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Research Article

Effect of intercropping alfalfa on physiological and biochemical parameters of young grapevine plants cultivated on agricultural and contaminated soils

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Abstract

Our research aimed to reveal the capacity of intercropping with *Medicago sativa*-rhizobia in the amelioration of grapevine growth in agricultural and a Cd/Pb contaminated soils. A local variety of grapevine was cultivated in monocropping and in intercropping with *Medicago sativa* inoculated or not with its associated rhizobia. Intercropping with alfalfa induced a significant increase in shoot and root biomass of grapevine in the agricultural soil. However, in the contaminated soil, a slight increase in root biomass was observed. Concerning photosynthesis apparatus, we showed that the presence of Cd and Pb in the soil induced a significant decrease in both CO_2 assimilation rate and stomatal conductance. Interestingly, intercropping with alfalfa only and with rhizobia alleviate this effect. Similar results are obtained for chlorophyll and carotenoid content. This was associated with a significant decrease in the malondialdehyde level in leaves and roots of grapevine cultivated in intercropping with alfalfa with and without inoculation in the two soils as compared the monoculture treatment. Comparison between treatments revealed also that intercropping with alfalfa induced a decrease in the activities of some enzymes implicated in the defence to the oxidative stress such as catalase and superoxide dismutase. Regarding soluble protein content, it is needed to signal the improvement of this parameter with the intercropping system in the contaminated soil when compared to the monocropping treatment. This work highlights the importance of the use of legumes in intercropping with grapevine as intercrop plant non-competitive for soil nutrient and proving N supply for associated plants.

Keywords: antioxidant enzyme; grapevine; growth; heavy metals; intercropping; *Medicago sativa*

Introduction

The viticulture is one of the most important and strategic culture in Tunisia that occupied about 21500 ha (Bouagga *et al.*, 2019). Furthermore, the grapevine (*Vitis vinifera*) is an important economic and agronomic

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fruit crop in the world (Cui *et al.*, 2016). However, the viticulture is exposed to multiples biotic and abiotic stress such as drought (Monteiro and Lopes, 2007; Medrano *et al.*, 2015), salinity, heavy metal stress (Angelova *et al.*, 1999; De Conti *et al.*, 2019) and viral and fungal attacks (Kennelly *et al.*, 2005). Hence, it was observed an expansion of the use of pesticides in this type of culture (Brunetto *et al.*, 2016). As consequences, the quality of soils was affected and being more and more not useful to agriculture.

Regarding the problems exposed to viticulture, sustainable agriculture becomes the principal goal to scientists, farmers and agronomists (Medrano *et al.*, 2015). Intercropping was known as an agricultural practice used in this condition and becomes the subject of many research works (Li *et al.*, 2014). Hence, the intercropping is the plantation of two or more plants species simultaneously in the same field (Vandermeer 1989). However, intercropping showed contrasting effects depending on species, genotypes, density, season and environmental conditions. The main disadvantage in some intercropping systems is the competition for nutrient, radiation and water use efficiency (Black and Ong, 2000; Sillon *et al.*, 2000).

Several models of intercropping were reported in the literature between different plants species with multiple benefits for cultures. Hauggaard-Nielsen and Jensen (2005) present a list of the top ten of intercrop species which, seven of them are legumes. Indeed, legumes in symbiosis for nitrogen fixing with rhizobia provide beneficial input for complimentary plant. Legumes increase the N quantity in soil, inducing its availability for intercrop plant (Xiao *et al.*, 2004; Li *et al.*, 2009) and increase the microbial dynamic and density in the soil by secretion of root exudates (Hinsinger *et al.*, 2009). Moreover, frequently, legumes are not competitive for water availability because most of them are known as drought tolerant, having deep, and pivoting roots (Darkwa *et al.*, 2016; Defez *et al.*, 2017).

The intercropping alfalfa/maize showed an increase in grain yield and economic incomes (Sun *el al.*, 2014). Similar results are obtained in intercropping clover, fenugreek or bean with the palm dates (Nagwa *et al.*, 2014). Concerning the intercropping legumes/grapevine, few reports were noted. It was shown that intercropping of two grapevine cultivars with clover and pea provide environmental benefits such as enhancing microbiological activity, promoting yield and increasing farmer income (Gaser *et al.*, 2017). Recently, Contreras *et al.* (2019) showed that subterranean clover in intercropping with grapevine stimulate the roots exudates and plant-plant interaction and it can improve the industrial grapevine performance.

Moreover, agricultural areas are threatened by trace metal pollution. This pollution causes different ecological and environmental problems (Liu *et al.*, 2018; Yadav *et al.*, 2018). In agronomy, the phytotoxicity, enhanced by trace metal, damaged life cycle of plants, may be transmitted in the food and affect the health human, and became a pervasive environmental problem (Abdelkrim *et al.*, 2018). On the other hand, grapevine is frequently cultivated on soils exposed to high risk of erosion (Garcia-Ruiz, 2010) and poor level of organic carbon (Salomé *et al.*, 2016). Intercropping could be a solution for remediation of heavy metal contaminated soils, especially via phytostabilisation technique. In fact, legumes with deep roots can stabilize metals in the rhizosphere and decelerate its absorption by complimentary plant (Erakhrumen *et al.*, 2007).

In this study, we investigate the effect of intercropping alfalfa on several physiological and biochemical parameters of grapevine cultivated on soil samples. Two types of soils were used in this work: an agricultural soil and a Cd/Pb contaminated one.

Materials and Methods

Soil characterisation

Two types of soil were used: soil of Mateur (m) which is an agricultural soil retrieved from coordinates 37° 2' 24" north 9° 39' 59" east of Tunisia; and soil of Gzala (g) from 37°04'13,6" north 9°31'38,4" east of Tunisia. The latter one is a contaminated soil that comes from areas in proximity of mine industry. Chemical parameters of two soils are presented in the Table 1. We determine the concentrations of some mineral elements, Zinc (Zn), Magnesium (Mg), Iron (Fe), Calcium (Ca), Copper (Cu), Lead (Pb) and Cadmium (Cd).

Concentrations of mineral elements were measured using the aqua regia method. Thus, 0.5 g of soil was weighed and dissolved in 8 ml of an acid mixture of 37% HCl and 63% HNO_3 (3/1 (v/v)).

The mixture was heated at 100 °C on a hot plate until total evaporation. After cooling, 20 ml of HNO_3 (N/7) were added, and it was filtered with a Whatman filter paper. Essential nutrients concentrations were measured by atomic absorption spectroscopy (AAS) (PERKIN ELMER Analyst 300). The pH values were determined using the pH meter. Similar values of pH were shown for the two soils, 7.86 for Gzala and 7.65 for Mateur.

Table 1. Chemical characteristics of the two types of soils used in the experiment

Chemical characteristics	Gzala soil (g)	Mateur soil (m)
pH	7.86	7.65
[Pb ($g\ Kg^{-1}$)]	2.6	0.3
[Cd ($g\ Kg^{-1}$)]	0.1	nd
[Ca ($g\ Kg^{-1}$)]	23.83	83
[Zn ($g\ Kg^{-1}$)]	20.55	1.4
[Mg ($g\ Kg^{-1}$)]	2.4	3.5

Plant materials and intercropping test

The main crop is a wild variety of grapevine named 1103 Paulsen (1103 P) and it is the most used rootstock in Tunisia. It has several characteristics that allow it to adapt to Tunisian soils. The intercrop plant is a local cultivar of alfalfa called 'wethref' originated from Gabès and commonly cultivated in the south of Tunisia. Grapevine plants are cultivated in 20 cm diameter plastic pots in association with alfalfa. One plant of grapevine and 100 alfalfa plants were used in each pot. The culture was carried out in a greenhouse (28 °C temperature, 70% humidity and 12h/12h photoperiod).

The experiment was conducted with three treatments on each type of soil:

*On Mateur soil: The monoculture treatment (Vm) refer to grapevine cultivated alone. In the intercropping treatment (VLm) grapevine is intercropped with alfalfa and for the treatment (VLRm) grapevine is intercropped with the alfalfa rhizobia couple.

*On Gzala soil, Grapevine sole (Vg); grapevine/alfalfa (VLg) and grapevine/alfalfa/rhizobia (VLRg).

The crop lasts four months. The alfalfa was sown 15 days before the establishment of grapevine plants. The inoculation was done with the RCR2011, reference strain of *Sinorhizobium meliloti* (about 10^{10} cells / pot). Bacterial culture was multiplied in a liquid yeast extract mannitol medium (YEM) (Vincent 1970) and grown in an incubator with controlled growth conditions (150 rpm, 28 °C). The first inoculation was done one week after sowing alfalfa, the second was realised after 15 days of the first inoculation.

Chlorophyll content

Leaf chlorophyll (a, b and total) and carotenoids concentrations ($mg.g^{-1}$ fresh weight (FW)) of grapevine leaves were spectrophotometrically determined according to Lichtenthaler and Wellburn (1983). The absorbance was measured at three wavelengths, 470, 646 and 663 nm.

Determination of photosynthetic apparatus

After four months of growth, assimilation rate (A), stomatal conductance (gs), intracellular CO_2 concentration (Ci), transpiration rate (E) and water use efficiency [WUE (the ratio between carbon gain in photosynthesis (A) and water loss in transpiration (E)] were determined on grapevine leaves by using potable infrared CO_2/H_2O gas exchange system (LCPro+, Bio-Scientific, Great Amwell, Herts, UK).

MDA determination

Lipid peroxidation was evaluated by determining the malondialdehyde (MDA) contents in the fresh tissues (Heath and Packer, 1968). We homogenize 0.5 g of fresh leaves and roots in 5 ml of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 12.000 g for 15 min at 4°C. 0.5 ml aliquot of the supernatant was mixed with 0.5 ml of 0.5% thiobarbituric acid (TBA) prepared in TCA 20%, and heated at 100°C for 25 min. the samples were emerged in an ice bath for stopping the reaction and were centrifuged at 10.000 g for 5 min.

After centrifugation, the absorbance of the supernatant was measured at 532 nm and 600 nm. The amount of MDA was calculated by the following ratio:

MDA equivalents (nmol ml^{-1}) = $[(A_{532}-A_{600})/155000]10^6$.

Total MDA content was expressed in nmol g^{-1} FW.

Antioxidant enzyme activities measurement

Soluble protein content

Soluble protein content was determined by the method of Bradford using bovine serum albumin as standards (Bradford, 1976). At 4 °C, shoots and roots of grapevine were homogenized, in a mortar, with 50 mg polyvinyl-pyrrolidone (PVP) with 1mM of phenylmethylsulfonyl fluoride (PMSF), 10 mM dithio-DL-threitol DTT, 0.1 mM EDTA, in 50 mM potassium phosphate buffer pH 7.8 (Gorcena *et al.*, 1997).

The mixture was centrifuged at 12.000 rpm for 20 min at 4 °C and the supernatant was used as enzyme extract.

Superoxide dismutase (SOD, EC 1.15.1.1)

According to the method of Beauchamp and Fridovich (1971), SOD was assayed by monitoring the inhibition of photochemical reduction of nitrobluetetrazolium chloride (NBT). 1 ml of reaction mixture was homogenized with 50 mM potassium phosphate buffer (pH 7.8), 2 mM riboflavin, 13 mM methionin, 75 mM NBT and enzyme extract. One unit of SOD activity was expressed as the amount of enzyme required to cause 50% inhibition of reduction of NBT as measured at 560 nm.

Catalase (CAT, EC 1.11.1.6)

According to the method of Aebi (1984), CAT activity was measured at λ= 240 nm. In reaction mixture 1 ml containing 50 mM potassium phosphate buffer (pH, 7.0) and enzyme extract. At 240 nm, the decomposition of H_2O_2 was followed for 1 min by absorbance decrease. Addition of H_2O_2 started the reaction (ξ_{240}=0.036 mM^{-1} cm^{-1}).

Glutathione peroxidase (GPX, EC 1.11.1.7)

During 1 min (ξ_{470}= 26.6 M^{-1} cm^{-1}), and at 470 nm, GPX activity was assayed by following the evolution of the kinetics of tetraguaiacol production from guaiacol containing reaction mixture by using hydrogen peroxide (H_2O_2) as electron donor (MacAdam, 1992).

Statistical analysis

All data presented are the mean values of at least three replicates. Analysis of variance (ANOVA I) for all measured variables was performed by SPSS Ver. 20, Inc., Chicago, USA. The treatment means were separated using TUKEY's multiple range test taking $P \leq 0.05$ as significant.

Results

Plant biomass

There is a significant difference in shoot and root dry weight of grapevine plants between treatments on the agricultural soil (Figure 1). In fact, intercropping grapevine with both alfalfa (VLm treatment) and the alfalfa-rhizobium couple (VLRm treatment) increased significantly shoot grapevine biomass by 27.4 and 54.2% respectively as compared to the monoculture treatment Vm where grapevine is cultivated only. Root biomass was similar between the two treatments VLm and VLRm which were significantly higher than the Vm biomass treatment.

Concerning the contaminated soil, an increase in root biomass was observed without being significant. On the other hand, there was a slight reduction (10%) in aerial biomass in the VLg treatment (Figure 1). Our results showed also that shoot and roots biomass of grapevine plants cultivated in the control monoculture treatment Vg in Gzala soil decreased substantially as compared to plants cultivated in the monoculture Vm treatment (Mateur soil).

Photosynthesis apparatus

The effect of intercropping grapevine/alfalfa-rhizobia on the photosynthetic parameters of grapevine on the two soils are presented in Table 2. Generally, the five tested parameters showed a significant difference between treatments and soils.

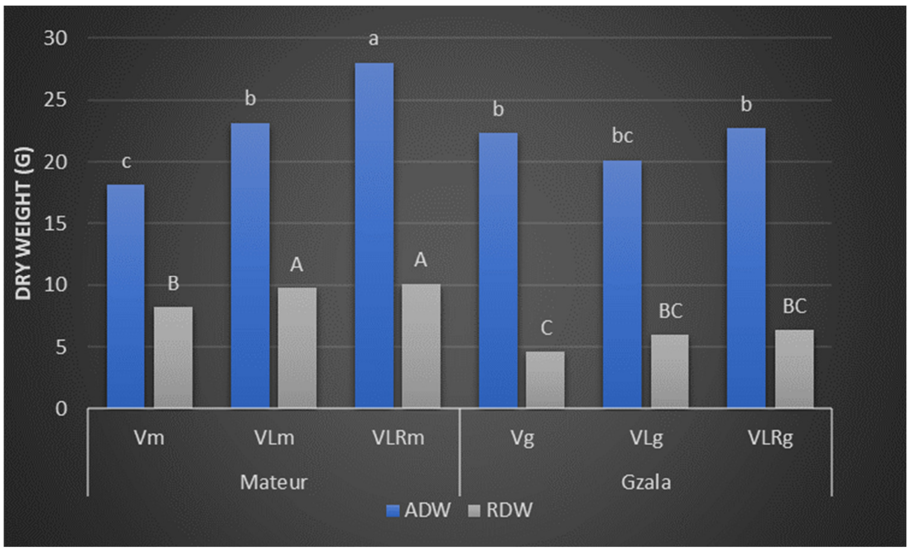

Figure 1. Variation of aerial and roots dry weights of grapevine plants cultivated on Mateur and Gzala soils
ADW: Aerial dry weight. RDW: Root dry weight. The unit is the gram (g). The data followed by different letters are significantly different according to TUKEY test, $p<0.05$. Vm: grapevine cultivated on Mateur soil. VLm: grapevine with *Medicago sativa* cultivated on Mateur soil. VLRm: grapevine, *Medicago sativa* and associated rhizobia on Mateur soil. Vg: grapevine cultivated on Gzala soil. VLg: grapevine with *Medicago sativa* on Gzala soil. VLRg: grapevine, *Medicago sativa* and associated rhizobia on Gzala soil.

The intracellular concentration of CO_2 (Ci) increases from the control treatments Vm and Vg to the treatment VLRm and VLRg with the same way in the two soils. The increase in CO_2 concentration induced by the addition of alfalfa and the associated rhizobia was about 20%. Furthermore, there is no difference between soils related to this parameter.

Table 2. Variation of gas exchange parameters of grapevine plants cultivated on Mateur and Gzala soils

	Mateur		Gzala	
Ci[μmol(CO_2)mol^{-1}]				
	Vm	236 c	Vg	239 c
	VLm	248 b	VLg	248 b
	VLRm	286,5 a	VLRg	288 a
E[mmol(H_2O)mol^{-2}s^{-1}]				
	Vm	1.75 b	Vg	1.2 d
	VLm	1.56 c	VLg	1.61 c

	VLRm	1.83 a	VLRg	1.89 a
gs[mmol(CO_2) mol^{-2} s^{-1}]				
	Vm	0.073 b	Vg	0.05 c
	VLm	0.073 b	VLg	0.1 a
	VLRm	0.077 b	VLRg	0.097 a
A[μmol(CO_2) mol^{-2}s^{-1}]				
	Vm	5,727 c	Vg	3.65 e
	VLm	5,113 d	VLg	6.67 b
	VLRm	5,123 d	VLRg	7.85 a
WUE[μmol(CO_2) mol(H_2O)$^{-1}$]				
	Vm	3.27 b	Vg	3.04 c
	VLm	3.27 b	VLg	4.14 a
	VLRm	2.8 d	VLRg	4.15 a

(Ci): Intracellular CO_2 content, (E): Transpiration rate, (gs): Stomatal conductance, (A): photosynthesis rate, (WUE): Water Use Efficiency. The data followed by different letters are significantly different according to TUKEY test, $p<0.05$. Vm: grapevine cultivated on Mateur soil. VLm: grapevine with *Medicago sativa* cultivated on Mateur soil. VLRm: grapevine, *Medicago sativa* and associated rhizobia on Mateur soil. Vg: grapevine cultivated on Gzala soil. VLg: grapevine with *Medicago sativa* on Gzala soil. VLRg: grapevine, *Medicago sativa* and associated rhizobia on Gzala soil.

For transpiration rate (E), in the agricultural soil, differences between treatments were significant. A slight decrease with 10% was shown in the VLm treatment but the inoculation with associated rhizobia (VLRm treatment) induced a significant increase even more than the control treatment Vm (Table 2). In the contaminated soil, the results showed a significant increase from the control treatment to the VLRg treatments. A significant difference between the two soils was observed by comparing the two control treatments.

Concerning the stomatal conductance rate (gs), there is no differences between treatments in the agricultural soil. Interestingly, in the contaminated soil, our results showed that alfalfa and associated rhizobia induced a significant increase in stomatal conductance of grapevine plants when cultivated in an intercropping culture system (Table 2). Agricultural soil was more adapted than contaminated one for the culture of grapevine only (treatments Vm and Vg). Indeed, grapevine plants cultivated alone in the agriculture soil exhibited higher stomatal conductance than grapevine plants grown only in the contaminated soil. Conversely, at intercropping system (VLm/VLg and VLRm/VLRg treatments) the contaminated soil was more efficient.

Concerning the CO_2 assimilation rate (A), we observed a significant decrease of this parameter when grapevine was cultivated in intercropping system with alfalfa and/or associated rhizobia on the agricultural soil (Table 2). However, in the contaminated soil, we showed a significant increase of this parameter in the VLg and VLRg treatments as compared to the control treatment (Vg) where grapevine is cultivated only. The comparison between the two soils showed that the contaminated one present the higher values.

Regarding the water use efficiency ratio (WUE), intercropping system with alfalfa on the agricultural soil has no significant effect on this parameter compared to the control treatment Vm where grapevine is cultivated only. However, the inoculation with the associated rhizobia in the intercropping system (VLRm) induced a decrease in this parameter (Table 2). In the contaminated soil, intercropping grapevine with alfalfa only and in association with rhizobium inoculation increased significantly WUE by approximately 36% as compared to the monoculture treatment Vg.

Chlorophyll and carotenoid contents

The data obtained for grapevine pigments showed that there is no effect of the intercropping system with alfalfa on chlorophyll and carotenoid content neither in the agricultural soil (Mateur) nor in the contaminated one (Gzala) (Table 3). However, the inoculation of alfalfa with the associated rhizobia induced a significant increase in the total chlorophyll and carotenoid contents in grapevine plants in both types of soils (Table 3).

Lipid peroxidation (MDA determination)

The malondialdehyde content was measured in grapevine leaves and roots. Interestingly, our results showed that intercropping with alfalfa-rhizobia (VLR) significantly reduces MDA content in roots and leaves of grapevine in both types of soils (Figure 2). Comparison between the two soils showed that leaf MDA content of grapevine plants was more pronounced in the contaminated soil.

Table 3. Total chlorophyll and carotenoid content of grapevine plants cultivated on Mateur and Gzala soils

Soil	Treatments	Total Chlorophyll (mg.g^{-1} FW)	Carotenoid (mg.g^{-1} FW)
Mateur	Vm	1.60 ± 0.09b	0.38 ±0.003c
	VLm	1.70 ± 0.24b	0.44 ±0.04c
	VLRm	4.80 ± 0.15a	1.11±0.07a
Gzala	Vm	1.08 ± 0.12b	0.27±0.01d
	VLm	1.60 ± 0.07b	0.38±0.01c
	VLRm	4.47 ± 0.53a	0.94±0.02b

The data followed by different letters are significantly different according to TUKEY test, $p<0.05$. FW: Fresh weight. Vm: grapevine cultivated on Mateur soil. VLm: grapevine with *Medicago sativa* cultivated on Mateur soil. VLRm: grapevine, Medicago sativa and associated rhizobia on Mateur soil. Vg: grapevine cultivated on Gzala soil. VLg: grapevine with *Medicago sativa* on Gzala soil. VLRg: grapevine, *Medicago sativa* and associated rhizobia on Gzala soil.

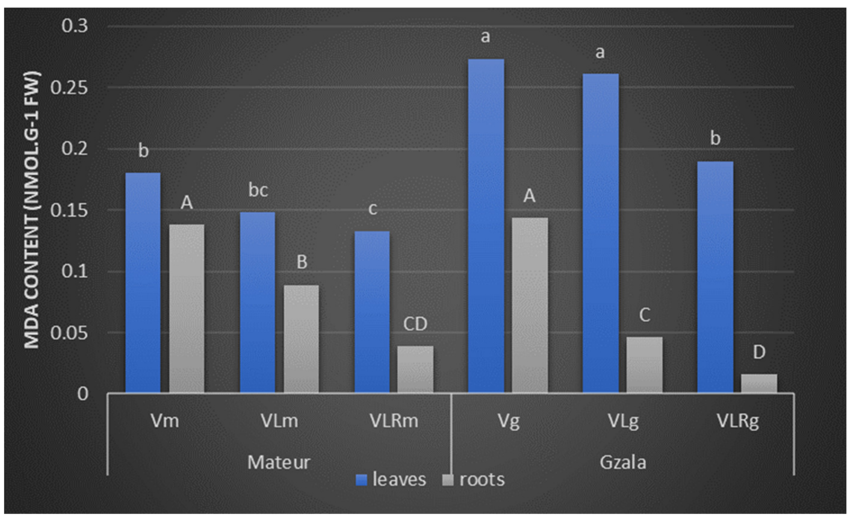

Figure 2. Variation of malondialdehyde contents (MDA) on grapevine leaves and roots on Mateur and Gzala soils

FW: Fresh weight. The data followed by different letters are significantly different according to TUKEY test, $p<0.05$. Vm: grapevine cultivated on Mateur soil. VLm: grapevine with *Medicago sativa* cultivated on Mateur soil. VLRm: grapevine, *Medicago sativa* and associated rhizobia on Mateur soil. Vg: grapevine cultivated on Gzala soil. VLg: grapevine with *Medicago sativa* on Gzala soil. VLRg: grapevine, *Medicago sativa* and associated rhizobia on Gzala soil.

Soluble protein content

In the agricultural soil of Mateur, soluble protein content values in roots showed a descendent evolution from the control treatment (Vm) to the intercropping system with alfalfa and associated rhizobia treatment (VLRm) (Figure 3i). In leaves, soluble protein content values were similar in the two treatments Vm and VLm. However, the VLRm treatment showed a significant decrease compared to the other treatments. However, in the contaminated soil, the evolution of soluble protein content values was mostly conversed. In fact, values evolved significantly from the control treatment (Vg) to the intercropping treatment with alfalfa (VLg) (Figure 3). Concerning leaves, we observed an ascendant evolution of soluble protein content from Vg to VLRg treatment. The comparison between soils showed an inversed situation for leaves and roots values (Figure 3i).

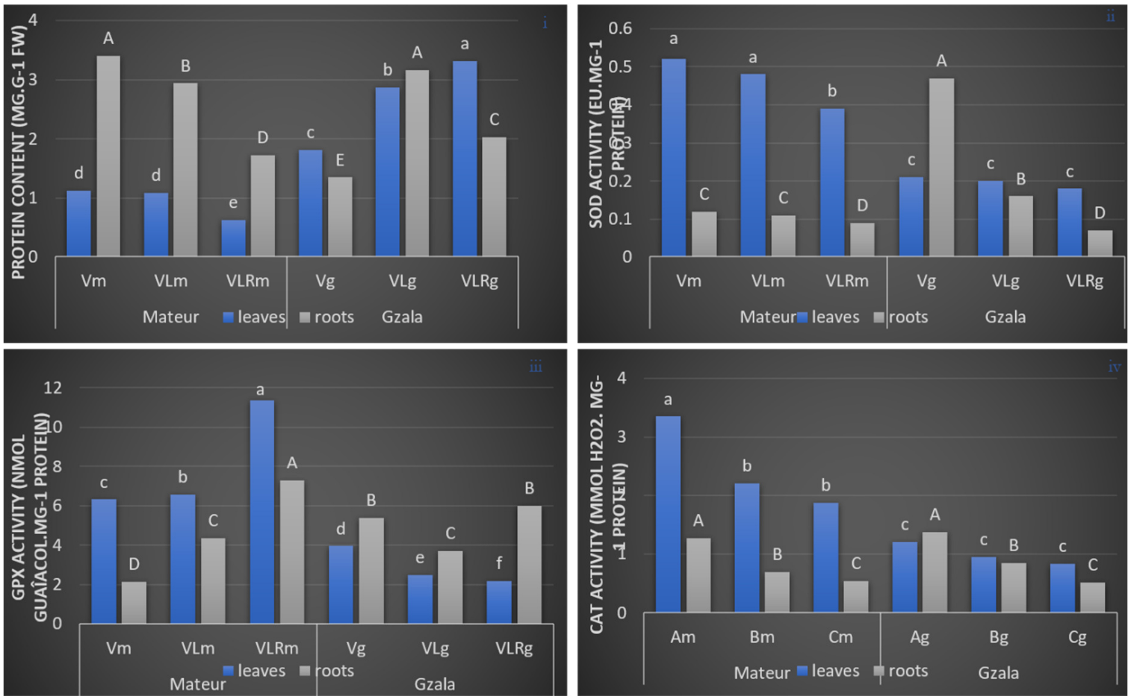

Figure 3. Variation of protein contents and enzymatic activities on grapevine leaves and roots on Mateur and Gzala soils

FW: Fresh weight. i: Proteins contents, ii: superoxide dismutase activities (SOD), iii: glutathione activities (GPX) and iv: catalase activities (CAT). The data followed by different letters are significantly different according to TUKEY test, $p<0.05$. Vm: grapevine cultivated on Mateur soil. VLm: grapevine with *Medicago sativa* cultivated on Mateur soil. VLRm: grapevine, *Medicago sativa* and associated rhizobia on Mateur soil. Vg: grapevine cultivated on Gzala soil. VLg: grapevine with *Medicago sativa* on Gzala soil. VLRg: grapevine, *Medicago sativa* and associated rhizobia on Gzala soil.

Antioxidant response

Concerning SOD activity of grapevine plants cultivated in the agricultural soil of Mateur, results showed that values in leaves and in roots were similar between the two treatments Vm and VLm (Figure 3ii). However, we remarked a significant decrease in this parameter in both leaves and roots in the treatment VLRm. In the contaminated soil of Gzala, there is no differences in SOD activity between treatments. However, in roots results showed a significant decrease from control treatment (Vg) to the intercropping treatment with alfalfa (VLg) (about three times) and from VLg to the intercropping treatment with alfalfa and associated rhizobia (VLRg). The comparison between soils showed a conversed situation for roots and leaves between control treatments (Vm and Vg).

Concerning GPX activity of grapevine plants cultivated in in the agricultural soil of Mateur, results showed significant differences between treatments in leaves with a higher value for the treatment VLRm (Figure 3iii). In roots, the difference was more pronounced between treatments than in leaves with a higher value in the VLRm treatment. In the contaminated soil of Gzala, the GPX activity in leaves was higher in the Vg treatment than in VLg and VLRg treatments (Figure 3iii). In roots, the GPX activity values were similar in Vg and VLRg treatments and they were significantly higher than in VLg treatment.

Concerning CAT activity, our results showed that in the agricultural soil, the control treatment Vm present the highest value in leaves, which is significantly different from the two other treatments VLm and VLRm (Figure 3iv). Similar results obtained in roots. In the contaminated soil, there is no difference in CAT activity in leaves between treatments. In roots, the control treatment Vg showed the highest value of CAT activity and VLRg showed the lowest value (Figure 3iv).

Discussion

Legumes are known as potential source for human and animal alimentation and as green fertilizers for soils by its principal characteristic, the symbiotic nitrogen fixation (Crews and peoples, 2004; Stagnari *et al.*, 2017). Furthermore, legumes could be beneficial for associated agro-system in rotation cropping system (Pokhrel and Pokhrel, 2013; Rahman *et al.*, 2014) and/or intercropping system (Brooker *et al.*, 2015; Contreras *et al.*, 2009). In this work, we investigate the effect of intercropping between grapevine and *M. sativa* and associated rhizobia on grapevine physiology. Seeing the positive role of the rhizobia – legumes symbiosis face to heavy metal stress in soils moderately contaminated as indicated in several reports (Zribi *el al.*, 2015; Abdelkrim *et al.*, 2018; Raklami *et al.*, 2018), we test this effect in agricultural and contaminated Tunisian soils.

The increase of biomass in grapevine plants could be related to the increased Nitrogen (N) availability because of the supply of this nutrient by its release in soil by legumes (Li *et al.*, 2013; White *et al.*, 2013b). Shoeib (2012) showed similar results where the intercropping with peas or clover was beneficial for grapevines culture. The beneficial of legumes in intercropping was also indicated with other types of culture. Nagwa *et al.* (2014) showed that, growth, yield and nutritional status of date Palms were increased by the intercropping with three types of legumes separately (Egyptian clover, Balady fenugreek or field bean). Furthermore, the positive effect of alfalfa intercropping was detected with corn and cereal cultures (Sun *et al.*, 2014; Xiao-hong *et al.*, 2016; Sun *et al.*, 2019; Shao *et al.*, 2020).

However, some studies showed that the cover cropping on grapevine are frequently conflicting especially with non-legumes species (Colugnati *et al.*, 2004; Guerra and Steenwerth, 2012). This could be resulted to the water and nutrient element competition (Mercenaro *et al.*, 2014).

Similarly, to our results in the contaminated soil, it was shown that legumes / rhizobia symbiosis could be used in the rehabilitation of soils moderately contaminated. Indeed, the phytostabilisation of some heavy metals (Zribi *el al.*, 2015) increased the microbial dynamic in soil (Pajuelo *et al.*, 2007; Rajkumar *et al.*, 2012) and the availability of nutrient to the plant (Nouairi *et al.*, 2015). Therefore, the alfalfa intercropping could be beneficial for grapevine growth in contaminated soil. This result was shown using non-legumes species for young grapevine in Cu-contaminated soil (De Conti *et al.*, 2019).

Regarding photosynthesis parameters, the reduction in CO_2 assimilation rate (A) and stomatal conductance (gs) of monocropped grapevine in contaminated soil could be related to the high quantities of Pb and Cd present in this soil. It is well known that Cd and Pb are two metals non-essential and mostly toxic for plants (Clemens and Ma, 2016). Furthermore, this harmful effect could frequently reduce photosynthesis parameters (Vassilev *et al.*, 2011; Verbruggen *et al.*, 2013). Xue *et al.* (2013) showed that net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration were decreased with the increasing Cd concentration in soybean leaves. Similar results are shown in *Arabidopsis arenosa* and *Arabidopsis halleri* (Szopinski *et al.*, 2019). However, studies about the effect of Cd or Pb on grapevine photosynthesis are scare. The effect of Cu was studied by De Conti *et al.* (2019) who showed that Cu supply reduces CO_2 assimilation rate in young grapevines.

Concerning the effect of the intercropping alfalfa on contaminated soil, our results confirm the beneficial effect of intercropping alfalfa and others legumes for physiological, biochemical and agronomic parameters of grapevine (Monteiro and Lopes, 2007; Contreras *et al.*, 2019). Similarly, we remarked that total chlorophyll and carotenoid content were reduced in the contaminated soil and the recovery was assured by the intercropping with alfalfa especially when inoculated with associated rhizobia. These two parameters were known as two indicators for metal stress in various plants (Sitko *et al.*, 2017; Paunov *et al.*, 2018).

The accumulation of heavy metals such as Cd and Pb in soils could affect physiological and biochemical mechanisms in plants. Hence, there is a production of reactive oxygen species inducing an oxidative stress in the plant (Sandalio *et al.*, 2009; Moura *et al.*, 2012). Face to this constraint, an arsenal of enzymes such as catalase, superoxyde dismutase are activated (Rusinowski *et al.*, 2019). In our study, intercropping grapevine with alfalfa with and without rhizobium inoculation decreased the activities of these two enzymes resulting in the reduction of the oxidative stress caused by metal accumulation. The same schema was shown with the MDA rate, which is a result of lipid degradation in condition of high oxidative stress level (Bouazizi *et al.*, 2010, Szopinski *et al.*, 2019). Zhao *et al.* (2017) showed that, in intercropping, the roots secretion of legumes is implicated to alleviate the stress implicated on crop in association. In this study, the decrease in the MDA content and SOD and CAT activities may be related to the presence of the couple alfalfa/rhizobia in intercropping system.

Concerning soluble proteins content, it was mostly demonstrated that abiotic stress including heavy metals induce a decrease in this parameter in different plants (Farghali and Quronfulah, 2016; Acemi *et al.*, 2020). The same result was shown in our work in the contaminated soil. Furthermore, the intercropping with alfalfa alleviate this effect and induce an increase in soluble protein content. It seems that intercropping induces the non-enzymatic way essentially in roots to alleviate the heavy metals stress.

Conclusions

In summary, we revealed in this work the beneficial role of alfalfa intercropping on physiological and biochemical parameters of grapevine. Furthermore, this effect of intercropping legumes species was shown in Cd/Pb contaminated soil. This result indicates that intercropping with alfalfa helps associated plant, grapevine in this condition, to alleviate the metal stress damages. In addition, we highlighted in this work the role of the associated rhizobia of alfalfa in the intercropping system.

Authors' Contributions

SJ, FM, FL and SC executed the experiences, and examine obtained results with KZ. IN, HM and KZ planned the study and guided the experiments. KZ, SJ and IN wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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