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EFFECT OF A COMMONLY USED VETERINARY ANTIBIOTIC ON OXIDATIVE STRESS AND ROOT TRANSPORTERS OF EDIBLE LEGUMES AND LEAFY CROPS

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EFFECT OF A COMMONLY USED VETERINARY ANTIBIOTIC ON OXIDATIVE STRESS AND ROOT TRANSPORTERS OF EDIBLE LEGUMES AND LEAFY CROPS

Abstract

Antibiotic accumulation in soil and plants is a rising problem in agriculture creating a serious threat to living organisms in the environment, hence needing huge attention. To this end, glasshouse pot experiments were conducted to simulate contamination by veterinary antibiotic at 150 mg kg^{-1} and 4800 mg kg^{-1} in a virgin soil in which lentil (*Lens culinaris Medik.),* chickpea (*Cicer arietinum L.),* arugula *(Eruca sativa Mill.)* and cress *(Lepidium sativum L.)* were grown, aiming at evaluating the potential toxicity of antibiotic in plants roots during their growth period. Biomarkers of toxicity such as malondialdehyde and proline levels and antioxidative enzyme activity (superoxide dismutase, SOD; catalase, CAT; and guaiacol peroxidase, POD) were analyzed in the roots of the four species. In addition, gene expression level of the antioxidant enzymes Cu/Zn-SOD and CAT4, IFS /IFR that are key enzymes in the isoflavone pathway, and four ABC transporters MRP2, MRP4, TT12, and PDR11 that are involved in detoxification processes were evaluated.

Among all four vegetables, chickpea had the highest antioxidant activity with reduced lipid peroxidation in roots treated with the highest antibiotic concentration suggesting its antibiotic tolerance. Cu/Zn-SOD was not the key player in SOD activity. High antibiotic concentration inhibited the antioxidant activity in lentil, arugula, and cress implying their sensitivity. In treated arugula, SOD and POD activities decreased synergistically while CAT increased; whereas, in treated cress, POD and CAT were induced at low antibiotic concentration and inhibited with the high one. Gene expression displayed tolerance of chickpea and sensitivity of arugula to the antibiotic added. Our results reveal toxic effect of antibiotic on lentil, arugula, and cress with chickpea exhibiting higher tolerance to high antibiotic concentrations

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Keywords

Antioxidant enzyme, ABC plant transporter, veterinary antibiotic, tolerant species, proline, lipid peroxidation.

1. INTRODUCTION

Starting early 1950, antibiotics were introduced in animal feed to enhance their growth and preventing or controlling infectious diseases (Ogle, 2013). Nowadays, the practice became a global trend (Zhang et al., 2021) with antibiotic uses grown to more than 100,000 tons per year worldwide (Zhao et al., 2019). Despite their economically beneficial effects in animal feeds, antibiotics are continuously used in a chaotic manner, with no supervisions regarding their release in the environment (Gomes et al., 2020; Pedrosa et al., 2020).

Animal excreta are discharged in tons on a daily basis and are mostly used in farming to spare their handling treatment's costs (Tasho & Cho, 2016). Major quantities of the administered antibiotics are excreted in considerable amounts in these manures that are used as fertilizers especially in organic farms (Gomes et al., 2020; Lundborg & Tamhankar, 2017). Runoffs from soils create pits of antibiotics that have been detected in water reservoirs (Pedrosa et al., 2020; Gomes et al., 2017). Unfortunately, there are no current guidelines for livestock waste managements before field's distribution.

In this connection, plants, more importantly the consumable ones, will be exposed to these antibiotics or their byproducts when grown in soils treated with the antibiotic rich-manure affecting their growth and development and hence altering their yields and quality (Gull et al., 2019; Pessarakli, 2015). Data on antibiotics-potential phytotoxic impacts on agricultural production are scarce (Rocha et al., 2021). Until now, it remains unclear how antibiotics persisting in soil have biological and physiological effects on plant growth and development. Antibiotics absorbed by plants will contaminate food crops and threaten human health (Cheong et al., 2020; Hanson & Gregory, 2011; Klotz, 1944).

The accumulation and the effects of veterinary antibiotics on soybeans, rice, lettuce, sweet oat, alfalfa, wheat, has been broadly studied where it was shown that plants are capable to accumulate antibiotics via water transport and passive absorption. Excessive levels of antibiotics in water or soil revealed many toxic impacts on their growth and biochemical activities (Hillis et al., 2011; Luo et al., 2011; Xie et al., 2011; Boonsaner & Hawker, 2010; F. Liu et al., 2009).

In 2021, Khan et al., explored the effects of tetracycline (TC), oxytetracycline (OTC) and norfloxacin (NF) on the growth, cell ultrastructure and metabolite pattern of *Brassica rapa ssp. chinensis* and found that plant growth, chlorophyll fluorescence, and antioxidant activities were negatively affected under all antibiotic treatments.

Hence, the introduction of various contaminants to plants causes physiological and biochemical changes in their metabolisms as well as genetic alterations (Wang et al., 2008; Choi et al., 2007). Plants protect themselves via enzymatic and non-enzymatic mechanisms to scavenge the reactive oxygen species (ROS) and diminish their harmful effects. The antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) play important roles in preventing oxidative stress by scavenging ROS and degrading the produced hydrogen peroxide (Riaz et al., 2017). The intracellular concentration of malondialdehyde (MDA) indicates the level of lipid peroxidation. Moreover, the osmoregulant proline has been used as well to assess oxidative stress (Xie et al. , 2011; Scandalios, 2005; Demiral & Türkan, 2005).Therefore, studying the changes of antioxidant enzyme activities in plants before and after antibiotics exposure may decipher their toxic mechanisms.

When exposed to abiotic stress, plants also overproduce polyphenols, such as phenolic acids and flavonoids that scavenge ROS (Sharma et al., 2019). The phenylpropanoid biosynthetic pathway is triggered under abiotic stress conditions. Isoflavonoids, derived from the phenylpropanoid pathway, require the activity of several enzymes such as phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), isoflavone synthase *(IFS*) and isoflavone reductase (*IFR*) (Sharma et al., 2019). Isoflavone synthases initiate the first specific step in the biosynthesis of the isoflavone phytoalexins in legumes (Saviranta et al., 2010) where liquiritigenin and naringenin are hydroxylated by 2-hydroxyisoflavanone synthase. Research proved that transcriptional activation of isoflavonoids may be a key protective function against oxidative stress-induced cell damage (Palareti et al., 2016).

Root absorption is the main route of antibiotics uptake by plants. Antibiotics are then distributed to the various plant tissues affecting the plant's physiology (Rocha et al., 2021). While large accumulation of antibiotics in roots could limit their negative effects on photosynthesis, their accumulation can disrupt root growth causing severe effects on mineral nutrition and water uptake

(Gomes, Richardi, et al., 2019). Understanding the antibiotics' entry route into the plant cell and the transporters involved in their translocation among plant tissues may help in the production of crops with low antibiotic accumulation, hence reducing their entrance in the food chain or increasing their phytoremediation capacity in some plant species (Rocha et al., 2021).

Since the ability of plants to avoid toxins is limited, they have adapted strategies to clear toxic compounds through a class of transporters known as ABC. These proteins were originally identified as transporters involved in the final detoxification process (Jasinski et al., 2003). ABC transporters have frequently been shown to be involved in processes such as pathogen response, surface lipid deposition, phytate accumulation in seeds, and transport of the phytohormones auxin and abscisic acid. Therefore, ABC transporters play an important role in organ growth, plant nutrition, development, reaction to abiotic stress, and their interaction with its environment (Kang et al., 2011). In addition, genetic, biochemical, and molecular evidence has implicated the Multidrug And Toxin Efflux (*MATE)-type* transporters in flavonoids transport across membranes (Zhao et al., 2011a). *TT12*, a MATE transporter, plays a role in vacuolar transport (Chen et al., 2015). In addition, the multidrug resistance-associated protein (*MRP* or *ABCC*)-type ABC transporters were reported to be involved in vacuolar sequestration, and in the detoxification and storage of glucosides, glutathione, polar β-D-glucosides and amino acids (Yazaki, 2006) conjugated with endogenous compounds and xenobiotics (Borghi et al., 2019).

The present study was conducted to investigate how plants responds to antibiotic toxicity in the roots of lentil (*Lens culinaris* Medik.*)*, chickpea (*Cicer arietinum* L.*)*, arugula *(Eruca sativa* Mill.*)* and cress *(Lepidium sativum* L.*)*. Comparative effects of stress were investigated at the level of lipid peroxidation, proline content and antioxidant enzyme activities in roots. To assess the antibiotic toxicity-tolerance and sensitivity amongst the 4 crops, gene expression of the antioxidant enzymes *Cu/Zn-SOD* and *CAT4*, and of the enzymes involved in the isoflavone pathway *IFS /IFR*, and four ABC transporters *MRP2*, *MRP4*, *TT12* and *PDR11* involved in detoxification processes were assessed.

2. MATERIALS and METHODS:

2.1 Selected Species:

Two Lebanese indigenous annual legumes species -chickpea (*Cicer arietinum* L.) and lentil *(Lens culinaris* Medik.), and two leafy annual crops from the Brassica family -cress (*Lepidium sativum* L.) and arugula (*Eruca sativa* Mill.) of Italian origin (Sgaravatti & Sementi S.p.a.) – were selected in this study.

2.2 Composition of the Selected Antibiotic:

The water-soluble antibiotic mixture consisted of 4 main classes, the macrolide (erythromycin), the tetracycline (oxytetracycline), the aminoglycoside (streptomycin and neomycin) and the polymyxin (colistin). The components of this antibiotic mixture include 1-Erythromycin thiocyanate (3,500 mg), 2-Furaltadone HCl (6,000 mg), 3-Oxytetracycline HCl (5,000 mg), 4-Streptomycin sulphate (3,500 mg), 5-Neomycin sulphate (1,000 mg), 6-Colistin sulfate (25,000,000 IU), in addition to vitamins. This mixture is water soluble, used as a preventive or as treatment against both Gram-positive and Gram-negative bacteria in livestock animals.

2.3 Experimental design of Pot Experiment:

The soil used in this study was collected from a virgin agricultural land in Kfarmelki (South Lebanon, 33°30'21.6"N, 35°29'30.7"E) at depth of 0-50 cm. The soil was air dried and passed through a 7-mm mesh sieve.

The pot experiment was conducted at Beirut Arab University (BAU) DEBBIEH campus in a climate- controlled glasshouse to approach field growth conditions. The growth conditions adopted were day/night (14/10 h), humidity (70%) and diurnal temperatures varied between 10-22°C.

Each pot (30x60 cm in surface area and 30 cm in depth) was filled with 30 kg of sieved soil. Three pots were used for each crop species, lentil (100g per pot), chickpea (100g per pot), arugula (0.4 g per pot) and cress (0.4 g per pot). In addition to three pots lacking any seeds served as negative control.

The dissolved antibiotic mixture was added once at the beginning of the experiment. Three concentrations were used based on the results of germination and plant growth tests (Nassar & Borjac, 2022). Applied treatments were as follow for each species:

A_0: untreated soils used as control (watered only with distilled water); **A_1**: (0.5 g L^{-1}) equivalent to 150 mg kg^{-1} of soil; **A_6**: (16 g L^{-1}) equivalent to 4800 mg kg^{-1} of soil. To avoid leaching of the antibiotics from pots, a saucer was placed under each plant at all time.

From each of the treated pots, 5 sampling points were performed for the lentil and chickpea species, where 3 group of roots were collected, rinsed with distilled water and directly grounded with liquid nitrogen and stored in -80 ^{0}C freezer for further analysis. Whereas for arugula and cress, only one sampling was made due to the low amount of plant material obtained at earlier weeks. Samples were run in triplicates for each antibiotic concentration in each species.

2.4 Determination of the Malonyldialdehyde Content

To measure the extent of lipid peroxidation in roots, the thiobarbituric acid (TBA) test used to determine the levels of MDA, the end product of lipid peroxidation, was used. Roots were homogenized at a ratio of 1:4 (w:v) in 0.1 % TCA solution. The homogenate was centrifuged at 12 000 g for 15 min. To a 500 µL of the supernatant, 1.5 mL of 0.5 % (w:v) TBA diluted in 20 % TCA were added. The mixture was incubated in water bath at 95 ^{0}C for 30 min. The reaction stopped by placing the reaction tubes in an ice bath. Tubes were briefly vortexed, then the absorbance of supernatant was read at 532 nm, the value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated using the Lambert-Beer law with an extinction coefficient (εM) of 155 $mM.cm^{-1}$ (Murshed et al., 2013): MDA (mM) = $\Delta A_{532\text{-}600}/155$.

Results are presented as µmol MDA g^{-1}FW.

2.5 Proline Determination

Determination of Proline content was performed according to a modified method by Carillo et al., (2011), a cold extraction procedure. Fresh roots (20-50 mg) were suspended in ethanol: water (40:60 v/v) was at ratio of 50:1 (w/v), the mixture was left overnight at 4°C, and then centrifuged at 14000x g for 5 min. Supernatants or standards (50 µl) were mixed with 100 µl of the reaction mixture consisting of 1% (w/v) ninhydrin in 60% (v/v) acetic acid and 20% (v/v) ethanol. Tubes were then sealed, mixed and incubated in a water bath at 95°C for 20 min. After cooling to room temperature, 100 µL of the mixture was transferred and measured at 520 nm in a microplate reader. Proline concentration was determined from the standard curve.

2.6 Extraction and Estimation of Antioxidant enzyme Activities

To determine the SOD, CAT and POD activities, frozen powdered root tissues (400 mg) were homogenized in 10 mM (1X) of freshly prepared phosphate buffer solution (pH 7.4) containing 1 mM PMSF at a ratio of 1/10 (w/v). Samples were centrifuged at 4^{o}C for 20 min. at 15000xg.

The principle of SOD activity assay was based on the inhibition of nitroblue tetrazolium (NBT) reduction. Illumination of riboflavin in the presence of O_2 and electron donor generates superoxide anions that reduce the yellow NBT to give blue color whose absorbance is measured at 560 nm. One unit of SOD activity is defined as that amount of enzyme required to inhibit 50% of nitroblue tetrazolium photoreduction (Stoica & Artenie, 2008). In brief, 1 ml of the reaction mixture contained 41 µM NBT, 1 µM riboflavin, 7 mM methionine, 16 µM EDTA in 100 mM sodium phosphate buffer (pH 7.8) were added to 50 µL root extract. The samples were first illuminated with 78 µmol photons $s^{-1}m^{-1}$ (6W fluorescent tube) for 15min and the absorbance of the generated color was measured at 560 nm. A non-irradiated reaction mixture that does not develop any color served as control.

The activity of CAT was determined following the method of Aebi (1984). The decomposition of H_2O_2 by CAT was monitored through the decline in absorbance at 240nm per unit time. To 1 ml of reaction mixture containing 13mM H_2O_2 in 100 mM sodium phosphate buffer (pH 7.0), 50 µL of root extract were added to initiate the reaction. One unit

of CAT activity is defined as the enzyme amount that decreases 0.1 of absorbance at 240 nm per minute.

For POD determination, the method of Guaiacol oxidation was followed. The reaction solution contained 1 ml of 325 µM Guaiacol, 120 µM H_2O_2 in 100 mM of sodium phosphate buffer (pH 7.0) and 50 µL root extract. One unit of POD is defined as the amount of enzyme that cause an absorbance increase of 0.01 per min at 470 nm.

Protein content was determined by a spectrophotometric method according to Warburg-Christian equation: Protein concentration (mg/ml) = 1.55 x A_{280} - 0.76 x A_{260}.

All enzymes' concentrations were reported as unit/mg protein.

2.7 Analysis of Gene Expression Using Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was used to determine the expression of 8 genes in chickpea and arugula plants treated with high concentration of antibiotic (4800 $mg.kg^{-1}$ of soil). These genes comprised 2 antioxidant-enzymes encoding genes (*CAT4*, and *Cu/Zn-SOD*) and 4 ABC transporters genes (*MRP2, MRP4, TT12* and *PDR11*) and the key enzymes in isoflavone biosynthetic pathway IFS/IFR.

Total RNA was isolated from 100 mg of frozen roots powder using ISOLATE II RNA Plant Kit (cat# BIO-52077, Bioline Reagents Ltd, Meridian Bioscience Inc.). The purity and concentration of RNA were spectrophotometrically assessed at 260 and 280 nm. RNA integrity was verified on 1 % agarose gels. The elimination of contaminating DNA was done within the kit. The first-strand of cDNA was synthesized starting with 1 µg of mRNA using the SensiFAST cDNA Synthesis Kit (cat # BIO-65053, Bioline Reagents Ltd, Meridian Bioscience Inc.). As for the qRT-PCR, it was performed following the SensiFAST™ SYBR® No-ROX kit (cat# BIO-98005, Bioline Reagents Ltd, Meridian Bioscience Inc.) according to the manufacturer's recommendations. The reaction was performed in a total reaction volume of 10 µL under the following conditions: polymerase activation at 95 °C for 2 min; 45 cycles of 95 °C for 5 s, 58 °C for 10 s and 72 °C for 20 s.

Gene-specific primers sequences were designed based on sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov) and tested in this study are listed in **Tab.1**. GAPDH served as a housekeeping gene, and the relative expression levels were determined using $2^{-\Delta\Delta}$ Ct method.

Table 1: Gene-specific primers sequences used in RT-PCR

Gene name	Gene description	Primer sequence	Amplicon length (bp)
SOD Cu-Zn	Superoxide dismutase	^{253}5'-CGACATGCTGGCGATTTAGG-3' ^{412}5'-CGTGACCACCTTTCCCAAGA-3'	160
Cat4	Catalase 4	^{1257}5'-CTCCCAAGTGTGCTCACCAT-3' ^{1430}5'-CCTTGGGACGTATGCACCTT-3'	174
CYP93C3/IFS	isoflavone synthase	^{1653}5'-GGTGCTGCTGCTAAACTCCT-3' ^{1794}5'-GCCCATGTACCATCACTAGCA-3'	142
IFR	isoflavone reductase	^{593}5'-AATTCGACGCCACTGAACCT-3' ^{722}5'-AATGTCCTGGGGTCATTTGCT-3'	130
ABCC2-like/ MPR2	ABC transporter C family member 2-like	^{109}5'-CCACTCGCTCACATTTCATTCA-3' ^{273}5'-AGCAACCGGCTGACAATACC-3'	165
ABCC4-like/ MPR4	ABC transporter C family member 4-like	^{1716}5'-CGTGGTGACAACTCGACAGA-3' ^{1901}5'-AACTTGGAAAGCCACCCGAA-3'	186
ABCG39-like/ PDR11	ABC transporter G family member 39-like	^{2364}5'-ATGAGCCCACAATTGGGAAGG-3' ^{2556}5'-TCCATGTCAACACCTTCTTCCA-3'	193
MATE/TT12	Multi antimicrobial extrusion protein	^{720}5'-ATGTTGCACTTAGGGTGGGG-3' ^{841}5'-CCAGTCCAAGCTTCACCACA-3'	122
GAPDH (reference)	Cytosolic glyceraldehyde-3-phosphate dehydrogenase	^{618}5'-ACCACCGTCCATTCCATCAC-3' ^{731}5'-AGCTCCGGTACTGCTAGGAA-3'	114

2.8 Statistical Analysis

The statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS 26.0 and by Tukey's HSD post-Hoc test at $p<0.05$ is considered as significant. Pearson correlation analysis was performed to investigate the relationships between experimental parameters. The values are the mean with standard deviation (n=3).

3. RESULTS

3.1 Effect of Antibiotic on MDA Levels

The effect of the antibiotic on MDA levels in the roots of lentil, chickpea, arugula and cress is shown in **Fig.1**. In lentils, significant increase in MDA level was observed with increasing antibiotic concentration during the first 6 weeks of treatment with 26% ($p<0.01$), 52% ($p<0.01$) and 20% ($p<0.01$) upon treatment with high amount of antibiotic on *w1*, 4 and 6 respectively. While on *w8*, a significant decrease in MDA was noticed (17.4%, p=0.001). However, the significant increase in MDA at low antibiotic concentrations was observed only on *w4* (25%, p=0.002). On the other hand, time dependent increase in the MDA level was observed after the 2nd week with treatment reaching 171% ($p<0.01$) for lentil with low antibiotic and 107% ($p<0.01$) for lentil with high antibiotic. Similar significant time-dependent increase in MDA was observed even in the absence of added antibiotic (170%, $p<0.01$). In chickpea roots, the levels of MDA decreased significantly at high concentration of antibiotic during the first four weeks of treatment compared to control with 21.4% ($p<0.01$), 10% (p=0.004), and 12.6% (p=0.016) respectively on *w1*,*w2* and *w4*. However the decrease in chickpea treated with low amount of antibiotic was only significant on *w2* (26%, $p<0.01$). The drop in the level of lipid peroxidation was the most significant between the 2nd and the 4th week and ranged from 31- 47% ($p<0.01$ all) in all treated chickpea as well as the control one. After that, no significant changes were observed at both concentrations on *w6* and *w8*.

On the other hand, arugula and cress roots were more sensitive to both antibiotic concentration used where significant increase 99.5% (p=0.004) and 230% ($p<0.01$) in MDA levels were observed in arugula after 8 weeks and 47% (p=0.002) and 64% ($p<0.01$) in cress after 10 weeks for plants treated with low and high amount of antibiotic respectively.

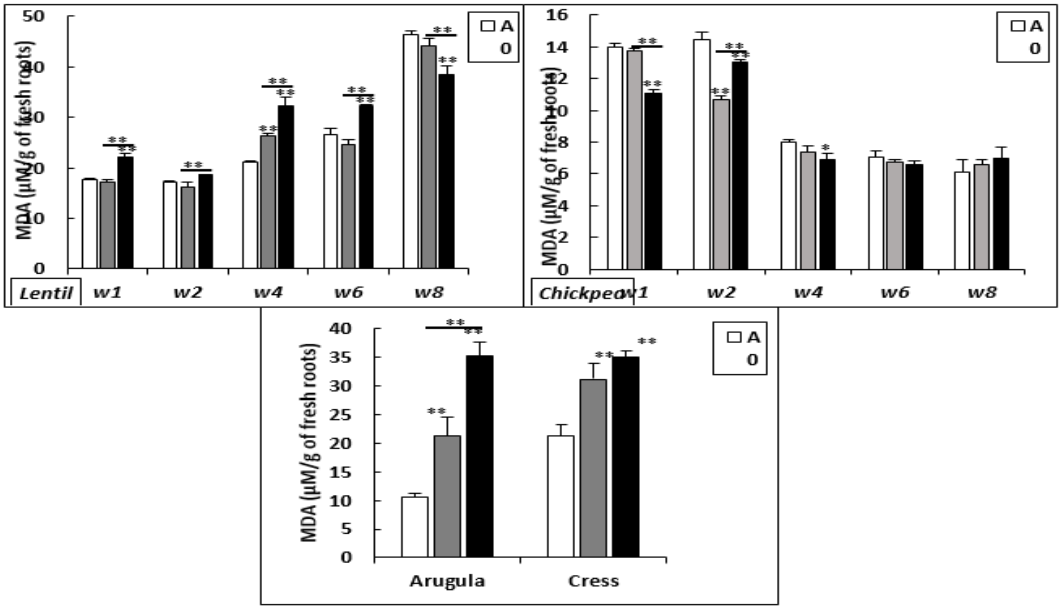

Fig.1: Effect of antibiotic on MDA Levels. MDA levels (µM/g of fresh roots) in lentil, chickpea, arugula and **cress** after week1 (***w1***), week2 (***w2***), week4 (***w4***), week6 (***w6***) and week8 (***w8***) in lentil and chickpea and after week 8 and week 10 in arugula and cress respectively. $\mathbf{A_0}$: control species; $\mathbf{A_1}$: species treated with low concentration of antibiotic; $\mathbf{A_6}$: species treated with high concentration of antibiotic. The asterisk (*****) indicates significant difference ($p < 0.05$), (******) indicates significant difference ($p<0.01$) between control and treated plants. Data are presented as mean values ± standard deviation (n=3).

3.2 Effect of Antibiotic on Proline Levels

Overall, a significant dose-dependent increase in proline levels was observed in all studied species with both antibiotic concentrations used as shown in **Fig.2**. In lentil, the increase of proline level ranged from 12.24% (p=0.002) to 51.1% (p=0.005) when treated with low antibiotic concentration, while it ranged from 39.47% (p=0.001) to 88.75% (p<0.05) with high antibiotic treatment.

In chickpea, the proline level ranged from 12.8% (p=0.008) to 137.2% (p<0.01) with low antibiotic treatment, while it ranged from 58.57% (p<0.05) to 92.83% (p<0.05) at high concentration. In arugula, the proline level increased 94.2% (p<0.01) vs 231% (p<0.01) on *w8*, while in cress the increase was 100 % (p=0.001) vs 183.4% (p<0.01) on *w10* at low and high antibiotic concentration respectively.

On the other hand, a significant time-dependent decrease in proline levels were observed between *w1* and *w8* of 35.9% (p=0.001) in control lentil, 46.28% (p<0.01) at low antibiotic concentration and 43.35% (p<0.01) at high antibiotic concentration. However, the observed proline decrease between *w1* and *w8* in chickpea was of 74.1% (p<0.01) in control chickpea, 62.87% (p<0.01) at low antibiotic concentration and 74.98% (p<0.01) at high antibiotic concentration.

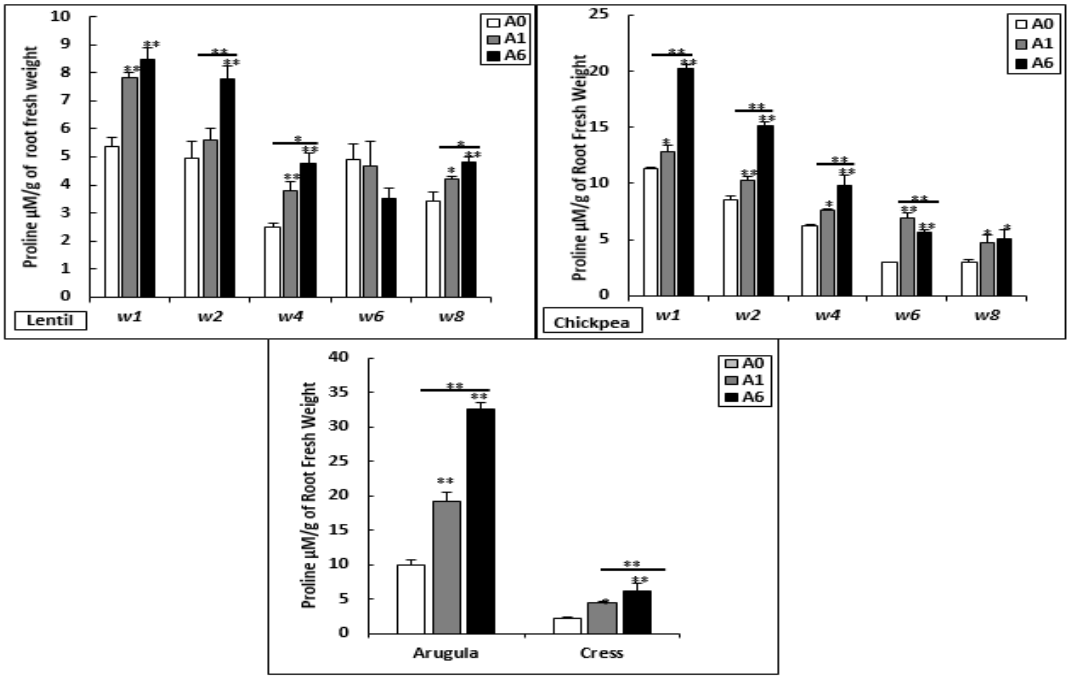

Fig. 2: Effect of Antibiotic on proline content in roots. Proline (µM/g of fresh roots) was measured in lentil, chickpea, arugula and cress, after week1 (w1), week2 (w2), week4 (w4), week6 (w6) and week8 (w8) in lentil and chickpea and after week 8 and week 10 in arugula and cress respectively. A_0: control species; A_1: species treated with low concentration of antibiotic; A_6: species treated with high concentration of antibiotic. The asterisk (*) indicates significant difference ($p < 0.05$), (**) indicates significant difference ($p<0.01$) between control and treated plants. Data are presented as mean values ± standard deviation (n=3).

3.3 Effect of antibiotic on antioxidant enzymes levels

The effect of the antibiotic of the activities of the antioxidant enzymes SOD in all studied species with both antibiotic concentrations used is shown in **Fig.3**. In lentils, SOD activity started to significantly increase at *w2* at low antibiotic concentration A_1 (13.87%, p=0.019) compared to the high antibiotic concentration A_6 and to control. The increase continued till the 4th week with 10.9% (p=0.017).

At *w6* and *w8* continuous significant increase in SOD at A_6 compared to control was observed by 63.8% (p<0.01) and 17.74% (p= 0.043) respectively. However, this increase was lower than that observed during the first 4 weeks.

In chickpeas' roots, an increase in the SOD activity ranging from 11.84% (p=0.05) to 45.83% (p=0.005) was observed SOD at all time points with *w4* showing the highest activity. However, significant decrease in SOD activity was observed at A_1 (23.4%, p<0.01) compared to control at *w4*.

In arugula**,** the activity of SOD decreased by 24.3% (p=0.003) when treated with A_6. While in cress, a significant decrease in SOD activity by 15.3% (p=0.01) was observed at A_1 with significant increase at A_6 (46.5%, p<0.01).

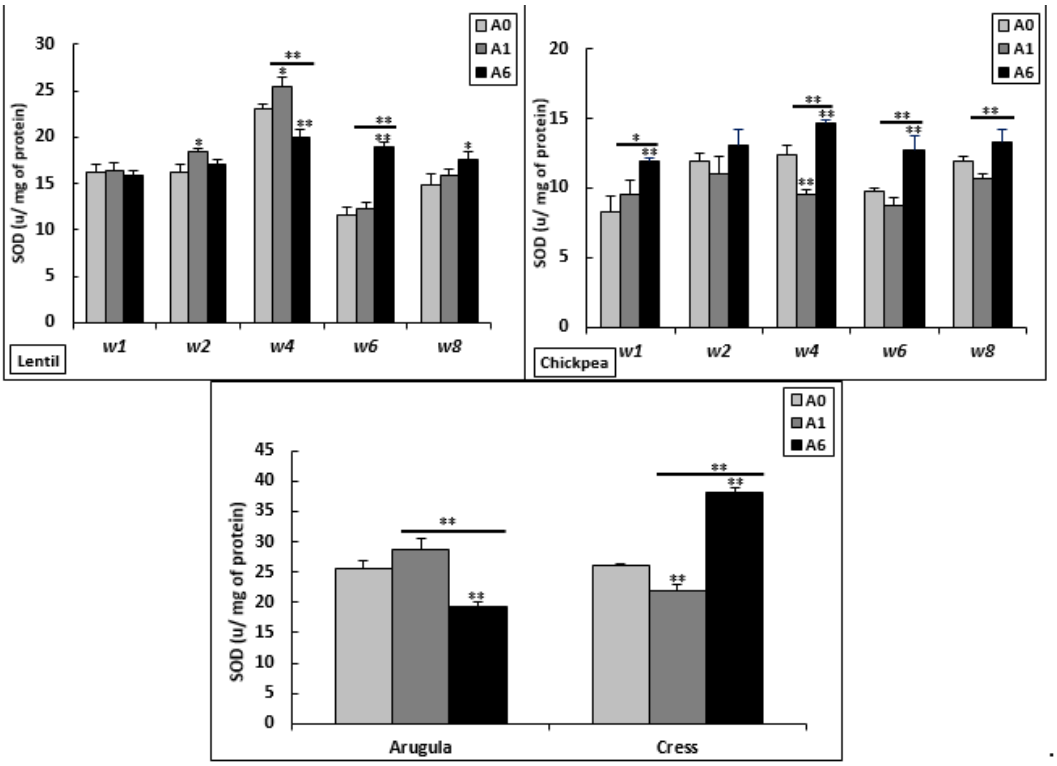

Fig. 3: Effect of Antibiotic on SOD activity in **lentil, chickpea, arugula** and **cress** roots**,** after week1 (***w1***), week2 (***w2***), week4 (***w4***), week6 (***w6***) and week8 (***w8***) in lentil and chickpea and after week 8 and week 10 in arugula and cress respectively. **A_0**: control species; **A_1**: species treated with low concentration of antibiotic; **A_6**: species treated with high concentration of antibiotic. The asterisk (*****) indicates significant difference (p < 0.05), (******) indicates significant difference (p<0.01) between control and treated plants. Data are presented as mean values ± standard deviation (n=3).

Regarding the catalase activity, in lentil, significant increase in activity was observed with A_1 at all time points with pronounced activity at *w1* and *w2* with a 638% and 164.5% increase (p<0.01) compared to control **(Fig.4).** While at A_6, CAT activity was reduced by 360% (p<0.01) at *w1* followed by an increase at *w2* then gradual decrease till *w6*.

In chickpea's roots, A_1 caused a significant increase in the catalase activity with 84% (p<0.01) and 51% (p<0.01) on the 4th and 6th week respectively. On the other hand, A_6 induced a significant 60% decrease (p<0.01) in catalase activity at *w1* followed by significant increase ranging between 13.9% (p<0.01) and 97.4% (p<0.01) compared to control. Overall, the catalase activity in all treated chickpea peaked on the 2nd week.

As for arugula's roots, significant increase in catalase activity of 34.76% and 31% (p<0.01 both) at A_1 and A_6 respectively was observed. While in cress, the activity at A_1 increased 20% (p<0.01) with a 36% decrease (p<0.01) at A_6.

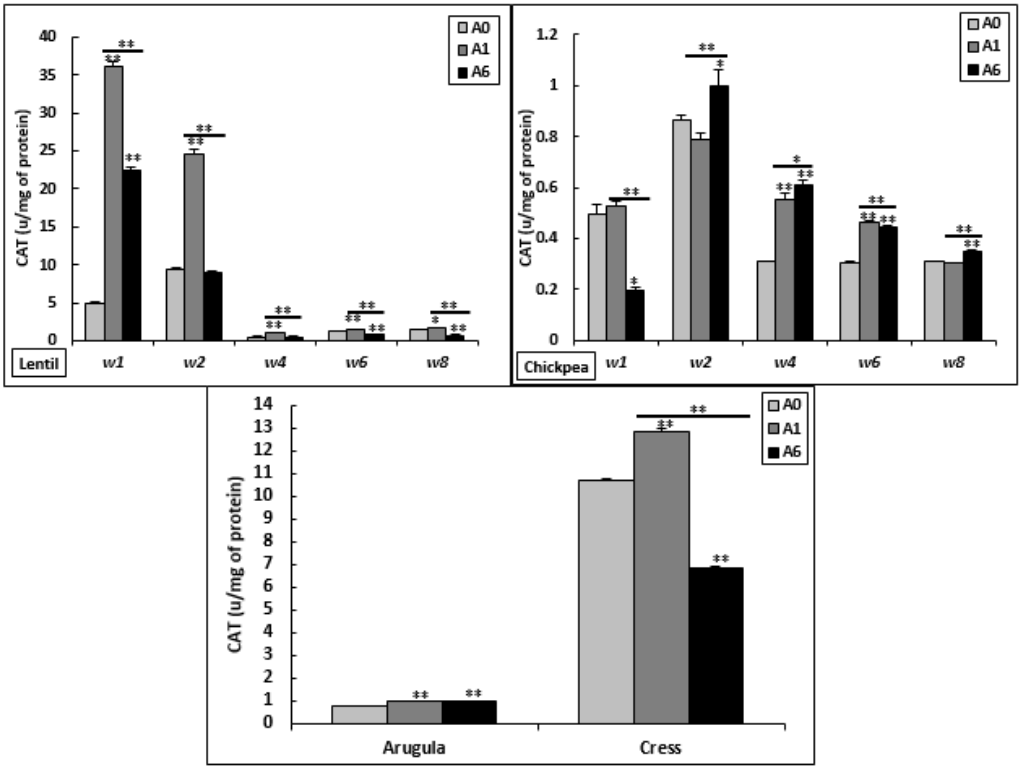

Fig.4: Effect of Antibiotic on CAT activity in lentil, chickpea, arugula and cress roots, after week1 (w1), week2 (w2), week4 (w4), week6 (w6) and week8 (w8) in lentil and chickpea and after week 8 and week 10 in arugula and cress respectively. A_0: control species; A_1: species treated with low concentration of antibiotic; A_6: species treated with high concentration of antibiotic. The asterisk (*) indicates significant difference ($p < 0.05$), (**) indicates significant difference ($p<0.01$) between control and treated plants. Data are presented as mean values ± standard deviation (n=3).

POD activity in the roots of all studied species is shown in **Fig.5**. In lentils' roots, POD activity increased at all time-points. It was the highest with A_1 with a 34.77% (p=0.029) and 32% (p=0.003) increase at 4th and 6th week respectively. On the other hand, lentil treated with A_6 showed significant decrease ranged between 26.77% (p=0.003) and 49.36% (p=0.011) compared to control.

In chickpeas' roots, POD significantly increased with A_1 and A_6 treatment only on *w2* with 41% (p=0.003) and 27% (p=0.024) respectively.

On the other hand, treating arugula and cress with A_1 did not induced any change in POD activity. However, significant decrease in POD activity of 34.4% (p=0.002) and 41.5% (p=0.001) was observed in arugula and cress respectively when treated with A_6 compared to control.

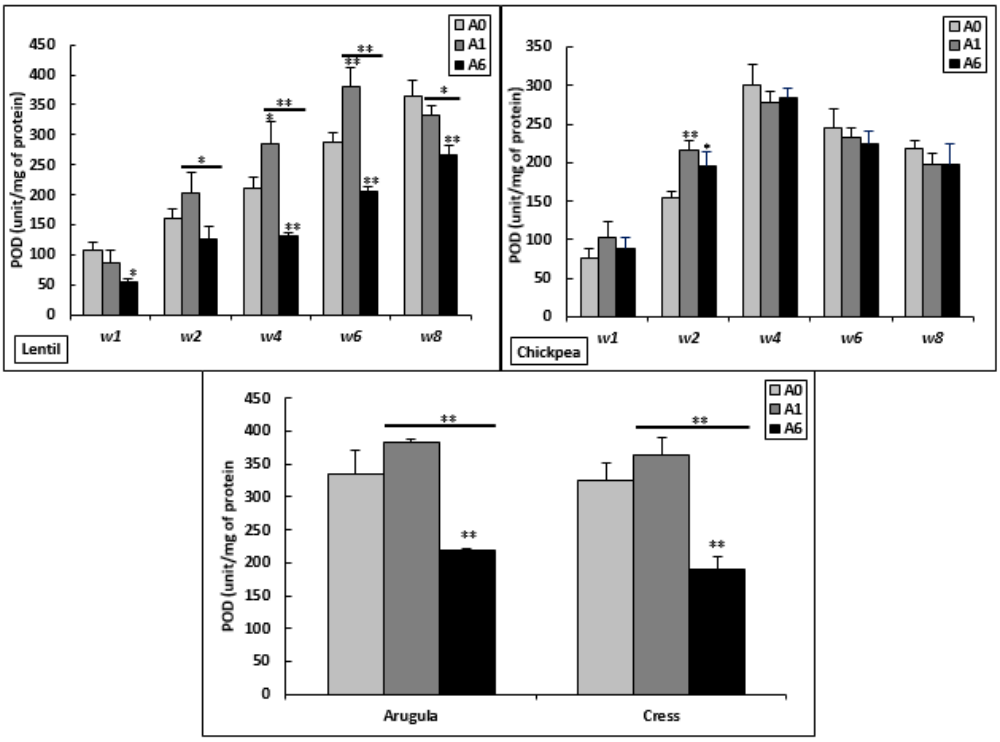

Fig.5: Effect of Antibiotic on POD activity in lentil, chickpea, arugula and cress roots, after week1 (*w1*), week2 (*w2*), week4 (*w4*), week6 (*w6*) and week8 (*w8*) in lentil and chickpea and after week 8 and week 10 in arugula and cress respectively. A_0: control species; A_1: species treated with low concentration of antibiotic; A_6: species treated with high concentration of antibiotic. The asterisk (*) indicates significant difference ($p < 0.05$), (**) indicates significant difference ($p<0.01$) between control and treated plants. Data are presented as mean values ± standard deviation (n=3).

3.4 Effect of antibiotic on gene expression levels in chickpeas and arugula

The time-dependent changes in mRNA expression levels of *SOD*, *CAT*, *IFS* and *IFR* are shown in **Fig.6**. In *w1*, chickpeas treated with A_6 showed increase in *SOD* mRNA by only 0.04 (p=0.044) while it decreased on *w2* by 0.47 (p=0.001). After that, the *SOD* mRNA increased by 0.9fold (p=0.002) on *w4* and peaked on *w6* by a 23fold (p=0.001). On *w8*, mRNA levels dropped remarkably to 0.7fold (p=0.002).

On the other hand, *CAT* mRNA levels decreased by 0.68fold (p=0.015) on *w1* compared to control, then a 3fold increase was observed on *w2* (p=0.001), a 6fold ($p<0.01$) on *w4* and peaked to 21fold ($p<0.01$) on *w6*. Similar to *SOD* mRNA levels, *CAT* mRNA levels dropped to 1.7fold ($p<0.01$) on *w8*.

Regarding the 2 enzymes involved in isoflavonoids synthesis, i.e. *IFS* and *IFR*, their mRNA levels decreased on *w1* and *w2* reaching 0.25fold (p=0.001) and 0.14fold ($p<0.01$) respectively at *w2*. At *w4*, a 1.3fold (p=0.025) increase in *IFS* mRNA level was observed. The expression levels of both genes peaked on *w6* reaching a 4.9fold ($p<0.01$) and 5.5fold ($p<0.01$). On *w8*, the expression levels dropped significantly to 1.23fold (p=0.05) in *IFS* and 0.93fold (p=0.008) in *IFR*.

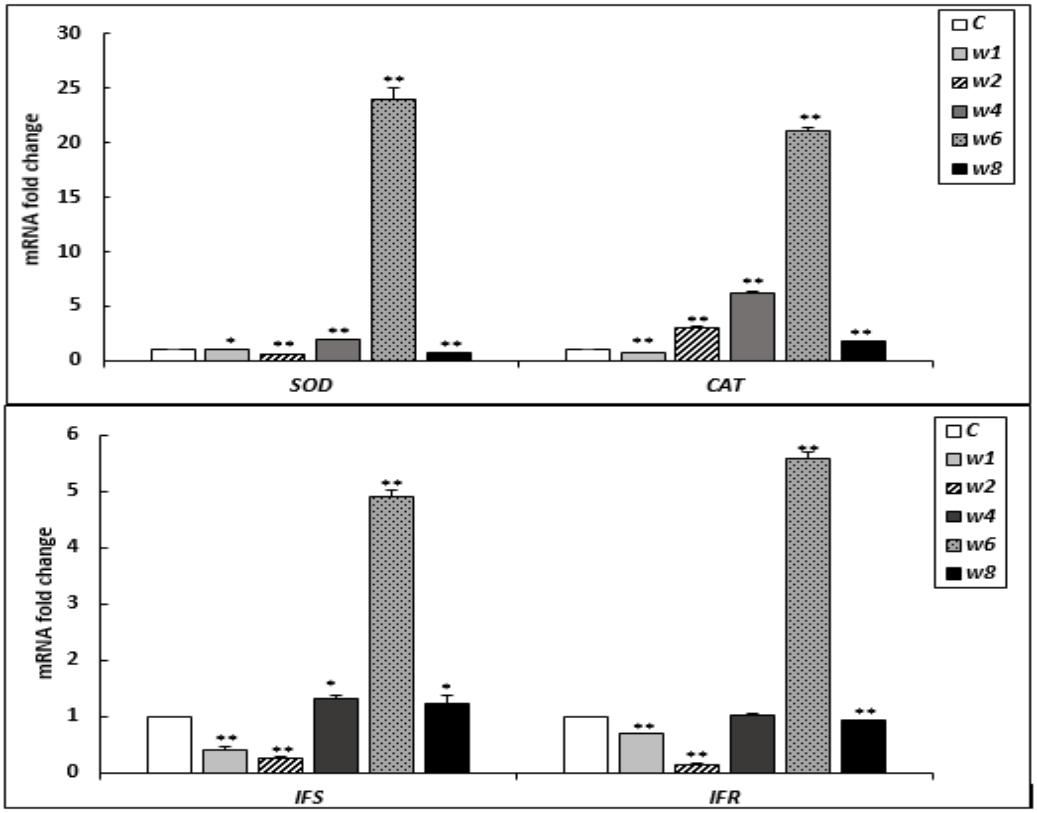

Fig.6: Expression patterns of SOD, CAT, IFS and IFR genes in chickpea roots treated with high concentration of antibiotic. RNA samples isolated from root were analyzed by real-time RT-PCR using specific primers as described in Experimental Procedures in week1 (w1), week2 (w2), week4 (w4), week6 (w6) and week8 (w8).

Regarding the ABC transporters **(Fig.7),** *MRP2* mRNA levels showed a time dependent increase ranging between 2 and 2.5fold ($p<0.01$). On the other hand, *ABCG39* expression decreased a 0.25fold ($p<0.01$) at *w1* then gradually increased reaching a 2fold change ($p<0.01$) at *w6* and *w8*. For the *TT12* transporter, a 0.17fold ($p<0.01$) decrease was observed on *w1*, followed by a 0.36fold increase ($p=0.001$) and 0.45 ($p=0.004$) on *w2* and *w4* respectively, then reaching its peak on *w6* by a 3.6fold ($p=0.007$) increase. On *w8*, a drop to 0.19fold was observed ($p<0.01$).

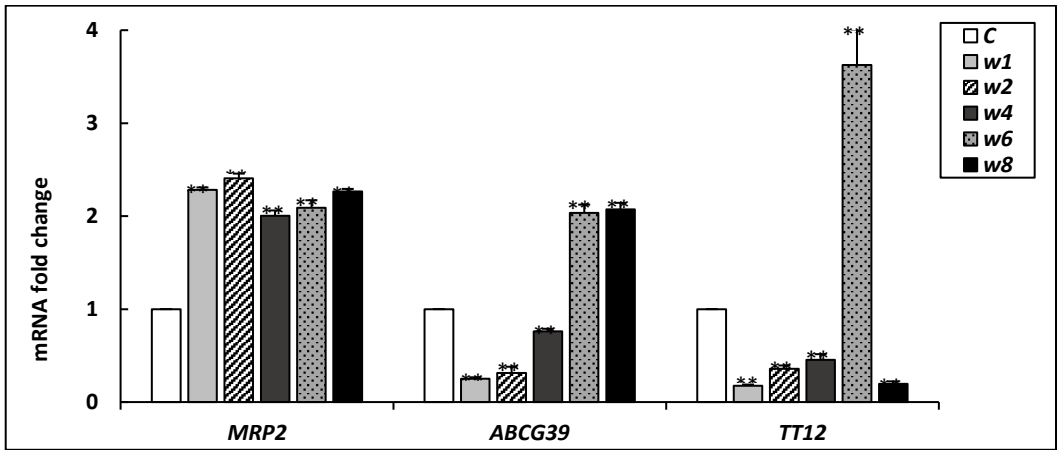

Fig.7: Expression patterns of MRP2, ABCG39 and TT12 genes in chickpea roots treated with high concentration of antibiotic. RNA samples isolated from root were analyzed by real-time RT-PCR using specific primers as described in Experimental Procedures on week1 (w1), week2 (w2), week4 (w4), week6 (w6) and week8 (w8).

In the case of arugula, the expression levels of tested genes were upregulated with a fold change of 5.9, 4.2, 2.9, 5.11, 16.5, 4.8 and 3.2 ($p<0.01$) for *SOD, CAT, IFS, IFR, MRP4, ABCG39* and *TT12* respectively. Only the expression level of *MRP2* decreased to 0.36fold ($p=0.006$) as shown in **Fig. 8**.

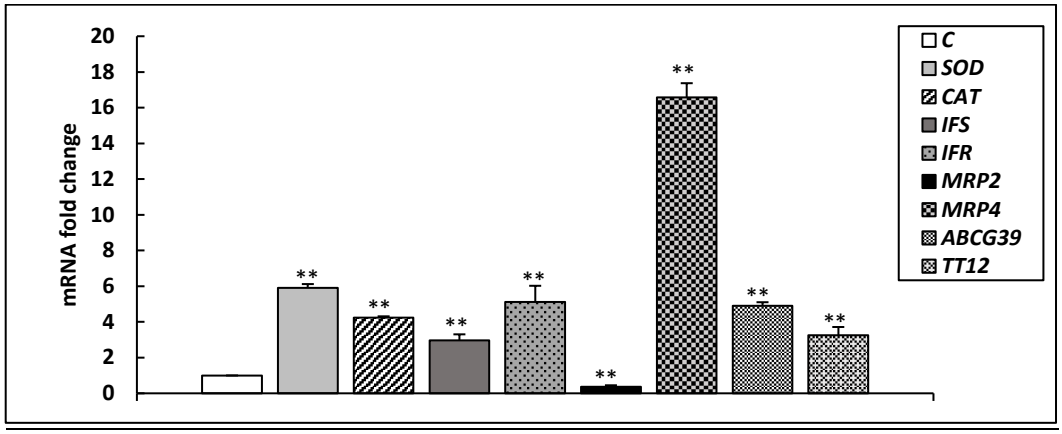

Fig.8: Expression patterns of SOD, CAT, IFS, IFR, MRP2, MRP4, ABCG39 and TT12 genes in arugula roots treated with high concentration of antibiotic. RNA samples isolated from root were analyzed by real-time RT-PCR using specific primers as described in Experimental Procedures in week 8.

4. DISCUSSION

The effect of antibiotic on the roots of four edible species was evaluated in this study through the assessment of antioxidant enzymes levels and gene expression. MDA, an indicator of lipid peroxidation content, has been used to assess oxidative stress in plants. In lentil, the MDA levels in untreated control significantly increased with time reaching high levels on *w8*. Treating with high antibiotic concentration induced stress translated with increase lipid peroxidation and hence MDA levels implying absorbance of the antibiotic at the root levels. Our results correlate with those of Xie et al., (2011) who showed that lipid peroxidation varied in a dose-dependent manner in the roots of wheat seedlings. The decrease in the level of lipid peroxidation on *w2* may indicate the possibility of translocation of the antibiotic to upper part of the plants and hence lowering their toxic effect at the root level. Based on previous studies, the translocation of antibiotics in crops depends on their physicochemical properties, the crop species and the concentration of antibiotics applied to the soil (Pan & Chu, 2017; Dodgen et al., 2015). At *w4*, MDA levels increased with both antibiotic concentration used along with the control which can be referred to as a developmental stage in plants as in accordance to previous studies (Cai et al., 2011). At low antibiotic concentration, the significant increase in MDA in lentil roots could be due to the accumulation of the antibiotic in the roots as seen by Liu et al who showed time dependent accumulation of pharmaceuticals in rice roots (Liu et al., 2009). The decrease in MDA levels on *w8* could imply to either its degradation or its translocation to upper plant parts as also shown by Zhao et al. (Zhao et al., 2019).

In chickpeas, the suppression of lipid peroxidation on the 1st week upon high antibiotic treatment, indicated that the accumulation of the antibiotic within the roots may have inhibited lipid peroxidation similar to what has been observed by Szentmihályi et al. (2021) where metronidazole solution was able to reduce MDA levels in sunflower oil exposed to 150 ^{0}C for10 min in a dose dependent manner. Our results confirm this effect in a time dependent manner as MDA levels decreased in roots on *w2* at low antibiotic concentration. The high level of MDA in control chickpea in the first 2 weeks could be due to germination as also observed by Cai et al. during seed germination and early seedlings growth of *Jatropha curcas* L. (Cai et al., 2011). On the 2nd week, the increase of MDA level in chickpeas treated with high amount of antibiotic in chickpea indicated its translocation from the roots, hence lowering its effect. While the decrease of MDA level in chickpea treated with low antibiotic may indicate that the inhibition of lipid peroxidation during germination may require a threshold level of antibiotic accumulation within

the roots. Our results agrees with Cai's study who confirmed that lipid peroxidation in plants was the highest during germination and early developmental stages and drops thereafter (Cai et al., 2011). This is also observed in our current study where after *w4*, the significant decrease in MDA levels in control chickpea was related to their growth level. The similar levels of lipid peroxidation in both control and treated chickpeas roots support our postulation about the disappearance of antibiotic from the roots after the 4th week.

In arugula and cress, dose dependent increase in MDA levels was observed at *w8* and *w1*0. These results implied the sensitivity of the two leafy species to the stress induced by the added xenobiotic and the limitation of antibiotic translocation from their roots.

Proline is one of the most common solute that accumulates in plants during undesirable environmental disorders and plays an important role in plant stress tolerance (Kaur & Asthir, 2015; Szabados & Savouré, 2010). The decrease in proline level in lentil and chickpea in control plants during our experimental period time was also proven by others who confirmed that proline accumulation in plants tissue is higher in seedlings stages and tends to decrease to basal levels post plant development (Verslues & Sharma, 2010). On the other hand, proline accumulation increased with the increased antibiotic concentration in all treated species. Our findings are in accordance with results of Tasho et al. (2020) who proved that antibiotic dose-dependent increase in proline accumulation in carrot, lettuce and pepper species. The significant increase of proline in the 6th week in control and lentil treated with low amount of antibiotic was coupled with the appearance of the first flower as also demonstrated by Mattioli et al. in Arabidopsis where association between the accumulation of proline and flowering process (Mattioli et al., 2008). Similarly, the flowering process in lentil grown in soil treated with high amount of antibiotics was observed on the 8th week and this can be explained through the same previous reason where the decrease in proline accumulation on the 6th week caused a delay in the flowering stage. The difference in the proline level accumulation among species may be species dependent differences. Its buildup upon abiotic pressures was seen in a wide number of various plants species (Meena et al., 2019; Chun et al., 2018).

The fluctuations of the levels of the antioxidant enzymes in all control species during the experimental period might be related to different developmental phases in diverse species. Several studies showed that plant responses to abiotic stresses (drought, UV radiation, O_3 …) vary mainly depending on the intensity and duration of stress as well as plant species, cultivar and growth stage (Jaleel et al., 2008 a, b; Schützendübel & Polle, 2002; Burkey et al., 2000). No clear induction of SOD was observed on the first week in lentils under all conditions, indicating that changes in SOD activity are more prominent after lentil germination. SOD production was induced on *w2* and *w4* in plants treated with low antibiotic concentration to resume similar activity as the control plants thereafter reflecting the decline of antibiotic stress in roots. Meanwhile, the decrease in the SOD activity in lentil treated with high amount of antibiotic on *w4*, then its increase from *w6* till the end of experiment indicated that high antibiotic concentration inhibited the enzyme and but the activity was regenerated post antibiotic stress termination. Selote & Khanna-Chopra (2010) demonstrated that ending drought stress by rewatering led to a rapid enhancement of all the antioxidant defense components in roots of wheat seedlings, suggesting that severe water stress conditions might have inhibited or down-regulated the antioxidant enzymes.

The activities of CAT and POD in lentil was induced at low antibiotic concentration but inhibited at high one. Similar results were observed in *Indigofera tinctoria* L. seedlings and *Corallina officinalis* L. exposed to UV-B stress that caused inhibition of the antioxidant enzymes under high abiotic stress and activation under moderate abiotic stress (Ravindran et al., 2010, Li et al., 2010),

Meanwhile in chickpea, the high amount of antibiotic caused the activation of the SOD at all time points whereas low antibiotic treatment didn't induce SOD activation. These results reflected the tolerance and the antioxidant capacity of chickpea to overcome antibiotic stress even at high concentrations. Recent studies revealed, that antibiotics have been inducing changes in the oxidative statuses of exposed plants via activation of antioxidant enzymes (Gomes et al., 2019; Singh et al., 2018; Vilvert et al., 2017). Those alterations indicated that the antibiotics can induce oxidative responses in aquatic macrophytes, and that antibiotic tolerance in plants is related to the activation of antioxidant defenses.

CAT activity increased in chickpea treated with high antibiotic concentrations after the 2nd week and after the 4th week in chickpeas treated with low antibiotic concentrations reflecting the

presence of stress within roots due to antibiotic accumulation. Whereas, POD activity wasn't affected after the 2nd week in all treated chickpea.

In arugula, SOD and POD activities in roots synergistically decreased in treated plants with high amount of antibiotic except for the CAT activity that was induced in both treatments. Our results agrees with those of Nie et al. (2013), where antioxidant responses of the green microalga *Pseudokirchneriella subcapitata* was tested in response to different concentrations of three antibiotics. Wang et al. (2019) also showed that the combination of two antibiotics synergistically increased SOD and POD activities but inhibited CAT activity in *Chlorella vulgaris*.

On the other hand, in cress, POD and CAT levels varied together at both antibiotic concentrations used while SOD did not. At low antibiotic concentration, CAT and POD were activated while SOD was inhibited. At high antibiotic concentration, SOD activity increased while CAT and POD were inhibited. Similar findings were observed by Saad El-Beltagi et al. (2010) where the specific activity of CAT increased in the roots of *Raphanus sativus* L. when exposed to 25 ppm of Cd and increased when it was exposed to high Cd concentration (50 ppm). Similarly, Qureshi et al. (2007) showed a dose-dependent increase in SOD and APX activities in Pb-treated plants with decrease in CAT activity under severe stress, i.e. 500 mM Pb-acetate. In wheat, the APX and SOD activities decreased at low concentrations of As, and increased at high concentrations of As, while CAT activity increased when the concentration of As was lower than 1 mg kg^{-1} then decreased (Li et al., 2007). Our findings indicated, that the difference in the activity of each antioxidant enzyme was due to the capacity of each species to tolerate antibiotic stress.

At the gene level, investigation of gene expression of the antioxidant enzyme could provide insight into molecular modification of chickpea and arugula to response against antibiotic stress. In this study, *Cu/Zn SOD* and *CAT4* genes encoding antioxidant enzymes exhibited different expression patterns in response to high antibiotic stress and also differed between the two studies species, i.e. chickpea and arugula. In chickpea, high antibiotic stress slightly increased expression level of *Cu/Zn SOD* in the first week, despite the increase in SOD activity. The gene expression of Cu/Zn SOD was reduced in the 2nd week with no significant change in the SOD activity. This fluctuation in transcript level were not consistent with the SOD activity. It indicated that *Cu/Zn SOD* was not the main enzyme causing the total SOD activity. Similar results were obtained by Xu et al., where the gene expression pattern of *Cu/Zn SOD* did not follow the enzyme activities changes in Kentucky bluegrass (Xu et al., 2011). Vaseva et al. (2012), suggested that *Cu/Zn SOD* was suppressed by *Fe SOD* in oat seeds. On the 4th and 6th week, the increase in SOD activity is correlated with increase in the level of the transcript. The fluctuation between the level expressions of *Cu/Zn SOD* within the last 2 weeks of experiment, didn't affect the activity of the SOD. The fluctuation between gene expression and enzyme activity seen in chickpea with high antibiotic treatment indicated that changes in mRNA levels may not always imply changes at the translational level. According to Merchante et al. (2017), the increase in the amount of mRNA does not necessarily correlate with the amount of protein produced. On the other part, the inconsistency in *Cu/Zn SOD* transcript levels with the SOD activity, indicated that it was not the main isoform contributing to total SOD activity induced by antibiotic stress. Such result was observed in arugula, where the transcript level of *Cu/Zn SOD* was not consistent with the declined activity of SOD in high antibiotic-treated arugula. However, the transcript level of *CAT4* was in parallel with the catalase activity in both species, suggesting that the *CAT4* isoform was a major player in the induction of catalase antioxidant activity.

Concerning *IFS* and *IFR* gene expression, a time dependent expression was observed in both species. Isoflavone synthase encoded by *IFS* gene catalyzes the first step in the phenylpropanoid pathway that induces the synthesis of isoflavone. Isoflavone reductase encoded by the *IFR* gene catalyzes the reduction of the isoflavone into isoflavonoids (Sharma et al., 2019). Being legume-specific specialized metabolites, isoflavonoids play an important role in plant-environment interaction. They act as chemoattractant to rhizobia and facilitate their symbiosis with legume plants (Garcia-Calderon et al., 2015). In addition Isoflavones are highly affected by environmental conditions. Dayde et al. (2004) reported that low temperatures associated with late watering increased the isoflavone content (Gutierrez-Gonzalez et al., 2010). In our study, both enzymes were downregulated in chickpea treated with high antibiotic concentration during the first two and the last weeks, whereas in arugula both enzymes were overexpressed. The results are in agreement with the previous data of the study, where the rhizobacterial count decreased in chickpea and increased in arugula (Nassar & Borjac, 2022). Their remarkable overexpression on

the 6th week in chickpea, might be related to the developmental stage of the chickpea and this is in agreement with Da Silva et al. (2021) who showed that long-term drought conditions reduced isoflavone content in soybean mostly at later development stages.

Plants have evolved diverse defense mechanisms to cope with heavy metals, such as extrusion, chelation, vacuolar sequestration and regulation of distribution. *AtABCC1* and *AtABCC2* conferred tolerance to As(III), Cd(II) and Hg(II), but not to Pb(II) (Borghi et al., 2019; Park et al., 2012). The continuous overexpression of the *MRP2* transporter in chickpea treated with high antibiotic concentration during the experimental timepoints implied that the *MRP2 (ABCC2*) buildup some tolerance against the antibiotic. This suggestion is supported by the downregulation of the transporter expression in the arugula treated with high concentration of antibiotic. Park et al showed that the vacuolar sequestration of the PC–Cd (II) complexes by *AtABCC1* and *AtABCC2* decreases the cytosolic concentration of the metal in root cells, in that way, reducing translocation to the shoot (Park et al., 2012). In agreement with this explanation, we previously showed that above ground part growth parameters in chickpea treated with high amount of antibiotic was not affected; while in intolerant arugula, severe effect on the leaves growth was observed (Nassar & Borjac, 2022).

Data on the *ABCG39-like (PDR11*) transport in chickpea is scarce. Our results showed inhibition in the level of *ABCG39- like* expression in the first 3 weeks and upregulation in its expression on the last 2 weeks of the experiment. A study on cadmium uptake and translocation in peanut roots showed that in the presence of Cd, *ABCG39* gene expression was downregulated among 18 other genes (Chen et al., 2019). The induction of *PDR11* in *Arabidopsis thaliana* by various stress treatments proposed that *AtPDR11* may be involved stress responses, and its expression may be regulated by oxidative signaling (Xi et al., 2012). Same result was observed in arugula, where the *ABCG39-like* transporter was induced at high antibiotic concentration.

In recent years, cumulative genetic, biochemical, and molecular biological evidences have implicated that Multidrug And Toxin Extrusion (*MATE*) transporters are known to be responsible for the transport of several flavonoids across membranes (Zhao et al., 2011b). Furthermore, in plant kingdoms, few members were reportedly involved in broad range of biological activities (Chen et al., 2015) such as protection of plant cells from inhibitory compounds and aluminum tolerance (Liu et al., 2009; Magalhaes et al., 2007), detoxification of heavy metals (Legong Li et al., 2002) and plant development (Burko et al., 2011). The *TT12* and *ABCC4* were downregulated in chickpea treated with high amount of antibiotic, while they were only overexpressed in the 6th week. Their overexpression might be accompanied with the flowering induction, given that, the first flower in chickpea treated with high antibiotic concentration appeared on the 8th week. However, the *ABCG39* and *TT12* in arugula were overexpressed in plants treated with high amount of antibiotic.

5. CONCLUSIONS

It can be concluded that released veterinary antibiotic causes oxidative stress as evidenced by proline accumulation within roots and increased lipid peroxidation showing the sensitivity of lentil, arugula and cress. In chickpea species, the high levels antibiotic inhibited lipid peroxidation conferring some tolerance. Moreover, the data demonstrated a significant increase in the activities of three major enzymes involved in the detoxification process, i.e., SOD, CAT and POD in chickpea. However in lentil, the antioxidant enzymes activities were inhibited in the presence of high antibiotic concentration. In treated arugula, SOD and POD activities synergistically decreased while CAT increased, whereas in treated cress, POD and CAT worked together and were induced at low antibiotic concentration and inhibited at high antibiotic levels.

Our findings indicated, that the magnitude of each antioxidant enzyme activity was due to the intrinsic tolerance capacity of each species, the antibiotic concentration and time of exposure.

The gene expression analysis showed that *Cu/Zn SOD* was not a main factor in total SOD activity in both chickpea and arugula, suggesting that changes in mRNA levels might be due to post-transcriptional or to post-translational modification. While, *CAT4* isoform was a major player in the induction of catalase antioxidant activity in both species. The expression pattern of *IFS, IFR, ABCC2, ABCC4, ABCG39-like* and *TT12* transporter were significantly affected by the presence of antibiotic, where their expression implied tolerance of chickpea against antibiotic stress while indicated sensitivity in arugula.

Additionally, although detailed information is still unclear, larger levels of ABC transporter genes might be induced or suppressed upon antibiotic exposure, indicating that further study of this family would be helpful to understand the mechanism of antibiotic uptake, transport and crop tolerance. Therefore, chickpea species could also be included in antibiotic tolerance breeding programs.

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