

Physiological and biochemical responses of argan (*Argania spinosa* (L.)) seedlings from containers of different depths under water stress

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Abstract

Plant species characteristic of arid and semi-arid zones, such as *Argania spinosa* (L.) Skeels, have a taproot that allows them to reach the soil horizons more quickly. Unfortunately, in the nursery, the containers of culture used for the production of seedlings do not support an excellent development of the root architecture that can be able to resist the shock of transplantation, in particular of the hydric stress. This study aimed to evaluate the physiological and biochemical behavior of *Argania spinosa* seedlings grown in containers of different depths under water stress. An experiment was conducted with 90 seedlings from the different containers (P1 for depth of 16 cm, P2 for depth of 30 cm, and P3 for depth of 60 cm), and three watering treatments (well-watered 100% of field capacity, moderate stress with 50% of field capacity and severe stress with 25% of the field capacity). Our results showed that seedlings from the 16 cm container had lower values of water status. Malondialdehyde content, electrolyte leakage, hydrogen peroxide, and superoxide radical content gave higher values on seedlings from the shallow container. The benefits of increasing the container depth of nursery seedlings contribute to the improvement of physiological and biochemical responses of seedlings under water stress. To fully validate our findings, a long-term field study must be conducted.

Keywords: *Argania spinosa*; biochemical characteristics; container depth; physiological characteristics; water stress

Introduction

In the Mediterranean ecosystem, plants are exposed to continuous and severe stress (Nogués and Baker 2000; Chakhchar *et al.*, 2015; Chakhchar 2015; Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021). These conditions, combined with anthropogenic activities, may threaten some forests in this ecosystem such as the argan grove (Defaa *et al.*, 2015), including the flagship and endemic species (*Argania spinosa*). *Argania spinosa* is one of the

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most important Moroccan forest species and extends over the arid and semi-arid areas of Morocco (Msanda *et al.*, 2005). During the last decades, a regression of the argan tree areas and aging of the populations because of an almost absent regeneration has been recorded (Defaa *et al.*, 2015; El Mrabet *et al.*, 2014; Hachemi *et al.*, 2021). To remedy this plague and thus preserve this endemic species, a vast program of assisted regeneration was launched in the late 1990s by the Forestry Department (Defaa *et al.*, 2015). However, several reforestation projects have failed (El Mrabet *et al.*, 2014; Defaa *et al.*, 2015). The difficulty of seedlings to adapt to the prolonged drought conditions of these extreme areas may partly explain these failures (El Mrabet *et al.*, 2014). In addition, seedling quality can also influence reforestation success in extreme areas (Bengough *et al.*, 2011; Bainbridge 2012). Defaa *et al.* (2015) pointed out that the seedling quality of *Argania spinosa* is very often linked to low success rates after transplanting.

Nevertheless, several authors have indicated that the quality of the seedlings can be corrected in the nursery by the use of deep culture containers, optimizing their development of the taproot structure (Chirino *et al.*, 2008; Comas *et al.*, 2013; Muñoz *et al.*, 2014; Ovalle *et al.*, 2015; Zine El Abidine *et al.*, 2016). Growing long taproot seedlings in deep containers can withstand transplant shocks and thus ensure successful reforestation (Bainbridge, 2012; Muñoz *et al.*, 2014). Other studies conducted in arid, semi-arid, and dry tropical areas have proven the positive effect between root length and seedling survival after transplanting (Markesteyn and Poorter, 2009; León *et al.*, 2011; Ovalle *et al.*, 2015). De La Fuente *et al.* (2017) pointed out that a seedling with a long taproot has adequate morphology and biomass distribution allowing it to colonize deep soil layers. These characteristics thus improve the physiological response of seedlings to water stress conditions (Chirino *et al.*, 2008). Therefore, the design of the containers determines the morphological, physiological, and biochemical characteristics of the seedlings, mainly concerning their root system (Aphalo and Rikala, 2003; Dominguez-Lerena *et al.*, 2006; Chirino *et al.*, 2008).

In semi-arid and arid zones, at the beginning of field planting, seedlings are exposed to various abiotic stresses, in particular water stress (Chakhchar *et al.*, 2015; Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021). Under water stress, some species, as *Argania spinosa* develop adaptive mechanisms to tolerate the effects of stress (Chakhchar *et al.*, 2015; Chakhchar, 2015; Hachemi *et al.*, 2021). *Argania spinosa* has been shown to reduce stomatal conductance, leaf water potential, and relative leaf water content under drought (Chakhchar *et al.*, 2015). In addition, under water stress, it has been shown that chlorophyll is degraded (Chakhchar, 2015). Several studies have indicated that the accumulation of osmolytes such as proline and soluble sugars is one of the well-known adaptive mechanisms in *Argania spinosa* against water stress (Chakhchar, 2015; Jafarnia *et al.*, 2018). According to Ben Ahmed *et al.* (2009), these osmolytes maintain water in the cytoplasm. Hessini *et al.* (2009) pointed out that osmolytes prevent protein denaturation and cell membrane damage, which induces structural stability of enzyme proteins to preserve their activity.

Furthermore, for *Argania spinosa*, one of the first biochemical responses to water stress is the production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) (Chakhchar *et al.*, 2015). In stress conditions, the high production of (ROS) can cause cellular damage due to oxidative stress and increased lipid peroxidation (Chakhchar *et al.*, 2015; Chakhchar, 2015; Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021). The increase in lipid peroxidation can be determined by the malondialdehyde (MDA) content (Chakhchar *et al.*, 2015; Chakhchar, 2015; Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021).

To regulate the excessive production of ROS and prevent oxidative stress, plants such as *Argania spinosa* have developed enzymatic systems. These enzyme systems include superoxide dismutase (SOD), peroxidase (POD), and polyphenol oxidase (PPO) (Chakhchar *et al.*, 2015; Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021).

Although there are several studies on the responses of *Argania spinosa* to water stress, there is little or no information on the physiological and biochemical responses of seedlings from deep containers. The objective of this study was to evaluate the physiological and biochemical behavior of *Argania spinosa* seedlings from containers of different depths. We sought to determine the depth of the growing container that would allow argan seedlings to better resist water stress. We hypothesized that deep containers reduce the adverse effects of water stress and improve resistance mechanisms in *Argania spinosa* seedlings. The answer to this

problem will allow the nursery managers to choose the depth of the ideal containers to produce argan seedlings resistant to the shock of transplantation. This will also optimize the success of reforestation-based argan.

Materials and Methods

Plant material and nursery growth

The experiment was conducted at the Regional Forestry Research Center of Marrakesh, Morocco (31°40'04 "N 7°58'04 "W). Ripe argan fruits were collected in July 2017 from twenty-five argan trees spaced more than 20 meters apart at various sites in Essaouira (31°32'07.8" N 9°28'34.4" W). On average we collected 5 to 10 kg of seeds per tree. The ripe fruits were sun-dried and stored at room temperature in a storage room. Around the end of April 2018, the dry fruits were shelled and the seeds were recovered. After that, the seeds were soaked in tap water for 48 h. Then, the soaked seeds were spread in sterilized sand [105 °C in an incubator (BINDER Model ED 56) for 48 h] and covered with a plastic film until the seed coat burst and the radicle appeared (Ferradous, 2018). The control container of our study is the one used for the production of argan seedlings in the nursery. It is made of 28 cells having the shape of a regular trapezium with the smaller base directed downwards. Inside each alveolus, we distinguish the grooves and a hole. Containers are placed on supports. In the absence of the same type of container with the desired depths, we opted for black polyethylene polybags slightly rigid (Figure 1 and Table 1). 720 healthy and uniform seeds were selected and sown in three containers of different depths (16 cm, 30 cm, and 60 cm) on 18th of May, 2018 (240 seeds for each container). The used substrate was air-dried forest soil (same origin as the seeds with a silty clay-sandy texture (16.5-29-55%) and the following physico-chemical characteristics: pH=8; electrical conductivity = 0.56 ms/cm; total nitrogen (N)= 0.03%; assimilable phosphorus (P)= 13.38 mg.kg⁻¹, potassium (K)= 30.06 mg.kg⁻¹, total organic carbon = 2.2%, and organic matter = 3.41%) and commercial peat (TS3) mixed (1:3, v/v). The mixture had a pH_{water} of 7.5 and contained an average of 8.21% total nitrogen, 129.67 mg kg⁻¹ of available phosphorus, and 115.67 mg kg⁻¹ of potassium, 1.14 ms.cm⁻¹ of electrical conductivity, 9.49% of total organic carbon, and 16.26% organic matter. Fertilization was applied weekly with a 20-20-20 fertilizer solution at a rate of approximately 100 mg per plant for each nutrient (nitrogen, phosphorus, and potassium) for the duration of the nursery growth period (Ferradous 2018). All seedlings remained for 15 months in the nursery growth phase. The seedlings were covered with a net allowing 60% sunlight (i.e. 40% shading) and watered daily.



Figure 1. Illustrative of the supports (containers/polybags)

Table 1. Characteristics of the container and polybags used in the different treatments

Container / Polybag	Depth (cm)	Diameter (cm)	Circumference/peri meter (cm)	Volume (cm ³)
Container (P1)	16	7 cm on a side	28 of perimeter	500
Polybag 1 (P2)	30	10	31.42	2355
Polybag 2 (P3)	60			4710

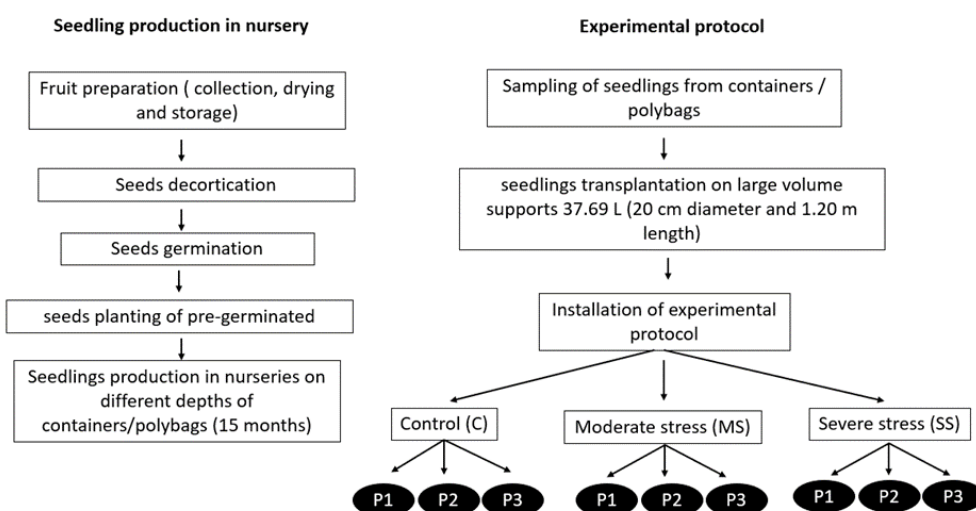
Experimental protocol and methodology

After the period of growth in the nursery, the seedlings of the argan tree were transplanted on supports of 37.69 L (20 cm in diameter and 1.20 m in length). The used soil is that of the mixture (same origin as seeds). The seedlings were divided into three equal groups for each container (16 cm, 30 cm, and 60 cm) to study the effect of the hydric stress according to the depth of the container of seedlings. Table 2 represents the morphological characteristics of the seedlings before transplantation. The experimental design was fully randomized, for 3 months (from 5th of August to 3rd of November 2019) with two factors (seedling container depth and watering regime). Three watering regimes were applied on each of the depths (16 cm, 30 cm, and 60 cm), well-watered (T), medium stress (SM), and severe stress (SS), respectively 100%, 50%, and 25% of the field capacity. Soil moisture was measured daily by time reflectometry (FieldScout TDR 200 Soil Moisture Meter, Spectrum Technologies Inc. Plainfield, IL, USA) with 20 cm probes to maintain the moisture content of each treatment. Each treatment was composed of three blocks and each block had 10 argan seedlings (with 90 seedlings in total/ replicate). The methodological approach describing the experimental protocol followed to elaborate for the present study is illustrated in Figure 2.

Table 2. Morphological characteristics of seedlings before transplanting

	H (cm)	RCD (mm)	SW (g)	RW (g)	DQI
P1 (16 cm)	47.46 ± 3.42 a	6.03 ± 0.68 a	4.15 ± 0.78 a	3.23 ± 0.38 a	0.80 ± 0.09 a
P2 (30 cm)	57.06 ± 8.05 ab	8.27 ± 0.94 b	11.71 ± 1.81 b	9.92 ± 2.24 b	2.69 ± 0.63 ab
P3 (60 cm)	62.50 ± 6.20 b	9.33 ± 1.34 b	16.25 ± 5.64 b	14.68 ± 4.70 b	4.20 ± 2.16 b
p-value	**	***	***	***	**

H: height, RDC: root collar diameter, SW: shoot weight, RW: root weight, DQI: Dickson quality index. ** and ***: significant at $p \leq 0.01$ and $p \leq 0.001$. Values with the same letter are not significantly different at $p < 0.05$. Data represent means ± SE (n = 5). Letters represent significant differences.

**Figure 2.** Experimental protocol elaborated for the present study

Leaf water status of seedlings

Leaf water potential (Ψ_{pd})

Leaf water potential was measured before dawn (04:30 to 06:00 h) using a pressure chamber (Skye Instruments, Powys, UK) in the absence of transpiration (stomata closed in the dark) on the upper third of the plants. Measurements were taken in the upper part of the stem (3 cm) bearing five developed leaves. We opted for 3 replicates per treatment (one plant per replicate).

Stomatal conductance (gs)

Stomatal conductance was determined between 10.00 and 12.00 h using a leaf porometer (model SC-1, Decagon Devices, Pullman, USA). The measurements were made on the lower side (abaxial side) of two leaves per plant. We opted for 3 replicates per treatment (one plant per replicate).

Leaf relative water content (RWC)

At the end of the experiment, briefly, a sample of five leaves per seedling was weighed to obtain the fresh mass (FM). Sampled leaves were placed in test tubes containing distilled water in the dark at 4°C for 24 hours to obtain the turgid mass (TM). Then they were dried in an oven at 105°C for 48 hours to obtain the dry mass (DM). We opted for 3 replicates per treatment (one plant per replicate). The relative water content was calculated as follows according to the formula described by Saura-Mas and Lloret (2007) :

$$RWC (\%) = \frac{FM-DM}{(TM-DM)} \times 100 \quad (1)$$

Pigment, free proline, and soluble sugar content

Free proline

At the end of the experiment, free proline was determined by the method of Bates *et al.* (1973). Briefly, a 100 mg sample of the fresh leaf mass was finely ground for each treatment. A 4 mL solution of 3% sulfosalicylic acid solution was used to homogenize these samples. The extract was centrifuged at 15000 x g for 15 min. A 2 mL sample of the produced supernatant was treated with 2 mL of glacial acetic acid and 2 mL of 2.5% ninhydrin solution and boiled for 1 h. The reaction mixture was then extracted with toluene (3 mL). Toluene was used as a blank, and the absorbance was read at 520 nm. L-proline was used as a standard to determine the proline concentration. We opted for 3 replicates per treatment (one plant per replicate).

Soluble sugars

At the end of the experiment, the content of soluble sugars was determined according to Dubois *et al.* (1956). Samples of 100 mg of fresh leaves were homogenized in 10 mL of 80% ethanol. After recovery of the supernatant, it was treated with a solution of phenol (5%) and concentrated sulfuric acid. Total soluble sugar was calculated using glucose as a standard and absorbance read at 490 nm. We opted for 3 replicates per treatment (one plant per replicate).

Pigment content

At the end of the experiment, chlorophyll pigment contents were determined spectrophotometrically. Briefly, 100 mg of leaf material samples were ground in 10 mL of compound solution (75% acetone + 25% ethanol). The supernatant was recovered after centrifugation of the plant extract at 3000 rpm for 10 min. For chlorophyll a and b, absorbance was read at 663 nm and 645 nm, respectively. For carotenoids, the absorbance was read at 460 nm. We opted for 3 replicates per treatment (one plant per replicate). The content of carotenoids and chlorophylls (a and b) was calculated according to the equations proposed by Arnon (1949):

$$Chl\ a(\mu g/mL) = 12.7\ DO\ (663) - 2.69\ DO\ (645) \quad (2)$$

$$Chl\ b(\mu g/mL) = 22.9\ DO\ (645) - 4.68\ DO\ (663) \quad (3)$$

$$\text{Chl (a + b) } (\mu\text{g/mL}) = \text{Chl a} + \text{Chl b} \quad (4)$$

$$\text{Car } (\mu\text{g/mL}) = 5 \text{ DO (460)} - \frac{(3.18 \cdot \text{Chl a}) + (130.3 \cdot \text{Chl b})}{200} \quad (5)$$

Results were expressed as $\mu\text{g/g}$ fresh matter.

Electrolyte leakage (EL) and MDA and H₂O₂ levels

At the end of the experiment, the MDA content was determined according to the method of Hernandez and Almansa (2002). It was calculated based on its extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$. Absorbance was recorded at 532 nm and 600 nm for non-specific turbidity correction. Briefly, fresh samples (0.1 g) were homogenized in 2 mL of 0.1% (w/v) TCA and centrifuged at $15,000 \times g$ for 10 min at 4°C . 0.5 mL of the supernatant was then recovered and mixed with 1.5 mL of 0.1% (w/v) TCA. Then, another 0.5 mL of the supernatant was then collected and mixed with 1.5 mL of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA. The mixture was incubated in a water bath at 90°C for 20 minutes. After stopping the reaction in an ice bath, the samples were centrifuged at $10000 \times g$ for 5 min. We opted for 3 replicates per treatment (one plant per replicate).

Electrolyte leakage (EL) was determined from 100 mg of fresh leaf samples cut into 5 mm lengths and placed in test tubes containing 10 mL of deionized distilled water. The tubes were placed in a water bath and kept at a constant 32°C . After two hours, the initial electrical conductivity of the medium (EC1) was measured using an electrical conductivity meter. The samples were placed in an oven at 120°C for 120 minutes and then cooled to 25°C . The final electrical conductivity (EC2) was measured. We opted for 3 replicates per treatment (one plant per replicate). The EL was then calculated by the following equation (Nayyar, 2003):

$$\text{EL } (\%) = \frac{\text{EC1}}{\text{EC2}} \times 100 \quad (6)$$

The H₂O₂ content was measured by spectrophotometry according to Velikova *et al.* (2000). An amount of 100 mg of fresh plant material (leaves) powder was homogenized with 5 mL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at $10000 \times g$ for 10 min at 4°C . A 0.5 mL sample of supernatant was collected and 1 mL of 1 M potassium iodide and 0.5 mL of 10 mM potassium phosphate buffer, pH 7, were added. After 1 h of incubation in the dark at room temperature, the absorbance was determined at 390 nm and plotted on a standard H₂O₂ curve. We opted for 3 replicates per treatment (one plant per replicate).

Antioxidant enzymes

At the end of the experiment, the leaf samples were immediately put in liquid nitrogen to obtain a fine powder that will be stored at -20°C . The homogenization of the latter is used to extract the antioxidant enzymes. 100 mg sample of the finely ground powder was homogenized in 50 mM potassium phosphate buffer ($\text{K}_2\text{HPO}_4 / \text{KH}_2\text{PO}_4$) (pH 7.8) containing 5 mM 2-mercaptoethanol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) polyvinylpyrrolidone (PVP), 0.1 mM phenylmethanesulfonyl fluoride (PMSF) solution, and 0.2% (v/v) Triton X100 for enzyme (SOD and POD) determination. Regarding PPO, ($\text{K}_2\text{HPO}_4 / \text{KH}_2\text{PO}_4$) buffer (pH 5.5) was used. After centrifugation at $15,000 \times g$ at 4°C for 15 minutes, the supernatant was used to determine the enzyme activities, and the total protein content was determined using bovine serum albumin (BSA) as a standard (Bradford, 1976).

The enzymatic activity of SOD was determined by its ability to inhibit the photochemical reduction of nitrotetrazolium blue chloride (NBT) at 560 nm by the method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme that induces 50% inhibition of NBT reduction.

According to the method described by Lin *et al.* (2019), the activity of SOD was determined, based on the rate, of tetraguaiacol production. The extinction coefficient of $25.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 436 nm was adopted. According to Moore and Flurkey (1990), PPO activity was measured at 410 nm by following the oxidation of

catechol. PPO activity is expressed in enzyme units ($U\ mg^{-1}\ protein$) due to the uncertainty in the molar extinction coefficient of the catechol oxidation product. We opted for 3 replicates per treatment (one plant per replicate).

Statistical analysis

To evaluate the effects of watering and container depth treatments and their interaction on physiological and biochemical variables. The data were subjected to several analyses of variance (ANOVA). Separation of the means was performed by Tukey's post-hoc test at $p \leq 0.5$. Before ANOVA, the data were checked for normality and variance homogeneity. All treatments were made in triplicate. Principal component analysis (PCA) was performed to be able to determine the container depth that best responds to water stress. All analyses were performed using R Studio software version 4.1.0.

Results

Leaf water status of seedlings

The water stress treatments significantly affected the water status of argan seedlings from the three containers (16, 30, and 60 cm) (Table 3). No differences were reported on the interaction between container depth and watering regime (Table 3). Compared to well-watered seedlings (T), the water stress treatments (SM and SS) showed decreases in leaf water potential (Ψ_{pd}), stomatal conductance (gs), and relative water content (RWC) of seedlings from the three containers at different depths (Table 4). However, seedlings from the 16 cm deep container represented low stomatal conductance (gs) and relative water content (RWC) values and consequently lower leaf water potential (Ψ_{pd}) values (Table 4).

Table 3. Results of a two-way ANOVA on the effects of container depth, water stress and their interaction on physiological and biochemical of *Argania spinosa*: Ψ_{pd} , gs, RWC, electrolyte leakage (EL), Chl (a+b), Car, soluble sugars, free proline, hydrogen peroxide (H_2O_2), Malondialdehyde (MDA), Superoxide dismutase (SOD), Peroxidase (POD), and Polyphenol oxidase (PPO).

Variables	Squares means and their significances		
	Container depth	Water stress	Container depth * Water stress
Ψ_{pd} (MPa)	0.14***	3.67***	0.02 ns
Gs	357.1***	12364.7***	45.4 ns
RWC	183.75***	5063.9***	29.45 ns
Chl (a+b)	70766 ***	898154 ***	7852.5 ns
Car	7935 ***	49531.5 ***	7530 ***
Soluble sugars	117.9 ***	3981.9 ***	23.05 **
Free proline	0.1 ***	3.28 ***	0.03 ***
H_2O_2	155.25 ***	5312.96 ***	106.95 ***
MDA	190.4 ***	8174.65***	57.05***
EL	59.5***	9541.85***	5.9 ns
SOD	190638.5 ***	7106679.5 ***	69821 ***
POD	0.13 ***	1.77 ***	0.04 ns
PPO	30122 ***	4954538.5 ***	9430 *

Note: ns not significant * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, all treatments were performed in triplicate.

Table 4. Mean values of leaf water potential (Ψ_{pd}), stomatal conductance (gs) and relative water content (RWC) of *Argania spinosa* seedlings grown on three containers of different depths (P1, P2, and P3 respectively 16 cm; 30 cm and 60 cm deep) exposed to three water regimes [well-watered (C), medium stress (MS) and severe stress (SS), respectively 100%, 50% and 25% of field capacity].

Depths	Water regimes	Ψ_{pd} (MPa)	gs ($\text{mmol m}^{-2} \text{s}^{-1}$)	RWC (%)
P1	C	-2.96 ± 0.04 e	108.66 ± 4.68 d	90.09 ± 0.75 e
	MS	-3.92 ± 0.08 bc	65.23 ± 0.45 c	62.54 ± 0.72 c
	SS	-4.25 ± 0.01 a	33.43 ± 2.13 a	37.75 ± 2.56 a
P2	C	-2.82 ± 0.07 e	113.23 ± 2.69 d	93.44 ± 1.20 e
	MS	-3.76 ± 0.04 cd	71.26 ± 1.66 c	70.24 ± 2.36 d
	SS	-4.08 ± 0.10 ab	44.00 ± 1.64 b	47.43 ± 1.98 b
P3	C	-2.81 ± 0.08 e	123.83 ± 4.42 e	94.06 ± 1.02 e
	MS	-3.59 ± 0.08 d	73.30 ± 3.90 c	71.73 ± 5.11 d
	SS	-3.97 ± 0.10 bc	47.90 ± 1.96 b	50.10 ± 2.45 b

Note: Means for each trait followed by the same letter are not significantly different at the 5% level (Tukey test).

Pigment, free proline, and soluble sugar content

Chlorophyll and carotenoid contents of seedlings decreased in response to water stress and container depth (Tables 3 and 5). However, the lowest values were recorded on the seedlings from the short depth container (16 cm) (Table 5). The concentration of proline and soluble sugar increased on the seedlings from the containers of different depths under moderate and severe water stress (Table 5). Nevertheless, the proline content was much more pronounced in the seedlings from the 16 cm deep container during the moderate and severe stress. Seedlings from the 30 and 60 cm deep containers showed decreased proline levels even at severe stresses compared to those found in the 16 cm deep containers. Soluble sugar concentration increased in response to water stress. In contrast, the highest value was recorded in seedlings from the 16 cm deep container under severe stress (Table 5).

Table 5. Mean values of chlorophyll (a+b) (Chl (a+b)), carotenoids (Car), free proline, and soluble sugars of *Argania spinosa* seedlings grown on three containers of different depths (P1, P2 and P3 respectively 16 cm; 30 cm, and 60 cm deep) exposed to three water regimes [well-watered (C), medium stress (MS), and severe stress (SS), respectively 100%, 50%, and 25% of field capacity].

Depths	Water regimes	Chl (a+b) ($\mu\text{g/g}$ fr wt)	Car ($\mu\text{g/g}$ fr wt)	Soluble sugars (mg/g fr wt)	Free proline (mg/g fr wt)
P1	C	1238.59 ± 3.09 d	226.77 ± 7.58 bc	29.76 ± 1.15 a	0.46 ± 0.003 b
	MS	824.80 ± 11.90 b	139.69 ± 1.74 a	47.46 ± 1.02 c	1.19 ± 0.01 d
	SS	558.34 ± 7.48 a	128.54 ± 4.66 a	74.77 ± 0.18 e	1.81 ± 0.004 f
P2	C	1256.51 ± 12.94 de	275.83 ± 3.21 de	28.16 ± 0.96 a	0.43 ± 0.006 ab
	MS	966.14 ± 33.14 c	205.78 ± 23.83 b	40.85 ± 3.81 b	0.92 ± 0.003 c
	SS	634.51 ± 93.32 a	130.33 ± 3.27 a	67.73 ± 0.97 d	1.55 ± 0.02 e
P3	C	1361.03 ± 50.66 e	308.49 ± 33.59 e	26.88 ± 0.32 a	0.41 ± 0.009 a
	MS	1020.29 ± 33.80 c	254.10 ± 9.79 cd	37.86 ± 1.02 b	0.92 ± 0.005 c
	SS	771.31 ± 9.28 b	107.28 ± 4.07 a	66.24 ± 0.32 d	1.57 ± 0.01 e

Note: Means for each trait followed by the same letter are not significantly different at the 5% level (Tukey test).

Electrolyte leakage (EL) and MDA and H_2O_2 levels

EL, MDA and H_2O_2 contents were evaluated in *Argania spinosa* leaves as a metabolic indicator of their oxidative stress status. Water stress significantly impacted H_2O_2 and MDA and EL levels in seedlings from the three containers at different depths (Table 6). Compared to the (well-watered) treatments, the water stress treatments (MS and SS) increased H_2O_2 , MDA, and EL content. Seedlings from the deeper containers (30 and 60 cm) showed low values of H_2O_2 , MDA, and EL content in all water stress treatments (Table 6). The

interaction of container depth and water stress had a significant effect on H₂O₂ and MDA levels except for EL (Table 3).

Table 6. Mean values of electrolyte leakage (EL), hydrogen peroxide (H₂O₂), and malondialdehyde (MDA) of *Argania spinosa* seedlings grown on four containers of different depths (P1, P2, and P3 respectively 16 cm; 30 cm, and 60 cm deep) exposed to three water regimes [well-watered (C), medium stress (MS), and severe stress (SS), 100%, 50%, and 25% of field capacity respectively].

Depths	Water regimes	EL (%)	MDA (nmol /g FW)	H ₂ O ₂ (nmol /g FW)
P1	C	36.33 ± 1.32 a	33.37 ± 0.30 b	50.44 ± 0.31 b
	MS	65.10 ± 0.91 b	63.13 ± 1.81 e	82.05 ± 0.21 d
	SS	100.43 ± 3.04 c	99.78 ± 0.78 h	119.01 ± 0.43 g
P2	C	32.87 ± 1.87 a	33.03 ± 0.51 b	49.47 ± 0.58 ab
	MS	59.67 ± 0.56 b	54.19 ± 0.51 d	77.35 ± 0.50 c
	SS	96.76 ± 2.85 c	90.15 ± 0.78 g	107.86 ± 0.99 f
P3	C	30.51 ± 1.87 a	30.62 ± 0.78 a	47.97 ± 0.26 a
	MS	59.95 ± 3.94 b	50.58 ± 0.51 c	77.65 ± 0.50 c
	SS	97.36 ± 1.56 c	86.70 ± 0.51 f	100.44 ± 1.08 e

Note: Means for each trait followed by the same letter are not significantly different at the 5% level (Tukey test).

Antioxidant enzymes

The effects of container depth and watering regime and their interaction on the activities of antioxidant enzymes (SOD, PPO, and POD) are presented in Table 3. Compared with seedlings grown under well-watered conditions, regardless of container depth, moderate or severe water stress significantly increased the activities of these enzymes (Table 7) ($p < 0.001$). Water stress (MS and SS) significantly increased the activities of antioxidant enzymes on seedlings from short container depths (16 cm) (Table 7).

Table 7. Mean values of superoxide dismutase (SOD), peroxidase (POD), and polyphenol oxidase (PPO) of *Argania spinosa* seedlings grown on four containers of different depths (P1, P2, and P3 respectively 16 cm; 30 cm, and 60 cm deep) exposed to three water regimes [well-watered (C), medium stress (MS), and severe stress (SS), 100%, 50%, and 25% of field capacity respectively].

Depths	Water regimes	SOD (U mg ⁻¹ protein)	POD (nmol min ⁻¹ mg ⁻¹ protein)	PPO (U mg ⁻¹ protein)
P1	C	491.49 ± 41.81 a	556.84 ± 57.95 a	872.78 ± 13.22 a
	MS	1592.97 ± 14.57 c	1099.13 ± 66.85 bc	1750.71 ± 26.05 b
	SS	2457.03 ± 85.38 e	1590.24 ± 47.20 e	2370.96 ± 41.51 d
P2	C	464.17 ± 28.45 a	571.39 ± 112.62 a	817.14 ± 17.15 a
	MS	1242.98 ± 38.83 b	1089.61 ± 66.77 bc	1689.76 ± 7.46 b
	SS	2149.63 ± 6.75 d	1447.09 ± 91.55 de	2300.71 ± 28.36 d
P3	C	449.20 ± 27.70 a	511.38 ± 73.03 a	793.74 ± 2.24 a
	MS	1248.88 ± 46.86 b	818.46 ± 237.80 ab	1696.74 ± 42.53 b
	SS	2097.44 ± 11.81 d	1248.50 ± 100.12 cd	2205.60 ± 61.20 c

Note: Means for each trait followed by the same letter are not significantly different at the 5% level (Tukey test).

Principal component analysis (PCA)

The principal component analysis (PCA) performed is based on the use of average data values to establish similarities between different treatments (watering regime) and different variables (Figure 3). The results showed that 98.2% of the total observed variability was explained by the first two dimensions (Dim1 and Dim 2). Dimension 1 (Dim 1) accounted for 96.8% of the variability while 1.4% of the variability was represented by dimension 2 (Dim 2) (Figure 3 (C) and (D)). Antioxidant enzymes, H₂O₂, MDA, soluble sugar, and proline were positively correlated with the first dimension (Dim1) (Figure 3 (A)). Chlorophylls (a+b), leaf

water potential (Ψ_{pd}), stomatal conductance (gs), and carotenoid content (car), on the other hand, were negatively correlated with the first dimension (Dim 1) (Figure 3 (A)). PCA provided a clear visualization between seedlings from containers of different depths associated with different treatments (watering regime) (Figure 3(B)). The results revealed that the container depth responds better to severe stress. Indeed, the severe stress treatment associated with seedlings from the 16 cm (P1_SS) and 30 cm (P2_SS) deep containers were characterized by higher values of antioxidant enzymes (SOD, POD and PPO), H_2O_2 , soluble sugar, and proline on the left side, while seedlings from the 60 cm deep container (P3_SS) were less correlated by these variables. The medium stress treatment associated with the 16 cm deep seedlings showed more effect of water stress compared to the seedlings from the 60 cm deep container.

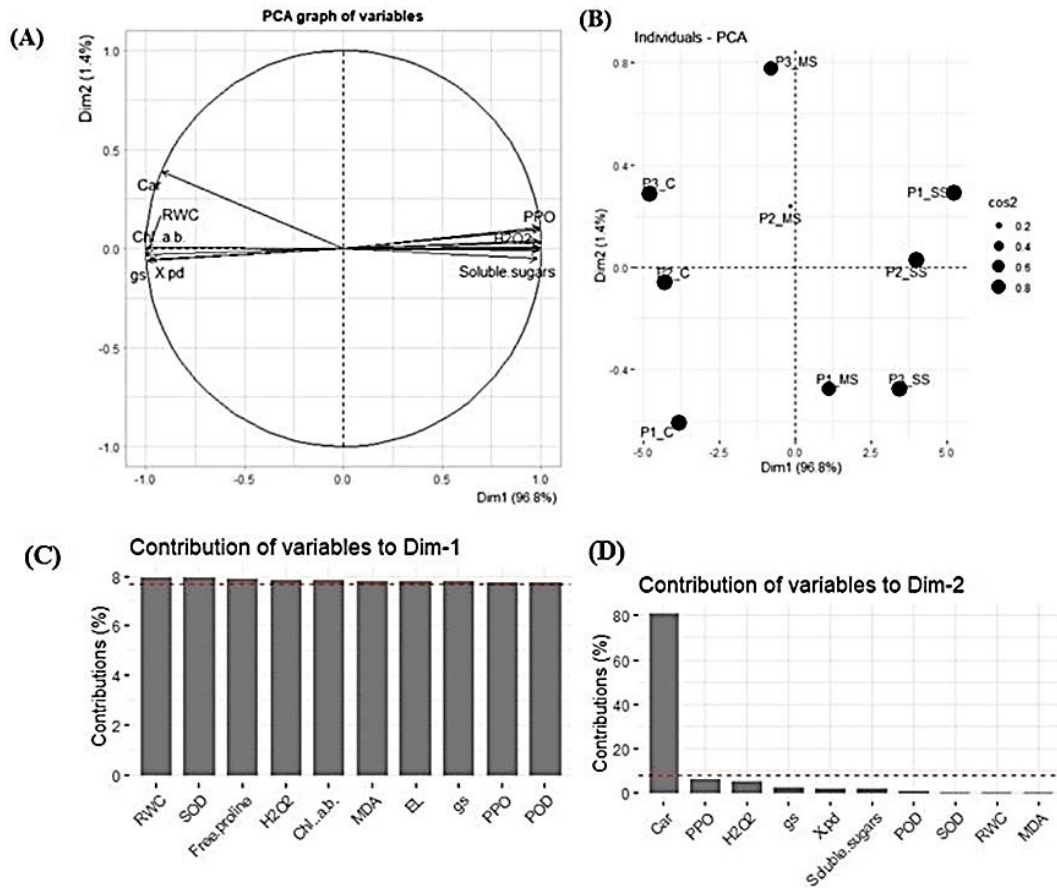


Figure 3. Principal component analysis of the studied variables (A) and applied treatments (B) on *Argania spinosa* seedlings from the containers of different depths after 3 months of transplanting and subjected to the watering regime treatment (water stress). Contribution of variables to dimension 1 (C) and dimension 2 (D)

Ψ_{pd} : leaf water potential, gs : stomatal conductance, RWC: relative water content, Car: carotenoids, EL: electrolyte leakage, SOD: superoxide dismutase, POD: peroxidase, PPO: polyphenol oxidase, H_2O_2 : hydrogen peroxide, MDA: malondialdehyde, Chl (a+b): chlorophyll (a+b), free proline, soluble sugars. P1_C: seedlings from the 16 cm deep container and well-watered, P1_MS: seedlings from the 16 cm deep container and medium stress, P1_SS: seedlings from the 16 cm deep container and severe stress, P2_C: seedlings from the 30 cm deep container and well-watered, P2_MS: seedlings from the 30 cm deep container and medium stress, P2_SS: seedlings from the 30 cm deep container and severe stress, P3_C: seedlings from the 60 cm deep container and well-watered, P3_MS: seedlings from the 60 cm deep container and medium stress, P3_SS: seedlings from the 60 cm deep container and severe stress.

Discussion

Deep containers improve the water transport capacity of the root system, which contributes to a better water status under drought stress conditions (Chirino *et al.*, 2008). The present study allowed us to evaluate the physiological and biochemical responses of *Argania spinosa* seedlings from containers of different depths to water stress. Water stress and shallow containers reduced water status and total chlorophyll and carotenoid contents, and increased osmolyte accumulation (Proline and soluble sugars), H₂O₂, EL, and MDA contents, and seedling antioxidant enzyme activities. In this study, under water stress, a relationship between leaf water potential and stomatal conductance and between stomatal conductance and relative water content was found in all seedlings produced in containers of different depths. In particular, this relationship indicated stomatal closure which was very important in preventing water loss. Several studies have shown that changes in leaf water potential and/or soil water content significantly cause stomatal closure (Pita *et al.*, 2005; Chakhchar, 2015; Chakhchar *et al.*, 2015). Our results showed that all stressed *Argania spinosa* seedlings exhibited more negative leaf water potential values and more closed stomata with increasing water stress. These results are in agreement with those of several studies in which qualitative relationships between leaf water potential and stomatal closure have been described (Wahbi *et al.*, 2005; Chakhchar *et al.*, 2015; Chakhchar, 2015). Nevertheless, the most negative values of leaf water potential and small values of stomatal conductance were recorded in the seedlings produced in the short depth containers (16 cm) compared to the seedlings in the other two long depth containers. This indicates that seedlings produced in deep containers (30 and 60 cm) had reduced adverse effects of water stress compared to seedlings produced in short containers (16 cm). Chirino *et al.* (2008) showed that *Quercus suber* (L) seedlings produced in deep containers (30 cm) expressed higher stomatal conductance under drought conditions. According to the same authors, these results are related to a longer taproot, a higher root hydraulic conductance, and a higher number of new roots colonizing the deepest layers offering greater water availability. Leaf relative water content (RWC) is considered a good indicator for assessing the water status of seedlings (Garnier *et al.*, 2001; Chakhchar *et al.*, 2015; Jafarnia *et al.*, 2018). Our results showed that the lowest RWC values were recorded in seedlings produced from short containers (16 cm) under the moderate and severe water stress treatments. This suggests that producing seedlings in deeper containers improves the water status of seedlings under water stress. Pemán *et al.* (2006) pointed out that the deep container indeed promotes a more efficient root system for water transport compared to the short container.

Pigment content is a good indicator of the plant's condition (Fassnacht *et al.*, 2015; Jafarnia *et al.*, 2018). According to them, the pigment content can be used for the evaluation of the photosynthetic capacity and activity of plants. Smirnoff (1993) pointed out that under water stress, reduction in chlorophyll content is a common response due to photooxidation or chlorophyll degradation. Our results showed very significant decreases in total chlorophyll (Chl a+b) and carotenoid contents in *Argania spinosa* seedlings produced in all studied containers under water stress treatments. Similarly, several studies have highlighted the reduction in chlorophyll and carotenoid content under water stress conditions (Ben Ahmed *et al.*, 2009; Chakhchar, 2015; Jafarnia *et al.*, 2018). However, in this study, the lowest chlorophyll and carotenoid contents are recorded in seedlings produced in the 16 cm deep containers. This suggests that under water stress, the photosynthetic capacity and activity of seedlings produced in shallower containers are likely to be much more affected by water stress than those produced in deeper containers (30 and 60 cm).

Based on our results, all stressed *Argania spinosa* seedlings (medium and severe stress) recorded a significant accumulation of proline as stress increased. Also, other studies have shown the increase of proline under water stress in *Argania spinosa* and other tree species (Chakhchar, 2015; Jafarnia *et al.*, 2018). Nevertheless, seedlings produced in the short depth containers (16 cm) recorded the highest proline content compared to seedlings produced in the other two deep containers. Likewise, this shows the advantages *Argania spinosa* seedlings produced in deep containers gain compared to those produced in short containers. Furthermore, Szabados and Savoure (2010) pointed out that proline also acts as an osmotic molecule and thus

protects the plant cell from ROS. It also plays an important biological role in the stress response by reducing dehydration damage (Deligoz and Gur, 2015).

As for proline, soluble sugars accumulation is also related to water stress resistance in plants (Chakhchar, 2015; Jafarnia *et al.*, 2018). Our results showed a strong accumulation of soluble sugars as water stress increased in seedlings produced in all containers. Soluble sugars accumulation under water stress has been observed repeatedly in *Argania spinosa* and other species (Chakhchar, 2015; Jafarnia *et al.*, 2018). However, under water stress, seedlings produced in the 16 cm deep containers had the highest soluble sugars contents compared to those produced in the deep containers (30 and 60 cm). Ashraf and Harris (2004) pointed out that under water stress, the accumulation of soluble sugar protects membranes and proteins in cells exposed to stress. Morkunas and Ratajczak (2014) suggested that sugars enhance the oxidation process and increase cell wall lignification.

Electrolyte leakage is considered to be an indicator of membrane stability and cellular integrity reflecting the degree of stress damage to the plant (Kocheva *et al.*, 2004; Jafarnia *et al.*, 2018). In addition, to measure the degree of lipid peroxidation caused by oxidative stress, the MDA content is evaluated (Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021). In the present study, MDA content increased with increasing water stress in stressed seedlings produced in the three containers and was concomitant with increasing electrolyte leakage. These results indicate that lipid peroxidation increased cell membrane permeability. Studies have shown that lipid peroxidation indicates changes in the cell membrane (Santos *et al.*, 2005; Jafarnia *et al.*, 2018). These changes cause increased electrolyte leakage and osmotic imbalance (Santos *et al.*, 2005). Several previous studies have reported similar results in increased MDA content and electrolyte leakage in plants under water stress (Cotrozzi *et al.*, 2016; Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021). Nevertheless, we found that under water stress, seedlings produced in short depth containers (16 cm) have the highest MDA and EL contents compared to seedlings produced in deep containers (30 and 60 cm). This suggests that *Argania spinosa* seedlings produced in deep containers (30 and 60 cm) have fewer adverse effects of oxidative stress than seedlings produced in 16 cm deep containers. Under water stress, ROS (including hydrogen peroxide) are one of the primary messengers in plants (Talbi *et al.*, 2015; Jafarnia *et al.*, 2018). Thus, their increase is a response to water stress and this has been proven (Shi *et al.*, 2015; Jafarnia *et al.*, 2018; Hachemi *et al.*, 2021). In the present study, H₂O₂ content increased with increasing water stress intensity in all stressed seedlings produced in all containers. Nevertheless, seedlings produced in short containers (16 cm) recorded the highest H₂O₂ contents compared to seedlings produced in deep containers (30 and 60 cm). These results indicate that *Argania spinosa* seedlings produced in the 16 cm deep containers are exposed to oxidative stress compared to seedlings produced in the deep containers.

Various defense mechanisms, including enzymatic antioxidant mechanisms, can prevent excess ROS and increased lipid peroxidation (Chakhchar, 2015; Hachemi *et al.*, 2021). In this study, the activities of antioxidant enzymes, including SOD, POD, and PPO increased in *Argania spinosa* seedlings with the intensity of water stress. In *Argania spinosa*, studies have shown a significant increase in the activities of these enzymes, confirming their importance as effective defense mechanisms against oxidative damage (Chakhchar, 2015; Chakhchar *et al.*, 2015; Hachemi *et al.*, 2021). The most powerful antioxidant enzyme and the first line of defense against ROS are SOD (Sayfzadeh *et al.*, 2011). POD plays a very important role in preventing oxidative damage and maintaining the integrity of the cell membrane by eliminating MDA and decreasing the H₂O₂ content (Chakhchar *et al.*, 2015). The catalysis of the o-hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to quinones in the presence of oxygen is performed by PPO (Rivero *et al.*, 2001). Our results also indicate that seedlings produced in deep containers recorded lower antioxidant enzyme activities compared to seedlings produced in 16 cm deep containers. These results indicate that producing seedlings in deep containers optimizes water stress tolerance strategies in *Argania spinosa* seedlings.

The use of deep rigid containers will increase the costs of seedling production (container, amount of substrate, irrigation, etc.) as well as those of transplanting (suitable transportation, preparation of deep pots, number of seedlings planted/hour, etc.) (Zine El Abidine *et al.*, 2016). However, deep containers play a significant role in producing physiologically and biochemically robust seedlings that can better tolerate the

shock of transplanting in semi-arid and arid zones, especially water stress (medium and severe stress). This can allow significant survival rates in reforestation programs in semi-arid and arid areas. Nevertheless, the manufacture of deep rigid containers must take into account the practical aspect (in the nursery and during transport). For example, by increasing the depth of the rigid containers, manufacturers can decrease the number of cells so that when filling the substrate, the containers are not too heavy. The key is to have containers that allow the root systems of taproot forest plants such as the argan tree to develop well. This will allow nursery managers to have morphologically, physiologically and biochemically balanced plants.

Conclusions

The depth of the nursery container is an important factor in the production of seedlings of satisfactory quality that can withstand transplanting shock in semi-arid areas. In the present study, we evaluated the physiological and biochemical behavior of *Argania spinosa* seedlings from containers of different depths to water stress to test their resistance capacity. Overall, our results showed the existence of a systematic relationship between the responses to water stress and the depth of the container. Indeed, under water stress conditions (MS and SS), seedlings from the deep containers recorded, as container depth increased, better leaf water status, higher chlorophyll pigment levels, free proline, soluble sugars, H₂O₂, and MDA levels, lower electrolyte leakage, and reduced antioxidant enzyme activities compared to seedlings from the short containers (16 cm). In addition, PCA showed that seedlings from the deep containers (30 and 60 cm) tolerated water stress better than those from the 16 cm containers. These results confirmed our hypothesis that container depths contribute to better adaptation of seedlings, such as *Argania spinosa*. These findings suggest that the production of argan seedlings requires deep containers to have better quality seedlings that can withstand the transplant shock of semi-arid areas. However, to fully confirm these findings, further long-term field research is needed.

Authors' Contributions

Conceptualization and methodology: OSA, AH and SEM; Data curation and Formal analysis OSA and AH; Writing - original draft: OSA and AH; Writing - review and editing: OSA, AH, AM, AL, TB, and SEM. All authors read and approved the final manuscript.

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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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