Acta Sci. Pol. Hortorum Cultus, 16(3) 2017, 3–11

www.acta.media.pl ISSN 1644-0692 DOI: 10.24326/asphc.2017.3.1

ORIGINAL PAPER

Accepted: 20.10.2016

THE EFFECT OF SIMULTANEOUS APPLICATION OF NITROGEN AND COPPER ON YIELD AND STEROIDAL SAPOGENIN PRODUCTION IN *Trigonella foenum graecum* L.

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ABSTRACT

Diosgenin (a steroidal saponin of fenugreek) has long been used as a raw substance for the manufacturing of steroid drugs. In order to evaluate the nutritional effect of different nitrogen (0, 50, 100, 150 and 200 kg ha^{-1}) and copper (0, 10, 20 and 30 kg ha^{-1}) doses on the diosgenin content, growth and yield of fenugreek (var. Ardestan), an experiment was carried out in a factorial arrangement based on complete randomized block design. The diosgenin content in the plant was monitored by a high performance liquid chromatography. Lower doses of copper increased yield, yield component and diosgenin content, but its higher level (30 kg ha^{-1}) had negative effects on the plant yield. The simultaneous application of nitrogen and copper ameliorate the diosgenin production, yield and yield component. The result obtained from correlation and stepwise regression analysis showed that traits such as leaf area index, pods number per plant, and dry weight have significantly direct relation to diosgenin production levels in plants, while dry weight has the highest effect on diosgenin production compared to other traits. According the results, we also can conclude that nitrogen application can decrease copper toxicity in fenugreek in the contaminated soils.

Key words: diosgenin, copper, fenugreek, nitrogen, yield

INTRODUCTION

Copper is one of the essential micronutrient, required for several different physiological and biochemical pathways in plants as well as mammalian nutrition [Stern et al. 2007]. Its deficiency has an adverse effect on human being [Stern et al. 2007]. In fact, copper is one of the essential nutrient required for secondary metabolite production, but its higher concentration cause some pr oblems in soil and restrict plant growth and development [Panou Filotheou et al. 2001]. Copper through fertilization, pesticides, municipal wastewater and industrial wastewater may contaminate soils. However, it has been reported to be a complex interaction between nitrogen and copper in legumes [Marschner 2012]. Marschner [2012] determined that the toxicity of copper could be decreased as nitrogen supply increased.

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Nitrogen is an essential element for plant growth and reproduction, which is a component of chlorophyll and thus necessary for photosynthesis [Alburquerque et al. 2013, Britto et al. 2014]. It is also the basic element of plant and animal proteins, including the genetic material DNA and RNA, and is important in periods of rapid plant growth. Previous studies indicated that nitrogen applications increased the number of pods per plant, seeds per pod and 1000 seed weight in fenugreek [Tunçtürk et al. 2011]. However, high levels of these elements, copper and nitrogen might have toxic effects on plant growth [Britto et al. 2014].

Fenugreek belongs to the Fabaceae family; its leaves and seeds are used as a source of health in Iran and used as a spice. It has three main components such as steroidal sapogenins, galactomanans and isoleucine, which has pharmacological character [Aradhana et al. 1992]. The presence of saponins has been reported in most species. Diosgenin belongs to the steroidal saponin group which is the starting material of industrial interest in the synthesis of many steroidal drugs. Fenugreek with its easy cultivation has considered as a source for diosgenin production [Sautour et al. 2007]. However, the plants yield depends on environmental condition such as abiotic and biotic factors [Berova et al. 2000, Nurzyńska-Wierdak et al. 2015, Shams et al. 2016, Sorkheh et al. 2016]. The abiotic factors such as copper, methyl jasmonate (MeJA) and ethylene can influence the diosgenin production in plants [Narula et al. 2005, Diarra et al. 2013, Chaudhary et al. 2015].

Copper is one of the factors in the production of secondary metabolites and performance of the plants. High levels of this element in plants can be toxic [Roy et al. 2016], but nitrogen has an important role in reducing the toxicity of copper in the fenugreek. A review of literature reveals that there is no much investigation on copper acclimation of fenugreek treated with nitrogen fertilization. Also there is little knowledge in the field of copper and nitrogen interaction on fenugreek yield and steroidal sapogenin production. Therefore, the purpose of this study was to evaluate the effect of different rates of nitrogen and copper on the growth and yield of fenugreek and the diosgenin production.

MATERIALS AND METHODS

Chemicals and materials. Acetonitrile (HPLC grade, Merck, Darmstadt, Germany) and deionized water purified through a Millipore system (Milford, Connecticut, USA) were used. All other reagents were of analytical grade and used without further purification. Diosgenin was purchased from Sigma (Buchs, Switzerland). Seeds of fenugreek (Ardestan variety) were obtained from the Seed and Plant Improvement Institute (SPII) of Karaj, Iran.

Field experiments. Field experiment was carried out at the research station of Zanjan University, Zanjan, Iran, in 2009 and 2010. Soil was sandy loam and contain 1.65% organic matter, 0.08% of total nitrogen, 29 mg kg^{-1} of P_2O_5, 285 mg kg^{-1} of K_2O, 0.26 ppm of Cu, 20% of $CaCO_3$, 1.72 ppm of Mn, 4 ppm of Zn, while soil saturation extract (ECe) had value 1.01 dS m^{-1} and pH value was 7.66 on top soil.

Seeds were sown at 10^{th} of April 2009 and 2010 in 2 × 3 m plots and the plant density was about 50 plant m^{-2}. The Sprinkled irrigations were supplied regularly and the weeding operation was conducted manually. Treatments included nitrogen application (N1 = 0, N2 = 50, N3 = 100, N4 = 150 and N5 = 200 kg ha^{-1} of Urea 46% N Fertilizer) and copper (Cu1 = 0, Cu2 = 10, Cu3 = 20 and Cu4 = 30 kg ha^{-1} of Copper Sulfate ($CuSO_4 \cdot 5H_2O$)), both mixed with top soil before seed planting.

Data collection and analysis. The experiment was conducted in a factorial arrangement based on a complete randomized block design with three replicates for each treatment. In order to diminish margin effect, samples were selected randomly from the middle of the plots. Sampling was performed in five different growth stage (25, 45 and 65 days old plants, full bloom and pod ripening) to determine and calculate dry matter content, leaf area index and diosgenin. The seed number per pod and pods number per plant were measured at full maturity. The Pearson correlation and stepwise regression analysis were used to determine important characteristics affecting (independent variables) diosgenin production rate (dependent variable). Statistical analysis was carried out by SPSS version 19 at $P > 0.05$, and the mean separation was done following Duncan's Multiple Range Test.

Extraction and determination of diosgenin. The diosgenin content of leaves and fruits was measured by the methods of [Saxena et al. 1996, Shams et al. 2013]. HPLC analyses were performed on a Knauer (Berlin, Germany) HPLC system equipped with a Wellchrom K-1001 pump, a Wellchrom K-2600 UV-Visible detector and a Nucleosil RP C18 column (125 mm length, 4 mm internal diameter and 5 µm particle size, Knauer, Berlin, Germany).

RESULTS

Growth, yield and yield component. The result showed that the separately application of copper at third level (Cu3, 20 kg h^{-1} copper sulfate) and nitrogen at third level (N3, 100 kg h^{-1} urea) had the highest effect on increasing LAI, dry weight, number of pods per plant and number of seeds per pod (tabs 1, 2). Copper at forth level (Cu4, 30 kg h^{-1} copper sulfate) had a negative effect on yield and yield component. However, simultaneous application of Cu4 (30 kg h^{-1} copper sulfate) and N3 (100 kg h^{-1} urea) had the most significance effect on the investigated characters rather than control (tab. 4). The copper had no significant effect on 1000 seed weight (tab. 2)

The results obtained from our study showed that as the nitrogen level increased in the soil (up to 100 kg h^{-1} urea) the number of pods per plant, seed number per pod and 1000 seeds weight increased, but high application of nitrogen in the soil (150 and 200 kg h^{-1} urea) decreased them (tab. 2).

According to the regression analysis, dry weight (independent variable) had the greatest effect on diosgenin production (dependent variable), and their relation was direct (tab. 5). This means that increasing the plant growth and dry matter accumulation can ameliorate the diosgenin production of in plants.

Diosgenin. The increasing of concentrations of Cu^{2+} in the media ameliorated the accumulation of diosgenin, but upper levels of Cu (30 kg h^{-1} copper sulfate) caused a decline in the production of diosgenin in fenugreek (tab. 1).

Nitrogen application had a significant effect on the production rate of diosgenin. As nitrogen concentration in culture medium increased, production rate of diosgenin enhanced. Moreover, nitrogen at third level (N3, 100 kg ha^{-1} urea) had a significant effect on diosgenin and increased its content up to 32 percent compared to control (tab. 1). Higher concentrations of N (150 and 200 kg h^{-1} urea) in the media decreased diosgenin production.

Simultaneous application of nitrogen at third level (N3, 100 kg ha^{-1} urea) and copper at forth level (Cu4, 30 kg ha^{-1} $CuSO_4$) had the highest effect on diosgenin in 45-day old leaves and improved its content up to 88 percent compared to control group (tab. 3).

There was a significant correlation between diosgenin production and dry weight (tab. 5), but the correlation between diosgenin production and leaf area index, 1000 seed weight, number of pods per plant was not significant. The Results of stepwise regression analysis showed that among all independent parameters, dry weight had a direct relation and the highest effect on diosgenin production.

DISCUSSION

Growth, yield and yield component. Our findings pointed out that lower levels of Cu increased dry matter, but upper levels of Cu decreased dry matter accumulation due to its toxicity effect. This may be due to preferable inhibitory effects of Cu on leaf elongation [Alaoui Sossé et al. 2004]. This result confirms previous findings about toxicity effects of Cu in cucumber Alaoui Sossé et al. [2004] and beans [Elleuch et al. 2013]. On the other hand, dry weight decline is attributed to the effects of copper and iron antagonist eventually, which causes chlorophyll decomposition and reducing plant total photosynthesis [Marschner 2012]. By increasing the copper concentration (70 µm) in the Murashige and Skoog medium in *Dioscorea bulbifera* L. the dry weight decreased [Narula et al. 2005].

Increasing leaf area index, number of pods per plant, seed number per pod and 1000-seeds weight by nitrogen application at optimum level (N3) is consistent with the findings of [Mehta et al. 2010], but its higher concentration had a negative effect. High levels of nitrogen enhanced vegetative growth, which inhibited light penetration into the canopy and

Table 1. Effects of nitrogen and copper nutrients on diosgenin production in 25, 45 and 65 day old leaves (DOL), leaves at flowering stage (LFS) and in fruits of fenugreek. Data followed by a different letter were significantly different ($P \leq 0.05$) according to the Duncan Multiple Range Test. DOL (day old leaves), FS (flowering stage) and SRS (seed ripening stage). There were no significant differences between 2009 and 2010

Treatment		Dry matter yield (g m^{-2})					Diosgenin content (µg g^{-1}) dry weight				
		25 DOL	45 DOL	65 DOL	FS	SRS	25 DOL	45 DOL	65 DOL	FS	SRS
Nitrogen levels (kg ha^{-1})	N1 = 0	27 E	76 D	166 C	413 D	382.5 D	6833 D	8070 D	7106 D	5673 D	3613 D
	N2 = 50	31 D	80.5 C	171 B	442.5 C	397 C	9610 C	12530 C	10326 C	7593 C	4730 C
	N3 = 100	34 B	84.5 A	184 A	488.5 A	437.5 A	13043 B	14483 B	13746 A	13230 A	7020 A
	N4 = 150	34 B	82 B	171.5 B	457.5 B	422 B	13096 AB	14596 AB	12690 B	7953 B	4853 B
	N5 = 200	38 A	77 D	164.5D	402 E	377.5 E	13166 A	8103 D	7093 D	5693 D	4710 D
Copper levels (kg ha^{-1})	Cu1 = 0	27 C	76 C	166 C	413 C	382.5 C	6833 D	8070 D	7106 D	5673 D	3613 D
	Cu2 = 10	31 B	84 B	183 B	453.5 B	437 B	11443 C	15760 C	11850 C	9096 C	5270 C
	Cu3 = 20	34 A	86.5 A	187 A	643 A	554 A	18523 A	29803 A	22696 A	20790 A	10430 A
	Cu4 = 30	31.5 B	84 B	167 C	408D	381 D	13056 B	23563 B	20213 B	16270 B	5946 B

Table 2. Effects of copper and Nitrogen nutrients on leaf area index, pods $plant^{-1}$, seeds pod^{-1}, 1000 seed weight of fenugreek. Data followed by a different letter were significantly different ($P \leq 0.05$) according to the Duncan Multiple Range Test. DOP (day old plants), FS (flowering stage) and SRS (seed ripening stage). There were no significant differences between 2009 and 2010

Treatment		Leaf area index					Pods plant	Seeds pod	1000 seed weight (g)
		25 DOP	45 DOP	65 DOP	FS	SRS			
Nitrogen level (kg ha^{-1})	N1 = 0	0.102 D	0.402 D	0.611 D	1.12 D	0.388 D	10 C	13 C	16.34 C
	N2 = 50	0.125 C	0.426 C	0.631 B	1.22 B	0.428 B	12 B	15 B	17.75 B
	N3 = 100	0.146 B	0.438 A	0.635 A	1.26 A	0.440 A	15 A	17 A	18.84 A
	N4 = 150	0.147 A	0.434 B	0.631 B	1.23 B	0.428 B	13 B	15 B	17.76 B
	N5 = 200	0.146 B	0.428 C	0.614 C	1.15 C	0.385 C	11C	13 C	16.22 C
Copper level (kg ha^{-1})	Cu1 = 0	0.102 D	0.402 D	0.611 D	1.12 C	0.388 C	10 C	13 C	17.34 A
	Cu2 = 10	0.113 C	0.418 B	0.624 B	1.15 B	0.427 B	12 B	15 A	17.84 A
	Cu3 = 20	0.127 A	0.433 A	0.675 A	1.25 A	0.467 A	14 A	16 A	17.14 AB
	Cu4 = 30	0.118 B	0.406 C	0.621 C	1.07 D	0.428 B	11 C	13 C	17.33 A

Table 3. Effects of copper and nitrogen interaction on total diosgenin content and dry matter of fenugreek. Data followed by a different letter were significantly different ($P \leq 0.05$) according to the Duncan Multiple Range Test. DOP (day old plants), DOL (day old leaves), FS (flowering stage) and SRS (seed ripening stage). There were no significant differences between 2009 and 2010

Nitrogen level (kg ha^{-1})	Copper level (kg. ha^{-1})	Dry mater yield (g m^{-2})					Diosgenin content (µg g^{-1}) dry weight				
		25 DOP	45 DOP	65 DOP	FS	SRS	25 DOL	45 DOL	65 DOL	FS	SRS
N1 = 0	Cu1 = 0	27 G	76 G	166 IJ	413 MN	382.5 N	6833 N	8070 O	7106 N	5673 Q	3613 N
	Cu2 = 10	31 FG	84 CDEF	183 EF	453.5 K	437 J	11443 L	15760 L	11850 K	9096 N	5270 K
	Cu3 = 20	34 EFG	86.5 CDE	187 DE	643 C	554 D	18523 G	29803 F	22696 C	20790 D	10430 D
	Cu4 = 30	31.5 FG	84 CDEF	167 IJ	408 N	381 N	13043 J	23563 H	20213 E	16270 H	5946 J
N2 = 50	Cu1 = 0	31 FG	80.5 EFG	171 HI	442.5 L	397 L	9610 M	12530 N	10326 M	7593 P	4730 M
	Cu2 = 10	35.5 DEF	80 DEFG	180 EFG	503 I	439.5 J	11800 K	17023 K	12733 J	12453 J	7146 G
	Cu3 = 20	36.5 CDEF	88 CD	190.5 CD	643.5 C	573 C	20466 F	31080 D	17710 G	21366 C	10686 C
	Cu4 = 30	37 CDEF	87.5 CD	191.5 CD	566 H	525 F	17333 H	27333 G	21360 D	17740 F	6990 G
N3 = 100	Cu1 = 0	34 EFG	84.5 CDE	184 EF	488.5 J	437.5 J	13056 J	14596 M	13746 I	13230 I	7020 G
	Cu2 = 10	39 CDE	88 CD	193.5 C	606.5 F	534 E	15600 I	23610 H	19343 F	17853 F	10116 E
	Cu3 = 20	39.5 BCDE	98.5 B	210.5 B	687.5 B	678.5 B	25443 C	34633 C	27540 B	23593 B	12376 B
	Cu4 = 30	41.5 BCD	103 A	270.5 A	762 A	706.5 A	26156 B	64966 A	41100 A	34213 A	14623 A
N4 = 150	Cu1 = 0	34 EFG	81.5 DEFG	171.5 HI	457.5 K	422 K	13096 J	14596 M	12690 J	7953 O	4883 L
	Cu2 = 10	41.5 BCD	81.5 DEFG	174.5 GH	577 G	459 I	15590 I	22850 I	17046 H	11640 K	5946 J
	Cu3 = 20	42.5 C	87.5 CD	182.5 EF	613.5 E	483.5 G	25033 D	30760 E	20180 E	17120 G	7010 G
	Cu4 = 30	57 A	89 C	186 DE	635 D	472 H	27243 A	42560 B	22773 C	18586 E	8560 F
N5 = 200	Cu1 = 0	35.5 CDEF	77.5 FG	164.5 J	402 O	377.5 N	13166 J	8103 O	7093 N	5693 Q	4710 M
	Cu2 = 10	39 CDE	80 EFG	178 FG	411 MN	381.5 N	15576 I	15793 L	10340 M	9073 N	6203 I
	Cu3 = 20	42.5 C	81.5 DEFG	179 FG	414.5 M	389 M	24310 E	21426 J	11716 L	10846 M	6303 HI
	Cu4 = 30	48 AB	82.5 CDEFG	179 FG	414 M	389 M	26133 B	22780 I	11940 K	11130 L	6323 HI

Table 4. Effect of copper and nitrogen interaction on leaf area index, pods plant^{-1}, seeds pod^{-1}, and seed 1000 weight (g) of fenugreek. Data followed by a different letter were significantly different ($P \leq 0.05$) according to the Duncan Multiple Range Test. DOP (day old plants), FS (flowering stage) and SRS (seed ripening stage). There were no significant differences between 2009 and 2010

Nitrogen level (kg ha^{-1})	Copper level (kg ha^{-1})	Leaf area index					Pods/plant	Seeds/pod	Seed 1000 weight (g)
		25 DOP	45 DOP	65 DOP	FS	SRS			
N1 = 0	Cu1 = 0	0.102 N	0.402 Q	0.611 N	1.12 L	0.388 K	10 J	13 G	16.34 EFG
	Cu2 = 10	0.113 M	0.418 O	0.624 K	1.15 K	0.427 J	12 HI	15 EF	17.84 EFG
	Cu3 = 20	0.127 J	0.433 L	0.675 C	1.25 G	0.467 D	14 FG	16 DE	17.14 EFG
	Cu4 = 30	0.118 L	0.406 P	0.621 L	1.07 N	0.428 J	11 IJ	13 G	17.33 EFG
N2 = 50	Cu1 = 0	0.125 K	0.426 N	0.631 I	1.22 H	0.428 J	12 HI	15 EF	17.75 EFG
	Cu2 = 10	0.142 I	0.437 JK	0.642 G	1.28 E	0.452 H	14 FG	17 D	18.13 CDE
	Cu3 = 20	0.154 G	0.455 G	0.654 F	1.32 CD	0.464 E	16 DE	20 C	19.42 CB
	Cu4 = 30	0.168 D	0.468 C	0.662 D	1.12 M	0.454 G	18 BC	14 FG	19.15 CBD
N3 = 100	Cu1 = 0	0.146 H	0.438 J	0.635 IJ	1.26 FG	0.44 I	15 EF	17 D	18.84 CD
	Cu2 = 10	0.163 F	0.466 C	0.657 E	1.33 C	0.473 C	17 CD	19 C	18.72 CD
	Cu3 = 20	0.177 C	0.482 B	0.688 B	1.39 B	0.477 B	19 B	23 B	20.18 B
	Cu4 = 30	0.185 B	0.515 A	0.722 A	1.43 A	0.483 A	22 A	25 A	23.72 A
N4 = 150	Cu1 = 0	0.147 H	0.434 K	0.631 J	1.23 H	0.428 J	13 GH	15 EF	17.76 EFG
	Cu2 = 10	0.166 E	0.457 F	0.641 GH	1.27 EF	0.451 H	15 EF	17 D	17.14 FGH
	Cu3 = 20	0.178 C	0.464 E	0.655 F	1.32 CD	0.462 F	17 CD	17 D	18.15 CDE
	Cu4 = 30	0.196 A	0.483 B	0.661 D	1.31 D	0.465 E	17 CD	19 C	18.14 CDE
N5 = 200	Cu1 = 0	0.146 H	0.428 M	0.614 M	1.15 K	0.385 M	11 IJ	13 G	16.22 H
	Cu2 = 10	0.167 DE	0.433 L	0.623 K	1.18 I	0.384 M	11 IJ	13 G	16.7 GH
	Cu3 = 20	0.177 C	0.448 H	0.64 H	1.17 IJ	0.391 L	13 GH	15 EF	17.72 EFG
	Cu4 = 30	0.184 B	0.446 I	0.641 GH	1.16 JK	0.392 L	13 GH	15 EF	17.71 EFG

Table 5. Stepwise regression analysis between diosgenin production (dependent variable) and dry matter and pod. plant^{-1} (independent variable) at final model

Model	Unstandardized coefficients		Standardized coefficient	t	Sig.
	B	Std. error	Beta		
Constant	-16.63	1.77	–	-9.03	0.00
Dry matter	4.34	0.68	0.65	6.34	0.00
Pod	0.53	0.20	0.27	2.63	0.01

therefore reduced photosynthesis rate. As a result, lower leaves become wilted, lose their chlorophylls and fall down, therefore, leaf area index and the yield capacity of plants is decreased [Mehta et al. 2010].

The interaction of copper with high concentrations of nitrogen (30 kg h^{-1} copper sulfate and 100 kg h^{-1} urea) increased the growth, yield and yield component. It is due to Cu^{+2} accumulation in fenugreek Rhizobium's and reduce the toxicity of copper in plant.

Increase in the number of pods in fenugreek is attributed to Cu active role in flower formation and avoiding flower abortion. Copper at its optimum level, by increasing carbohydrate content in plants, prevent flower abortion and pods number per plant [Marschner 2012].

Diosgenin. According to the findings of our study, there was a direct relation between dry matter production and diosgenin production. This means that the diosgenin production would increase in plants by means of the accumulation of dry matter. Similarly, the secondary metabolic production in ginger was increased by the accumulation of dry matter [Ghasemzadeh et al. 2011]. There was an evident relationship between carbohydrates production and dry matter accumulation in plants [Ghasemzadeh and Jaafar 2011].

Dry matter accumulation occurred at the same time with the carbohydrate production of plants. Carbohydrates are among the constituents of pyruvic acid cycle and are the basic components of diosgenin production in plants [Veeresham et al. 2004].

Copper is required for several biochemical and physiological pathways and thus is considered essential for carbohydrate production and plant growth [Veeresham and Kokate 2004]. Therefore, increasing accumulation of $CuSO_4$ in media enhanced the diosgenin production.

Higher concentrations of Cu^{2+} in the media inhibited the formation of diosgenin in fenugreek. Our results reflect the impact of copper on diosgenin production in okra (*Hibiscus esculenthus*) reported by [Narula et al. 2005]. Due to complex interaction between nitrogen and copper in legumes, the simultaneous application of higher level of copper and nitrogen, improved steroidal sapogenins production.

Higher concentrations of NO_3 in the media supported the increased accumulation of diosgenin in vitro cultures of fenugreek [Ravishankar et al. 1991]. In contrast, in this study higher concentration of nitrogen at field conditions, along with increased leaf area index (Cell size expansion and not significant effect on dry matter accumulation) declined diosgenin production.

CONCLUSIONS

High copper amount in tissue culture surveys has been shown to induce lower yields and secondary metabolite accumulation. On the contrary, in field cases with situation respect to the extreme demand of root to Cu for nitrogen-fixing, nodules partly reduced copper toxicity so copper had no significant effect on yield decline. As the dry weight of plant increased, secondary metabolite production elevated, it is due to the increase of carbohydrate accumulation. It is necessary to conduct further researches to evaluate the effects of other nutrients on the yield and production of secondary metabolites in plants.

ACKNOWLEDGEMENTS

The authors acknowledge the Institute for Advanced Studies in Basic Science and Zanjan University for the financial supports. In addition, Dr. Behzad Nikkhah from Biology Department of Tehran University are gratefully acknowledged for their helpful guidance.

REFERENCES

Alaoui Sossé, B., Genet, P., Vinit Dunand, F., Toussaint, M.L., Epron, D., Badot, P.M. (2004). Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. Plant Sci., 166, 1213–1218.

Alburquerque, J.A., Salazar, P., Barrón, V., Torrent, J., Del Campillo, M.D., Gallardo, A., Villar, R. (2013). Enhanced wheat yield by biochar addition under different mineral fertilization levels. Agron. Sustain. Dev., 33, 475–484.

Aradhana, Rao, A.R., Kale, R.K. (1992). Diosgenin a growth stimulator of mammary gland of ovariectomized mouse. Indian J. Exp. Biol., 30, 367–370.

Berova, M., Zlatev, Z. (2000). Physiological response and yield of paclobutrazol treated tomato plants (*Lycopersicon esculentum* Mill.). Plant Growth Regul., 30, 117–123.

Britto, D.T., Balkos, K.D., Becker, A., Coskun, D., Huynh, W.Q., Kronzucker, H.J. (2014). Potassium and nitrogen poising: physiological changes and biomass gains in rice and barley. Can. J. Plant Sci., 94, 1085–1089.

Chaudhary, S., Chikara, S.K., Sharma M.C., Chaudhary, A., Alam Syed, B., Chaudhary, P.S., Mehta, A., Patel, M., Ghosh, A., Iriti, M. (2015). Elicitation of diosgenin production in *Trigonella foenum-graecum* (Fenugreek) seedlings by Methyl Jasmonate. Int. J. Mol. Sci., 16, 29889–29899.

Diarra, S.T., He, J., Wang, J., Li, J. (2013). Ethylene treatment improves diosgenin accumulation in *in vitro* cultures of *Dioscorea zingiberensis* via upregulation of CAS and HMGR gene expression. Electron. J. Biotechnol., 16(5). DOI: 10.2225/vol16-issue5-fulltext-9.

Elleuch, A., Chaâbene, Z., Grubb, D.C., Drira, N., Mejdoub, H., Khemakhem, B. (2013). Morphological and biochemical behavior of fenugreek (*Trigonella foenum-graecum*) under copper stress. Ecotoxicol. Environ. Saf., 98, 46–53.

Ghasemzadeh, A., Jaafar, H. (2011). Effect of CO_2 enrichment on synthesis of some primary and secondary metabolites in ginger (*Zingiber officinale Roscoe*). Int. J. Mol. Sci., 12, 1101–1114.

Marschner, H. (2012). Mineral Nutrition of Higher Plants. Academic Press, San Diego.

Mehta, R.S., Patel, B.S., Meena, S.S., Meena, R.S. (2010). Influence of nitrogen, phosphorus and bio-fertilizers on growth characters and yield of fenugreek (*Trigonella foenum-graecum* L.). J. Spices Aromat. Crops, 19, 23–28.

Narula, A., Kumar, S., Srivastava, P.S. (2005). Abiotic metal stress enhances diosgenin yield in *Dioscorea bulbifera* L. cultures. Plant Cell Rep., 24, 250–254.

Nurzyńska-Wierdak, R., Zawiślak, G., Kowalski, R. (2015). The Content and composition of essential oil of *Origanum majorana* L. grown in Poland depending on harvest tme and method of raw material preparation. J. Essent. Oil-Bear. Plants, 18, 1482–1489.

Panou Filotheou, H., Bosabalidis, A.M., Karataglis, S. (2001). Effects of copper toxicity on leaves of oregano (*Origanum vulgare* subsp. hirtum). Ann. Bot., 88, 207–214.

Ravishankar, G.A., Grewal, S. (1991). Development of media for growth of *Dioscorea Deltoidea* cells and in vitro diosgenin production: Influence of media constituents and nutrient stress. Biotechnol. Lett., 13, 125–130.

Roy, S.K., Kwon, S.J., Cho, S.-W., Kamal, A.H.M., Kim, S.-W., Sarker, K., Oh, M.-W., Lee M.-S., Chung, K.-Y., Xin, Z., Woo, S.-H. (2016). Leaf proteome characterization in the context of physiological and morphological changes in response to copper stress in sorghum. Bio Metals, 1–19.

Sautour, M., Mitaine-Offer, A.-C., Lacaille-Dubois. M.-A. (2007). The Dioscorea genus: a review of bioactive steroid saponins. J. Nat. Med., 61, 91–101.

Saxena, A., Singh, D.V., Joshi, N.L. (1996). Autotoxic effects of pearl millet aqueous extracts on seed germination and seedling growth. J. Arid Environ., 33, 255–260.

Shams, M., Yildirim, E., Ekinci, M., Turan, M., Dursun, A., Parlakova, F., Kul, R. (2016). Exogenously applied glycine betaine regulates some chemical characteristics and antioxidative defence systems in lettuce under salt stress. Hortic. Environ. Biotechnol., 57, 225–231.

Shams, M., Haghighi, B., Abbassi Niasar, M., Zandi Esfahan, E. (2013). Effect of copper and nitrogen nutrients on diosgenin production in fenugreek. Arch. Agron. Soil Sci., 60, 1115–1124.

Sorkheh, K., Amirbakhtiar, N., Ercisli, S. (2016). Potential Start Codon Targeted (SCoT) and Inter-retrotransposon Amplified Polymorphism (IRAP) markers for evaluation of genetic diversity and conservation of wild pistacia species population. Biochem. Genet., 54(4), 368–387.

Stern, B.R., Solioz, M., Krewski, D., Aggett, P., Aw, T.C., Baker, S., Crump, K., Dourson, M., et al. (2007). Copper and human health: biochemistry, genetics, and strategies for modeling dose-response relationships. J. Toxicol. Environ. Health, Part B, 10, 157–222.

Tunçtürk, R., Esen Çelen, A., Tunçtürk, M. (2011). The effects of nitrogen and sulphur fertilizers on the yeild and quality of fenugreek (*Trigonella foenum-graecum* L.). Turk. J. Field Crops, 16, 69–75.

Veeresham, C., Kokate, C.K. (2004). Medicinal plant biotechnology. CBS Publishers & Distributors, New Delhi.