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Modulation of nickel toxicity by glycinebetaine and aspirin in *Pennisetum typhoideum*

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ABSTRACT Germinated *Pennisetum typhoideum* seeds were grown under phytotoxic amount of nickel (Ni) and its combinations with aspirin (Asp) and/or glycinebetaine (GB). The results revealed that exposure to Ni caused reduced growth and membrane stability index of *P. typhoideum*, which were correlated with the accumulated Ni and reactive oxygen species. Oxidative stress markers; malondialdehyde, 4-hydroxy-2-nonenal and lipoxygenase were also elevated by Ni, while were diminished significantly by exogenously applied Asp and/or GB. However, considerable loss in protein and DNA contents were discernible in Ni subjected tissues, but were stimulated largely in the Asp and/or GB applied radicles. Additionally, alteration in the activities and native-PAGE profiles of antioxidant enzymes (superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase) were discernible in response to Ni, which are reputed to counterbalance the oxidative condition. However, exogenous addition of Asp and/or GB activated the defense system and uplifted proline accumulation in stressed *P. typhoideum*. The results approved that combined addition of Asp and GB performed far better in Ni-stress mitigation than their alone application. Conducted study indicated that combined application of Asp and GB served as complementary tool to confer tolerance by up-regulating the antioxidant enzymes and thus can be implicated in the mitigation of Ni-toxicity.

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Introduction

Nickel (Ni) is necessarily required by the plants for their normal growth/development and completion of life cycle, but is equally toxic in excess leading to altered growth and metabolism (Negi et al. 2014). It is essentially required by the enzyme urease that metabolizes the nitrogenous compounds like urea, inside the plant (Hussain et al. 2013). Therefore, its deficiency not only lowers down the nitrogen assimilation rate, but also results superoxide ($O_2^{\cdot-}$) accumulation (Negi et al. 2014). In normal course, Ni is required by the plants within 0.05 to 10 µg g^{-1} dry mass (DM) for their optimum growth and development, synthesis of anthocyanin, and to resist diseases (Stanisavljevic et al. 2012). However, over accumulation of it alters metabolic status and uptake of both water and nutrients (Gajewska et al. 2012). Nickel also causes hindrance in the activities of key enzymes like amylase, protease and ribonuclease, thereby inhibited seed germination and growth responses. The most obvious symptoms of Ni-toxicity includes decreased root and shoot growth, chlorosis, necrosis, wilting,

altered mineral nutrition and water relations, photosynthesis, respiration, nitrogen and carbohydrate metabolism in plants (Kazemi et al. 2010; Hussain et al. 2013).

Although, Ni is not a redox-active metal, even though studies indicate that toxicity of it is associated with production of reactive oxygen species (ROS) like; $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) consequently oxidative stress in plants. Regardless of this fact, studies on antioxidant system in Ni-stressed plants confirmed that it interferes with the defense responses (Hussain et al. 2013). Therefore, over accumulation of ROS prompts degenerative modifications in nucleic acids, proteins and lipids (Chandrakar et al. 2016a, 2017). Poly unsaturated fatty acids (PUFAs) of membrane lipid are prone to ROS assault and lipoxygenase (LOX: EC 1.13.11.12), which releases aldehydic cytotoxic products after like malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) (Chandrakar et al. 2016a). To adjust with oxidative condition plants developed an integrated network of defense system comprising both enzymatic and non-enzymatic candidates, former includes superoxide dismutase (SOD: EC 1.15.1.1), catalase (CATEC: in between CAT and EC. 1.11.1.6), ascorbate peroxidase (APX: EC 1.11.1.11) and guaiacol peroxidase (POD: EC 1.11.1.7), while later consists of α-tocopherol, ascorbic acid, glycinebetaine (GB), and pro-

line, which possesses free radical scavenging ability (Jafari et al. 2015; Chandrakar et al. 2016b; Yadu et al. 2017).

In the past, efforts have been made to restore normal growth and development in plants exposed to abiotic stresses including Ni (Kazemi et al. 2010; Siddiqui et al. 2013). Even though, roles of GB and/or aspirin (Asp) in the Ni-stress mitigation in plants are still to be established completely. Glycinebetaine, a compatible solute, largely accumulates in stressed tissues (Lou et al. 2015). Moreover, its exogenous application awards stress tolerance to the affected plants by protecting the photosynthetic machinery, stabilizing proteins and scavenging ROS (Nusrat et al. 2014). Till now, involvement of GB in salinity and few of the metal stress mitigation in plants has only been shown (Kaya et al. 2013; Nusrat et al. 2014; Ali et al. 2015), however its role in Ni-stress amelioration is still to be resolved fully. Correspondingly, Asp provides stress tolerance to the plants by controlling the ROS and up-regulating antioxidants (Zhen et al. 2010). Till this date, it has been exploited to modulate adverse effects of both salinity and drought (Senaratna et al. 2000; Daneshmand et al. 2009), but its mitigation efficiency against metal stress, particularly Ni, remains to be obscure. Considering the protective functions of GB and Asp, in the present study an effort has been made to investigate the protective roles of these two compounds on growth of Ni subjected *P. typhoideum*. Application of GB and/or Asp may improve the growth performance of tested radicles by lowering the Ni uptake, ROS generation and lipid peroxidation, while improving vital metabolic activities such as viability, membrane stability index (MSI), antioxidant enzymes and contents of protein, proline and DNA.

Materials and Methods

Seed germination, treatments and growth analysis

Seeds of *Pennisetum typhoideum* were disinfected with sodium hypochlorite solution (0.1%, v/v) for 3 min, and washed thoroughly (5 times) with MilliQ water (MW) (Millipore, Gradient A-10, USA). Sterilized seeds were placed over two layers of filter paper pre-soaked with MW, in germination boxes of 26 x 16 x 6 cm size (Chandrakar et al. 2016a). These boxes were kept in the dark at room temperature (RT, 26-28 °C) until the emergence of 1 mm sized radicles in seeds.

The 1 mm radicle bearing seeds were now supplemented with prefixed treatments: MW (control), 135 ppm Ni (NiCl$_2$, as source), 100 μM each of GB and Asp separately, GB + Asp, Ni + GB, Ni + Asp and Ni + GB + Asp. On 5th day of growth, radicles were carefully harvested for the assessment of different parameters. Initially, change in the radicle length (of 10 radicles) was noted, and then to monitor DM, five sets

of randomly selected 10 radicles each, were oven dried at 103 °C for 48 h and weighed electronically. Left over excised radicles were ground in liquid nitrogen (LN$_2$) and stored at -80 °C in sterile vials for further usage. All the evaluations were done in five replicates and repeated twice.

Membrane stability index

To monitor MSI, 1 g of radicles was taken in a test tube, containing 10 mL of MW and then divided into two sets. Out of these, one set was heated at 40 °C for 30 min, and second was at 100 °C for 10 min in water baths, and electrical conductivity of the solutions were recorded as T_1 and T_2 respectively, using EC-TDS analyzer (CM-183, Elico, India) (Chandrakar et al. 2017). Membrane stability index was calculated using the formula: MSI (%) = $[1-T_1/T_2] \times 100$.

Determination of viability

For tissue viability, 0.2 g of radicles were overnight soaked in 2 mL 0.5% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) solution in the dark at RT (Lakon 1949). Thereafter, tissues were extracted with ethanol (2 mL) and centrifuged (5000 rpm, 10 min). Absorbance of the supernatants were read at 520 nm using an UV-spectrophotometer (Lambda-25, Perkin Elmer, USA), and values were expressed as A_{520} g^{-1} FM (fresh mass).

Estimation of Ni

Oven dried (65 °C for 24 h) tissues were digested with a mixture of HNO$_3$:H$_2$SO$_4$:H$_2$O$_2$ (4:1:1) at 80 °C for 3 h. Digested samples were filtered through Whatman paper (42), and their volumes were adjusted by MW (Zheljazkov and Nielson 1996). For quality assurance, standard reference (NIST, USA) was included. Concentration of Ni was determined using an atomic absorption spectrophotometer (AA 8000, Labindia, India), and values were presented as μg g^{-1} DM.

Determination of ROS

The LN$_2$ crushed samples (0.2 g) were homogenized in 2 mL chilled sodium phosphate buffer (0.2 M, pH 7.2) consisting 0.001 M diethyldithiocarbamate and centrifuged (12000 rpm, 15 min, 4 °C). To the supernatant (200 μL), 2.4 mL sodium phosphate buffer (0.2 M, pH 7.2) and 100 μL nitroblue tetrazolium (NBT, 2.5 x 10^{-4} M) were added and absorbance was recorded at 540 nm (Sangeetha et al. 1990). The O$_2$$^{·-}$ content was expressed as μmol min^{-1} g^{-1} FM. For determination of H2O2, the samples (0.2 g) were homogenized with 0.1% (w/v) trichloroacetic acid (TCA, 2 mL), and then centrifuged (10000 rpm, 15 min). The 0.5 mL of sodium phosphate buffer (10 mM, pH 7.0) and 1 mL of potassium iodide (1 M)

were added to the supernatant (0.5 mL), and absorbance was recorded at 390 nm (Velikova et al. 2000). Content of H_2O_2 was expressed as µmol g^{-1} FM. For ·OH, 0.2 g sample was homogenized in 2 mL sodium phosphate buffer (10 mM, pH 7.4) comprising 15 mM 2-deoxy-ribose and centrifuged (11000 rpm, 15 min). Supernatant was incubated at 37 °C for 2 h. To this (0.7 mL), added 3 mL of thiobarbituric acid {TBA, 0.5% (w/v) in 5 mM NaOH}, and 1 mL of glacial acetic acid, incubated at 100 ºC for 30 min and then cooled at 4 °C. Absorbance was read at 532 nm and corrected for non-specific absorbance at 600 nm (Chandrakar et al. 2016a). Content of ·OH was expressed as nmol g^{-1} FM.

Monitoring of MDA and 4-HNE

The LN_2 crushed radicles (0.2 g) were homogenized with 2 mL of 0.5% (w/v) TBA {prepared in 20% (w/v) TCA} (Velikova et al. 2000). The homogenate was incubated at 95 °C for 30 min, then quickly cooled in an ice bath (10 min), and centrifuged (10000 rpm, 10 min). The absorbance of the supernatant was recorded at 532 nm and non-specific absorbance was read at 600 nm. Amount of MDA was expressed as mmol g^{-1} FM. For 4-HNE estimation, 0.2 g samples were extracted with 2 mL cold borate buffer (0.2 M, pH 7.4), and 1.5 mL 10% (w/v) TCA, then centrifuged (11000 rpm for 15 min). Supernatant (1 mL) was mixed with 1 mL of 2,4-dinitrophenyl hydrazine (1 mg mL^{-1}, in 0.5 M HCl) and allowed to stand for 2 h at RT. Now, the sample was extracted with hexane and dried under LN_2. After cooling, added 2 mL of methanol and absorbance was read at 350 nm against methanol as blank (Ray et al. 2007). The level of 4-HNE was expressed as mmol g^{-1} FM.

Estimation of LOX

Lipoxygenase was extracted by homogenizing 0.2 g samples in 2 mL cold borate buffer (0.2 M, pH 7.4) followed by centrifugation (11000 rpm, 15 min at 4 °C). Substrate was prepared by adding 10 µL of linoleic acid in 25 mL of 0.1% (w/v) sodium tetraborate containing 0.1% (v/v) Tween-20. The 0.1 mL of substrate was suspended in 2.9 mL of sodium phosphate buffer (0.1 M, pH 4.5), and shaken vigorously. To initiate reaction, 0.1 mL extracted enzyme was added and absorbance at 234 nm was recorded (Chandrakar et al. 2016a). Activity of LOX was expressed in terms of µmol min^{-1} g^{-1} FM.

DNA: extraction and estimation

Genomic DNA was isolated following the CTAB method (Doyle and Doyle 1987). DNA content was expressed as mg g^{-1} FM.

Extraction of protein and enzymes

Radicles (0.5 g) were extracted with 10 mL of cold potassium phosphate buffer (10 mM, pH 7.2) containing 1 mM EDTA, 2 mM DTT and 0.2% (v/v) Triton X-100, and centrifuged (11000 rpm, 20 min, 4 °C). The supernatant was used as source of both protein and enzymes.

Estimation of protein and proline

Protein was assayed following Bradford (1976). Bovine serum albumin was used as standard and content of protein was expressed as µg g^{-1} FM. Proline was quantified following the method of Bates et al. (1973). The L-proline was used as standard and content of proline was expressed as mg g^{-1} FM.

Determination of antioxidant enzymes

Following Marklund and Marklund (1974), the activity of SOD was monitored by measuring percent inhibition of pyrogallol auto-oxidation by the enzyme at 420 nm. Enzyme activity was expressed as units of SOD min^{-1} g^{-1} FM. CAT was assayed spectrophotometrically at 240 nm by the procedure of Chance and Maehly (1955) and its activity was expressed as nmol min^{-1} g^{-1} FM. Activity of POD was measured after Chance and Maehly (1955) by estimating oxidation of guaiacol in presence of H_2O_2 at 470 nm. Activity of it was expressed as µmol min^{-1} g^{-1} FM. APX was assayed following the method of Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. Enzyme activity was expressed as mmol min^{-1} g^{-1} FM.

Isozyme analysis

Electrophoretic performance of SOD, CAT, POD and APX were analyzed over Native-PAGE gels (10%) using Tris-Glycine buffer (5 mM, pH 8.3) (in case of APX, running buffer consisted of 4 mM ascorbate), at 4 °C for 2 h with a constant current of 20 mA, using Mini-Protean tetra cell (BioRad, USA). After running, gels were imaged and analyzed using Gel-Doc (BioRad, USA). To determine the SOD activity, gels were incubated in dark for 20 min in NBT (2.45 mM) solution, and then immersed in 36 mM dipotassium hydrogen phosphate (pH 7.8), containing 28 µM riboflavin and 28 mM TEMED, until the gel turns blue, except the region(s) showing SOD activity (Chandrakar et al. 2016a). Gel for CAT activity was stained following the method of Woodbury et al. (1971). Initially, the gels were incubated in 0.03% (v/v) H_2O_2 solution for 10 min. Thereafter, they were rinsed quickly in MW and stained with 1% (w/v) each of potassium ferricyanide and ferric chloride, sequentially. As soon as a green color began to appear, generally within 5-10 min, gels were washed with MW. For APX detection, gels were equilibrated with 50 mM

Table 1. Effects of different combinations of nickel (Ni), aspirin (Asp) and glycinebetaine (GB) on growth, viability, membrane stability index (MSI) and Ni content of *P. typhoideum* radicles. Data represents mean (± SD) of five replicates. Each value followed by small alphabets indicates significant differences at 0.05% level.

Treatments	Radicle length (mm)	Dry mass (mg)	Viability (A$_{520}$ g^{-1} FM)	MSI (%)	Ni content (μg g^{-1} DM)
Control	41^c ± 3	13.13^d ± 0.25	6.75^d ± 0.5	20.9^c ± 0.6	ND
Ni	19^g ± 4	7.13^h ± 0.75	1.7^h ± 0.2	5.60^f ± 1.7	5.60^a ± 0.23
GB	48^b ± 1	18.20^b ± 0.26	9.932^b ± 0.06	29.13^b ± 2.2	ND
Asp	41^c ± 1	15.03^c ± 0.58	7.82^c ± 0.22	22.7^c ± 0.6	ND
GB + Asp	53^a ± 3	21.30^a ± 0.36	12.75^a ± 0.5	32.01^a ± 2.75	ND
Ni + GB	36^e ± 2	10.16^f ± 0.32	3.73^f ± 0.15	12.45^d ± 0.2	4.02^c ± 0.14
Ni + Asp	33^f ± 1	8.73^g ± 0.35	2.62^g ± 0.05	8.50^e ± 0.7	4.75^b ± 0.09
Ni+ GB + Asp	37^d ± 1	11.46^e ± 0.58	4.67^e ± 0.05	13.57^d ± 0.1	2.19^d ± 0.30

ND = Not determined

sodium phosphate buffer (pH 7) containing 4 mM ascorbate for 20 min (Mittler and Zilinskas 1993). Afterwards, the gels were incubated with 50 mM sodium phosphate buffer (pH 7) containing 4 mM ascorbate and 4 mM H$_2$O$_2$ for 20 min. Finally, gels were washed twice with sodium phosphate buffer (50 mM, pH 7) and stained in 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 10-15 min. Isozymes of POD were revealed following Srivastava and Huystee (1977). Initially, the gels were equilibrated with 100 mM sodium phosphate buffer (pH 6.5) for 15 min and then stained for 10 min with 12.5 mM guaiacol solution consisting benzidine (1.7 mM) and H$_2$O$_2$ (12 mM). After gentle shaking, brown colored bands appeared against a clear background.

Statistical analysis

Data were analyzed applying one-way ANOVA to unravel interaction of Ni with GB and/or Asp. Means were compared using Duncan's multiple range tests using SPSS (Ver. 16.0) and expressed as mean ± SD of five replicates. Significance difference was tested at $P < 0.05$.

Results

Growth traits

Physiological traits of *P. typhoideum* radicles were affected adversely, compared to the control when was subjected to Ni, but addition of Asp and/or GB in combination, revealed enhancement in these parameters (Table 1). Data witnessed that Ni remarkably reduced length (53%), biomass (36%), tissue viability (74%) and MSI (73%) of seedlings, compared to the controls. However, addition of Asp or GB into Ni sig-

nificantly (35-88%) alleviated these effects. Moreover, joint application of Asp and GB enhanced growth and vitality of stressed radicles far better (up to 93%) than their alone addition (Table 1).

Nickel content

Considerable amount (5.6 μg g^{-1} DM) of Ni was measured in the *P. typhoideum* radicles exposed to 135 ppm Ni. However, exogenous Asp and/or GB allowed limited (15-60% lesser) accumulation of it in the Ni-stressed samples (Table 1).

Oxidative stress markers

Treatment of Ni aggravated the production of all the three ROS in *P. typhoideum* radicles, compared to control (Table 2). Enhancement of around 206%, 112% and 160% in the O$_2^-$,·OH and H$_2$O$_2$ generation respectively, were observed in response to Ni. On the other hand, blending of Ni with Asp or GB resulted in limited accrual (42-124%) of ROS. Moreover, joint addition of Asp and GB permitted least accumulation of ROS in Ni-stressed seedlings (Table 2).

In Ni exposed radicles, levels of MDA, 4-HNE and LOX raised considerably (109%, 98% and 58% respectively) than their respective controls. Exogenous Asp or GB permitted low abundance of MDA, 4-HNE and LOX activity in Ni-stressed *P. typhoideum*. Moreover, combined addition of Asp and GB revealed least levels of MDA, 4-HNE and LOX (Table 2).

Exposure to Ni caused lowering of both protein (37%) and DNA (38%) in *P. typhoideum* radicles, compared to their respective controls (Table 3). However, these reductions were significantly (75-91%) alleviated by exogenous Asp or GB. Additionally, joint application of Asp and GB uplifted both protein and DNA (88-95%) turnover in Ni subjected radicles.

Table 2. Changes in superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) and lipoxygenase (LOX) in *P. typhoideum* radicles subjected to nickel (Ni), aspirin (Asp) and/or glycinebetaine (GB). Each data represents the mean (± SD) of five observations. Mean followed by different letters indicate significant differences at the 0.05% level compared with the control.

Treatments	$O_2^{\cdot-}$ (µmol min^{-1} g^{-1} FM)	$\cdot OH$ (nmol g^{-1} FM)	H_2O_2 (µmol g^{-1} FM)	MDA (mmol g^{-1} FM)	4-HNE (mmol g^{-1} FM)	LOX (µmol min^{-1} g^{-1} FM)
Control	92.5^d ± 2.16	0.25^{de} ± 0.02	2.00^{de} ± 0.22	48.60^d ± 3.0	2.28^d ± 0.17	8.42^{cd} ± 2.0
Ni	283.75^a ± 10.11	0.53^a ± 0.01	5.21^a ± 0.20	101.62^a ± 4.1	4.53^a ± 0.1	13.34^a ± 2.0
GB	77.5^f ± 1.94	0.24^d ± 0.01	1.68^{ef} ± 0.14	32.64^f ± 1.1	1.72^e ± 0.1	7.01^{de} ± 0.65
Asp	81.25^e ± 1.28	0.18^d ± 0.03	1.71^e ± 0.26	38.82^e ± 3.5	2.22^d ± 0.1	8.02^{cd} ± 1.0
GB+Asp	57.5^g ± 1.75	0.14^e ± 0.02	1.49^f ± 0.10	25.34^g ± 1.2	1.24^f ± 0.3	3.67^g ± 0.6
Ni + GB	145^c ± 8.84	0.40^c ± 0.03	2.86^c ± 0.10	63.67^c ± 2.1	3.75^b ± 0.2	10.32^{bc} ± 2.0
Ni + Asp	208.75^b ± 9.12	0.43^b ±0.02	3.86^b ± 0.25	80.58^b ± 4	4.04^b ± 0.2	10.86^b ± 1.0
Ni + GB + Asp	93.75^d ± 2.34	0.34^d ± 0.02	2.16^d ± 0.05	61.26^c ± 3.2	2.91^c ± 0.15	5.71^{ef} ± 0.25

Table 3. Variations in the levels of proline, DNA and protein in the *P. typhoideum* radicles exposed to different combinations of nickel (Ni), aspirin (Asp) and glycinebetaine (GB). Each value represents mean (±SD) of five replicates. Values possessing different letters are statistically significant at 0.05% probability level.
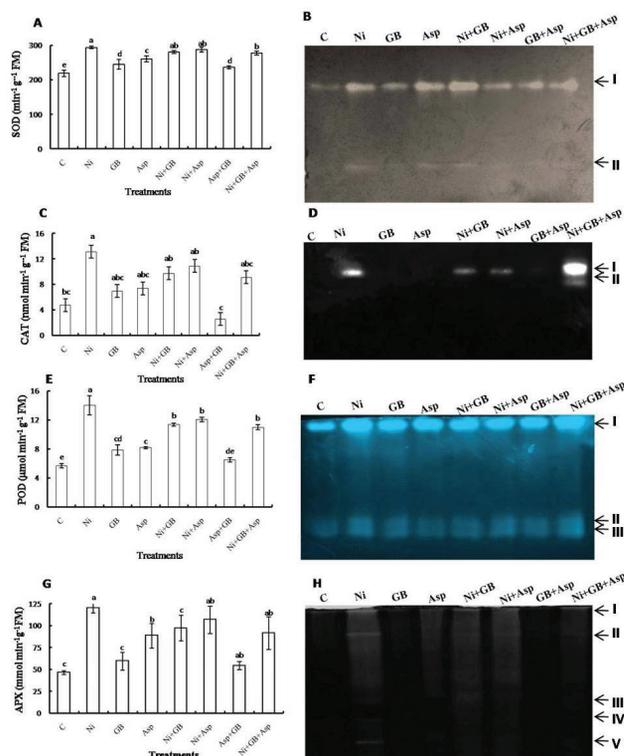
Treatments	Proline (mg g^{-1} FM)	DNA (mg g^{-1} FM)	Protein (µg g^{-1} FM)
Control	2.4^h ± 0.10	6.26^c ± 0.03	279.4^{bcd} ± 61.92
Ni	5.3^d ± 0.12	3.85^f ± 0.1	173.56^d ± 36.97
GB	2.8^g ± 0.07	7.19^b ± 0.3	366.34^{ab} ± 51.22
Asp	3.7^f ± 0.13	6.98^b ± 0.03	345.56^{bc} ± 65.48
GB + Asp	3.9^e ± 0.05	7.94^a ± 0.1	400.95^a ± 26.72
Ni + GB	5.7^c ± 0.04	4.92^e ± 0.01	251.68^{cd} ± 0.44
Ni + Asp	6.1^b ± 0.26	4.64^e ± 0.4	250.52^{cd} ± 35.19
Ni + GB + Asp	6.6^a ± 0.15	5.5^d ± 0.3	264.92^{bcd} ± 33.41

Discussion

In the present study, *P. typhoideum* radicles subjected to deleterious amount of Ni revealed inhibited growth in terms of reduced length and DM accumulation, which might be

Proline and antioxidant enzymes

Remarkable increase (120%) in proline content was discernible in Ni-stressed *P. typhoideum* radicles than that of control (Table 3). However, application of either Asp or GB along with Ni enhanced proline accumulation by 154% and 137% respectively, as compared to the control. Maximum (174%) proline was measured in both Asp and GB blended Ni-exposed *P. typhoideum* radicles. Activities of SOD, CAT, POD and APX were increased drastically (34, 175, 147 and 159%, respectively) by Ni in *P. typhoideum* radicles, compared to non-treated controls. Exogenous Asp and/or GB-induced only very small changes in the activities of tested enzymes (Fig. 1). These observations were in coherence with the electrophoretic profiles of tested antioxidants, which revealed two distinct isoforms each of SOD and CAT, three of POD, and five isoforms of APX, respectively. In general, all these isoforms were not only appeared, but also more pronounced in the Ni-stressed samples than of any other treatments, including control.

Figure 1. Spectrophotometric and Native-PAGE analyses of (A-B) superoxide dismutase (SOD), (C-D) catalase (CAT), (E-F) guaiacol peroxidase (POD) and (G-H) ascorbate peroxidase (APX) of *P. typhoideum* under nickel (Ni) stress alone or along with aspirin (Asp) and/or glycinebetaine (GB). Each bar represents mean (± SD) of five separate observations. Small letters correspond to significant difference at 0.05% level.

Table 4. Pearson's correlation coefficients of the studied parameters in the *P. typhoideum* radicles exposed to nickel (Ni), aspirin (Asp) and/or glycinebetaine (GB). Positive R and P < 0.05 meant the positive correlation (bold) between the two variables. Negative R and P < 0.05 meant the negative correlation (italic) between the two variables. P > 0.05 meant that there was no significant relationship between the two variables.

	DM	TTC	MSI	$O_2^{·-}$	·OH	H_2O_2	MDA	4-HNE	LOX	Proline	DNA	SOD	CAT	APX	POD
RL R	**0.895**	**0.896**	**0.918**	*-0.927*	*-0.909*	*-0.935*	*-0.966*	*-0.923*	*-0.858*	*-0.722*	**0.926**	-0.364	-0.371	*-0.842*	-0.457
P	**0.003**	**0.003**	**0.001**	*0.001*	*0.002*	*0.001*	*0.000*	*0.001*	*0.006*	*0.043*	**0.001**	0.376	0.366	*0.009*	0.254
DM R		0.988	**0.970**	*-0.798*	*-0.873*	*-0.797*	*-0.912*	*-0.938*	*-0.851*	-0.620	**0.948**	-0.091	-0.051	*-0.808*	-0.146
P		**0.000**	**0.000**	*0.018*	*0.005*	*0.018*	*0.002*	*0.001*	*0.007*	0.101	**0.000**	0.831	0.904	*0.015*	0.730
TTC R			**0.989**	*-0.825*	*-0.926*	*-0.827*	*-0.938*	*-0.967*	*-0.838*	*-0.722*	**0.979**	-0.153	-0.088	*-0.762*	-0.211
P			**0.000**	*0.012*	*0.001*	*0.011*	*0.001*	*0.000*	*0.009*	*0.043*	**0.000**	0.717	0.835	*0.028*	0.616
MSI R				*-0.856*	*-0.937*	*-0.863*	*-0.962*	*-0.978*	*-0.810*	*-0.784*	**0.984**	-0.238	-0.145	*-0.756*	-0.294
P				*0.007*	*0.001*	*0.006*	*0.000*	*0.000*	*0.015*	*0.021*	**0.000**	0.570	0.731	*0.030*	0.479
$O_2^{·-}$ R					**0.924**	**0.998**	**0.953**	**0.917**	**0.898**	**0.792**	*-0.895*	0.269	0.240	0.668	0.373
P					**0.001**	**0.000**	**0.000**	**0.001**	**0.002**	**0.019**	*0.003*	0.519	0.567	0.070	0.363
·OH R						**0.931**	**0.969**	**0.966**	**0.841**	**0.873**	*-0.979*	0.303	0.259	0.645	0.402
P						**0.001**	**0.000**	**0.000**	**0.009**	**0.005**	*0.000*	0.466	0.535	0.084	0.324
H_2O_2 R							0.961	0.918	0.877	**0.810**	*-0.902*	0.300	0.271	0.662	0.408
P							**0.000**	**0.001**	**0.004**	**0.015**	*0.002*	0.471	0.517	0.074	0.316
MDA R								0.968	0.854	0.815	*-0.976*	0.267	0.222	**0.718**	0.364
P								**0.000**	**0.007**	**0.014**	*0.000*	0.523	0.597	**0.045**	0.375
HNE R									**0.882**	**0.828**	*-0.987*	0.284	0.197	**0.756**	0.344
P									**0.004**	**0.011**	*0.000*	0.496	0.640	**0.030**	0.403
LOX R										0.563	*-0.852*	0.067	0.109	**0.755**	0.155
P										0.146	*0.007*	0.876	0.796	**0.030**	0.713
Proline R											*-0.813*	0.560	0.378	0.448	0.599
P											*0.014*	0.149	0.356	0.265	0.116
DNA R												-0.250	-0.198	*-0.718*	-0.335
P												0.550	0.639	*0.045*	0.418
SOD R													**0.917**	0.413	**0.972**
P													**0.001**	0.309	**0.000**
CAT R														0.411	**0.955**
P														0.312	**0.000**
APX R															0.411
P															0.311

4-HNE = 4-hydroxy-2-nonenal; APX = ascorbate peroxidase; CAT = catalase; DM = dry mass; H_2O_2 = hydrogen peroxide; LOX = lipoxygenase; MDA = malondialdehyde; MSI = membrane stability index; $O_2^{·-}$ = superoxide; ·OH = hydroxyl radical; POD = guaiacol peroxidase; RL = radicle length, SOD = superoxide dismutase and TTC = 2,3,5-triphenyl tetrazolium chloride.

the consequence of Ni-imposed inhibition in photosynthesis, respiration and uptake of nutrients and water affecting energy metabolism, and rate of cell division and elongation (Yusuf et al. 2012). Our results are in coherence with the findings of Yusuf et al. (2012) and Siddiqui et al. (2013). However, Asp and/or GB caused improvement in growth, which may be due to enhanced rate of nutrient uptake, photosynthesis and overall metabolic activities (Zhen et al. 2010; Nusrat et

al. 2014). Under abiotic stresses, addition of Asp and GB has been revealed to enhance expressions of genes responsive to ROS scavenging, photosynthesis, and energy utilization (Senaratna et al. 2000; Malekzadeh 2015). Radicles of *P. typhoideum* subjected to Ni accumulated considerable amount (5.6 µg g^{-1} DM) of it, which later resulted in altered integrity of membranes and loss of tissue viability. Leakage of cellular constituents is a key indicator of membrane deterioration

that occurs due to peroxidation of PUFA fractions (Kaya et al. 2013). Exposure to Ni adversely affected MSI and tissue viability (Tables 1 and 4). However, exogenous Asp and GB maintained membrane integrity and thereby ionic balance inside the stressed cells (Senaratna et al. 2000; Nusrat et al. 2014). Our results approved the mitigation potential of both Asp and GB by measuring alleviation (65-70%) in the MSI and cell vitality, and reduced accumulation of Ni, in stressed radicles. Similar kind of Asp- and GB-induced modulation in the MSI and ion uptake system has previously been reported by Daneshmand et al. (2009) and Nusrat et al. (2014).

In general, ROS are produced as off-spins of various metabolic pathways operative in sub-cellular compartments of plants (Stanisavljevic et al. 2012). Many abiotic factors, including Ni exposure are designated to produce ROS in abundance (Yusuf et al. 2012; Siddiqui et al. 2013). In congruent, we have noted remarkable upsurge (206-112%) in ROS in Ni-exposed *P. typhoideum* and were quite low up on addition of Asp and/or GB. Exogenous GB protects RUBISCO, a CO_2 fixing enzyme, which minimizes ROS production under abiotic stresses (Malekzadeh 2015). On the other hand, Asp led to reduced turnover of ROS *via* enhanced activities of antioxidant enzymes in stressed plants (Daneshmand et al. 2009). Studies have proven that LOX plays crucial role in the catabolism of phospholipids by initiating a lipolytic cascade in the cellular membranes consequently MDA and 4-HNE production in stressed cells (Yadu et al. 2016). Our results exemplified high levels of MDA, 4-HNE and LOX in Ni-treated samples (Table 2). Similar rise in LOX mediated peroxidative products has also been reported by Kazemi et al. (2010). While, addition of Asp and GB demonstrated lower activity of LOX, consequently enhance stability of the membrane lipids (Daneshmand et al. 2009; Ali et al. 2015).

In stressed plants, LOX and ROS alter lipid composition of membranes (Yadu et al. 2017). A relationship among lipid peroxidation, ROS generation and Ni application has been well reported (Stanisavljevic et al. 2012). The PUFAs of membranes are highly vulnerable to ROS assault and give rise to cytotoxic products (MDA and 4-HNE), which interact with cellular macromolecules and adversely affects to the membranes (Gajewska et al. 2012). The results indicated an association of ROS with MDA (R = 0.961), 4-HNE (R = 0.933) and MSI (R = -0.885) (Table 4). On the contrary, Asp and GB stabilize the membrane lipid and reduced the levels of MDA and 4-HNE, which are well related with previous reports (Daneshmand et al. 2009; Lou et al. 2015).

Like lipids, proteins are also equally vulnerable to ROS by several mechanisms *viz*.; glycation, carbonylation, nitrosylation and hydroxylation (Chandrakar et al. 2016b). Nickel leads to depletion of protein bound thiols thereby making it non-functional. Oxidation of proteins in response to Ni resulted in reduced amount of active proteins (Negi et al.

2014). Our results for Ni-stressed *P. typhoideum* coincide well with this observation (Table 3). Conducted study revealed an inverse association (R = -0.754) between protein and ROS content. Exogenous application of Asp and GB stabilized the protein structure and increased the protein content (Zhen et al. 2010; Lou et al. 2015). Moreover, these compounds also reduced the rate of protein carbonylation, and increased the level of reduced glutathione (Ali et al. 2015). ROS promoted DNA damage has widely been shown to occur under abiotic stresses (Erturk et al. 2014; Chandrakar et al. 2017). ROS-imposed deleterious changes frequently results permanent deformity in the DNA strand *via* inactivating the repair systems (Erturk et al. 2014). In this study, considerable fall (38%) in the DNA content of Ni subjected *P. typhoideum* radicles was discernible, and was related inversely with ROS (R = -0.925) (Table 4). Content of DNA was raised in presence of Asp and/or GB, which suggests that they protect DNA either by up-regulating DNA polymerase activity or by direct scavenging of ROS in stressed cells.

Additionally, synthesis of proline has been shown as one of the strategies needed to tune the cell with stressed condition (Kazemi et al. 2010; Siddiqui et al. 2013). It stabilizes proteins and cell membranes, scavenges free radicals, functions as molecular chaperons, and enhances the activities of antioxidants (Yusuf et al. 2012). In this study, enhanced synthesis of proline was discernible after Ni, and Asp and/or GB addition in *P. typhoideum* radicles. Increased proline was largely shown to protect the plants from abiotic stress by scavenging ROS (Kaya et al. 2013).

In general, plants respond to oxidative condition by improving the activities of defensive enzymes (Daneshmand et al. 2009; Malekzadeh 2015). In this study, activities of SOD, CAT, POD and APX were enhanced in *P. typhoideum* under Ni-stress (Figs. 1A, C, E, G). It is known that these enzymes consume ROS as their substrate, therefore, with the rise of it, their activities also increased (Yusuf et al. 2012). Although, there was a rise in the antioxidants in Ni subjected *P. typhoideum*, but rise might not be sufficient to detoxify such a high amount of ROS, which caused oxidative condition inside the cells. Moreover, exogenous Asp and GB restored the antioxidants under varied abiotic stresses possibly due to lesser accrual of ROS (Daneshmand et al. 2009; Lou et al. 2015). Isoenzyme analyses of SOD, POD and APX revealed that isoforms of these enzymes appeared in the Native-PAGE gel, but their intensities varied in response to Ni (Figs. 1B, F, H). Interestingly, isoform(s) of CAT appeared only in the presence of Ni, which suggests the activation of enzymes to counteract the toxicity (Fig. 1D). Above observations were in coherence with their spectrophotometric results. Restoration in the enzyme activities in response to ROS scavenger, are in line with Mandal et al. (2013).

Conclusions

The results of the present study revealed that exogenous Asp and/or GB prevented the *P. typhoideum* radicles against Ni-stress by improving biomass accumulation, viability, MSI, contents of protein, DNA and proline, restoring the activities of antioxidant enzymes, and controlling LOX and lipid peroxidation. We found that Asp and/or GB restored the activities/improved the isoforms of SOD, CAT, POD and APX, which are crucial to minimize Ni accumulation. Moreover, it is noteworthy that addition of Asp and/or GB inhibited the LOX, reduced the MDA and 4-HNE, and enhanced proline accumulation. However, information about the participation of Asp and GB in heavy metal uptake by varied plant species, and precise mechanisms of stress tolerance are limited, hence need to be studied further.

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