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Cupper Treatments and Their Effects on Growth, Carbohydrates, Minerals and Essential Oils Contents of *Rosmarinus officinalis* L.

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Abstract: Pot experiments were carried out in the greenhouse of of sandy loamy type at Taif University, S.A. in order to investigate the effect of copper concentrations (0,50,200,800 and 3200 ppm) on the dry matter accumulation, sugar metabolism, chemical composition and essential oils content of *Rosmarinus officinalis* L. It was observed that application of Cu at rates of 0,50 and 200 ppm were gradually increased the dry matter accumulation and sugar fraction. However, high rate up to 3200 ppm, the dry matter gradually diminished as well as sugar fractions. Data revealed that both non- reduced sugar (T.R.V) and reduced sugar (D.R.V.) were gradually increased under different Cu concentrations. Data also indicated that high rates of Cu added the total N , p and k were significantly decreased. Results revealed that application of Cu treatments were significantly increased the essential oil content and also produced alternation in the oil composition i.e increased the concentration of of verbenone and 1, 8-cineole in the same time α-pinene were also decreased.

Key words: *Rosmarinus officinalis* · cupper · growth creature and essential oil

INTRODUCTION

Rosmarinus officinalis L. is a dominant shrub species of calcareous Mediterranean communities that has increased its presence in wide areas due to fire frequency increase and field abandonment [1]. It is an obligate seed germinator with abundant flowering. Its sunlit character and its high reproductive effort allow it to colonise the bare landscape [2]. *R. officinalis* is one of the most interesting aromatic species of Mediterranean flora from the economic point of view [3] and antoxidative fraction [4] that can be utilized. The effect of soil properties and iron on yield and chemical composition of *Rosmarinus officinalis* essential oil was studied by Moretti and Peana [5].

Sugars considered important metabolites in plant metabolism, not only because the first complex organic compounds formed in the plant as a result of photosynthesis, but also provides a major source of respiratory energy [6]. Also sugars play a number of ecological roles in plant protection against wounds and infection, as well as in the detoxification of foreign substances [7].

Cupper is not an essential nutrient and at high concentration it inhibit plant growth [8-10]. This heavy metal happens to be an important environmental pollutant [11] and causes perturbations in various plant processes such as development of chloroplasts [12], than chlorophyll biosynthesis [13] as well as photosynthesis [14, 15]. The effect of Cu on N-nutrations was divergent and linked to the type of soil [16]. Cupper toxicity is a problem of both agricultural and environmental significance. Sources of Cu contamination include mining and smelting, urban, industrial and agricultural wastes and the use of agrochemicals. Cupper is present in many forms in soils, with free Cu^{2+} activity considered to be the best indicator of bioavailability [17]. Soil solution Cu concentrations are generally extremely low, with more than 98% Cu in solution bound to soluble organic matter, irrespective of pH [18]. Adsorption of Cu is highly pH dependent and bioavailability of Cu increases with decreasing pH. Due to its high affinity for organic matter, Cu is not readily leached from the soil profile and tends to accumulate in the surface soil [19].

The aim of the present work was to elucidate the role of cupper toxicity on growth, sugar metabolism, minerals and oil contents of *Rosmarinus officinalis* L.

MATERIALS AND METHODS

Plant materials: *Rosmarinus officinalis* L. cuttings about 15 cm in height were obtained from the Botanical Garden

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at Faculty of Pharmacy, Zagazig Univ. Egypt, to reached the homogeneous plantlets. Three cuttings were planted in plastic pots each containing about 5 kg soils of sandy loamy type at Taif University, S.A. Three replicated of each were carried out. The experiment was carried in the period from November, 2005 to February, 2006. The irrigation with 300 ml of half Hoagland solution supplemented with various concentrations of Cu (0, 50, 200, 800, 3200 ppm) added as cupper sulphate. The nutrient solutions were renewed every third day and the pH was adjusted to 6.8-7.3. Samples of leaf were collected for the different measurements. The fresh weight, dry weight and degree of succulence were determined in the same time.

Sugar analysis: The soluble carbohydrates extracted with 5% TCA and determined according to the method of Nelson [20], that modified by Naguib [21]. After extraction of soluble carbohydrates the residue was dried at 80°C till constant weight for determination of polysaccharides as adopted by Naguib [22].

Tissue nutrient concentration: Elemental composition was determined calorimetrically according to Jackson [23].

Essential oil analysis: Oils were extracted and fatty acids were identified using gas liquid chromatography as described by El-Shintinawy and Selim [24]. The fresh leaves, used for oil extraction. The equivalent fresh weight to 100g dry weight was used for each treatment. Equipment was in conformity with F.U.I.IX standards [5, 25]. The oil yields reported in Table (5) are the mean values of three distillations at least. The separated oil concentrated by a stream of N_2 gas at room temperature to known volume. A sample of 5 µL of the concentrated oil was injected into a GLC-Variant 6000. Flame ionization detector, 2 m column 3.2 mm internal diameter, packed with 15%. OV-295, Chrom P/Aw/80-100 mesh operating at 180°C, injection temperature 230°C, detector temperature 250°C. The flow rate was 0.42 ml/s. Fatty acids were identified comparing with standards (Polscience Corporation kit no. 61 Cx) and the quantities were calculated from the peak areas obtained by the KLB-2220 Recording Integrator.

Statistic method: Analysis of variance followed by the Tukey test comparing control values with the different treatments a.p value of 0.05 was carried out [26].

RESULTS AND DISCUSSION

Soil characteristics: The data in Table (1) shows the chemical and physical properties of the soil, in which *Rosmarinus officinalis* was grown. It was cleared that the granulometric of soils are mainly of sand and the clay recorded 16.8%. There were also intermediated values of $CaCO_3$ (11.2%) and relative high value of organic matter (2.5%). The conductibility and salinity were 1.6 mS and 0.12%. The soil reaction was slightly alkaline with pH value of 7.4.

Biomass production: Biomass production of Cu-treated plants increased at lower treatments (00 to 200 ppm) and decreased gradually above 800 ppm Cu. Consequently at low Cu concentrations moisture content and degree of succulence were more pronounced. In addition *R. officinalis* growing with low-Cu concentrations tended to increase biomass production (Table, 2). Costa and Spitz [27] suggesting that response effect of heavy metal, increase of biomass production, to certain level (200 ppm) which was associated to an accumulation of some heavy metals in the plant organs but not effects on CO_2 assimilations rate. At concentration above 200 ppm Cu, biomass was reduced, our results in agreements with Barcelo and Poschenrieder [28]. Sancenón *et al.,* [29] cleared that the *Arabidopsis* copper transporter COPT1 functions in root elongation and pollen development.

Table 1: Soil physical & chemical analysis

Physical characteristics	%	Chemical characteristics	%
Gravels	1.3	$CaCO_3$	11.2
Coarse sand	12.6	Organic matter	2.5
Thin sand	50.4	Conductibility	1.6mS
Silt	18.9	Salinity	0.12%
Clay	16.8	pH	7.4
Porosity	43.9%	N total	0.13%
		C/N	11.9

Table 2: Biomass production of *Rosmarinus officinalis* as a function of Cu concentration after 9 months

Conc. (ppm)	F.W g	DW g	D.S.	M.C%
0		7.8	2.56	61
50		8.3*	2.41*	58.5
200	20g	9.2**	2.17**	54*
800		6.1*	3.28*	69.5*
3200		4.23**	7.73**	78.85**

D.S. = Degree of succulenceM.C.= Moisture content.

Table 3: Carbohydrates content of *R. officinalis* as a function of Cu treatments

Conc. ppm Fraction %	0	50	200	800	3200
D.R.V.	0.63±0.05	1.12*±0.08	1.62**±0.09	1.54±0.1	1.42±0.1
T.R.V	1.83±0.1	2.36**±0.11	2.54**±0.12	2.00*±0.13	1.95±0.14
Polysacch.	11.26±0.8	17.21**±1	19.25***±1.2	14.6±0.8	9.21*±0.7
Total carbohyd.	13.72±0.8	20.69**±1.2	23.41***±1.4	18.14**±1	12.58±0.9

Statistical significant: * = $P \le 0.1$ ** = $P \le 0.05$ *** = $P \le 0.01$ D.R.V. = Reduced sugar.T.R.V. = Non-reduced sugar

Table 4: The element contents of *R. officinalis* shoot system as a function of Cu treatments concentration after 9 months. Values are the means±SEM of three replicated measurements

Treatment ppm Cu	Element N	P	K	Na	Ca	Fe	Zn
	mg/100 g air dry weight)					ppm	
0	1.04±.2	1.29±.06	3.91±.2	.42±.05	2.4±.3	422±5	130±2
50	1.04±.2	1.24±.05	1.87±.1**	1.84±.2***	2.28±.3	355±4	141±2
200	1.14±.3	.91±.03**	1.81±.1**	1.52±.1***	2.05±.3	295±4***	140±1
800	1±.3**	.67±.04***	1.49±.4	.71±.03*	1.8±.2**	390±4**	100±1**
3200	.76±.2***	.33±.01***	.64±.05***	.5±.2*	1.53±..3**	315±4**	95±2***

Statistical significant: * = $P \le 0.1$ ** = $P \le 0.05$ *** = $P \le 0.01$

The results cleared that the degree of succulence and moisture content increased with the increase of Cu-treatment, i.e. the highest values (4.73 and 78.85%) were obtained at 3200 ppm treated samples respectively. Generally the degree of succulence increased with the increase of environmental stress. In this connection Dahmash [30] stated that succulence increased as a response to increase cupper stress which regarded as a protective adaptive character. El-Monayeri *et al.* [31] reported that the transpiration rate decreased with increase soil salinity, such reduction may be attributed to decrease in the permeability of leaf cells to water.

Carbohydrates content: The concentrations of D.R.V (reduced sugars), T.R.V (non-reduced sugars), polysaccharides and total carbohydrate at the end period were shown in Table (3). It is cleared that all carbohydrate fractions increased under cupper treatments until 200 ppm as compared with control. At 800 ppm Cu the fractions decreased as compared with 200 ppm treatment plants as well as control plants. At 3200 ppm treatments polysaccharides and total carbohydrates decreased greatly as compared with the control, but the reverse was true for D.R.V and T.R.V.

According to the results obtained in this work *Rosmarinus officinalis* subjected to low concentration of Cu treatments exhibited an increase in the total carbohydrate values. However, the reverse was true at high concentration. The interaction of Cu toxicity with photoinhibitory and recovery processes on PSII has been also investigated [32, 33]. They demonstrate that Cu enhances the adverse effects of light. The photosynthetic activity decreases when oxygenic organisms are exposed to prolonged illumination with high light intensities. This process, which includes the functional impairment of PSII electron transport and the structural damage of the PSII reaction center, is known as photoinhibition [34].

Cu increases susceptibility to photoinhibition in isolated thylakoids [35, 36] or PSII-enriched membrane preparations [37]. Considering that Cu is an efficient catalyst in the formation of reactive oxygen species (ROS), it was suggested that the increased Cu toxicity by light during photoinhibition is due to production of hydroxyl radicals [37]. A different proposal was given by Pätsikkä *et al.* [38] to explain the severe effects caused by the presence of high Cu concentrations during photoinhibition *in vivo*. These authors suggested that the reduced chlorophyll content observed in plant leaves grown in the presence of high Cu concentrations made leaves more susceptible to photoinhibition as a consequence of a Cu-induced Fe deficiency.

However stimulatory effects of D.R.V and T.R.V were detected due to Cu addition to the nutrient medium. The inhibitory effect Cu on the carbohydrate contents at high concentration (3200 ppm) attributed to the increase of respiration of the plant organs. Ahmed [39] found that treatment of corn plant with lead increased respiration rates of its organs and reduced the photosynthetic rates. The increase of D.R.V and T.R.V attributed to enhance of amylolytic enzyme by Cu addition. Mohamed [40] suggested that the reducing sugars accumulate in leaves of plants subjected to heavy metals as Mn, Zn and Fe, due to enhance the amylolytic activity and consequently led an increase of soluble sugars rather than polysaccharides. Moreover, the stimulatory effect of suitable concentration of Cu on the soluble carbohydrates may be due to the stimulate photosystem I and II. In this connection Mohamed [41], found a relation between Cu and ferrodoxin on the reducing site of PS I, where Cu^{++} stimulate the rate of overall electron transfer from water to NADP. Costa and Spitz [27], showed that at lower concentration of some heavy metals (200 ppm), the carbohydrate and amino acids levels increased in shoots, but not in roots. At high concentration, all primary metabolites decreased. This could by explained by the reduction of CO_2 fixation in heavy metal treated plants. But for lower concentrations, the photosynthetic measurements were not affected by treatment and thus could explain the carbohydrate increase.

Tissue nutrient concentration: The increase in the concentration of Cu in the growth medium from 50 ppm to 3200 ppm was accompanied by modification of the shoot concentration of nutrient cations Table (4). Increasing Cu concentration from 50 to 3200 ppm in the medium decreased the N, P, K, Ca and Fe content of *R. officinalis* shoot system. In agreement, El-Shafey *et al.,* [42] found that the increased of stress in the growth medium was accompanied with a marked decrease in Ca and K content. This inhibition of uptake may be due to increased competition. At high Cu concentrations, where severe root damage was observed, reduced uptake of these elements may be due to breakdown of membrane function. The concentrations of N, K, P and Ca in treatment were below the concentrations considered adequate for plant growth [43]. This was despite the concentration of these elements in solution being within the range considered sufficient for plant growth in solution culture. The effect of Cu toxicity again resembles Al toxicity, in that Al is a strong inhibitor of Ca and Mg uptake [44]. A slight reduction in K concentration in the shoot was observed. This may be due to K efflux as part of a mechanism of Cu tolerance [45].

Table 5: Oil fraction (fatty acids) of *Rosmarinus officinalis* plant under different Cu concentration in the nutrient medium (%w/w of oil)

	Cu treatments				
Components	0 ppm	50 ppm	200 ppm	800 ppm	3200 ppm
a-pipene	36.5	30.0	28.63	27.0	25.7
Camphene	6.12	6.32	7.0	6.33	4.55
B-pipene	3.17	4.12	4.32	5.21	4.55
Phellandrene	6.52	7.23	7.81	8.1	8.2
Myrcene	0.48	0.42	0.40	0.35	0.3
Limonene	3.61	3.46	3.32	3.0	3.30
Y-terpine	1.2	1.1	1.1	1.0	1.0
P-cymane	1.16	0.8	0.8	0.7	0.50
B-caryophyllene	1.36	1.63	1.82	1.99	2.10
Sum of hydrocarbons	60.12	55.08	55.20*	53.7*	49.20**
1,8-cineole	12.26	13.03	13.22	15.0	15.55
Camphor	9.28	11.21	10.0	9.26	9.00
Verbenone	6.53	7.28	8.00	9.00	11.26
Sum of ketones	28.07	31.52	31.22	33.26**	35.81***
Bornyl acetate	7.03	8.21	8.52	8.71	10.26
Linaiool	0.78	0.63	0.55	0.55	0.51
Borneol	3.52	4.12	4.00	3.42	3.86
Geraniol	0.46	0.44	0.43	0.40	0.36
Sum of alcohols	11.7	13.4	13.50*	13.08**	14.99**
Total oil (ml/100 g)	2.71	3.61**	3.82**	4.12***	4.24***

Statistical significant: * = $P \leq 0.1$ ** = $P \leq 0.05$ *** = $P \leq 0.01$

Essential oil yield and compositions: Table (5) show the composition of the oil obtained from *Rosmarinus officinalis* under Cu-treatments in the nutrient medium. The data revealed that yield in control reached 2.71 ml/100 g. In general the yield of fatty acids increased with increase in the Cu concentrations. The 3200 ppm treatment samples give the highest yield of fatty acids (4.24 ml/100 g). It cleared that differences in the hydrocarbon content of the plant oil examined. In the low Cu-treatment only a slight difference could be observed, while in the high concentration treatment (3200 ppm) marked significantly decrease was observed. The most marked decrease was in the α-pinene content. All Cu treatment showed an increase in the 1, 8-cineole content from 10% to 40% as compared with the control. In all Cu-treatment samples, the ketenes and alcohols component showed an increase in the Cu treated compared with control. But the increment was insignificant in the 50 ppm treated samples and significant in the 3200 ppm treated samples. Data on the ketenes, shows that the verbenone-camphor ratio progressively increased from 0 Cu treatment (control) to 3200 ppm treated plants due to a marked rise in the verbenone concentration from 6.53% to 11.26% and moderate reduction in the camphor concentration especially at high levels of Cu-treatments (800 ppm 3200 ppm). Such increment in ratio showed a significant decline in the relative contents of the unsaturated fatty acids which would be expected since double bonds are more labile to oxidative stress [46]. This reduction depended on the concentration of cupper.

At this stage of investigation the results showed that Cu affected oil yield and its composition. On the other hand Cu treatment affected the composition of the oil through an increase in the concentration of verbenone and 1, 8-cineole with a reduction in the concentration of monoterpenic hydrocarbons, particularly α-pinene. This effect of Cu could be explained as an intensification of the oxidative metabolic processes, which produce verbenylic derivatives from α-pinene [47, 25]. From a practical point of view, treatment *R. officinalis* with cupper could prove of economic importance, since relatively high concentrations of verbenone together with lower hydrocarbons and camphor content increase the value of rosemary oil for the perfume industry [48].

This would indirectly confirm that the increase of the oil yield and alternation of the oil composition parallel with the decrease in carbohydrates content, could be explained the reduction in the metabolic activity resembled by carbohydrate content increased secondary metabolites due to lack of water availability. In this connection Mule *et al.* [49] demonstrated that the highest oil yield of *R. officinalis* at the conditions of the stress. The data also showed that the predominant influence of Cu treatments (stress condition) on metabolism of *R. officinalis*. Thus there were alternation between the effect of Cu on the primary products (sugar) and secondary one (oil yield and its composition). Moretti and Peane, [25] demonstrated that the need to increase the amount of acids and phenols to compensate nutrient deficiencies in the soil causes an alternation in the modes of synthesis used in the formation of terpenic hydrocarbons by oxygenated intermediates.

Thus it can be said that Cu causes its toxicity by inducing a failure of the membrane protection from the oxidative damage judged by increasing the ratio of saturated to unsaturated fatty acids. Such findings coupled with a decline in the biomass and carbohydrate contents that may act synergistically to reduce the energy transfer of photosystem II retarding the reaction centre to receive light energy. Consequently, the whole process of photosynthesis would be suppressed. Our data shows alternations associated with a significant reduction in the biomass and total carbohydrates, reflect the dramatic inhibitory, effect of Cu on the plant yield and productivity.

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