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OPTOMETRIC, PHYTOCHEMICAL AND GENE EXPRESSION ANALYSIS OF ERGASTIC CRYSTAL LOAD IN PLANT PARTS OF PURPLE AMARANTH (*Amaranthus cruentus* L.)

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ABSTRACT

The present work was a trio-experimental approach focused on the omics-based gene expression pattern for ergastic crystal formation in vegetative parts of purple amaranth (*Amaranthus cruentus***) and compared with physio-chemical estimation methods and optometric image analysis. Plant samples, root (S1), stem (S2), mature leaf (S3), young leaf (S4), insect attacked leaf (S5), senescent leaf (S6) were analysed. The gene expression analysis was carried out using qRT-PCR for two oxalate synthesizing genes SoGLO and SoOXAC, and two oxalate degrading genes SoOXO and SoOXDC and the area occupied by calcium oxalate crystals were plotted with the help of IMAGE J and ZEN 4 software in optometric device and the same was quantified using chemical analysis method. Transcriptional level analysis of both sets of gene pointed to the regulation of oxalate levels by complex pathways and could help in generating oxalate-less hybrids in leafy vegetables. Total oxalate was estimated to be 20% more in S6 than S1, and ranged from 25.0-50.2 mg 100 g^{-1} sample which falls under high to very high oxalate permissible content in foods. Area occupied by druse crystals was maximum in S5, followed by S6 and least was found in S1 ranging from 0.0013-0.0284 µm $µm^{-2}$ sample. Gene expression of SoGLO gene was comparable to the total oxalate content; whereas the area occupied by CaOx crystals showed variations owing to the presence of soluble oxalate content in it.**

Keywords: *Amaranthus cruentus*, druses, ergastic crystals, gene expression, image analysis, oxalate load, qRT-PCR.

INTRODUCTION

The existence of ergastic crystals such as calcium oxalate, calcium carbonate and calcium phosphate in various plant parts was a snag when its levels were elevated in food. Lersten and Horner (2006) reported the presence of calcium oxalate crystals in 215 families of higher plants including angiosperms and gymnosperms. Though in earlier times they were considered as waste products, but now are attributed to be involved in many physiological functions in plants (Tooulakou *et al.*, 2016). Among the ergastic crystals, calcium oxalate can be crucial as the oxalate has anti-nutrient effect in causing renal issues when consumed in higher quantities (Nguyen and Savage, 2013). The permissible amount of dietary oxalate has been standardized to 40-50 mg day^{-1} by American Dietetic Association for a normal healthy person (2005). About 70% renal stones were of calcium oxalate origin (Vijaya *et al.*, 2013). The prolonged and excessive consumption of starfruit (*Averrhoa carambola*) caused oxalate poisoning, leading to the renal failure because of its extremely high oxalate content (Abeysekera *et al.*, 2015). The case studies on Angus cows and sheep reported rapid livestock deaths

due to oxalo-nephropathy after grazing a weed *Halogeton glomeratus* which contained 30% oxalate to its dry weight (Rood *et al.*, 2014) revealing the fatal effect of anti-nutrients present in plants. Many vegetables like spinach, rhubarb, amaranth, purslane, parsley, soybean, etc. induced high oxalate levels when consumed raw (Hesse and Honow, 2002).

Purple amaranth (*Amaranthus cruentus* L.) is the most popular, easily available and economical leafy vegetable consumed by common masses in India. High nutrition value of amaranths (Nascimento *et al.*, 2014) and its anti-oxidant properties due to β-cyanin pigments has made it a preferred leafy vegetable around the globe. Purple amaranth was used as a natural source of iron, calcium and antioxidant (Tyszka-Czocharam *et al.*, 2016) and is considered as an antimicrobial agent (Al-Mamun *et al.*, 2016). Ghosh and Savage (2013) reported very high oxalate load in Indian spinach dishes. The antinutrient oxalate load in different vegetative parts of purple amaranth is scantily studied. The study on physical, biochemical and gene expression analysis of purple amaranth was to enunciate the regulation of oxalate metabolism in the plant. The study was expected to fill the gap in oxalate gene expression levels to its actual gene product in various parts of purple amaranth consumed as leafy vegetable.

MATERIALS AND METHODS

The purple amaranth sample series were selected at several biotic potential-based on a detailed pilot study conducted at field level for 9 months (March-November, 2021) and The plant samples/parts were labelled as root (S1), stem (S2), mature leaf (S3), young leaf (S4), insect attacked leaf (S5), and senescent leaf (S6). All the chemical and biochemical studies were carried out in the Research Lab at Union Christian College, Aluva, India. Voucher herbarium specimen from the original collection of *Amaranthus cruentus* UCCH 512 was deposited at the Botanical Survey of India, Southern Circle, India under Accession No: MH 178183.

Gene expression analysis

The total RNA extraction kit (RNA iso Plus, TaKARa Bio, Shiga, Japan) was used for rapid purification of RNA from all the samples. Qubit®4.0 fluorometer was used to quantify the RNA yield. Thermo Scientific Revert Aid First Strand cDNA synthesis kit was used for cDNA synthesis. Gene expression of oxalate synthesizing genes SoGLO (glycolate oxidase), SoOXAC (oxaloacetate acetyl hydrolase) and oxalate degrading genes SoOXO (oxalate oxidase) and SoOXDC (oxalate decarboxylase) were carried out in samples.

Estimation of total oxalate

The estimation of oxalate load was done by permanganometric titration with standardized $KMnO_4$ as per AOAC (2016) norms. To extract the total oxalate from sample, 1 g homogenized sample was added to 30 mL of 0.5 N sulphuric acid and boiled in water bath for 15 min. The extract was filtered through Whatman filter paper No. 1 and equal volume of deionized water added to prepare the sample extracts. Oxalate ions are extracted from the plant parts by boiling them with 0.5 N sulphuric acid. The oxalate concentration was estimated volumetrically by titrating the extract with standard 0.05N $KMnO_4$ solution.

$$MnO_4^- + + 5C_2O_4 + 8H^+ \rightarrow Mn_2^+ + 10CO_2 + 4\,H_2O$$

Sample extract (1 mL) was added to 40 mL dil. H_2SO_4 in a conical flask and titrated against standard $KMnO_4$ (0.05N) until pink colour appeared and stayed for at least 15 sec. The end point was noted and the amount of oxalate present in mg sample^{-1} estimated stoichiometrically (AOAC, 2016).

Image analysis

Image J and ZEN 4 software were used for image analysis in purple amaranth samples. Microscopic preparations were digitally photographed by Axiocam attachment on Carl Zeiss Primostar

microscope. All CaOx crystals were measured under 10 X magnification microscopic field. Mean area occupied by the crystals were calculated using IMAGE J software. One-way analysis of variance was performed. The physical area occupied by druse crystals in unit area of the sample vegetative parts were analysed (Schindelin *et al.*, 2012).

RESULTS AND DISCUSSION

Transcriptional level analysis of genes was compared with total oxalate load in purple amaranth by permanganometric titration for quantification. The surface area data on oxalate crystals in microscopic field was recorded for S1 to S6 study samples with ZEN 4 and IMAGE J software. The amount of oxalate in food consumed determined the chances of kidney stone formation in human (Mitchell *et al.*, 2019).

Gene expression analysis

In gene expression analysis, root was considered as control because it's TO value was least, and the rest vegetative samples were compared with it for fold induction of gene. Both oxalate synthesizing and degrading genes worked in tandem to regulate oxalate metabolism in plants (Nakata and He, 2010). Nakata (2012) reported techniques to create transgenic plants with reduced oxalate content by regulating SoGLO genes. Fold induction of two oxalate synthesizing genes (SoGLO and SoOXAC) (Fig. 1 and 2) and two oxalate degrading genes (SoOXO and SoOXDC) (Fig.3 and 4) were analysed in different plant part samples.

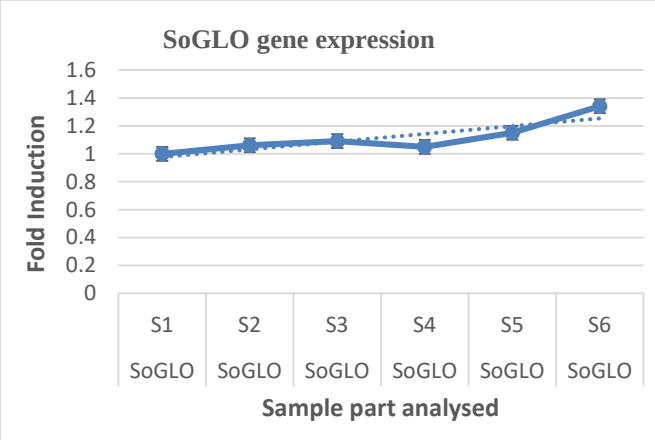

Fig. 1: SoGLO gene expression in samples S1 to S6 of *Amaranthus cruentus* S1 - root, S2 - stem, S3 - mature leaf, S4 - young leaf, S5 - insect attacked leaf, and S6 - senescent leaf.

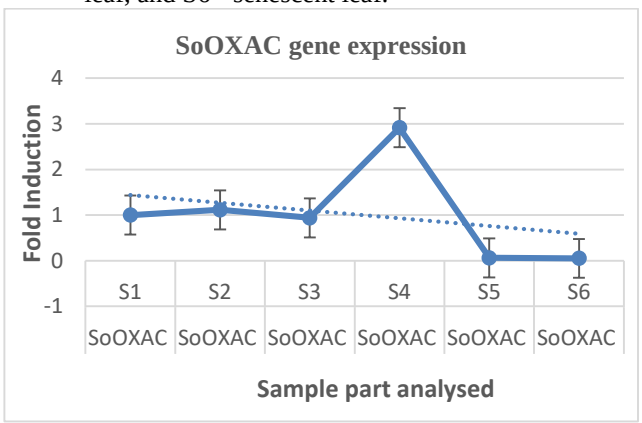

Fig. 2: SoOXAC gene expression in samples S1 to S6 of *Amaranthus cruentus* S1 - root, S2 - stem, S3 - mature leaf, S4 - young leaf, S5 - insect attacked leaf, and S6 - senescent leaf.

The oxalate synthesizing SoGLO gene showed maximum fold induction in senescent leaves and minimum in root thus pointing to the differential level of gene expression in vegetative parts of the plant. This was in agreement with the report of Joshi *et al.* (2021) that the presence of differentially expressed genes (DEGs) regulated oxalate metabolism in spinach. Oxalate synthesizing SoOXAC gene showed maximum fold induction in young leaf sample and minimum in insect attacked and senescent leaf samples (Fig. 2) and hence indicated high transcription levels of SoOXAC gene expression in young leaf which is not in accordance with the other oxalate synthesizing SoGLO gene. Root and stem samples showed similar pattern in fold induction of SoOXAC gene to that of SoGLO gene.

Permanganometric TO value corresponded to the SoGLO gene level

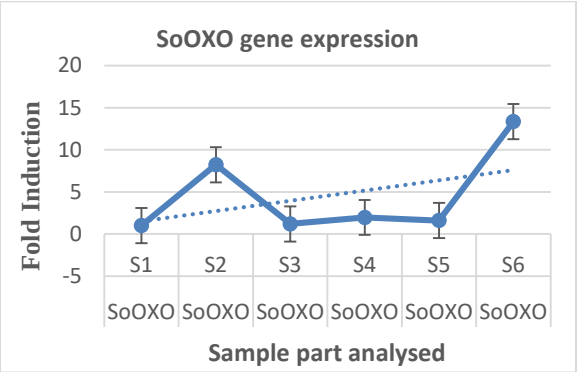

Fig. 3: SoOXO gene expression in samples S1 to S6 of *Amaranthus cruentus* S1 - root, S2 - stem, S3 - mature leaf, S4 - young leaf, S5 - insect attacked leaf, and S6 - senescent leaf.

Fig. 4. SoOXDC gene expression in samples S1 to S6 of *Amaranthus cruentus* S1 - root, S2 - stem, S3 - mature leaf, S4 - young leaf, S5 - insect attacked leaf, and S6 - senescent leaf.

expression in senescent (S6) samples. Image analysis pointed very high area occupancy of oxalate crystals unit^{-1} area in S5 and S6. Errakhi *et al*. (2008) and Kim *et al*. (2008) reported that high oxalate levels were responsible for death in plants. The high SoGLO gene expression in insect attacked (S5) and senescent (S6) leaves was in accordance with them.

SoOXAC gene showed maximum fold induction in young leaf which indicated high level of oxalate synthesis but TO value of young leaf was more than root, stem and mature leaf and less than insect attacked and senescent leaf samples. This clearly confirmed that the chemical interactions of different oxalate pathways maintain the intermediate TO levels in young leaves. Low oxalate levels pointed to the low transcriptional levels of oxalate synthesizing genes as well as high transcription levels of oxalate degrading genes. Stem samples showed high transcription level of oxalate degrading genes SoOXO and SoOXDC and hence had very low TO values. Senescent leaf samples showed high transcription levels of SoOXO (Fig. 3) and SoOXDC (Fig. 4) despite the low TO values which indicated the presence of overlapping concentrations of gene products (Svedruzic *et al*., 2005) and also the environmental factors such as soil nitrate and ammonia content appear to have affected the altered gene products (Xiaofeng *et al*., 2018).

Total oxalate (TO) in vegetative parts of purple amaranth

The Table 1 and Fig. 5 show the TO analysis of vegetative parts of purple amaranth. ANOVA was performed to analyse the variance between and among the TO of selected leaf or its vegetative parts. Homogeneity of variance, assessed by Levene test, showed that the group variances were homogenous. The mean values, standard deviation (SD), coefficient of variation (CV) of total oxalate content in vegetative parts were calculated. The results were subjected to the analysis of variance so as to assess the differences in total oxalate content, and the means were compared by Tukey test at 0.05 significance level. The statistical analysis was performed using the IBM SPSS software package. The classification of foods to low oxalate content (<10 mg 100 g^{-1}), moderate oxalate content (10-25

Table 1: Ox2 (TO **mg g^{-1} sample) in *Amaranthus cruentus* estimated by chemical analysis method**

Sample vegetative part	Minimum	Maximum	Mean ± SD	CV (%)
Root (S1)	0.15	0.35	0.2458 ± 0.059^{d}	24.003
Stem (S2)	1.05	1.45	1.2800 ± 0.131^{c}	10.234
Mature leaf (S3)	3.05	4.30	3.6667 ± 0.529^{b}	14.427
Young leaf (S4)	3.60	4.00	3.8375 ± 0.130^{b}	3.388
Insect attacked leaf (S5)	4.25	5.30	4.8667 ± 0.414^{a}	8.507
Senescent leaf (S6)	4.50	5.65	5.0208 ± 0.438^{a}	8.724

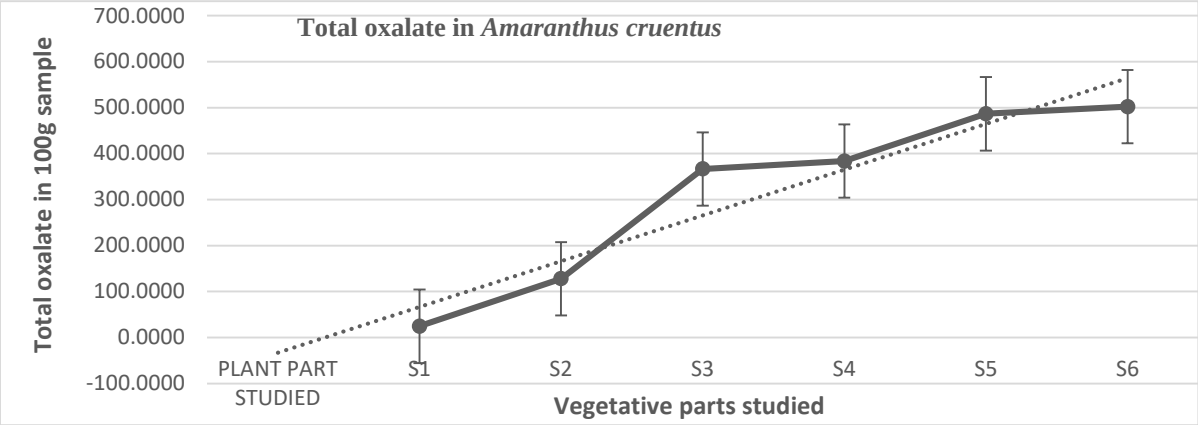

Fig. 5: Total oxalate (TO) in 100 g sample^{-1} of vegetative parts of *Amaranthus cruentus*; S1 - root, S2 - stem, S3 - mature leaf, S4 - young leaf, S5 - insect attacked leaf, and S6 - senescent leaf.

mg 100 g^{-1}), high oxalate content (26-99 mg 100 g^{-1}) and very high oxalate content (100-900 mg 100 g^{-1}) according to the total oxalate content of vegetables was done by Abdel-Moemin (2014). This could be helpful in understanding and monitoring the food consumed by renal patients in managing hyperoxaluria and urolithiasis. Foods containing > 50 mg 100 g^{-1} sample of oxalate were reported to be rich in oxalate (Massey, 2007). Estimation of TO in vegetative parts root (S1), stem (S2), mature leaf (S3), young leaf (S4), insect attacked leaf (S5) and senescent leaf (S6) by permanganometry revealed that they all fell under oxalate rich category. Samples projected TO in an order as follows S6 > S5 > S4 > S3 > S2 > S1. Senescent and insect attacked leaves accommodated 20% higher TO than root. Young and mature leaves had 15.32 and 14.4% higher TO than roots. Mishra *et al.* (2017) reported high TO in mature leaves than young leaves in *Anagallis arvensis* which corresponded to purple amaranth. Stem exhibited 5% increase in TO than root. The result showed differential TO levels in the vegetative parts studied. Similar results were reported by Savage and Vanhanen (2015) in curly and flat leaf cultivars of parsley. The TO values when compared to gene expression analysis showed that it was not exclusively genetically determined or overlapping of gene products were responsible for disparity in actual TO and gene fold expressions.

Image analysis

Micro-preparations of S1, S2, S3, S4, S5 and S6 revealed the presence of calcium oxalate druse crystals which were globose with uneven spiny surfaces. Area occupied by CaOx crystals per 10X microscopic field can be inferred as S5> S6> S3> S4> S2> S1 (Table 2) in unit area of leaf analysed (Fig. 6).

The result indicated that the amount of TO need not be proportional to the area occupied by insoluble CaOx crystals in the analysed vegetative parts of *A. cruentus* as the proportion of soluble and insoluble oxalate can vary depending on the external environmental conditions as well (Lester, 2013). Area occupied by druses and TO values were low in young leaf samples (Fig. 8) than mature leaf samples (Fig. 7) and could be important information for renal patients to opt low oxalate diet. Least area occupied by crystals were observed in root followed by stem and maximum area occupied by crystals were noted in insect attacked samples than senescent leaf samples.

Maximum coefficient of variation was observed in insect attacked and senescent leaf samples and minimum in young leaf samples. Kim *et al.* (2008) reported positive effect of oxalic acid on ethylene accumulation post-infection in plants which is in accordance with the present result of increased oxalate load in insect attacked samples. Role of calcium oxalate as a signal molecule to stress response indicated by very high oxalate load in insect attacked leaf samples. Oxalic acid reportedly acts as an elicitor of apoptosis and programmed cell death in plants (Errakhi *et al.*, 2008). The high calcium oxalate load in senescent leaves when compared to young and mature leaves indicated that calcium oxalate had a potential role in programmed cell death and senescence of leaves as a signal molecule.

Table 2: Image analysis data on *Amaranthus cruentus* L.

Sample vegetative part	Minimum (µm²)	Maximum (µm²)	Mean ± SD	CV (%)
Root (S1)	1.87	2.15	1.99 ± 0.14[d]	7.035
Stem (S2)	4.32	5.12	4.77 ± 0.41[d]	8.595
Mature leaf (S3)	19.42	21.70	20.74 ± 1.18[b]	5.690
Young leaf (S4)	15.17	16.39	15.76 ± 0.61[c]	3.871
Insect attacked leaf (S5)	31.85	44.08	37.33 ± 6.21[a]	16.635
Senescent leaf (S6)	24.77	36.75	29.27 ± 6.5[a]	22.207

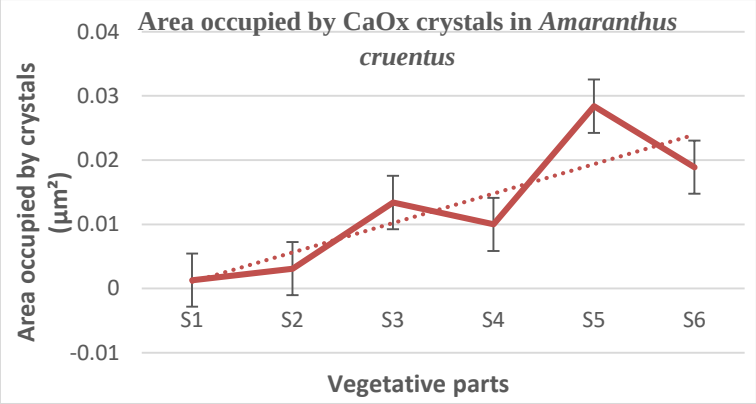

Fig. 6: Image area of *Amaranthus cruentus* CaOx druses: Surface area occupied by druse crystals per µm² sample: root (S1), stem (S2), mature leaf (S3), young leaf (S4), insect attacked leaf (S5) and senescent leaf (S6))

Conclusions*:* Purple amaranth (*Amaranthus cruentus*) had high oxalate content in various plant parts at various biotic phases, so care should be taken when consumed raw. The prolonged and excess dietary intake of purple amaranth is questionable in patients with renal disorders and infections. Young leaves contain more total oxalate than mature leaves and underground root had moderate oxalate levels only. The area occupied by CaOx druses were not in accordance with the total oxalate values of

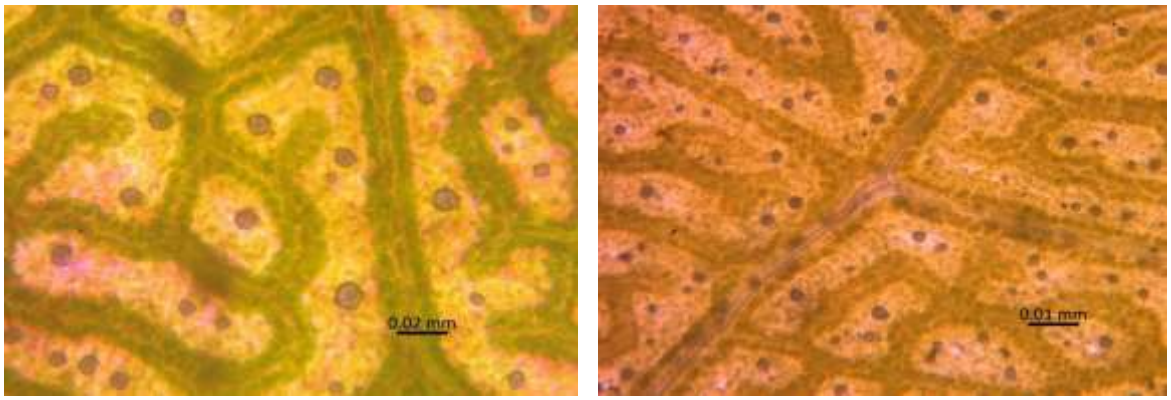

Fig. 7: *Amaranthus cruentus* CaOx druses in mature and young leaves; Grey spiny globose druses interspersed with photosynthetic tissue in mature lamina (left hand side), and young lamina with druses interspersed in photosynthetic tissue (right hand side)

vegetative parts studied. Mature leaves showed more area occupied by druse crystals than young leaves. The oxalate synthesizing gene SoGLO expressed transcriptional levels in different samples in accordance to total oxalate levels of the same, whereas SoOXAC gene and oxalate degrading genes (SoOXO and SoOXDC) expressed transcription levels not comparable to total oxalate levels in the vegetative parts studied. The result may be beneficial to healthcare, medical references and nutritional sciences for further studies and interpretations.

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Conflicts of Interest: The authors declare that they do not have any conflict of interest.

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