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Effects of irrigation with urban treated wastewater on the morpho-physiology, accumulation of heavy metals and biochemical traits of *Casuarina glauca* Sieb

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Abstract - Waste water recycling is a strategy used to preserve fresh water resources, which are becoming increasingly scarce, particularly in Tunisia. Long-term reuse of treated waste water may have negative effects on plant growth and the environment, but short-term reuse may boost crop growth due to their high organic and mineral content. Irrigation of tree species by these unconventional waters during higher evapotranspiration periods for reforestation and biomass production could be a critical alternative for the recovery of these waters. The purpose of this research is to assess the effects of treated waste water (TWW) on the morpho-physiological behavior of *Casuarina glauca* over a period of 90 days. One-year-old seedlings were split into two groups, the first with drinking water (controls) and the second with treated wastewater (TWW). TWW had a positive effect on *C. glauca* plant growth, , dry mass production, gas exchange, and the synthesis of total chlorophyll and primary and secondary metabolites. Furthermore, the findings demonstrated that this species has the ability to phytostabilize heavy metals, specifically Cu, Cd, and Pb .

Key words: *Casuarina glauca*, treated wastewater, gas exchanges, growth, heavy metals.

1. Introduction

Tunisia, like other Mediterranean North African countries, is experiencing a water scarcity, which poses a threat to long-term development (Slaimi et al. 2021). Thus, the annual renewable water resources of these countries in 2018 were 525m^3/capita which just above thethresholdfor absolute water scarcity that is 500m^3/capita (Frascari et al. 2018). Population growth, rising living standards, increased urbanization, desertification, overexploitation of groundwater, seawater intrusion into aquifers, deterioration of water quality and the intensification of the effects of climate change worsen this situation (Slaimi et al. 2021). Additionally, in the Mediterranean climatic zone such as North African country, rainfall was decreased as a result of climate change (Abouabdillah et al., 2010). Among the strategies that are included for the mobilization and development of water resources in Tunisia is the reuse of treatedwastewater in the agricultural sector where irrigation uses in Tunisia up to 80% of the water consumed (El Ayni et al. 2011). The total treated wastewatervolumewas estimated about 243 million m^3 witch present about 5% of mobilized water resources at the national level (Zouari et al. 2019). Their exploitation is still low because approximately 70% of their volume is removed in the sea and valleys, and the rest is used for various sectors, including agriculture, irrigation of green areas, golf courses, and groundwater recharge (Zouari et al. 2020). The use of TWW would be a strategy for crop production due to its high content of nutrients essential for plant growth. However, high mineral salts, heavy metals and organic compounds in this kind of water can deteriorate soils, crops and human health (Shehzadi et al.2014). As a result, its use for irrigation must be done with caution to mitigate its negative effects (Slaimi et al. 2021). Irrigation with TWW for the production of forage or food crops in lands with limited freshwater supplies is a well-known solution (Cornel and Weber 2004; Raes et al. 2009). In addition its use as irrigation supply for afforestation of marginal lands could support the rehabilitation of such degraded area which represents around 75 % of the Tunisian's land area (Béjaoui et al. 2008). Failures of tree or shrub plantations are mostly related to the low survival rate of young plants due to the lack of water after transplantation (Béjaoui et al. 2008), irrigation with treated wastewater could enhance the successof these plantations by providing water supply for seedlings, particularly during the early phase of growth. In addition, afforestation of degraded soils could contribute to the conservation of water and soil, the fight against global warming through carbon sequestration and mitigation of greenhouse gas emissions. Also, It could improve water quality by filtration and absorption of harmful chemicals, including metals, organic compounds such as fuels by transforming them to less harmful substances stored in the roots, stems or leaves (Kuzovkina and Quigley, 2005; Tan and Yeo, 2009).One of the most solutions for the restoration of degraded soils is the use of woody shrubs or trees, highly resilient to harsh environmental conditions (Diem and Dommergues, 1990).The use of trees for phytoremediation and forest creation becomes increasingly essentialaslow-cost ecological strategies to rehabilitate and improve ecosystem benefits (e.g., timber production, firewood, and fodder) (Ghazouani et al. 2020)and to mitigate climate change effects over the next decades (El Moussaoui et al., 2019). Among the woody species witch able to establish the degraded soil figure the *Casuarina* species. They have a fast growth and cangrow up to 3m per year to reach a final height of 30 m (Ghazouani et al. 2020). They also present an economic and ecological impact through the use of their wood for the constructionand the production of plywood (Zhong et al. 2010). These Australian origin species not widespread in the Mediterranean basin are known by their high tolerance of various environmental constraints (Ghazouani et al. 2020). Several studies have shown its potential for adaptation to various conditions of environmental stress such as flooding, drought, salinity, alkalinity, acidity, and mineral or organic pollution (Béjaoui et al. 2008; Bargali, 2011; Ghazouani et al. 2020). Historically, these woody plants were planted on poor and contaminated soils in order to increase their fertility (Duhoux and Franche, 2003). *Casuarina* trees are frequently used for windbreaks, erosion control, dunes stabilization in particular in lands where the water resources are limited (Albouchi et al. 1989). *Casuarinaceae* family form symbiotic N-fixing associations with actinomycetes (*Frankia*) as well as ectomycorrhizal and endomycorrhizal fungi. (Diem 1996). This symbiotic enhances the resistance and root plasticity system of *Casuarina* species in harsh conditions or degraded soils (Duhoux and Franche, 2003).

Casuarina glauca was the candidate species for this study due to its wide using in agroforestry, for woody production programs and soil rehabilitation (Zhong et al. 2013, Slaimi et al. 2021). In order to ensure the tolerance of *C. glauca* used for afforestation, it would be necessary to evaluate their behavior after their irrigation with treated wastewater. The aim of this study is (i) to evaluate the treated wastewater effects of on *C. glauca* morpho-physiological behavior after 90 days (ii) to determine to accumulate and translocate heavy metals.

2. Materials and methods

2.1. Experimental setup and culture Conditions

The study was carried out at the nursery of the National Institute of Research in Rural Engineering, Waters and Forests of Tunisia (INRGREF) in a semi-arid bioclimate (36 °50 N, 10 ° 14 E and 3 m above sea level). The annual average of rainfall was around 475 mm and the monthly average of temperatures varied between 7.2 °C and 34.8°C. The experiment was conducted under semi-controlled conditions using one year old *C. glauca* plants. Homogeneous seedlings with the same height growth have been put in plastic pots of 50 cm in diameter and 60 cm in depth, containing 10kg of substrate consisting of a mixture of sand and loam (30% – 70%). They were divided into two batches, the first one was irrigated with tap water (control C) and the second with urban secondary treated waste water (TWW) collected from the outlet of wastewater treatment plant Kalâat El Andalous of Tunisia (N: 36° 53' 51''.55 E: 10° 12' 01''.71). This is an ecological wastewater treatment plant that provides secondary effluents treated with lagoons. The main physicochemical and microbiological characteristics of TWW are shown in table 1. The characteristics of the tap water used for the control are (61 mg/l of $Ca^{2+,}$ 23 mg/l of $Mg^{2+,}$ 15.1 mg/l K+, 0.569 mg/l of PO43, 314.11 mg/l of $Cl^{-}$and 0.066 mg/l Fe3+). The plants in both treatments were irrigated every 2 days according to the water holding capacity of the soil to prevent water leakage from pots throughout the experiment, which lasted 90 days. At the end of this period (90 days), several morphological, physiological, biochemical parameters and concentration of heavy metals in plant tissues have been measured. The adopted experimental design type was a random full. Each plant that is represented individually by a pot has been considered as a repetition in a completely randomized design which contained 20 repetitions per treatment.

Table1: Microbiological and physicochemical properties of treated wastewater (TWW) used according to the standards Tunisian for the discharge of effluents into hydraulic natural flows (NT 106.02).

Parameters	Units	TWW	NT 106.02
Total coliforms	UFC per 100ml	4.3×10^3	-
Fecalcoliforms	UFC per 100 ml	3.5×10^3	2000
Fecal streptococcus	UFC per 100ml	2.5×10^5	1000
pH		7. 94 ± 0.01	6.50–8.50
Conductivity	mS/cm	5.32±0.02	7
Salinity	g/l	3.8± 0.2	-
Temperature	°C	20 ±0.6	25
BOD_5	mg O_2/l	37± 0.03	30
COD	mg/l	385 ±0.11	90
Suspendedmatter	mg/l	35±0.04	30
Nitrogen	mg/l	22.58 ± 0.17	30
Phosphorus	mg/l	2.38± 0.19	0.05
Ortho phosphate(PO_4^{3-})	mg/l	18.396±0.53	0.05
Magnesium	mg/l	139.9±0.005	200
Sodium	mg/l	1192±0.01	300
Calcium	mg/l	338±0.02	500
Sulfate	mg/l	750 ± 0.01	100
Potassium	mg/l	166. 8 ±0.04	50
Chloride	mg/l	1532 ± 0.03	600
Cadmium	mg/l	0. 2145 ±0.01	0.005
Manganese	mg/l	0. 16597± 0.09	0.5
Copper	mg/l	0.1181± 0.007	0.5
Lead	mg/l	0.3114±0.12	0.1
Nickel	mg/l	0.531±0.005	0.2
Zinc	mg/l	0.9043±0.02	5

2.2. Growth and biomass of plants

The growth of the *C. glauca* plants irrigated with TWW and fresh water hasbeen assessed by the measurement of the height of the main stem and weighed shoots and roots biomass. In order to evaluate the effect of TWW treatmenton the growth of seedlings, the relative growth rates (RGR) of plantheight was calculated using 10 plants per treatment according to the equation described by Salim and Pitman (1983):

$RGR = (\ln W2 - \ln W1) / (t_2 - t_1)$

Where W2= Height of plant (cm) at time t_2

W1 = Height of plant (cm) at time t_1

t_1 and t_2(separatedby days depending on doubling time)

At the end of the experiment, the dry mass produced in the aerial and theroot part was measured on 10 plants per treatment. The roots were extracted after sievingof the substrate using a mesh of 2 mm. The biomass of plants was determined by weighing after dryingat 70°C for 48 hours.

2.3. Measurements of gas exchange, chlorophyll content and relative water content

Net photosynthesis (A), stomatal conductance(gs), transpiration (E)and intercellular CO2 concentration(Ci)were determined between 09:15 and 11:15 am usingan infrared analyzer the LCPRO + (LCpro + model, ADC Bio Scientific LTD., Hoddesdon, England) on five seedling selected randomly by treatment, under the following conditions: an active photosynthetic flux of photons (PPFD) of 980 ± 64µmol.mol^{-1}m^{-2}s^{-1}, a CO_2 concentration of350 µmol.mol^{-1} and a foliar temperature of 30 ± 2 ° C.The intrinsic water use efficiency (iWUE)has been calculated as the ratio of net photosynthesis to the stomatal conductance(Farquhar et al. 1982).

For determining chlorophyll concentration concentrations, 100 mg of fresh needle was crushed in 10 ml of 80 percent acetone. The samples were incubated at 4°C overnight before being centrifuged at 10,000g for 10 minutes. The extract was collected, and the supernatant's absorbance was measured at 645 and 663 nm with a spectrophotometer (Shimadzu UV-1800 PC model Kyoto, Japan). The total chlorophyll content was calculated using the equation of Arnon (1949) and expressed as mg. g-1 dry mass.

The water status of the plants for each treatment was estimated through the measurement of the relative water contents of treated and control leaves. This parameter was measured on ten needlesused by the same plants used for the gas exchange and chlorophyll measurements. The relative water contents (RWC) was calculated according to the formula:

$RWC= (P_f-P_s)/(P_{turg} - P_s) x 100$

With:

P_f : the fresh mass determined immediately after excision of shoots.

P_{turg} : the saturation mass determined after immersion of the sectioned petiole in distilled water and its passage to the dark at 25°C for 24 h.

P_s : the dry mass determined after drying at 70°C for 48 hours.

2.4. Determination of soluble sugars and proline

2.4.1. Soluble sugars extraction

The extraction of soluble sugars was performed according to the technique described by Albouchi (1997). These measures have been carried out on five samples of mature needles which were randomly selected for each treatment. For each repetition, 100 mg of leaf dry matter finely crushed were mixed with 10 ml of 80%ethanol. Themixture was heated in a water bath at 70 ° C for 30 minutes and then centrifuged at 10 000 g for 15 minutes. The soluble sugars in the supernatant were assayed by 0.2% anthrone in the presence of 80% ethanol. The optical density was measured at 640 nm in a spectrophotometer (Lambda 40, Perkin Elmer, USA). The concentrations of soluble sugars determined using glucose standard and expressed as mg g^{-1}dry matter.

2.4.2. Proline extraction

Proline was extracted according to the method described by Monneveux and Nemmar (1986), using L-proline as a standard. One hundred mg of needles dry powder were placed in a test tube, to which 2 ml of methanol (40%) have been added. The mixture was heated in a water bath at 85 ° C for 60 minutes. After cooling, 1 ml of the extract was tacked and mixedwith 1 ml of acetic acid, 25 mg of ninhydrinand 1 ml of a mixture containing (120 ml of distilled water, 30 ml of acetic acid and 80 ml of orthophosphoric acid). The tubes were thenincubated in a water bath at 100 C for 30 min. After cooling, 5 ml of toluene were added and taken for centrifugation. The supernatant containing the proline was recovered and dried by addition of Na_2SO_4. The optical density was measured at 528 nm in a spectrophotometer (Lambda 40, Perkin Elmer, USA). The concentrations of proline are expressed as µmol g^{-1} of dry matter.

2.5. Determination of phenolic compounds

The extraction of phenolic compounds was performed according to the method of Mau et al. (2001), by mixing 2.5 g of needles or roots dried matter with 25 ml of distilled water. The mixture was agitated for 30 min and kept at rest for 24 hours at 4°C in darkness. Finally, the mixture is filtered on paper Wattman. The obtained extracts were stored at 4°C for later use in the dosage of total polyphenols and flavonoids.

2.5.1. Determination of total polyphenols

A volume of 125 µl of the plant extract diluted 10 times was mixed with 500µl of distilled water and 125µl of Folin-Ciocalteureagent. After vigorous agitation of the mixture followed by a rest 3min, a volume of 1250 µl of $CO_3(Na)_2$ (7 %) was added. Finally the mixture was adjusted by distilled water to 3 ml. After resting for 90 min in the dark, reading the absorbance was performed using a spectrophotometer (Lambda 40, Perkin Elmer, USA) at 760 nm. The standard range was prepared with gallic acid at concentrations ranging from 50 to 500 $mg.L^{-1}$. The levels of polyphenols wereexpressed as mg of gallic acid equivalent per gram of dry matter (mg $EAG.g^{-1}$ MS). (Bettaib Rebey et al, 2016).

2.5.2. Determination of total flavonoids

A volume of 0.25 ml of extract was diluted for 5 times with 0.075 ml of $NaNO_2$ (5%). The mixture was standing for 6 min in temperature room before adding 0.15 ml of aluminum chloride (AlC_{l3}, 10%), freshly prepared. A second incubation for 5 min at room temperature was performed and also followed by the addition of 0.5 ml of NaOH (1M). The mixture is subsequently adjusted with distilled water to a final volume of 2.5 ml. The absorbance reading was made at 510 nm. The standard range was prepared with catechin at increasing concentrations ranging from 50 to 500 $mg.L^{-1}$. The concentrations of flavonoids were expressed as mg equivalent of catechin per gram of dry matter (mg $EC.g^{-1}$ MS).

2.6. Heavy metals analyses

The assay was performed on shootsand roots samples of five plants randomly selected for each treatment previously rinsed with distilled water and then dried at 80 °C for 72 h. The dried samples were ground and digested in the presence of three acids solution (HNO_3:$HClO_4$: H_2SO_4; 10: 4: 1) on a hot plate at 300 °C for 2 h. The extracts, were also filtered on filter paper (Whatman No. 1) without ash. The heavy metals concentrations in filtrate were determined by using atomic absorption spectrophotometry (Model COULD 9100, Philips, Cambridge,UK). The concentrations of heavy metals were expressed as $mg.kg^{-1}$. dry mass (DM).

The metals transfer capacity of *C. glauca* from roots to shoots was evaluated at the end of the experiment with the calculation of translocation factor (TF) (Mackay and Fraser 2000) according to the following formula:

$TF = Metal\ concentration\ in\ shoot / Metal\ concentration\ in\ root$

2.7. Statistical analysis

The data were subjected to statistical analysis using the software SPSS, 16.0 (SPSS Institute Inc., 252 Chicago, Illinois, USA). The comparison of the averages for the different measured variables was carried out using the test Student at the threshold of 5%.

3. Results

3.1. Growth, biomass production and gas exchange of seedlings.

After 30 days, the RGR of plants height (RGRh) was similar in control and treated plants which areabout 0.006 $cm.d^{-1}$ (Figure 1). The RGRh of treated plants increased by 20% and 24% respectively after 60 and 90 days of irrigation with TWW compared to control. Then, after 90 days of irrigation, shoots and roots dry mass of treated plants increased by 26% and 24%, respectively, compared to control plants. Also, in treated plants, net assimilation (A), stomatal conductance (gs), transpiration(E) and CO2 intercellular (Ci) increased significantly ($p < 0.05$) by 57%, 24%, 68% and 22%, respectively compared to control plants (Figure 2). Furthermore, net assimilation (A) oftreated plantswas 2.4 times greater than their stomatal conductance (gs). Similarly, treated plants have higher intrinsic water use efficiency (WUEi) than control plants (Figure 2).

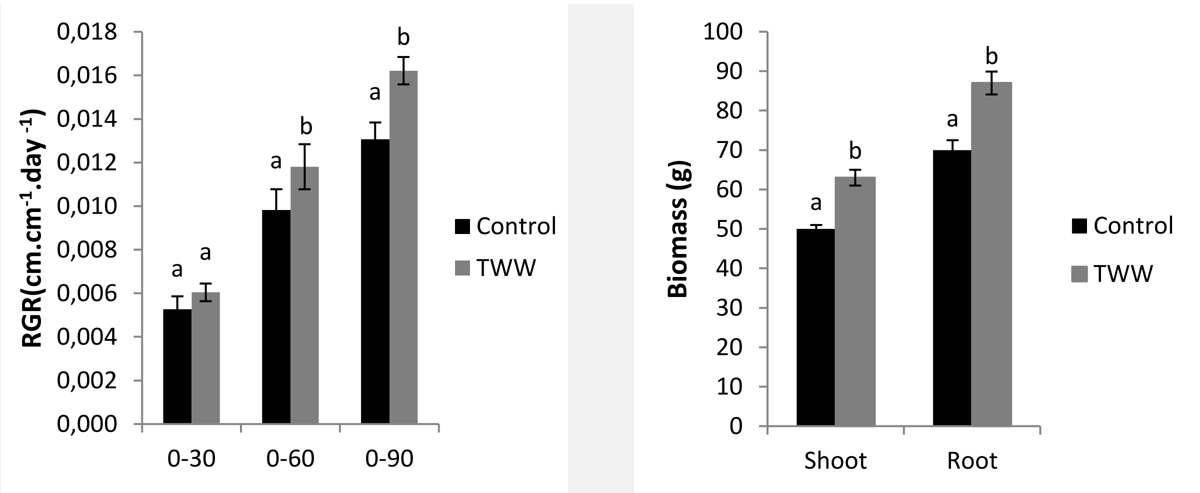

Figure 1. Relative growth rate of plant height and biomass production of shoots and roots dry mass of C*asuarina glauca* irrigated with tap water (control) and secondary treated wastewater (TWW) after 90days.For each parameter, the bars represent the mean values ± SE (n = 5) associated with different letters (a,b) indicate significant differences among the two treatments. The confidence intervals were calculated at5 % threshold.

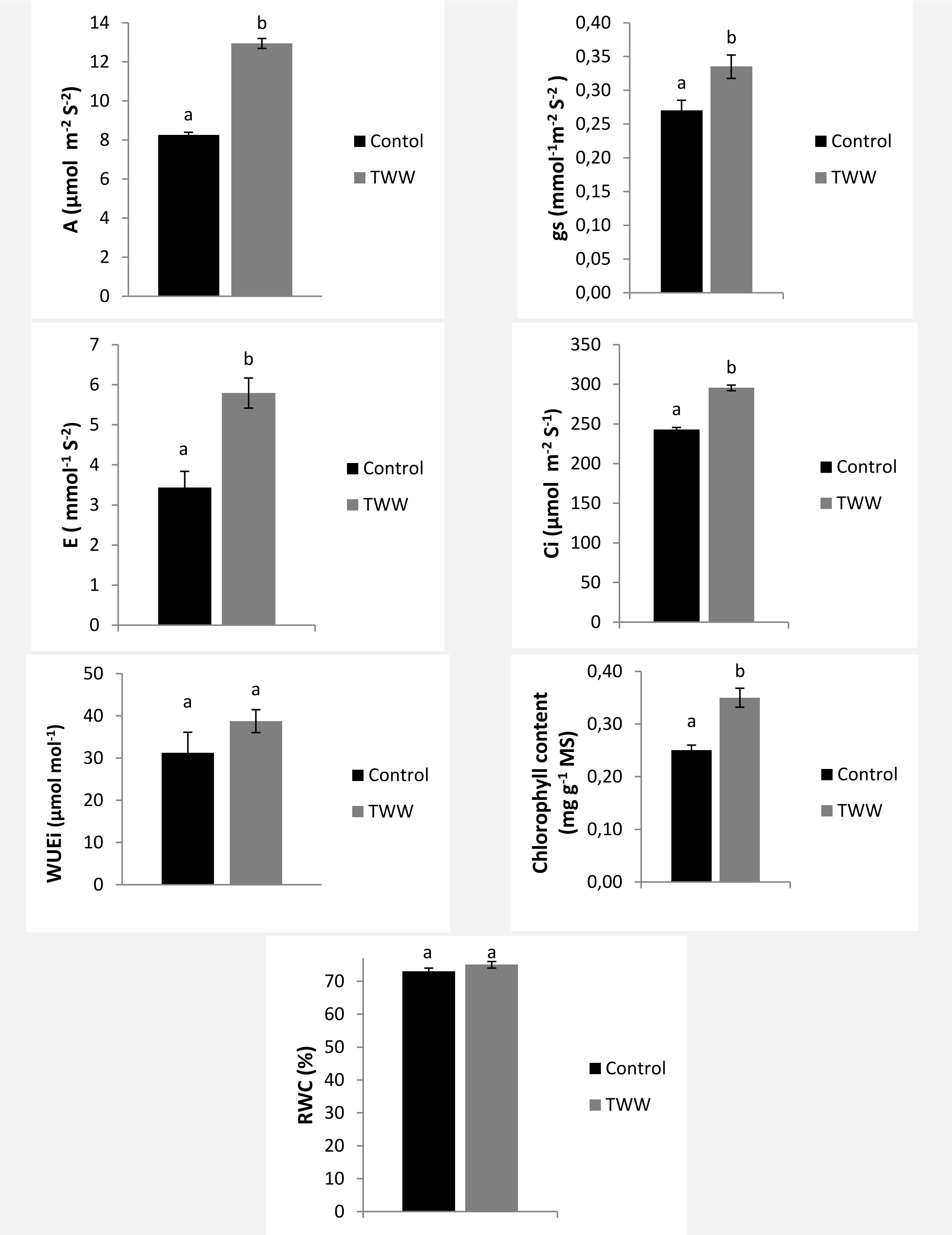

Figure 2. Net photosynthesis rate (A), stomatal conductance (gs), transpiration (E), intracellular CO2 (Ci), Intrinsic Water Use efficiency (WUEi), total chlorophyll and relative water of *Casuarina glauca* irrigated during 90 days with tap water (control) and secondary treated wastewater (TWW). For each parameter, the bars represent the mean values ± SE (n = 5)associated with different letters (a,b) indicate significant differences among the two treatments. The confidence intervals were calculated at 5 % threshold.

3.2. Water content and biochemical Changes in *C. glauca*

The relative water content (RWC) of treated plants has not significantly changed after 90 days compared to controls indicating adequate tissue hydration (Figure 2). Total chlorophyll content was also significantly

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enhanced ($p< 0.05$) after irrigation with TWW during 90 days, it was higher 40% in treated than in control plants of *C. glauca* (Figure 3). The phenolic compounds of plants irrigated with TWW were significantly greater ($p< 0.05$) than in *C. glauca* control plants (Figure 3). In addition, total polyphenols and flavonoids in shoots of treated plants were 2.3-fold and 1.5-fold higher than in controls plants. However, the soluble sugars in plants irrigated with TWW were significantly higher than in control plants after 90 days. In contrast, there was no significant differenceof proline concentration between treated and control plantsof *C. glauca*.

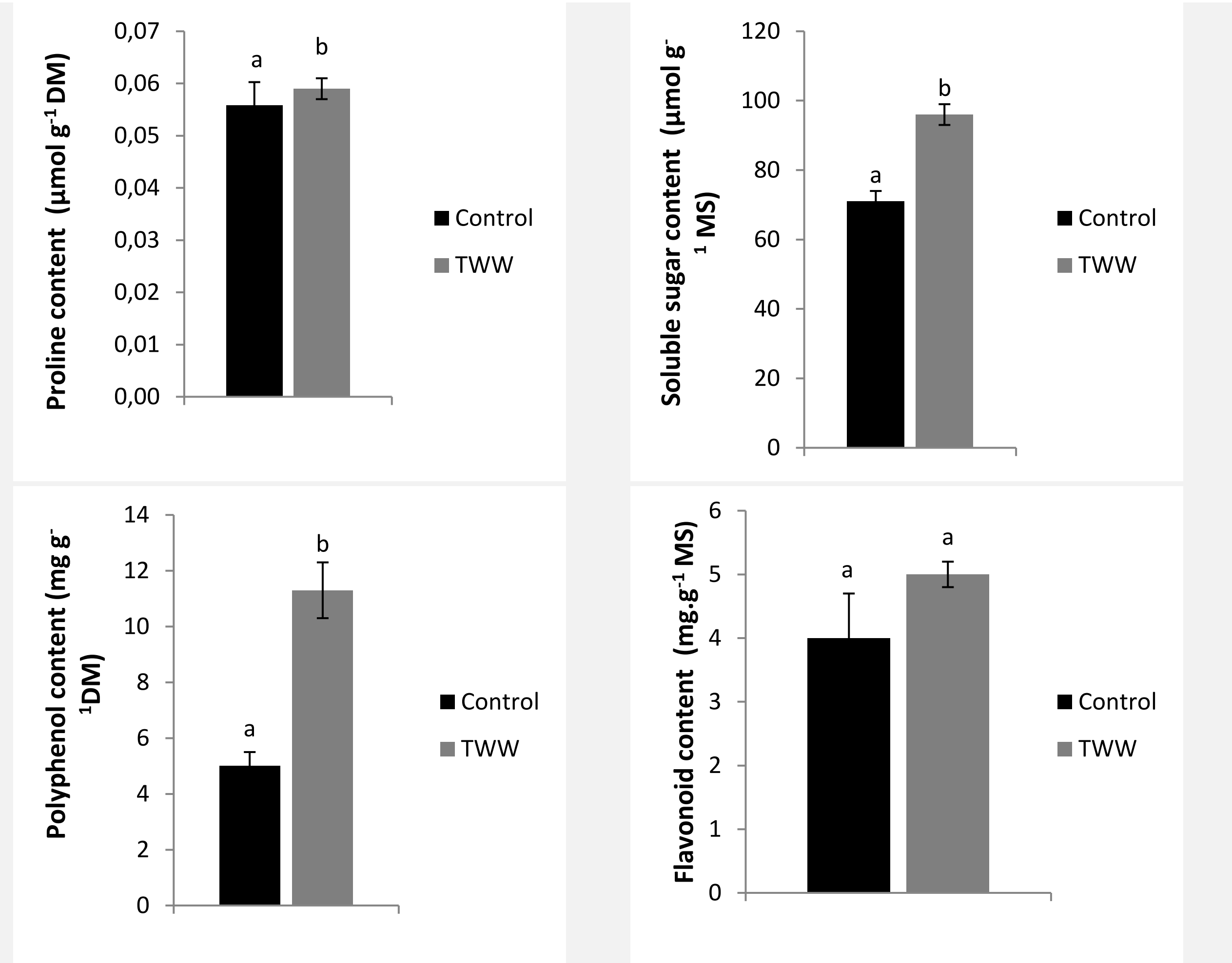

Figure 3. Proline content, soluble sugar, polyphenol and flavonoids content of *Casuarina glauca* irrigated during 90 days with tap water (control) and secondary treated wastewater (TWW). For each parameter, the bars represent the mean values ± SE (n = 5) associated with different letters (a,b,c,d) indicate significant differences among the two treatments. The confidence intervals were calculated at 5 % threshold.

3.3. Effect of treated wastewater on accumulation and translocation of heavy metals

The results showed higher heavy metal accumulation in roots than in shoots of *C. glauca* irrigated with, such as Cu, Zn, Cd, Ni, and Pb, except for Mn that is more accumulated in shoots (Figure 4). Their concentrations in roots were (3,2; 1,8; 4; 3.5 and 3.5-fold) higher than in shoots, while the Mn concentration in shoots was 3 times higher than in roots. The irrigation with TWWduring90 days has significantly ($P <0.05$) increased the concentration of these metals in plants of *C. glauca*. Cd is accumulated in higher proportion in the two parts of treated plants. It was 4 times higher in shoots and 8.8 times higher in roots compared to control plants. The translocation factor (TF) of heavy metals in *C. glauca*is shown in table 2. The highest TF (> 1) was recorded for Mn, related to the preferential translocation of this element to the leaves. The lowest TF (0,12) has also been noted for the Cd. Furthermore, the TF of Zn, Cu, Ni, and Pb ranged from 0.56 to 0.28.

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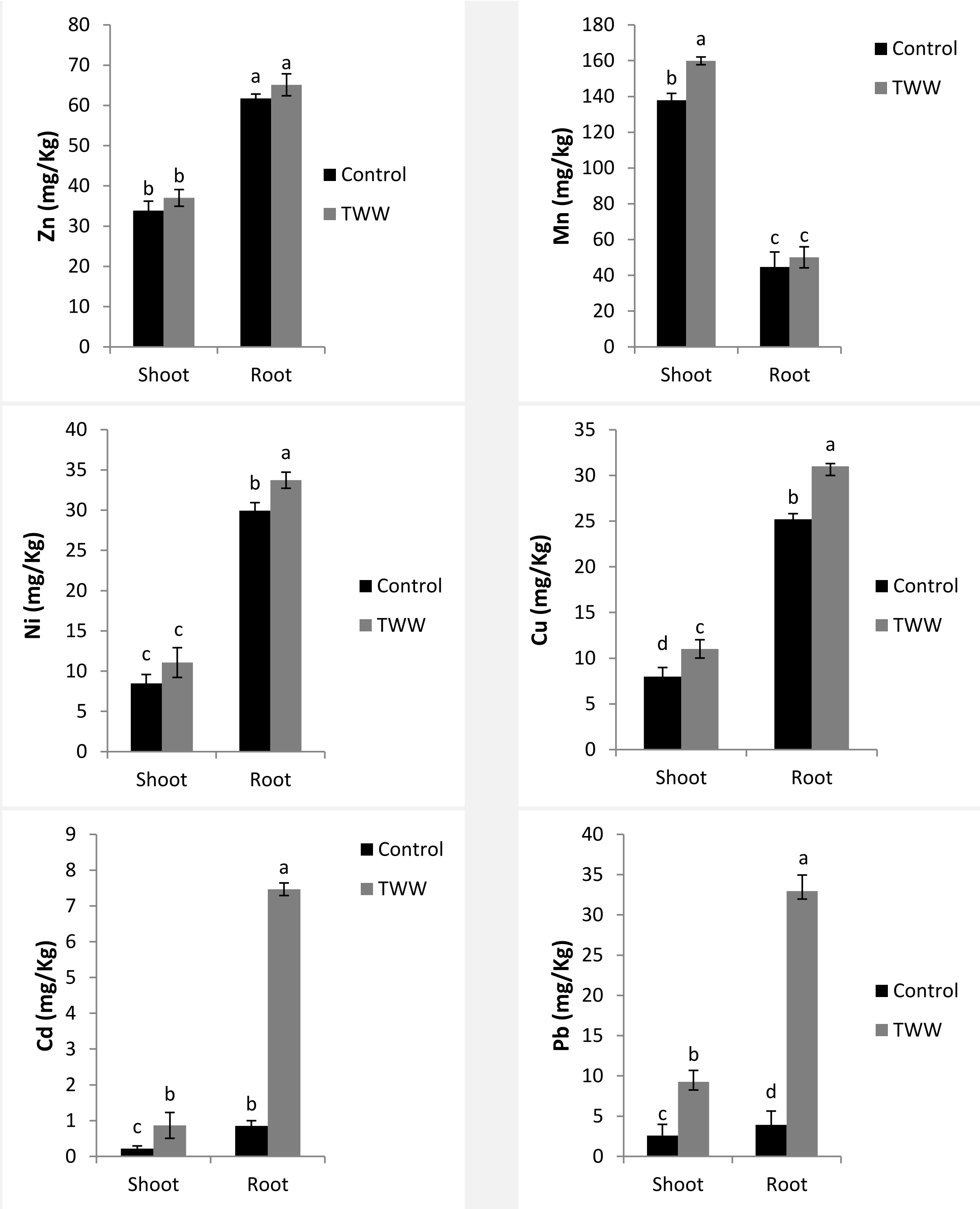

Figure 4. Concentrations of zinc (Zn), manganese (Mn), nickel (Ni), cupper (Cu), cadmium (Cd), lead (Pb), in shoots and roots of *Casuarina glauca* plants irrigated during 90 days with tap water (control) and secondary treated wastewater (TWW). For each parameter, the bars represent the mean values ± SE (n = 5) associated with different letters (a,b,c) indicate significant differences among the two treatments. The confidence intervals were calculated at 5 % threshold.

Table 2: Translocation factor (TF) of heavy metals in *Casuarina glauca* irrigated with secondary urban treated wastewater after 90 days.

Métaux lourds	Zn	Mn	Cu	Ni	Pb	Cd
TF	0.56± 0.02 b	3.19± 0.01 a	0.35± 0.03 c	0.32± 0.03 c	0.28± 0.02 d	0.12± 0.01 e

The averages followed by the same letter are not significantly different according to the Newman and Keuls test ($p < 0.05$).

4. Discussion

Irrigation with urban secondary TWW for 90 days significantly increased the growth of C. glauca compared to control plants which were irrigated with fresh water. The TWW increased the height of the RGR of treated plants (fig.1). The same effects of irrigation with TWW have been demonstrated on the biomass production of treated plants (fig.1). Thus, the TWW stimulated biomass production in the aerial parts as well as roots (fig.1). This growth was related to the nutrient content in TWW, such as mineral nitrogen, phosphorus, potassium, and micronutrients (Rais et al. 2009). These elements, which are essential for growth, stimulated the biomass productivity of *C. glauca* (Karnosky, 2003). Taking into account the fertilizer supply of phosphorus, nitrogen, potassium, and macronutrients, some authors believe that TWW could play the same role as fertilizer, depending on the concentration of these elements in water, the type of crop, and the level of soil fertility (Singh and Agrawal, 2010; Justin et al. 2010). The results also indicate that irrigation with TWW for 90 days stimulated the production of biomass without any negative effects of pollutants. These findings are consistent with Kumar and Reddy's (2010) findings of positive growth in *Casuarina equisetifolia* trees irrigated with TWW for 13 months. In contrast, Zarati et al. (2015) reported that the irrigation with TWW for 60 days reduced the growth and thephotosynthesis rate of *P. nigra*. The positive responses of physiological characteristics of plants irrigated by treated wastewater with high levels of sodium and chloride, which are respectively 4 and 2.6 times higher than the norm (Table 1), suggest that *C. glauca* seedlings tolerate the salinity of waterwhich is also confirmed byScotti-Campos et al, (2016).However, no significant differences ($p > 0.05$) of relative water contents (RWC)were observed between treated and control plants, reflecting an osmotic adjustments to prevent oxidative damage (Hachani et al. 2021). Total chlorophyll was higher in plants irrigated withTWW. The stimulation of chlorophyll production could berelated to the preferential compartmentalization of Mn in leaf cell organelles (Emamverdian et al. 2015). The Mn has several metabolic roles in different cellular compartments that induce plant growth (Emamverdian et al. 2015). It is an essential cofactor of several biochemical reactions in plants. Moreover, the most metabolically dependent plant on Mn is the water-splitting reaction in photosystem II (Alejandro et al. 2020). In contrast to the present study, several studies reported that heavy metal accumulation decreased chlorophyll production (Padmaja et al. 1990; Mysliwa-Kurdzieland Strzalka, 2002). Moreover, the higher concentrations of heavy metals such as Cd and Pb in the needles of treated plants did not affect the chlorophyll synthesis, which indicates the metal stress tolerance of *C. glauca* during the experimental time.The higher chlorophyll concentration of treated plants increased the photosynthetic rate and, indirectly, the growth and biomass production. Similarly, other studies reported that irrigation with TWW increased the photosynthetic rate of plants such as *Populus nigra*, *Salix nigra*, and *Populus canadensis* (Borghi et al. 2007; Singh and Agrawal, 2010; Zarati et al., 2015). In addition, the higher availability of nitrogen (N) in TWW has stimulated the photosynthetic performance of plants. This stimulation is linked to the involvement of N in the production of essential molecules for the photosynthetic system such as chlorophyll and chloroplast composition (Deroche, 1983; Bassi et al. 2018). Yet, another study showed a positive correlation between nitrogen supply and the increase in photosynthetic rate in sugarcane (Bassi et al. 2018). The higher stomatal conductance (gs) of plants irrigated with TWW as compared to ground water irrigated ones (Fig. 3) could be due to potassium availability in TWW (Table 1). Indeed, potassium is one of the main osmotically active solutes involved in the stomatal opening which leads to the increase in CO_2 uptake and photosynthesis (Dietrich et al. 2001). In addition, a slight increase of WUEi was noted in plants irrigated with TWW that indicates plants tolerance to wastewater pollutants. The higher accumulation of solutes such as anions, nutrient compounds in treated plants than in control plants can prevent plants against a greater water relations reduction by regulating cellular osmotic potential and stomatal closure (Gómez-Bellotet al. 2020). In fact, water is the exchange unit that is required for the acquisition of CO_2 for plants. Thus, the WUEi of treated plants reflects a lower water amount spent per CO_2 molecule assimilated (Farooq et al., 2009). In the current experiment, primary metabolites such as proline and soluble sugar content increased in plants after irrigation with TWW. The high intracellular levels of accumulation of these osmoprotective compounds regulates the osmotic balance of cells and preserves the subcellular structures for better water absorption under stress (Zhang et al. 2010; Sharma et al. 2012). However, the accumulation of proline is a possible mechanism to detoxify plants through ROS scavenging against oxidative damage to mitigate the metallic stress effects (Hassan et al. 2018). Irrigation with TWW has increased specific phenolic compounds in plants, such as total polyphenols and flavonoids. These secondary metabolites have several physiological functions, such as antioxidant properties such as the protection of cells from potential damage related to heavy metal accumulation (Agati et al. 2012; Zhang et al. 2015). Higher accumulation of heavy metals (Zn, Cu, Ci, Pb and Cd) in roots of *C. glauca plants* than in shoots has been observed. The accumulation of metals differs between organs and tissues of the same plant (Rotkittikhun et al. 2006). The heavy metals amount was significantly enhanced in root for Cu, Ni, Pb, and in particular for Cd which increased 8.8-fold compared to in control plants. These amounts indicate that *C. glauca* is non-hyper accumulative species because all heavy metals concentrations were less than 1000 $mg.kg^{-1}$(Baker and Brooks, 1989). Hyper accumulative plant species have a strong capacity to extract metals by transferring them to aerial part, but the tolerant plants tend to restrict this transfer to soil-root or root-shoot system (Yoon et al. 2006)**.** The ability of plants to transfer the metals from the root to the aerial part is determined by the translocation factor (TF). ThusTF < 1 indicates a low heavy metals transfer from roots to the aerial part suggesting that these plants accumulate the metals in their roots or rhizomes more than in their aerial part (Usman et al, 2019). This case applies to *C. galuca* plants which have shown an FT < 1 for the elements (Zn, Cu, Ni, Pb, and Cd). In addition, the very low translocation of the Pb (TF = 0,28) and, in particular, Cd (TF = 0,12) indicates that the *C. glauca* plants would have limited the transfer of these elements from the roots to the leaves in order to decrease their toxicity. These two elements are not essential for the development of the plant, their accumulation could be toxic to photosynthetic activity, the synthesis of chlorophyll and the activities of anti-oxidant (Slaimi et al. 2021; Touati et al. 2019). The sequestration of these metals in the roots, which limited their toxic effects on leaves, would be an adaptive mechanism of *C. glauca* to mitigate the metal stress. This mechanism could also be a metal-exclusive character in *C. glauca* (Mganga et al. 2011; Ghazouani et al. 2020). In addition, our study showed the phytostabilisation capacity of *C. glauca* for these metals in order to reduce their mobility and their diffusion in the soil (Susarla et al. 2002). It reduces the mobility of metals and their leaching into groundwater which limits their bioavailability and their passage into food chains (Yoon et al, 2006). The stabilization and the extraction of heavy metals by plants are the result of the ability of root exudates to change physicochemical characteristic of the rhizosphere. Moreover, these exudates are able to modify the soil pH which increased the chelating, the solubilization and the uptake of heavy metals and micronutrients (Shen et al. 2017). Moreover, the strong *C. glauca* tolerance of Cd high level in roots may be due to the richness of root exudates with secondary metabolites with low molecular weight. These metabolites are capable to create a Cd-phytochelatin complex at the soil-root interface which allowing the plants tolerate the Cd toxicity (Bali et al. 2020). This mechanism could provide efficient conditions for natural removal or stabilization of pollutants from soil (Yoon et al. 2006).

5. Conclusion

The current study investigated the fertilizing effect of irrigation with urban treated wastewater on *C. glauca* seedlings over a 90-day period. This was demonstrated by a faster increase in height growth as well as an increase in biomass production. C. glauca was recommended for the rehabilitation of marginal and degraded lands due to its rapid growth, higher biomass production, adaptation to extreme conditions, and phytostabilization potential of heavy metals. The use of TWW as a source of water supply to ensure the success of the early phase of plantation could be an advantage of this process. However, on-site planting testing is required to evaluate this species in natural conditions and over a longer period of time. Previous studies have shown the effectiveness of the symbiosis *C. glauca*–Frankia in the bio-remediation of soils polluted with industrial effluents. The interaction between *C. glauca* and mycorrhizae, which could affect the uptake and translocation of metals, deserves further investigation. The use of TWW as a source of water supply to ensure the early phase of plantation success could be an advantage of this process. However, in order to evaluate this species in natural conditions and over a longer period of time, on-site planting testing is required. Previous research has demonstrated the efficacy of the *C. glauca–Frankia* symbiosis in the bio-remediation of soils polluted with industrial effluents. The interaction between *C. glauca* and mycorrhizae, which may affect metal uptake and translocation, warrants further study.

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References

Agati G, Azzarello E, Pollastri S, &Tattini M (2012) Flavonoids as antioxidants in plants: location and functional significance. Plant science, 196, 67-76

Albouchi, A., Chatty, Y., &Benzarti, J. (1989) Des arbres et des arbustes ausages multiples pour réaliser des brise-vent en Tunisie. *Min. Agric. IRESA-INRF, Tunis*

Albouchi A, Ghrir R, & El Aouni M H (1997) Hardness drought and accumulation of soluble carbohydrates and free amino acids in the phyllodes of Acacia cyanophyllaLindl. In Annals of forest science (Vol. 54, No. 2, pp. 155-168). EDP Sciences

Alejandro, S., Höller, S., Meier, B., &Peiter, E. (2020) Manganese in plants: from acquisition to subcellular allocation. Frontiers in Plant Science, 11, 300

Arnon D I (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant physiology, 24(1), 1

Baker, A. J., & Brooks, R. (1989) Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. *Biorecovery.*, *1*(2), 81-126

Bali, A. S., Sidhu, G. P. S., & Kumar, V. (2020) Root exudates ameliorate cadmium tolerance in plants: a review. *Environmental Chemistry Letters*, *18*(4), 1243-1275

Bargali, K. (2011) Screening of leguminous plants for VAM association and their role in restoration of degraded lands. *Journal of American Science*, *7*(1), 7-11

Bassi, D., Menossi, M., &Mattiello, L. (2018) Nitrogen supply influences photosynthesis establishment along the sugarcane leaf. *Scientific reports*, *8*(1), 1-13

Bejaoui, Z., Albouchi, A., Lamhamedi, M. S., & El Aouni, M. H. (2008) Effet d'un assèchement édaphique sur la croissance, l'allocation de biomasse et les relations hydriques chez *Casuarina glauca*. *Botany*, *86*(11), 1242-1251

BettaiebRebey I, Sriti J, Besbess B, MkadminiHammi K, HamrouniSellami I, Marzouk B, &Ksouri R (2016) The effect of the origin and solvent extraction on the content of phenolic compounds and the potential antioxidant seeds of fennel (Foeniculumvul-station Mill.). Journal of New Sciences, 27, 1478-1487

Borghi M, Tognetti R, Monteforti G, &Sebastiani, 2007 Responses of Populus× euramericana (P. deltoides× P. nigra) clone Adda to increasing copper concentrations. Environmental and ExperimentalBotany, 61(1), 66-73.

Cornel P,& Weber B (2004) Water reuse for irrigation from waste water treatment plants with seasonal varied operation modes. Water Science and Technology, 50(2), 47-53

Deroche, M. E. (1983) Relations entre la photosynthèse et l'assimilation de l'azote. *Bulletin de la Société Botanique de France. Actualités Botaniques*, *130*(1), 85-98

Diem HG (1996) The mycorrhizal plants actinorhiziennes. Actabotanicagallica, 143(7), 581-592

Diem, H. G., &Dommergues, Y. R. 1990. Current and potential uses and management of *Casuarinaceae* in the tropics and subtropics. The biology of *Frankia* and actinorhizal plants, 317-342

Dietrich, P., Sanders, D., &Hedrich, R. (2001) The role of ion channels in light-dependent stomatal opening. *Journal of Experimental Botany*, *52*(363), 1959-1967

DUHOUX, E., &Franche, C. (2003) Les nodules actinorhiziens de Casuarina. *Biofutur (Puteaux)*, 235, 45-49.

El Ayni, F., Cherif, S., Jrad, A., &Trabelsi-Ayadi, M. (2011) Impact of treated wastewater reuse on agriculture and aquifer recharge in a coastal area: Korba case study. *Water Resources Management*, *25*(9), 2251-2265

El Moussaoui, T., Wahbi, S., Mandi, L., Masi, S., &Ouazzani, N. (2019) Reuse study of sustainable wastewater in agroforestry domain of Marrakesh city. Journal of the Saudi Society of Agricultural Sciences, 18(3), 288-293

Emamverdian, A., Ding, Y., Mokhberdoran, F., & Xie, Y. (2015) Heavy metal stress and some mechanisms of plant defense response. The Scientific World Journal, 2015

Farooq, M., Wahid, A., & Lee, D. J. (2009) Exogenously applied polyamines increase drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties. ActaPhysiologiaePlantarum, 31(5), 937-945

Frascari, D., Zanaroli, G., Motaleb, M. A., Annen, G., Belguith, K., Borin, S., ...& Ortega, C. V. (2018) Integrated technological and management solutions for wastewater treatment and efficient agricultural reuse in Egypt, Morocco, and Tunisia. *Integrated environmental assessment and management*, *14*(4), 447-462

Ghazouani, S., Béjaoui, Z., Spiers, G., Beckett, P., Gtari, M., &Nkongolo, K. (2020) Effects of Rhizobioaugmentation with N-Fixing Actinobacteria*Frankia* on Metal Mobility in *Casuarina glauca*-

Soil System Irrigated with Industrial Wastewater: High Level of Metal Exclusion of *C. glauca*. Water, Air, &Soil Pollution, 231(8), 1-17

Ghazouani, S., Béjaoui, Z., Michael, P., Spiers, G., Beckett, P., Gtari, M., &Nkongolo, K. (2020) Rhizobioaugmentation of Casuarina glauca with N-fixing actinobacteria*Frankia*decreases enzymatic activities in wastewater irrigated soil: effects of Frankia on *C. glauca* growth. *Ecotoxicology, 29(4),* 417-428

Gómez-Bellot, M. J., Lorente, B., Sánchez-Blanco, M. J., Ortuño, M. F., Nortes, P. A., &Alarcón, J. J. (2020) Influence of mixed substrate and arbuscularmycorrhizal fungi on photosynthetic efficiency, nutrient and water status and yield in tomato plants irrigated with saline reclaimed waters. *Water*, 12(2), 438

Hachani, C., Lamhamedi, M. S., Zine El Abidine, A., Abassi, M., Khasa, D. P., & Béjaoui, Z. (2021) Water Relations, Gas Exchange, Chlorophyll Fluorescence and Electrolyte Leakage of Ectomycorrhizal*Pinushalepensis* Seedlings in Response to Multi-Heavy Metal Stresses (Pb, Zn, Cd). *Microorganisms, 10(1)*, 57

Hassan, F. A. S., Ali, E. F., &Alamer, K. H. (2018) Exogenous application of polyamines alleviates water stress-induced oxidative stress of Rosa damascena Miller var. trigintipetalaDieck. *South African Journal of Botany*, *116*, 96-102

Justin MZ, Pajk N, Zupanc V, &Zupančič M (2010) Phytoremediation of landfill leachate and compost wastewater by irrigation of Populus and Salix: biomass and growth response. Waste management, 30(6), 1032-1042

Karnosky, D. F. (2003) Impacts of elevated atmospheric CO2 on forest trees and forest ecosystems: knowledge gaps. Environment international, 29(2-3), 161-169

Kumar AY, & Reddy VM (2010) Effects of municipal sewage on the growth performance of Casuarina equisetifolia (Forst. &Forst.) it is sandy soil of the East Coast at Kalpakkam (Tamil Nadu, India). Applied Ecology and Environmental Research, 8(1), 77-85

Kuzovkina, Y. A., & Quigley, M. F. (2005) Willows beyond wetlands: uses of Salix L. species for environmental projects. Water, Air, and Soil Pollution, 162(1), 183-204

Mackay, D., & Fraser, A. (2000) Bioaccumulation of persistent organic chemicals: mechanisms and models. *Environmental pollution*, *110*(3), 375-391

Mganga, N., Manoko, M. L. K., &Rulangaranga, Z. K. (2011) Classification of plants according to their heavy metal content around North Mara gold mine, Tanzania: implication for phytoremediation. *Tanzania journal of science*, *37*

Mau JL, Chao GR, & Wu KT(2001) Antioxidant properties of methanolic extracts from several ear mushrooms. Journal of Agricultural and Food Chemistry, 49(11), 5461-5467

Monneveux P,&Nemmar M (1986) Contribution to the study of drought resistance in wheat (Triticumaestivum L.) and durum wheat (Triticum durum Desf.): study of proline accumulation during the development cycle

Myśliwa-Kurdzie lB, & Strzałka K (2002) Influence of metals on biosynthesis of photo synthetically pigments. In Physiology and biochemistry of metal toxicity and tolerance in plants (pp. 201-227). Springer, Dordrecht

Padmaja K, Prasad DDK, & Prasad ARK (1990) Inhibition of chlorophyll synthesis in Phaseolus vulgaris L. seedlings by cadmium acetate. Photosynthetica, 24(3), 399-405

Qadir, M., Bahri, A., Sato, T., & Al-Karadsheh, E. (2010) Wastewater production, treatment, and irrigation in Middle East and North Africa. *Irrigation and Drainage Systems*, *24*(1), 37-51

Raes D, Steduto P, Hsiao TC, &Fereres E (2009) AquaCrop—the FAO crop model to simulate yield response to water: II. Main algorithms and software description. Agronomy Journal, 101(3), 438-447

Raïs, M. T., Sifi, S., &Xanthoulis, D. (2009) Valorisation des eaux usées traitées pour l'irrigation de complément de l'olivier (oleaeuropaea L.): effets sur les comportements végétatif et productif de l'arbre et sur la qualité sanitaire des fruits. Durable rég. méditerr, 142-155

Salim, M., &Pitman, M. G. (1983) Effects of salinity on ion uptake and growth of mung bean plants (Vignaradiata L.). FunctionalPlantBiology, 10(5), 395-407

Scotti-Campos, P., Duro, N., da Costa, M., Pais, I. P., Rodrigues, A. P., Batista-Santos, P., ...&Ribeiro-Barros, A. I. (2016) Antioxidative ability and membrane integrity in salt-induced responses of Casuarina glauca Sieber ex Spreng.in symbiosis with N2-fixing FrankiaThr or supplemented with mineral nitrogen. *Journal of plant physiology*, *196*, 60-69

Sharma P, Jha AB, Dubey RS, &Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of botany, 2012

Shehzadi, M., Afzal, M., Khan, M. U., Islam, E., Mobin, A., Anwar, S., & Khan, Q. M. (2014). Enhanced degradation of textile effluent in constructed wetland system using Typha domingensis and textile effluent-degrading endophytic bacteria. *Water research*, *58*, 152-159.

Shen, Z. J., Chen, Y. S., & Zhang, Z. (2017) Heavy metals translocation and accumulation from the rhizosphere soils to the edible parts of the medicinal plant Fengdan (Paeoniaostii) grown on a metal mining area, China. *Ecotoxicology and environmental safety*, *143*, 19-27

Singh, A., &Agrawal, M. (2010) Effects of municipal waste water irrigation on availability of heavy metals and morpho-physiological characteristics of *Beta vulgaris* L. *Journal of Environmental Biology*, *31(5)*, 727

Slaimi, R., Abassi, M., & Béjaoui, Z. (2021) Assessment of *Casuarina glauca* as biofiltration model of secondary treated urban wastewater: effect on growth performances and heavy metals tolerance. *Environmental Monitoring and Assessment*, *193*(10), 1-16

Susarla, S., Medina, V. F., & McCutcheon, S. C. (2002) Phytoremediation: an ecological solution to organic chemical contamination. *Ecological engineering*, *18*(5), 647-658

Tan, H. T., & Yeo, C. K. (2009) The Potential of Native Woody Plants for Enhancing the Urban Waterways and Waterbodies Environment in Singapore. Raffles Museum of Biodiversity Research and Singapore-Delft Water Alliance, National University Singapore, Singapore, 14

Touati, M., Bottega, S., Castiglione, M. R., Sorce, C., Béjaoui, Z., &Spanò, C. (2019) Modulation of the defence responses against Cd in willow species through a multifaceted analysis. *Plant Physiology and Biochemistry*, *142*, 125-136

Usman, K., Al-Ghouti, M. A., & Abu-Dieyeh, M. H. (2019) The assessment of cadmium, chromium, copper, and nickel tolerance and bioaccumulation by shrub plant Tetraenaqataranse. *Scientific reports*, *9*(1), 1-11

Yoon, J., Cao, X., Zhou, Q., & Ma, L. Q. (2006) Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site. *Science of the total environment*, *368*(2-3), 456-464

Zarati H, Mguis K, Abassi M, Jouili H, Albouchi A, Youssef A, ... &Béjaoui Z (2015) Growth, gas exchanges and accumulation of inorganic matter of Populusnigra L. in responses to treated wastewater. Journal of Biodiversity and Environmental Sciences, 6, 297-305

Zhang B, Deng Z, Ramdath DD, Tang Y, Chen PX, Liu R, ... &Tsao R (2015) Phenolic profiles of 20 Canadian lentil cultivars and their contribution to antioxidant activity and inhibitory effects on α-glucosidase and pancreatic lipase. Food Chemistry, 172, 862-872

Zhong C, Zhang Y, Chen Y, Jiang Q, Chen Z, Liang J, ... &Bogusz D (2010) Casuarina research and applications in China. *Symbiosis*, 50(1-2), 107-114.

Zouari M, Bloem E, Souguir D, Schnug E, Hanchi B, &Hachicha M (2020) Assessment of selected plant species under drained saline and waterlogged conditions in pots when irrigated with treated wastewater. Water and Environment Journal, (0),1-10