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Assessment of Foliar Application of Copper and Zinc to Elevate the Defence Response of *Bacopa monnieri* (L.)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Each of the nutrients is needed in different amounts and carries specific functions in the plant**.** Also, nutrients do not work in isolation; therefore, balanced nutrition is needed to optimize crop quality. Nutrient interactions may be synergistic or antagonistic and also possible to have no interactions. However, nutrients also stimulates the antioxidant activity in medicinal plants that improves the secondary metabolite production. To investigate the Zn and Cu response in *Bacopa monnieri*, an

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experiment was consisted of $ZnSO_4$ and $CuSO_4$ (BM0: CONTROL; BM1:1ppm Cu; BM2:2.5ppm Zn; BM3:5ppm Zn; BM4:0.5ppm Cu+5ppm Zn; BM5:1ppm Cu+2.5ppm Zn; BM6:1ppm Cu+5ppm Zn) during two successive years (2019-2020). Two foliar applications was given at 30 days of interval and samples were collected and analysed after 10 days of each spray. Most of the results showed that Zn concentration increased with BM6 treatment and Cu content recorded a rise with BM1 and BM6 treatments respectively, compared with control plants. Also, MDA content and H_2O_2 activity were observed higher in the presence of BM6. Meanwhile, all the treated plants induced the activity of SOD that found maximum at BM6 while APOX activity mostly increased at BM3 and BM6. The highest proline accumulation took place BM1 and BM3. The result also showed 2.5ppm Zn treatment gave the highest content of bacoside-A (0.31%). From the research it seems that all the parameters were significantly increased when compared to control. It was suggested from the present experiment that foliar spray of Zn and Cu accelerated antioxidative activities that provides *Bacopa* to compensate the oxidative damage along with Zn and Cu content along with Bacoside production.

Keywords: Bacoside; nutrients; medicinal plants.

ABBREVIATION

MDA : Malondialdehyde;
SOD : Superoxide dismutase;
H_2O_2 : Hydrogen peroxide;
APOX : Ascorbate peroxidase

1. INTRODUCTION

Micronutrient elements such as Zn and Cu are essential for plant growth and development. Zn is a major metal component that is required in a large number of enzymes and plays an essential role in DNA synthesis and transcription. Other functions of Zn include: catalyzing the process of oxidation in plant cell and is vital for the transformation of carbohydrates; and influencing the formation of chlorophyll and auxins, the growth promoting compounds. On the other hand, Cu in a constituent of enzyme system which brings about oxidation-reduction reactions in the plant, it regulates respiration, photosynthesis, reduction of nitrates and sulphates. In case with other plant micronutrients Zn and Cu limit plant growth when they are present both in low concentrations and in excessive concentrations due to deficiency and toxicity respectively [1]. *Brahmi* is a succulent, non-aromatic herb, botanically known as *Bacopa monnieri.* In *Ayurveda* it is categorized as *'Medhya Rasayana'* meaning 'memory enhancer'. Main bioactive compound of this herb are saponins called Bacosides mainly Bacoside A and B, internationally in demand as a brain tonic for enhancing memory. Various commercial formulations such as; Brahmighritam, Brahmirasayanam, Mentat plus and Mentat are some of the prominent Ayurvedic medications made from this plant [2]. It is mostly found in damp or marshy areas near streams or on the border of ponds throughout India approx. upto 1300 m elevation. It is a native of India, Bangladesh and Southern Asia. A temperature range between 30-40° C with relative humidity of 65-80% good for its optimum vegetative growth. In india, it is found naturally growing in the states of Uttar Pradesh, Haryana, Punjab, Bihar, Bengal, Tamil Nadu, Kerala, Karnataka, foothills of Himachal Pradesh and Uttarakhand [3].

Medicinal herbs are the rich source of secondary metabolites, thus nutrient management approach helps in increasing the bioactive compounds that improves the herb quality. Interaction of nutrient mainly occurs at root surface or within the plant when ions capable of forming chemical bonds compete for adsorption, absorption, transport and function sites with ions with similar chemical characteristics. Zn improve the expression level of the major enzymes of bacoside pathway. It has been discovered that Bacopa has two distinct biosynthetic routes for triterpenoid saponins: Mevalonic acid pathway (cytosol) and methyl-D-erythritol 4-phosphate pathway in the plastid. The key regulatory enzymes of bacoside synthesis are 3- hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), mevalonate diphosphate decarboxylase (MDD) and squalene synthase (SQS) which supplies carbon for its synthesis [4,5]. Bacoside-A consist of four compounds namely, bacoside A3, bacopaside II, jujubogenin isomer of bacopasaponin C (bacopaside X), and bacopasaponin C [6].

Scarce information is available in the literature regarding the effect of the Zinc and Copper and their combine effect with *Bacopa* yield. Because whole herb mainly aerial portion of *B. monnieri*

have been reported to contain active principle and therefore, the present research highlights: to assess the potential of Zn and Cu accumulation as well as to study antioxidant strategies of *Bacopa* plant after singly/combination of different level of Zn and Cu.

2. MATERIALS AND METHODS

2.1 Site of Experiment

The present investigation was carried out in the experimental site of department of Plant Physiology, GBPUA&T, Pantnagar, Uttarakhand (29^0 N latitude, 79^0 29'E longitude and at an altitude of 243.8 meter above from the mean sea level) during two successive years 2019 and 2020. This area falls under Tarai belt about 30 km southwards of foothills of Shivalik range of Himalayas. The climate of the site retains humid subtropical with hot and dry summers and cool winters. The soil is sandy loam with uniform fertility. The pre-experimental soil pH and organic carbon was 6.9 and 1.5%. The content values of ($mgKg^{-1}$) for N, P, K, Zn, Cu, Fe and Mn were 313.6, 25.1, 20, 0.8 0.5 7.4 and 0.6, respectively.

2.2 Experimental Details

The experiment was laid out in complete randomized block design with three replications. The field size (10X5 m^2) was levelled and divided into 75X50 cm^2 plot. The cuttings of *Bacopa monnieri* (variety CIM-Jagriti), were obtained from CSIR-CIMAP, Lalkuan, Uttarakhand. Three stolons of uniform thickness (5-10 cm) with 2-3 nodes were planted in pits of 5 cm depth at a spacing of 25 cm in each plot in the month of July as first year experiment (2019). The planting was done in the morning hours for minimum desiccation. Frequent irrigation was provided after monsoon to maintain the optimum moisture level and weeding was done at regular intervals. After harvest of 2019 crop, regenerates were allowed to grow further for second year experiment (2020). Pest and disease problems were not observed during experiment. Foliar treatments consist of freshly prepared $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$: [BM0: (CONTROL); BM1: (1ppm $CuSO_4$), BM2: (2.5ppm $ZnSO_4$); BM3: (5ppm $ZnSO_4$); BM4: (0.5ppm $CuSO_4$+5ppm $ZnSO_4$); BM5: (1ppm $CuSO_4$+2.5ppm $ZnSO_4$); BM6: (1ppm $CuSO_4$+5ppm $ZnSO_4$)]. Here, BM signifies the name of plant. Foliar treatments were applied twice in 30 days of interval. For biochemical parameters samples were analysed after 7-10 days of each spray while micronutrient estimation, plant samples were analysed after harvesting i.e., 30 days after each spray.

2.3 Micronutrient Estimation

Zinc and Copper were determined by [7]. 0.5 g dried leaf sample was taken and 10 ml tri- acid mixture (10:1:4v/v) was added overnight. Next day, the flask was heated until brown fumes appear than 10 ml of TCA was added and heated until appeared transparent. Add 5 ml 6 N HCl solution. Filter it and makeup volume up to 50 ml with distilled water. The digested samples were analysed with the help of atomic absorption spectrometer (AAS). The respective elements were estimated in test sample by preparations standard curve.

2.4 Malondialdehyde Assay

The lipid peroxidation was estimated by the method described by [8]. 0.2 grams of fresh leaves from each replication were taken and homogenize with 2 ml of TBA (0.25% TBA in 10 % TCA (Trichloro Acetic Acid). The homogenate was heated at 95ºC for 30 minutes and cooled rapidly in ice bucket. The content was centrifuged at 10,000g for 30 minutes and supernatant was collected. The absorbance was recorded at 532nm and 600 nm against blank.

2.5 Hydrogen Peroxide

The hydrogen peroxide was estimated by following the method of [9]. Leaf tissues (500 mg) were homogenized in ice bath with 5 ml 0.1% (w/v) TCA. The homogenate was centrifuged at 12000 rpm for 15 min and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI. The absorbance of supernatant was read at 390 nm. A graph of absorbance vs. concentration for standard solutions of H_2O_2 was plotted and the amount of H_2O_2 in the samples was calculated from the standard curve.

2.6 Superoxide Dismutase Activity

Superoxide dismutase was assayed by using procedure given by [10]. Fresh samples (2.0 g) grounded with 2ml of 0.1M potassium phosphate buffer of pH 7. The grinded sample was centrifuged at 16000 g for 15 minutes under 4ºC, after centrifugation supernatant was collected. 3mL of the reaction mixture containing 50mM potassium phosphate buffer (pH 7.8), 13mM

methionine, 2µM riboflavin, 0.1 µM EDTA, 75 µM NBT and 50µl crude enzyme extract (volume make up with DW) were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1mL riboflavin (60µM) and placing the tubes below a light source of two 15W florescent lamps for 15 minutes. Reaction was stopped by switching off the light and covering the tubes by black cloth. Tubes without enzyme extract developed maximum colour. A blank without enzyme and NitroBlue Tetrazolium chloride was set to calibrate spectrophotometer recorded at 560nm.

2.7 Estimation of Ascorbate Peroxidase Activity

Ascorbate peroxidase activity was estimated according to the method of [11]. 100 mg of leaves was homogenised in phosphate buffer and centrifuged at 16000 rpm for 20 min at 40^0 C and the supernatant was used as enzyme extract. The reaction mixture for the peroxidase contained 1.5 ml of potassium phosphate buffer (pH 7.5), 300 µl ascorbate, 600 µl H_2O_2, and 600 µl enzyme extract in a total volume of 3ml. It was based on the decrease in absorbance at 29 0nm as ascorbate was oxidized with time. The reaction was started by adding the enzyme and H_2O_2 simultaneously and the decrease in absorbance was recorded 30 seconds after this addition at 290 nm.

2.8 Proline Content

Proline content was determined in plants by the method as described by [12]. Leaf sample (0.5g) was homogenized in 5 mL of sulphosalicylic acid (3%) and centrifuged at 8000g for 10 minutes and supernatant was saved. Residue was again extracted twice with 5mL of 3% aqueous sulphosalicylic acid. 2mL of the extract was taken in the test tube and 2mL ninhydrin reagent and 2mL glacial acetic acid were added. After immediate cooling the reaction mixture was added with 4mL toluene. Thereafter the mixture was shaken vigorously and the proline was quantified spectrophotometrically 520 nm.

2.9 Bacoside Content

For Bacoside content three treatments were selected: (bm0: CONTROL; bm1:1ppm $CuSO_4$ and bm2:2.5ppm $ZnSO_4$) and one-gram dried leaf samples were sent to CSIR-CIMAP for HPLC analysis.

2.9.1 Sample preparation

One gram of Bacopa leaves were powdered and 100 mg of each sample were extracted three times in 10 ml of methanol with sonication for 30 min at room temperature. The extract was filtered, and the filtrate was evaporated to dryness. Obtained residue was dissolved in 1 ml of HPLC grade methanol for analysis.

2.9.2 HPLC analysis conditions

The mobile phase was A- water, B- acetonitrile, 70: 30 (v/v) (A: B system) (Isocratic). The flow rate was 1.5 ml/min, Column: Chromolith Performance RP-18e (100 X 4.6 mm, 5 µm), 10 µL of Injection volume, run time of 30 min and detection was done at 205 nm.

2.10 Statistical Analysis

Experiment was performed in completely randomized block design (CRBD) with three replications for each treatment. One way ANOVA used to analyse the Zn and Cu effect on *Bacopa*. Duncan multiple range test were performed to analyse the differentiate significant means of treatments. For the Pearson correlation analysis average values of each spray were taken and conducted to reveal the relationships between different parameters. Means were compared with least significant difference (LSD) at 5% probability level ($P \leq 0.05$).

3. RESULTS

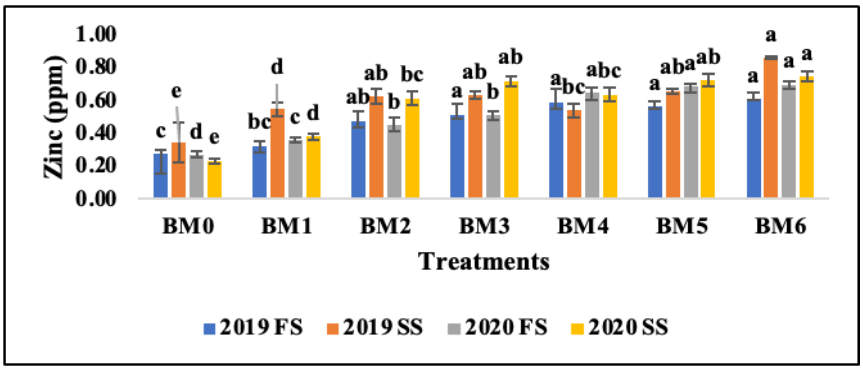
3.1 Micronutrient Estimation

To understand the nutrient uptake, we determined changes in mineral nutrient element concentrations in leaves of *Bacopa* plants and found content of Cu and Zn significantly increased at different level of Cu and Zn individual/combination application, represented in (Fig. 1). Compared with the control (0.28ppm), Zn content increased after exposure to BM6 that recorded (0.61 and 0.86ppm Zn) along with BM3 (0.52ppm), BM4 (0.59ppm Zn) and BM5 (0.56ppm Zn) respectively, more that of control after first spray, while after second spray BM6 (0.86ppm Zn) followed by BM2 (0.63ppm Zn), BM3 (0.63ppm Zn) and BM5 (0.66ppm Zn) respectively, than control (0.34ppm Zn) during 2019. Similar trend observed in the year 2020, after first foliar treatment Zn content increased almost in BM6 (0.70 ppm Zn) followed by BM4 (0.65ppm Zn) and BM5(0.68ppm Zn) when

compared to control (0.27ppm Zn). The most effective level was BM6 (0.72ppm Zn) along with BM3 and BM5 (0.72ppm Zn) noticed after second foliar spray and minimum content found in control (0.23ppm Zn). During 2019, it is clearly showed that, after first spray Cu content increased and all the treated plants revealed closed to maximum value at BM4 (0.29ppm Cu) whereas after second spray BM2 (0.29ppm Cu) reported higher Cu content. While control plants had only a small amount of Cu in their leaves (0.23 and 0.19ppm Cu). During 2020, foliar spray of BM1 (0.33ppm) along with BM3 and BM6 recorded highest Cu content (0.31ppm) after first spray whereas BM6 and BM4 (0.36 and 0.34ppm Cu) observed increase after second spray. In case of untreated plants (0.24 and 0.21ppm Cu) lower values of Cu content was noticed.

3.2 Oxidative Indicators

Lipid peroxidation level in leaves of *Bacopa* plants, measured as MDA content and H_2O_2 activity, given in **(**Fig. 2). Plant treated with increasing doses indicating free radical generation in *Bacopa* leaves. During 2019, it was demonstrated that maximum average value of MDA content was reported in BM6 (10.08 and 10.55 µ mol g^{-1} FW) along with BM3 (9.40 and 9.94 µ mol g^{-1} FW) and BM1 (9.35 and 9.54 µ mol g^{-1} FW) w.r.t control (4.13 and 4.94 µ mol g^{-1} FW) after each foliar treatment. During 2020, treatments of BM5 (12.53 µ mol g^{-1} FW) followed by BM1 (11.58 µ mol g^{-1} FW), BM3 (11.59 µ mol g^{-1} FW) and BM6 (11.58 µ mol g^{-1} FW) respectively, resulted higher MDA content after first foliar spray. MDA content was significantly increased by foliar spraying of BM6 (14.65 µ mol g^{-1} FW) along with BM3 (13.34 µ mol g^{-1} FW) after second foliar treatment. During 2019, accumulation of H_2O_2 recorded higher mean values in BM1 and BM6 (12.11 and 11.54 µ mol g^{-1} FW) applied plants in leaves of *Bacopa* after first spray whereas after second spray BM6 and BM4 (15.58 and 14.11 µ mol g^{-1} FW) over control (4.26 µ mol g^{-1} FW). In the year 2020, H_2O_2 content found maximum mean values under the BM6 (5.87 and 6.13 µ mol g^{-1} FW) treatment with respect to control (1.62 and 4.41 µ mol g^{-1} FW).



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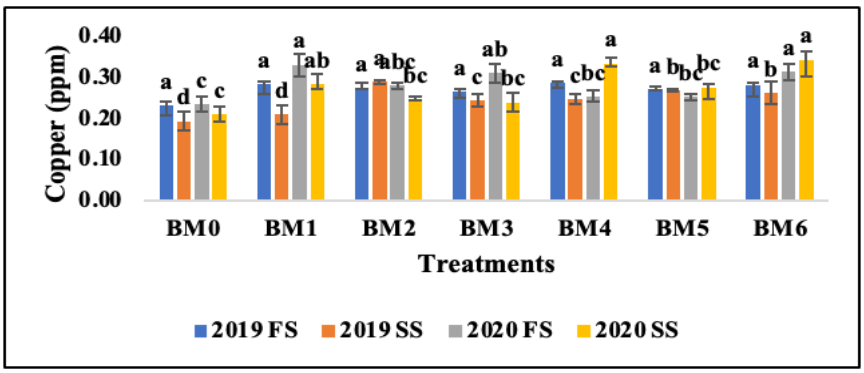
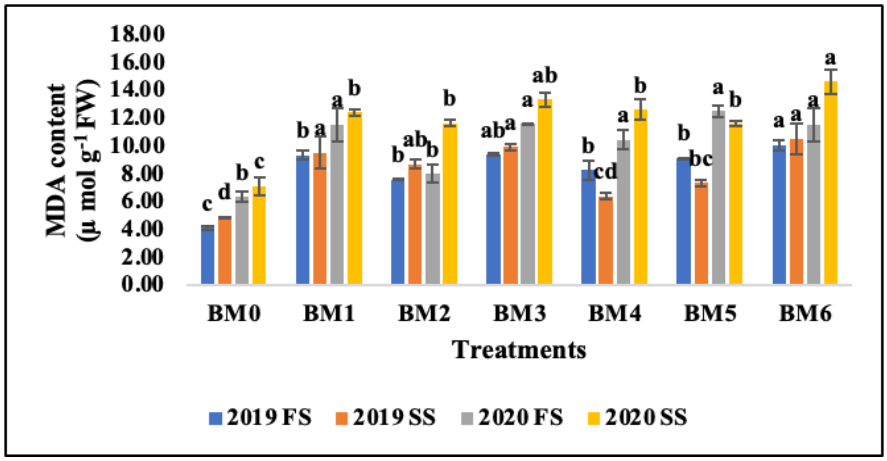


(B)

Fig. 1. Effect of Cu and Zn sole/combination on (A) zinc content and (B) Cu content in dried leaves of *Bacopa monnieri* after first and second foliar spray application during two successive year (2019-2020)

(Letter belong to each treatment separately and data are shown as means SE of three replicates using DMRT. Bars represented standard error.)



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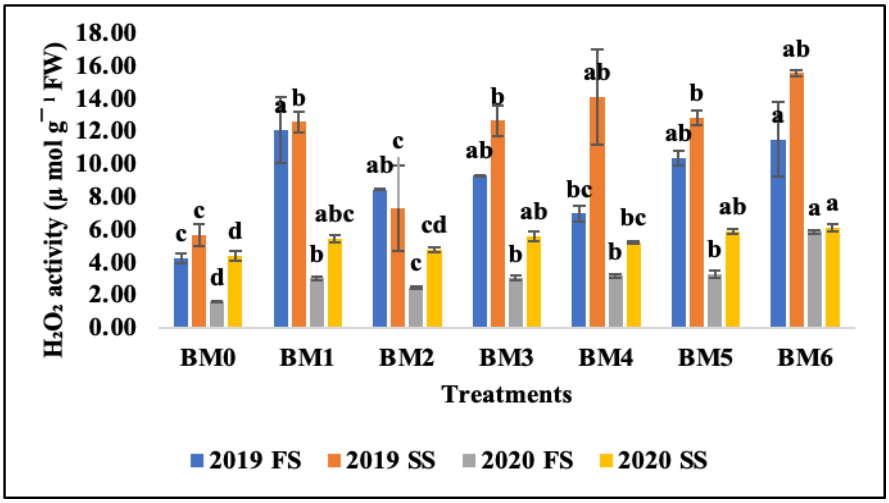


(B)

Fig. 2. Effect of Cu and Zn sole/combine (A) MDA content (µ mol g^{-1} FW); (B) H_2O_2 activity (µ mol g^{-1} FW) in fresh leaves of *Bacopa monnieri* after first and second foliar spray application during two successive year (2019-2020)

(Letter belong to each treatment separately and data are shown as means SE of three replicates using DMRT. Bars represented standard error.)

3.3 Antioxidant Activity and Osmolyte Accumulation

Bacopa plant applied with several Zn and Cu concentrations singly/combination revealed a significant difference among treatments that triggered activity of antioxidant activities after foliar spray, represented in (Fig. 3). According to our investigation, during 2019 when the BM6 (0.50 units/min/g F.W) along with BM2 and BM3 (0.49 units/min/g F.W) concentration given the activity of SOD increased after first foliar spray. Result showed that BM3 and BM6 (0.48 units/min/g F.W) significantly increased in SOD activity and similar values were noticed after second foliar spray. While lower values obtained in untreated plants (0.33 and 0.31 units/min/g F.W). In 2020, after first foliar treatment maximum SOD activity showed at BM6 (0.52 units/min/g F.W) which is statistically at par with BM3 and BM5 (0.51 units/min/g F.W) that of control (0.35 units/min/g F.W). In our study, when plants were subjected to BM6 (0.54 units/min/g F.W) reported maximum APOX activity along with BM3 (0.53 units/min/g F.W) with respect to control (0.34 units/min/g F.W). On the other hand, during 2019 BM3 (16.90 units/min/g F.W) and BM6 (16.81 units/min/g F.W) followed by BM4 (15.79 units/min/g F.W) were significantly increased in APOX activity over control (9.74 units/min/g F.W) after first foliar spray. Similar pattern observed after second foliar spray in

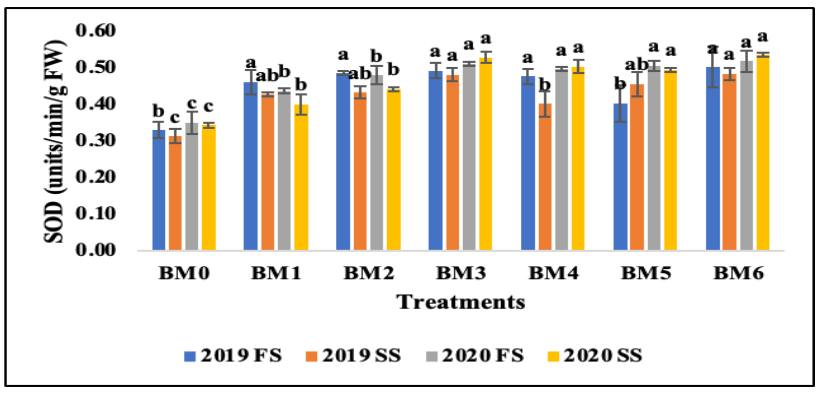
Bacopa leaves, BM6 (13.83 units/min/g F.W) and BM3 (13.32 units/min/g F.W) along with BM1 (12.99 units/min/g F.W) and BM4 (12.57 units/min/g F.W). During 2020 experiment, APOX activity peaked at BM3 (2.63 units/min/g F.W), BM6 (2.54 units/min/g F.W) and BM4 (2.15 units/min/g F.W) respectively, after first foliar treatment. It can also be seen that second foliar spray showed almost same mean values BM3 and BM6 (1.41 units/min/g F.W). In untreated plants APOX activity is less (0.82 and 0.79 units/min/g F.W). It is evident from the result that plant treated with BM3 and BM6 appeared higher SOD and APOX activity among treatments.

Data presented in (Fig. 3), During 2019 it was noticed that the level of proline in leaves recorded maximum when exposed to BM3 (2.48 µmoles per gram) and BM1 (2.37 µmoles per gram) after first spray over control (1.98 µmoles per gram tissue). Plants applied to BM6 and BM1 (2.69 µmoles per gram) along with BM3 (2.67 µmoles per gram) that showed maximum proline content after second spray with respect to control (1.86 µmoles per gram). During 2020, at BM3 (4.65 µmoles per gram) along with BM1 and BM2 (3.76 µmoles per gram) proline content was maximum after first foliar treatment. Proline content at BM3 and BM1 (3.65 and 3.22 µmoles per gram) increased after second foliar spray. The lower values identified in control (1.23 and 1.13 µmoles per gram). Plants applied to BM4 treatments showed minimum value that reduced upto 11.02% that of control.

3.4 Bioactive Compounds

As shown in chromatogram of different constituents of bacoside-A, treatment bm2 led to lower (21.83%) peak area of Bacoside A3 as compared to bm1 and bm0 that recorded similar peak area (22.33%). A minor difference between bm0 and bm1 was detected in Bacopaside II. Foliar treatment of bm2 have lesser peak area (26.97%) when compared with bm0 and bm1 i.e. (33.62%) and (34.00). For the jujubogenin isomer of Bacopasaponin C, it is observed bm0 appeared highest peak area (19.84%) followed by bm1 and bm0 that received (18.22%) and (17.25%), respectively. Comparing the peak area Bacopasaponin C found more in bm2 treated plants (31.37%) with respect to bm0 and bm1 peak area (26.55%) and (26.27%). Present findings result showed that bm2 treatment gave the highest content of bacoside-A (0.31%). However, bm0 and bm1 gave the same result of bacoside-A content (0.21%). The results suggested that Zn is more effective, increment of about 32.20% in Bacoside-A content than untreated and Cu treated plants, data and chromatograms, given in (Fig. 4.).

For the correlation analysis of different parameters average values of each spray were considered 2019-2020 (Supplementary material). The single and cotreatment of Cu and Zn at the lower amounts was the most significantly efficient treatment for increasing the Cu and Zn accumulation in both the years 2019 and 2020, showed in (Table. 1). During 2019, foliar application increased Zn and Cu content that observed highly significant positive correlation between two elements (r = 0.80*). Zn also showed highly significant correlation with oxidative indicators H_2O_2 (r= 0.76*), MDA (r=0.75) and enzymatic antioxidant activity SOD (r=0.82*), APOX (r=0.70). Moreover, there was a significant positive correlation between Cu with H_2O_2 (r= 0.73*) MDA (r=0.76) and antioxidant production SOD (r=0.80*). In 2020, Zn recorded highly positive correlation with H_2O_2 (r= 0.76*), MDA (r=0.78*), SOD (r=0.97**), APOX (r=0.79*). Similar trend also noticed with Cu between H_2O_2 and MDA (r=0.84*) as well as APOX (r=0.77*). During both year proline accumulation showed lesser response with micronutrient and other parameters.



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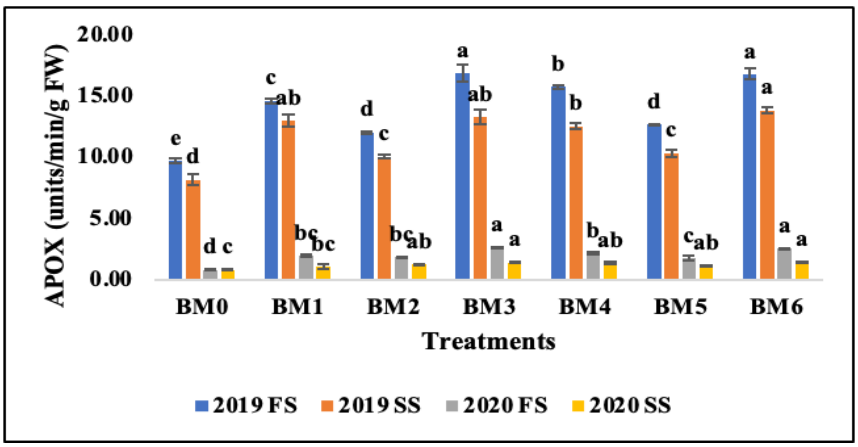

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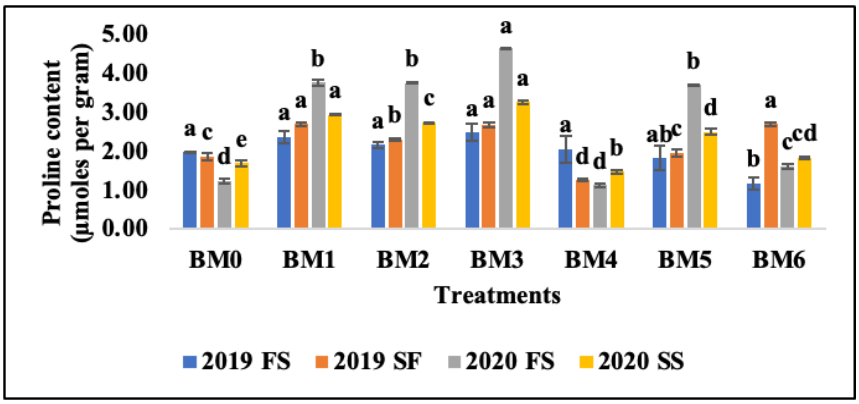
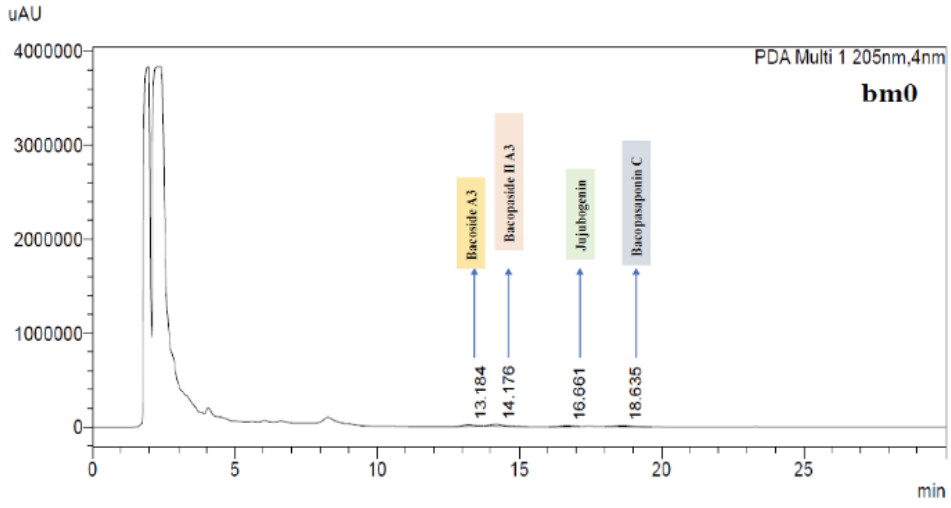


(C)

Fig. 3. Effect of Cu and Zn sole/combination on (A) SOD (units/min/g F.W); (B) APOX (units/min/g F.W) and (C) Proline content (µmoles per gram) in fresh leaves of *Bacopa monnieri* after first and second foliar spray application during two successive year (2019-2020)

(Letter belong to each treatment separately and data are shown as means SE of three replicates using DMRT. Bars represented standard error.)



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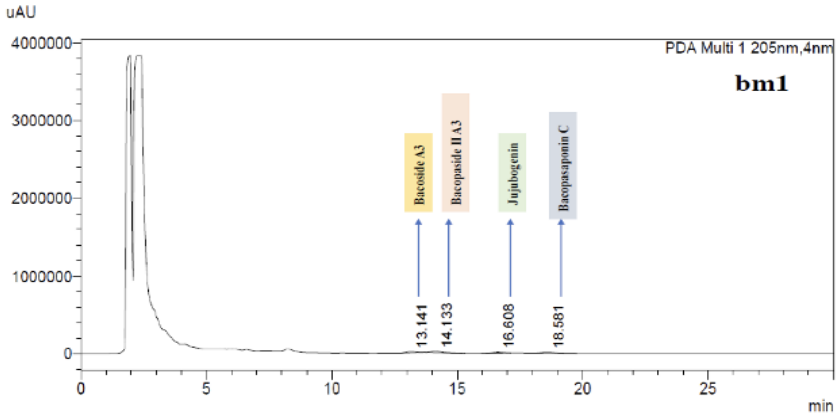

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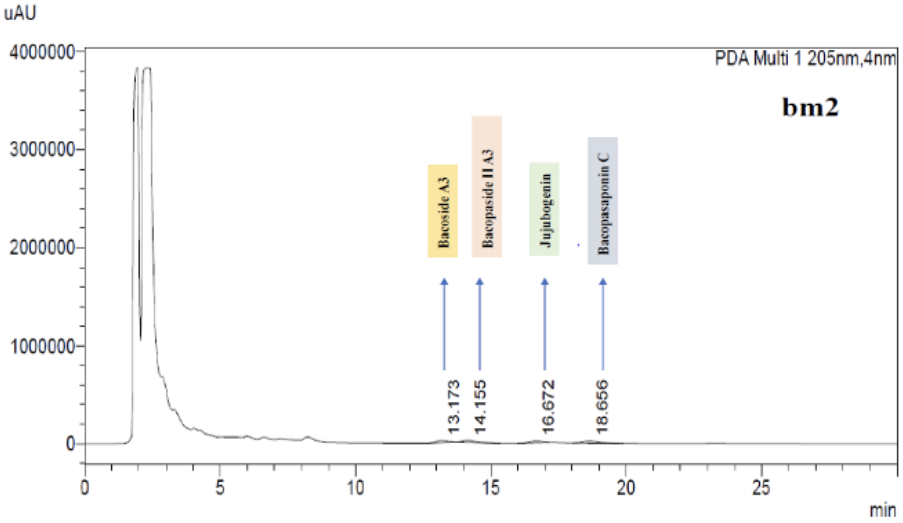

(C)

Fig. 4. HPLC chromatogram of *Bacopa monnieri* Pearson Correlation coefficient (r) between micronutrient content, oxidative indicators, antioxidant activity and proline accumulation of *Bacopa monnieri* under different level of Zn and Cu during 2019-2020

Table 1. Pearson Correlation coefficient (r) between micronutrient content, oxidative status, antioxidant activity and proline accumulation of *Bacopa monnieri* under different level of Zn and Cu during 2019

	Zn	Cu	H_2O_2	MDA	SOD	APOX	PA
Zn	1						
Cu	0.80*	1					
H_2O_2	0.76*	0.73	1				
MDA	0.75	0.76	0.88**	1			
SOD	0.82*	0.80	0.76*	0.93**	1		
APOX	0.70	0.50	0.84*	0.83*	0.83*	1	
PA	-0.16	0.06	0.16	0.47	0.35	0.25	1

*Correlation is *Significant at $p < 0.05$ and **Significant at $p < 0.01$.*
Note: Zn (zinc); Cu (copper); MDA (malondialdehyde); H_2O_2 (hydrogen peroxide); SOD (superoxide dismutase), APOX (ascorbate peroxidase) and PA (proline accumulation)

Table 2. Pearson Correlation coefficient (r) between micronutrient content, oxidative status, antioxidant activity and proline accumulation of *Bacopa monnieri* under different level of Zn and Cu during 2020

	Zn	Cu	H_2O_2	MDA	SOD	APOX	PA
Zn	1						
Cu	0.53	1					
H_2O_2	0.76*	0.84*	1				
MDA	0.78*	0.84*	0.84*	1			
SOD	0.97**	0.62	0.77*	0.86*	1		
APOX	0.79*	0.76*	0.73	0.88**	0.92**	1	
PA	0.29	0.25	0.03	0.56	0.44	0.55	1

*Correlation is *Significant at p < 0.05 and **Significant at p < 0.01.*
Note: Zn (zinc); Cu (copper); MDA (malondialdehyde); H_2O_2 (hydrogen peroxide); SOD (superoxide dismutase), APOX (ascorbate peroxidase) and PA (proline accumulation).

4. DISCUSSION

Because the levels were low in this study, no antagonistic or competitive behaviour between Cu and Zn was found in *Bacopa* leaves and most of the results showed Zn and Cu accumulation was improved when applied in combination of both. The finding revealed that the nutrient in plant cells was well distributed and accumulated as a result of Zn and Cu exterior exposure. The beneficial effect of foliar micronutrient spray on crop growth could be attributed to the crop's improved ability to absorb nutrients, photosynthesis and improved sink source connections, all of which are important in various biochemical processes. These findings are in conformity with the studies of [13]. Because Cu regulates redox reactions and is a component of multiple enzymes, it is well recognized that Zn and Cu have substantial metabolic effects. The effect of Zn on metabolism, on the other hand, is linked to its impact on gene expression and DNA replication [14]. During years of research in A. *millefolium, T. vulgare, E. purpurea* each Kg of Zn fertiliser increased Zn content in plants by 0.30 and 0.38 $mgKg^{-1}$, Cu by 0.07, 0.05, 0.03 $mgKg^{-1}$, respectively while 1 Kg of Cu fertiliser increased Cu content by 0.47, 0.07 and 0.23 $mgKg^{-1}$ and Zn by 1.40, 2.32 and 1.26 $mgKg^{-1}$, respectively. The elements Zn↔Cu and Cu↔Zn have been found to have synergistic interaction [15]. In comparison with control leaves, foliar application of ZnO nanoparticles (NPs) considerably boosted the nutrient content of Sweet Basil leaves namely, Zn, and Cu [16]. Our findings are more consistent with a prior study on *Zea mays* L., which found that plants sprayed with Cu and Zn or both had increased Cu content [17].

Cassia angustifolia seeds were germinated on Knop's medium with Zn and Cu at varied concentration (0, 1, 10, 50, 100 and 200 mgL^{-1}) during research. Lipid peroxidation was shown to begin even at extremely low concentrations of Zn (1 mgL^{-1}), and its increased 1.24-fold when compared to control. Further increasing Zn levels increased MDA content by 1.89, 2.12, 2.21 and 2.5-fold respectively, over control. Cu stress had similar effects on MDA content, which increased by1.33, 2.06, 2.30, 2.6 and 3.33 respectively, that of control [18]. It was noted that both deficient and excess amounts of Zn resulted in greater levels of MDA, H_2O_2, $O_2^{\bullet-}$ and electrolyte leakage in *Camellia sinensis* when compared to the optimal condition [19]. Similarly, with increasing Zn concentrations MDA, H_2O_2, $O_2^{\bullet-}$ and electrolyte leakage elevated Zn (25 and 50 µM) for 60 days in Cotton plant [20]. The same result reported in ryegrass (*Lolium perenne* L.), MDA, H_2O_2, $O_2^{\bullet-}$ generation rate *was* significantly high under 200 µM Cu condition for 14 days [21].

As the result of the Cu-Zn SOD enzyme's role in the conversion of ROS to H_2O_2 and oxygen, cells are protected from ROS. In the area of the PS-I, the enzymes SOD and APOX are connected to the chloroplastic thylakoids. APOX protects cellular lipids from peroxidation by converting H_2O_2 to water [22]. According to previous study, different concentrations of Zn and Cu (Zn1:2ppm, Zn2:4ppm, Zn3:6ppm, Cu1:0.1ppm, Cu2:0.3ppm and Cu3:0.5ppm) were used in *Centella asiatica* results revealed that SOD activity increases with Zn1 and Zn2 and decrease at high level Zn3 (0.88, 0.95 and 0.78 nmol/mg/g) whereas APOX noted increase with the increasing level of Zn singly (0.28, 0.47 and 0.95 nmol/mg/g). However, Zn and Cu in combination, Zn1Cu1, Zn2Cu2 and Zn3Cu3 showed higher SOD (0.98, 1.03 and 1.11 nmol/mg/g) and APOX activity with Zn1Cu1, Zn2Cu2 (0.61, 0.97 nmol/mg/g) except Zn3Cu3 (0.25 nmol/mg/g). APOX and SOD activity in leaves were increased when Zn was added

together with Cu [23]. Earlier research considered with four treatments with different concentrations of Cu (50, 125, 250, 500 mgL^{-1}, nanoparticles) applied twice in tomato fruits result revealed that APOX enzyme showed a maximum value with 500 mgL^{-1} of Cu, since the 250 mgL^{-1} of Cu treatment generated the lowest value, 54.55% [24]. Proline as an amphiphilic (hydrophobic-hydrophilic) amino acid can bind to hydrophobic protein moieties, creating more hydrophilic domains on their surface, allowing water molecules to bind. In this way, high proline levels contribute to maintaining the integrity and functionality of proteins [25]. Earlier investigation was conducted in seven-day-old bean seedlings (*Phaseolus vulgaris* L.) that were applied with $CuCl_2$ (0.1, 0.2 and 0.3 mM) showed with the increase in concentration proline content increased by 12.2% (0.1 mM Cu), 21.3% (0.2 mM Cu) and 30.9% (0.3 mM Cu) [26]. Previously, under the presence of Cu and Zn showed higher proline in plants at (500 and 1000ppm) copper and (1500 and 3000ppm) zinc that of control plants [27]. Other authors have also confirmed increase in proline content in different plant species after treatment with different concentrations of heavy metals (for example cadmium, lead, mercury, copper, and zinc) [28,29].

The role of elicitors and salt stress influenced the involvement of various enzymes in Bacoside production in *B. monnieri* [30]. The finding supports the theory that Zn can improve steviol glycosides (SVglys) quality. In terms of total SYglys identified, it was found that ZnO NPs had the highest content at 1 mg/l, whereas CuO NPs had highest at 10 mg/l of NPs concentrations [31]. The present study accordance, ZnO NPs supplementation can affect the transcriptional modulation of the HMG reductase enzyme for bacoside A production in *Bacopa* plant [32]. Thus, our finding suggest that Zn could upregulate the HMGR, MDD and SQS may be the explanation of higher bacoside content. According to previous findings Cu had positive impact on bacoside production [33,34]. In our experiment copper doesn't appear effective in terms of Bacoside-A content but it was recorded Bacoside A3 and Bacopaside II appeared higher peak area when compared with bm2 and bm0. It is suggested that the higher amount of Bacopasaponin C in bm2 responsible for increased Bacoside A content when compared to other compounds in dried leaves of Bacopa. The difference between these compounds might be due to inherent or external Cu and Zn that may varied in its synthesis or prolonged stored sample caused deterioration of the bioactive constituents. Generally, the content of Bacoside is 0.8- 1.5% and it may vary with post-harvest condition, harvesting time, soil factors and free bacosides are degraded [35].

5. CONCLUSION

The elevated level of MDA and H_2O_2 suggested that Zn and Cu were responsible for the generation of free radicles, leading to membrane damage and lipid peroxidation that activate the antioxidant system. From the results, it may be concluded that both elements significantly influenced each other accumulation too that triggers the antioxidant system and an optimum dose of Zn and Cu result in a better content of Bacoside A which is a demand of herbal drugs market. Therefore, a study is required for the confirmation of specific micronutrient involved in expression of key enzymes of bacoside pathway. However, further research with application rates may be needed to establish optimal and toxicity levels of these two elements and other micronutrient strategies to improve nutrient status and antioxidant activity of *Bacopa* or other medicinal herbs.

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DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

SUPPLEMENTARY MATERIALS

Supplementary materials available in this link: https://journaljsrr.com/media/JSRR122150.pdf

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Jin-Zhao M, Min Z, Zhi-Guang L, Min W, Sune S, Wem-Kui Z, Hua L. Copper-based-zinc-boron foliar fertilizer improved yield, quality, physiological characteristics, and microelement concentration of celery (*Apium graveolens* L.), Environment pollutants and bioavailability. 2019;31:261–271.
   Available:https://doi.org/10.1080/26395940.2019.1668859
2. Smitha GR, Kalaivanan D, Sujatha S. Influence of shade and organic nutrition on growth, yield and quality of memory enhancing herb, Brahmi (*Bacopa monnieri* L.), Medicinal Plants. 2021;13:72-90.
3. Production and post harvest technology of brahmi. ICAR-IIHR, Bengaluru, Extension folder no. 117.
   Available: https://ww.iihr.res.in
4. Vishwakarma RK, Patel K, Sonawane P, Kumari U, Singh S, Ruby Abbassi S, Agrawal DC, Tsay HS, Khan BM. Squalene Synthase Gene from Medicinal Herb *Bacopa monniera*: Molecular Characterization, Differential Expression, Comparative Modelling, and Docking Studies, Plant Molecular Biology Reports. 2015;33(6):1675-1685.
5. Gupta R, Singh A, Srivastava M, Singh V, Gupta MM, R. Pandey, Microbial modulation of bacoside A biosynthetic pathway and systemic defense mechanism in *Bacopa monnieri* under Meloidogyne incognita stress. Scientific reports. 2017;7(1):1-11.
6. Jahan S, Tamta S, Shankhdhar SC, Shankhdhar D. Salicylic acid potential to reversing drought induced oxidative stress in *Bacopa monnieri* (L.) through enhancement of bioactive compound (Bacoside-A) and antioxidants including physio-biochemical attributes. South African Journal of Botany. 2023;161: 617-626.
7. Hussain J, Khan AL, Rehman N, Hamayun M, Shinwari ZK, Ullah W, Lee IJ. Assessment of herbal products and their composite medicinal plants through proximate and micronutrients analysis. Journal of Medicinal Plants Research 2009;3:1072-1077.
8. Health RL, Packer L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation, Archives of biochemistry and biophysics. 1968;125:189-198.
9. Velikova V, Yordanov I, Edreva A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. Plant Science. 2000, 151, 59–66.
10. Beauchamp, C., Fridovich, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry. 1971;44(1):276- 287.
11. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and cell physiology. 1981;22:867-880.
12. Bates LS, Waldren RP, Teare LD. Rapid determination of free proline for water stress studies. Plant and Soil. 1973;39: 205-207.
13. Nasiri Y, Zehtab-Salmasi S, Nasrullahzadeh S, Najafi N, Ghassemi-Golezani K. Effects of foliar application of micronutrients (Fe and Zn) on flower yield and essential oil of chamomile (*Matricaria chamomilla* L.). Journal of Medicinal Plants Research. 2010; 1733-1737.
14. Marschner H. (Ed.). Marschner's mineral nutrition of higher plants. Academic press; 2011.
15. Zharkova NN, Suhotskaya VV, Ermohin YI. Impact of the use of essential trace elements (zinc, copper) on the chemical composition of perennial medicinal plants in southern Western Siberia. In IOP Conference Series: Earth and Environmental Science. 2021;624: 012179.
16. El-Kereti MA, El-feky SA, Khater MS, Osman YA, A El-sherbini ES. ZnO nanofertilizer and He Ne laser irradiation for promoting growth and yield of sweet basil plant, Recent Patents on Food Nutrition Agriculture. 2013;5:169–181.
17. Iqbal MN, Rasheed R, Ashraf MY, Ashraf MA, Hussain I. Exogenously applied zinc and copper mitigate salinity effect in maize (*Zea mays* L.) by improving key physiological and biochemical attributes, Environment Science and Pollution Research. 2018;25: 23883-23896.

18. Nanda R, Agrawal V. Elucidation of zinc and copper induced oxidative stress, DNA damage and activation of defence system during seed germination in Cassia angustifolia Vahl., Environmental and Experimental Botany. 2016;125:31-41.
19. Mukhopadhyay M, Das A, Subba P, Bantawal P, Sarkar B, Ghosh P, Mondal TK. Structural, physiological, and biochemical profiling of tea plants under zinc stress, Biologia Plantarum. 2013;57: 474–480.
20. Anwaar SA, Ali S, Ishaque W, Farid M, Farooq MA, Najeeb U, Abbas F, Sharif M. Silicon (Si) alleviates cotton (*Gossypium hirsutum* L.) from zinc (Zn) toxicity stress by limiting Zn uptake and oxidative damage, Environmental Science and Pollution Research. 2015; 22:3441–3450.
21. Dong Y, Xu L, Wang Q, Fan Z, Kong J, Bai X. Effects of exogenous nitric oxide on photosynthesis, antioxidative ability, and mineral element contents of perennial ryegrass under copper stress, Journal of Plant Interactions. 2014;9:402–411
22. Pandey ST, Ardeep; Negi, M.S.; Reena. Brahmi: An emerging medicinal crop for Tarai region of Uttarakhand, Indian Farmers' Digest. 2018;51:17-18
23. Ong GH, Yap CK, Maziah M, Tan SG. The effect of cu exposure on the bioaccumulation of Zn and antioxidant activities in different parts of Centella asiatica, Asian Journal of Microbiology Biotechnology and Environmental Sciences. 2011;13:387-392
24. López-Vargas ER, Ortega-Ortíz H, Cadenas-Pliego G, de Alba Romenus K, Cabrera de la Fuente M, Benavides-Mendoza A, Juárez- Maldonado A. Foliar application of copper nanoparticles increases the fruit quality and the content of bioactive compounds in tomatoes, Applied Sciences. 2018;8.
DOI:10.3390/app8071020
25. Sharmila P, Saradhi PP. Proline accumulation in heavy metal stresses plants: an adaptive strategy. M. N. Prasad, K. Strzałka (Eds.), Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants, Springer Science & Business Media. 2002;179-199.
26. Zengin FK, Munzuroglu O. Effects of some heavy metals on content of chlorophyll, proline and some antioxidant chemicals in bean (Phaseolus vulgaris l.) seedlings, Acta biologica cracoviensia Series Botanica. 2005;47/2:157–164.
27. Kulbat-Warycha K, Georgiadou EC, Mańkowska D, Smolińska B, Fotopoulos V, Leszczyńska J. Response to stress and allergen production caused by metal ions (Ni, Cu and Zn) in oregano (*Origanum vulgare* L.) plants, Journal of Biotechnology. 2020;324: 171-182.
28. Dinkar N, Nagajyothi PC, Suresh S, Damodharam T, Suresh C. Cadmium induced changes on proline, antioxidant enzymes, nitrate and nitrite reductases in *Arachis hypogaea* L., Journal of Environmental Biology. 2009;30:289–294.
29. Handique GK, Handique AK. Proline accumulation in lemongrass (Cymbopogon flexuosus Stapf.) due to heavy metal stress, Journal of Environmental Biology. 2009;30:299–302.
30. Murthy HN. Biotechnological production of bacosides from cell and organ cultures of *Bacopa monnieri*. Applied Microbiology and Biotechnology. 2022;106:1799–1811.
31. Javed R, Usman M, Y€ucesan B, Zia M, G€urel E. Effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *Stevia rebaudiana* Bertoni. Plant Physiology and Biochemistry. 2017;110.
DOI: 10.1016/j.plaphy.2016.05.032
32. Bhardwaj P, Goswami N, Narula P, Jain CK, Mathur A. Zinc oxide nanoparticles (ZnO NP) mediated regulation of bacosides biosynthesis and transcriptional correlation of HMG-CoA reductase gene in suspension culture of *Bacopa monnieri*. Plant Physiology and Biochememistry. 2018;130:148-156.
33. Sharma M, Ahuja A, Gupta R, Mallubhotla S. Enhanced bacoside production in shoot cultures of Bacopa monnieri under the influence of abiotic elicitors. Natural product Research. Formerly Natural Product letters. 2015;29(8):745-749.
34. Chung IM, Thiruvengadam, M. Impact of Copper Oxide Nanoparticles on Enhancement of Gymnemic Acid and Phenolic Compounds Using Cell Suspension Culture of *Gymnema sylvestre* (Retz.). Applied sciences. 2019;9: 2165.
DOI: 10.3390/app9102165

35. Silpa SG, Smitha GR, Ranjitha K. Drying and packaging methods impact the bacoside profile and microbiological quality of Brahmi herb (*Bacopa monnieri* L.) during storage. Industrial Crops & Products. 2021; 159.
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