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Expression analysis of diosgenin pathway genes and diosgenin accumulation in fenugreek sprouts after exposure to copper sulfate

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ABSTRACT

Trigonella foenum-graecum L. is an annual herb belonging to the family Fabaceae commonly called Fenugreek. It is rich in various secondary metabolites such as alkaloids, flavonoids, phenolic compounds, and steroidal saponins. In recent years, diosgenin has much attention in the cosmetic, functional food, and pharmaceutical industries. In this study we aimed to examine the effect of different concentrations of copper sulfate ($CuSO_4$) on growth, diosgenin biosynthetic (DB) gene expression, and diosgenin accumulation in *T. foenum-graecum* sprouts. Results showed that the seed germination, fresh weight, shoot length, and root length were gradually decreased with increasing the $CuSO_4$ concentrations. In contrast, the expression level of DBGs i.e., *TfSQS*, *TfSQLE*, *TfCAS*, and *TfSTRL* were gradually upregulated with increasing the $CuSO_4$ concentrations. Among all those tested concentrations, the expression levels of all those genes were significantly higher in 0.5 mM $CuSO_4$ treated sprouts. The highest expression level was obtained in the *TfCAS* gene, which was 3.25-fold higher than the unexposed sprouts. The diosgenin content was significantly influenced in the $CuSO_4$ exposed sprouts. The highest diosgenin content was achieved in the 5.0 mM followed by 1.0, 10.0, and 0.5 mM $CuSO_4$ exposed concentrations, with a reduction of 41%, 39%, 36%, and 35%, respectively. From these results, it is shown that exposure of fenugreek sprout to $CuSO_4$ is one of the suitable strategies to enhance the accumulation of diosgenin content.

KEYWORDS: $CuSO_4$, Diosgenin, Fenugreek sprouts, Gene expression

INTRODUCTION

Trigonella foenum-graecum L. (Fabaceae), is an annual herb and it is otherwise known as Fenugreek (Snehlata and Payal, 2012). Fenugreek contains various secondary metabolites, such as alkaloids, flavonoids, phenolic compounds, steroidal saponins, and other compounds (Laila and Murtaza, 2015; Wani and Kumar, 2016). The major bioactive chemical compound present in fenugreek is diosgenin, which is structurally similar to cholesterol and other steroids (Rahimzadeh *et al.*, 2011).

Diosgenin belongs to the group of triterpenes. It is a naturally occurring phytosteroid sapogenin present in *Dioscorea* species (yams), fenugreek, and *Costus speciosus* (Kumar *et al.*, 2014; Liu *et al.*, 2012). Diosgenin is mainly used in the pharmaceutical industry as a key precursor for the synthesis of many different active ingredients, including, immunomodulatory effectors, lipid metabolism facilitators, anti-carcinogenic, neuroprotective, glucose-lowering, anti-inflammatory, anti-oxidant, and anti-diabetes agents. In addition, it also acts as a cardiovascular protector, cholesterol absorption suppressor, estrogenic, skin protective, and male fertility promoter (Raju and Bird, 2007; Son *et al.*, 2007; Chen *et al.*, 2015; Mischitelli *et al.*, 2016).

Heavy metals are naturally occurring elements. Some are biologically important, such as Fe, Mo, and Mn; in contrast, many others are toxic even at low concentrations -although useful in a narrow concentration range, i.e., Zn, Ni, Cu, V, and Co; still, others can be lethal even in trace amounts, such as Ag, As, Cd, Hg, Pb, and Sb. However, the biological function of these

heavy metals were not been studied well (Schutzendubel, 2002). At optimal concentrations, these heavy metals have beneficial functions in plant growth and development, senescence, energy-generating processes, and productivity (Shah *et al.*, 2010; Arif *et al.*, 2016) while, they can severely inhibit photosynthesis metabolism and many other physiological processes in plants (Siedlecka, 1995). Some heavy metals induced the production and accumulation of secondary metabolites, such as phenolic compounds and glucosinolates, and increased amino acids as well (Mourato *et al.*, 2015). Copper (Cu) is a micronutrient for plants that is an essential component of the photosynthetic machinery. A slight excess of Cu in the growing medium though, induced stress and caused injury to plants. This heavy metal can regulate growth retardation, leaf chlorosis, and oxidative stress (Nagajyoti *et al.*, 2010).

In this study, we investigated the different growth parameters of fenugreek sprouts after being exposed to copper sulfate ($CuSO_4$). In addition, we analyzed the DB gene expression and diosgenin content by using quantitative real-time PCR (qRT-PCR) and high-performance liquid chromatography (HPLC), respectively.

MATERIALS AND METHODS

Plant Materials

The seeds of *T. foenum-graecum* L. were sown in plastic pots filled with vermiculite and kept in a growth chamber under a 16 h photoperiod (300 µmol/m^2s) at 25°C, 50 seeds were sown in each pot.

Copper Sulfate Treatment of Fenugreek Sprouts

Different concentrations of $CuSO_4$ (0, 0.5, 1.0, 5.0, and 10.0 Mm) were prepared and daily poured equal amounts into each pot. All sprouts were harvested 10 days after seed germination. Seed germination rate, fresh weight, shoot length, and root length were recorded and then, sprouts were harvested and immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

RNA Isolation and cDNA Synthesis

Total RNA was isolated by using a Plant Total Mini Kit (Generaid Taiwan) according to the manufacturer's instructions. Trizol reagent was used to homogenize cells and then ice-cold chloroform: isopropanol was added for precipitating the RNA. Concentration and purity of RNA were measured by using NanoVue Plus spectrophotometer (GE Healthcare Bio-Science Crop, USA) and RNA integrity was checked by agarose gel electrophoresis. The cDNA was converted by using a ReverTra Ace-α kit (Toyobo, Japan) according to the manufacturer's instructions. The resulting cDNA template was used for qRT-PCR analysis.

Real-time PCR Analysis

Primers for squalene synthase (*SQS*), squalene monooxygenase (*SQLE*), and cycloartenol synthase (*CAS*) were designed using the free online software Primer 3 http://bioinfo.ut.ee/primer3-0.4.0/). Sterol-3-ß-glucosyl transferase (*STRL*) primer was obtained from the transcriptome data of the GujaratMethi-1 variety (Chaudhary *et al.*, 2015). As a housekeeping gene, the 18S gene was used (Table 1). qRT-PCR was performed in a BIO-RAD CFX96 Real-time PCR system (Bio-Rad Laboratories, USA) and the conditions were as follows: pre-denaturation for 15 s at 95°C, followed by 20 s at 95°C; annealing for 40 s at 55°C followed by 40 cycles and elongation for 20 s at 72°C. The final extension was done at 72°C for 10 min.

HPLC Analysis

A hundred milligrams of freeze-dried were mixed with 5 ml of 20% H_2SO_4 containing 70% isopropanol and incubated in a water bath at 80°C for 8 h. Every 30 min the samples were vortexed vigorously. After incubation, the mixer was centrifuged at 12,000 rpm for 10 min at 4°C. The resulting supernatant was collected into a new falcon tube and added 5 ml n-hexane and vortexed vigorously. The mixer was centrifuged at 12,000 rpm for 10 min at 4°C. The resulting supernatant was collected in a new falcon tube and this step was repeated thrice. Extracts were then evaporated at 40°C by using a Rotovac evaporator. Dried crude extracts were mixed with 1 ml acetonitrile and filtered by using a 0.45 µm PTFE syringe filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for HPLC analysis. The diosgenin content was analyzed by using an Agilent HPLC system 1100 series coupled with a C_{18} reverse-phase column (250 mm × 4.6 mm, 5 µm; RStech, Daejeon, Korea) and detected at 203 nm with a UV detector. Solvent A consisted of water and solvent B consisted of acetonitrile. The initial ratio of the mixer was 10% of solvent A and 90% of solvent B with a flow rate of 1.0 ml/min. The identification and quantification of diosgenin content were done by matching with the retention time and corresponding calibration curve of the standard.

Statistical Analysis

All experiments were done in triplicate for each treatment. The results were analyzed using IBM SPPS Statistic V24. The

Table 1: Real-time PCR primers used in this study

Genes	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)
Tf18S rRNA	GTCTCAACCATAAACGATGCCGACCA	ACCTGGTAAGTTTCCCCGTGTTGAGT
TfSQS	GGACTTGTTGGGCTGGGTTT	GTGGCCAAAACATGCGTGAC
TfSQLE	TGCTGGTATTGCTGGTGCTG	CCACCGGGTTGTAGCAACTC
TfCAS	TCCCAACTCCGGATCTCCAC	CGGTATCACTTCGCCCATCG
TfSTRL	TGGAAAATGAGGATGGGGTA	TTGCATTGGATCAGGAACAA

analyses were done by using Tukey's Multiple Range Test using one-way ANOVA at the 5% significance level.

RESULT AND DISCUSSION

Effect of $CuSO_4$ on Growth Parameters

The effects of different concentrations of $CuSO_4$ (0, 0.5, 1, 5, and 10 mM) on the growth of 10-days-old *T. foenum-graecum* sprouts are presented in Figure 1. The results indicated that all the growth parameters such as seed germination, fresh weight, shoot length, and root length were gradually decreased with increasing the $CuSO_4$ concentrations. Among the different parameters, the germination rate was markedly decreased with the reduction of 81.67, 73.33, 66.67, and 63.33 at 0.5, 1.0, 5.0, and 10 mM $CuSO_4$ concentrations, respectively (Figure 2A). The fresh weight of $CuSO_4$ exposed sprouts ranges between 0.14-0.06 g. The highest fresh weight was obtained at the 0.5 mM $CuSO_4$ exposed sprout, whereas the lowest was achieved at the 10mM $CuSO_4$ exposed sprout (Figure 2B). The shoot and root lengths results showed a similar trend as that of the germination rate and fresh weight results. The shoot length of $CuSO_4$ exposed sprouts ranges between 6.26-4.26 cm. A higher level of shoot length was found in 0.5 mM, followed by 1.0, and 5.0 mM with 6.26, 5.56, and 4.72 cm, respectively. The root length showed a slight difference in all the $CuSO_4$ exposed sprouts, the highest root length was achieved in the 0.5 mM $CuSO_4$ concentration (1.64 cm), whereas the lowest was obtained in 10 mM $CuSO_4$ concentration (0.3 cm) (Figure 2C). In all the growth parameters, the control sprout (not exposed to $CuSO_4$) showed the highest growth rate when compared to the $CuSO_4$ treated sprouts. Similar results were obtained in several plants (*Arabidopsis thaliana*, *Artemisia annua*, *Limoniastrum monopetalum*, *Nicotiana plumbaginifolia*) that increase in the Cu concentrations leads to a decrease in the growth parameters (Chipeng *et al.*, 2010; Lequeux *et al.*, 2010; Cambrollé *et al.*, 2013; Saghirzadeh Darki *et al.*, 2019). This might be due to the exposure of plants to the highest Cu concentration can

Figure 1: Fenugreek sprout grown under different concentrations $CuSO_4$

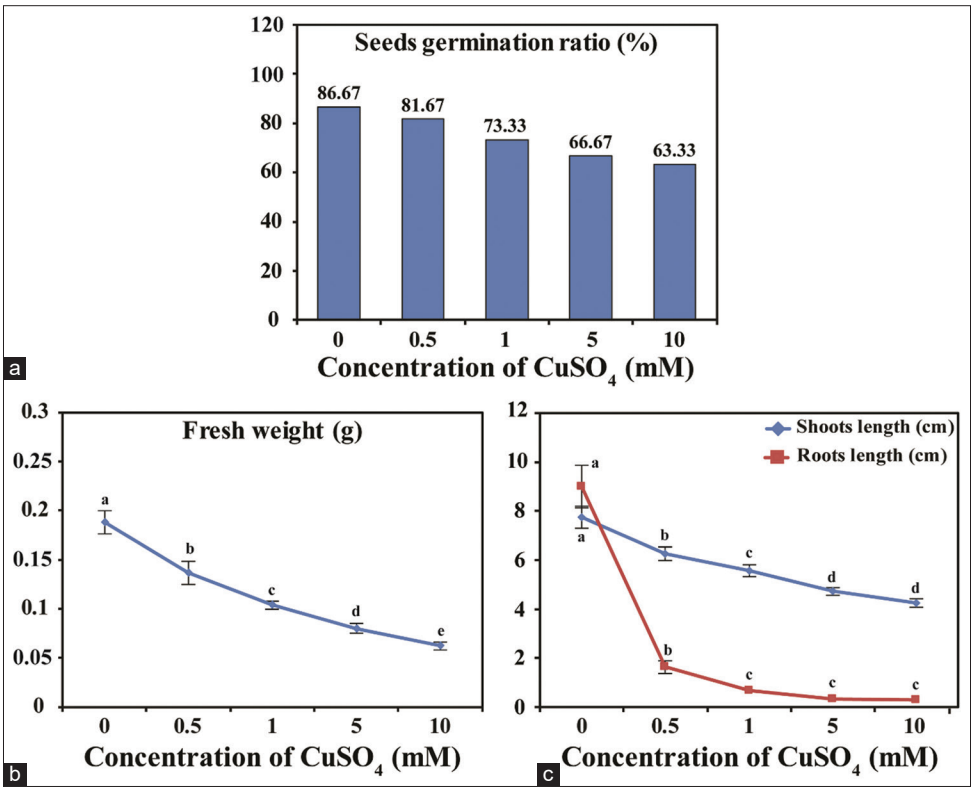

Figure 2: (a) Seed germination ratio (%), (b) Fresh weight, and (c) Shoot and root length of fenugreek sprouts exposed to various concentrations of $CuSO_4$ [0 (control)], 0.5, 1, 5, and 10 mM) ($n = 3$). Different letters indicate that significantly different from each other at $P \leq 0.05$ according to Tukey's Multiple Range Test.

cause damage to the photochemical apparatus which leads to the reduction in the photosynthetic carbon assimilation. From these results, it is shown that the $CuSO_4$ has a significant effect on the growth parameters of fenugreek sprouts.

Effect of $CuSO_4$ on Gene Expression

The results of qRT-PCR showed that the DB genes were highly expressed in the $CuSO_4$ treated sprout when compared to that of the control. But the expression levels were different based on the $CuSO_4$ concentrations (Figure 3). Among all those tested genes, the highest gene expression was achieved in *TfCAS* followed by *TfSQS*, *TfSQLE*, and *TfSTRL*. The expression level of *TfSQS* and *TfSQLE* were highly expressed with increasing the $CuSO_4$ concentration and then gradually decreased at the highest $CuSO_4$ concentration (10 mM). This result supports the growth parameters result of this study. The expression patterns of both *TfCAS* and *TfSTRL* were different than that of *TfSQS* and *TfSQLE*. The *TfSQS* expression was increased up to 1 mM and then gradually decreased in 5 and 10 mM $CuSO_4$ treated sprout. The expression level of *TfSQS* was elevated in 1 mM, which was 1.10-, 1.10-, and 2.04- times higher than that in the 0.5, 5.0, and 10.0 mM $CuSO_4$ exposed sprout. In addition, the *TfSQLE* were highly upregulated in 5 mM CuSO4 treated sprouts when compared to 0.5, 1.0, and 10.0 mM $CuSO_4$ exposed sprouts. The *TfCAS* and *TfSTRL* showed a similar expression pattern, at 0.5 mM $CuSO_4$ concentration the expression level was increased and then decreased at 1 mM $CuSO_4$ concentration, thereafter revived at 5 mM, and then the expression level was decreased sharply at 10 mM $CuSO_4$ concentration. In both genes, the highest gene expression was achieved at 0.5 mM, whereas the lowest was achieved in the 10 mM $CuSO_4$ exposed sprout. The expression level of *TfCAS* and *TfSTRL* were highest at 0.5 mM, which was 3.25- and 1.15- times higher than that in the control (Figure 3). The expression level of all those tested genes showed the lowest expression in 10 mM $CuSO_4$ exposed sprouts. Similar results were obtained in the marine green algae *Ditylum brightwellii* (Guo *et al.*, 2013), *Montastraea franksi* (Venn *et al.*, 2009), *Prorocentrum minimum* (Guo *et al.*, 2012), and *Tetraselmis suecica* (Sathasivam *et al.*, 2018; Sathasivam and Ki, 2019a, b) that expression level of carotenoid pathway genes and heat shock protein genes were considerably increased with increasing the Cu concentration and then gradually decreased at the highest Cu concentration. From these results, it is shown that the $CuSO_4$ significantly induces the DB pathway gene expression in fenugreek sprouts.

Effect of $CuSO_4$ Treatments on Diosgenin Accumulation

Diosgenin content was quantified from *T. foenum-graecum* sprouts after exposure to different $CuSO_4$ concentrations (Figure 4). The highest level of diosgenin content was detected in the 5 mM $CuSO_4$ treatment followed by 1.0, 10.0, and 0.5 mM $CuSO_4$ treatment, which was 41%, 39%, 36%, and 35% higher than that in the control, respectively. This result indicates that a low concentration of $CuSO_4$ treatment is the most suitable concentration to enhance the diosgenin content. A similar result was obtained in the *Dioscorea bulbifera* at the lowest copper concentration the diosgenin content was increased (Narula *et al.*, 2005). In addition, it has been reported that Cu also enhances the betalains and artemisinin production in *Beta vulgaris* and *Artemisia annua*, respectively (Trejo-Tapia *et al.*, 2001; Saghirzadeh Darki *et al.*, 2019). From these results, it is shown that Cu enhances the secondary metabolite content in plants.

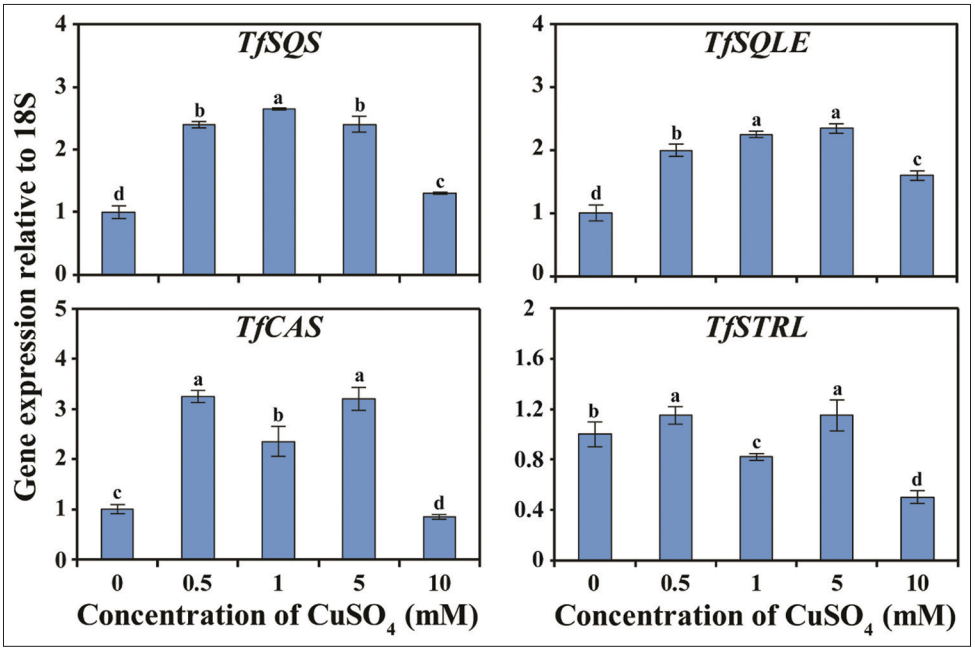

Figure 3: The expression level of DB genes in fenugreek sprouts treated with various concentrations (0 (control), 0.5, 1, 5, and 10 mM) of $CuSO_4$. Different letters indicate that significantly different from each other at $P \leq 0.05$ according to Tukey's Multiple Range Test.

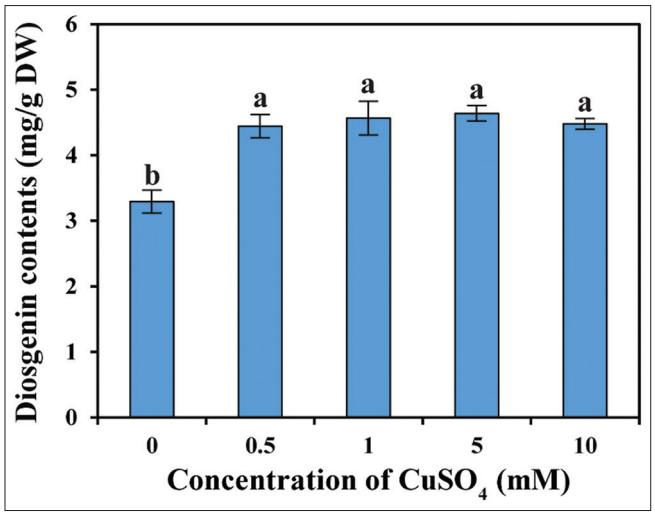

Figure 4: Diosgenin content (mg/g DW) in the 10-day-old fenugreek sprouts treated with different concentrations of $CuSO_4$ (0, 0.5, 1, 5, and 10 Mm) ($n = 3$). Different letters indicate that significantly different from each other at $P \leq 0.05$ according to Tukey's Multiple Range Test.

CONCLUSIONS

In the pharmaceutical industry, diosgenin is the key precursor compound for the synthesis of several synthetic steroidal drugs. It is a promising bioactive compound showing various biological properties, like anti-inflammatory, antioxidant, antiproliferative, hypoglycaemic, and hypolipidemic activities. Fenugreek sprouts treated with $CuSO_4$ showed decreased germination rate, fresh weight, shoot length, and root length. However, this treatment enhanced the upregulation of DB genes and increase the diosgenin content in the $CuSO_4$ treated fenugreek sprouts. It is shown that the highest level of diosgenin accumulation might be due to the upregulation of the DB genes, which agreement with the result of numerous studies. From this study, it is suggested that exposure of fenugreek sprout to $CuSO_4$ is one of the suitable methods for increasing the yield of diosgenin content.

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AUTHOR'S CONTRIBUTIONS

S.U.P. and B.B.P. designed the experiments. D.Y.K., R.S., Y.B.K., H.-H.K., performed the experiments and analyzed the data. D.Y.K. and R.S. wrote the manuscript. S.U.P. and B.B.P revised the manuscript. All authors read and approved the final manuscript.

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