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Effect of treated wastewater irrigation and mycorrhizal inoculation on *Olea europaea*: Physiological, anatomical and morphological responses

Ameni Ben Hassena [a,b,d], Lina Trabelsi [c], Mohamed Zouari [c,d], Raphaël Decou [d], Mohamed Ali Triki [a], Wahid Khabou [a], Pascal Labrousse [d,*], Nacim Zouari [b]

[a] *Laboratory LR16IO01, Olive Tree Institute of Sfax, University of Sfax, Sfax, Tunisia*
[b] *Higher Institute of Applied Biology of Medenine, University of Gabes, Medenine, Tunisia*
[c] *Laboratory LR16IO02, Olive Tree Institute of Sfax, University of Sfax, Sfax, Tunisia*
[d] *University of Limoges, E2Lim UR 24133, Limoges, France*

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ABSTRACT

This study evaluated the effects of irrigation with saline treated wastewater (TWW) (EC = 5.84 dS m^{-1}) and inoculation with arbuscular mycorrhizal fungi (AMF) (*Glomus deserticola*, *Gigaspora margarita* or a combined inoculum of *G. deserticola* and *G. margarita*) on the physiological, anatomical and morphological performance of young olive plants (*Olea europaea* L. cv. Chetoui) over a 12-month period. Irrigation with TWW resulted in an increase in leaf water saturation deficit and water uptake capacity, along with a decrease in the membrane stability index, compared to control plants irrigated with tap water. Consequently, these changes negatively affected gas exchange parameters and total chlorophyll content, which were consistent with corresponding changes in leaf anatomy and morphology. However, principal component analysis (PCA) demonstrated that mycorrhizal symbiosis improved leaf water status and photosynthetic capacity, as compared to non-inoculated plants. These benefits were closely associated with enhanced anatomical and morphological traits in mycorrhizal plants. Overall, the results suggest that inoculated olive plants were less stressed and more resilient to the salinity stress caused by TWW irrigation.

1. Introduction

The agricultural sector is highly vulnerable to the impacts of climate change [1]. The dramatic increase in average global temperature and the variation in precipitations (in both frequency and intensity) affect agricultural productivity and, consequently, global food security [2]. Some countries, such as Tunisia, where freshwater resources are limited, are particularly threatened and highly vulnerable [3]. To meet the water demand for agricultural purposes, the reuse of treated wastewater (TWW) may provide a viable solution [1,3,4]. In Tunisia, TWW is authorized for irrigating various fruit trees, including lemon tree (*Citrus limon* L.), date palm (*Phoenix dactylifera* L.), grapevine (*Vitis vinifera* L.) and olive (*Olea europaea* L.) [5]. Although TWW is rich in nutrients, it can contain higher levels of Na^{+} and Cl^{-}, compared to conventional water sources [4,6]. Many studies have investigated the effect of TWW on physiological, morphological and/or anatomical performance of various plant species, although discrepancies have been reported [4,7–11]. The responses of plants irrigated with TWW depend on several factors, including plant species, chemical composition of TWW, duration of irrigation, irrigation system, and environmental conditions [12–14].

Due to its significant economic and social importance, the olive tree is one of the most widely cultivated crops in Mediterranean countries [15,16]. The olive tree is a rustic plant able to grow and produce fruits under various environmental conditions [17]. However, olive trees are increasingly exposed to stresses, such as prolonged droughts, as a result of climate change. Consequently, irrigation is essential to ensure crop survival and productivity under these conditions. To deal with this challenging situation, irrigation with TWW may offer a promising solution for olive cultivation. Nevertheless, it is well established that olive tree is slightly tolerant to salinity. Indeed, irrigation water with an electrical conductivity (EC) exceeding 5.5 dS m^{-1} can cause physiological disruptions in plants [18]. As a result, depending on the salt concentration, TWW irrigation may induce salt stress in olive trees. According to the literature, olive trees can cope with this stress through

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[*] Corresponding author. Université de Limoges, E2Lim UR 24 133, Laboratoire de Botanique et cryptogamie, Faculté de Pharmacie, 2, rue du Dr Marcland, Cedex, 87025, Limoges, France.

E-mail address: pascal.labrousse@unilim.fr (P. Labrousse).

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physiological, morphological and anatomical adaptations [16, 19, 20, 21] [][][]. However, the capacity for these adaptations is limited. Inoculation of plant with arbuscular mycorrhizal fungi (AMF) is an effective strategy to enhance salt stress tolerance [22]. AMF are ubiquitous soil microorganisms that form symbiotic relationships with the majority of plant species, including the olive tree [15,23]. Under normal conditions, AMF enhance water and nutrient uptake through extra-radical hyphae, leading to improved plant growth [24,25]. Under saline conditions, AMF help host plants cope with stress through various physiological and morphological mechanisms, including (a) improving osmotic homeostasis; (b) regulating gas exchange parameters; (c) modulating stomatal aperture, and (d) enhancing growth parameters [22,26].

To date, no studies have investigated the anatomical response of Chetoui olive tree subjected to TWW irrigation and AMF inoculation. Therefore, the main objective of this investigation was to study the impact of TWW and AMF inoculums (*Glomus deserticola*, *Gigaspora margarita* or a combined inoculum of *G. deserticola* and *G. margarita*) on various physiological parameters (water relations and gas exchange), as well as on morphological and anatomical traits of olive leaves.

Can these parameters serve as biomarkers for stress adaptation? Could AMF play a pivotal role in mitigating the effects of saline TWW on olive plant development? These are the two main questions this study aims to address.

2. Materials and methods

2.1. Plant material and treatments

Experiments were carried out at the Olive Tree Institute in Sfax, Tunisia (34°43′N, 10°41′E). One-year-old *Olea europaea* L. cv. Chetoui plants were transplanted into 20-L plastic pots, filled with sieved and sterilized sandy loam soil (75.88 % sand, 16.29 % silt and 7.83 % clay). The soil was sterilized by autoclaving (Tuttnauer, model 2540, USA) at 121 °C for 30 min. The soil pH was 8.1, its electrical conductivity (EC) was 0.63 dS m^{-1}, and its organic matter content was 0.92 %. The olive plants were inoculated with 5 g of commercial arbuscular mycorrhizal fungal (AMF) inoculum consisting of spores and hyphal tissue fragments, supplied by Agrauxine-Biorize, (Dijon, France). Control plants were supplemented with a mixture of 2.5 g of *G. deserticola* and 2.5g of *G. margarita* inoculum, previously autoclaved. For treated plants: either 5g of *G. deserticola* (Gd treatment) or 5g of *G. margarita* (Gm treatment) or a mixture of 2.5g *G. deserticola* and 2.5 g *G.margarita* (Gd + Gm treatment) powder were placed below the roots of each plant. To confirm the establishment of mycorrhizal symbiosis, the roots of each plant were inspected before and after the experiment using the staining method of Phillips and Hayman (1970) [27]. At the beginning of the experiment, the mycorrhizal colonization rates, assessed as mycelium, vesicles, or arbuscules, was 67.78, 66.67, and 78.89 %, respectively, in plants inoculated with *G. deserticola*, *G. margarita* and a combined inoculum of *G. deserticola* and *G. margarita*. By the end of the experiment, the accumulation of Na^+ and Cl^- in the soil slightly decreased these colonization rates by 26.23, 21.67 and 21.13 %, respectively. As expected, non-inoculated plants, had no colonized roots [28].

The cultures were maintained in the field, and the pots were covered with plastic film to prevent rain interference during the experiment. During the culture period, air temperature ranged between 17 ± 3 and 32 ± 3 °C and relative humidity varied from 53 to 74 %. To facilitate the establishment of symbiosis, olive plants were irrigated twice a week for two months with tap water, applying an amount below the weight at field capacity (90 % field capacity). After this acclimation period, plants were irrigated with either tap water or treated wastewater (TWW) for 12 months according to the following treatments (7 olive plants per treatment).

1. M^- TW: without mycorrhiza (M^-) and irrigated with tap water (TW), referred as control plants;
2. M^- TWW: without mycorrhiza (M^-) and irrigated with treated wastewater (TWW);
3. Gd TWW: inoculated with *Glomus deserticola* (Gd) and irrigated with TWW;
4. Gm TWW: inoculated with *Gigaspora margarita* (Gm) and irrigated with TWW;
5. Gd + Gm TWW: inoculated with a mixture of *G. deserticola* and *G. margarita* (1:1) (Gd + Gm) and irrigated with TWW.

TWW was supplied by the Southern Sfax wastewater treatment plant (Tunisia) where it underwent secondary biological treatment. The chemical properties of tap water and TWW used in the experiment are presented in Table 1.

During the experiment, to prevent drainage, all plants were irrigated twice a week at 90 % of field capacity. According to Ben Hassena et al. (2021) [13], the weight of each pot at field capacity was recorded before the experiment began. Each pot was irrigated with an excess volume of water to saturate the soil and reach its water holding capacity. The pots were left to equilibrate overnight until drainage became negligible, then weighed to determine the weight at 100 % field capacity. Prior to each irrigation, all pots were weighed, and the difference between the weight at 100 % field capacity and the current pot weight was measured. This difference was used to calculate the volume of irrigation water needed to reach field capacity and, subsequently, the volume required to maintain 90 % field capacity.

The lack of drainage over an extended period leads to salt accumulation in the soil, allowing for the examination of the response of young olive plants and AMF to such conditions. During the experimental period, drainage occurred only once.

2.2. Leaf water relation measurements

The water status of olive leaves was assessed through the water saturation deficit (WSD) and the water uptake capacity (WUC). These parameters were calculated, at the end of the experiment, using the following equations [29].

$$\text{WSD}\ (\%) = (\text{TW} - \text{FW})/(\text{TW} - \text{DW}) \times 100$$

$$\text{WUC} = (\text{TW} - \text{FW})/\text{DW}$$

Table 1
Chemical properties of the water sources employed for irrigation in the experiment.

Parameter	Tap water	Treated wastewater
pH	7.74 ± 0.34	7.95 ± 0.27
EC (dS m^{-1})	2.28 ± 0.06	5.84 ± 0.40
SO_4 (mg L^{-1})	471.90 ± 25.15	916.36 ± 19.01
HCO_3 (mg L^{-1})	368.95 ± 8.13	888.00 ± 2.82
NO_3 (mg L^{-1})	1.49 ± 0.25	14.88 ± 1.24
NH_4 (mg L^{-1})	2.02 ± 0.07	66.50 ± 2.37
P_{tot} (mg L^{-1})	0.01 ± 0.00	4.23 ± 0.29
K (mg L^{-1})	10.00 ± 1.00	66.67 ± 2.89
Na (mg L^{-1})	370.00 ± 14.14	730.00 ± 10.00
Cl (mg L^{-1})	592.07 ± 10.64	1465.39 ± 16.38
Ca (mg L^{-1})	130.00 ± 5.00	266.67 ± 5.77
Mg (mg L^{-1})	64.33 ± 6.03	105 ± 9.54
Zn (mg L^{-1})	<0.01	1.02 ± 0.18
Fe (mg L^{-1})	0.08 ± 0.00	0.98 ± 0.10
Cu (mg L^{-1})	0.02 ± 0.00	0.10 ± 0.01
Mn (mg L^{-1})	0.031 ± 0.00	0.09 ± 0.00
Cd (μg L^{-1})	0	<0.01
Pb (μg L^{-1})	0	<0.01
Cr (μg L^{-1})	0	0
Co (μg L^{-1})	0	0

Values represent the means of 3 replications ± SD.

where TW, FW and DW represent turgid, fresh and dry weights, respectively. The fresh weight (FW) was measured immediately after harvest using an Ohaus Pioneer TM PA214 balance (NJ, USA). Turgid weight (TW) was determined after immersing the leaves in distilled water for 24h in the dark. Dry weight (DW) was measured after oven-drying the leaves at 70 °C (ON-02G Jeio Tech, Seoul, Korea) until a constant weight was reached.

At the end of the experiment, the membrane stability index (MSI) was determined on fresh olive leaves using the method described by Lutts et al. (1996) [30]. Briefly, 0.2 g of leaf samples were incubated in 10 mL of distilled water for 24h at room temperature with agitation. Then, the initial electrical conductivity (EC_1) was measured, followed by the measurement of the second electrical conductivity (EC_2) after a 20 min incubation at 100 °C. The MSI was calculated using the following formula:

$$\text{MSI}\ (\%) = [1 - (EC_1/EC_2)] \times 100$$

2.3. Determination of leaf gas exchange and chlorophyll content

Leaf gas exchange parameters were measured on a sunny day between 9:00 and 11:00 a.m. using a handheld photosynthesis system (CI-340, CID Bio-Science Inc., Camas, WA, USA) [13]. The intercellular to ambient carbon dioxide concentration ratio (C_i/C_a) and the water use efficiency (WUE), defined as the ratio of net carbon dioxide (CO_2) assimilation rate to transpiration rate, were determined.

The total chlorophyll content (Chl a+b) was measured as described by Lichtenthaler and Buschmann (2001) [31]. 0.2 g sample of fresh leaves was homogenized in 5 mL of pure acetone using a pestle and mortar. After filtration through cheesecloth, the extract was diluted with pure acetone to a final volume of 20 mL. The absorbance was measured at 661.6, 644.8 and 470 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

2.4. Leaf anatomy

At the end of the experiment, three uniform, well-exposed and fully expanded leaves from the middle third of the shoot were sampled for each treatment. Squares approximately 0.3 × 0.3 cm in size were excised from the center of the leaves to measure the thickness of the palisade and spongy parenchyma, as well as the upper and lower epidermis with the cuticle. The leaf samples were fixed overnight at 4 °C in 2.5 % glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH = 7.2). The samples were then carefully washed in the same buffer. Tissue dehydration was carried out through a graded ethanol series (10, 30, 50, 90, 95 and 100 %), followed by infiltration with 100 % Micro-bed resin (EMS). Tissues were embedded in gelatin capsules filled with fresh Micro-bed resin. Polymerization was carried out using a thermal cure protocol at 55 °C for 24–48h [32]. Semithin sections (3 μm thickness) were obtained using an MT-990 microtome (RMC Boeckeler, AZ, USA) equipped with a glass knife, set to a cutting speed of 1.5 mm/s. The sections were stained with Toluidine Blue. Microphotographs of five thin sections from each leaf sample (in total 15 sections per treatment) were captured using a Leica station (Leica Microsystems Ltd, Germany) with Leica Application Suite Software. The tissue thickness was measured using Mesurim Pro (Version 3.2) Software.

At the end of the experiment, trichome and stomatal densities (per square millimeter) were measured as described by Guerfel et al. (2007) and Bader et al. (2015) [33,34]. Briefly, for three fresh leaf samples per treatment, trichomes were carefully removed from the abaxial face of the leaves using adhesive tape and placed directly on a microscope slide. Additionally, for each leaf, five imprints were made using nail polish (in total 15 imprints per treatment), to determine stomatal density using the WinDIAS image analysis system (Delta-T Devices Ltd, Cambridge, UK).

2.5. Leaf growth determination

At the end of the experiment, the leaf growth parameters were assessed. The leaf production rate (LPR) was calculated as the percentage of leaves produced during the experiment. The leaf perimeter (LP) was measured using a leaf-area-meter AM 300 (ADC Bioscientific Ltd., UK). For each treatment, LP was measured on five uniform, well-exposed and fully expanded leaves from the middle third of the olive shoot of three plants. A total of 15 leaves per treatment were analyzed. Leaf fresh weight (LFW) was determined using an Ohaus Pioneer TM PA214 balance (NJ, USA), and the leaves were then oven-dried at 70 °C (ON-02G Jeio Tech) until constant weight was achieved, allowing the determination of leaf dry weight (LDW).

2.6. Statistical analysis

Statistical analysis was performed using SPSS 20.0 statistical software. After verification of normal distribution, a one-way analysis of variance (ANOVA) was performed using the Duncan test (significance level set at $p \leq 0.05$) to compare the mean values of all parameters.

Additionally, a principal component analysis (PCA) was carried out on all studied parameters, using the Analyse-it software for Microsoft Excel.

3. Results

3.1. Leaf water relation parameters

The water saturation deficit (WSD), water uptake capacity (WUC) and membrane stability index (MSI) of young olive plants are presented in Fig. 1. Under treated wastewater (TWW) irrigation, both mycorrhizal and non-mycorrhizal plants exhibited increased WSD and WUC compared to control treatment (M^- TW), with a more pronounced increase in non-mycorrhizal plants. Indeed, in M^- TWW-treated plants, WSD and WUC increased significantly by 54.29 and 66.66 %, respectively, compared to M^- TW-treated plants. However, the increases in WSD were about 22.85, 35.48 and 10.10 % in Gd TWW, Gm TWW and Gd + Gm TWW treatments, respectively, compared to M^- TW treatment. For the WUC, all mycorrhizal treatments showed a 3.33 % increase, in comparison to the control. In addition, a significant decrease in MSI was observed in the leaves of plants subjected to TWW irrigation. Compared to the control plants, MSI decreased by about 34.21 % in M^- TWW-treated plants. Interestingly, mycorrhizal inoculation improved the membrane stability index in all TWW-treated plants. Indeed, the MSI increased by 21.59, 24.88 and 31.22 % in the leaves of Gd TWW, Gm TWW and Gd + Gm TWW-treated plants, respectively, as compared to M^- TWW treatment.

3.2. Leaf gas exchange and chlorophyll contents

Results presented in Table 2 show that TWW irrigation affected the leaf gas exchange parameters (except for Ci/Ca) and the chlorophyll content of young olive plants. A slight decrease in leaf intercellular carbon dioxide concentration (C_i) was noted in all plants irrigated with TWW for 12 months. Nevertheless, no significant differences in C_i values were observed between mycorrhizal and non-mycorrhizal plants. The ratio of intercellular to ambient carbon dioxide concentration (C_i/C_a) remained constant across all treatments, with values close to one. Moreover, under TWW irrigation, the water use efficiency (WUE) was significantly increased only in non-mycorrhizal plants, with no significant effect observed in mycorrhizal ones. In M^- TWW-treated plants, the increase in WUE was about 76.07 %, as compared to M^- TW treatment. The total chlorophyll content (Chl a+b) was significantly influenced by both the quality of irrigation water and the establishment of mycorrhizal symbiosis across different treatments. Irrigation with TWW resulted in reduced chlorophyll synthesis or increased chlorophyll degradation

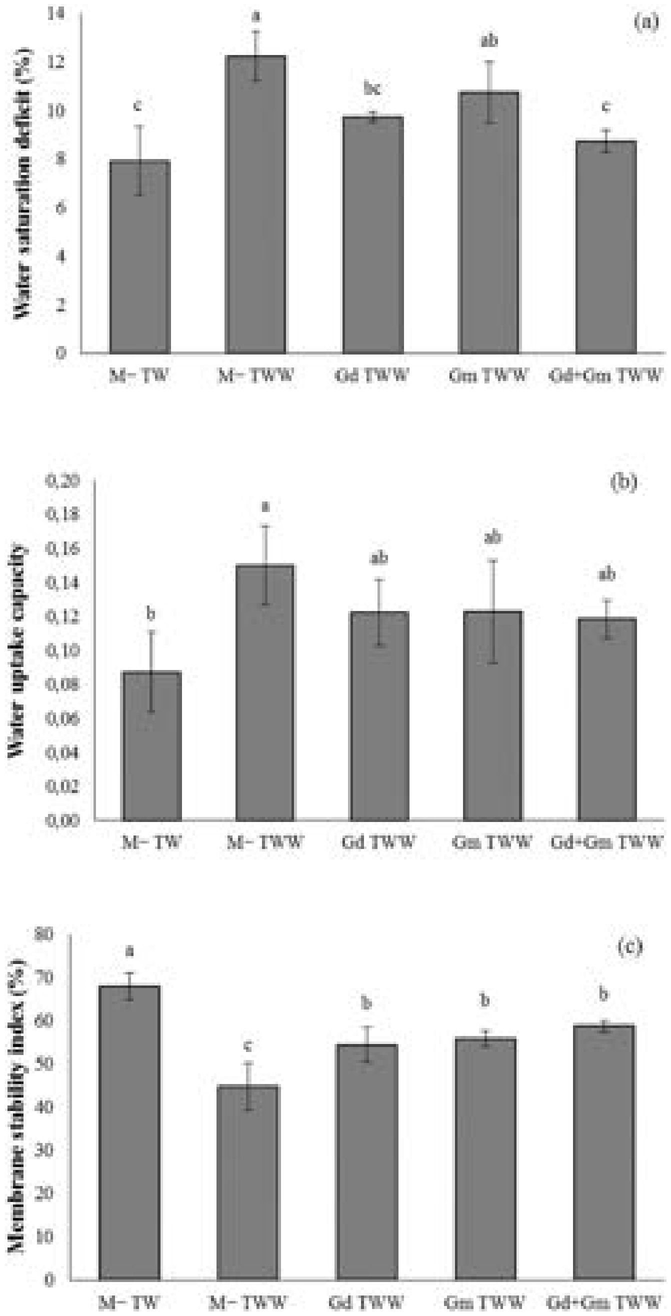

Fig. 1. Water saturation deficit (WSD), water uptake capacity (WUC) and membrane stability index (MSI) of young olive plants irrigated with treated wastewater and inoculated with different arbuscular mycorrhizal fungi. Values represent the means of 3 replications per treatment ± SD. Lowercase letter represent the significance difference, or not, by Duncan test ($p \leq 0.05$). M^- TW: non-mycorrhizal plants irrigated with tap water (control plants); M^- TWW: non-mycorrhizal plants irrigated with treated wastewater; Gd TWW: plants inoculated with *G. deserticola* and irrigated with treated wastewater; Gm TWW: plants inoculated with *G. margarita* and irrigated with treated wastewater; Gd + Gm TWW: plants inoculated with both *G. deserticola* and *G. margarita* and irrigated with treated wastewater.

compared to the control. A greater decline was noted in non-mycorrhizal plants compared to mycorrhizal ones. In comparison to M^- TW treated-plants, the decline in Chl *a*+*b* content was about 55.32 % in M^- TWW treated-plants, while it was only about 39.21, 34.95, and 22.19 % in Gd TWW, Gm TWW and Gd + Gm TWW treated-plants, respectively. Moreover, a significant increase in chlorophyll *a*/*b* ratio was noted in non-inoculated plants irrigated with TWW and to a lesser extent in plants inoculated with the two fungal species.

3.3. Leaf anatomy measurements

At the end of the experiment, both TWW irrigation and inoculation with arbuscular mycorrhizal fungi (AMF) significantly affected all the anatomical parameters of olive leaves (Fig. 2; Table 3). Compared to the control (M^- TW), TWW irrigation for 12 months reduced the thickness of the spongy parenchyma, but increased the thickness of the palisade parenchyma and both the upper and lower epidermis in non-mycorrhizal olive leaves. For instance, the thickness of spongy parenchyma in M^- TWW treated-plants decreased by about 9.58 %, compared to control olive plants, while the thickness of palisade parenchyma increased by 40.28 % in the same treatment. In addition, the number of stomata per mm^2 of leaf surface was lower in non-mycorrhizal olive plants irrigated with TWW, compared to those irrigated with tap water. In mycorrhizal plants, stomatal density increased progressively from Gd to Gm + Gd inoculated plants. However, the number of trichomes increased in all TWW-treated plants, with a 10.20 % rise in trichome density in leaves of non-mycorrhizal olive plants irrigated with TWW (M^- TWW) compared to control ones.

These changes in anatomical parameters due to TWW irrigation were mitigated by fungal inoculation. Indeed, the thickness of the spongy parenchyma decreased by 14.32, 6.88 and 5.62 % in Gd TWW, Gm TWW and Gd + Gm TWW-treated plants, respectively, compared to control plants (M^- TW). Moreover, the thickness of the palisade parenchyma increased by only 24.56, 28.58 and 19.56 % in Gd TWW, Gm TWW and Gd + Gm TWW-treated plants, respectively, compared to M^- TW treatment. Regardless of the AMF inoculum, the inoculation of young olive plants resulted in an increase of the number of trichomes per mm^2 of leaf surface, compared to non-mycorrhizal plants. This increase was about 9.26, 9.88 and 27.16 % in leaves of Gd TWW, Gm TWW and Gd + Gm TWW-treated plants, respectively, compared to M^- TWW-treated plants. Finally, under TWW irrigation, mycorrhizal olive plants developed a slightly higher number of stomata per mm^2 of leaf surface, compared to non-mycorrhizal ones (except for Gd TWW). The increase was about 8.18 % in the leaves of Gd + Gm TWW-treated plants, compared to M^- TWW treatment.

3.4. Leaf growth measurements

The leaf growth parameters, including leaf production rate (LPR), leaf perimeter (LP), leaf fresh weight (LFW) and leaf dry weight (LDW), were measured at the end of the experiment (Fig. 3). Irrigation with TWW significantly reduced all leaf growth parameters in non-mycorrhizal plants, compared to those irrigated with tap water. The decrease in leaf production rate and leaf perimeter was 49.00 and 18.47 %, respectively, in M^- TWW-treated plants, compared to M^- TW treatment. Additionally, the fresh and dry weights of olive leaves were reduced by 28.38 and 36.83 %, respectively, in the same treatments. Interestingly, an overall increase in growth parameters was observed in plants subjected to AMF inoculations. Although no significant differences were found between all mycorrhizal treatments, the values of leaf growth parameters were slightly higher in plants inoculated with a combined inoculum of *G. deserticola* and *G. margarita* (with the exception of LFW for Gd inoculum).

3.5. Principal component analysis

The analyzed parameters were used for principal component analysis (PCA). The PCA results revealed that two principal components explained 72.8 % of the total variation. The first principal component (PC 1) accounted for 57.9 % of the variance, while the second principal component (PC 2) explained 14.9 % of the variance (Fig. 4).

The PCA-biplot showed that the PC 1 axis separated the salinity effect induced by TWW irrigation, while the PC 2 axis tended to separate

Table 2
Intercellular carbon dioxide concentration (C_i, μmol mol^{-1}), intercellular to ambient carbon dioxide concentration ratio (C_i/C_a), water use efficiency (WUE, μmol CO_2 mmol H_2O^{-1}), total chlorophyll (Chl *a*+*b*, mg g^{-1} FW) and chlorophyll *a*/*b* ratio (Chl *a*/*b*) in young olive plants irrigated with treated wastewater and inoculated with different arbuscular mycorrhizal fungi.

	M^- TW	M^- TWW	Gd TWW	Gm TWW	Gd + Gm TWW
C_i	398.23 ± 2.74^a	392.78 ± 1.41^b	392.99 ± 1.38^b	394.00 ± 1.06^b	394.54 ± 1.10^b
C_i/C_a	1.01 ± 0.00^a	1.01 ± 0.00^a	1.01 ± 0.00^a	1.01 ± 0.00^a	1.01 ± 0.00^a
WUE	5.85 ± 0.52^b	10.30 ± 1.93^a	5.79 ± 0.80^b	6.96 ± 0.26^b	6.61 ± 1.11^b
Chl *a*+*b*	3.29 ± 0.02^a	1.47 ± 0.11^d	2.00 ± 0.10^c	2.14 ± 0.13^c	2.56 ± 0.11^b
Chl *a*/*b*	1.28 ± 0.04^c	1.66 ± 0.06^a	1.21 ± 0.04^c	1.25 ± 0.07^c	1.38 ± 0.02^b

Values represent the means of 3 replications per treatment ± SD. Means in the same column followed by the same letter do not differ significantly by Duncan test ($p \leq 0.05$). M^- TW: non-mycorrhizal plants irrigated with tap water (control plants); M^- TWW: non-mycorrhizal plants irrigated with treated wastewater; Gd TWW: plants inoculated with *G. deserticola* and irrigated with treated wastewater; Gm TWW: plants inoculated with *G. margarita* and irrigated with treated wastewater; Gd + Gm TWW: plants inoculated with both *G. deserticola* and *G. margarita* and irrigated with treated wastewater.

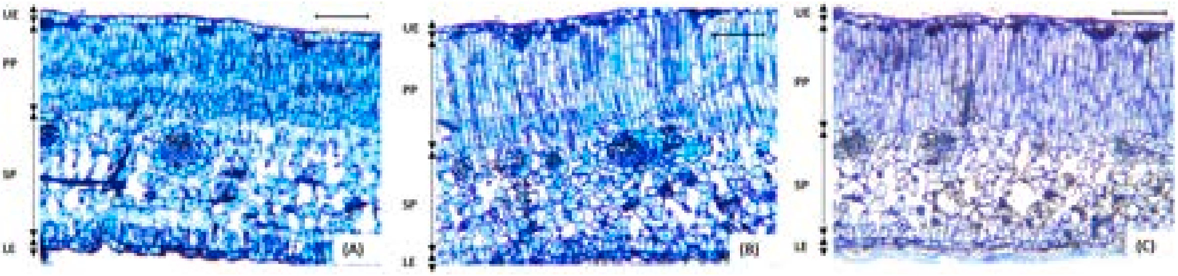

Fig. 2. Anatomy of leaves sections of young olive plants. (A) non-mycorrhizal plants irrigated with tap water (control plants, M^-TW); (B): non-mycorrhizal plants irrigated with treated wastewater (M^-TWW); (C): plants inoculated with both 2.5g *G. deserticola* + 2.5g *G. margarita* and irrigated with treated wastewater (Gd + Gm TWW); UE: Upper epidermis; LE: Lower epidermis; PP: Palisade parenchyma; SP: Spongy parenchyma.Scale bar represent 100 μm.

Table 3
Leaf anatomical characteristics of young olive plants irrigated with treated wastewater and inoculated with different AMF.

	M^- TW	M^- TWW	Gd TWW	Gm TWW	Gd + Gm TWW
Upper epidermis (μm)	16.69 ± 0.75^b	20.46 ± 1.53^a	17.11 ± 0.70^b	16.97 ± 1.21^b	16.80 ± 0.70^b
Lower epidermis (μm)	13.99 ± 0.54^c	16.73 ± 1.19^a	14.92 ± 0.79^b	14.89 ± 0.67^b	14.54 ± 1.00^{bc}
Palisade parenchyma (μm)	154.77 ± 4.38^e	217.11 ± 7.06^a	192.78 ± 9.38^c	198.99 ± 6.60^b	185.05 ± 7.27^d
Spongy parenchyma (μm)	252.63 ± 6.69^a	228.43 ± 12.86^b	216.44 ± 11.48^c	235.24 ± 19.22^b	238.42 ± 12.89^b
Trichomes density (trichomes/mm^2)	175.00 ± 20.47^d	192.86 ± 18.11^c	210.71 ± 22.59^b	211.90 ± 22.25^b	245.24 ± 19.64^a
Stomatal density (stomata/mm^2)	400.00 ± 24.21^a	331.25 ± 35.04^{cd}	318.75 ± 44.51^d	347.92 ± 42.37^{bc}	358.33 ± 23.23^b

Values are means of 15 replications per treatment ± SD. Means in the same row followed by the same letter do not differ significantly by Duncan test ($p \leq 0.05$). M^- TW: non-mycorrhizal plants irrigated with tap water (control plants); M^- TWW: non-mycorrhizal plants irrigated with treated wastewater; Gd TWW: plants inoculated with *G. deserticola* and irrigated with treated wastewater; Gm TWW: plants inoculated with *G. margarita* and irrigated with treated wastewater; Gd + Gm TWW: plants inoculated with both *G. deserticola* and *G. margarita* and irrigated with treated wastewater.

the effect of AMF inoculation. The control treatment (M^- TW, yellow cluster) and the M^- TWW treatment (violet cluster) were clearly segregated, positioned on opposite sides of the origin along the PC1 axis. Furthermore, the PCA-biplot showed that the three AMF treatments were clearly separated from the uninoculated ones. The clustering of AMF treatments suggests a similar effect. These treatments were positioned centrally between the control treatment (M^- TW, yellow cluster) and the M^- TWW treatment (violet cluster). In addition, Gd TWW (blue cluster) and Gm TWW (orange cluster) showed distinct, through somewhat overlapping distributions, implying related but distinguishable traits. However, among the three AMF groups, the combined inoculation with *G. deserticola* and *G. margarita* (green cluster) appears slightly closer to the control along both PC1 and PC2 axes than the others AMF groups. This positioning may suggest that the combined AMF inoculation has a somewhat more effective role in enhancing the physiological response of olive plants compared to single AMF inoculation. This hypothesis is statistically supported by the absence of significant differences between the control treatment and the Gd + Gm TWW treatment for several studied parameters (WUE, C_i/C_a, LFW, LDW, LP, UE, LE …). As presented in Fig. 4 (indicated by arrows) and Table 4, the variables contributing to segregation along PC1 axis included leaf fresh weight (LFW), leaf dry weight (LDW), membrane stability index (MSI), total chlorophyll content (Chl *a*+*b*), net carbon dioxide assimilation rate (A), leaf production rate (LPR), palisade parenchyma thickness (PP), and water saturation deficit (WSD). In contrast, the variables contributing to segregation along the PC 2 axis were leaf production rate (LPR), chlorophyll *a*/*b* ratio (Chl *a*/*b*), water use efficiency (WUE), net carbon dioxide assimilation rate (A), stomatal density (SD), spongy parenchyma thickness (SP), upper epidermis thickness (UE), trichome density (TD) and leaf fresh weight (LFW).

4. Discussion

The effect of irrigation with saline treated wastewater (TWW) (EC = 5.84 dS m^{-1}; Na^+ = 730.00 mg L^{-1}; Cl^- = 1465.39 mg L^{-1}) on the leaf water status of young olive plants was first assessed. Determining water relations characteristics is crucial to reveal the physiological effects and to understand the salinity tolerance mechanisms of the plant [35,36]. In this study, irrigating with TWW over a 12-month period resulted in higher levels of leaf water saturation deficit (WSD) and water uptake capacity (WUC) in both mycorrhizal and non-mycorrhizal plants, compared to control plants irrigated with tap water. The WSD measures

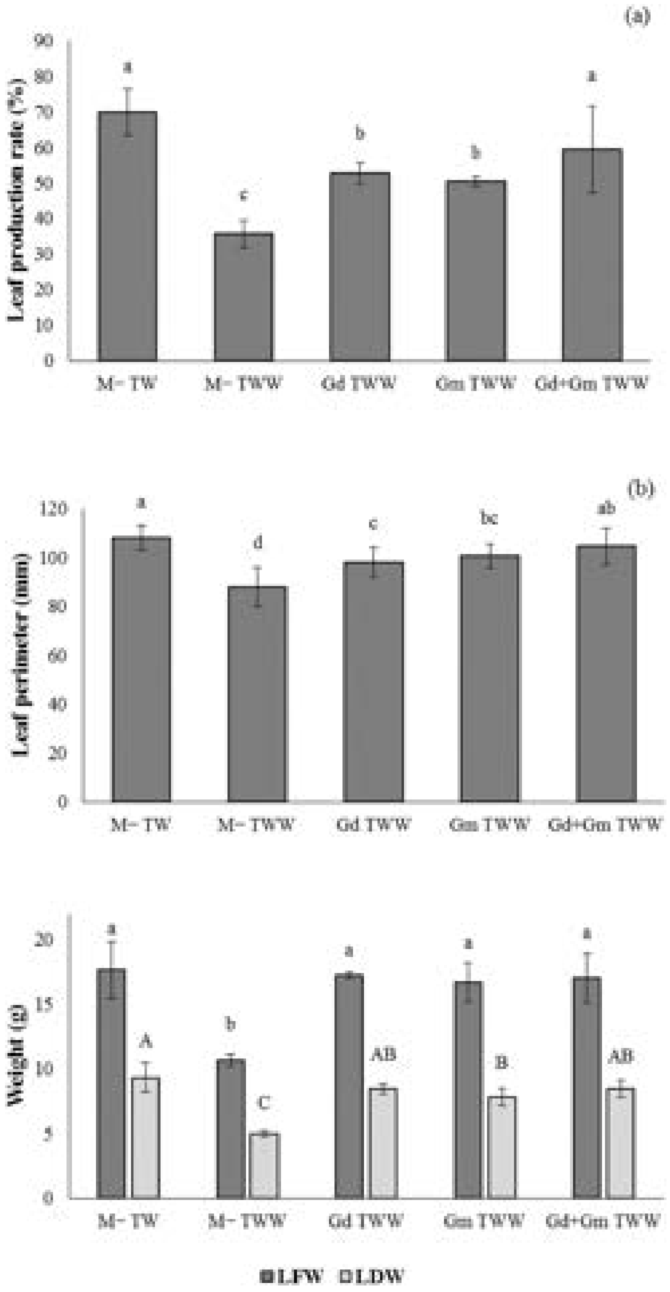

Fig. 3. Leaf production rate, leaf perimeter, leaves fresh weight (LFW) and leaves dry weight (LDW) of young olive plants irrigated with treated wastewater and inoculated with different arbuscular mycorrhizal fungi. Data of leaf production rate, leaves fresh weight and leaves dry weight are means of 3 replications per treatment ± SD. Data of leaf perimeter are means of 15 replications per treatment ± SD. Means in the same column followed by the same letter do not differ significantly by Duncan test ($p \leq 0.05$). M^- TW: non-mycorrhizal plants irrigated with tap water (control plants); M^- TWW: non-mycorrhizal plants irrigated with treated wastewater; Gd TWW: plants inoculated with *G. deserticola* and irrigated with treated wastewater; Gm TWW: plants inoculated with *G. margarita* and irrigated with treated wastewater; Gd + Gm TWW: plants inoculated with both *G. deserticola* and *G. margarita* and irrigated with treated wastewater.

the extent of water deficit in plants, while the WUC quantifies the plants' ability to absorb water per unit dry weight, in relation to their turgid weight [37]. The obtained results suggest that young olive plants experienced water stress when irrigated with saline TWW. The observed increase in WUC under TWW treatments may indicate that these plants required more water to reach full turgidity. In our previous work [6], an accumulation of salts in the soil was noted under TWW irrigation by the end of the experiment. According to the same study, the soil EC ranged between 11.39 and 12.13 dS m^{-1} under TWW treatments. This increase in salinity may lower the water osmotic potential of soil solution and disrupt the water absorption process by the roots. Indeed, when soil water availability to the roots decreases, it results in a limited water supply to the leaves. Similar results were reported by Sultana et al. (2019) [38] and Larbi et al. (2020) [39] for potted mungbean (*Vigna radiata* L.) and young olive plants, respectively, under saline conditions (100 and 200 mM NaCl).

Furthermore, the results showed a decrease in the membrane stability index (MSI) in the leaves of young olive plants irrigated with TWW, compared to the control. It is well-known that the cell membrane serves as a semi-permeable barrier facilitating the movement of solutes. The high concentrations of Na^+ and Cl^- lead to increased production of reactive oxygen species (ROS), such as hydrogen peroxide, which interact with membrane lipids, causing lipoperoxidation, severe membrane damage, and electrolyte leakage from the cells [4,40,41]. This reaction has been widely observed in olive plants under saline conditions and is considered an early indicator of stress [21,39,42].

Interestingly, both the WSD and WUC showed a decreasing tendency in mycorrhizal olive plants, compared to non-mycorrhizal ones. Furthermore, the results revealed a higher membrane stability index in inoculated olive plants, in contrast to non-inoculated ones. This suggests that arbuscular mycorrhizal fungi (AMF) may help in protecting the cell membrane from damage. According to the literature, AMF are able to improve water relations in host plants through several mechanisms [24,25,43]. In fact, AMF are known to form an extra-radical mycelium network that functions similarly to root hairs, enabling deeper soil exploration for more efficient water extraction [24,25,44]. Additionally, the AMF can activate the plant's antioxidant defense system [37] and regulate the osmotic potential of inoculated plants by accumulating osmolytes, such as proline and soluble sugars [45], which may improve the cellular water balance. Moreover, AMF produce glomalin, a glycoprotein that enhance the stability of soil aggregates and increase the soil water holding capacity, which potentially could lead to better water uptake by plants [25,46].

In addition to disrupting plant water status, the high salt concentration in irrigation water can also affect gas exchange parameters and photosynthetic pigment synthesis in plant leaves. The obtained results showed a decrease in leaf intercellular carbon dioxide concentration in non-mycorrhizal plants irrigated with TWW, compared to control plants. Furthermore, according to our previous study [13], the net carbon dioxide (CO_2) assimilation rate (A) was also reduced in the same treatments. Indeed, the CO_2 assimilation rate was about 12.86 μmol of CO_2 m^{-2} s^{-1} in control plants, while it was only about 8.08 μmol of CO_2 m^{-2} s^{-1} in non-inoculated plants irrigated with TWW. A similar effect has been documented by several authors in various species irrigated with saline TWW [4,10,11]. For instance, Hajihashemi et al. (2020) [11] noted a remarkable decrease in net photosynthesis and intercellular CO_2 concentration in wheat plants (*Triticum aestivum* L.) in response to varying concentrations (25, 50 and 100 %) of saline TWW. According to Gómez-Bellot et al. (2013) [12] and Hussain et al. (2018) [47], under salt conditions, plants close their stomata to reduce water loss. This behavior limits transpiration and CO_2 diffusion via the stomata of stressed plants, which in turn decreases the leaf intercellular carbon dioxide concentration and then the net photosynthesis rate. Furthermore, the ratio of intercellular to ambient carbon dioxide concentration (C_i/C_a) remained unchanged across all plants in this experiment, suggesting an impaired functioning of the Calvin cycle due to decreased Rubisco activity. This can lead to an overloading of the photosynthetic electron transport chain and increased excitation energy within chloroplasts [16]. Similarly, Tang et al. (2022) [25] linked the disturbance of gas exchange parameters in stressed plants to the detrimental effect of ROS, which reduce the supply of ATP and NADPH to the Calvin cycle.

In this work, the changes in gas exchange parameters were also

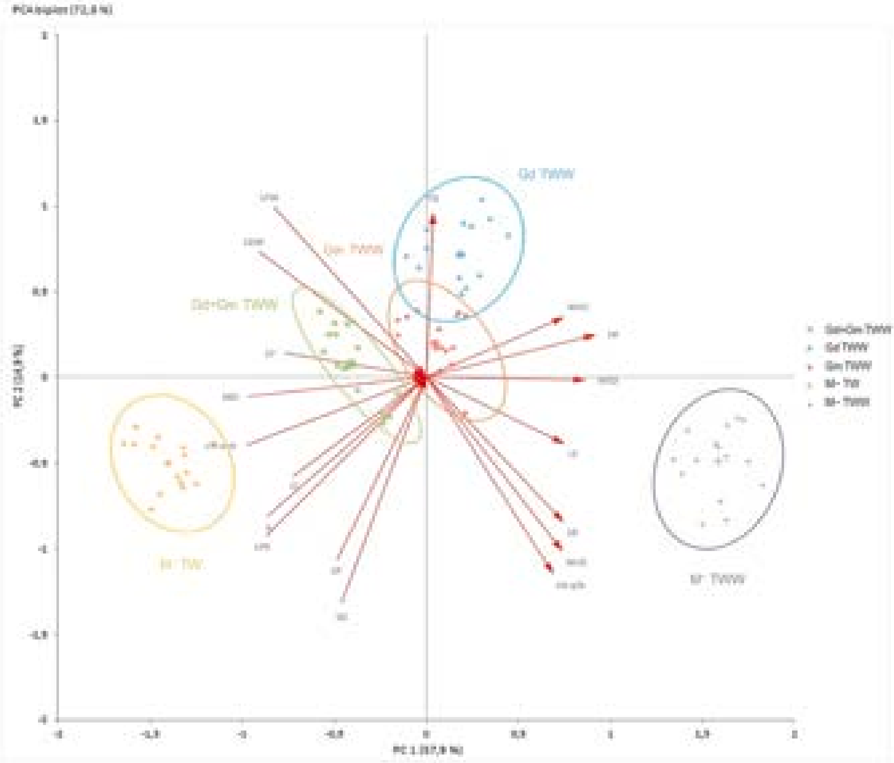

Fig. 4. Principal components analysis (PCA) of physiological, morphological and anatomical parameters of young olive plants irrigated with treated wastewater and inoculated with different arbuscular mycorrhizal fungi. PC 1: first principal component axis; PC 2: second principal component axis; M^- TW: non-mycorrhizal plants irrigated with tap water (control plants); M^- TWW: non-mycorrhizal plants irrigated with treated wastewater; Gd TWW: plants inoculated with *G. deserticola* and irrigated with treated wastewater; Gm TWW: plants inoculated with *G. margarita* and irrigated with treated wastewater; Gd + Gm TWW: plants inoculated with both *G. deserticola* and *G. margarita* and irrigated with treated wastewater. Arrow label: A: Net carbon dioxide assimilation rate; Ci: Intercellular carbon dioxide concentration; WUE: Water use efficiency; Chl *a*+*b*: Total chlorophyll; Chl *a*/*b*: Ratio chlorophyll *a*/b; LFW: Leaves fresh weight; LDW: Leaves dry weight; WSD: Water saturation deficit; WUC: Water uptake capacity; MSI: Membrane stability index; LPR: Leaf production rate; UE: Upper epidermis; LE: Lower epidermis; PP: Palisade parenchyma; SP: Spongy parenchyma; TD: Trichomes density; SD: Stomatal density; LP: Leaf perimeter.

Table 4

Percentage of contribution of physiological, morphological and anatomical parameters of young olive plants irrigated with treated wastewater and inoculated with different arbuscular mycorrhizal fungi to the two principal components (PC 1 and PC 2).

	UE	LE	PP	SP	TD	SD	LP	A	Ci
PC 1	5.199	5.291	7.931	2.177	0.012	1.981	5.423	6.932	4.841
PC 2	6.639	1.405	0.583	10.593	8.578	16.060	0.189	6.154	3.081
	WUE	**Chl a+b**	**Chl a/b**	**LFW**	**LDW**	**WSD**	**WUC**	**MSI**	**LPR**
PC 1	5.123	8.860	4.388	6.306	7.618	7.009	5.254	8.579	7.076
PC 2	9.616	1.442	12.273	9.096	5.003	0.003	1.146	0.115	8.024

A: Net carbon dioxide assimilation rate; Ci: Intercellular carbon dioxide concentration; C_i/C_a: Intercellular to ambient carbon dioxide concentration ratio; WUE: Water use efficiency; Chl *a*+*b*: Total chlorophyll; Chl *a*/*b*: Ratio chlorophyll *a*/b; LFW: Leaves fresh weight; LDW: Leaves dry weight; WSD: Water saturation deficit; WUC: Water uptake capacity; MSI: Membrane stability index; LPR: Leaf production rate; UE: Upper epidermis; LE: Lower epidermis; PP: Palisade parenchyma; SP: Spongy parenchyma; TD: Trichomes density; SD: Stomatal density; LP: Leaf perimeter.

accompanied by an important increase in water use efficiency (WUE), consistent with the results of Acosta-Motos et al. (2017) [10] in *Eugenia myrtifolia* plants irrigated with saline reclaimed water. However, contradictory results were reported by Ansari et al. (2019) [48] and Hajihashemi et al. (2020) [11] in wheat plants subjected to varying NaCl concentrations and saline TWW irrigation, respectively. In this study, the increase in WUE may reflect an adaptation strategy of olive plants to maintain homeostasis. This could occur through several physiological mechanisms, such as reduced transpiration and stomatal conductance, while maintaining photosynthetic capacity, to conserve water and enhance WUE. In fact, our previous work [13] showed that under TWW irrigation, young olive plants experienced reductions in stomatal conductance and transpiration by approximately 53 % and 63 %, respectively, compared to control olive plants. However, the decrease in net photosynthesis was only about 37 %. Similar results were recently reported by Liao et al. (2022) [49] and Hou et al. (2023) [50] in maize (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) respectively, who showed that the increase in WUE under saline conditions is mainly due to a greater reduction in transpiration and stomatal conductance than in the photosynthetic rate. Moreover, the results revealed a notable reduction in total chlorophyll content (Chl *a*+*b*) in the leaves of non-colonized olive plants irrigated with TWW, compared to the control treatment. Chlorophyll *a* and chlorophyll *b* are the primary pigments involved in the conversion of light energy into chemical energy during photosynthesis. Thus, the decline of chlorophyll content could negatively impact the photosynthetic capacity of plants, which is in agreement with the present findings. Similar observations were made by Vicente-Sánchez et al. (2014) [7] in lettuce (*Lactuca sativa* L.) irrigated with saline TWW (EC = 4.19 dS m^{-1}) and by Hou et al. (2023) [50] in cotton under saline conditions (200 mM NaCl). These authors attributed the decrease in chlorophyll content to the degradation of chlorophyll by the degrading enzyme chlorophyllase, which is triggered by oxidative stress. On the other hand, many macro- and micro-nutrients, such as nitrogen (N), magnesium (Mg^{2+}), and zinc (Zn^{2+}), are known to enhance photosynthetic activity and promote chlorophyll biosynthesis [9]. Nevertheless, in the present study, the nutrient richness of TWW did not appear to improve these parameters. In fact, our previous study [6], found that the content of macro- and micronutrients was slightly increased in the tissues of olive plants irrigated with TWW. According to the same work, the high concentrations of Na^+ and Cl^- in the soil solution decreased the availability of essential nutrients due to competitive uptake, leading to nutrient imbalance and disruptions in physiological process [6].

The negative effect of saline TWW on gas exchange parameters and total chlorophyll content observed in this experiment were mitigated by mycorrhizal colonization. This suggests that the photosynthetic machinery of the host plants may function more efficiently under saline conditions. In accordance with these results, the improvement of leaf water status in inoculated plants, due to the extra-radical mycelium, may facilitate stomatal opening, thereby increasing CO_2 diffusion and improving stomatal conductance [51,52]. Thus, the availability of both water and CO_2 can stimulate the photosynthetic process. However, in this experiment and compared to non-mycorrhizal plants irrigated with TWW, a slight increase in C_i values was noted in mycorrhizal treatments, but differences were not statistically significant. In fact, we suggest that the damage to the photosynthetic process, caused by salt stress under TWW irrigation, could be gradually alleviated not only through stomatal regulation but also through other mechanisms. The increase in CO_2 assimilation rate in inoculated olive plants can be attributed to the effective role of AMF in maintaining ion homeostasis, reducing the uptake of Na^+ and Cl^-, and thereby limiting damage to Photosystem I (PSI) and Photosystem II (PSII) as it was observed by Zong et al. (2023) in *Xanthoceras sorbifolium* [52]. Recently, Lorente et al. (2022) [4] studied the effect of irrigation with saline TWW (EC of 3.4–4.0 dS m^{-1}; Na^+ = 170.20 ppm; Cl^- = 210.46 ppm) in *Salvia officinalis* (L.) and *Asteriscus maritimus* (L.) plants inoculated with *Glomus iranicum* and concluded that AMF played a positive role in increasing the PSII photochemical efficiency, which led to an improvement in gas exchange parameters in mycorrhizal plants. In addition to limiting the uptake of Na^+ and Cl^-, AMF are recognized for their ability to improve the absorption of nutrients, which plays a crucial role in regulating stomatal opening (K^+), the stimulating chlorophyll synthesis (Mg^{2+}), and thereby improving the photosynthetic capacity of host plants [41,53,54]. Our previous study [6] demonstrated that inoculated olive plants exhibited lower Na^+ and Cl^- contents, but higher contents of macro- and micro-nutrients in their leaves and roots, as compared to uninoculated plants. For instance, in the same study, the Na^+, Cl^-, and K^+ contents in the leaves of M^- TWW-treated plants were 0.55, 0.35 and 0.77 % dry weight (DW), respectively. However, the content of these nutrients in the leaves of Gd + Gm TWW-treated olive plants were about 0.42, 0.20 and 1.01 % DW, respectively. These results may consequently explain the improvement in gas exchange parameters observed in mycorrhizal olive plants. Furthermore, the observed improvement in chlorophyll content could be attributed to the beneficial role of AMF in reducing ROS formation, limiting the chlorophyllase activity, as well as maintaining the activity of enzymes involved in chlorophyll synthesis [25].

The examination of leaf anatomical characteristics showed that TWW irrigation and AMF colonization induced several changes. Compared to the control treatment, TWW irrigation resulted in an increased trichome density, accompanied by a decrease in the number of stomata per mm^2 of leaf surface in non-mycorrhizal olive plants. It is well known that trichomes are a protective barrier against ultraviolet irradiation, excessive temperatures and water loss [55,56]. These appendages cover the stomata and facilitate gas exchange between the plant and the surrounding atmosphere by regulating stomatal aperture [57]. The changes in trichomes and stomatal densities observed in this experiment may represent an important adaptive mechanism to reduce water loss through transpiration and regulate stomatal conductance, helping to prevent dehydration and optimize water use efficiency under saline conditions [34,58]. In addition, these changes could explain the disturbance in leaf water status and gas exchange parameters observed in non-mycorrhizal plants irrigated with TWW. These results are in accordance with previous studies of Bader et al. (2015) [34], Mehdi-Tounsi et al. (2016) [58] and Hamani et al. (2021) [59] conducted on olive, pistachio (*Pistacia vera* L.) and cotton plants, respectively, under saline conditions. In this context, Hamani et al. (2021) [59] reported that regulating gas exchange through stomatal adjustments, including stomatal closure and changes in stomatal density, can be considered as an important mechanism for improving plant resistance to various stresses, particularly salt stress.

Mycorrhizal colonization resulted in a significant increase in both trichomes and stomatal density in olive leaves under TWW treatments, compared to non-mycorrhizal plants. These findings are consistent with those of Zhu et al. (2018) [26], who observed similar effects in potted wheat plants inoculated with *Rhizophagus irregularis* and subjected to three NaCl levels (0, 1 and 2 g kg^{-1} dry soil). According to the previous studies of Chitarra et al. (2016) [60] and Zhu et al. (2018) [26] in tomato (*Solanum lycopersicum* L.) and wheat, AMF have the ability to alter the genes controlling stomatal development, thereby influencing the stomatal behavior of host plants under varying environmental conditions. In the current study, it seems that different AMF inoculums, particularly the combined inoculation with *G. deserticola* and *G. margarita*, stimulated the development of additional trichomes and stomata, as a strategy to alleviate the deleterious effect of Na^+ and Cl^- present in TWW. This mechanism may help explain the observed physiological improvements in mycorrhizal plants, particularly the enhancement of leaf water relations and gas exchange parameters. It is well known that the increased trichomes density may reduce the leaf temperature and limit the water loss by increasing the resistance of the boundary layer between the leaf and the surrounding air, thus maintaining better water status in the plants [61,62]. In addition, as reported in our previous study [13], mycorrhizal symbiosis significantly enhanced stomatal conductance from 8.98 **mmol of H_2O m^{-2} s^{-1} in M^- TWW to 8.46, 9.58 and 11.05 mmol of H_2O m^{-2} s^{-1} in** Gd TWW, Gm TWW and Gd + Gm TWW treatments, respectively. Moreover, similar results were observed for net photosynthesis, which increased from 11.42 **μmol of CO_2 m^{-2} s^{-1}** in M^- TWW to 17.06, 17.15 and 19.71 **μmol of CO_2 m^{-2} s^{-1}** in Gd TWW, Gm TWW and Gd + Gm TWW treatments, respectively. Based on these findings, we suggest that the increase in the number of stomata and trichomes per mm^2 of leaf surface helped the young olive plants reduce the water loss through transpiration, thereby improving the regulation of stomatal conductance to prevent dehydration and optimize water use efficiency. On the other hand, other anatomical alterations were recorded. Compared to control plants, leaves of non-mycorrhizal olive plants irrigated with TWW exhibited increased thickness of both the upper and lower epidermis (including the cuticle). These plants also showed a thicker palisade parenchyma, while the spongy parenchyma was less developed. Similar results were recorded by Gómez-Bellot et al. (2015) [8] in laurustinus plants (*Viburnum tinus* L.) irrigated with saline TWW (EC = 4 and 6 dS m^{-1}). According to these authors, such anatomical changes could help sustain photosynthetic activity. Indeed, the increased thickness of the palisade parenchyma may enhance the number of chloroplasts, thereby promoting the synthesis of chlorophyll. This effect could, in turn, improve the photosynthetic capacity of stressed plants and reduce the damage caused by saline TWW [63,64]. Additionally, Guerfel et al. (2009) [19] suggested that anatomical variations in olive leaves under stressful conditions could improve water relations and provide better protection for inner tissues. Indeed, leaf thickening contributes to more effective control of the transpiration rate, which may increase the plant's water retention capacity as demonstrated by Xao et al. (2023) in *Lycium barbarum* [64]. Generally, an increase in the thickness of the palisade parenchyma is associated with a decrease in the thickness of the spongy parenchyma. This change may facilitate the CO_2 diffusion to the chloroplasts, even under reduced stomatal aperture conditions as evidenced in *Lantana* and *Ligustrum* [65,66]. Such adaptations could serve as an important adaptive mechanism for improving the salt tolerance of young olive plants.

The leaf anatomical observations of mycorrhizal plants irrigated with TWW showed minor changes in tissues thickness, compared to non-mycorrhizal ones, suggesting that mycorrhizal olive plants experienced less stress. In accordance with the physiological observations, it seems that mycorrhizal inoculation partially alleviated the negative effects of saline TWW by regulating water relations and gas exchange parameters which, in turn, contributed to the preservation of favorable leaf anatomical characteristics. According to Shen et al. (2022) [63] and Chen et al. (2023) [67], anatomical characteristics gradually develop during the plant's growth process and are closely linked to its physiological performance. The anatomical features observed in this study are similar to those found by Gómez-Bellot et al. (2015) [8] in laurustinus plants subjected to saline TWW irrigation and inoculated with *Glomus iranicum*. These authors reported that AMF counteracted the negative effects of saline TWW by promoting the antioxidant activity and improving the physiology of the host plants. However, Li et al. (2019) [68] noted a compaction of leaf tissues and an increase in the thickness of palisade layer in *Fraxinus mandshurica seedling after inoculation with Glomus mosseae* and *G. etunicatum*. This response could serve as an adaptive strategy for host plants to limit water loss through transpiration. Based on these observations, the protection of internal plant tissues by AMF under stressful conditions may depend on several factors, such as the specific AMF species, their interaction with host plants, and the type and duration of the stress. In this experiment, TWW irrigation reduced all leaf growth parameters (leaf production rate, leaf perimeter, leaves fresh and dry weight), compared to control plants. Similarly, Gómez-Bellot et al. (2013) [12] observed a decrease in total biomass, leaf number, and total leaf area in Euonymus (*Euonymus japonica* Thunb.) plants subjected to saline TWW ($EC = 4$ dS m^{-1}) for twenty weeks, compared to control. Likewise, Valdés et al. (2015) [69], showed that irrigation with saline TWW ($CE = 5, 5.5, 6$ and 6.5 dS m^{-1}) reduced the number of leaves and leaf area, and caused foliar damage, such as burning or chlorosis, in old geranium (*Pelargonium* × *hortorum* L.H.), as compared to control plants. Several authors have reported that the salinity of TWW can induce ion toxicity, osmotic stress and nutritional imbalances, which in turn limit water and nutrient uptake by the roots of various plant species [4,11,12,69]. Such physiological disorders can directly impact growth by reducing cell division and elongation rates [24,69,70]. In the present study, the reduction in leaf production rate and perimeter under TWW irrigation may be considered as an avoidance strategy employed by young olive plants to reduce transpiration [4]. Under TWW treatments, the observed changes in leaf water status, gas exchange parameters, total chlorophyll content, and stomatal density may, in part, be attributed to the leaf morphological modifications.

Interestingly, AMF inoculation positively influenced the growth and development of young olive plants under TWW irrigation, compared to non-mycorrhizal plants. In accordance with the physiological observations, mycorrhizal olive plants exhibited better leaf water status and enhanced photosynthetic performance under TWW treatments compared to non-mycorrhizal ones. These ameliorative effects may, consequently, act in coordination to stimulate the growth of host plants [22]. Similar results have been observed in plants subjected to TWW [7] or saline water irrigation [71–73] and inoculated with AMF. These studies attributed the improved growth parameters of host plants to the enhanced water and nutrient uptake via the extraradical mycelium network, as well as the reduced uptake of Na^+ and Cl^-. Furthermore, it is well established that AMF can modify the structure and architecture of colonized roots, thereby increasing water and nutrient acquisition, which in turn significantly boosts plant growth [22,25]. However, in the few available studies dealing with inoculation of established trees (Citrus, pines, maples), the reported results seem inconclusive or only partial improvement was achieved [74,75].

5. Conclusions

In this study, irrigation with saline TWW for 12 months caused physiological, anatomical and morphological changes in potted young olive plants (*Olea europaea* L. cv. Chetoui).

The results showed that TWW irrigation decreased leaf intercellular carbon dioxide (CO_2) concentration and net photosynthesis, compared to the control treatment (tap water). These physiological alterations were accompanied by morphological and anatomical modifications in olive plants, particularly at the leaf level. These adaptations, which well-known to be induced in plant under stress to help them cope with stressful conditions, include: 1/an increase in trichome density, as well as the thickness of both the upper and lower epidermis and the palisade parenchyma; 2/a decrease in the thickness of the spongy parenchyma and the number of stomata per mm^2 of leaf surface. In addition, the leaf growth rate and the leaf perimeter were reduced under TWW irrigation, compared to the control. Interestingly, all of these alterations were attenuated in mycorrhizal olive plants. Indeed, mycorrhizal symbiosis enhanced CO_2 diffusion which, thereby improving the photosynthetic capacity of the olive plants. Furthermore, AMF inoculation increased trichomes and stomatal density and improved all the studied anatomical and morphological parameters, which could serve as potential biomarkers.

This study highlights the beneficial role of AMF in mitigating the deleterious effects of TWW and improving the physiological, anatomical and morphological responses of potted young olive plants. In the context of climate change, the production of mycorrhizal plants in nurseries could serve as an alternative strategy to enhance plant resilience and vigor. This approach may represent a promising management strategy and a sustainable agricultural practice for reducing the adverse effects of challenging environmental conditions. Furthermore, based on our results, we suggest that applying AMF in olive fields could be highly beneficial to reduce the detrimental effects of various environmental stresses, particularly water shortage and salinity. Future studies investigating the effects of mycorrhizal symbiosis on olive trees under field conditions, where multiple factors will be considered, could further validate our findings. However, it should be noted that, in the few available studies dealing with inoculation of established trees, the results seem inconclusive or only partial improvement were observed. Consequently, many further studies are needed to assess the effect of all these combined factors. Indeed, in olive orchard, like in other established tree conditions, natural factors and management practices are likely to influence the response of mycorrhizal olive trees, compared to potted experiment. Interaction between exogenous mycorrhizal species and native soil microorganisms (mycorrhizal, endophytic …) or amendment practices could be important factors to explore first and foremost in future research.

CRediT authorship contribution statement

Ameni Ben Hassena: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lina Trabelsi:** Investigation, Formal analysis. **Mohamed Zouari:** Methodology. **Raphaël Decou:** Investigation. **Mohamed Ali Triki:** Visualization. **Wahid Khabou:** Supervision. **Pascal Labrousse:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Nacim Zouari:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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