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## MICROMORPHOLOGICAL OBSERVATION OF EUCALYPTUS SEEDS, MULTIVARIATE STATISTICAL ANALYSES AND MODELING OF THEIR GERMINATION UNDER SALT STRESS AND OSMOTIC CONSTRAINT

MECHERGUI, K.; JAOUADI, W.; NAGHMOUCHI S.; ALSUBEIE, M.; KHOUJA, M. L. Micromorphological observation of Eucalyptus seeds, multivariate statistical analyses and modeling of their germination under salt stress and osmotic constraint. **CERNE**, v. 25, n. 2, p.156-171, 2019.

### HIGHLIGHTS

Eucalyptus is a very important reforestation species in Tunisia, it is from North to South of the country.

Micromorphological characters provide basis for classification and delimitation of genus *Eucalyptus*.

Micromorphological features study of seeds of 19 species of eucalyptus reforested in Tunisia facilitated the identification of these species.

The hybrid *E. gomphocephala* x *E. cornuta* was more tolerant to salt stress and osmotic potential than the others *Eucalyptus* species studied.

### ABSTRACT

Micromorphological characters including surface, length and width of seeds were recorded for 19 species of Eucalyptus (Myrtaceae) using stereomicroscope to determine the importance of seed morphological characters as an additional tool for identification. With the aim of selecting and valorizing abiotic-stress-tolerant species with landscape and industrial values and would be a potential solution for valorizing dry arid area and development of land degradation, we launched the assessment of the performance of five Eucalyptus species (*E. torquata*, *E. sargentii*, *E. gillii*, *E. gomphocephala* x *E. cornuta* and *E. microtheca*) under salinity and osmotic potential constraints. Several NaCl concentrations (0, 3, 6, 9, 12, and 15 g·L<sup>-1</sup>) and several osmotic potentials (0, -0.03, -0.1, -0.7, -1 and -1.6 MPa) were applied to seeds cultivated in petri dishes for a period of one month. Germination rates, means time of germination and coefficient of velocity were evaluated to better understand the salt stress and osmotic potential effect on germination. Univariate and multivariate analyses were used to identify the major characteristics pertaining to salinity tolerance. Modeling of germination under both constraints saline and osmotic stress is carried out to predict the behavior of the species. The hybrid *E. gomphocephala* x *E. cornuta* was more tolerant to salt stress (15 % at 12 g·L<sup>-1</sup>) and osmotic potential (61 % at -1.6 MPa) than the others species of Eucalyptus; it showed a higher germination percentage under all tested potentials, when compared to the not hybrid species of Eucalyptus. Our analyses of seeds morphology revealed that the observations shown diversity of morphological characters in seeds. Area, length and width of seeds vary significantly between species of Eucalyptus. Micromorphological characters can provide basis for classification and delimitation of genus Eucalyptus.

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## INTRODUCTION

The genus *Eucalyptus* is a member of the Myrtaceae family. There are more than 700 species of *Eucalyptus*, mostly originating from the Australian continent, with a very small number found on the neighboring islands of Papua New Guinea, Indonesia, and Philippines (Yang et al., 2016). The total plantation area of *Eucalyptus* covers more than 19 million ha, and representing one of the most common plantation forest species in the world (Wen, 2008). China ranks second in the world in the plantation area of *Eucalyptus*, with a plantation area of more than 4.5 million ha in southern China (Li, 2015).

Currently, the plantation area of *Eucalyptus* forests in Hainan (China) is nearly 200.000 ha, covering 18 cities and counties throughout the island (Yang et al., 2016). *Eucalyptus* name originates from the Greek word «*Eucalyptol*» which means «well covered» (Ishag et al., 2018). Leaf extracts of *Eucalyptus* have been approved as food additives (Gilles et al., 2010). Because of their rapid growth rates (Cossalter and Pye-Smith, 2003), wide adaptability (Johansson and Tuomela, 1996; Gardner, 2007) and high productivity (Singh and Toky, 1995), *Eucalyptus* plantations generate large economic returns.

Nevertheless, there is continuing controversy about their ecological functions (Zhang and Fu, 2009). *Eucalyptus* occupies one of the largest planted areas in Brazil and presents high productive and technological potential, and its wood is used in the pulp and paper industry, for the production of fiber panels and agglomerates, in the manufacture of furniture, as well as in firewood and in sawmills (IBÁ, 2015; Lopes et al., 2011; Soares et al., 2003; Souza et al., 2004). *Eucalyptus* spp. are indigenous to Australia; they have been widely introduced into countries throughout the world because of their rising demand for paper and plywood (Turnbull, 1999; Cossalter and Pye-Smith, 2003). It is used in the fabrication of pulp and paper, in sawn lumber for structural purposes, engineering wood products, and flors (Rezende et al., 2018). *Eucalyptus* monoculture provides distinct products, such as wood, charcoal, resins, plywood, cellulosic ethanol, cellulose and paper (Takahashi et al., 2004). *Eucalyptus* species might be exploited as natural antibiotic for the treatment of several infectious diseases (Ishag et al., 2018).

*Eucalyptus* have been successfully introduced worldwide; it is used for ornamentation, afforestation, or to obtain timber, gum, pulp and paper and it's also known by its cosmetic and medicinal values. Essential oil is used in food, perfumery, beverage and pharmaceutical industry

(Batish et al., 2008; Leicach et al., 2012; Vecchio et al., 2016). In Tunisia, the total area planted with *Eucalyptus* is about 29 000 ha (Oueslati, 2005). In 1957, total 117 *Eucalyptus* have been introduced in Tunisia. They were used essentially as fire wood, for the production of mine wood and against the erosion (Khouja et al., 2001). *Eucalyptus camaldulensis* and *Eucalyptus gomphocephala* account for more than 80% of the *Eucalyptus* stand.

The governorate of Bizerte in Tunisia owns 10806 ha, 37% of the total area, the majority being located in the region of Sejenane in Bizerte (Oueslati, 2005). In Tunisian folk medicine, inhalation of *Eucalyptus* sp.'s essential oil has been traditionally used to treat respiratory tract disorders such as pharyngitis, bronchitis and sinusitis (Boukef, 1986). Many studies have been demonstrated their antibacterial, antifungal and antiviral activities of *Eucalyptus* sp.'s essential oil against a wide range of microorganisms (Elaissey findi et al., 2015 ; Su et al., 2006; Cermelli et al., 2008; Gilles et al., 2010; Jha et al., 2014). Few investigations were reported on the biological activities of *Eucalyptus* oils worldwide (Elaisi et al., 2012). Antitryptical, anticoagulant and hemagglutinating activities of *Eucalyptus* sp. seeds were identified (Queiroz dos Santos et al., 2019). Seed germination is used to calculate sowing rate, evaluate the physiological quality of a lot, and establish criteria for commercialization (Martins et al., 2008; Tomaz et al., 2016).

Nevertheless, the increase of the soil salinisation is a consequence of the irrigated cultivations, resulting in salt accumulations that are harmful for the plants (Lopes da Silva et al., 2012) leading to a reduced productivity and lost of agricultural practices. Effects of salt stress on various *Eucalyptus* species have been reported in (Woodward and Bennett, 2005; Merchant et al., 2007; Nasim et al., 2008; Zohar et al., 2010; Silva et al., 2012; Feikema et al., 2012; Cha-um et al., 2013). It is supposed that *Eucalyptus* species could be one of the bio-economic plants suitable to grow in salt affected soils and arid climatic conditions (Nasim et al., 2008; Zohar et al., 2010; Feikema et al., 2012; Silva et al., 2012; Cha-um et al., 2013).

According to the latest forest inventory in Tunisia (INF, 2010), the area of *Eucalyptus* in was estimated at 41 397 ha almost 3.63% of the total area of the Tunisian forest. Given the importance of this species, this area should be protected and valued for the multiple use of this species. Indeed, soils affected by salinity in Tunisia cover about  $1.5 \times 10^6$  ha, representing 10% of the area of the country (Hachicha, 2007). The alternative

approach is using plants that display salt tolerance and adaptation behavior, and its yields remain satisfactory versus high levels of salt (Ayadi et al., 218). However, little is known about seed germination of these five species of *Eucalyptus* under these harsh environmental conditions. The objective of this experiment was to study the micromorphological features i.e. seed length, width and area, for 19 species of *Eucalyptus* in Tunisia to facilitate the identification of these species and evaluate the effect of salt stress and osmotic pressure on five *Eucalyptus* species: *Eucalyptus torquata*, *Eucalyptus sargentii*, *Eucalyptus gillii*, *Eucalyptus gomphocephala* x *Eucalyptus cornuta* and *Eucalyptus microtheca* in order to understand the adaptation of *Eucalyptus* species to the different abiotic constraints and to develop a salinity and drought tolerance classification, which are important criteria for forest managers, nurseries and foresters in the choice of species for seedling production, landscape enhancement or reforestation program in order to plan, in a further attempt, its introduction for forest and urban forest cultivation.

## MATERIAL AND METHODS

This work was carried out in the Forest Ecology Laboratory and Laboratory of Management and Valorization of Forest Resources from the National Research Institute of Rural Engineering, Water and Forests in Tunisia.

### Plant material

All seeds used in this study were collected from the *Eucalyptus* population in Rtiba Arboretum (seed orchard) in Nabeul Governorate in north of Tunisia, it occupies an area of about 100 ha at an altitude of 450 m. Five *Eucalyptus* species seeds were collected for germination under salt stress and osmotic potential constraints: *E. torquata*, *E. sargentii*, *E. gillii*, *E. gomphocephala* x *E. cornuta* and *E. microtheca*. Nineteen *Eucalyptus* species were collected for seed morphometric measurements: *E. sargentii*, *E. saligna*, *E. cornuta*, *E. gomphocephala*, *E. accedens*, *E. leucoxydon*, *E. microtheca*, *E. Stoutei*, *E. platypus*, *E. perriniana*, *E. lehmannii*, *E. viminalis*, *E. gillii*, *E. diversifolia*, *E. torquata*, *E. dumosa*, *E. angulosa*, *E. burracoppinensis* and *E. pimpiniana*.

### Seed morphometric measurements

We collected 20 capsules from 10 randomly selected individuals of nineteen *Eucalyptus* species and allowed them to ripen in the laboratory for 10 days, exposed to the air. Only 20 fruits were collected from

the capsules of every species and was harvested. Seeds were subsequently manually extracted from dried fruits. After extraction, 20 seeds were randomly selected and the length, width, and area were individually measured to the nearest 0.01 mm using strero-microscope (leica DM 205-C) in the Laboratory of Management and Valorization of Forest Resources.

### Germination procedure of five *Eucalyptus* species under salt stress and osmotic potential constraints

Collected seeds offive *Eucalyptus* species were processed in an air blower, stored in plastic containers and placed in a cool chamber until use. Seeds were placed in Petri dishes on perlite containing a polyethylene glycol solution (PEG 6000) to germinate under osmotic potentials of 0, -0.03, -0.1, -0.7, -1 and -1.6 MPa and kept in incubator at 25 °C. Seeds were placed to germinate under NaCl induced salt stress, at 0.0 (control sample), 3, 6, 9, 15 and 15 g of salt/L.

Germination was evaluated daily for 30 days, in order to determine germination rate (%) mean time of germination (days) and germination velocity index (VI). For each type of stress, the experiment was conducted in completely randomized design, using five replications of 20 seeds comprising 6 concentrations of NaCl salt and 6 concentrations of osmotic potential. Seeds were considered to be germinated when the root length reached the seed length and shoot length reached half of the seed length (Cheng et al., 2014 ; Wang et al., 2014). The number of germinated seed was counted every day for 30 days. The standard germination test ended at 30 days because from this date on, no normal seedlings emerged in any treatment for three successive counts (Affonso et al., 2018)

The parameters of the germination rate (GR), the meantimeofgermination(MTG)andthecoefficientofvelocity (CV) of Kotowski (1962), were calculated as follows, Here,  $n$  is the total number of germinated seeds and  $N$  is the total number of tested seeds. Where  $D$  is the number of days counted from the date of sowing and  $N$  is the number of seeds germinated on day  $D$ . Coefficient of velocity: equation 1, where,  $n_i$  is the number of seeds newly germinating on day  $i$  and  $D_i$  is the number of days from sowing

$$GR(\%) = n / N \cdot 100 \quad [1]$$

$$MGT = \sum DN / \sum N \quad [2]$$

$$CV = \sum (n_i \times D_i) / \sum n_i \quad [3]$$

**Statistical analysis**

The data of the different parameter values were subjected to one-way ANOVA with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The differences between the means were tested with the *Tukey test* and values of  $P \leq 0.05$  were considered significantly different (Sokal and Rohlf, 1995). Principal component analysis (PCA) of germination parameters data was performed with the R language. An absolute value of 0.50 was used in the loading matrices to select the characteristics in a particular principal component (PC). Correlations between variables were calculated with Spearman correlation coefficients. Multiple linear regression (MLR) analyses based on least-square procedures are usually used for estimating the variable effects involved in a model. The success of MLR can be measured by evaluating the magnitude of the adjusted  $R^2$ , the residual standard error (RSE) for the regression, and the results of the *Student t-test* for the individual predictor variables.

**RESULTS**

**Seed morphometric measurements of nineteen *Eucalyptus* species**

Morphological traits of seeds of nineteen species of *Eucalyptus* were observed under stereo microscope (Figure 1). Area, length and width of seeds varied significantly

between the different species (Table 1). This indicates that in the same family of Myrtaceae and in the same genus and species, the morphological characteristics of the seeds vary significantly. Seed morphometric measurements of nineteen *Eucalyptus* species were represented in Table 2. *Eucalyptus* seeds had a length between  $1.35 \pm 0.15$  mm and  $2.70 \pm 0.41$  mm, width between  $1.09 \pm 0.11$  mm and  $1.84 \pm 0.29$  mm, and area seeds between  $1.32 \pm 0.32$  mm<sup>2</sup> and  $3.65 \pm 0.66$  mm<sup>2</sup>. *E. angulosa* had the greatest area of seed and *E. dumosa* had the highest length of seed. Length and width of seeds were positively correlated ( $P < 0.001$ ; Pearson coefficient = 0.685) and in the simple linear regression ( $y = ax+b$ )  $R^2=0.469$  and  $AIC_c=27,866$ , as well length and area of seeds were positively correlated ( $P < 0.001$ ; Pearson coefficient=0.914;  $R^2=0.836$ ;  $AIC_c =63.206$ ) also that width and area of seeds were positively correlated ( $P<0.001$ ; Pearson coefficient=0.822;  $R^2=0.675$ ;  $AIC_c=121,45$ ). The interactions between morphological seed traits were tested by multivariate analysis, PCA. The first two components (F1 and F2) explained 97% of the total variation. The first component (axis 1) explained 99.60 % of the variation, followed by 1.15 % for the second component

**TABLE 1** Analysis of variance of seeds parameters of 19 species of *Eucalyptus*.

Parameters of seeds	Df	Sum	Sq	F value	Pr(>F)
Area of seeds	18	276.71	15.373	65.02	<2e-16 ***
Length of seeds	18	72.39	4.022	50.74	<2e-16 ***
Width of seeds	18	26.30	1.4612	28.34	<2e-16 ***

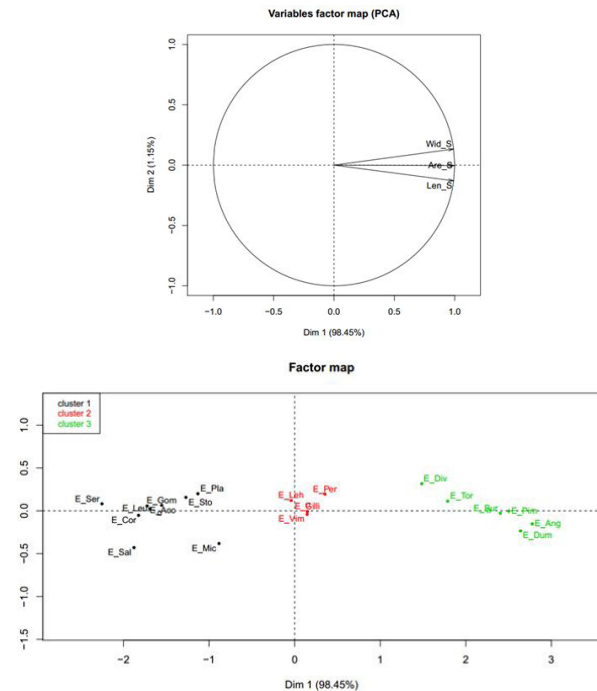


**FIGURE 1** Seeds of *Eucalyptus* species observed under stereomicroscope.

**TABLE 2** Descriptive statistique of morphometric seeds parameters of 19 species of *Eucalyptus*.

	Area of seeds (mm <sup>2</sup> )			Length of seeds (mm)			Width of seeds (mm)		
	mean	SD	CV	mean	SD	CV	mean	SD	CV
<i>E. accedens</i>	1.42	0.25	0.17	1.50	0.19	0.12	1.20	0.12	0.10
<i>E. angulosa</i>	3.65	0.66	0.18	2.69	0.34	0.12	1.84	0.29	0.15
<i>E. burracoppinensis</i>	3.40	0.37	0.11	2.58	0.31	0.11	1.82	0.22	0.12
<i>E. cornuta</i>	1.53	0.19	0.12	1.46	0.20	0.14	1.14	0.26	0.22
<i>E. diversifolia</i>	3.01	0.46	0.15	2.22	0.22	0.09	1.74	0.28	0.16
<i>E. dumosa</i>	3.52	0.52	0.14	2.70	0.41	0.15	1.82	0.22	0.12
<i>E. gillii</i>	2.25	0.35	0.15	2.01	0.26	0.13	1.49	0.15	0.10
<i>E. gomphocephala</i>	1.45	0.72	0.50	1.55	0.41	0.26	1.24	0.28	0.23
<i>E. lehmannii</i>	2.17	0.43	0.19	1.92	0.23	0.11	1.48	0.18	0.12
<i>E. leucoxydon</i>	1.49	0.41	0.27	1.50	0.23	0.15	1.19	0.18	0.15
<i>E. microtheca</i>	1.71	0.28	0.16	1.88	0.20	0.11	1.27	0.23	0.18
<i>E. perriniana</i>	2.30	0.51	0.22	2.02	0.19	0.09	1.56	0.27	0.17
<i>E. pimpiniana</i>	3.16	0.57	0.15	2.55	0.26	0.10	1.82	0.30	0.16
<i>E. platypus</i>	1.62	0.16	0.10	1.63	0.13	0.08	1.33	0.17	0.13
<i>E. saligna</i>	1.32	0.32	0.24	1.61	0.27	0.17	1.09	0.11	0.10
<i>E. sargentii</i>	1.17	0.14	0.12	1.35	0.15	0.11	1.13	0.11	0.09
<i>E. stoatei</i>	1.51	0.28	0.18	1.61	0.24	0.15	1.31	0.20	0.15
<i>E. torquata</i>	3.16	0.68	0.21	2.36	0.35	0.15	1.74	0.21	0.12
<i>E. viminalis</i>	2.31	0.95	0.41	2.02	0.42	0.21	1.47	0.28	0.19

(axis 2) (Figure 2). Width, length and area seeds were strongly correlated (Figure 2). Principal component analysis (Figure 2 and 3) showed three groups of *Eucalyptus* seeds: Group 1 consists of: *E. sargentii*, *E. saligna*, *E. cornuta*, *E. gomphocephala*, *E. accedens*, *E. leucoxydon*, *E. microtheca*, *E. stoatei* and *E. platypus*. Group 2 consists of *E. perriniana*, *E. lehmannii*, *E. viminalis* and *E. gillii*. Group 3 consists of

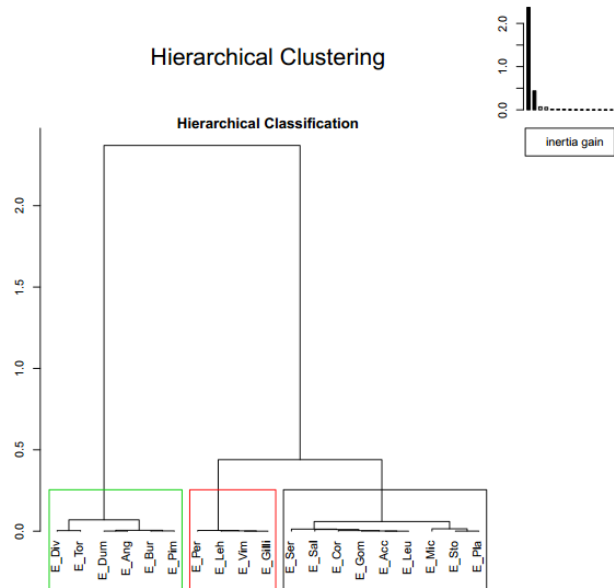


**FIGURE 2** Principal component analysis, circle of correlation of seeds morphological traits of 19 *Eucalyptus* species in the factorial plane F1 × F2 (Wid\_S : width of seeds ; Are\_S : area of seeds ; Len\_S : length of seeds) /19 *Eucalyptus* species : *E. accedens* : *E. angulosa* : *E. burracoppinensis* : *E. cornuta* : *E. diversifolia* : *E. dumosa* : *E. gillii* : *E. gomphocephala* : *E. lehmannii* : *E. leucoxydon* : *E. microtheca* : *E. perriniana* : *E. pimpiniana* : *E. platypus* : *E. saligna* : *E. sargentii* : *E. stoatei* : *E. torquata* : *E. viminalis*.

*E. diversifolia*, *E. torquata*, *E. dumosa*, *E. angulosa*, *E. burracoppinensis* and *E. pimpiniana*.

**Effect of salt stress on germination of five *Eucalyptus* species**

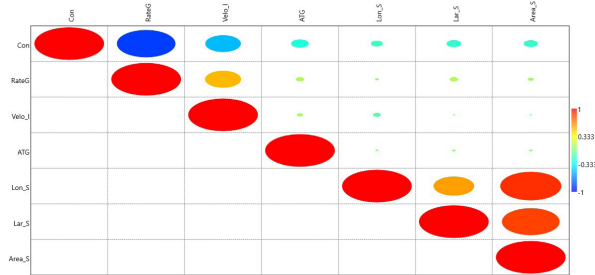
The results for the germination behavior of the five *Eucalyptus* seeds in terms of germination rate under the effect of different NaCl concentrations are shown in Table 3. Interestingly, germination was affected by salinity and seeds were not able to germinate at up to 9, 12 and 15 g·L<sup>-1</sup> NaCl. At 12 g·L<sup>-1</sup> the rates varied between 4% for *Eucalyptus microtheca* and 15% for *Eucalyptus gomphocephala*, respectively. This indicates that the five *Eucalyptus* species had lower germination rate even in high salt stress. According to the statistical analysis, there was a significant difference between the control and the plants subjected to 12 and 15 g·L<sup>-1</sup> NaCl treatments, that their germination rate did not reach 16%. Salinity reduced the rate of *Eucalyptus* germination, the latter dropped to 0 for *Eucalyptus gillii*, 3% for *Eucalyptus microtheca* and *Eucalyptus gomphocephala* when treatment reached 15 g·L<sup>-1</sup> NaCl. The results of the analysis of variance confirmed that the salinity levels influenced the percentage of germination, the mean germination time and velocity index. Mean germination rate comparison did not reveal



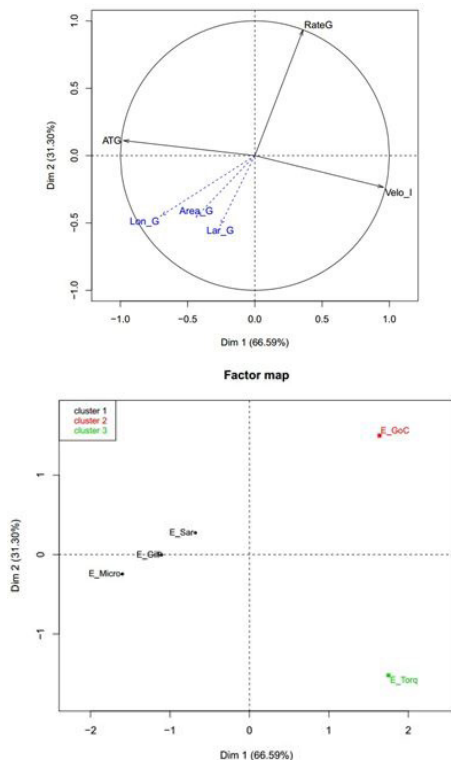
**FIGURE 3** Hierarchical classification of morphological traits of seeds of 19 *Eucalyptus* species (*E\_Acc* : *E. accedens* / *E\_Ang* : *E. angulosa* / *E\_Bur* : *E. burracoppinensis* / *E\_Cor* : *E. cornuta* / *E\_Div* : *E. diversifolia* / *E\_Dum* : *E. dumosa* / *E\_Gilli* : *E. gillii* / *E\_Gom* : *E. gomphocephala* / *E\_Leh* : *E. lehmannii* / *E\_Leu* : *E. leucoxydon* / *E\_Mic* : *E. microtheca* / *E\_Per* : *E. perriniana* / *E\_Pim* : *E. pimpiniana* / *E\_Pla* : *E. platypus* / *E\_Sal* : *E. saligna* / *E\_Ser* : *E. sargentii* / *E\_Sto* : *E. stoatei* / *E\_Tor* : *E. torquata* / *E\_Vim* : *E. viminalis*)



correlated ( $P < 0.001$ ; Pearson coefficient = 0.822), also length and area of seeds were positively correlated ( $P < 0.001$ ; Pearson coefficient = 0.886). Velocity index and average time of germination were negatively correlated ( $P < 0.001$ ; Pearson coefficient = -0.752). The morphological

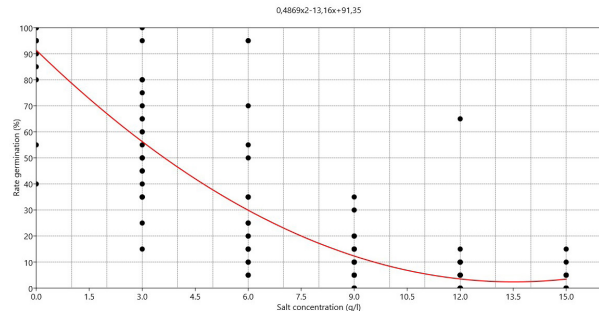


**FIGURE 4** Matrice of correlation of seeds morphological traits and germination parameters of 5 *Eucalyptus* species under salt stress (Con : concentration of salt stress ; RateG : germination rate ; Velo\_I : velocity coefficient ; ATG ; mean time of germination ; Lar\_S : width of seeds ; Are\_S : area of seeds ; Lon\_S : length of seeds).



**FIGURE 5** Principal component analysis, circle of correlation of seeds morphological traits and germination parameters under salt stress of 05 *Eucalyptus* species in the factorial plane F1 × F2 (morphological traits of seed : Long\_G; length of seed, Larg\_G; width of seed, Area\_G; area of seed, germination parameters: ATG; means time of germination, RateG; germination rate, Velo\_I; velocity index, *Eucalyptus* species :E\_Micro ; *Eucalyptus microtheca*, E\_Gilli ; *Eucalyptus gillii*, E\_Sar : *Eucalyptus sargentii*, E\_Tor ; *Eucalyptus torquata*, E\_GoC : *Eucalyptus gomphocephala* x *Eucalyptus cornuta*).

done to determine the best non linear combination of the constructs for predicting attitude (Figure 6). The value of the determination coefficient ( $R^2 = 0.798$ ), the combination of the variables significantly predicted the dependent variable ( $F = 290.13$ ;  $P < 0.05$ ).



**FIGURE 6** Relationship between germination rate of seeds of five *Eucalyptus* species and salt concentration.

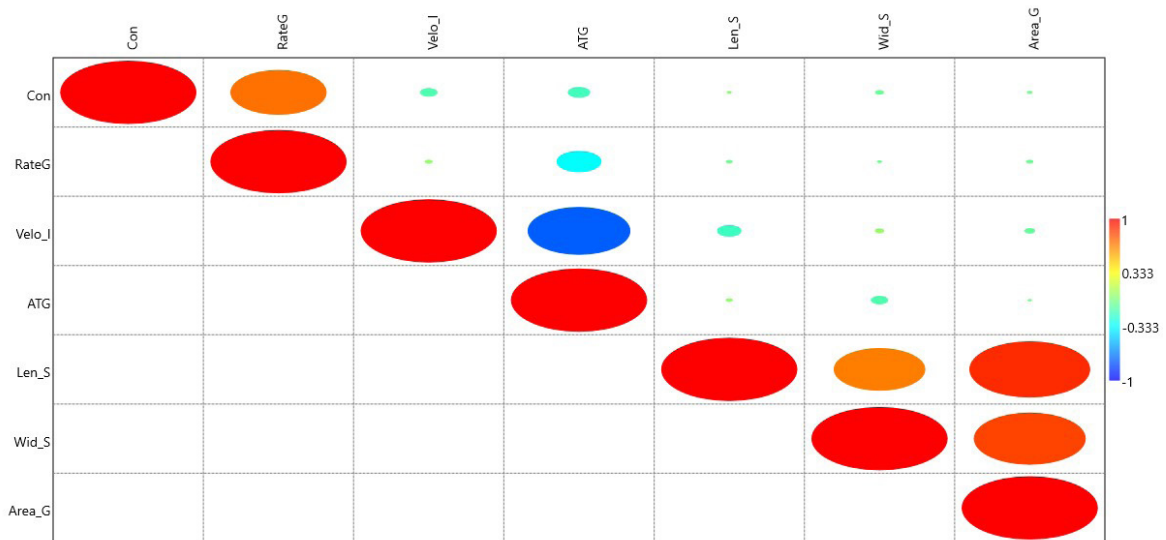
### Effect of osmotic potential on germination of five species of *Eucalyptus*

The results of the germination rate of *Eucalyptus* seeds under different osmotic potentials are shown in Table 4. *E. gomphocephala* x *E. cornuta* germination rate was slightly affected by osmotic stress and seeds were able to germinate at up to -1 and -1.6 MPa at the rates of 72% and 61%, respectively. This indicates that the *gomphocephala* x *E. cornuta* seeds have high germination capacity even under high osmotic stress. *E.gillii*, *E.microtheca*, *E.sergenti* and *E.torquata* were significantly affected by stress germination between 9 and 27% at osmotic stress of -1.6 MPa. The statistical analysis showed that *E. gomphocephala* x *E. cornuta* had no significant difference between the control and the plants subjected to 0 and -1 MPa treatments, where their germination rate reached 72%. However, although salinity slowly reduced the rate of *E. gomphocephala* x *E. cornuta* germination, the latter dropped to 61% when the treatment reached -1.6 MPa. The results of the analysis of variance confirmed that the salinity levels influenced significantly the percentage of germination of *E.gillii*, *E.microtheca*, *E.sergenti* and *E. torquata*. For 5 species of *Eucalyptus*, mean time of germination did not reveal any significant difference between 0 to -1.6 MPa. Means time of germination were around 0.56 to 13.98 and velocity index was maintained elevated even under high NaCl concentrations, showing the velocity of germination (Table 1). The correlation matrix between the morphological traits of the seeds of the five species of *Eucalyptus* studied and the germination characteristics of the seeds (GR, MTG and VC) are shown in Figure 7. Osmotic stress and rate germination of seeds were positively correlated ( $P < 0.001$ ; Pearson coefficient = 0.704), as well length and width of seeds were positively correlated ( $P < 0.001$ ; Pearson coefficient = 0.670), also that width and area of seeds were positively

**TABLE 4** Analysis of variance and multiple comparison of means of germination rate, Means time of germination and velocity index of seeds of five *Eucalyptus* species after transfer from 0, -0.03, -0.1, -0.7, -1, and -1.6 MPa NaCl at 25 °C. Data are means ± SD. Different letters indicate significant differences between treatments (osmotic potential MPa) at P < 0.05 according to the Tukey test.

Species	Parameters	0 MPa	-0.03 MPa	-0.1 MPa	-0.7 MPa	-1 MPa	-1.6 MPa	Pr(>F)
<i>Eucalyptus Torquata</i>	Germination rate (%)	90 ± 10.00c	96 ± 4.18c	72 ± 21.67bc	57 ± 12.54b	19 ± 14.74a	9 ± 4.18a	0.000***
	Means time of germination (Days)	6.58 ± 1.60a	7.81 ± 1.21a	7.91 ± 1.63a	5.59 ± 1.11a	13.70 ± 11.27a	8.53 ± 8.81a	0.374
	Velocity index	16.03 ± 4.39a	13.09 ± 2.41a	13.06 ± 2.62a	18.43 ± 3.64a	12.77 ± 8.63a	20.16 ± 11.70a	0.356
<i>Eucalyptus Sergenti</i>	Germination rate (%)	84 ± 17.10b	91 ± 5.47b	78 ± 15.24b	60 ± 11.72ab	59 ± 39.27ab	27 ± 27.52a	0.000***
	Means time of germination (Days)	5.66 ± 0.95a	5.10 ± 1.41a	5.40 ± 1.01a	5.74 ± 1.33a	4.74 ± 1.49a	10.15 ± 9.97a	0.370
	Velocity index	18.04 ± 2.83a	20.81 ± 5.75a	19.01 ± 3.29a	19.21 ± 4.94a	22.86 ± 7.31a	19.01 ± 15.10a	0.934
<i>Eucalyptu Gillii</i>	Germination rate (%)	85 ± 22.63c	87 ± 13.50c	73 ± 16.04bc	32 ± 21.96a	45 ± 15.81ab	24 ± 23.82a	0.000***
	Means time of germination (Days)	9.83 ± 1.56a	7.34 ± 0.85a	7.50 ± 1.74a	7.26 ± 1.83a	6.76 ± 1.53a	13.98 ± 8.74a	0.051
	Velocity index	10.39 ± 1.73a	13.76 ± 1.59a	13.85 ± 2.92a	14.43 ± 3.28a	15.18 ± 2.47a	10.23 ± 6.47a	0.140
<i>Eucalyptus Gomphocornuta</i>	Germination rate (%)	88 ± 11.51a	92 ± 11.51a	82 ± 14.40a	78 ± 24.13a	72 ± 27.74a	61 ± 28.56a	0.238
	Means time of germination (Days)	20 ± 3.06a	17.84 ± 2.91a	16.91 ± 3.19a	16.44 ± 3.67a	18.03 ± 4.23a	17.10 ± 4.00a	0.674
	Velocity index	8.58 ± 2.27a	10.67 ± 1.78a	11.87 ± 1.29a	10.23 ± 2.34a	14.29 ± 1.98a	11.69 ± 4.41a	0.618
<i>Eucalyptus Microtheca</i>	Germination rate (%)	77 ± 16.04c	67 ± 15.24c	59 ± 14.74bc	37 ± 4.47b	35 ± 16.20b	9 ± 4.18a	0.000***
	Means time of germination (Days)	6.47 ± 1.45a	7.96 ± 1.42a	8.46 ± 2.20a	5.95 ± 0.98a	7.40 ± 0.56a	5.33 ± 2.29a	0.037 *
	Velocity index	16.19 ± 4.21ab	12.90 ± 2.45ab	12.48 ± 3.25a	17.24 ± 3.32ab	13.57 ± 1.02ab	21.83 ± 9.18b	0.035 *

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1



**FIGURE 7** Matrice of correlation of seeds morphological traits and germination parameters of 05 *Eucalyptus* species under osmotic stress (Con: concentration of osmotic stress; RateG: germination rate; Velo\_I: velocity coefficient; ATG: mean time of germination; Wid\_S: width of seeds; Are\_G : area of seeds; Len\_S : length of seeds).



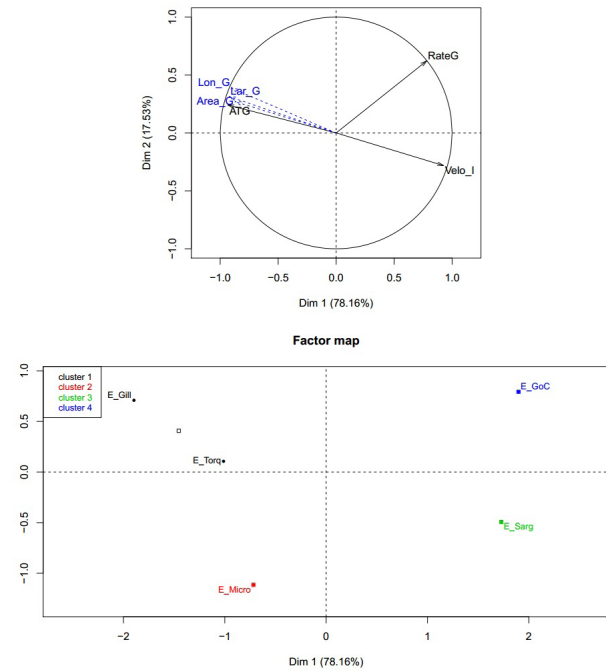
traits of seeds had no effect on the germination rate, average germination time or the velocity coefficient (Figure 7). The interactions between germination parameters and morphological traits of seeds under osmotic potentials were investigated by multivariate analysis, PCA. Considering 6 parameters, the first two components (F1 and F2) explained 95,69% of the total variation. The first component (axis 1) explained 78,16% of the variation, followed by 17,53 % for the second component (axis 2) (Figure 7). The effect of osmotic potential on the germination of *Eucalyptus* seeds showed 4 groups (Figure 8): group 1 formed by *E.gomphocephala* x *E.cornuta*, group 2 consists of *E. Sargenti*, group 3 contains *E.microtheca* and group 4 contains *E. gillii* and *E. Torquata*. Group 1 composed by *E.gomphocephala* x *E. cornuta* is not affected by osmotic potential and remains the least sensitive compared to other *Eucalyptus* species, group 4 is most sensitive to osmotic potential. These results were confirmed by modeling analysis. The results from multiple linear regression were obtained using germination rate as the independent variable and osmotic stress. This was done to determine the best non linear combination of the constructs for predicting attitude (Figure 9). The value of the determination coefficient ( $R^2= 0.500$ ), the combination of the variables significantly predicts the dependent variable ( $F=73.745$ ;  $P<0.05$ ).

## DISCUSSION

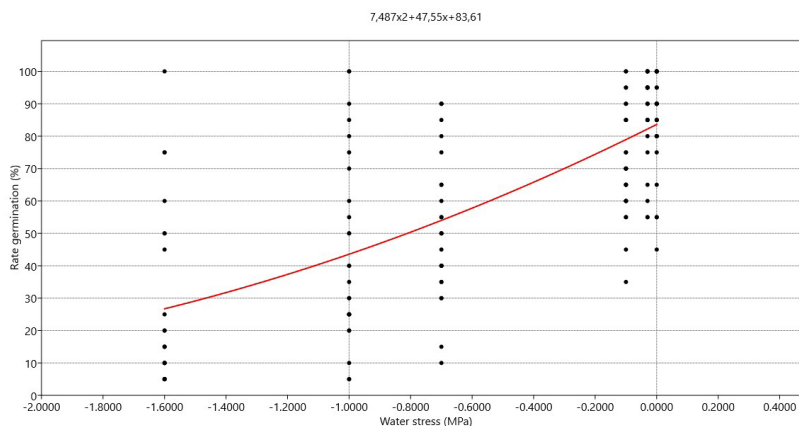
### Micromorphological observation of seed coat of nineteen *Eucalyptus* species

The first phylogenic study of Myrtaceae was carried out by Johnson and Briggs (1984) using inflorescence. Similarly, Wilson et al. (2005) explored the taxonomy and morphology of Myrtaceae using molecular data. External morphology is the basis for classification of many plants such as fruits, flowers, buds, etc (Luqman et al., 2018). Thus, the portrayal of morphological

characters for the progression of scientific classification of Myrtaceae is essential for the scientific categorization (Barroso & Peron, 1994). The present study describes the macromorphological features such as, size, surface of the seeds for nineteen species of *Eucalyptus* in Tunisia. Seed dimensions (length, width, area) of at least 20 seeds of nineteen species of a sample were measured using a stereo microscope (leica DM 205-C). Our analyses



**FIGURE 8** Principal component analysis, circle of correlation of seeds morphological traits and germination parameters under osmotic stress of 05 *Eucalyptus* species in the factorial plane F1 × F2 (morphological traits of seed : *Long\_G* ; length of seed, *Larg\_G*; width of seed, *Area\_G*; area of seed, germination parameters: *ATG*; means time of germination, *RateG*; germination rate, *Velo\_I*; velocity index, *Eucalyptus* species: *E\_Micro*; *Eucalyptus microtheca*, *E\_Gilli*; *Eucalyptus gillii*, *E\_Sar*; *Eucalyptus sargenti*, *E\_Tor*; *Eucalyptus torquata*, *E\_GoC*; *Eucalyptus gomphocephala* x *Eucalyptus cornuta*).



**FIGURE 9** Relationship between germination rate of seeds of five *Eucalyptus* species and osmotic stress.

of seeds morphology revealed that the observations showed a high diversity of morphological characters in seeds. Micromorphological characters along with seed shape and size are diagnostically important characters to separate species (Luqman et al., 2018). This study clearly illustrated the variability among species in seed size. Seed length ranged from 1.35 to 2.70 mm. Three groups were distinguished: (I) range between 1.35 and 1.88 mm, (II) varying between 1.92 and 2.02 mm, and (III) size from 2.22 to 2.70 mm (Table 2 and Figure 2). *E. dumosan*, *E. diversifolia*, *E. torquata*, *E. pimpiniana*, *E. burracoppinensis*, *E. angulosa* with larger seed length among the nine species belonged to Group III (2.22–2.70 mm); *E. Gillii*, *E. lehmannii*, *E. perriniana* and *E. viminalis* came under Group II (1.90–2.02 mm); and the rest of the species belonged to Group I (1.35–1.88 mm). The measurements on seed width and area revealed that they can be classified into three classes (Table 2 and figure 2). The seed coat morphological features had had many morphological variations according the species (Newell & Hymowitz, 1978). Our results are similar to other studies on the micromorphological observation of seed coat of nine *Eucalyptus* species, which found that *Eucalyptus* showed a relatively diverse morphological characters as seed length, width, and weight and the variability among species in seed size (Seed length ranges from 1.21 to 2.39 mm) (Luqman et al., 2018). Luqman et al. (2018) have also pointed out that three groups of *Eucalyptus* seed: (I) ranged between 1.20 and 1.60 mm, (II) varying between 1.60 and 2.00 mm, and (III) size from 2.00 to 2.40 mm. *Eucalyptus pruinosa* with larger seed length among the nine species is placed in Group III (2.00–2.40 mm); *Eucalyptus melanophloia*, *Eucalyptus microtheca*, and *Eucalyptus tereticornis* come under Group II (1.60–2.00 mm); and rest of the species (*Eucalyptus Amplifolia*, *Eucalyptus camaldulensis*, *Eucalyptus globulus*, *Eucalyptus kitsoniana* and *Eucalyptus pellita*) belong to Group I (1.20–1.60 mm). Luqman et al. (2018) observed that the measurements on seed width revealed that they can be classified into three classes, with *Eucalyptus microtheca* belonging to a class ranging from 1.50 to 2.00 mm, *Eucalyptus pruinosa* to a class varying between 1.00 and 1.50 mm, and the remaining species are placed between 0.50 and 1.00 mm width class. To study the relationship at generic and infra-generic level, Whiffin and Tomb (1972) studied the importance of seed characteristic features at length and found a relationship among certain angiosperm groups, particularly Melastomataceae. According to Chuang and Heckard (1972), seed surface pattern had high significance in the taxonomic delimitation

of certain species of *Cordylanthus* at subsectional and sectional level. This study has depicted that numerous morphological features in seed can be used as additional supporting features for the identification of taxa. Seed surface characters are different and vary from one another. These variations are observed in the surface, width and length.

### Effect of salt stress and osmotic potential on germination of 5 species of *Eucalyptus*

The utilization of *Eucalyptus* species as a salt-tolerant industrial crop could be useful to recover salinized land and to valorize its fiber components for numerous industrial applications (paper and pulp, fabrics, textiles, biocomposites, insulation mats, absorption materials, animal bedding, etc.). *Eucalyptus* represents an alternative crop that may be a feasible source of economically viable and ecologically friendly cellulose. Seed germination is known to be a critical point in seedling establishment, subsequent plant vigor, and ultimately the obtaining of successful crop production. Furthermore, the phenotypic response of seeds to salinity could also be an indicator of plant behavior for the later stages of development (Ayadi et al., 2018). Therefore, our study was conducted as a first attempt in Tunisia to assess the germination behavior of these five species under salt and osmotic stress in order to plan its introduction in forest and urban forest cultivation. Thereafter, multivariate analyses and modeling germination were used to evaluate the salt and osmotic tolerance of *Eucalyptus*. These statistical methods have numerous advantages. PCA allowed a simultaneous analysis of several parameters to elevate the accuracy of the plant status at different salt levels. Moreover, modeling is an excellent tool to predict whether a model shows interactions between parameters. It appeared that the five *Eucalyptus* species were not able to germinate at up to 12 and 15 gL<sup>-1</sup> NaCl (4 to 15%). Similar results obtained by José et al. (2016) who showed that under salt stress, germination continuously decreased, reaching 39% and 13% at -1.0 MPa for *E. urophylla* and *E. urophylla* x *E. grandis*, respectively. Seed germination and germination speed index decreased as the water potential of the germination medium decreased (José et al., 2016). However, *E. urophylla* was more tolerant to salt stress; it showed a higher germination percentage under all tested potentials, when compared to the *E. urophylla* x *E. grandis*. José et al. (2016) noted that salt stress reduced the germination percentage and speed of *E. urophylla* x *E. grandis* and *Eucalyptus urophylla* seeds. Our result

showed that a low germination rate (0%) was detected on *Eucalyptus gillii* in  $15 \text{ g}\cdot\text{L}^{-1}$ . This was not in agreement with the findings of Lopes da Silva et al. (2012) who found that *Eucalyptus saligna* presented higher growth and larger tolerance to the salinity. The largest tolerance of salinity of the *Eucalyptus saligna* can be due to the larger produced amount of sugar soluble and proline (Lopes da Silva et al., 2012). The osmotic conditioning in PEG enabled an increase in the final germination speed and percentage under salt stress for both species (José et al., 2016). The five *Eucalyptus* species were able to germinate at up to  $-1 \text{ MPa}$  (19 to 72 %) with a notable rate of *Eucalyptus gomphocephala* x *Eucalyptus cornuta* (61%) at  $-1.6 \text{ MPa}$  NaCl. For the hybrid, this germination solution potential caused a marked drop in the germination final percentage from 77% at  $-0.5 \text{ MPa}$  to 35% at  $-0.75 \text{ MPa}$  (José et al., 2016). The germination of *E. urophylla* drastically decreased only when seeds were placed under  $-1.0 \text{ MPa}$  salt stress. In this case, germination was reduced from 90% at  $-0.75 \text{ MPa}$  to 39% at  $-1.0 \text{ MPa}$  (José et al., 2016). The conditioning potential of  $-1.5 \text{ MPa}$  was more effective than  $-1.0 \text{ MPa}$  to induce seed tolerance to germination under moderate salt stress ( $-0.75 \text{ MPa}$ ). Martins et al., (2014) who studied *E. grandis* and *E. urophylla*, verified that the  $-0.8 \text{ MPa}$  potential already inhibited germination in practically all species, reducing germination to null or close to zero values, both in water and salt stress. Feikema and Baker, 2011 reported that *E. grandis* was only proper for low salinity soils, since it presented high mortality rate and low volume increment along the years, when submitted to high salinity soils. Interspecies genetic variations for salt tolerance have been related for the *Eucalyptus* genus (Niknam and McComb, 2000). The *Eucalyptus camaldulensis* Dehnh. is highly recommended to combat salinity, because it can tolerate high levels of NaCl-salinity as compared to other tree species (Marcar, 1993; Akilan et al., 1997; Van der Moezel et al., 1988). Salinity up to a level of  $160 \text{ mM}$  NaCl did not affect the survival of *E. camaldulensis*; however, it can affect plant growth and dry-matter production negatively (Rawat and Banerjee, 1998; Grieve et al., 1999). Nawaz et al. (2016) showed that survival of the *Eucalyptus camaldulensis* plants was not affected by high NaCl salinity or higher levels of Pb in nutrient solutions. *E. camaldulensis* plants survived the NaCl-salinity stress at  $200 \text{ mM}$  NaCl. These results were in agreement with previous studies (Marcar, 1993; Rawat and Banerjee, 1998; Van der Moezel et al., 1988; Gomes et al., 2012; Peng et al., 2012) who have shown that *E. camaldulensis* tree species can successfully grow on salt-

affected soils as well as Pb-polluted soils. According to Pulavarty et al. (2016) *Eucalyptus camaldulensis* under NaCl is affected significantly on the morphological features after 2 months exposure. Salt stress has detrimental effect on plant growth and development (Pulavarty et al., 2016). Plant-water relationship in *Eucalyptus* is specific, because of its high water uptake ranging from 50 to even  $90 \text{ L/day/plant}$  and the particular tolerance of several eucalypts to drought (Joshi & Palanisami, 2011). Ouyanga et al., 2016 suggested that the cumulative annual water use by *E. urophylla* in sandy soil is about  $3200 \text{ L/tree}$ ; the parameters that explain this high water consumption are in order: root uptake, leaf transpiration and soil evaporation. Joshi & Palanisami (2011) reported that eucalypt roots can grow even up to  $6\text{--}9 \text{ m}$  in stress conditions and extract more water. Indeed, roots of some species can grow to  $30 \text{ m}$  in depth and extract the ground water (FAO, 2011). *Eucalyptus* species are generally characterized by a great ability of water absorption, as well as a great resistance to the dry conditions. This quality is manifested in morphological and physiological parameters, including root development and changes in osmotic potential, stomatal conductance, gas exchange, transpiration rates and photosynthesis (Saadaoui et al., 2017). The use of *Eucalyptus* for reforestation under reduced water conditions, essentially with climate change impacts, would pose problems related mainly to its water consumption, biodiversity reduction and environment degradation (Zhang, 2012). The tolerance to the saline stress can be due to the control of the acquisition and for the allocation of sodium inside the plant, or for the osmotic readjustment and other physiologic processes (Cheeseman, 1988). The inhibition of the growth and the plant production is due to the reduction in the osmotic potential caused by the excess of salts and/or to the toxicant effect of the same ones (Silva et al., 2000). Fredj et al. (2013), when studying field conditioning levels in coriander cultivars (0, 2, 4, 6 and  $9 \text{ g}\cdot\text{L}^{-1}$  NaCl) for three duration periods (12, 24 and 36 hours), found that the higher the duration, the lower the benefits of conditioning and that a conditioning saline solution in the  $4 \text{ g}\cdot\text{L}^{-1}$  concentration provided the best germination results, decreasing significantly in higher concentrations. In the Mediterranean basin, salinity is one of the major constraints impeding germination and affecting seedling and plant growth (Gupta and Huang, 2014). Salt concentration is the most important factor in regulating the germination of non-dormant seeds at the beginning of the growth season. Abiotic stress, depending on its intensity, may severely limit the growth and development of plants (Harfouche et al., 2014). Salinity and water deficit are the main factors affecting the

productivity of cultures around the world, especially in arid and semi-arid regions (Jisha et al., 2013; Gholami et al., 2015). NaCl reduces the efficiency of use of the nutrients, although its translocation is not affected (Silva et al., 2000; Rego et al., 2011). Several morphological characteristics and growth comparison studies of *Eucalyptus camaldulensis* and *E. citriodora* are affected by drought stress and the *Eucalyptus* height gradually decreased as the irrigation interval increased and root length varied drastically against the influence of the drought (Yousaf et al., 2018). The present study aimed to model seed germination in response to salt and water. R-square ( $r^2$ ) values of regression coefficients established for five *Eucalyptus* varied from 0.798 (salt concentration) to 0.500 (osmotic potential).

## CONCLUSION

In conclusion, the hybrid *E. gomphocephala* x *E. cornuta* was more tolerant to salt stress (15 % at 12 g L<sup>-1</sup>) and osmotic potential (61 % at -1.6 MPa) than the others species of *Eucalyptus*; it showed a higher germination percentage under all tested potentials, when compared to the not hybrid species of *Eucalyptus*. Seeds morphology revealed that the observations shown diversity of morphological characters in seeds. Area, length and width of seeds vary significantly between species of *Eucalyptus*. Micromorphological characters can provide basis for classification and delimitation of genus *Eucalyptus*. Our results suggest that extensive work is needed to be carried out on the seed micromorphology of genus *Eucalyptus* considering more species. These characters can play an important role in a delimitation and accurate identification of complex or closely related species.

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