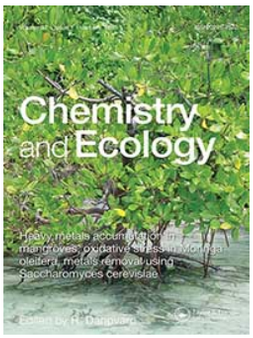

Chemistry and Ecology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gche20

Spectral variations associated with anthocyanin accumulation; an apt tool to evaluate zinc stress in *Zea mays* L.

E. Janeeshma, Vijisha K. Rajan & Jos T. Puthur

To cite this article: E. Janeeshma, Vijisha K. Rajan & Jos T. Puthur (2021) Spectral variations associated with anthocyanin accumulation; an apt tool to evaluate zinc stress in *Zea mays* L., Chemistry and Ecology, 37:1, 32-49, DOI: 10.1080/02757540.2020.1799993

To link to this article: https://doi.org/10.1080/02757540.2020.1799993

CHEMISTRY AND ECOLOGY
2021, VOL. 37, NO. 1, 32–49
https://doi.org/10.1080/02757540.2020.1799993

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Spectral variations associated with anthocyanin accumulation; an apt tool to evaluate zinc stress in *Zea mays* L.

E. Janeeshma[a], Vijisha K. Rajan[b] and Jos T. Puthur[a]

[a]Plant Physiology and Biochemistry Division, Department of Botany, University of Calicut, Thenhipalam, India; [b]Department of Chemistry, University of Calicut, Thenhipalam, India

ABSTRACT

The correlation between anthocyanins content and accumulation of zinc (Zn) in leaves of *Zea mays* was investigated and this was considered as a mode of assessing the intensity of Zn toxicity. Characterisation and quantification of leaf pigments were done in 45 d old maize plants exposed to different concentrations of Zn (0.0, 0.65, 1.30, 1.95 g Zn Kg^{-1} soil), and the enhancement in the accumulation of anthocyanins and degradation of chlorophyll was detected by the spectral analysis performed in the leaf extract of plants as the Zn content in the shoot increases. The role of anthocyanins in safeguarding photosynthesis and chelation of Zn was critically evaluated using chlorophyll *a* fluorescence responses and computational DFT-B3LYP structural analysis of anthocyanins-Zn complex has been performed using 6-311++ G (df, p) basis set. It was concluded that the accumulation of anthocyanins can act as an indicator of the intensity of Zn stress and this anthocyanins have a lesser potential to maintain photosynthetic efficiency in *Z. mays*, but plays a prominent role in metal chelation as the anthocyanins-Zn complex is a stable molecule.

ARTICLE HISTORY
Received 24 April 2020
Final Version Received 20 July 2020

KEYWORDS
Anthocyanins; fluorescence; heavy metals; metal chelation; Zinc

Introduction

In the present scenario, arable lands are getting contaminated with different heavy metals (HMs) due to various reasons such as solid waste deposition, usage of agricultural chemicals, and effluents of industrial wastes, *etc* [1]. The concentration of Zn in agricultural land is increasing day by day and acts as a threat to all organisms including plants due to its biomagnification [2,3]. One of the prominent responses of plants against Zn stress is the production of ROS (reactive oxygen species) like superoxide; hydrogen peroxide; hydroxyl radical; hydroxyl ions which are potentially toxic to plants [4]. Plants encounter this situation by producing different free radicals scavenging compounds, including anthocyanins [5].

Anthocyanins are natural, water-soluble, vacuolar polyphenol pigments synthesised in the cytosol, and it is responsible for the red, purple, blue colours of flowers, leaves, fruits, and stems of plants [6,7]. Anthocyanins are belong to the group flavonoids, have a wide

CONTACT Jos T. Puthur jtputhur@yahoo.com Plant Physiology and Biochemistry Division, Department of Botany, University of Calicut, C.U. Campus P.O, Thenhipalam 673635, Kerala, India
Supplemental data for this article can be accessed https://doi.org/10.1080/02757540.2020.1799993.

spectrum of distribution in higher plant groups as compared to lower forms. Soil elements have a strong correlation with the accumulation of leaf anthocyanins and it was studied by Rahimi and coworkers [8] in coriander leaves. They reported that the application of macroelements like nitrogen, phosphorus, and potassium in soil reduces the anthocyanins content in leaves of plants growing over there. But microelements like magnesium, iron, molybdenum and boron treatments increased anthocyanins content in coriander leaves and a drastic increase was observed only with Zn treatment in the soil [8]. Similarly, Zare et al. [9] reported that anthocyanins content increased in leaves of mentha with an increase in Zn concentration.

The antioxidant and photoprotective potential of anthocyanins was well studied by many researchers [10,11]. The stress-induced ROS generation elicit the accumulation of anthocyanins in *Arabidopsis*, denoting that there was a strong cross-regulation between ROS and anthocyanins production [12]. The potential of cytosolic anthocyanins to scavenge the superoxide radicle produced by chloroplast and the strong reducing capacity of vacuolar anthocyanins were proved in *Lactuca sativa* [13]. Even the exogenous application of leaf extract, rich in anthocyanins prepared from red cabbage proved beneficial against Cd toxicity in *Egeria* plants [14]. The accumulation of anthocyanins was observed in *Arabidopsis* with the increase in the concentration of various metals and out of these metals, copper was found to be the most potent elicitor of anthocyanins. This work also showed that Zn induced elevation in anthocyanins synthesis and the accumulation of the same was augmented with the increase in concentration of Zn [15].

The present study focuses on its metal chelation capacity, and for analysing the interaction between anthocyanins and Zn computational methodologies were employed which is considered as a green method, less time consuming and easy to perform [16,17]. The theoretical calculations were employed greatly by the advancement of density functional theory (DFT) to design molecular structures and evaluation of its properties at the molecular level which was not based on the wave function, but the density of electrons. Theoretical investigations of the physical and chemical properties of anthocyanins help to disclose the ability of anthocyanins to complex with different metal ions [16–18]. In this study, we are extending this theoretical knowledge to study the complexity of *in vivo* synthesised anthocyanins with bioaccumulated Zn in maize plants.

Some plants show tolerance towards the high level of Zn content, which can be evaluated by analysing various physiological and biochemical responses of plants [9,19]. Accumulation of anthocyanins content is one such kind and has the capacity to modify the absorption spectrum of plant leaf extract [20]. Therefore, tracking this secondary metabolite could be useful to analyse Zn stress intensity as well as metal tolerance potential of the plants.

We hypothesise that the accumulation of anthocyanins alleviates the photo-oxidative stress induced by Zn stress and thus could maintain the photosynthetic efficiency of *Zea mays* even under higher concentrations of Zn. To test this hypothesis, the main focus of the present study was to evaluate the metal chelation potential of anthocyanins based on the molecular orbital analysis of anthocyanins-Zn complex and to characterise the Zn induced variations in the absorption spectrum of leaf extract which can be used as an indicator to analyse the Zn stress tolerance potential of *Z. mays*.

Materials and method

Collection of plant material

Maize (variety CoHM 6) seeds were collected from Centre for Plant Breeding and Genetics, Department of millets, Tamil Nadu Agriculture University (TNAU), Coimbatore, India.

Experimental design

Maize seeds were surface sterilised with 0.1% $HgC1_2$ solution for 5 min and three seeds were placed at 8 cm below the sterilised soil filled in each polythene bag (18 × 13 cm). Polythene bags were filled with 1 kg of sterilised soil (soil: sand in 1:1) and the soil was sterilised according to the method of Raj and Sharma [21]. These polythene bags were kept in polyhouse maintained at 60 ± 2% relative humidity and 25 ± 2°C temperature. Thinning was carried out on 15 d and 30 d, and finally, a single healthy plant was maintained in a bag. After 45 d of growth, plants were treated with 40 mL of aqueous solutions of four different concentrations of $ZnSO_4.7H_2O$ accounting to 0.0, 0.65, 1.30, 1.95 g Zn Kg^{-1} soil. The second lower leaf of the maize plants was taken for various analyses on 6 d after the treatment.

Leaf extracts preparation and spectral analysis

0.2 g of fresh leaf sample was extracted with 5 mL of acidified methanol (1: 99, HCl: methanol, v/v) for 24 h at 3–5°C, with continuous shaking. The extracts were cleared by filtration and the content was made up to 10 mL. The visible spectrum of the leaf extracts was analysed from 420 to 750 nm using a UV-visible spectrophotometer (Shimadzu 1601, Kyoto, Japan).

Fourier-transform infrared spectroscopy (FTIR)

For sample preparation, the leaf extracts were concentrated in a fume hood for 72 h at 25° C to 2 mL and further mixed with potassium bromide (KBr) in a ratio of 1:150 mg (sample: KBr) with 10 ton of hydraulic pressure. FTIR analysis of the samples was carried out at mid-infra-red region of 400–4000 cm^{-1} (Jasco 4100, Shanghai, China).

Pigments quantification

Estimation of photosynthetic pigment content

The estimation of chlorophyll *a* and *b*, total chlorophyll and carotenoids content were done according to Arnon [22].

Anthocyanins

Anthocyanins content was determined according to the method of Mancinelli et al. [23] with some modifications. Fresh leaf samples (0.2 g) were homogenised and extracted in 5 mL of acidified methanol (1: 99, HCl: methanol, v/v) using a mortar and pestle. The

extract was kept at 4°C for 24 h and the content was made up to 10 mL. The absorbance of the leaf extract was measured at 530 and 657 nm against reagent blank and the anthocyanins content was determined based on the molar extinction coefficient of 29600 L mol^{-1} cm^{-1} for cyanidin-3-glucoside equivalents.

Estimation of malondialdehyde (MDA) content

The intensity of lipid peroxidation was measured in terms of MDA content as described by Heath and Packer [24].

Moisture content %

The tissue water status was determined by measuring the fresh and dry weights of the leaves. The dry weight was recorded after drying the tissue at 100°C in the hot air oven for 1 h and later transferring it into an oven maintained at 60°C. The dry weight was recorded on every alternate day until the weights became constant. Moisture content percentage (MC %) was calculated using the following equation [25].

$$\text{Moisture content \%} = [(FW - DW)/FW] \times 100$$

Total proline content

The total proline content of the maize leaves was estimated according to the method of Bates et al. [26] using L-proline as the standard.

Estimation of bioaccumulated Zn in leaves

The leaves of *Z. mays* plants were harvested and dried in an oven at 100°C for 1 h and then at 60°C until a constant weight was achieved. The same was used for Zn bioaccumulation studies according to the method of Allan [27]. From each sample, 1 g was digested by refluxing in 5:3 ratio of nitric: perchloric acid until the solution became colourless using Kjeldahl flasks heated in a heating mantle (60°C). Subsequently, the digest was transferred to a standard flask, and volume was made up to 100 mL. Atomic absorption spectrophotometer (Shimadzu AA-7000, Kyoto, Japan) was used for the estimation of Zn present in the digested samples.

Anthocyanins-zinc interactions

In the present work, we have employed a DFT based computational calculation to predict the probable structure of the anthocyanins (cyanidin)-Zn complex and some of its structural parameters, which will enable the study of Zn interactions with cyanidin. The computational calculation starts with the geometry optimisation of the complex followed by the analysis of various structural parameters. Both cyanidin and its Zn complex were optimised with the DFT-B3LYP level of theory and 6-31+ G (d, p) as a basis set. The term 'B3LYP' stands for the functional in DFT which consists of Becke's exchange functional [28] in conjunction with Lee-Yang–Parr correlational functional [29]. All the computational works were carried out in Gaussian 09 software package [30]. The electronic excitations in a

compound can be easily analysed from its UV-Visible spectrum. Here also, we have analysed the UV-Visible spectra of both the cyanidin and its Zn complex theoretically by the same software.

Chlorophyll a fluorescence measurement

Chlorophyll *a* fluorescence emission was measured by a Plant Efficiency Analyser Hansatech-Handy PEA, Norfolk, UK. All measurements were performed on leaves by giving a dark adaptation period of 20 min using the leaf clips provided by the manufacturer. Maximal fluorescence was induced at the peak wavelength of 650 nm with ultra-bright red LEDs, at a maximum intensity of 3000 µmol photon m^{-2} s^{-1} at the leaf surface. Chlorophyll *a* fluorescence transients were analysed and JIP-test was conducted [31]. Phenomenological fluxes or activities per exciting cross-section (CS) were visualised in the leaf pipeline model using Biolyzer (Biolyzer v.4.0.30.03.02, University of Geneva, Switzerland). Major parameters derived from the Chl *a* fluorescence transients using JIP-test were ABS/CSm (absorption flux per CS), TR/CSm (trapped energy per CS) ET/CSm (electron transport flux per CS) and DI/CSm (dissipated energy flux per CS), PI_{abs} (performance index on absorption basis), t for Fm (time taken to reach Fm), Area (area above the fluorescence induction curve), Fv/Fm (maximum quantum yield of PSII), Fv/Fo (maximum efficiency of water splitting complex) and N (Turn over number of Q_A).

Statistical analysis

Statistical analyses of the results were carried out according to the Duncan test at 5% probability level. One-way ANOVA was applied using the SPSS software (SPSS 16.0, Chicago, USA) to evaluate the significant difference in the traits among control and $ZnSO_4$ treated plants. Pearson's correlation analysis was performed to evaluate the relationships between the most important variables obtained in *Z. mays* under Zn toxicity. The data represent mean ± standard error (SE) and the values are the average of recordings from three independent experiments, each with three replicates (i.e. $n = 9$).

Results

Absorption spectra of leaf extract

The absorption spectra of leaf extract of the control plants showed a prominent peak in the visible region at 420 nm and a small peak at 670 nm (Figure 1). In the case of Zn treated plants, the emergence of a new peak was observed at visible region (λ=530 nm) and a significant reduction in the peak area at 420 and 670 nm was also observed.

FTIR spectra

FTIR spectra of methanolic leaf extracts from all the leaves samples have strong absorption peaks at 3423, 1638 and 1444 cm^{-1} (Figure 2). But the absorption peak at 1204 cm^{-1} is characteristic of Zn treated plants (Figure 2B).

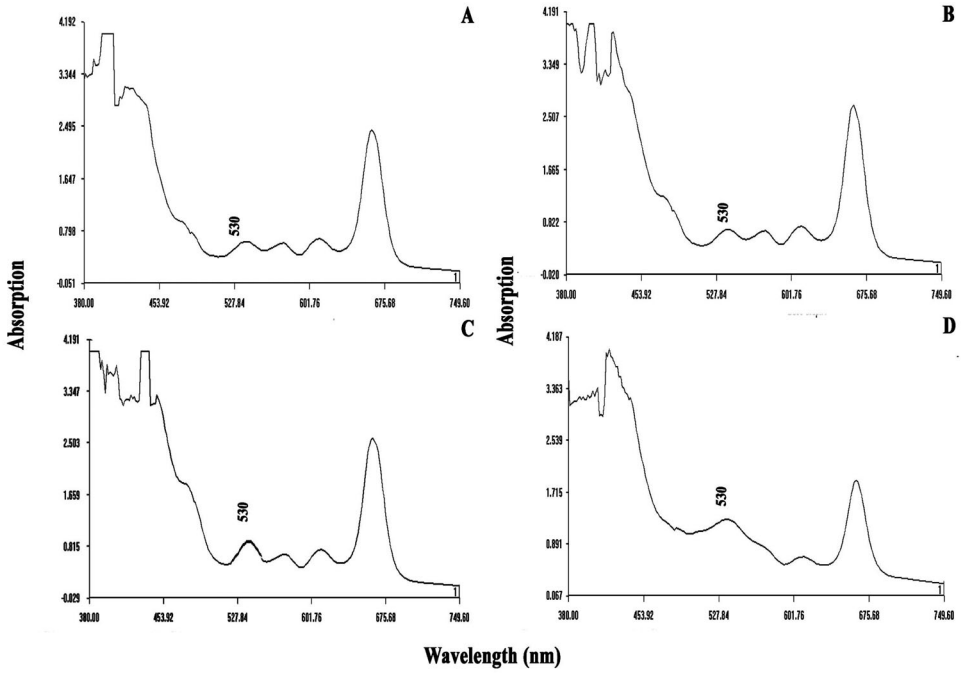

Figure 1. Visible spectra of leaf extracts of control (A), 0.65 (B), 1.30 (C) and 1.95 g Zn Kg^{-1} (D) treated plants, isolated in acid methanol from *Zea mays* on 6 d of exposure.

Chlorophyll and carotenoids

Plants subjected to the higher concentration of Zn (1.95 g Kg^{-1}) showed a reduction (19–30%) in chlorophyll *a* and *b* content as compared to the control (Table 1). But at lower concentrations (0.65 and 1.30 g Kg^{-1}), chlorophyll *b* content was increased to 13–18% as compared to control. Carotenoid content also got increased in plants exposed to the higher concentration of Zn (18–40%) as compared to the control. At the lower concentrations (0.65 and 1.30 g Kg^{-1}) of Zn treatment, the chlorophyll *a/b* ratio was decreased as compared to the control.

Quantification of anthocyanins

In *Z. mays,* a gradual increase in the anthocyanins content was observed in leaves of the plant exposed to increasing concentration of Zn and it was to the extent of 56% in the higher concentration of Zn (1.95 g Kg^{-1}) treated plants as compared to the control plants (Figure 3A).

Stress indicators

Stress indicators (MDA and proline) were increased in the leaves of *Z. mays* as the concentration of Zn treatment increases (Figure 3). The MDA content was drastically increased to the extent of 147% in the leaves of plants treated with the higher concentration of Zn

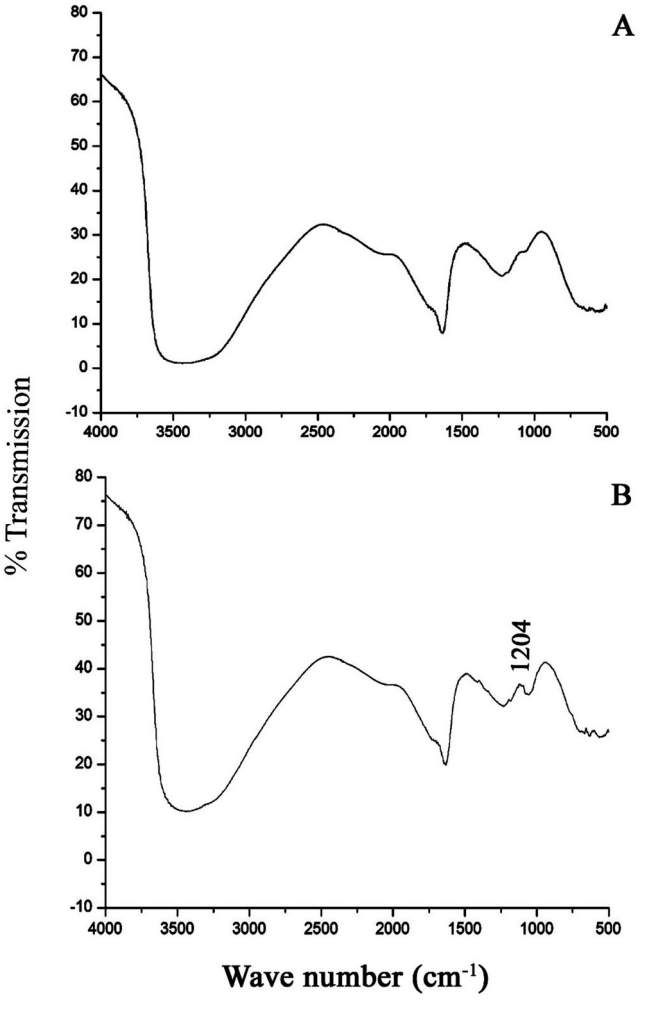

Figure 2. FTIR spectra of leaf extracts of control (A) and Zn (1.95 g Zn Kg^{-1}) treated (B) plants, isolated from *Zea mays* on 6 d of exposure.

Table 1. Photosynthetic pigment content in leaves of *Z. mays* under Zn toxicity.

Zinc treatments (g/Kg soil)	Chlorophyll *a* (µg/g f.w)	Chlorophyll *b* (µg/g f.w)	Total Chlorophyll (µg/g f.w)	Carotenoids (µg/g f.w)	Chl a/*b*
0	283.02 ± 14.15 b	56.33 ± 2.82 b	331.66 ± 16.58 b	48.75 ± 2.44 c	5.02 ± 0.34 a
0.65	294.67 ± 14.73 a	66.67 ± 3.33 a	347.64 ± 17.38 a	61.31 ± 3.07 b	4.42 ± 0.28 b
1.3	276.67 ± 13.83 b	63.67 ± 3.18 a	339.85 ± 16.99 ab	88.32 ± 4.42 a	4.35 ± 0.19 b
1.95	191 ± 9.55 c	45.33 ± 2.27 c	242.14 ± 12.11 c	60.18 ± 3.01 b	4.21 ± 0.2 b

Note: Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$). The data is an average of recordings from three independent experiments each with three replicates (i.e. n = 9). The data represent mean ± standard error

(1.95 g Kg^{-1}) as compared to the control. At the same time, proline showed a 102% increase in the leaves of Zn (1.95 g Kg^{-1}) treated plants. The moisture content of leaves was reduced to an extent of 24% under the exposure of Zn (1.95 g Kg^{-1}) toxicity.

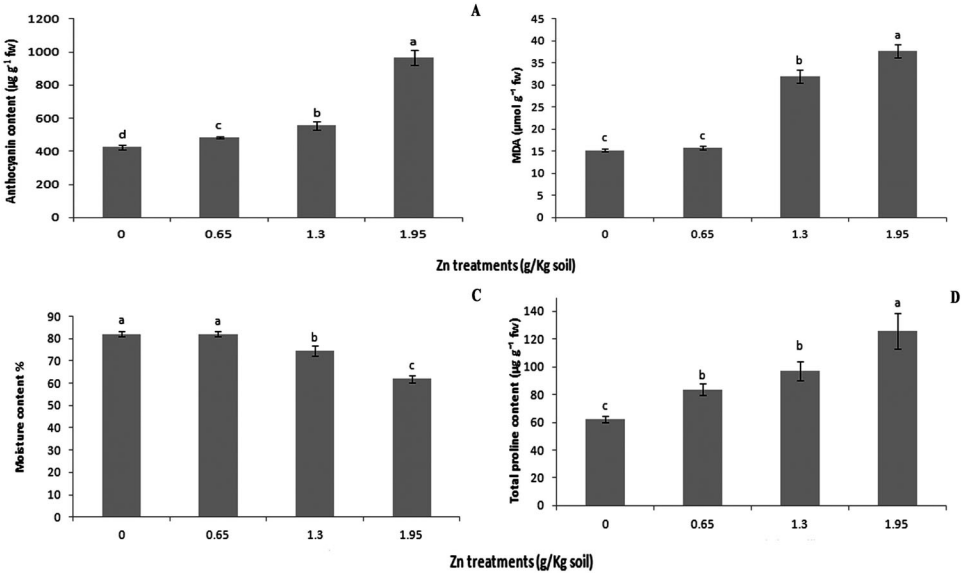

Figure 3. Anthocyanins content (A), MDA content (B), moisture content % (C) and Total proline content (E) in leaves of *Zea mays* subjected to Zn stress. Different letters on each bar indicate significant differences among the treatments assessed by Duncan test, at $P \leq 0.05$.

Bioaccumulation of Zn

In roots and leaves of *Z. mays,* a gradual increase in the Zn accumulation was observed with an increase in the concentration of Zn treatment and it was to the extent of 2–5 fold in the higher concentration of Zn (1.95 g Kg^{-1}) treated plants as compared to the control plants (Figure 4). A strong positive correlation was observed between anthocyanins content and accumulation of Zn in the leaves of *Z. mays* and it was significant in all Zn treatments ($R^2 = 0.797$ and $P \leq 0.01$). The correlation between anthocyanins content and accumulation of Zn in the leavs is represented in the supplementary Table 1.

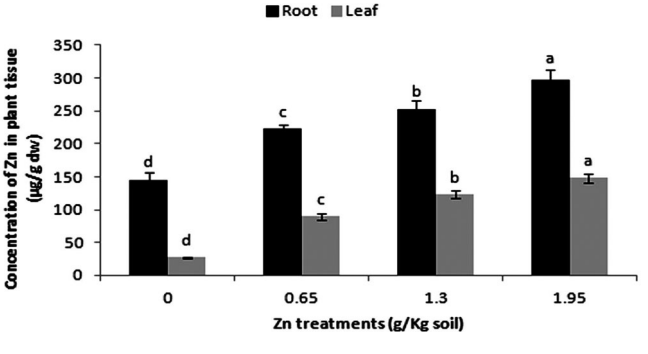

Figure 4. Zinc concentration in leaves of *Zea mays* subjected to Zn stress. Different letters on each bar indicate significant differences among the treatments assessed by Duncan test, at $P \leq 0.05$.

Anthocyanins-zinc interactions

The geometry of cyanidin and its Zn complex were optimised by DFT/B3LYP/6-31+ G -(d, p). The optimised stable structures of cyanidin and cyanidin-Zn complex are shown in Figure 5. The LUMO (lowest unoccupied molecular orbital) and HOMO (highest occupied molecular orbital) of cyanidin were delocalised over the entire cyanidin molecule.

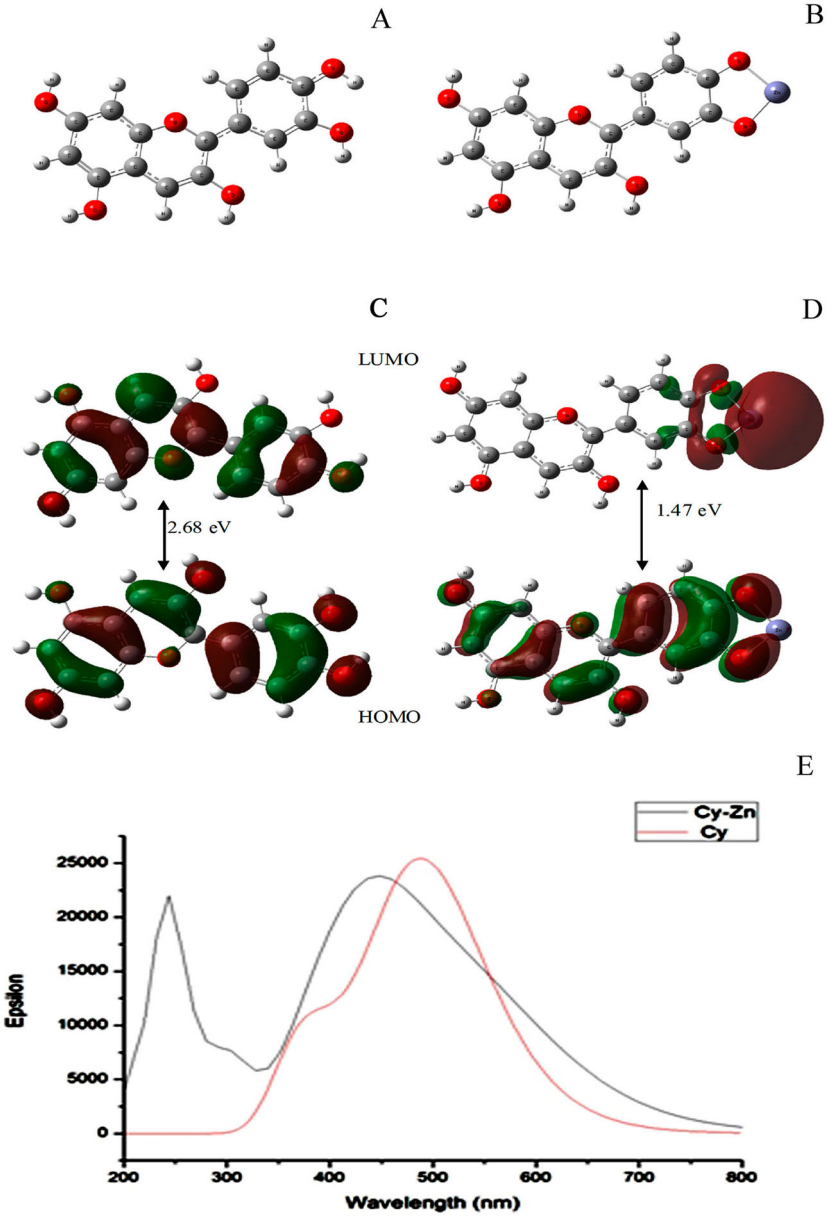

Figure 5. Interaction between anthocyanins and zinc; optimised structures of Cyanidin (A) and Cyanidin-Zinc complex (B); Energy gap in Cyanidin (C) and Cyanidin-Zinc complex (D); and UV-VIS spectrum of Cyanidin and Cyanidin-Zinc complex (E).

However, this structure changes when cyanidin interacts with Zn. Even though the HOMO of the complex is delocalised over the entire molecule, the LUMO is not so; it localises over the Zn ion and half of the aromatic ring of cyanidin. Cyanidin has a bandgap of 2.68 eV and it gets reduced to 1.47 eV in the cyanidin-Zn complex (Figure 5). The computed UV-visible spectra of both cyanidin and its Zn complex are shown in Figure 5(E), wherein the λ_{max} of cyanidin was observed at 491 nm, while it was at 455 nm for its Zn complex. There is a shoulder peak for cyanidin at 378 nm, but the shoulder peak is at 245 nm for the cyanidin-Zn complex.

Chlorophyll a fluorescence

The energy leaf models of *Z. mays* under different treatments were used to visualise the variations in the phenomenological energy fluxes. Area above the induction curve, Fv/Fm, Fv/Fo, N, and PI_{abs} were reduced at the higher concentration of Zn (1.95 g Kg^{-1}) treatment, but all these parameters were enhanced in the lower concentration of Zn (0.65 g Kg^{-1}) treatment (Table 2).

The density of active reaction centres was decreased to a greater extent in the high concentration of Zn (1.95 g Kg^{-1}) treated plants as compared to the control (Figure 6). The absorbance per cross-section (ABS/CSm) and ET**o**/CSm decreased significantly in the high concentration of Zn (1.95 g Kg^{-1}) treated plants, and the reduction was 29 and 67% respectively. Similarly, TR**o**/CSm decreased to a greater extent in the higher concentration of Zn treatment (44%) as compared to the control plants. At the same time, DI**o**/CSm was increased to 24% in plants exposed to the high concentration of Zn (1.95 g Kg^{-1}). All Chl *a* fluorescence parameters indicated by phenomenological energy flux were increased in plants subjected to the lower concentration of Zn (0.65 g Kg^{-1}) treatment. A strong negative correlation was observed between anthocyanins content and PI_{abs} in Zn treated *Z. mays* plants ($R^2 = -0.877$and $P \leq 0.01$). The correlation between anthocyanins content and PI_{abs} of the leaf is represented in the supplementary Table 1.

Discussion

Analysis of the visible spectrum gave evidence to the pigmental variation in leaves induced by Zn toxicity. The peaks emerging at 530 nm in Zn (1.95 g Kg^{-1}) treated plants corresponds to total anthocyanins compound, which generally have a characteristic peak at wavelength region 490–550 nm and another peak at 420 nm, which indicates chlorophyll [32]. But one of the catabolic products of chlorophyll, 'red chlorophyll' has the same spectral characters of anthocyanins with maximum absorption at 530 nm. For this reason, the presence of anthocyanins in Zn treated maize leaves was confirmed with the help of FTIR analysis. The absorption peaks at 1638 and 1444 cm^{-1} corresponds to the skeletal vibration of aromatic and heterocyclic rings. The absorption peak at 1204 cm^{-1} is characteristic of C–O–C group, which has a strong stretching vibration and corresponds to anthocyanins and not to red chlorophyll, because the latter does not have a C–O–C group in its structure [33]. Of the different anthocyanins, cyanidin is the common one and it seems to be spread over in all parts of maize; in the seed coat, leaves, kernels, and flowers [34,35]. If visible spectra of leaf extract have a characteristic

Table 2. Chlorophyll *a* fluorescence parameters in leaves of *Z. mays* subjected to Zn stress.

Zinc treatment (g/Kg soil)	t for Fm	Area	Fv/Fm	Fv/Fo	N	ABS/CSm	DIo/CSm	TRo/CSm	ETo/CSm	PI_{abs}
0	280 ± 14 a	23006 ± 1150 b	0.793 ± 0.04 a	3.835 ± 0.19 a	33.487 ± 1.67 b	1574 ± 78.73 a	326 ± 16.3 c	1248 ± 62.43 a	686 ± 34.3 a	2.069 ± 0.1 a
0.65	290 ± 14.5 a	26066 ± 1303 a	0.786 ± 0.04 a	3.678 ± 0.18 a	37.341 ± 1.87 a	1672 ± 83.63 a	357.5 ± 17.88 bc	1315 ± 65.75 a	718.5 ± 35.93 a	1.852 ± 0.09 b
1.3	245 ± 12.25 b	18219.5 ± 910 c	0.767 ± 0.04 b	3.322 ± 0.17 b	29.154 ± 1.46 c	1596 ± 79.8 a	371 ± 18.55 ab	1225 ± 61.25 a	600 ± 30 b	1.343 ± 0.07 c
1.95	270 ± 13.5 a	8291 ± 414 d	0.582 ± 0.03 c	1.801 ± 0.09 c	24.471 ± 1.22 d	1106 ± 55.3 b	407 ± 20.35 a	699 ± 34.95 b	221.5 ± 11.08 c	0.336 ± 0.02 d

Note: The data is an average of 10 recordings from three independent experiments. Values are expressed as mean ± SE of three independent experiments. Different letters following values indicate a significant difference between treatments (Duncan's test, $P \leq 0.05$). The data is an average of recordings from three independent experiments each with ten replicates (i.e. $n = 30$).

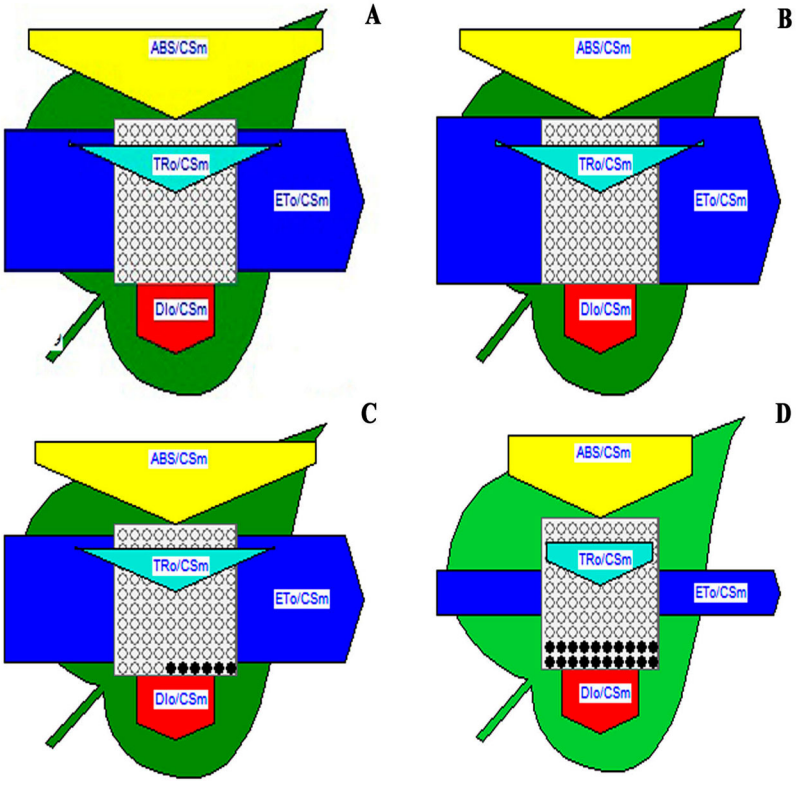

Figure 6. Leaf model of Chl *a* fluorescence related parameters recorded in the leaves of *Zea mays* under $ZnSO_4$ treatment in control (A), 0.65 (B), 1.30 (C) and 1.95 g Zn Kg^{-1} (D) treated plants. Leaf pipeline model showing the proportion of phenomenological energy flux parameters with in a leaf calculated per cross section (CS) approximated by maximal fluorescence (Fm) where ABS/CSm- absorption flux per CS, TR/CSm-trapped energy per CS, ET/CSm- electron transport flux per CS and DI/CSm- dissipated energy flux per CS. The width of the arrow represents the relative values of each parameter and empty and dark circles represent active and non active reaction centres, respectively.

peak of 515–530 nm, it points to the presence of cyanidin [36]. Therefore, the peak observed in the region 530 nm can be confirmed of cyanidin presence in maize leaves.

The peak at 420 nm was reduced in plants subjected to the higher concentration of Zn (1.95 g Kg^{-1}) as compared to the control plants, because of the inhibition in the biosynthesis and/or uncontrolled stress-induced degradation of chlorophyll. Heavy metal-induced leaf chlorosis due to the inhibition in the activity of photochlorophyllide reductase was reported by Stobart and coworkers [37]. So the visible spectrum of the leaf extract can be used as a tool to find out the exposure of Zn toxicity as it causes anthocyanins accumulation and chlorophyll degradation which were detectable in the visible spectrum.

The Zn treated plants exhibited anthocyanins accumulation and the significance of this accumulated anthocyanins was further scrutinised in this study. The drastic increase of anthocyanins content in *Z. mays* seedlings subjected to Zn stress, points directly towards the free radical scavenging and metal chelating potential of anthocyanins [38–42]. The antioxidation potential of anthocyanins was observed in *Ocimum basilicum*

under boron toxicity, where the direct correlation between anthocyanins accumulation and metal tolerance was shown by the purple-leaved 'Red Rubin' [43].

The ROS accumulated during heavy metal stress has a crucial role in the signalling of anthocyanins synthesis. A study conducted in ten *Arabidopsis* mutants showed that the ROS molecules trigger the up-regulation of anthocyanins biosynthesis and the corresponding regulatory genes, resulting in increased production of anthocyanins [12]. In the present study, elevation in the ROS content of maize leaves under Zn toxicity was evidenced by the increased MDA content. The accumulated ROS content was responsible for the augmentation in the biosynthesis of anthocyanins. As the concentration of Zn was increased in the leaves, the MDA and anthocyanins content was also increased. Therefore, the rate of accumulation of anthocyanins can be directly related to the intensity of the oxidative stress caused due to the increase in Zn concentration [44,45].

Anthocyanins are powerful antioxidant and it also interacts with metal ions by acting as a chelator and forming coloured complexes; where the concentration of metal ions has a crucial role in the colour intensity of the complex formed [46–49]. When *Brassica juncea was exposed to molybdenum and tungsten, the development of blue crystals was observed in their epidermal cells as a result of anthocyanins-metal ions complexation* [50,51]. As per these reports, the purple-red colour developed in the leaves of maize plants in the present study could be also due to the complexation between anthocyanins and excess Zn ions. Cyanidin, delphinidin, and petunidin are the three major anthocyanins with considerable metal chelation potential as they have adjacent hydroxyl groups [52]. In the present study, cyanidin was taken as the representative of anthocyanins, to evaluate the affinity towards Zn ion. The charge transfer complex reactions were analysed with the help of molecular orbital analysis. Based on the frontier molecular orbital (FMO) theory, HOMO and LUMO of a molecule determine the chemical reactivity of a molecule [17]. The difference in the delocalisation pattern of LUMO in cyanidin and its Zn complex showed that the LUMO of cyanidin interacts with the Zn ion by accepting electrons, thereby forming a stable complex. The reduction in the bandgap between cyanidin and cyanidin-Zn complex also indicates the stabilisation of cyanidin with a metal ion [53]. The charge transfer between the cyanidin molecule and the metal ion resulted in the stabilisation of the cyanidin-Zn complex. The positive charge in the cyanidin makes them good electron acceptors, which further supports the charge transfer from Zn to cyanidin in the studied complex. Thus as assessed from the above observation, there is every possibility for the accumulated anthocyanins in the leaves of maize plants under Zn stress to act as a strong metal chelator, which helps the plant to tolerate the higher Zn concentrations.

According to Park et al. [54] the leaf colour modification arising out of the accumulation of anthocyanins was the result of the translocation of Zn to the leaves. The translocation of metal ions to the leaves gets increased with an elevation in the availability of the metal ions. In the present study, synthesis of anthocyanins was increased with an increase in the bioaccumulation of Zn and these two parameters exhibited a strong positive correlation. It clearly gives evidence to the potential of anthocyanins to act as an indicator of Zn stress intensity. The bioaccumulated Zn ions potentially increases the ROS accumulation, which ultimately signals the production of anthocyanins.

The photoprotective role of accumulated anthocyanins was assessed with the help of chlorophyll *a* fluorescence. Inactivation of reaction centres is the best indicator to assess

the decline in the quantum efficiency of PS II [55,56] and it occurred in the plants exposed to higher concentrations of Zn where anthocyanin content was also high. Simultaneous decrease in chlorophyll content and photosynthetic efficiency was also observed in plants exposed to the higher concentration of Zn. The decrease in chlorophyll content and photosynthetic efficiency was observed in durum wheat exposed to the higher concentration of Zn [55] and it supports the results obtained from the present study. Even though Zn induced an increase in the chlorophyll *b* content, the photosynthetic efficiency of *Z. mays* was decreased at 1.30 g Kg^{-1} Zn treatment. But at 0.65 g Kg^{-1} Zn treatment, the chlorophyll content and photosynthetic efficiency of plants increased and it indicates that at low concentration, Zn promotes the growth as it is an essential element.

The ABS/CSm was reduced in plants under the higher concentration of Zn (1.95 g Kg^{-1}) stress and this parameter is commonly applied for analysing the absorption efficiency of RC [57]. The reduction in chlorophyll content in $ZnSO_4$ treated plants was reflected in energy absorption potential as assessed from low ABS/CSm of these plants. The reduction in the absorbance may be due to the chlorophyll shielding effect of anthocyanins or reduction in accessory pigments [13]. The fluorescence analysis indicated that the PI_{abs} (photosynthetic efficiency) of Zn (1.95 g Kg^{-1}) treated plants was negatively affected, which accumulated more anthocyanins as compared to control [58,59]. Similar to our results, the maximal quantum yield of PSII in *Miscanthus* × *giganteus* plants grown under Zn stress was reduced [60]. In UV-B and high light stress, the tolerance level of plants was directly related to the anthocyanins content in the leaves which helps the optical masking of chlorophyll and thus reduces photo-oxidative stress and thus could optionally maintain the photosynthetic rate [61,62]. The photoprotective role of anthocyanins was also experienced in *Ocimum basilicum* under boron toxicity with a combination of highlight stress [63]. Boron toxicity resulted in the accumulation of anthocyanins in *Ocimum basilicum,* and when these plants were exposed to highlight stress it showed better tolerance, because of the light filtering action of accumulated anthocyanins. In the present study, it was conclusively established that Zn treated plants have higher anthocyanins (cyanidin) content used for chelation of the metal and accumulation of the same would reduce the photosynthetic efficiency and it has to be explored whether the anthocyanins accumulated has a shielding effect as in the case of high light/UV stress to prevent oxidative stress. Moreover, it can be used as an indicator of the presents of high concentrations of Zn ions in the plants.

Conclusion

Anthocyanins accumulation in leaves was considered as a marker to evaluate the intensity of Zn toxicity, as it was easily detected in the visible spectrum of leaf extracts. The role of anthocyanins in Zn stress was scrutinised using molecular orbital analysis and results supported the high metal chelation potential of anthocyanins, which helps in the stabilisation of Zn ions. Moreover, the reduction of PI_{abs} in the anthocyanins accumulated leaves confirmed the lower potential of anthocyanins to maintain the photosynthetic efficiency as the concentrations of Zn increases, possibly by hindering the photosynthetic machinery by the exceeded Zn ions in leaves.

Acknowledgment

Edappayil Janeeshma greatly acknowledges the technical assistance provided by Department of Chemistry, Calicut University.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the University Grant Commission (UGC), India in the form of JRF under [grant number 319492].

Notes on contributors

E Janeeshma performed the analysis, processed the experimental data, interpreted the results, drafted the manuscript and designed the figures.

Vijisha K. Rajan performed the computations and derived the models

Jos T. Puthur provided critical feedback and helped shape the research and analysis aided in interpreting the results and worked on the manuscript.

References

[1] Shiyu QIN, Hongen LIU, Zhaojun NIE, et al. Toxicity of cadmium and its competition with mineral nutrients for uptake by plants: A review. Pedosphere. 2020;30:168–180.
[2] Herawati N, Suzuki S, Hayashi K, et al. Cadmium, copper, and zinc levels in rice and soil of Japan, Indonesia, and China by soil Type. Bull Environ Contam Toxicol. 2000;64:33–39.
[3] Brus D, Li ZB, Temminghoffd EJM, et al. Predictions of spatially averaged cadmium contents in rice grains in the Fuyang Valley. P.R. China. J Environ Qual. 2009;38:1126–1136.
[4] Morina F, Jovanovic L, Mojovic M, et al. Zinc-induced oxidative stress in *Verbascum thapsus* is caused by an accumulation of reactive oxygen species and quinhydrone in the cell wall. Physiol Plant. 2010;140:209–224.
[5] Moustaka J, Tanou G, Giannakoula A, et al. Anthocyanin accumulation in poinsettia leaves and its functional role in photo-oxidative stress. Environ Exp Bot. 2020;175:104065.
[6] Trojak M, Skowron E. Role of anthocyanins in high-light stress response. World Sci *News*. 2017;81:150–168.
[7] Piccolo E, Landi M, Giordani T, et al. Can anthocyanin presence ameliorate the photosynthetic performance of Prunus saplings subjected to polyethylene glycol-simulated water stress? Photosynthetica. 2020;58:799–807.
[8] Rahimi AR, Babaei S, Mashayekhi K, et al. Anthocyanin content of coriander (*Coriandrum sativum* L.) leaves as affected by salicylic acid and nutrients application. Int J Biol Sci. 2013;3:141–145.
[9] Zare DS, Asrar Z, Mehrabani M. Effect of zinc on growth and some physiological and biochemical parameters of spearmint (*mentha spicata* L). Iran J Biol. 2007;20:230–241.
[10] Landi M. Can anthocyanins be part of the metal homeostasis network in plant. Amer J Agri Biol Sci. 2015;10:170–177.
[11] Gould KS, Jay-Allemand C, Logan BA, et al. When are foliar anthocyanins useful to plants? Re-evaluation of the photoprotection hypothesis using Arabidopsis thaliana mutants that differ in anthocyanin accumulation. Environ Exp Bot. 2018;154:11–22.

[12] Xu Z, Mahmood K, Rothstein SJ. ROS induces anthocyanin production via late biosynthetic genes and anthocyanin deficiency confers the hypersensitivity to ROS-generating stresses in *Arabidopsis*. Plant Cell Physiol. 2017;58:1364–1377.
[13] Neil SO, Gould KS. Anthocyanins in leaves: light attenuators or antioxidants? Funct Plant Biol. 2003;30:865–873.
[14] Maleva M, Garmash E, Chukina N, et al. Effect of the exogenous anthocyanin extract on key metabolic pathways and antioxidant status of Brazilian elodea (*Egeria densa* (Planch.) Casp.) exposed to cadmium and manganese. Ecotox Environ Safe. 2018;160:197–206.
[15] Baek SA, Han T, Ahn SK, et al. Effects of heavy metals on plant growths and pigment contents in *Arabidopsis thaliana*. Plant Pathol J. 2012;28:446–452.
[16] Payán-Gómez SA, Flores-Holguín N, Pérez-Hernández A, et al. Computational molecular characterization of the flavonoid morin and its Pt (II), Pd (II) and Zn (II) complexes. J Mol. 2011;17:979–985.
[17] Rajan VK, Muraleedharan K. A computational investigation on the structure, global parameters and antioxidant capacity of a polyphenol, Gallic acid. Food Chem. 2017;220:93–99.
[18] Fedenko VS, Shemet SA, Landi M. UV–vis spectroscopy and colorimetric models for detecting anthocyanin-metal complexes in plants: An overview of in vitro and in vivo techniques. J Plant Physiol. 2017;212:13–28.
[19] Garg N, Aggarwal N. Effects of interactions between cadmium and lead on growth, nitrogen fixation, phytochelatin, and glutathione production in mycorrhizal *Cajanus cajan* (L.) Millsp. J Plant Growth Regul. 2011;30:286–300.
[20] Merzlyak MN, Chivkunova OB, Solovchenko AE, et al. Light absorption by anthocyanins in juvenile, stressed, and senescing leaves. J Exp Bot. 2008;59:3903–3911.
[21] Raj S, Sharma SD. Integration of soil solarization and chemical sterilization with beneficial microorganisms for the control of white root rot and growth of nursery apple. Sci Hortic. 2009;119:126–131.
[22] Arnon DT. Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 1949;24:1–15.
[23] Mancinelli AL, Yang CPH, Lindquist P, et al. Photocontrol of anthocyanin synthesis: III. The action of streptomycin on the synthesis of chlorophyll and anthocyanin. Plant Physiol. 1975;55:251–257.
[24] Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophysi. 1968;125:189–198.
[25] Lokhande VH, Nikam TD, Patade VY, et al. Effects of optimal and supra-optimal salinity stress on antioxidative defence, osmolytes and in vitro growth responses in *Sesuvium portulacastrum* L. Plant Cell Tiss Org. 2011;104:41–49.
[26] Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil. 1973;39:205–207.
[27] Allan JE. The preparation of agricultural samples for analysis by atomic absorption spectrometry. Varian Techtron Bulletin. 1969;SIS Edn:12-69.
[28] Becke AD. Density-functional thermochemistry. III. The role of exact exchange. J Chem Phys. 1949;98:5648–5652.
[29] Lee C, Yang W, Parr RG. Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. Phys Rev B. 1988;37:785.
[30] Frisch MJ, Trucks GW, Schlegel HB et al. Gaussian 03, revision C. 02. Wallingford, CT: Gaussian, Inc. 4, 2004; 26.
[31] Strasser RJ, Tsimilli-Michael M, Srivastava A. Analysis of the fluorescence transient. In: GC Papageorgiou, Govindjee, editors. *Chlorophyll-a fluorescence: A signature of photosynthesis advances in photosynthesis and respiration*. Rotterdam: Academic Publishers; 2004. p. 321–362.
[32] Merzlyak MN, Solovchenko AE, Gitelson AA. Reflectance spectral features and non-destructive estimation of chlorophyll, carotenoid and anthocyanin content in apple fruit. Postharvest Biol and Technol. 2003;27:197–211.
[33] Moser S, Müller T, Holzinger A, et al. Fluorescent chlorophyll catabolites in bananas light up blue halos of cell death. P Natl Acad Sci USA. 2009;106:15538–15543.

[34] Fossen T, Slimestad R, Øvstedal DO, et al. Anthocyanins of grasses. Biochem Syst Ecol. 2002;30:855–864.
[35] Khoo HE, Azlan A, Tang ST, et al. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food Nutr Res. 2017;61:1361779.
[36] Da Silva FL, Escribano-Bailón MT, Alonso JJP, et al. Anthocyanin pigments in strawberry. LWT - Food Sci Technol. 2007;40:374–382.
[37] Stobart AK, Griffiths WT, Ameen-Bukhari I, et al. The effect of Cd2+ on the biosynthesis of chlorophyll in leaves of barley. Physiol Plant. 1985;63:293–298.
[38] Radovanović B, Radovanović A. Free radical scavenging activity and anthocyanin profile of Cabernet Sauvignon wines from the Balkan region. Molecules. 2010;15:4213–4226.
[39] Lima ADJB, Correa AD, Saczk AA, et al. Anthocyanins, pigment stability and antioxidant activity in jabuticaba [*Myrciaria cauliflora* (Mart.) O. Berg]. Rev Bras Frutic. 2011;33:877–887.
[40] Dai LP, Dong XJ, Ma HH. Molecular mechanism for cadmium-induced anthocyanin accumulation in *Azolla imbricata*. Chemosphere. 2012;87:319–325.
[41] Kumchai J, Huang JZ, Lee CY, et al. Proline partially overcomes excess molybdenum toxicity in cabbage seedlings grown *in vitro*. Genet Mol Res. 2013;12:5589–5601.
[42] Pardossi A, Romani M, Carmassi G, et al. Boron accumulation and tolerance in sweet basil (*Ocimum basilicum* L.) with green or purple leaves. Plant Soil. 2015;395:375–389.
[43] Landi M, Pardossi A, Remorini D, et al. Antioxidant and photosynthetic response of a purple-leaved and a green-leaved cultivar of sweet basil (*Ocimum basilicum*) to boron excess. Environ Exp Bot. 2013;85:64–75.
[44] Shackira AM, Puthur JT, Salim EN. *Acanthus ilicifolius* L. a promising candidate for phytostabilization of zinc. Environ Monit Assess. 2017;189:282.
[45] Alia KV, Prasad SK, Saradhi PP. Effect of zinc on free radicals and proline in *Brassica* and *Cajanus*. Phytochemistry. 1995;39:45–47.
[46] Agati G, Azzarello E, Pollastri S, et al. Flavonoids as antioxidants in plants: location and functional significance. Plant Sci. 2012;196:67–76.
[47] Gould KS, McKelvie J, Markham KR. Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. Plant Cell Environ. 2002;25:1261–1269.
[48] Hughes NM, Neufeld HS, Burkey KO. Functional role of anthocyanins in high-light winter leaves of the evergreen herb *Galax urceolata*. New Phytol. 2005;168:575–587.
[49] Cortez R, Luna-Vital DA, Margulis D, et al. Natural pigments: stabilization methods of anthocyanins for food applications. Compr Rev Food Sci Food Saf. 2017;16:180–198.
[50] Hale KL, McGrath SP, Lombi E, et al. Molybdenum sequestration in *Brassica* species. A role for anthocyanins? . Plant Physiol. 2001;126:1391–1402.
[51] Hale KL, Tufan HA, Pickering IJ, et al. Anthocyanins facilitate tungsten accumulation in *Brassica*. Physiol Plant. 2002;116:351–358.
[52] Osawa Y. Copigmentation of anthocyanins. In: Markakis P, editor. Anthocyanins as food colors. New York, NY: Academic press; 1982. p. 41–68.
[53] Gildea LF, Williams JAG. Iridium and platinum complexes for OLEDs. In: Buckley A, editor. In Organic light-emitting diodes (OLEDs). Cambridge: Woodhead Publishing; 2013. p. 77–113.
[54] Park W, Han KH, Ahn SJ. Differences in root-to-shoot Cd and Zn translocation and by HMA3 and 4 could influence chlorophyll and anthocyanin content in *Arabidopsis* Ws and Col-0 ecotypes under excess metals. J Soil Sci Plant Nutr. 2012;58:334–348.
[55] Paunov M, Koleva L, Vassilev A, et al. Effects of different metals on photosynthesis: cadmium and zinc affect chlorophyll fluorescence in durum wheat. Int J Mol Sci. 2018;19:787.
[56] Krause GH, Weis E. Chlorophyll fluorescence as a tool in plant physiology. Photosynth Res. 1984;5:139–157.
[57] Fghire R, Anaya F, Ali OI, et al. Physiological and photosynthetic response of quinoa to drought stress. Chil J Agr Res. 2015;75:174–183.
[58] Zivcak M, Brestic K, Olsovska SP. Performance index as a Sensitive indicator of water stress in *Triticum Aestivum* L. Plant Soil Environ. 2008;54:133–139.

[59] Tsimilli-Michael M, Strasser RJ. In vivo assessment of stress impact on plant's vitality: applications in detecting and evaluating the beneficial role of mycorrhization on host plants. In: Varma A, editor. Mycorrhiza. Heidelberg: Springer; 2008. p. 679–703.
[60] Andrejić G, Gajić G, Prica M, et al. Zinc accumulation, photosynthetic gas exchange, and chlorophyll a fluorescence in Zn-stressed *Miscanthus*× *giganteus* plants. Photosynthetica. 2018;56:1249–1258.
[61] Chalker-Scott L. Environmental significance of anthocyanins in plant stress responses. Photochem Photobiol. 1999;70:1–9.
[62] Landi M, Tattini M, Gould KS. Multiple functional roles of anthocyanins in plant-environment interactions. Environ Exp Bot. 2015;119:4–17.
[63] Landi M, Guidi L, Pardossi A, et al. Photoprotection by foliar anthocyanins mitigates effects of boron toxicity in sweet basil (*Ocimum basilicum*). Planta. 2014;240:941–953.