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Effects of temperature and treated urban wastewater on seed germination and seedling growth in different populations of *Moringa oleifera* (Lam.)

Nidhal Marzougui[1‡]*, Arwa Hammami[1‡], Ferdaous Guasmi[2] & Saloua Rejeb[1]

[1]Research Laboratory Valorization of Non Conventional Waters, Institut National de Recherches en Génie Rural,
Eaux et Forets, Ariana, Tunisia
[2]Laboratory of Dry Land Farming and Oasis Cropping, Institut des Régions Arides, Medenine, Tunisia

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Drought is an environmental concern in North Africa. Hence, countries in this region promote treated wastewater reuse in agricultural irrigation as sustainable practice in reducing the increasing stress on water resources. Here, we evaluated the treated urban wastewater (TUW) impact on seed germination and seedling growth of four *Moringa oleifera* (MO) populations in different temperature conditions. Seeds were brought from Morneg and Kairouan in Tunisia and from India and Egypt. Germination tests were performed using different TUW concentrations at 25 and 35°C for 9 days under darkness and distilled water for control. Parameters such as germination percentage (GP), mean germination time (MGT), shoot length (SL) and TUW half-maximal effective concentration (EC_{50}) were measured. GP, MGT, and SL varied significantly with incubation temperature and seed source. Only SL did not show any significant variations in all TUW concentrations. Increased temperature had a positive influence on GP and SL; on the contrary, a negative impact on MGT was observed in Kairouan, Egyptian and Indian seeds. EC_{50} evaluation showed that TUW was less harmful on Indian *Moringa* seeds. Seeds incubated at 25°C were structured into two groups: the first containing Morneg, Indian and Egyptian seeds; and the second one with Kairouan seeds. At 35°C, Egyptian seeds left the first group and joined the second indicating that their germination didn't occur rapidly for temperature increase. Considering Tunisian climate conditions, results showed that *Moringa oleifera* is able to acclimatize to temperatures of this country.

Keywords: Climate change, Drumstick tree, Germination parameters, Treated sewage, Tunisia

Climate changes in the Middle East and North Africa region highlight general warming as well as economic and environmental consequences, especially in the regions where a decline of precipitation is observed and projected[1]. Mediterranean ecosystems are characterized by contrasting functional plant types competing for water and sensitive to warming and changes in water availability[2]. All these changes are expected to modify the vegetation patterns through the habitat loss of several plant species[3] and the impact on seed production[4]. Under such conditions, new species may occupy new territories and previously existing species in these areas may disappear[5]. The other major problem caused by climate changes in the entire Middle East and North Africa region is water shortage[6]. Some countries from this region such as Tunisia fall below the scarcity level of 500 m^3 of renewable water per person and per year[7]. Faced with the increased demand caused by the socio-economic development of these countries, almost all the accessible freshwater resources have been utilized and exhausted[8]. Hence, the need to use other resources such as treated wastewater (TWW) for agricultural irrigation is a feasible option for minimizing the pressure on conventional water resources[9,10].

TWW constitutes a reliable source of water throughout the year. It is increasingly used in agriculture in developing and industrialized countries due to the increasing scarcity of water resources, and the degradation of freshwater sources resulting from the incorrect disposal of wastewater and also by the demographic growth and increase in demand for food and fiber[11]. This water contains the nutrients needed for plant growth such as nitrogen and phosphorus[12]. Its reuse in agriculture constitutes a recycling form of water and nutrients and often reduces the environmental impact that it would have downstream on soils and water resources[13]. Further, it improves agricultural production and at the same time provides

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*Correspondence:
E-Mail: marzouguinidhal@gmail.com
‡Equally contributed

environmental protection by minimizing the use of chemical fertilizers[14].

Moringa oleifera Lam. (MO) is a fast growing medicinal tree tolerant to drought and able to tolerate poor soil conditions[15]. It is native to the sub-Himalayan regions of northwestern India and is currently found in tropical regions around the world[16]. This species is newly introduced to Tunisia, where several farmers are showing interest because of its multiple industrial and medicinal uses, and also as animal fodder[17,18]. Its leaves, fruits, flowers, and immature pods are used as highly nutritious vegetables because of their high vitamin and protein content[19], and also exploited for non-food purposes such as water purification and biofuel[20,21]. Seed germination in *Moringa* is hypogeal[22]. High temperatures favour the seed germination rate and seedling growth of *M. oleifera*[16]. In the present study, we evaluated the impact of using secondary treated urban wastewater on seed germination and seedling growth of four *M. oleifera* populations in different temperature conditions.

Materials and Methods

Plant material and wastewater sampling

The four MO populations, used in this study were collected in 2017 by the Unconventional Water Valorization Laboratory team (LRVENC-INRGREF) from Tunisian farmers and stored in hermetically sealed containers under ambient laboratory condition with temperature varying from 15 to 35°C in winter and summer seasons, respectively. Two of these populations were cultivated for 3 years in the regions of Morneg (Ben Arous Governorate) and Elbaten (North of Kairouan Governorate) (Fig. 1); the two others were imported from India and Egypt by the "General Agriculture" company localized at Kairouan Governorate, Tunisia. Authentication of seed samples was conducted by the LRVENC-INRGREF team according to the Organisation for Economic Cooperation and Development (OECD) seed schemes procedures. The MO populations' progress at different stages in the seed production process was checked as follows: (i) Examination of farmers' control plots using MO seed samples drawn from lots; (ii) Laboratory tests on MO seeds and seedlings, using seed samples drawn from lots; and (III) Field inspection of MO growing seed crops several times.

In the experiments described below, the water qualities used were distilled water (DW) used as control and 24 h average sample of treated urban wastewater (TUW) collected in polyethylene bottles from the wastewater treatment plant of Hammamet city (activated sludge with supply water of 90% domestic and 10% from tourism activity). The physicochemical characteristics of the water samples are shown in Table 1.

Table 1 — Mean concentration of physico-chemical parameters of the water samples used for germination tests

Parameters	Distilled water	Treated urban wastewater
pH	7.08	7.14
EC (mS/cm)	0.37	2.78
COD (mg/L)	-	47
BOD_5 (mg/L)	-	9
TSS (mg/L)	-	9
Na^+ (mg/L)	-	618.3
Mg^{2+} (mg/L)	-	32.75
Ca^{2+} (mg/L)	-	27.05
K^+ (mg/L)	-	26.5
NH_4^+ (mg/L)	-	23.78
Cl^- (mg/L)	-	452
HCO_3^- (mg/L)	-	1102.6
Cd^{2+} (mg/L)	-	0.008
Co^{2+} (mg/L)	-	0.016
Cr^{2+} (mg/L)	-	0.026
Cu^{2+} (mg/L)	-	0.004
Fe^{3+} (mg/L)	-	0.16
Mn^{2+} (mg/L)	-	0.04
Ni^{2+} (mg/L)	-	0.024
Pb^{2+} (mg/L)	-	0.039
Zn^{2+} (mg/L)	-	3.39

[EC, Electrical conductivity; COD, Chemical oxygen demand; BOD_5, Biochemical oxygen demand during 5 days; and TSS, Total suspended solids]

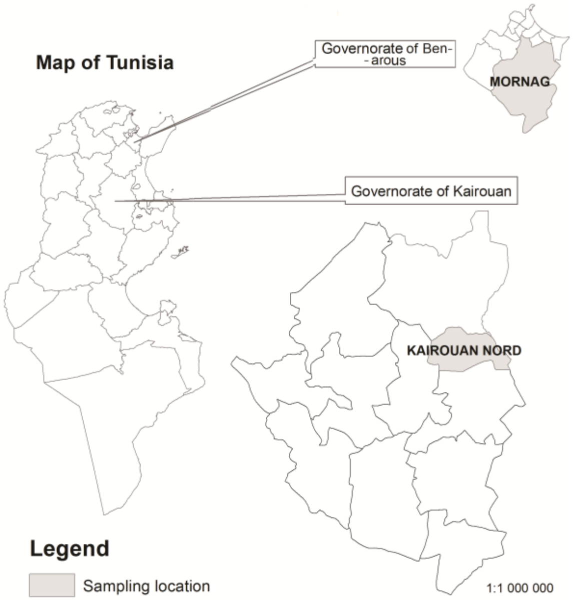

Fig. 1 — Location map of Tunisia regions of *Moringa oleifera* study populations (ArcGIS 10.0)

Germination tests

TUW samples were filtered through No.1 Whatman paper (filter mesh= 11 μm) and kept at 4°C. Healthy and uniform MO seeds were selected, sterilized with 0.1% $HgCl_2$ (Prolabo, Paris, France) solution, and thoroughly cleaned up with sterilized DW to avoid surface contamination. They were subsequently decorticated to accelerate germination[23] and then 20 seeds were placed equidistantly in sterile Petri dishes on cotton moistened with 2 mL of TUW at the concentrations of 100% (undiluted TUW), 80, 60, 40, 20 and 0% (control). Dilutions were performed using DW. Each treatment was performed in triplicate and placed in the dark in a growth chamber (Memmert, Schwabach, Germany) for 9 days at 25°C. The same experiment was done at 35°C[16]. Germination percentage was calculated following the formula: GP = [n×100]/N, where GP is the germination percentage; n, the number of germinated seeds; and N, the total number of seeds[24]. The mean germination time was determined according to the formula:

$$MGT = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

with t_i time from the experiment start, n_i number of seeds germinated[24]. The shoot length (SL) was recorded after 9 days.

Statistical analysis

The statistical model used incorporates the three independent factors “incubation temperature” (25 and 35°C), “seed source” (*Moringa* populations), and “wastewater dilution level” (0, 20, 40, 60, 80 and 100%) explaining the variations of the dependent quantitative variables GP, MGT and SL. Data normality of quantitative variables was assessed by means of the Shapiro-Wilks test. A three-way ANOVA was performed using SPSS software (IBM SPSS statistics, version 20) to study variability and interactions between the variables GP, MGT and SL. The ANOVA was complemented by a Duncan test of means comparison of population groups and wastewater concentration groups, two by two. Principal component analysis (PCA) was accomplished by the XLSTAT software (Adinosoft, version 2014.5.03) to graphically show similarities between populations and to specify the main parameters that determine variability. Hierarchical classification analysis (HCA) was carried out using SPSS software to confirm PCA results. The observed effect of TUW concentration on the germination of *Moringa* seeds was estimated by the calculation of the half-maximal effective concentration (EC_{50}) using XLSTAT software.

Results and Discussion

Impact of treated urban wastewater (TUW) on germination and seedling growth of *M. oleifera*

Germination controls plant populations and ensures their reproduction and their productivity[25]. In our study, results showed that the variation of *Moringa* seeds GP is highly significant depending on incubation temperature, seed source, and TUW concentration, considered separately, and according to the interaction between them when they are taken by pairs or by triplets ($P < 0.01$). The two highest GP were obtained in presence of the distilled water (control test) and the TUW diluted at 20% (GP = 99%); the lowest values of this parameter were recorded with undiluted TUW (GP = 29%) (Table 2). Zaouri *et al*.[26] confirmed these results when they studied the effect of several qualities of municipal wastewater on seed germination of tomatoes and lettuces. They showed that undiluted domestic wastewater affects negatively seed germination due to the heavy metal accumulation in germinated seeds. These toxic elements affect amylase, protease, and ribonuclease enzyme activities and cause the delay of seed germination and growth but it is not the case for all plant species[27,28]. In fact, Sleimi *et al*.[28] demonstrated improved germination percentage of the seeds of *Cucumis sativus* treated by increasing Ba doses. Such stimulation was also observed with *Cucurbita pepo* seeds treated by different copper concentrations. Akhkha *et al*.[29] recorded a *Calotropis procera* GP increase in all treatments that lasted 6 days. They showed that compared to the distilled water, treatment with wastewater had a significant effect ($P < 0.05$) on germination rate extending to a 20% increase for seeds treated with raw wastewater and primary and secondary treated wastewater. Results of our study showed also that the temperature increase favoured MO seed germination especially for Kairouan seeds which showed nil germination at 25°C, presented 98% GP at 35°C with TUW diluted at 20 and 60% (Table 2). Muhl *et al*.[16] also showed the positive influence of the temperature increase on germination percentage and rate of MO seeds. It could be attributed to the fact that temperature variation touches enzyme reactions of

Table 2 — Mean values of the germination parameters of the seeds of four *Moringa oleifera* populations at two different temperatures

Morneg seeds			Kairouan seeds			India seeds			Egypt seeds		
Wastewater concentration	Temperature condition		Wastewater concentration	Temperature condition		Wastewater concentration	Temperature condition		Wastewater concentration	Temperature condition	
	25°C	35°C		25°C	35°C		25°C	35°C		25°C	35°C
Germination percentage											
0%	74^{a}	99^{a}	0%	0^{d}	$97,6^{a}$	0%	85^{a}	97^{a}	0%	98^{a}	97^{a}
20%	99^{a}	78^{b}	20%	0^{e}	98^{b}	20%	75^{a}	53^{b}	20%	98^{b}	60^{b}
40%	97^{a}	95^{a}	40%	0^{d}	97^{a}	40%	74^{a}	98^{a}	40%	96^{a}	96^{a}
60%	94^{a}	96^{a}	60%	0^{d}	98^{a}	60%	74^{a}	98^{a}	60%	93^{a}	98^{a}
80%	51^{c}	96^{a}	80%	0^{d}	87^{a}	80%	72^{c}	97^{a}	80%	92^{a}	94^{a}
100%	29^{b}	54^{c}	100%	0^{d}	75^{b}	100%	66^{b}	28^{c}	100%	77^{a}	68^{c}
Mean germination time											
0%	6^{b}	2.25^{d}	0%	0^{a}	6^{b}	0%	9^{b}	6^{d}	0%	4.5^{e}	2^{a}
20%	6^{a}	4.5^{d}	20%	0^{a}	6^{c}	20%	9^{b}	8^{d}	20%	6.75^{e}	4^{a}
40%	9^{d}	6^{d}	40%	0^{a}	6^{ab}	40%	9^{b}	8^{d}	40%	9^{e}	6^{a}
60%	9^{a}	6^{d}	60%	0^{b}	6^{ab}	60%	9^{b}	8^{d}	60%	9^{e}	8^{a}
80%	9^{a}	6^{d}	80%	0^{a}	6^{ab}	80%	9^{b}	8^{d}	80%	9^{e}	8^{a}
100%	9^{c}	6^{d}	100%	0^{a}	6^{d}	100%	9^{b}	8^{d}	100%	9^{e}	8^{c}
Shoot length											
0%	3.3^{ef}	0^{f}	0%	0^{cd}	4.7^{f}	0%	4.7^{def}	4.6^{f}	0%	0^{a}	3.3^{bc}
20%	6.13^{cde}	0^{f}	20%	0^{a}	6.7^{f}	20%	2.4^{bcde}	11.3	20%	0^{cde}	6.9^{de}
40%	4.8^{bcde}	0^{f}	40%	0^{a}	9.2^{f}	40%	5.3^{def}	5.35^{f}	40%	0^{ab}	6.7^{ab}
60%	2.8^{abc}	0^{f}	60%	0^{a}	4^{f}	60%	6.3^{cdef}	6.37^{f}	60%	0^{cdef}	6.5^{ab}
80%	1.7^{cdef}	0^{f}	80%	0^{cd}	14^{f}	80%	0^{def}	5^{f}	80%	3.3^{bcde}	3.2^{abc}
100%	8.1^{cdef}	0^{f}	100%	0^{a}	6^{f}	100%	1.13^{bcd}	5.7^{f}	100%	0^{cdef}	0^{cdef}

[T: Temperature; 0%: control treatment; 20, 40, 60 and 80%: different dilutions levels; 100%: undiluted TUW. Different letters indicate significant differences at *P* ≤0.05 according to the Duncan-test]

physiological processes such as seed rate of deterioration and seed dormancy[30]. Contrarily, Hassanein & Al-Soqeer[31] did not notice a positive influence of the temperature increase on the germination percentage of MO seeds. They evaluated *Moringa* seed germination under seven temperature regimes in the laboratory (10, 15, 20, 25, 30, 35, and 40ºC) and observed that 20°C was the optimum temperature for *Moringa* germination allowing the GP of 87-90% within 5 days. They obtained also a satisfied GP (80%) with 15°C.

Wu *et al*.[32] considered that species distribution range is a determinant factor in germination response to the temperature treatment regardless of phylogeny. Widely distributed species are less sensitive than endemic species like MO[33] to temperature variation[34,35], and this is a handicap to colonize new areas or tolerate changes[36]. As of April 14, 2021, the World Bank Group listed on its website of Climate Change Knowledge Portal that in Tunisia the average monthly temperature for 1991-2016 varied from 10.3 to 28.86°C, and with the approved positive influence of temperature increase on *Moringa* germination, it seems that MO will be acclimated to temperatures in this country. According to the results in Table 2, the *Moringa* Egyptian population presented satisfactory GP with the different concentrations used of TUW and at the different incubation temperatures tested.

The parameter MGT expresses the average time which seeds take to germinate at each temperature and makes it possible to quantify the effect of temperature on seed germination[37]. Its variations were highly significant depending on incubation temperature, seed source, and TUW concentration, taken separately and also according to their interactions (*P* <0.01). The shortest MGT were obtained for Egyptian seeds with distilled water (2 days at 35°C) and TUW diluted to 20% (4 days at 35°C), while the seeds soaked with undiluted TUW showed longer MGT (8 days at 35°C and 9 days at 25°C, Table 2). Results showed also that incubation temperature influenced this parameter so that MGT decreased with increasing temperature (Table 2). Studies from *Moringa* incubator germination trials conducted within the Department of Plant Production and Soil Science at the University of Pretoria by Muhl *et al*.[16] endorse these findings, as they found the MGT to be 18.8 days at 20°C and only 11.4 days at 30°C.

Regarding seedling growth estimated through SL, statistical analysis showed that this parameter presents high significant variations according to the incubation temperature and seed source (*P* <0.01), whereas its variations were not significant on the basis of TUW concentration ($P = 0.17$). In contrast, Khaleel *et al*.[38] showed that seed germination and seedling growth of *Abelmoschus esculentus* varied significantly with different concentrations of dairy wastewater, which is more contaminated than urban wastewater used in this study. Divya *et al*.[39] proved that shoot length is dependable on the concentration of the effluent used when they studied the impact of treated sewage effluent on seed germination and vigor index of monocots and dicot seeds and showed that the 10% concentration of effluent was most effective and could be used for irrigation. Table 2 shows that the *Moringa* seed shoots of Morneg population could not grow at 35°C despite germination. Shoot lengths of Kairouan, Egyptian and Indian seeds grown at 35°C were taller than those grown at 25°C. The highest value of this parameter (14 cm) was obtained with *Moringa* seeds from Kairouan incubated at 35°C and watered with TUW diluted to 80% (Table 2). The "world weather online" site showed that Kairouan has a hot climate. During the period 2015-2020, the minimum and maximum values of the monthly average temperatures of this Tunisian region vary from 11-35°C. Thus, the Kairouan *Moringa* population is physiologically adapted to hot climate. Muhl *et al*.[16] confirmed in their work on *Moringa* seeds from Malawi the positive linear relationship between temperature and seedling growth after emergence. They found that seedlings germinated and grown at 30°C were on average 91.6% taller than those grown at 25°C, and 177% taller than the seedlings grown at 20°C. Moreover, it seems that using wastewater for crop irrigation has negative and positive effects. The positive effect is manifested by the improvement of the quality of certain soils and the increase in the production of certain plant species and varieties. Whereas, the negative effects are primarily caused by the presence of high dissolved and total suspended solids, high nutrient contents, and potentially toxic elements[39,40]. On the other hand, plant species and varieties have different capacities for accumulation and removal of potentially toxic elements from the soil, different compartmentation of these elements in plant parts, and even different expression of transporter proteins controlling their compartmentation[41].

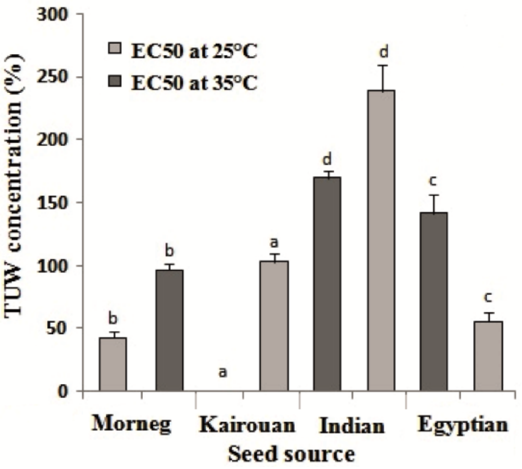

Fig. 2 — Variations in half-maximal effective concentration (EC_{50}) of urban treated wastewater with respect to seed germination of *Moringa oleifera* at different temperatures

Evaluation of half maximal effective concentration of urban treated wastewater

Changes in the half-maximal effective concentration (EC_{50}) of TUW, which generates germination inhibition of 50% of *Moringa* seeds as compared to control seeds irrigated with distilled water, are shown in Fig. 2. Given that a high EC_{50} is indicative of low toxicity of TUW and conversely a low EC_{50} reflects the great harmfulness of the TUW in question, it turns out that the TUW tested in this study was less harmful on *Moringa* seeds from India than seeds from the other sources (Fig. 2). Indeed the phytotoxic mechanisms of metallic elements, such as those present in TUW, involve different biochemical and genetic pathways in the different plant species[42,43]. In addition, tolerance to metal stress varies from one variety to another within the same plant species[44]. Although plant defense strategies exist to resist metal toxicity via reduced uptake into the cell, sequestration into vacuoles through complexes formation, phytochelatins binding, osmolytes synthesis, activation of antioxidants to combat reactive oxygen species (ROS), altered enzymes expression and overexpression of existing genes, the resistance mechanisms of germinated seeds to combat metallic stress remains unclear[43]. Results showed also that TUW harmfulness was lower at 35°C for Morneg, Kairouan, and Indian seeds, whereas for Egyptian seeds, incubation at 25°C was favourable to germination of seeds soaked by TUW (Fig. 2). This may be justified by variation of *Moringa* germinating seeds response to high-temperature conditions according to the studied populations. Plants react to high temperature conditions by the synthesis of small heat shock proteins (HSPs). In addition to the synthesis in response to stress, HSPs are also expressed during seed development[45]. Wehmeyer & Vierling[46] reported that during the germination stage, progressively regulated HSPs are abundant in the first days and then decline quickly.

Structuring of *M. oleifera* populations studied

Based on the parameters germination percentage, MGT and SL, the principal compound analysis (PCA) allows structuring of the four tested populations of MO and to graphically shows the degree of resemblance between them in its projection planes. Considering the germination conditions of *Moringa* seeds soaked in distilled water (control) at 25 and 35°C, the first two axes of the PCA absorb 98.62 and 96.2% of the total variability (3 initial variables), respectively. At 25°C, the first axis (59.3% of the total variation) is positively correlated with the MGT and SL parameters. The second axis with 39.25% of the total inertia is positively defined by the germination percentage parameter. These axes make it possible to group the four *Moringa* seed sources into two groups. The first group (G1) on the positive side of ax 2 contains Morneg, Indian, and Egyptian seed sources with the highest values of germination percentage (GP=74, 85 and 98%, respectively) and MGT (MGT=6, 9 and 4.5 days). The second group (G2) is located on the negative sides of the two axes 1 and 2 and formed by the seeds from Kairouan with null values in all studied parameters (GP=0%, MGT=0 and SL=0). For the seeds incubated at 35°C, the PCA classified them into two groups. The first group (G1) is formed by Morneg and Egyptian seeds characterized by high GP values (GP=99 and 97%, respectively) and the lowest values of MGT (MGT=2.25 and 2 days, respectively). The second group (G2), containing the seeds of India and Kairouan, is marked by high GP values (about 97%) and the highest values of the MGT parameter (6 days). These structures of MO seeds were confirmed by the dendrograms obtained by the hierarchical classification analysis (HCA). Results of HCA showed that *Moringa* seeds incubated at 25°C can be structured into two clusters; the first is formed by Morneg, Indian and Egyptian seeds and the second contains the seeds of Kairouan. Similarly, for seeds incubated at 35°C, HCA resulted in a two-cluster structure showing that Kairouan seeds were germinated at 35°C and joined Indian ones in G2 characterized by high GP and MGT values. Zhang *et al.*[47] observed that the germination occurs at optimal temperatures or an optimal temperature range which is the case of the Kairouan *Moringa* seeds at the conditioned incubation of 35°C.

Conclusion

Moringa oleifera is newly introduced in Tunisia. The agricultural reuse of treated urban wastewater (TUW) is a feasible solution in this water-stressed country. Results of this study have shown that the Egyptian population, one of the four *Moringa* populations tested, has satisfactory GP with the different TUW concentrations used and at the different incubation temperatures tested. Variation of the GP and MGT of *Moringa* seeds were significant according to incubation temperature, seed source, and TUW concentration. The temperature increase exercised a positive influence on GP and a negative influence on MGT. Considering the Tunisian average monthly temperature, it is possible that MO is acclimated to temperatures in this country. The parameter SL presented significant variations according to incubation temperature and seed source and no significant variation on the basis of TUW concentration. Shoots of Morneg population could not grow at 35°C despite having been able to germinate. Kairouan, Egyptian and Indian seeds SL showed a positive linear relationship between temperature increase and seedling growth after emergence. Evaluation of EC_{50} at the different dilution levels showed that TUW was less harmful on *Moringa* seeds from India than seeds from the other sources. TUW harmfulness was lower at 35°C for Morneg, Kairouan and Indian seeds, whereas for Egyptian seeds, incubation at 25°C was favourable to germination. These results should be supplemented by studying MO growth at 25 and 35°C, and under irrigation with TUW at different levels of dilution for evaluation of the vegetative development and production in such conditions.

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Conflict of interest

Authors declare no conflict of interests.

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