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GERMINATION, SEEDLING GROWTH, AND ANTIOXIDANT ACTIVITY IN FOUR LEGUME (*FABACEAE*) SPECIES UNDER COPPER SULPHATE FUNGICIDE TREATMENT

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Abstract

The experiment was fixed to investigate the effect of copper sulphate (0, 1.5, 3 and 4.5 g/l $CuSO_4$) used as a biological fungicide within fruit trees fields on germination, seedling growth, as well as some mechanisms involved in germination, growth and cell protection processes on the roots of four legume plants: faba bean (*Vicia faba*), common vetch (*Vicia sativa*), fenugreek (*Trigonella foenum-graecum*) and *Medicago truncatula*. Biometric and physiological results showed that higher copper doses (3 and 4.5 g/l) significantly decreased germination percentage, root growth, total sugar content in all analyzed species. Lipid peroxidation (MDA) content and the activity of some antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and guaïacol peroxidase (GPOX) were markedly increased concomitantly with the increasing copper stress. *V. sativa* showed relatively high germination percentage, low root and dry weight phytotoxicity compared to other species. It also showed a relatively higher induction of antioxidant enzymes mainly guaïacolcperoxidase. With these results, *V. sativa* is more copper sulphate-tolerant and could be proposed as suitable candidate used as cover soil for fruit trees fields.

Key words: Antioxidant systems; Copper sulphate; Legumes; Lipoperoxidation; Seed germination; Tolerance.

Introduction

Many fruit, vegetable and cereal crop are vulnerable to fungal attacks, highly altering crop yields. In intensive and modern agriculture, farmers are forced to use pesticides as crop protection chemicals, to prevent diseases development. The frequent and indiscriminate application of pesticide caused serious problems (Mishra *et al*., 2008) and could reduce soil fertility leading to some nutritional deficiencies. Nitrogen is a major element for plant growth and its deficiency induced soil fertility reduction and soil erosion aggravation and limit crop productivity (Fageria & Baligar, 2005). To overcome these problems, the intercropping of "legume" thanks to their symbiotic nitrogen fixation characteristic, has been suggested as a means of improving soil fertility. Intercropping system is defined as the agronomic method in which two or more crops are grown in the same field simultaneously (Miao *et al*., 2013; Vandermeer *et al*., 1998). Several workers were interested in studying this approach due to its advantages for agriculture like increasing the fertility of soil, prevent soil erosion, reduction of plant diseases, insects and weeds (Lithourgidis *et al*., 2011), improving crop biomass and productivity (Dong *et al*., 2013).

Legumes are a good source of nitrogen (Elsheikh *et al*., 2009; Ashworth *et al*., 2015) because they have the capacity to take nitrogen from the air and make it available to the plant by symbiosis with rhizobia via root nodules (Mhadhbi *et al*., 2009). Legumes showed great potential to enhance productivity system and could be used as soil cover (Thierfelder *et al*., 2012) and could fix N fertilizer to the following crop in the frame of crop rotation systems.

Among pesticides, copper sulphate is commonly used as a fungicide in biological agriculture, by farmers to prevent fungal diseases. This pesticide contains copper that exerts its toxic effect via cupric ion (Cu^{2+}). Copper as a heavy metal has been largely proposed in studding physiological and biochemical assays, because of its both essential and toxic effects on plants (An, 2006). This metal is an essential micronutrient needed for the development of plants in very small amounts (Singh *et al*., 2007). However, it is toxic when absorbed in excess (Guo *et al*., 2009; Azouz *et al*., 2012). The impact of Cu on germination and seedling growth are well documented (Mihoub *et al*., 2005; Sfaxi-Bousbih *et al*., 2010; Karmous *et al*., 2012; Bes *et al*., 2013). Excessive levels of copper metals in the soil environment adversely affect seed germination and plant growth (Gupta *et al*., 2001; Houshmandfar & Moraghebi, 2011). Seed germination and early seedling growth are rapidly influenced by some environmental factors. Seed germination is sensitive to metal pollution because of a lack of defense mechanisms (Xiong & Wang, 2005) and it is considered as a critical stage and sensitive process than the other stages of plant development. According to the same authors, poor germination limit life cycle of plant under stressful conditions. It has been also suggested that germination rate is an important biomarker to elucidate the toxicity effects on plants (Labra *et al*., 2006).

In plants copper can affect seedling development, stopping root growth by interfering with mitotic divisions (Lin *et al*., 2003; Yildz *et al*., 2009) or through nutrient shortage for the embryo due to low cotyledon reserve mobilization (Elleuch *et al*., 2013). Especially excess copper can cause leaf chlorosis (Bouazizi *et al*., 2010), reduction of chlorophyll content (Brahim & Mohamed, 2011), damage to root epidermal and root cells membrane (Ouzounidou *et al*., 1995; Wain Wright & Wolhouse, 1997).

Like others stresses, excess copper can catalyze, through Fenton and Haber-Weiss reactions (Connolly & Guerinot, 2002), the generation of extremely toxic free radicals and reactive oxygen species (ROS) (Azouz *et al*., 2012), responsible for lipoperoxidation (Valko *et al*., 2005). ROS include hydroxyl radicals ($HO^{\bullet}$), superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Posmyk & Kontek, 2009) which are highly reactive and induced membrane destabilization by affecting structural and functional organic molecules such as proteins, lipids, nucleic acids and carbohydrate (Mittler *et al*., 2004). However, plants have well-developed antioxidant systems including catalase (CAT), guaïacol peroxidase (GPOX), ascorbate peroxidase (APX) and superoxide dismutase (SOD) in order to avoid or repair the deleterious effects of ROS (Tewari *et al*., 2006; Sharma & Dietz, 2008; Zaefyzadeh *et al*., 2009). Antioxidant responses in plants to copper-induced oxidative stress are well documented (Lombardi & Sebastiani, 2005; Tewari *et al*., 2006; Zhang *et al*., 2010).

The aim of the present work is to identify candidate legume specie useful as soil cover in fruit tree cultivation fields frequently using copper sulphate as a fungicide. For this purpose, we evaluated copper sulphate toxicity on germination and seedling growth of four legumes species: *V. faba*, *V. sativa*, *T. foenum-graecum* and *M. truncatula*, as well as on the related metabolisms and stress response mechanisms (antioxidant enzymes) involved in seedling protection from the deleterious impact of fungicide.

Materials and Methods

Identical size of faba bean (*V. faba*), common vetch (*V. sativa*) and fenugreek (*T. foenum-graecum*) seeds were disinfected with mercuric chloride $HgCl_2$ (0.1%) for 1 min. Seeds of medicago (*M. truncatula*) were chemically scarified in concentrated sulfuric acid (H_2SO_4, 95%) for 8 min. Then, they were washed abundantly, using distilled water. Soaked seeds (for 6 h) were transferred in Petri dishes with filter paper moistened with various copper sulphate doses (0, 1.5, 3 and 4.5 g/l) and kept for germination at room temperature (20°C) in dark during 5 days. After 5 days, healthy seedlings were transferred and cultivated in other Petri dishes containing sterile sand irrigated with four copper sulphate levels (0, 1.5, 3, 4.5 g/l of $CuSO_4$). The seed was considered germinated when radical appeared and visible at length reached 2 mm. The radical length and fresh weight were immediately determined after the harvest (after 10 days), while the dry weight (DW) was determined after desiccation at 60°C until constant weight. Besides, fresh shoot and root samples from each seedling were stored at -80°C for the biochemical analysis.

Carbohydrate contents: Soluble carbohydrate content was measured following Yemm & Willis (1954). Soluble sugars were extracted by homogenizing 25 mg of dry tissue (two times) with 5 ml of ethanol (80%). Then the homogenate was heated during 30 min at 70°C and centrifuged for 30 min at 3000 g. 0.5 ml of sulfuric anthrone (0.5 g anthrone in 250 ml of 99% sulfuric acid) was added to 2.25 ml of ethanol (80%) and 250 µl of ethanolic extract. The reaction mixture was heated in boiling water bath for 10 min at 100°C and then rapidly cooled on ice. Absorbance of the extract was measured at 640 nm using a spectrophotometer. The content of total soluble sugar was expressed as $mg.g^{-1}$ DW through the calibration curve.

MDA determination: Lipid peroxidation was evaluated by determination of malondialdehyde (MDA) content in the fresh tissues (Alia *et al*., 1995). Fresh sample (0.5 g) was homogenized in 5ml of 5% trichloroacetic acid (TCA).The homogenate was centrifuged at 12.000 g for 15 min at 4°C. An aliquot of the supernatant (0.5 ml) was added to 0.5 ml of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA. The test tubes were heated at 95°C for 25 min and then rapidly cooled in ice bath. After centrifugation at 10.000 g for 5 min, the absorbance of supernatant was measured at 532 nm. Non-specific turbidity was corrected at 600 nm by subtracting the absorbance. The content of MDA was determined by using an extinction coefficient of 155 $mM^{-1} cm^{-1}$.

Antioxidant enzyme activities measurement: Protein rate was measured following the method of Bradford (1976) using bovine serum albumin as a standard. Fresh tissues (0.5 g) were ground at 4°C in mortar with 2% (w/w) polyvinyl-polypyrrolidone (PVP) and 1ml phosphate buffer (50 mM; pH 7.8) composed by 0.2 mM ethylene diaminetetra acetic (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), as protease inhibitor, and 0.1% (v/v) Triton X-100. Extracts were centrifuged at 12.000 rpm for 20 min at 4°C. The supernatant was used to determine enzyme activities.

Superoxide dismutase (SOD, EC 1.15.1.1) was assayed by using the photochemical NBT (nitroblue tetrazolium chloride) method (Beauchamp & Fridovich, 1971). The reaction mixture contained 50 mM K_2HPO_4/ KH_2PO_4 buffer (pH 7.8), 2 mM riboflavin, 13 mM methionine, 75 mM NBT and enzyme extract. One unit of SOD activity was expressed as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25°C.

Catalase (CAT, EC 1.11.1.6) activity was measured by following the decline of absorbance at 240 nm caused by the decomposition of the10 mM H_2O_2 during 1 min (ξ= 36 $M^{-1} cm^{-1}$) (Aeibi, 1984).

GPOX (EC 1.11.1.7) activity was determined by monitoring the formation of tetraguaïacol from guaïacol (9 mM) at 470 nm during 1 min (ξ= 26.6 $mM^{-1} cm^{-1}$), 19 mM H_2O_2 was added for starting the reaction (Mhadhbi *et al*., 2005).

Statistical analysis: All the values in this manuscript are presented as means value of at least three replicates± SE.Each result shown in a Table and Figure was the mean of at least three replicates. Analysis of variance (ANOVA) was done using STATISTICA software (http://www.statsoft.com) and the differences for all measured variable were performed by the Tukey HSD test with a significance of $p\leq 0.05$.

Results

Copper sulphate effects on seed germination: The effect of copper sulphate on seed germination varied according to the species and the pesticide concentration in the media (Table 1, Fig. 1). Indeed, this fungicide decreased significantly germination percentage in all legumes. This reduction is less pronounced in *V. sativa* and *T. foenum-graecum* (17% and 16%, respectively). However, it reaches 47% and 50%, respectively, for *V. faba* and *M. truncatula*.

Fig. 1. Morphology of *Vicia faba* (A), *Vicia sativa* (B), *Trigonella foenum-graecum* (C) and *Medicago truncatula* (D) roots under different copper sulphate concentrations (0, 1.5, 3 and 4.5 g/l $CuSO_4$).

Effect of copper sulphate on root development: The effects of copper sulphate on root development and dry weight in the different studied species are given in Table 2 and Fig. 1. Results indicated that radical length and biomass were reduced significantly with increasing concentration of copper. During copper stress, *V. sativa* maintained relatively a lower reduction rate of these both parameters, reaching 30% and 35%, respectively compared to severe decreases revealed in *V. faba* (70% and 26%, respectively), in *T. foenum-graecum* (28% and 51%, respectively) and *M. truncatula* (57% and 41%, respectively).

Effect of copper sulphate on soluble sugar and soluble protein contents: Carbohydrate metabolism is altered by adverse environmental factors. Soluble sugar contents were less influenced by the lowest copper concentration (1.5 g/l), then it decreased progressively with increasing concentrations of $CuSO_4$ (3 and 4.5 g/l). Results showed that soluble sugar content was reduced by 49%, 47%, 44% and 39% in *V. faba*, *V. sativa*, *T. foenum-graecum* and *M. truncatula*, at the highest copper level (4.5 g/l) (Fig. 2A). The same trend was obtained for protein content, which showed minor variations under 1.5 g/l $CuSO_4$ level, and then decreased drastically following the addition of 4.5 g/l $CuSO_4$ (Fig. 2B).

Effect of copper sulphate on lipid peroxidation: Results showed that the lowest concentration of $CuSO_4$ (1.5 g/l) slightly decreased MDA content in all the studied legumes compared to controls ones. However, $CuSO_4$ at higher dose (4.5 g/l), significantly increased MDA levels by 26%, 21%, 22% and 38% in *V. faba*, *V. sativa*, *T. foenum-graecum* and *M. truncatula*, respectively (Fig. 2C).

Antioxidant response to copper: In order to assess the influence of $CuSO_4$ on ROS-scavenging enzymes of legumes seedlings, the activities of some enzymes were examined. Under 1.5 g/l of copper sulphate treatment, SOD (Fig. 3A), CAT (Fig. 3B) and GPOX (Fig. 3C) of *V. faba*, *V. sativa*, *T. foenum-graecum* and *M. truncatula* seedlings have not a great changes in their activities compared to their respective controls.However at higher dose (4.5 g/l) of copper, SOD, CAT and GPOX activities increased markedly in *V. faba* (by 12%, 79% and 68%, respectively), in *V. sativa* (by 7%, 53% and 89%, respectively), in *T. foenum graecum* (by 65%, 42% and 56%, respectively) and in *M. truncatula* (by 84%, 120% and 99%, respectively).

Table 1. Effect of copper sulphate concentrations on germination percentage of *Vicia faba*, *Vicia sativa*, *Trigonella foenum-graecum* and *Medicago truncatula* seedlings.

Treatments		Germination (%)			
		V. faba	***V. sativa***	***T. foenum-graecum***	***M. truncatula***
Copper sulphate (g/l)	0	98.61 a	99.16 a	98.33 a	99.16 a
	1.5	97.22 a	99.16 a	99.16 a	95.83 a
	3	76.38 b	95 a	95.83 a	64.16 b
	4.5	51.66 c	81.66 b	82.5 b	49.16 c

Values are the means of at least three replications ± S.E. Bars with different letters are significantly different at $p \leq 0.05$ (Tukey HSD test)

Table 2. Effect of copper sulphate concentrations on radical length and dry weight (DW) of *Vicia faba*, *Vicia sativa*, *Trigonella foenum-graecum* and *Medicago truncatula* seedlings.

Treatments		Radical length (cm)				Dry weight (mg)			
		V. faba	***V. sativa***	***T. foenumgraecum***	***M. truncatula***	***V. faba***	***V. sativa***	***T. foenumgraecum***	***M. truncatula***
Copper sulphate (g/l)	0	6.01 a	9.14 a	9.69 a	8.85 a	10.28 a	10.26 ab	10.65 a	7.18 a
	1.5	4.76 b	9.05 a	9.30 a	6.66 b	10.13 a	11.56 a	7.07 b	6.96 a
	3	3.33 c	8.06 b	7.02 b	5.27 c	8.33 b	7.91 bc	6.06 c	5.88 b
	4.5	1.77 d	6.40 c	6.98 b	3.75 d	7.59 b	6.67 c	5.17 c	4.19 c

Values are the means of at least three replications ± SE. Bars with different letters are significantly different at $p \leq 0.05$ (Tukey HSD test)

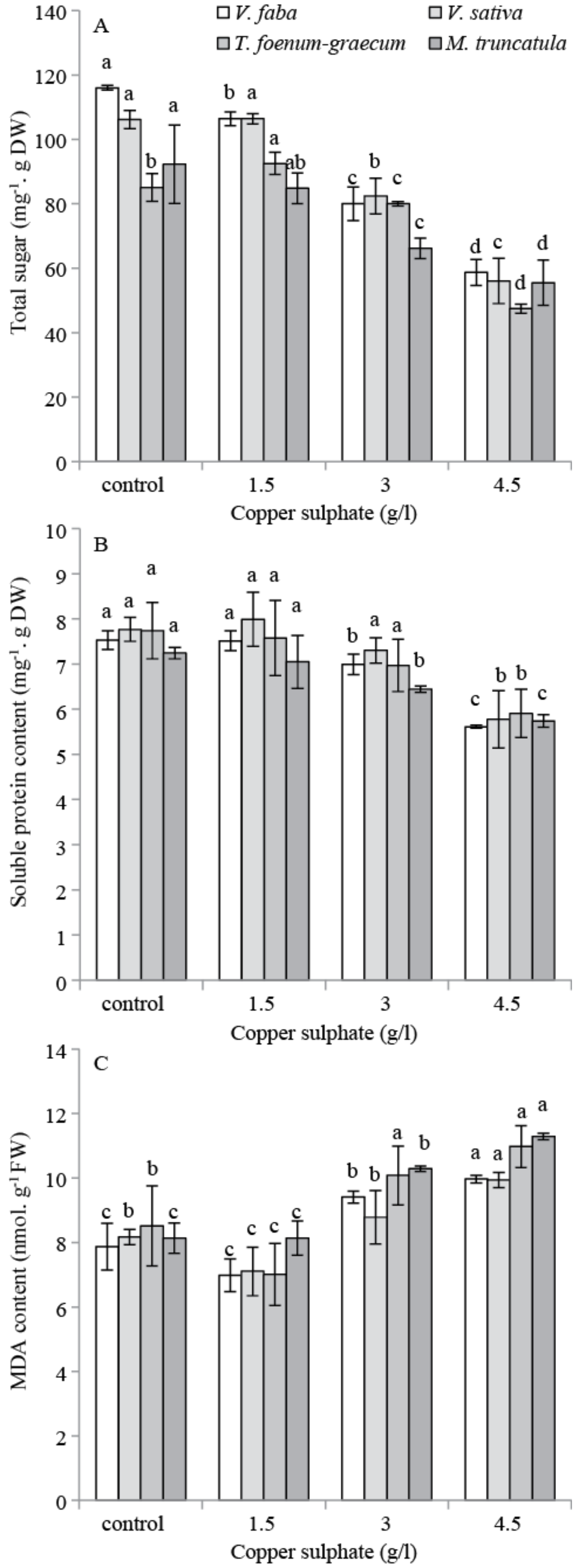

Fig. 2. Effect of copper sulphate concentrations on soluble sugar content (A), soluble protein content (B) and MDA (C) content in roots of 10-day old *Vicia faba*, *Vicia sativa*, *Triggonella foenum-graecum* and *Medicago trnucatula* seedlings. Values are the means of three replication ± SE. Data with the same letter are not significantly different at $p \leq 0.05$ (Tukey HSD test).

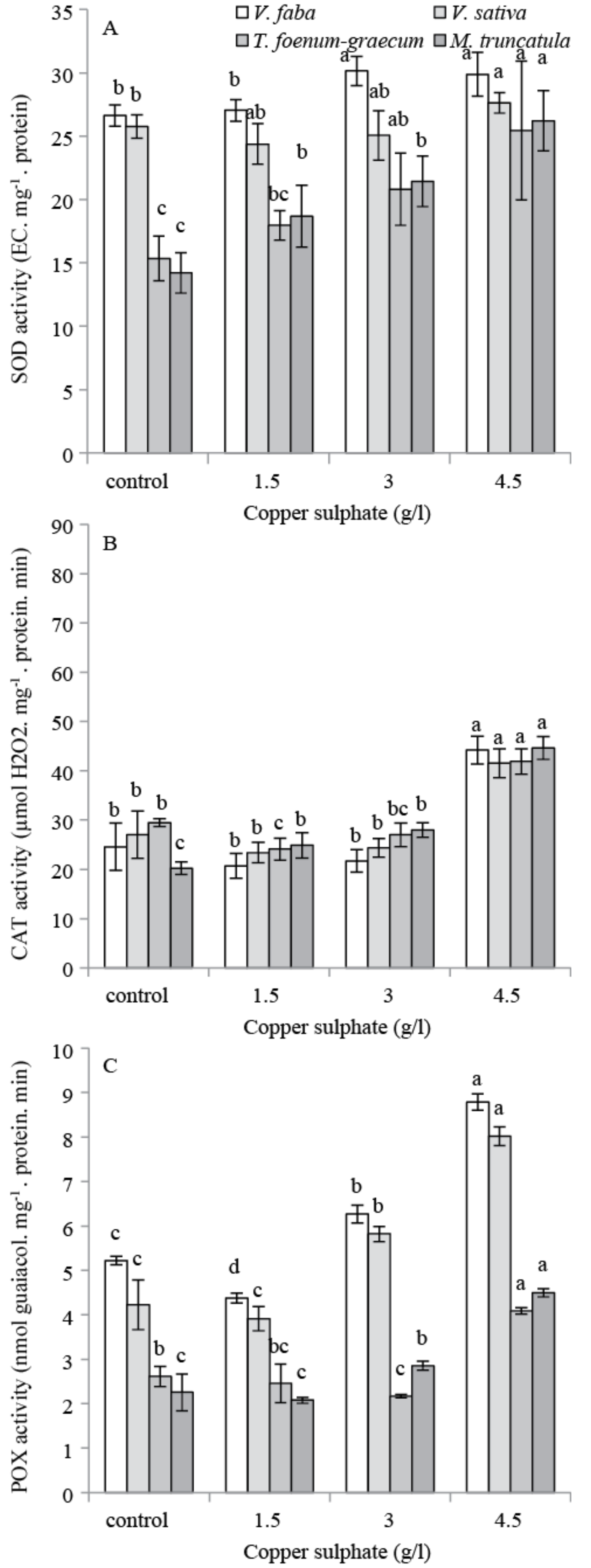

Fig. 3. Effect of copper sulphate concentrations on SOD (A) CAT (B) and GPOX (C) activities in roots of 10-day old *V. faba*, *V. sativa*, *T. foenum-graecum* and *M. trnucatula* seedlings. Values are the means of three replication ± SE. Data with the same letter are not significantly different at $p \leq 0.05$ (Tukey HSD test).

Discussion

Seed germination and early seedling growth constitute the highly sensitive physiological processes in plant and are rapidly altered by heavy metals (Shanker *et al*., 2005). Our experiment was designed to study the impact of $CuSO_4$ levels (0, 1.5, 3 and 4.5 g/l) on seed germination, physiological and biochemical changes in *V. faba*, *V. sativa*, *T. foenum-graecum* and *M. truncatula* species.

Under copper sulphate stress, the germination percentage, roots length and biomass exhibited differences between the four studied legumes. This difference depends on the available concentration of copper in solution (Tables 1 and 2). In this paper, legumes treated with low dose of copper (1.5 g/l) showed no considerable difference in germination percentage. Higher concentrations (3 and 4.5 g/l of $CuSO_4$) showed a decrease in these parameters in all studied species. But the relatively lower reduction germination percentage was recorded in *V. sativa* compared to the other legumes (Table 1). The depressive effects of cupric ion on germination percentage also has been reported in other species: in *Raphanus sativus* (Gupta*et al*., 2001), in red cabbage (Posmyk & Kontek, 2009), in *Vigna radiata* (Hema & Subramani, 2013), in *Lycopersicon esculentum* and *Phaseolus vulgaris*(Ashagre *et al*., 2013a, 2013b), in *Trigonella foenum-graecum* (Elleuch *et al*., 2013) and in *Vicia sativa* (Muccifora & Bellani, 2013). This inhibitory action of excess copper in seed germination may be in part associated with modification in appropriate quantity of water absorption needed to achieve the essential metabolic processes at this stage (Elleuch *et al*., 2013). In other study, Mihoub *et al*. (2005) and Singh *et al*. (2007) concluded that reduction in seed germination observed in pea seedlings after heavy metals including cadmium or copper treatments is not the consequence of modification in water absorption by seed tissue, but may be due to perturbation of reserve mobilization process from cotyledons. These authors give a number of hypotheses to explain this reduction: (1) negative interference of pollutant with mineral and organic reserves mobilization, (2) decrease in transport of micronutrients to the embryo. Furthermore, the decline in soluble total sugar content in this study (Fig. 2A), suggests that excess cupric ion may perturb sugar translocation from cotyledons to axis. Our results agreed with those of Thevenot *et al*. (1992) who reported that total sugar content in maize was affected under copper stress. Furthermore, Elleuch *et al*. (2013), investigated the deleterious effect of copper on fenugreek α-amylase, a vital enzyme needed for necessary metabolic function during germination (Mayer, 1977), which was quite sensitive to 20mM of Cu treatment.

Same behavior of total sugar content in plant seedling was revealed under others heavy metals as spanich plant (Malook *et al*., 2017).

Thus, the observed reduction in total sugar could be correlated to the low rate of the enzymatic hydrolysis of starch by amylase (which hydrolysis amylase and amylopectin to simple sugars) due to its decreased activity in seeds.

The length of the root and biomass are also decreased with elevated metal doses (3 and 4.5 g/l) (Table 2). The relatively highest value of radical length and dry weight were obtained in *V. sativa* specie, indicating a higher ability of this specie to tolerate this fungicide stress than the others seedlings. The hazardous effects of excess copper on root growth is largely documented in several other species: *Vigna radiata* (Verma *et al*., 2011), *Phaseolus vulgaris* (Karmous *et al*., 2012), *Lycopersicon esculentum* (Ashagre *et al*., 2013a), *Plantago psyllium* (Mohammadi *et al*., 2013), *Trigonella foenum-graecum* (Elleuch *et al*., 2013), *Vicia sativa* (Muccifora & Bellani, 2013) and *Triticum aestivum* (Atabayeva *et a*l., 2017). The reduction of root development and seedling growth may be due to copper interference with cell division or cell elongation (Souguir *et al*., 2008; Bandurska & Ciéslak, 2013; Choudhary & Agrawal, 2014), disturbance of water uptake (Souguir *et al*., 2008), modification of permeability and elasticity of cell wall (Naseer, 2001). Therefore, our finding showed that germination percentage and root development were less affected by Cu stress in common vetch, suggesting that this species could show better tolerance to copper stress compared to the other studied legumes.

Owing to its redox-active properties, copper at excess concentration catalyses the production of high toxic free radicals and reactive oxygen species (ROS) (Pinto *et al*., 2003; Azouz *et al*., 2012). These ROS can react with organic molecules such as lipids causing lipid peroxidation (as determined by MDA content). Our data showed that the highest dose of copper sulphate increased MDA level in all studied legumes as compared with control ones (Fig. 2C), but the lowest decrease in MDA content was recorded in *V. sativa* (by 21%). These results are in accordance with the results previously obtained in other species under copper treatment such as *Arabidospsis thaliana* (Skorzynska-Polit *et al*., 2010), *Triticum aestivum* (Azouz *et al*., 2012). This increased level of MDA might be the result of overproduction of ROS and severe oxidative injury caused by Cu^{2+}. The study conducted by Koca *et al*. (2007) reported that the more generation of MDA was closely correlated with the higher level of ROS in the plant when they were exposed to many stress.

In order to avoid and alleviate the stress intensity caused by the ROS, plants respond to a variety of environmental stresses by developing complex antioxidant defense systems include SOD, CAT and GPOX which have a vital role in keeping ROS levels under control instead of producing them in order to maintain metabolically compatible levels of H_2O_2 and $O_2^{\bullet-}$ (Alcher *et al*., 2002). In this work, copper sulphate-treated seedlings of each legume showed a significant stimulation of SOD, CAT and GPOX activities, especially when dose reached 4.5 g/l (Figs. 3A, B & C). These results are in accordance with other results previously described in other species such as *Triticum aestivum* (Azouz *et al*., 2012) and *Vigna radiata* (Verma *et al*., 2011). In several studies, it has been confirmed that the increase of the activity of these enzymes as a defense antioxidant system and is regarded as general response to excess heavy metals like copper and cadmium as well as other stresses

(Mittova *et al*., 2003; Nouairi *et al*., 2006; Srivastava *et al*., 2006; Bashir *et al*., 2007; Nouairi *et al*., 2012; Parween *et al*., 2012; Elleuch et *al*., 2013; Chen *et al*., 2017). The induction of antioxidant enzymes play a major role in ROS scavenging, which will confer to plants some adaptive response and tolerance to metal stress.

Conclusion

It could be summarized that copper sulphate fungicide was toxic at 3 and 4.5 g/l doses, these concentrations perturb seed germination, physiological and biochemical parameters in *V. faba*, *T. foenum graecum*, and *M. truncatula* seedlings. Consequently, copper sulphate could be used at safe concentrations, not more than 3 g/l. On the other hand, it was showed that *V. sativa* exhibited relatively copper sulphate tolerance at germination and seedling growth stages compared to other species. The relative tolerance of this specie was correlated to a relative higher increase in germination percentage and seedling growth parameters and the relatively maintain of MDA level. This specie is consequently suggested to be a suitable candidate as cover crop and intercrop with other species in sustainable agricultural system.

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