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Copper-Induced changes in the expression of *Dihydroflavonol-4-reductase* (*DFR*) and its role in anthocyanin production in *Plantago ovata* Forsk.

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Abstract: Copper is one of the most important micronutrients of plant but enhanced accumulation of it can cause toxic effects on plant. Dihydroflavonol-4-reductase plays pivotal role in anthocyanin production via phenylpropanoid and anthocyanin biosynthesis pathway. This present study is focused on elucidating the importance of Dihydroflavonol-4-reductase in production of anthocyanin and also its role in withstanding copper induced toxicity in *Plantago ovata*. Hence, this study describes how copper stress induces higher expression of *DFR* and thereby increases the anthocyanin production to withstand stress. Partial cds of *DFR* gene was isolated, sequenced and its expression pattern was studied under copper stress. Anthocyanin content was measured to get an idea about the effect of copper toxicity on its accumulation. Copper in plant cells is kept at lower concentrations because at higher concentrations it acts as a cytotoxic stress factor. Correlation between induction of *DFR* expression and anthocyanin synthesis to protect plant against copper induced oxidative stress was observed. This study would enable to understand the genetic regulation of *DFR* induction due to copper toxicity. The up regulation of its expression under copper stress confirms its important role in anthocyanin production. This study also depicts the protective role of anthocyanin in plants against copper stress.

Keywords: Anthocyanin, Antioxidant, Dihydroflavonol-4-reductase, Reverse transcription, Real-time PCR.

INTRODUCTION

Anthocyanins are a group of secondary metabolites which not only confer protection against environmental stress but also help to overcome and adapt with the changing environment[1]. Anthocyanin has diverse biological functions in plant and it attracts insects, birds and other pollinators for pollination and also provides protection against oxidative damage[2, 3].Anthocyanins are mainly synthesized via phenylpropanoid pathways[4]. Dihydroflavonol-4-reductase (DFR) is a pivotal enzyme for anthocyanin production[5].Anthocyaninsare water soluble pigments whose colours depend[6] on pH. Due to its crucial role in anthocyanin production it has been intensively studied in various plants[5, 7, 8]. Apart from that its transcriptional regulation has also been documented in several studies[6, 9], recently even in *Arabidopsis thaliana*[10]. Abiotic stress inducing factors not only play important role in induction of *DFR* expression but also enhance production of anthocyanin[11-13]. In some recent studies a direct relationship between up regulation of DFR expression and higher accumulation of anthocyanin had been documented[14]. Several studies have demonstrated that deactivation or lesser expression of the *DFR* gene results in the loss of anthocyanin accumulation in mutants of barley and Arabidopsis[15, 16]. Studies have reported that anthocyaninshave the ability to scavenge active oxygen radicals[17] due to its antioxidant activity. Anthocyanin as antioxidant protects plants under stressful conditions and also heals cytotoxic injury caused by copper ion[18]. In this present investigation the relation between *DFR* induction under copper stress and anthocyanin production was investigated.

Plantago ovata Forsk (Psyllium or Isabgol) is a winter annual medicinal herb which belongs to the family Plantaginaceae. It grows in arid and semi- arid sandy temperate regions of western India, mainly in the states of Gujarat and Rajasthan[19]. Its natural habitat is in the Mediterranean region of southern Europe and North Africa, Middle East, central Asia and southern Russia[20]. Excess of metals in the soil affects plant metabolism and plants have evolved different mechanisms to cope with it. Copper (Cu) is an essential plant micronutrient so it plays an important role in plant growth and in other physiological processes. Being a redox-active metal excess accumulation of Cu generates reactive oxygen species (ROS) [21]. In a study on *Hedera helix* L. it was shown that lack of *DFR* activity limits the production and accumulation of anthocyanin in plant tissue[22].

The main aim of the present research was, therefore, to determine *DFR* expression and anthocyanin accumulation in the roots and shoots of *P. ovata* seedlings exposed to low (5uM) to high (50 uM) doses of copper in the media. This study was conducted on one hand to examine the effect of sub lethal doses of copper on *DFR* expression and on the other hand to detect and quantify anthocyanin in metal-treated root and shoot tissue to establish its function in tolerance to metal stress.

MATERIALS AND METHODS

Plant materials and treatments: Seeds of *P. ovate* [(GI-2) Gujarat India-1] were procured from Gujarat Agricultural University, Anand, Gujarat, India. The seeds were surface sterilized with10% (v/v) commercial bleach (NaOCl) and germinated on agar-sucrose medium, containing 3% (w/v) sucrose

(Sisco Research Laboratory [SRL], Mumbai, India) and 0.9% (w/v) agar (SRL, Mumbai, India). In case of copper treatment 5, 10, 20 and 50 µ M solution of copper chloride ($CuCl_2$) (SRL, Mumbai, India) was added to the agar-sucrose medium. The seedlings were maintained for twelve days at standard laboratory conditions (temperature 22°C – 25°C, relative humidity 55 – 60% and illumination at 1500 Lux for 16/8 h duration of light/dark photoperiods). LD_{50}, the dose at which half survival is found to be 80µ M for $CuCl_2$ and therefore the above-mentioned doses were lesser than predetermined LD_{50}.

Determination of total anthocyanin content: Total anthocyanin content was determined by the method described by Zhang and Quantik[23]with minor modifications. 1 g of plant tissue was crushed in 5 ml 1% HCl- methanol solution and then filtered. The filtrate was diluted with 1% HCl-methanol solution to 10 ml. Absorbance was taken at 600 nm and 515 nm. Total anthocyanin content was expressed as the change of 0.1 unit difference between the absorbance at 530 nm and 600 nm.

Primer designing: The primers for *P. ovata DFR* (*PoDFR*) were designed from the published sequences in NCBI (National Centre for Biotechnology Information) databank. Reported sequences of DFR amino acids were aligned using ClustalW multiple sequence alignment tool and conserved domains were identified. Specific primers for *PoDFR* were designed from the conserved regions using primer3 software (Version 4.0).

Genomic DNA extraction and polymerase chain reaction: Genomic DNA was extracted from the seedlings by the method of Edward *et al.*,[24]. Amplification of *DFR* gene was performed by PCR using gene specific primers (**Table 1).** PCR reactions were carried out with a hot start at 94 ° C for 5 min. The reaction condition for *DFR* is shown in **Table 2**

Table1: Details of primers used for *DFR* sequencing

Gene	Forward primer (5´- 3´)	Reverse primer (5´- 3´)
DFR	GAGAATGAAGTGATCAAGCC 20 mer, Tm=49.7°C, GC content- 45%	GTGATTAAGCTAGGTGGGA 19 mer, Tm=49°C, GC content- 47%

Table 2: PCR conditions for *DFR*

Gene	Denaturation	Annealing	Elongation	Final extension	No. of Cycles
DFR	94°C for 1 min	50°C for 1 min	72°C for 1min 30 sec	72°C for 10 min	35

***PoDFR* expression analysis by Reverse transcription PCR and densitometry:** The relative expression pattern of *PoDFR* at different doses of copper treatment was determined densitometrically from the band intensity of the agarose gel. Total RNA was extracted from root and shoots treated with different doses of

copper in the culture media and the RNA samples were subjected to reverse transcription PCR using specific primer pair RTDF/RTDR. The thermal cycler conditions were as follows: reverse transcription at 50°C for 30 minutes followed by 40 cycles of amplification (95°C for 15 minutes, 94°C for 1minute, 60°C for 1 minute, 72°C for 1 minute 30 seconds) and a final extension at 72°C for 10 minutes. Densitometric analysis was performed with ImageJ software. Expression profile was normalized against *P. ovata* β-actin gene. Data was represented as mean ± SEM. Statistical significance was performed by one way ANOVA using Kyplot software. Group means were compared by Student's *t* test. Differences of the data at $P \leq 0.05$ were considered significant.

***DFR* expression analysis by real-time PCR:** The relative expression of *DFR* transcript at untreated and different treated conditions was measured using Real-Time PCR in a Step One plus Real-Time PCR thermocycler (Applied Biosystems, CA, and USA. Primers for Real-Time PCR of *DFR*, *ACTIN* were designed using primer express software (Version 3.0) and are listed in **Table 3**.

Table 3: Primers used for reverse transcription PCR and Real Time PCR

Primers	Forward primer (5´- 3´)	Reverse primer (5´- 3´)
DFR	RTDF TGCCAAAAGCGGATACAAACT 21 mer, Tm- 51°C, GC content- 43%	RTDR CGATTCGCGATTGCTGGAT 21mer, Tm=54°C, GC content- 52%
ACTIN	RTACTF CACGAGACCACCTACAACTCG 21 mer, Tm=56.3°C, GC content-57.1%	RTACTR CAACCTTAATCTTCATGCTGCTC 23mer, Tm=53.5°C, GC content- 44%

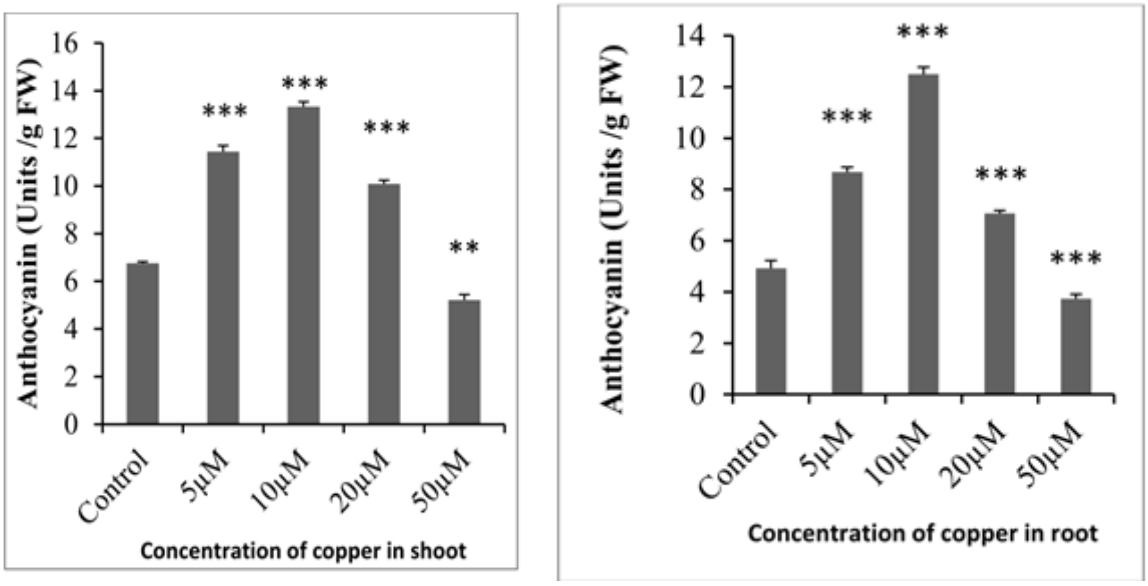
The thermal condition of the Real time PCR was as follows: 95 °C for 10 min (1 cycle) followed by 40 cycles at 95 °C for 30 s, annealing and elongation at 60 °C for 1 min. The gene encoding *Plantago* β-actin was used as endogenous control. The relative expression of *DFR* transcript was normalized with *P. ovata* β-actin gene expression in each sample using actin gene specific primers **(Table 3).**

The relative expression pattern of *DFR* was calculated using the $2^{-\Delta\Delta CT}$ method[25]. The results of relative gene expression were analyzed by Step One software (Version 2.1) (Applied Biosystems, CA, USA). All the experiments were performed in triplicate and calibrated to the expression in a 12day old seedling tissue. A negative control was maintained in each reaction. The results presented here are means of triplicates, and mean ± SEM was indicated by the bars. KyPlot software was used for analysis of statistical significance of the data by analysis of variance (ANOVA) using.

RESULT AND DISCUSSION

Total anthocyanin content: Anthocyanin content was also found to increase in low doses of copper treated (5 and 10 µM) samples. Anthocyanin content in untreated control was 6.76 units /g FW in shoot

and 4.92 units /g FW in root. Anthocyanin accumulation was higher in 5 µM copper treated samples which was 11.44 units /g FW and 8.92 units /g FW in shoots and roots respectively. Highest amount of anthocyanin accumulation was found in 10 µM copper treated seedlings. At higher (20 and 50 µM) copper treatment anthocyanin production decreased in both root and shoot tissue. The pattern of anthocyanin production showed that at higher concentration of copper treatment the total anthocyanin content decreased in both shoot and root **(Fig 1a and Fig 1b).**The results showed that there was higher anthocyanin accumulation in shoot as compared to the root tissue.



(a) **(b)**

Figure 1: Total anthocyanin content in *P. ovata* treated with different copper concentration.

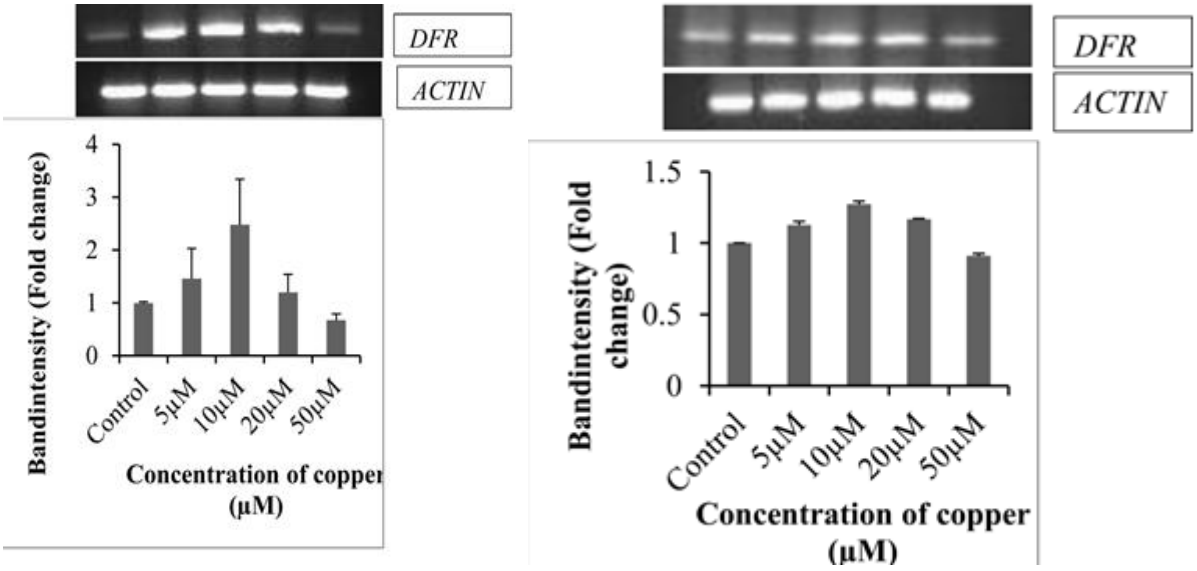
(a) Anthocyanin content in shoot at different copper treated concentrations;
(b)Anthocyanin content in root at different copper treated concentrations;

Data are represented as mean ± standard error. Asterisks indicate significance levels: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$(***).

Expression of *Plantago* DFR gene: A 639 bp fragment of DFR gene was obtained (Accession No. KU668976). *DFR* gene expression was found in both root and shoot; however, the pattern of expression was different. The expression level was determined from the relative band intensity of agarose gel. In case of copper treated samples, in shoot *DFR* was found to be expressed in an inducible manner but in root it showed a lower induction. Expression was found to increase 1.14fold in 5 µM copper treated roots than the control. Accumulation of *DFR* transcript was maximum (1.3fold) in 10 µM treated root sample and then declined (1.18 fold) in 20 µM treated sample and also further decreased and lesser then control in case of higher (50 µM) copper treated root samples **(Fig 2b).**

In case of shoot the change in expression was comparatively higher. In case of shoot, *DFR* gene expression was found to be increased greatly in 10 µM as well as 5 µM $CuCl_2$ treated samples as

compared to control. Expression increased 1.5 fold in 5 µM copper treated shoot and it further increased 2.5 fold in 10 µM copper treated shoot **(Fig 2a).** At higher doses there was a decline in *DFR* expression in copper treated shoot tissue.



(a) **(b)**

Fig 2a: Gel photograph of *DFR* expression in shoots treated with different copper concentrations and Graphical representation of band intensity represents the change in expression of *DFR* in shoots treated with different copper concentrations.

Fig 2b: Gel photograph of *DFR* expression in roots treated with different copper concentrations and Graphical representation of band intensity represents the change in expression of *DFR* in roots treated with different copper concentrations.

Expression analysis of *Plantago DFR* gene by Real time PCR: In order to precisely analyze the accumulation of *DFR* transcript, Real Time PCR was performed on samples treated with different concentrations of copper (Control untreated, 5, 10, 20 and 50 µM). Results of the real time PCR results showed the accumulation of *DFR* transcript in all the samples. However, expression of *DFR* was relatively low in untreated shoot and root samples.

Expression increased significantly in 5 µM copper treated roots which was 1.5-fold higher than the control. Accumulation of *DFR* transcript was maximum (3.2 fold) in 10 µM copper treated roots and then it declined (1.1 fold) in 20 µm copper treated root sample. Expression of *DFR* further decreased and became lesser then control in case of higher (50 µm) copper treated samples **(Fig 3a).**In case of shoots a similar kind of pattern was obtained but degree of change in expression was higher than that of roots. The results of real-time PCR clearly indicated stress induced accumulation of *DFR* transcript at lower doses. At lower doses (5 and 10 µM) the expression of *DFR* significantly increased (1.3 and 4.8 fold respectively) with respect to control but at higher dose (50 µM) a sharp decrease in expression has been observed **(Fig 3b).** Highest expression was observed in 10 μM treated sample both in shoot and roots.

Several studies have shown that polyphenols and anthocyanin are induced by stress and accumulate in different tissues due to various abiotic and biotic stresses. The importance of DFR in anthocyanin production can be understood from the fact that low expression of *DFR* results in complete absence or extremely low anthocyanin production[26].

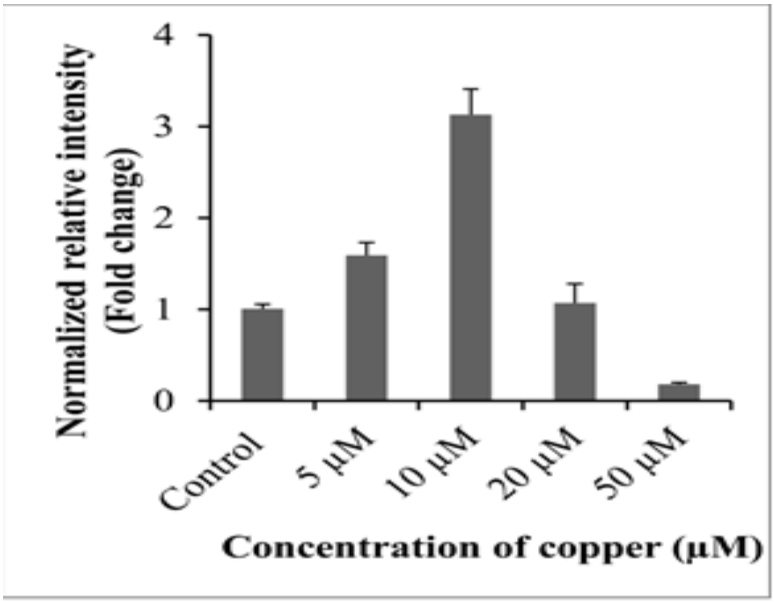


Fig 3a: Real Time PCR expression profile of *DFR* transcript in roots treated with different copper concentrations. Results are represented as the mean ± SEM.

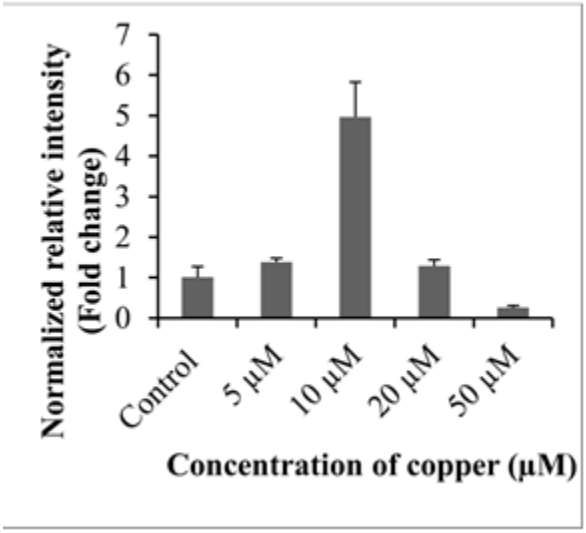


Fig 3b: Real Time PCR expression profile of *DFR* transcript in shoots treated with different copper concentrations. Results are represented as the mean ± SEM.

Transcriptional regulation is one of the main controlling steps of secondary metabolite pathway[27, 28]. These metabolites may also participate in ROS scavenging through the activation of different antioxidative enzymes. Plants have evolved defense system against oxidative stress by enhancing the activity of ROS scavenging enzymes[29]. In a study on potato it was observed that anthocyanin production can be increased almost 4 fold by the over expression[30] of *DFR*. The partial sequence of a *DFR* gene from *P. ovata* was determined in the present study. High *DFR* activity is very crucial in producing high amount of anthocyanin which gives the plant the ability to quench ROS, produced due to metal toxicity.In a study on rice the correlation between higher *DFR* expression under ABA, excess salt stress and enhanced production of anthocyanin was established[31].Our results showed an increase in *DFR*activity and anthocyanin accumulation mainly in shoots and comparatively lower in rots treated with low Copper concentration (5 and 10 µM). It was further observed that the effect was more prominent in the shoot tissues compared to that of Root. Studies showed that the increase of anthocyanin content is one of the ways to improve tolerance of plant to oxidative stress caused by higher salt concentration[32]. In a study on effect of copper stress in red cabbage seedlings it was observed that the negative effect of oxidative stress was reduced by higher production of anthocyanin[18]. Accumulation of anthocyanin increased in the low dose of copper treatment (5 and 10µM) which is in accordance with the increased *DFR* expression in those samples. A recent study on aluminium and boron stress a relation between metal stress and phenolic and anthocyanin metabolism had been established[33]. In a study on *Azolla imbricata* it was observed that cadmium (Cd) treatment significantly enhanced the expression of *DFR* suggesting that anthocyanin biosynthesis is regulated by the up regulation of *DFR* gene[34].

Similarly the present article showed that DFR gene up regulation lead to anthocyanin accumulation following copper stress. Anthocyanin plays an important role in strengthening antioxidative defense system against metal induced oxidative stress. Therefore, lower dosage of copper treatment in *P. ovata* showed an interesting pattern. Concentrations up to 10 µM of $CuCl_2$ resulted in gradual increase of anthocyanin accumulation as well as enhanced *DFR* expression which decreased when the dose was increased further. In a recent study on *Camellia sinensis* var. *assamica* it was observed that the expression of anthocyanin biosynthetic genes such as *DFR* can be regulated by the induction of various transcriptions factors and hence induces higher anthocyanin accumulation[35]. A similar mechanism may be conjectured to occur in plants treated with heavy metal stress. Similarly in a study onQuanhong Poplar lower cadmium treatment induced anthocyanin biosynthesis[36].

Our study shows at lower copper concentration there is a significant increase in *DFR* activity and total anthocyanin accumulation. Strong redox properties of Cu enable it to produce higher amount of ROS. Therefore, plants enhance phenolic metabolism, flavonoid, and anthocyanin production and related *DFR* activity aimed to reduce the toxic effects of metals and their uptake into shoot and root tissue. This can be supported by our observation that 10 µM Cu led to more than two fold higher anthocyanin accumulation in shoots and significant increase in roots as well as higher DFR expression. Altogether these findings may help us to find out the mechanism by which plants adapt themselves against copper toxicity by enhancing the production of anthocyanin mainly in shoots by primarily increasing *DFR* expression. The results evidenced in this article would provide better insight into the regulatory aspects of *DFR* induction and expression for making plant cells competent to synthesize higher amount of anthocyanin to withstand copper stress in *Plantago ovata.*

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

All the experiments have been conducted by Dr. Pratik Talukder in consultation with the guide Prof. Sarmistha Sen Raychaudhuri. The manuscript has been written based on the results of this experiment.

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