous site, had the highest (7.73 mm^2). For roundness, the highest value was at Ain Snoussi 2 (0.358) and the lowest was at Kebili (0.257), belonging to lower humid and upper Saharan climates, respectively. At the end, the highest J index (87.4) is at Bou Hedma and Gafsa (two nearby sites) and the lowest is at 76 at Ain Snoussi 2 (Table 2).

Table 2. Comparison between populations for morphological parameters

Population	N	Area (mm^2)	Roundness	J Index
Rimel	30	7.22g.h	0.273a.b.c	86.1b.c
Teskraya	30	7.73h	0.297a.b.c	85.2b.c
Bellif	18	4.74a.b	0.294a.b.c	85.4b.c
Ain Snoussi 1	30	6.15c.d.e.f	0.267a.b	84.1b.c
Ain Snoussi 2	30	4.44a	0.358d	76.0a
Sidi Bou Said	30	7.00f.g.h	0.280a.b.c	84.3b.c
Grombalia	30	6.35c.d.e.f.g	0.293a.b.c	85.8b.c
Sidi Khelifa	30	5.69b.c.d.e	0.293a.b.c	84.7b.c
Sousse	30	6.48d.e.f.g	0.283a.b.c	86.0b.c
Msaken	30	6.95f.g.h	0.267a.b	86.3b.c
Monastir	30	5.59b.c.d	0.283a.b.c	85.6b.c
Kairouan	30	5.37a.b.c	0.300a.b.c	83.1b.c
Hbira	30	5.35a.b.c	0.293a.b.c	85.1b.c
Sfax	30	6.31c.d.e.f.g	0.290a.b.c	83.4b.c
Bou Hedma	30	6.55d.e.f.g	0.283a.b.c	87.4c
Bir Ali Ben Khelifa	30	6.75f.g.h	0.312c	83.5b.c
Gafsa	30	4.76a.b	0.273a.b.c	87.4c
Skhira	30	6.03c.d.e.f	0.307b.c	82.6b
Nahel	30	6.40d.e.f.g	0.280a.b.c	87.3c
Hamma	30	6.63e.f.g	0.287a.b.c	84.6b.c
Ghandri	30	6.52d.e.f.g	0.293a.b.c	85.8b.c

Zerkine	30	6.34c.d.e.f.g	0.283a.b.c	84.3b.c
Medenine	30	6.29c.d.e.f.g	0.263a.b	83.9b.c
Kebili	30	6.66e.f.g	0.257a	85.3b.c

N: Number of Seeds
Significant differences (at 0.05 significant level) are indicated by different letters (a,b,c,d,e,f,g and h)

Effect of the category (spontaneous /cultivated)

A summary of results concerning the comparison between spontaneous and cultivated plants is presented in Table 3; a significant difference (P <0.05) was observed between these two categories regarding for the area, J Index, and germination rate. Seeds of the cultivated plants are smaller, and have a lower J index and higher germination rate.

On the other hand, the roundness and germination rate are without significant differences between spontaneous and cultivated sites.

Only location number 4 (Ain Snoussi, in the lower humid climate) contains spontaneous and cultivated plants, and also in this population seeds from cultivated plants are smaller, with higher roundness and lower J index values than the seeds obtained from spontaneous plants (Figure 3).

Table 3. Comparison between spontaneous (S) and cultivated (C) plants for area, roundness, J index and germination %

	Type	N	Mean	Standard deviation	Standard error	Significance
Area (mm^2)	S	38	6.48	1.44428	0.12295	0.005
	C	70	6.14	1.23909	0.05190	
Roundness	S	38	0.286	0.04233	0.00360	0.638
	C	70	0.288	0.05182	0.00217	
J Index	S	38	85.6	4.4436	0.3783	0.012
	C	70	84.5	5.3372	0.2236	
Germination (%)	S	38	49	50	4.3	0.001
	C	70	65	48	2.0	

N: Number of Seeds

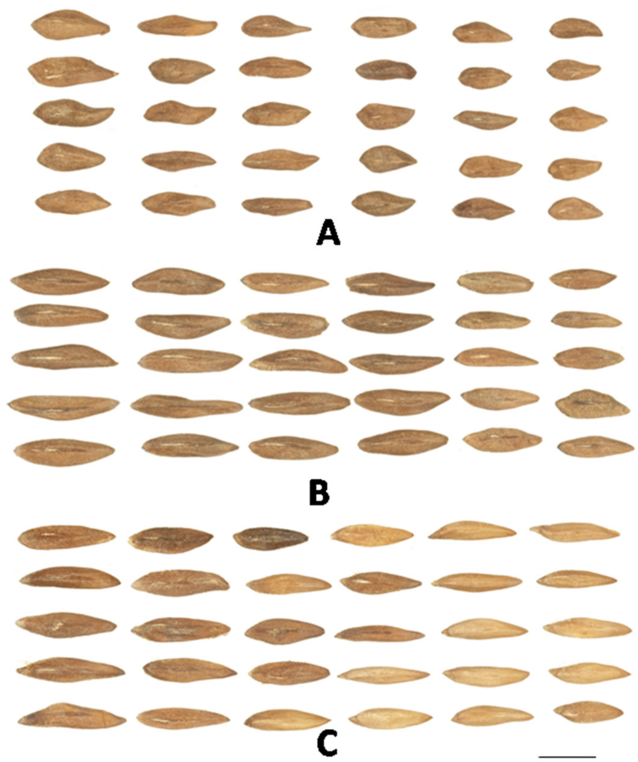

Figure 3. Representative seeds of the two populations grown in Ain Snoussi (cultivated (A) and spontaneous (B)) and Kebili's population(C) (Scale is 5 mm).

Effect of bioclimates

Bioclimates have a significant effect ($P < 0.05$) on the parameters of seed diversity. For the cultivated populations, a low area was recorded in lower humid and upper arid. However, the highest value was observed in the upper Saharan (Table 4 and Figure 3). For roundness, the highest value was recorded in lower humid, and the lower value was in upper Saharan. J index also varied. In fact, the lowest averages were in the lower humid, and high averages were observed in lower semi-arid, lower arid and upper Saharan. For germination, the lowest germination rate is in the upper Saharan (Table 4).

For the spontaneous populations, area, roundness, and germination rate were not significantly different. Only the J index was significantly different. Indeed, it was low in the lower humid and high in the upper and lower arid (Table 5).

Table 4. Comparison between climatic regions for cultivated plants

Bioclimate	N	Area (mm^2)	Roundness	J Index	Germination (%)
Lower Humid	60	5.83a	0.316c	81.0a	75b
Upper Semi-arid	90	6.35a. b	0.289b. c	84.9b	72b
Lower Semi-arid	90	6.34a. b	0.278a. b	85.9b	67b
Upper Arid	90	5.68a	0.294b. c	83.9a. b	72b
Lower Arid	210	6.17a. b	0.286b	84.8b	59a. b
Upper Saharan	30	6.66b	0.257a	85.3b	33a

Table 5. Comparison between climatic regions for spontaneous plants

Bioclimate	N	Area	Roundness	J Index	Germination (%)
Lower Humid	88	6.43a	0.285a	84.8a	58 a
Upper Arid	30	6.54a	0.283a	87.4b	43 a
Lower Arid	30	6.52a	0.293a	85.8a. b	33 a

Effect of altitude

Table 6 shows a significant difference for the values of the area, as well as those of the J index according to the altitude; indeed, the area is 6.87 mm^2 at low altitude and 5.47 mm^2 at high altitude, in the same way the average values of J index are high (85.29) at altitude below 30 m and low (83.71) for altitudes above 300 m. On the other hand, these differences according to the altitudes are not significant for the roundness and germination rate.

Table 6. Comparison between seeds grown in different altitudes

Parameters	Altitude	N	Mean	Standard deviation	Standard error	Significance
Area (mm^2)	Below 30 m	120	6.87	1.12	0.102	0.000
	Above 300 m	120	5.47	1.33	0.122	
Roundness	Below 30 m	120	0.28	0.050	0.004	0.147
	Above 300 m	120	0.29	0.059	0.005	
J Index	Below 30 m	120	85.29	4.49	0.410	0.037
	Above 300 m	120	83.71	6.94	0.634	
Germination (%)	Below 30 m	120	58	49.5	4.5	0.197
	Above 300 m	120	50	50.2	4.6	

Discussion

Among the two magnitudes used to quantify seed shape in *Nerium oleander*, roundness and J index, taking as the model an ellipse of a relationship between the major and minor axis of 3.7. The values of the J index based on this model give an accurate approach to seed shape useful and complementary to roundness values. Nevertheless, the values obtained for J index are still lower than those obtained with other geometric models applied to a range of seed species. Cardioid or cardioid-derived figures were applied to the model plant *Arabidopsis thaliana* (L.) Heynh (Cervantes *et al*., 2010), as well as to the model legumes *Lotus japonicus* and *Medicago truncatula* Gaertn (Cervantes *et al*., 2012) and *Capparis spinosa* L. (Saadaoui *et al*., 2013). Ellipse-based models were applied to the Euphorbiaceae *Ricinus communis* L. (Martín Gómez *et al.*2016; Saadaoui *et al.*, 2017) and *Jatropha curcas* L. (Saadaoui *et al.,* 2015). In the majority of these cases J index values were around 90 reaching values over 95. To make shape comparisons between genotypes or treatments, we find that values for J index of 90 or upper provide better results. In general, those high values are obtained for seeds that present simple shapes, similar to geometric figures, and without appendixes or specialized structures for dissemination. Seeds of *N. oleander*, in addition to the hairs that cover their surface, don't adjust completely to the ellipse because they tend to have elongated, sharpened and curled shapes, resulting in complex structures, diverging from simple geometric models. These parameters may be related to the complexity of seed dispersion (Herrera, 1991)

For cultivated plants, extreme climates (lower humid and upper Saharan) are characterized by extreme values for all three morphological parameters analyzed (J index, Roundness, and area; Table 4); this result points to the effect of climate on the development and morphology of the seed. There is no difference in seed area between the spontaneous populations, although aridity favors small seeds (Peco *et al.,* 2003; Saadaoui *et al.,* 2013). But seeds of the Saharan site have an elongated shape which may assist them in hiding in small holes or apertures in the soil to adapt to the arid desert conditions (Figure 3).

J index showed a considerable difference between studied bioclimates. For cultivated plants, the lowest is in the upper humid and the highest is in the lower semi-arid, followed by upper Saharan. Also, for the spontaneous plants, the lowest J index is in upper humid and the highest values are in upper arid, followed by lower arid. A correlation was observed between the spontaneous and the cultivated for the bioclimate effect on the J. index. Domestication reduces seed shape and J index, but the characters are variable with climatic regions.

Germination rate differs between cultivated sites; the highest values were recorded for upper humid, upper semi-arid, lower semi-arid, and upper arid and lower for lower arid and upper Saharan. The latter showed the lowest germination rate (33%). This shows the direct effect of climatic conditions on cultivated plants and the quality of their seeds. Indeed, abiotic stress, particularly elevated temperature and water stress will reduce seed yield and quality (Hampton *et al.*, 2016).

Seed size and shape is influenced by altitude. Plants grown at higher altitudes produce seeds smaller and with lower values of the J index. Bonnier (1890) in his classical experiments cultivating diverse plant species at varying altitudes showed that higher altitudes result in reduced plant size, which may also result in smaller seed size. Similarly, in cactus (*Gymnocalycium monvillei* (Lem.) Britton and Rose), Bauk *et al.* (2015) studied the effect of altitude on the seed parameters and concluded the presence of a relationship between altitude and seed height and shape. Also, Pluess *et al.* (2005) confirmed the effect of altitude on the seed size for several species studied in the Swiss Alps. Rawat and Bakshi (2011) studied *Pinus wallichiana* and recorded a negative correlation between altitude, 1000 Seed weight, and Germination percentage. This correlation is positive with the germination period. But, for *Abies pindrow* (silver fir), Bhat *et al.* (2018) obtained a negative correlation between altitude and percentage germination. Our results showed the effect of population, climate, and altitude on seed diversity, also, the plant culture acts on these variables. Other factors may be influencing the seed size and shape of *Nerium oleander* seeds.

Conclusions

This study was carried out at 23 *Nerium oleander* var. 'Villa Romaine' sites in different bioclimates of Tunisia. The analyses of variations within these localities by using morphological (Area, Roundness and J Index) and physiological (germination) parameters showed high variability. These variables reflected differences in size, shape and physiological behavior. The observed variations seemed to be directly related to populations, their nature (spontaneous or cultivated), and/or bioclimatic conditions and altitudes.

Authors' Contributions

Conceptualization of research (EC, ES, JJMG); Designing of the experiments (EC, JJMG); Contribution of experimental materials (ES, KY, JJMG); Execution of field/lab experiments and data collection (ES, KY, JJMG); Analysis of data and interpretation (EC, ES, JJMG, KY); Preparation of the manuscript (EC, ES, JJMG, KY). All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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