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Response of cauliflower (*Brassica oleracea* L.) to nitric oxide application under cadmium stress

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ABSTRACT

Soil contamination with cadmium (Cd) is a persistent threat to crop production worldwide. The present study examined the putative roles of nitric oxide (NO) in improving Cd-tolerance in cauliflower (*Brassica oleracea* L.). The present study was conducted using four different genotypes of *B. oleracea* named as FD-3, FD-4, FD-2 and Ceilo Blanco which were subjected to the Cd stress at various concentrations i.e., 0, 5, 10 and 20 μM with or without the application of NO i.e., 0.10 mM in the sand containing nutrient Hoagland's solution. Our results illustrated that the increasing levels of Cd in the sand, significantly ($P < 0.05$) decreased shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, germination percentage, germination index, mean germination time, time to 50% germination, chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents in all genotypes of *B. oleracea*. The concentration of malondialdehyde (MDA) and Cd accumulation (roots and shoots) increased significantly ($P < 0.05$) under the increasing levels of Cd in all genotypes of *B. oleracea* while antioxidant (enzymatic or non-enzymatic) capacity and nutritional status of the plants was decreased with varying levels of Cd in the sand. From all studied genotypes of *B. oleracea*, Ceilo Blanco and FD-4 was found to be most sensitive species to the Cd stress under the same levels of the Cd in the medium while FD-2 and FD-3 showed more tolerance to the Cd stress compared to all other genotypes of *B. oleracea*. Although, toxic effect of Cd in the sand can overcome by the application of NO which not only increased plant growth and nutrients accumulation but also decreased the oxidative damage to the membranous bounded organelles and also Cd accumulation in various parts of the plants in all genotypes of *B. oleracea*. Hence, it was concluded that application of NO can overcome Cd toxicity in *B. oleracea* by maintaining the growth regulation and nutritional status of the plant and overcome oxidative damage induced by Cd toxicity in all genotypes of *B. oleracea*.

1. Introduction

In recent decades, rapid increases in urbanization and

industrialization have caused the excessive release of heavy metals in farmlands with damaging effects on ecosystems (Al Jabri et al., 2022; Riaz et al., 2020). Contamination of agricultural soils with cadmium

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(Cd) has become one of the most toxic and widespread environmental problems (Afzal et al., 2020; Javed et al., 2017). Cd typically causes direct or indirect inhibition of various physiological processes, such as respiration, transpiration, photosynthesis, oxidative stress, cell elongation, nitrogen metabolism and uptake of mineral nutrition, finally resulting in growth retardation, leaf chlorosis and reduced biomass (Nabaei and Amooaghaie, 2019; Rehman et al., 2015; Rizwan et al., 2016a). In the case of Cd stress, the plants has involved several strategies that can resort to a number of defense systems, such as: (1) immobilization; (2) exclusion; (3) synthesis of phytochelatin; (4) compartmentalization; (5) synthesis of metallothioneins; (6) synthesis of stress proteins; (7) production of stress ethylene (Hoseini and Zargari, 2013; Imran et al., 2020; Rehman et al., 2017; Shanying et al., 2017). Moreover, higher Cd retention in plant cells/tissues triggers the production of reactive oxygen species (ROS), hydroxyl groups (OH), and superoxide radicals (O⁻), which either directly or indirectly affects the in planta metabolic pathways (Rehman et al., 2018; Tanwir et al., 2015; Zia ur Rehman et al., 2021). Over-production of ROS is toxic, and plants need to scavenge those immediately through an antioxidative defense system (Rizwan et al., 2012; Sager et al., 2020). Previously, antioxidative enzymes played a significant role in the reduction of Cd phytotoxicity in *Glycine max* (El-Esawi et al., 2020), *Solanum lycopersicum* (Alyemeni et al., 2018), *Pfaffia glomerata* (Pereira et al., 2018), *Oryza sativa* (Liu et al., 2017), *Boehmeria nivea* (Tang et al., 2015) and *Zea mays* (Abbas et al., 2020).

Nitric oxide (NO), a small, water and lipid soluble gas, has emerged as a major signaling molecule (He et al., 2014). NO has been implicated in a number of diverse physiological processes in plants, including seed germination, stomatal closure, maturation and senescence, and programmed cell death responses to biotic and abiotic stresses (Akram et al., 2018; Kaya et al., 2019). The protective role is based on its ability to regulate the level and toxicity of reactive oxygen species (ROS). In addition, it has also been reported that NO, a diffusible gaseous free radical can protect many vegetative plant species against Cd-induced oxidative stress (Arora and Bhatla, 2017; Gill et al., 2013; Qiu et al., 2021). On the other hand, Cd accumulation can also induce changes in antioxidative systems of a cell; e.g., in tomato, Cd enhanced antioxidative defense system with increased lipid peroxidation and hydrogen peroxide (H₂O₂) accumulation (Ahmad et al., 2018). Cauliflower, belonging to Brassicaceae, is a popular vegetable grown throughout the world for its abundant proteins, minerals, vitamins, and metabolites which protect mankind from heart diseases and certain cancers (Ma et al., 2021). Plant species related to Brassicaceae are generally considered as sensitive indicators or phytoremediators for their fast growth, higher biomass, and adsorptive ability of heavy metals (Ahmad et al., 2019). Previous studies have described the response of *Helianthus annuus*, *Arachis hypogaea*, and *Triticum aestivum* (Arora and Bhatla, 2017; Howladar et al., 2018; Kong et al., 2014), on Cd toxicity under the different applications. *B. oleracea* characteristics such as huge biomass and high tolerance to metal-stressed environment may be useful for the selection of best tolerant genotype, but plant efficacy for Cd stressed environment and core reactions of the antioxidant defense system under Cd stress are unrevealed. However, none of the previous studies reported the screening of different *B. oleracea* genotypes for Cd stressed soils. Therefore, the primary objectives of this study was to assess the effects of exogenous application of NO on growth, photosynthetic efficiency, oxidative stress and response of antioxidant compounds, nutritional status of the plants and Cd accumulation in the various part of the plants. In addition, we have determined the role of enzymatic and non-enzymatic antioxidant defense system in details under the toxic concentration of Cd in the soil. *B. oleracea* is a vegetable and sensitive to the Cd stress, therefore we have screened various genotypes of *B. oleracea* to obtain which variety will show maximum tolerance under the same condition. The study will provide useful information to sort out suitable *B. oleracea* genotype for the Cd contaminated sand.

2. Materials and methods

2.1. Experimental design and plant sowing

Seeds of four genotypes named as FD-3, FD-4, FD-2 and Ceilo Blanco were collected from vegetable section of Ayub Agricultural Research Institute (AARI) Faisalabad 38000, Pakistan. The seeds (5–8) were sown in plastic pots (25 × 35 cm²) having washed natural soil supplemented with 0, 5, 10 and 20 μM CdCl₂ containing the natural sand which was supplied by the Hoagland's solution. For the application of Cd stress, cadmium chloride (CdCl₂) salt was used. The composition of Hoagland's nutrient solution was as follows (μmol L⁻¹): Ca(NO₃)₂, 2000; KH₂PO₄, 100; KNO₃, 3000; MgSO₄, 1000; H₃BO₃, 50; MnCl₂·0.4 H₂O, 0.05; ZnSO₄·0.7H₂O, 0.8; CuSO₄·0.5 H₂O, 0.3; H₂MO₄·H₂O, 0.10; and FeNa-CA, 12.5. The physio-chemicals are nutritional properties of the sand used in this experiment are as follow: organic matter: > 70 % of the total solids; density: 350 kg/m³; pH: 7.6; electric conductivity: 20 mS/m; organic nitrogen: 1400 mg/L; nitrogen: g/m³; phosphorus: 75 g/m³; potassium: 160 g/m³; magnesium: 250 g/m³; calcium: 1600 g/m³; sulfur: 85 g/m³; copper: 2.5 g/m³; zinc: 1.8 g/m³; molybdenum: 2.7 g/m³ and iron: 5.6 g/m³. (Javed et al., 2020). The levels of Cd treatments were selected based on previous study (Jung et al., 2020). Sodium nitroprusside (SNP) was used as NO donor. The SNP dose (0.10 mM) was selected based on available literature (Kaya et al., 2020). The concentration of SNP (NO) application was sprayed to the plants after two weeks of the seed emergence. We established eight treatments in this study i.e., 0 mM CdCl₂ + 0 mM NO, 0 mM CdCl₂ + NO, 5 mM CdCl₂, 5 mM CdCl₂ + NO, 10 mM CdCl₂, 10 mM CdCl₂ + NO, 20 mM CdCl₂ and 20 mM CdCl₂ + NO. Various treatments of Cd was provided with the addition of the nutrient Hoagland's solution and this solution was maintained with the pH 6 using NaOH or HCl, once in the week. In addition, ½ strength Hoagland's was provided to the sand mixture, as this is the seedling stage experiment. The pots were placed in a growth chamber (Sanyo versatile Environmental Test Chamber MLR-351, UK) with a photoperiod of 16 h light/8 h dark with light intensity of 200 μmol m⁻² s⁻¹ belonging to the Department of Botany, Government College University, Faisalabad 38000, Punjab, Pakistan (31° 24'N, 73° 04'E). The day/night temperature was adjusted at 25 °C/23 °C with relative humidity of 70–72 %. After 14 days of the seed sowing the Hoagland's nutrient solution was added to each pot to avoid any nutrient deficiency in the sand mixture. The whole plants in triplicate were uprooted from each treatment pot at 6 leaf stage (after six weeks of seed sowing) for various morpho-physio and biochemical traits. During the entire experiment, we did not observe any symptoms of water-logging in both genotypes of *B. oleracea*. The experiment was conducted in three replicates following complete randomized design (CRD).

2.2. Morphological traits and germination attributes

All plants were harvested to measure different morphological and physiological attributes after 6 leaf stage of the life cycle of *B. oleracea*. Plants were rooted up in the July 2020 and analysis of different biological parameters were performed in Government College University, Pakistan. The sampled leaves were washed with distilled water, immediately placed in liquid nitrogen and stored in a freezer at low temperature (–80 °C) for further analysis. The whole plants were washed with distilled water and separated into roots and shoots. Shoot length and root length were measured using measuring scale and fresh biomass was measured using weighting digital balance. For dry biomass, the samples of roots and shoots were oven dried at 65 °C for 75 h. Germination index, time to 50% germination, and mean germination time was measured by following the method presented by Wiesner (1990), Coolbear et al. (1984) and Ruan et al. (2002) respectively. Germination percentage (%) was calculated by the following formula.

$$G\% = \text{No. of germinated seeds} / \text{Total number of seeds} \times 100$$

2.3. Determination of photosynthetic pigments

Chlorophyll contents were determined following (Arnon, 1949), by taking 0.1 g leaf sample extracted with 95.5% acetone and absolute ethyl alcohol in 1:1 ratio. This extraction was done in the dark place for 48 h.

2.4. Determination of malondialdehyde and antioxidant capacity

Lipid peroxidation was detected by MDA contents following the method described by (Heath and Packer, 1968). Briefly, 0.5 g leaf sample was taken and homogenized with 5 mL of 0.1 % trichloroacetic acid (TCA). Then, the obtained homogenate was centrifuged at 10,000 rpm for 5 min. Four milliliters of TCA (20 %) along with thiobarbituric acid (0.5 %) was added in every 1 mL of aliquot. Finally, MDA contents were determined using extinction coefficient of $155 \mu\text{M}^{-1} \text{cm}^{-1}$.

Catalase (CAT) activity was analyzed according to (Aebi, 1984). The assay mixture (3.0 mL) was comprised of 100 μL enzyme extract, 100 μL H_2O_2 (300 mM) and 2.8 mL 50 mM phosphate buffer with 2 mM EDTA (pH 7.0). The CAT activity was measured from the decline in absorbance at 240 nm as a result of H_2O_2 loss ($\epsilon = 39.4 \text{ mM}^{-1} \text{cm}^{-1}$).

Peroxidase (POD) activity in the leaves was estimated using the method of (Sakharov and Ardila, 1999) by using guaiacol as the substrate. Increases in the absorbance at 470 nm because of guaiacol oxidation were recorded for 2 min. One unit of enzyme activity was defined as the amount of the enzyme.

Superoxidase dismutase (SOD) activity was assayed in 3 mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7), 56 mM nitro blue tetrazolium, 1.17 mM riboflavin, 10 mM methionine and 100 μL enzyme extract. Finally, the sample was measured by using a spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad, Hercules, CA, USA). Enzyme activity was measured using a method by (Chen and Pan, 1996) and expressed as $\text{U g}^{-1} \text{FW}$.

2.5. Determination of non-enzymatic antioxidants

Plant ethanol extracts were prepared for the determination of non-enzymatic antioxidants and some key osmolytes. For this purpose, 50 mg of dry plant material was homogenized with 10 mL ethanol (80 %) and filtered through Whatman No. 41 filter paper. The residue was re-extracted with ethanol, and the 2 extracts were pooled together to a final volume of 20 mL. The determination of flavonoids (Pečkal and Pyszynska, 2014) and phenolics (Bray and Thorpe, 1954) was performed from the extracts. (Rosen, 1957) method was used to measure the total free amino acid. 0.5 mL extract was taken and allowed to react with 0.5 mL 4% ninhydrin and 0.5 mL of 2% pyridine. After cooling the test tubes distilled water is added to make the final volume 7.5 mL and absorbance was measured at 570 nm. Total soluble proteins (TSP) were measured from fresh leaf material. The leaf tissue (0.5 g) was crushed in 10 mL of chilled potassium phosphate buffer (50 mM; pH 7.5). The supernatant was used to determine proteins following the method of (Bradford, 1976).

2.6. Determination of Cd concentration and nutrient uptake

Plant dry material (0.1 g) was used to determine different elements from root and shoots (Allen et al., 1986). For this purpose, plant roots and shoots were washed twice in the distilled water, dipped in 20 mM EDTA for 3 s, and then, again, washed with the distilled water twice for the removal of adsorbed metal on the plants surface. The dried roots and shoots were digested by using a wet digestion method in HNO_3 : HClO_4 (7:3 V/V) until clear samples were obtained. Each sample was filtered and diluted with redistilled water up to 50 mL. The root and shoot

contents of Mn, Mg, Ca and K and were analyzed by using flame photometer (Sherwood, Model 360).

Finely ground samples were digested with pure HNO_3 at 190°C for 45 min (10 min pre-heating, 15 min heating, 20 min cooling) in a microwave oven (Mars 6, CEM Corporation, Matthews, NC, USA) with the settings described in details by (Jezek et al., 2015). Samples were diluted with 2 % HNO_3 and determined by inductively coupled plasma-mass spectroscopy (ICP-MS; Agilent 7700, Agilent Technologies Inc., USA).

2.7. Statistical Analysis

Data analysis was executed by using two-way analysis of variance (ANOVA) with computer based Co-stat version Cohorts Software 6.2, 2003 (Monterey, CA, USA). The differences among treatments were evaluated by least significant difference method (Fisher's LSD) at p value of ≤ 0.05 level. The data was standardized by logarithmic or inverse transformations prior to analysis. Principal component analysis (PCA) was executed to quantify relationship among different variables. The Pearson correlation coefficients and the principal component analysis between variables different genotypes of *B. oleracea* were also calculated using RStudio software.

3. Results

3.1. Response of morphological, germination and photosynthetic attributes

In the present study, different morphological traits such as root length, shoot length, root fresh weight, shoot fresh weight, root dry weight and shoot dry weight, various germination parameters such as germination percentage, germination index, mean germination time and time to 50 % germination and also photosynthetic pigments such as chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were decreased under the increasing levels of Cd in the sand (Table 1, Fig. 1). The maximum decreased in growth parameters and germination attributes were found at the highest level of Cd in the sand i.e., (20 μM), compared to the plants which were grown in the sand which was not contaminated with Cd. According to the results, it was also noticed that the Ceilo Blanco and FD-4 was found to be most sensitive species to the Cd stress under the same levels of the Cd in the medium while FD-2 and FD-3 showed more tolerance to the Cd stress compared to all other genotypes of *B. oleracea*. The negative impact of Cd toxicity was overcome by the application of NO, which significantly ($P < 0.05$) increased growth, germination and photosynthetic pigments in the plants grown under the various levels of Cd in the sand (Table 1, Fig. 1).

3.2. Response of malondialdehyde and antioxidant capacity

In the present study, we have measured the concentration of malondialdehyde (MDA) from the leaves of *B. oleracea* and presented in Fig. 3a. Results showed that the increasing levels of Cd in the sand (0, 5, 10 and 20 μM), caused a significant increase in the concentration of MDA in all genotypes of *B. oleracea*. However, in all genotypes of *B. oleracea* the activity of CAT was decreased but contrastingly; activity of SOD and POD were increased in all genotypes of *B. oleracea* under the varying levels of Cd in the sand. Although, non-enzymatic antioxidants (total soluble protein, flavonoid and phenolics) were decreased with the increasing levels of Cd in the nutrient solution (Fig. 4), but a minor change has been found in all genotypes of *B. oleracea* in their free amino acid under the treatment of different levels of Cd in the sand (Fig. 4a).

It was also noticed that the application of NO induced different results in different parameters for the various genotypes of *B. oleracea* under the treatment of Cd stress in the sand. Under the application of NO, the concentration of MDA was decreased significantly ($P < 0.05$) and also the activities of SOD, CAT were also decreased in all genotypes

Table 1

Effect of various application levels of nitric oxide and Cd toxicity tolerance on various growth parameters in various varieties of *B. oleracea* grown in various levels of Cd treatments in the medium.

	Variety	T0	T1	T2	T3	T4	T5	T6	T7
Shoot Length	FD-3	6.53 ± 0.01	6.66 ± 0.02	6.41 ± 0.01	7.4 ± 0.06	6.1 ± 0.06	7.10 ± 0.06	5.8 ± 0.06	6.13 ± 0.03
	FD-2	6.47 ± 0.03	6.78 ± 0.02	6.37 ± 0.02	7.87 ± 0.03	6.3 ± 0.03	7.83 ± 0.03	6 ± 0.06	7.47 ± 0.15
	FD-4	6.86 ± 0.03	7.2 ± 0.06	5.7 ± 0.09	6.5 ± 0.06	5.3 ± 0.12	6.53 ± 0.03	5 ± 0.06	5.97 ± 0.07
	Ceilo Blanco	5.87 ± 0.03	6.3 ± 0.06	5.5 ± 0.06	5.8 ± 0.06	5.3 ± 0.03	6.0 ± 0.03	5 ± 0.06	5.7 ± 0.03
Root Length	FD-3	1.90 ± 0.06	2.93 ± 0.09	1.67 ± 0.09	2.20 ± 0.06	1.4 ± 0.06	1.80 ± 0.06	1.10 ± 0.06	1.73 ± 0.12
	FD-2	1.80 ± 0.06	3.43 ± 0.07	1.72 ± 0.04	2.07 ± 0.03	1.03 ± 0.19	1.50 ± 0.06	0.60 ± 0.06	1.17 ± 0.09
	FD-4	2.47 ± 0.03	3.00 ± 0.06	2.30 ± 0.06	2.60 ± 0.06	1.53 ± 0.01	1.72 ± 0.01	0.37 ± 0.03	0.80 ± 0.06
	Ceilo Blanco	1.83 ± 0.09	2.80 ± 0.06	1.90 ± 0.06	2.60 ± 0.06	2.07 ± 0.09	2.47 ± 0.20	1.00 ± 0.06	1.70 ± 0.06
Shoot F.W	FD-3	0.64 ± 0.01	0.73 ± 0.01	0.64 ± 0.01	0.72 ± 0.01	0.64 ± 0.01	0.69 ± 0.00	0.64 ± 0.01	0.66 ± 0.01
	FD-2	0.60 ± 0.01	0.68 ± 0.01	0.60 ± 0.01	0.69 ± 0.0	0.60 ± 0.01	0.68 ± 0.0	0.60 ± 0.01	0.71 ± 0.0
	FD-4	0.62 ± 0.0	0.72 ± 0.01	0.62 ± 0.0	0.75 ± 0.01	0.62 ± 0.0	0.70 ± 0.01	0.62 ± 0.0	0.70 ± 0.0
	Ceilo Blanco	0.59 ± 0.01	0.66 ± 0.02	0.59 ± 0.01	0.64 ± 0.01	0.59 ± 0.01	0.66 ± 0.01	0.59 ± 0.01	0.64 ± 0.01
Root F.W	FD-3	0.18 ± 0.00	0.29 ± 0.00	0.17 ± 0.00	0.26 ± 0.01	0.15 ± 0.00	0.21 ± 0.01	0.10 ± 0.00	0.16 ± 0.00
	FD-2	0.19 ± 0.01	0.34 ± 0.01	0.19 ± 0.01	0.31 ± 0.00	0.15 ± 0.01	0.27 ± 0.01	0.11 ± 0.01	0.20 ± 0.00
	FD-4	0.24 ± 0.01	0.29 ± 0.00	0.22 ± 0.00	0.27 ± 0.01	0.17 ± 0.01	0.21 ± 0.01	0.13 ± 0.01	0.19 ± 0.00
	Ceilo Blanco	0.20 ± 0.00	0.28 ± 0.00	0.18 ± 0.00	0.21 ± 0.01	0.13 ± 0.01	0.19 ± 0.01	0.10 ± 0.01	0.16 ± 0.01
Shoot D.W	FD-3	0.24 ± 0.01	0.31 ± 0.01	0.23 ± 0.01	0.30 ± 0.00	0.17 ± 0.01	0.28 ± 0.00	0.11 ± 0.00	0.26 ± 0.00
	FD-2	0.22 ± 0.01	0.37 ± 0.01	0.21 ± 0.01	0.32 ± 0.01	0.14 ± 0.01	0.29 ± 0.00	0.10 ± 0.00	0.27 ± 0.01
	FD-4	0.25 ± 0.01	0.30 ± 0.01	0.24 ± 0.01	0.28 ± 0.01	0.16 ± 0.01	0.26 ± 0.01	0.12 ± 0.01	0.19 ± 0.01
	Ceilo Blanco	0.21 ± 0.01	0.29 ± 0.00	0.20 ± 0.01	0.21 ± 0.01	0.14 ± 0.01	0.19 ± 0.01	0.08 ± 0.01	0.13 ± 0.01
Root D.W	FD-3	0.14 ± 0.01	0.25 ± 0.00	0.13 ± 0.00	0.18 ± 0.00	0.10 ± 0.00	0.19 ± 0.00	0.07 ± 0.00	0.12 ± 0.01
	FD-2	0.12 ± 0.01	0.28 ± 0.00	0.09 ± 0.00	0.20 ± 0.01	0.05 ± 0.00	0.20 ± 0.01	0.01 ± 0.00	0.17 ± 0.01
	FD-4	0.09 ± 0.00	0.19 ± 0.01	0.08 ± 0.01	0.17 ± 0.01	0.04 ± 0.00	0.16 ± 0.01	0.01 ± 0.00	0.12 ± 0.01
	Ceilo Blanco	0.13 ± 0.01	0.18 ± 0.01	0.09 ± 0.00	0.14 ± 0.01	0.06 ± 0.00	0.13 ± 0.00	0.02 ± 0.00	0.10 ± 0.00

Means sharing similar letter(s) within a column for each parameter do not differ significantly at P < 0.05. Data in the tables are means of three repeats (n = 3) of just one harvest of *B. oleracea* varieties ± standard deviation (SD). Different abbreviations used in the table are as follow: T0= 0 μM CdCl₂, T1 = 0 μM CdCl₂ +NO, T2 = 5 μM CdCl₂, T3 = 5 μM CdCl₂ +NO, T4 = 10 μM CdCl₂, T5 = 10 μM CdCl₂ +NO, T6 = Control+ 20 μM CdCl₂, T7 = 20 μM CdCl₂ +NO.

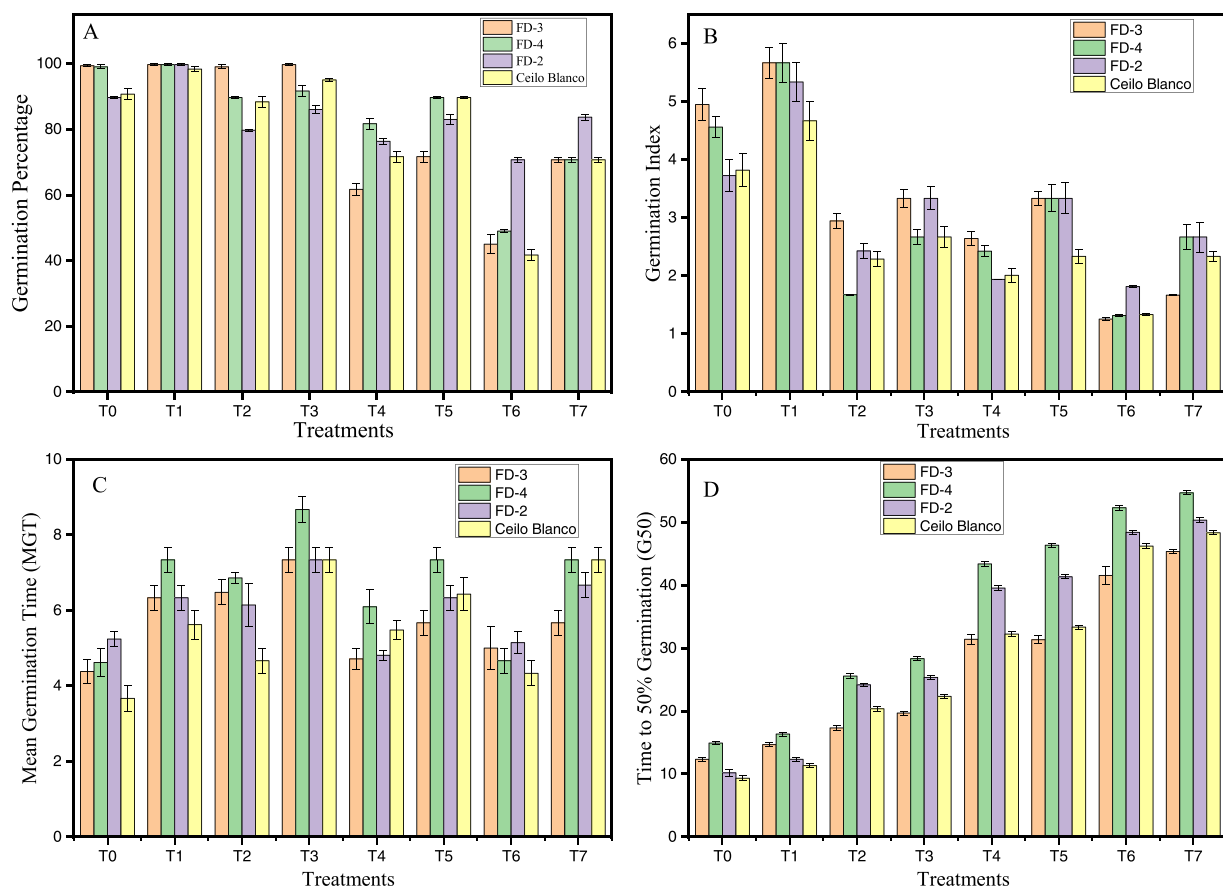


Fig. 1. Effect of Nitric oxide (NO) on germination attributes (A=Germination percentage, B=Germination index, C=Mean germination time, D=Time to 50% germination) in four genotypes of cauliflower (*Brassica oleracea* var. botrytis) under varying levels of cadmium stress. T0= 0 μM CdCl₂, T1 = 0 μM CdCl₂ +NO, T2 = 5 μM CdCl₂, T3 = 5 μM CdCl₂ +NO, T4 = 10 μM CdCl₂, T5 = 10 μM CdCl₂ +NO, T6 = Control+ 20 μM CdCl₂, T7 = 20 μM CdCl₂ +NO.

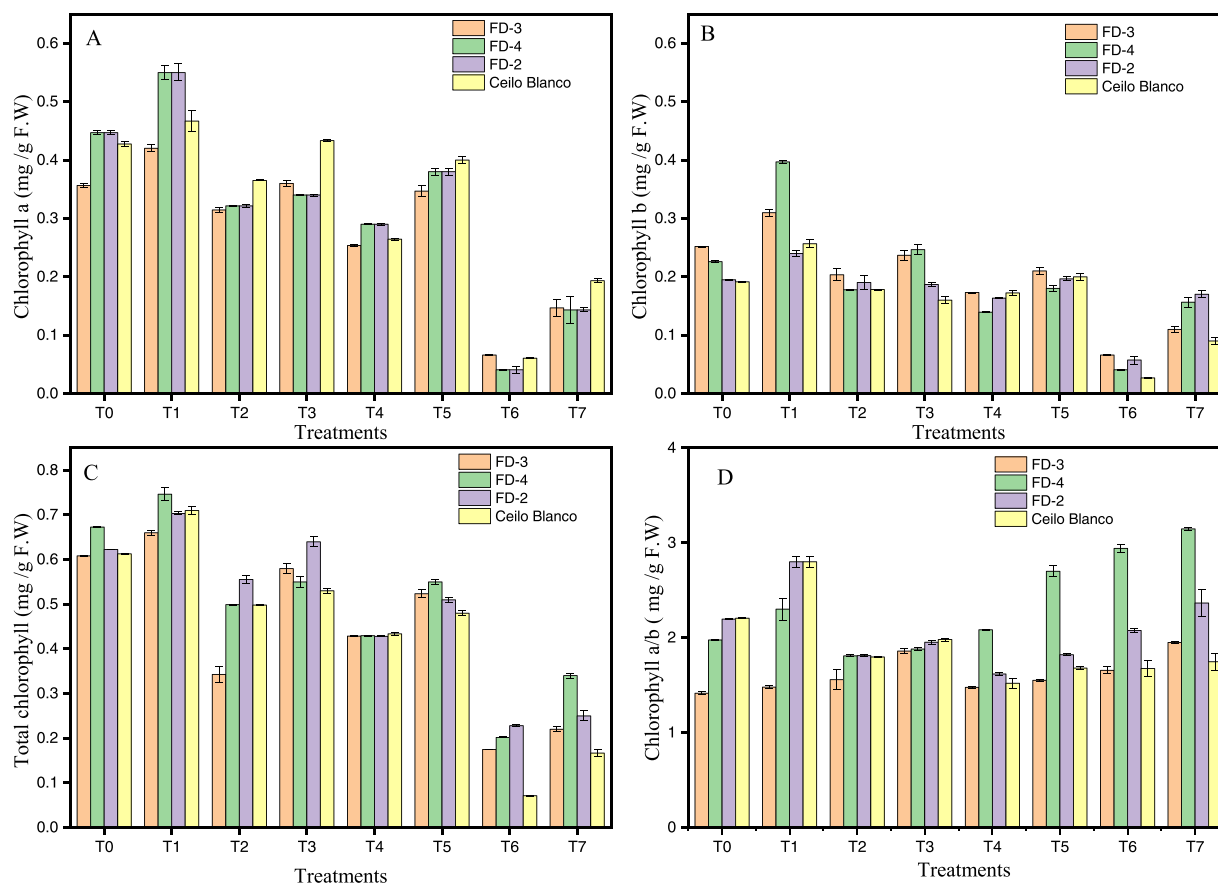


Fig. 2. Effect of Nitric oxide (NO) on chlorophyll contents (A=chlorophyll a, B=chlorophyll b, C=total chlorophyll, D=chlorophyll a/b) in four genotypes of cauliflower (*Brassica oleracea* var. botrytis) under varying levels of cadmium stress. T0= 0 μ M CdCl₂, T1 = 0 μ M CdCl₂ +NO, T2 = 5 μ M CdCl₂, T3 = 5 μ M CdCl₂ +NO, T4 = 10 μ M CdCl₂, T5 = 10 μ M CdCl₂ +NO, T6 = Control+ 20 μ M CdCl₂, T7 = 20 μ M CdCl₂ +NO.

of *B. oleracea*. It was noticed that the free amino acid, soluble protein, flavonoid and phenolic were increased under the application of NO in all genotypes of *B. oleracea*. Although, the activity of POD showed different responses under the application of NO.

3.3. Response of nutrient uptake and Cd accumulation

In the present study, we also measured the nutrients content (Mg, Mn, K and Ca) and Cd accumulation from the different parts of the plants body (roots and shoots) in all genotypes of *B. oleracea*. The data regarding the nutritional status of the plants is presented in Table 1S and the data regarding the Cd accumulation in the plants is presented in Table 2S. From the given results, we illustrated that the increasing concentration of Cd in the sandn caused a significant ($p < 0.05$) increased in Cd concentration in the roots and shoots of the plants (Table 2S) while a significant ($p < 0.05$) decreased in the nutrient contents (Mg, Mn, K and Ca) in the roots and shoots of the plants (Table 1S). The maximum concentration of Mg, Mn, K and Ca was observed in the FD-4 and Ceilo Blanco genotypes, while the maximum concentration of Cd was accumulated in the FD-2 and FD-3 genotypes, under the same level of the stress in the sand. Although, the exogenous application of NO decreased the content of Cd in the roots and shoots of all genotypes of the plants, compared to the plants which were grown without the application of NO. In contrast, exogenous application of NO increased the content of essential ions (Mg, Mn, K and Ca) in the roots and shoots of the plants, compared to the plants which were grown without the application of NO.

3.4. Relationship between Cd uptake with different attributes of the plants

A Pearson's correlation graph illustrates the relationship between various growth, morphological and physiological parameters of different varieties of *B. oleracea* under various application levels of nitric oxide grown under the various levels of Cd in the sand (Fig. 5). The Cd concentration in the roots was positively correlated with Cd concentration in the shoots, proline content, ascorbate peroxidase activity, manganese content in the roots, peroxidase activity, chlorophyll a/b content, malondialdehyde content, germination percentage, germination index, germination energy and means germination time while negatively correlated with potassium content in the roots, magnesium content in the shoots, calcium content in the shoots, magnesium content in the roots, potassium content in the shoots, intercellular CO₂, manganese content in the shoots, calcium content in the roots, flavonoid content, shoot dry weight, root dry weight, trifluoroacetic anhydride, catalase activity, transpiration rate, root length, superoxidase dismutase activity, chlorophyll a content, total chlorophyll content, chlorophyll b content, stomatal conductance, time to 50% seed germination, shoot fresh weight, carotenoid content, trisodium phosphate, phenolic content, shoot length and root fresh weight. This relationship depicts a close connection between plant growth and composition in different varieties of *B. oleracea* under various application levels of nitric oxide grown under the various levels of Cd in the sand.

3.5. Principal component analysis

A principal component analysis (PCA) was used to illustrate the effect of nitric oxide on various levels of Cd toxicity in various varieties of

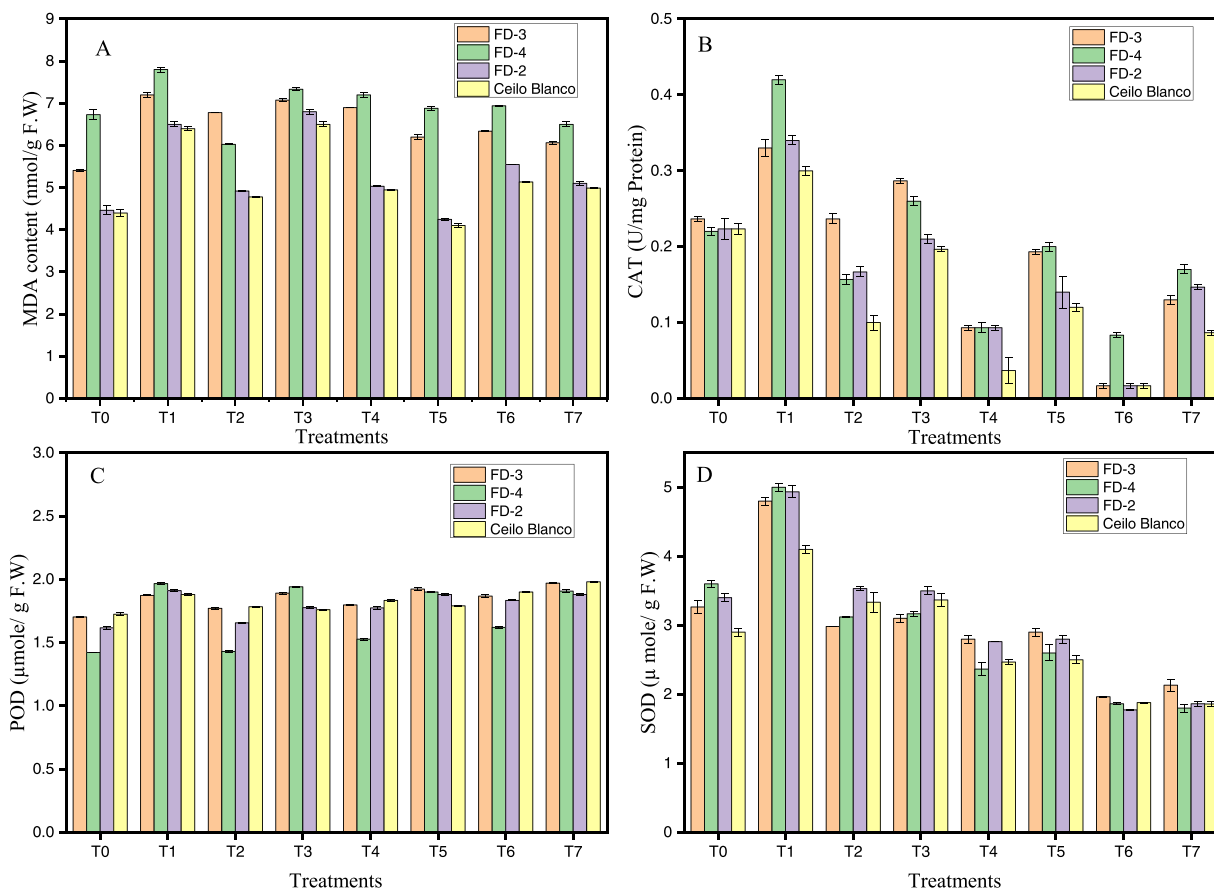


Fig. 3. Effect of Nitric oxide (NO) on enzymatic antioxidant contents (A=MDA, B=CAT, C=POD, D=SOD) in four genotypes of cauliflower (*Brassica oleracea* var. botrytis) under varying levels of cadmium stress. T₀= 0 $\mu\text{M CdCl}_2$, T₁ = 0 $\mu\text{M CdCl}_2$ +NO, T₂ = 5 $\mu\text{M CdCl}_2$, T₃ = 5 $\mu\text{M CdCl}_2$ +NO, T₄ = 10 $\mu\text{M CdCl}_2$, T₅ = 10 $\mu\text{M CdCl}_2$ +NO, T₆ = Control+ 20 $\mu\text{M CdCl}_2$, T₇ = 20 $\mu\text{M CdCl}_2$ +NO.

B. oleracea (Fig. 6). Dim1 and Dim2 exhibited maximum contribution and occupy more than 72 % of differences. Among which Dim1 exhibits (56.3 %) and Dim2 exhibits (16.1 %). All studied parameters distributed successfully in the database which is giving a clear indication that Cd stress causes a significant effect to the growth and physiology of all *B. oleracea* varieties. From the results it can be derived that Cd concentration in the roots, Cd concentration in the shoots, proline content, ascorbate peroxidase activity, manganese content in the roots, peroxidase activity, chlorophyll a/b content, malondialdehyde content, germination percentage, germination index, germination energy and means germination time were positively correlated in the database to all other parameters studied in this experiment. While, potassium content in the roots, magnesium content in the shoots, calcium content in the shoots, magnesium content in the roots, potassium content in the shoots, intercellular CO₂, manganese content in the shoots, calcium content in the roots, flavonoid content, shoot dry weight, root dry weight, trifluoroacetic anhydride, catalase activity, transpiration rate, root length, superoxidase dismutase activity, chlorophyll a content, total chlorophyll content, chlorophyll b content, stomatal conductance, time to 50 % seed germination, shoot fresh weight, carotenoid content, trisodium phosphate, phenolic content, shoot length and root fresh weight were positively correlated with all other studied attributes.

4. Discussion

In line with earlier studies, it was inferred that high absorption and translocation of Cd via phloem disrupt the water absorption resulting in abridged plant biomass that is major factor leading to reduced plant growth subjected to Cd stress (Imran et al., 2021; Qiu et al., 2021;

Valivand and Amooghaie, 2021a; b). Photosynthesis, respiration, cell division, water relations, opening and closing of stomata, nitrogen metabolism, and mineral nutrition are the main metabolic processes within the plants, which are negatively affected by Cd stress (Anwar, 2019). The mechanisms of NO as a signaling regulatory molecule and reactive oxygen scavenger in improving plant tolerance to Cd stress were poorly understood. In recent years, NO is an important signaling molecule and has gained a noticeable consideration due to its role in alleviating an abiotic and biotic stresses in plants (Kaya et al., 2019). This research demonstrated an insight into the role of NO donor SNP in regulating the physiological, biochemical and nutritional responses of *B. oleracea* genotypes under Cd stress. In the present study, the inhibitory effects were significantly alleviated by exogenous NO, and the mitigation effect of NO on all *B. oleracea* genotypes were noticed (Table 1). The stimulation of plant growth by NO has also been reported in *Lolium perenne* (Wang et al., 2013), *Oryza sativa* (Rizwan et al., 2018) and *Solanum lycopersicum* (Amooghaie and Nikzad, 2013). The alleviation of Cd stress by NO may be related to increased germination rate (Fig. 1), enhanced chlorophyll content (Fig. 2), improved nutrient balance (Table 1S), better regulated activities of antioxidant enzymes (Fig. 3), and inhibited Cd translocation from roots to the leaves (Table 2S), thus enhancing the tolerance of *B. oleracea* genotypes to Cd toxicity. Recent work has explained that NO is a phytohormone that influences many physiological processes of plants (Sun et al., 2018). NO acts on phospholipid bilayers which enhanced membrane fluidness, relax the cell wall, which ultimately stimulate cell enlargement and growth of plant (Gill et al., 2013; Kaya et al., 2019). It was also reported that the different crop varieties exhibited different germination percentage when grown under metal-contaminated soil (Saleem et al.,

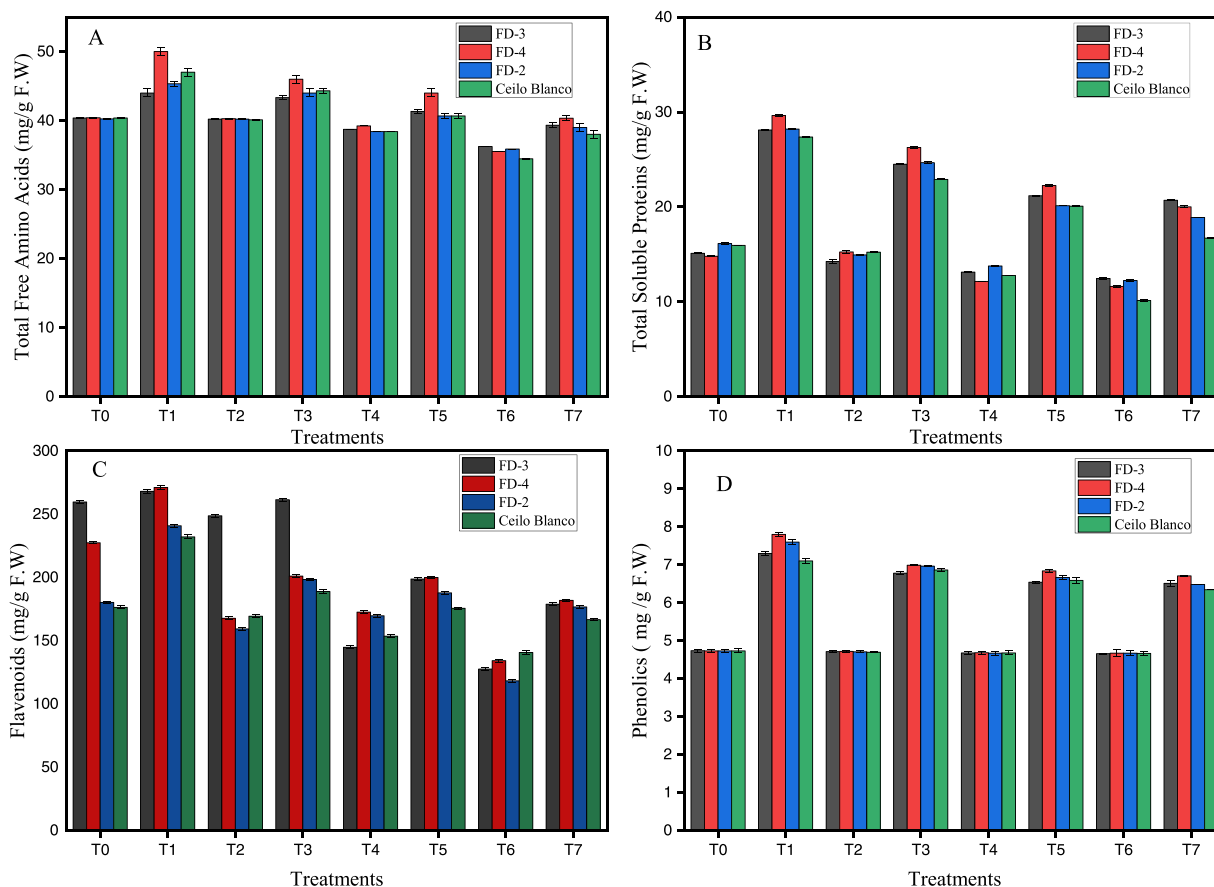


Fig. 4. Effect of Nitric oxide (NO) on non-enzymatic antioxidant contents (A=Total free amino acids, B=Total soluble proteins, C=Flavonoids, D=Phenolics) in four genotypes of cauliflower (*Brassica oleracea* var. botrytis) under varying levels of cadmium stress. T₀= 0 μ M CdCl₂, T₁ = 0 μ M CdCl₂ +NO, T₂ = 5 μ M CdCl₂, T₃ = 5 μ M CdCl₂ +NO, T₄ = 10 μ M CdCl₂, T₅ = 10 μ M CdCl₂ +NO, T₆ = Control+ 20 μ M CdCl₂, T₇ = 20 μ M CdCl₂ +NO.

2019). Nevertheless, the specific rate of germination could be due to the high concentration of Cd in the soil; thus, under the same Cd concentration, FD-2 and FD-3 exhibited maximum plant height and fresh and dry biomass compared with Ceilo Blanco and FD-4. Plant growth and biomass variation under the same environmental conditions in different *B. oleracea* genotypes might be due to low availability of water, poor stomatal regulation, and perturbed root architecture (Saleem et al., 2020b).

Stress conditions can disturb the dynamic equilibrium of reactive oxygen species (ROS) production and elimination under normal growth in plants (Javed et al., 2021), which promotes ROS accumulation and membrane lipid peroxidation, and disrupts the structure and function of the cell membrane system (Aziz et al., 2021; Gill et al., 2021). It was reported that an excess of Cd can increase lipid peroxidation and MDA, an oxidized product of membrane lipids, indicating the prevalence of oxidative stress and membrane damage (Rizwan et al., 2019). Accumulation of protein and amino acids is known to be important signal of stress tolerance under heavy metal stress and it also has a significant role in osmotic adjustment, macromolecule stabilization, ROS scavenging and cell protection from oxidative damage (Afzal et al., 2020). Siddiqui et al. (2013) concluded that NO has the capability of restoring and defending the cell membrane to alleviate the damage in the cell membrane system e.g., minimize the membrane permeability and membrane lipid peroxidation, hence preventing electrolyte leakage. This ROS accumulation in plants is removed by a variety of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) which were also increased in the plants grown in the toxic concentration of Cd (20 μ M), compared with the plants grown in Cd-free sand (Fig. 3). However, exogenous NO prevented Cd-induced increase

in the activities of antioxidant enzymes (SOD, POD and CAT) in the *B. oleracea* varieties. The regulation in the activities of antioxidant enzymes by SNP alleviated the stress of *B. oleracea* genotypes and scavenged the O₂ and H₂O₂ (Fig. 3), as well as MDA (Fig. 3a). So, the stimulation of antioxidant production may suggest that NO can stabilize the cell membranes, counteract oxidative damages and protect *B. oleracea* genotypes against stressful condition. Plants produce a variety of non-enzymatic compounds such as total free amino acids, total soluble proteins, flavonoids and phenolics that improve tolerance against metal toxicity (Rizwan et al., 2016b). Interestingly, NO-treatment caused a marked enhancement of Cd-induced soluble protein contents, which indicated that the *B. oleracea* genotypes were partially relieved from Cd stress.

Cd toxicity has been stated to lead the imbalance of water status and suppress the nutrient uptake, which could be the cause of decrease in leaf water potential, Mn, Mg, Ca and K under Cd stress, but these attributes were found to be enhanced with exogenously applied NO under Cd toxicity (Kaya et al., 2019). Mineral nutrients are needed for several key metabolic processes, such as plant growth and development, and water status. Adequate accumulation of minerals is vital to safeguard mechanical integrity of the plant and key physiological processes, and any changes in mineral uptake may markedly perturb plant metabolism (Mumtaz et al., 2021; Saleem et al., 2020a). NO has a substantial regulatory influence on the contents of plant mineral nutrients in plants and relieves stress by allocation to sustain those elements. Numerous reports demonstrated that the uptake and translocation of essential elements in plants were restricted under Cd stress (Abbas et al., 2020; Tanwir et al., 2015). It is well known that Cd toxicity in crops depends on the bioavailability of Cd in soils and the concentration of elements, which

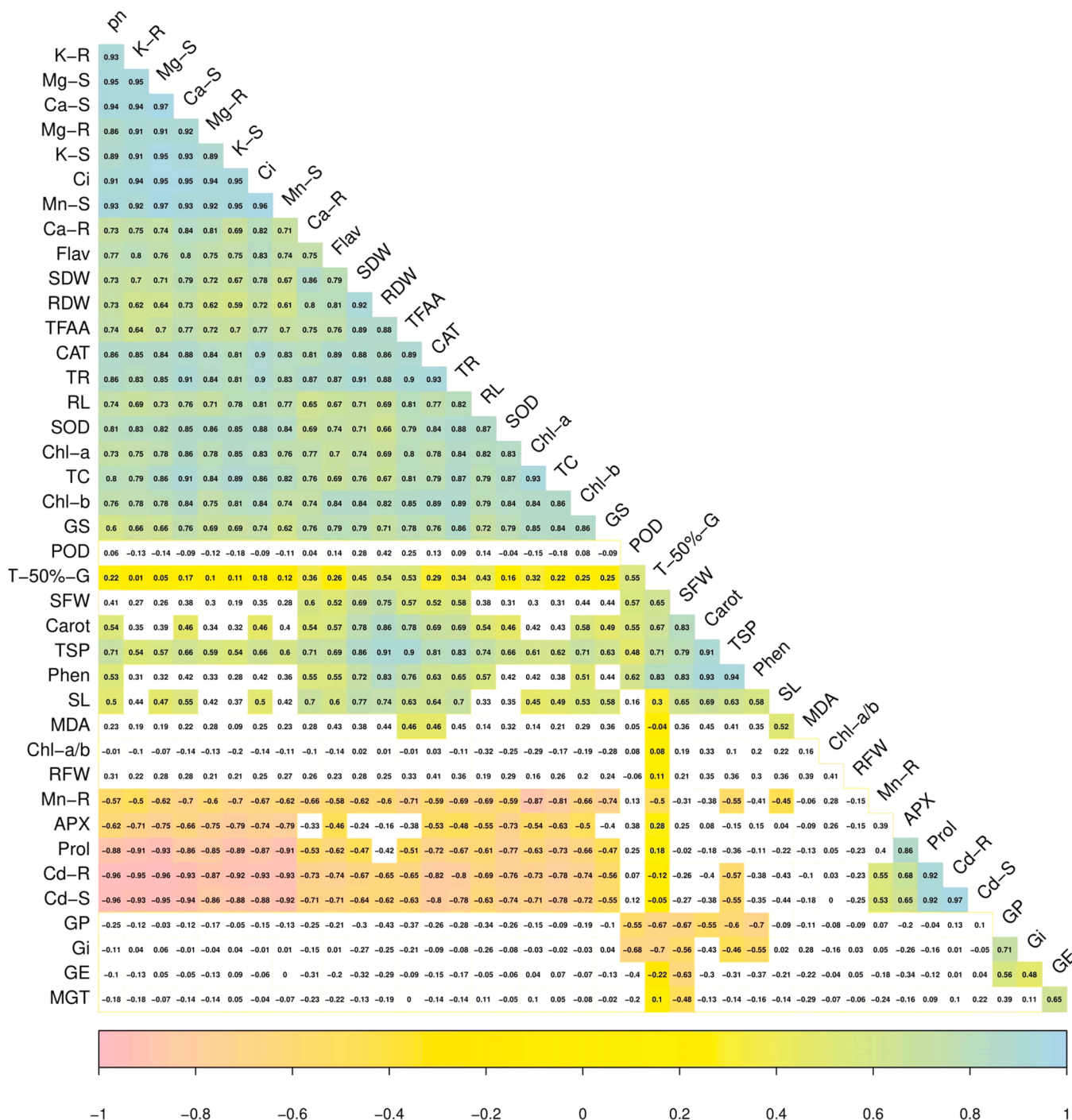


Fig. 5. Relationship between different varieties of *B. oleracea* under various application levels of nitric oxide grown under the Cd-contaminated soil. Different abbreviations used in the figure are as follow: K-R (potassium content in the roots), Mg-S (magnesium content in the shoots), Ca-S (calcium content in the shoots), Mg-R (magnesium content in the roots), K-S (potassium content in the shoots), Ci (intercellular CO₂), Mn-S (manganese content in the shoots), Ca-R (calcium content in the roots), Flav (flavonoid content), SDW (shoot dry weight), RDW (root dry weight), TFAA (Trifluoroacetic anhydride), CAT (catalase activity), TR (transpiration rate), RL (root length), SOD (superoxidase dismutase activity), Chl-a (chlorophyll a content), TC (total chlorophyll content), Chl-b (chlorophyll b content), GS (stomatal conductance), POD (peroxidase activity), T-50 %-G (time to 50 % seed germination), SFW (shoot fresh weight), Carot (carotenoid content), TSP (Trisodium Phosphate), Phen (phenolic content), SL (shoot length), MDA (malondialdehyde content), Chl-a/b (chlorophyll a/b content), RFW (root fresh weight), Mn-R (manganese content in the roots), APX (ascorbate peroxidase activity), Prol (proline content), Cd-R (Cd concentration in the roots), Cd-S (Cd concentration in the shoots), GP (germination percentage), Gi (germination index), GE (germination energy) and MGT (means germination time).

can compete with Cd during plant uptake (Shanying et al., 2017). In general, *B. oleracea* takes up Cd in the form of Cd²⁺ from the soils. Cd uptake in *B. oleracea* plants varies with soil pH and organic matter content present in the soils (Gill et al., 2013). Excess Cd decreased the Mn, Mg, Ca and K contents in the present study (Table 1S), which may

cause ions deficiency in plants. This shows that Cd stress inhibits transfer of these elements in plants from root to shoot. The ion stability in a cell is closely related to plant adaptation to heavy-metal toxicity. Although, it can be depicted that an increase in H⁺-ATPase activity (not measured in the present study) is the mechanism of protecting the integrity of plasma

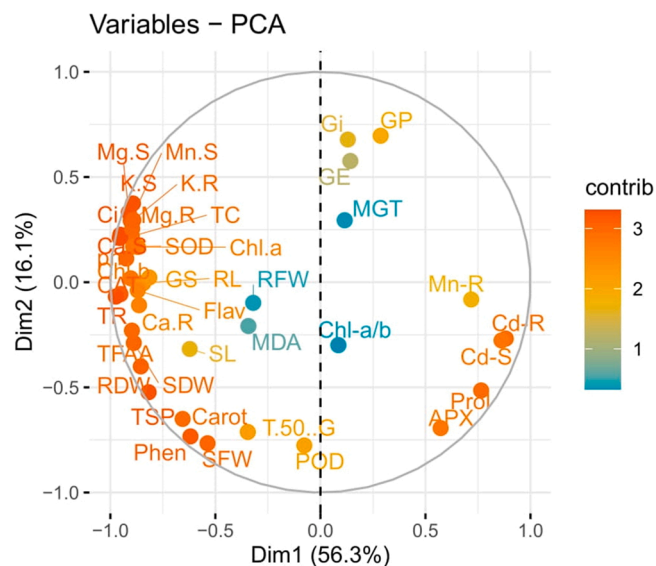


Fig. 6. Loading plots of Principal component analysis (PCA) on various attributes (morphological and physiological) of different varieties of *B. oleracea s* under the various application levels of nitric oxide in Cd-contaminated soil. Different abbreviations used in the figure are as follow: K-R (potassium content in the roots), Mg-S (magnesium content in the shoots), Ca-S (calcium content in the shoots), Mg-R (magnesium content in the roots), K-S (potassium content in the shoots), Ci (intercellular CO₂), Mn-S (manganese content in the shoots), Ca-R (calcium content in the roots), Flav (flavonoid content), SDW (shoot dry weight), RDW (root dry weight), TFAA (Trifluoroacetic anhydride), CAT (catalase activity), TR (transpiration rate), RL (root length), SOD (superoxidase dismutase activity), Chl-a (chlorophyll a content), TC (total chlorophyll content), Chl-b (chlorophyll b content), GS (stomatal conductance), POD (peroxidase activity), T-50%-G (time to 50% seed germination), SFW (shoot fresh weight), Carot (carotenoid content), TSP (Trisodium Phosphate), Phn (phenolic content), SL (shoot length), MDA (malondialdehyde content), Chl-a/b (chlorophyll a/b content), RFW (root fresh weight), Mn-R (manganese content in the roots), APX (ascorbate peroxidase activity), Prol (proline content), Cd-R (Cd concentration in the roots), Cd-S (Cd concentration in the shoots), GP (germination percentage), Gi (germination index), GE (germination energy) and MGT (means germination time).

membrane, which can improve the resistance to Cd toxicity. So the plants can absorb more mineral nutrients and maintain ionic homeostasis (Ahmad et al., 2018). A principal defense step to counteract Cd toxicity in plants is to prevent Cd accumulation in shoot tissues. It was documented by (He et al., 2014) that exogenous NO alleviated Cd toxicity in *Oryza sativa* by increasing pectin and hemicellulose contents in root cell walls, increasing Cd deposition in root cells and decreasing Cd accumulation in soluble fractions of leaves. Recently the application of NO scavenger in lupine is shown to reduce the level of Cd accumulation due to reduced endogenous NO levels (Gill et al., 2013; Panda et al., 2011). In this work the reduced uptake of Cd perhaps may also be a result of formation of metal Cd-NO complex, however this will need further experiments.

5. Conclusion

Outcomes of current study revealed that toxic level of Cd significantly affected plant growth and biomass, photosynthetic pigments, gaseous exchange traits, antioxidative machinery and minerals uptake by *B. oleracea* genotypes. Furthermore, Cd toxicity increased the oxidative stress indicators, Cd contents in plant organs. We also noticed that FD-4 and *Ceilo Blanco* genotypes showed higher tolerance to Cd stress while FD-2 and FD-3 showed more sensitive responses to the Cd stress induced into in the sand. Our results provided strong evidence that NO effectively alleviated Cd-induced reduction in growth and biomass,

which was mainly attributed to decrease in Cd uptake, protection of photosynthetic pigments and proteins, inhibition of ROS content by increasing the activities of several antioxidant enzymes as well as by maintaining the content of essential minerals. Overall, we have concluded that FD-4 and *Ceilo Blanco* showed higher resistance to the Cd stress under NO application and can be cultivated in metal stressed environment to produce higher yield and productivity. However, further genetic and molecular studies should be considered to gain a deeper insight into the better understanding of the detailed mechanisms of NO-induced Cd-stress tolerance in *B. oleracea* genotypes.

CRediT authorship contribution statement

Conceptualization, Mehwish, Muhammad Nawaz, methodology, Mehwish, Muhammad Nawaz; software Muhammad Hamzah Saleem, Mohammed Alsafran, Hareb Al Jabri; validation, Kamal Usman; formal analysis, Mehwish, Muhammad Nawaz; investigation, Mehwish; resources, Shafaqat Ali; data curation, Mehwish, Muhammad Nawaz; writing—original draft preparation, Jing Ma, Shafaqat Ali, Kamal Usman and Muhammad Hamzah Saleem; writing—review and editing, Jing Ma, Muhammad Hamzah Saleem, Mohammed Alsafran, Hareb Al Jabri, Shafaqat Ali; visualization, Mehwish, Muhammad Nawaz; supervision, Muhammad Nawaz; project administration, Jing Ma, Muhammad Hamzah Saleem; funding acquisition, Jing Ma, Muhammad Hamzah Saleem.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113969.

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