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Folic acid supplementation improves seed germination, seedling growth and cadmium uptake in a mining ecotype of *Solanum nigrum* L.

Zulfiqar Ali Sahito [a], Afsheen Zehra [a], Song Yu [a], Shaoning Chen [b], Mian Abdur Rehman Arif [c], Syed Turab Raza [d], Altaf Hussain Lahori [e], Mai Ali Mwaheb [f], Zhenli He [g], Xiaoe Yang [a,*]

[a] *Ministry of Education (MOE) Key Laboratory of Environmental Remediation and Ecosystem Health, College of Environmental and Resources Science, Zhejiang University, Hangzhou 310058, China*
[b] *College of Life Sciences and Medicine, Zhejiang Sci-Tech, University, Hangzhou 310018, China*
[c] *Plant Breeding and Genetics Division, Nuclear Institute for Agriculture and Biology, Jhang Road, Faisalabad, Pakistan*
[d] *Yunnan Key Laboratory of Plant Reproductive Adaptation and Evolutionary Ecology, Institute of Biodiversity, Yunnan University, Kunming, 650500, China*
[e] *Department of Environmental Sciences, Sindh Madressatul Islam University, Karachi 74000, Pakistan*
[f] *Department of Botany, Faculty of Science, Fayoum University, Fayoum 63514, Egypt*
[g] *University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, Fort Pierce, FL 34945, United States*

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ABSTRACT

Cadmium (Cd) contamination poses a significant threat to global agricultural production and hyperaccumulating plant species, such as *Solanum nigrum* (L.), face challenges in phytoremediation due to limited biomass production. Application of plant growth regulators such as, folic acid (FA) is a promising strategy to increase biomass production in these plant species. Therefore, a study was conducted to assess the interaction between FA and Cd in the mining ecotype of *S. nigrum*. The study involved two phases: germination and growth. In the first phase, seeds were exposed to different concentrations of $CdCl_2$ (i.e. 0, 10, 25, 50, 100, 200, and 400 μM) in the presence (0, 25, 50 and 100 μM) or absence of FA for ten days. Results showed that FA enhanced seed germination under Cd stress and acted as an antioxidant and stimulated germination and emergence at moderate concentrations. At higher concentrations, it reduced germination percentage. The study also found significant variations ($P < 0.05$) in growth attributes and Cd uptake in all FA concentrations. Cd was generally higher in shoots than roots, with the highest Cd concentration found in T6 (200 μmol Cd + 50 μmol/L FA) with a 170-fold difference (mean value: 1690 mg/kg) from control (10 mg/kg). Furthermore, an increase in exposure time led to a 2-fold increase in reduced glutathione (GSH) level in leaves at T3 (100 μmol/L Cd + 50 μmol/L FA).Therefore, we concluded that FA is a biostimulant that activates protective mechanism, alleviates oxidative stress and improves Cd uptake and accumulation in hyper-accumulating species.

* Corresponding author.
E-mail address: xeyang@zju.edu.cn (X. Yang).

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1. Introduction

In the recent years, cadmium (Cd) pollution has emerged as a significant public health and environmental concern around the globe (Sahito et al., 2022; Zehra et al., 2020). The proper remediation of contaminated soils is mandatory to protect agricultural production and human life from the detrimental effects of Cd exposure (Yu et al., 2024; Liu et al., 2022; Chaney et al., 2005). There are several methods available for soil remediation, but these are either costly or potentially harmful to the surrounding environment. As research advances, more sustainable and cost-effective methods are being developed. One commonly used approach is phytoremediation, which utilizes certain plant species that have ability to accumulate and detoxify heavy metals. This approach enhances environmental sustainability and promotes a healthier ecosystem for sustainable agriculture (Yu et al., 2023; Wang et al., 2020). Phytoremediation relies on two cornerstones, i.e. identifying plants with a high potential for metal hyperaccumulation and understanding the dynamics to optimize metal uptake and accumulation in these plant species (Yang et al., 2004; Ueno et al., 2008). The first criterion was probably confirmed by the identification of Cd hyperaccumulators such as *Sedum alfredii* (Hance) (Yang et al., 2004), *Arabidopsis halleri* (L.) (Zhao et al., 2006), *Solanum nigrum* (L.) (Han et al., 2020; Sun et al., 2008), *Bidens pilosa* (L.) (Sun et al., 2009), *Noccaea caerulescens* (j. & C. Presl) FK Mey (Rees et al., 2015) and *Lantana camara* (Linn) (Liu et al., 2019), which can uptake high concentration of Cd and display no toxicity symptoms (Verbruggen et al., 2013). These Cd hyperaccumulators are from different families and have adapted unique mechanisms for accumulating and tolerating high concentrations of Cd in their tissues. Among them, Chinese black nightshade (*Solanum nigrum* L.) is gaining attention due to its capacity to accumulate significant amounts of Cd in its above ground biomass (Wei et al., 2005; Han et al., 2020). This makes it an excellent option for cleaning of contaminated soils. Its farmland ecotypes are incorporated into landscaping and green spaces to help in improving the environmental quality of urban area. Secondly, large scale phytoremediation projects require enhanced growth performance and biomass production in these hyperaccumulating plant species. Therefore, researchers are exploring new methods and technologies, such as applications of growth regulators, intercropping techniques, gene manipulation, microbial inoculation and sustainable farming practices to accelerate the phytoremediation process (Tak et al., 2012; Lin et al., 2014; Rostami and Azhdarpoor, 2019). These methods reduce environmental impact while maintaining productivity, promoting soil health, erosion reduction, and water conservation. A more sustainable future in phytoremediation is not far if we employ an amalgam of these approaches.

One approach to improve the phytoremediation potential of plant species is to enhance antioxidant capacity (Zhang et al., 2022). Research on citrus (*Citrus aurantium* L.) and maize (*Zea mays* L.) has shown that high antioxidant levels can protect the photosynthetic system from oxidative damage (Zhang et al., 2015; Giannakoula et al., 2021). Folate (Folic Acid) is a part of the vitamin B-complex and is essential for both plants and animals for metabolic functioning of proper cell growth, cell division and DNA/RNA synthesis (Burguieres et al., 2007; Gorelova et al., 2017; Ozmen and Tabur, 2020; Stakhova et al., 2000). Folates are essential for proper cell functioning in stem cells and serve as coenzymes in several biological activities (Wagner, 2001; Ormazabal et al., 2015; Intlekofer and Finley, 2019; Kim et al., 2014). Vitamin B9 and folates are crucial for carbohydrate and nitrogen metabolism (Stakhova et al., 2000). FA has been found to regulate protein and nucleic acid synthesis, accelerate cell division, stimulate hormone production and improve nutrient uptake in plants (Khater et al., 2023; Selem et al., 2022). It positively affects photosynthesis and chlorophyll content (Khan et al., 2022). FA also enhances tolerance to metal stress (Poudineh et al., 2015; Kilic and Aca, 2016; Ibrahim et al., 2021; Alsamadany et al., 2022; Al-Elwany et al., 2022). Golizadeh et al., (2015) reported that FA exerts a strong dose-dependent effect on plant growth promotion. FA has been reported with dual roles in plant physiology: as an oxidant that stimulates plant growth, and as a pro-oxidant that causes oxidative damage depending on its optimal concentrations. FA-treated plants exhibit changes in gene expression and may be used as a pro-oxidant strategy to control plant growth under specific conditions. A dose and context based evaluation may be required to comprehend the complex role of FA in plant physiology (Xu et al., 2021). Therefore, we hypothesized that treating plants (*S. nigrum*) with FA at seed germination level and seedling growth phase may neutralize the harmful effects of Cd toxicity and improve the phytoextraction potential of hyperaccumulating plant species because of its antioxidant properties.

2. Materials and methods

2.1. Seed germination experiment

We used black nightshade (*Solanum nigrum* L.) seeds in our investigation. The seeds were initially sterilized with 1% NaOCl. Cadmium (Cd) concentrations were adjusted as 0.0, 10, 25, 50, 100, 200, and 400 μM) and folic acid ($C_{19}H_{19}N_7O_6$) concentrations were (0, 25, 50 and 100 μM) for ten days. Seeds were pre-soaked in distilled water (control) and FA (25, 50 and 100 μM) for 12 h at room temperature. After soaking period, the seeds were carefully, transferred onto petri dishes (25 seeds per plate) lined with sterilized filter paper and treated with distilled water as control and different Cd concentrations i.e. 10, 25, 50, 100, 200, and 400 μM) for 10 days. The petri dishes were placed in a growth chamber set at optimal temperature and humidity conditions for further observation and analysis. The solution was replaced after three days. The penetration of radicle through the seed coat was used as criteria for seed germination (Bewley, 1997). This method allowed us accurate measurement of successful germination. At the end of the experiment, germination (%) was counted. Several germination indices were used to calculate the means of germination index (%), mean germination time, seedling vigor index, energy of germination, and final germination percentage (%). These indices provided a comprehensive assessment of the germination process. The mean germination index (%) revealed the overall success rate of germination, while the mean germination time indicated the average duration for seeds to germinate. The seedling vigor index quantified the strength and vitality of the seedlings, while the energy of germination measured the metabolic activity during germination. Finally, the final germination percentage (%) represented the proportion of seeds that successfully completed the germination process.

Germination Percentage (GP):

$$GP = \frac{\text{Number of Total Germinated Seeds}}{\text{Total Number of seeds Tested}} \times 100 \quad (1)$$

Germination Index (GI):

$$GI = \frac{\text{Number of Germinated Seeds}}{\text{Day of First Count}} + \cdots\cdots\cdots\cdots + \frac{\text{Number of Germinated Seeds}}{\text{Day of Final Count}} \quad (2)$$

Mean Germination Time (MGT):

$$MGT = \sum dn \div \sum n \quad (3)$$

Where (n) is the number of seeds, which were germinated on days (d), and (d) is the number of days counted from the beginning of germination.

Energy of Germination (EG):

$$EG = \frac{\text{Number of Germinated Seeds after twenty days}}{\text{Number of Germination Days}} \times 100 \quad (4)$$

Seedling Vigor Index (SVI):

$$SVI = (\text{Average Shoot Length} + \text{Average Root Length}) \times \text{Germination \%} \quad (5)$$

2.2. Growth experiment

After seeds germination, seedlings were transplanted into 500 ml plastic pots filled with nutrient solution (HNS: Hogland and Arnon, 1938). Seedlings were allowed to grow up to three leaf stage and acclimatize in hydroponics for two weeks. The experiment involved a slightly modified water culture system, which included a container filled with a nutrient solution for plant growth (Gibeaut et al., 1997). The plants were placed in Styrofoam, which allows their roots to be submerged in the solution. This system ensured constant water and nutrient supply for plant growth. The modification aimed to optimize conditions for growth and maximize nutrient uptake efficiency. The experiment was conducted using a fully randomized design, consisting of four replicates of each treatment. The seedlings were exposed to 0, 100 and 200 μM of Cd ($CdCl_2$), in HNS supplemented with 0, 25 and 50 μM FA ($C_{19}H_{19}N_7O_6$). The solution was aerated continuously during the experimental time. The plants were grown in a growth chamber with an 8 h light period, 350 l mol m^{-2} s^{-1}light intensity, 25 °C temperatures and 60–70% relative humidity. The plants were collected 14 d and 28 d after treatments and washed, separated into roots, shoots and weighed. Shoots and roots were dried in an oven at 70 °C for 48 h to determine dry weight and Cd analysis. Fresh samples were frozen in liquid nitrogen and stored at −70 °C for further biochemical testing.

2.2.1. Morphological parameters

Shoot (Sl) and root (Rl) lengths were measured with the help of scale in (cm). Fresh shoot (FSW) and root (FRW) weights (g) were measured at the time of harvest after rinsing with tissue paper with the help of lab balance. For the purpose of dry shoot (DSW) and root (DRW) weights, the root and shoots were dried at 65 °C for 72 h in oven and then their weights (mg) were measured. The root related traits viz. root surface area (RSA), root diameter (ARD) and root tip number (RTN) were measured with the help of root automatism scanner (MIN Mac, STD1600+) connected with computer coupled WINRHIZO™ 2000 program software (Regent Instruments Inc., Quebec, Canada).

2.2.2. Cd analysis

Oven-dried leaves and roots were digested with conc. nitric acid using USEPA Method 3050B (USA.EPA, 1994) on a temperature controlled digestion block. Cadmium analysis was conducted using a graphite furnace atomic absorption spectrometer (Perkin-Elmer SIMAA 6000, Norwalk, CT). We used a standard reference material (SRM) and certified Cd standard solution for calibration. The analysis used matrix spikes on 10% of samples, which resulted in an average recovery of 94 ± 10%.

2.2.3. Lipid peroxidation

Thiobarbituric acid reacting substances (TBARS) are quantitative indicator of oxidative stress in living organisms. The lipid peroxidation was assessed following the Heath and Packer method (Heath and Packer, 1968; Ohkawa et al., 1979). Frozen tissues were crushed and homogenized with 2.5 ml of 5% (w/v) TCA (trichloro acetic acid) in a glass homogenizer using a cold mortar and pestle. The mixtures were mixed and placed in 50 ml Nalgene centrifuge tubes, which were then centrifuged at 10,000 g for 20 min at room temperature. The concentrations of lipid peroxide and oxidatively modified plant proteins were quantified and expressed in in terms of l mol g^{-1} fresh weight of plant sample, with total TBARS being calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.2.4. Reduced glutathione (GSH)

The GR-based quantification method is widely used for determination of GSH levels in living organisms (Gossett et al., 1994). Frozen plant samples (1 g) were pulverized with inert sand and ice-cold 6% m-phosphoric acid (pH 2.8) with 1 mM EDTA in a cold pestle and mortar. The homogenate mixture was centrifuged for 20 min at 22,000 g, the supernatant was extracted and the mixture

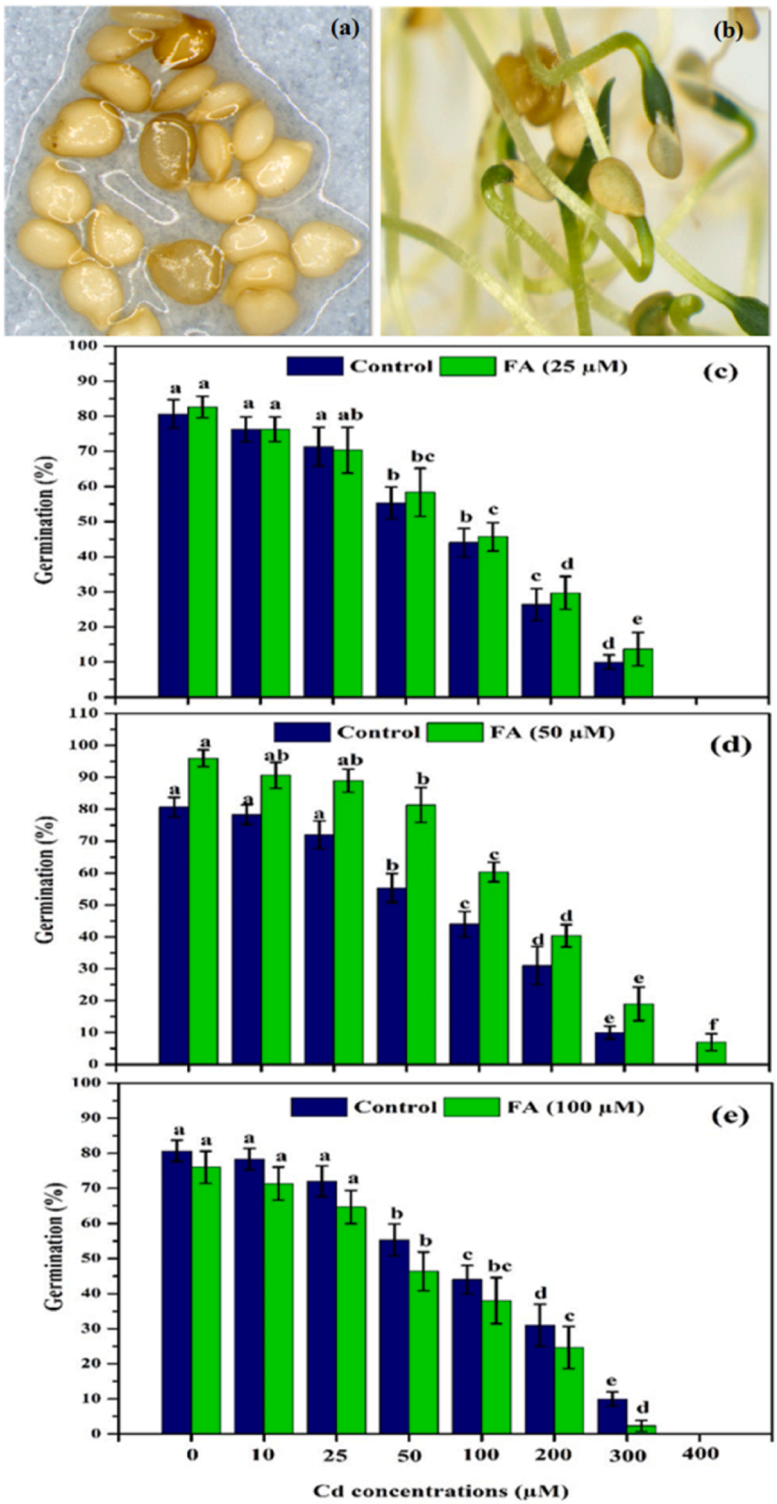

Fig. 1. Effects of Cd and Folic acid (FA) applications on seed germination. A: Seeds are germinating (B) Germinated seeds after seven days (C) Effect of different Cd concentration on seeds germination percentage (GP) in the presence of Folic acid (FA) FA 25=25 µM (D) Effect of different Cd concentration on seeds germination percentage (GP) in the presence of Folic acid (FA) FA 50=50 µM (E) Effect of different Cd concentration on seeds germination percentage (GP) in the presence of Folic acid (FA) FA 100=100 µM. Data are means ± SE (n=5).

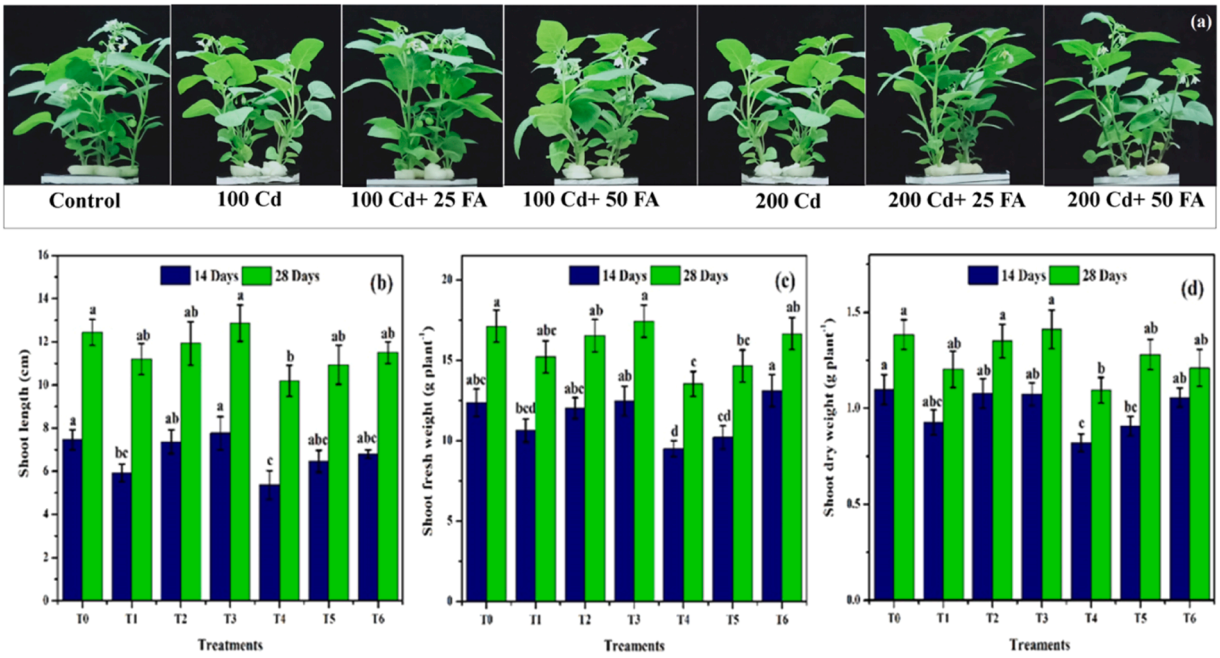

Fig. 2. Effects of Cd and Folic acid (FA) applications on shoot fresh weight and biomass of Solanum nigrum in hydroponics. A: Phenotypes of *Solanum nigrum*-mining ecotype under different treatments.Cd^{2+}=100μM, Cd^{2+}=200μM. Folic acid (FA) FA 25=25 μM, FA 50=50 μM. (B) Shoot length under different treatments (C) Shoot fresh weight under different treatments and different time periods (D), shoot biomass. Data are means ± SE (n=5). Different letters indicate significant differences at $P < 0.05$. Different treatments: T0 = (control); T1 = 100 μmol/L of Cd; T2 = 100 Cd+25FA μmol/L; T3 = 100 Cd+50FA μmol/L; T4 = 200 Cdμmol/L; T5 = 200 Cd+25FA μmol/L; T6 = 200 Cd+50FA μmol/L.

was filtered through a 0.45 mm ultrafilter. The mixture solutions were prepared: Solution A, composed of 110 mM Na2PO4 7H2O, 15 mM EDTA, 40 mM NaH2PO4 H2O, 0.3 mM 5, 0.4 ml L 1 BSA and 50 -dithiobis-(2-nitrobenzoic acid), and Solution B, composed to 0.2 ml L 1 BSA, 50 mM imidazole, 1 mM EDTA. The GSH was measured in the reaction mixture containing 400 ml of solution A, 320 ml of solution B, a 1:50 dilution of the extract in 5% Na^2HPO^4 (pH 7.5), and 80 ml of NADPH. To determine the reaction rate, a spectrophotometer was used to measure the change in absorbance at 412 nm over 5 min

2.2.5. Total non-protein thiol

The concentrations of non-protein thiol were determined using spectrophotometry method in frozen plant samples. The samples were pulverized with liquid nitrogen, and 100 mg of plant material was extracted using a mixture of 1 M NaOH and 1 g L^{-1} $NaBH_4$.The mixture was thoroughly mixed, centrifuged at 15,000 g for 10 min, and then 37% of concentrated HCl was added to 300 IL of supernatant. After vigorous shaking, the samples were recentrifuged at 14,000 g for 5 min the supernatant was added to 1 ml of a solution containing 300 lM 5,5-dithiobis(2-nitrobenzoic acid) in 100 mM potassium phosphate buffer, pH 7.5 (Ellman's reagent). Samples were incubated at room temperature for 10 min and the optical density of samples was recorded at 412 nm. Standard curve ($r^2 > 0.97$) was created using samples with varying GSH values (0–100 mmol per 20 μL). This method does not account total or conjugated thiols; it only measures free non-protein thiols.

2.3. Data analysis

All results were expressed as an average of four replications (mean ± standard deviation (SD). Treatment effects were determined by analysis of variance using SPSS Software (IBM SPSS platform for data science and data analysis). Duncan's test, with a 5% probability, was utilized for post hoc comparisons to distinguish treatment differences. Correlation diagram was visualized in R Studio build 2023.06.0 using the "qgraph" package (Epskamp et al., 2012) according to Arif et al. (2022)

3. Results

3.1. Seed germination

The seed germination is a very crucial stage in the life cycle of the plant which is greatly influenced by both biotic and abiotic factors that the germinating seeds may encounter (Arif et al., 2012). It highly affected various metrics in the germination process (Table 1). It also showed a significant impact on seed emergence and early plant growth (Table 2). The increase in Cd concentration

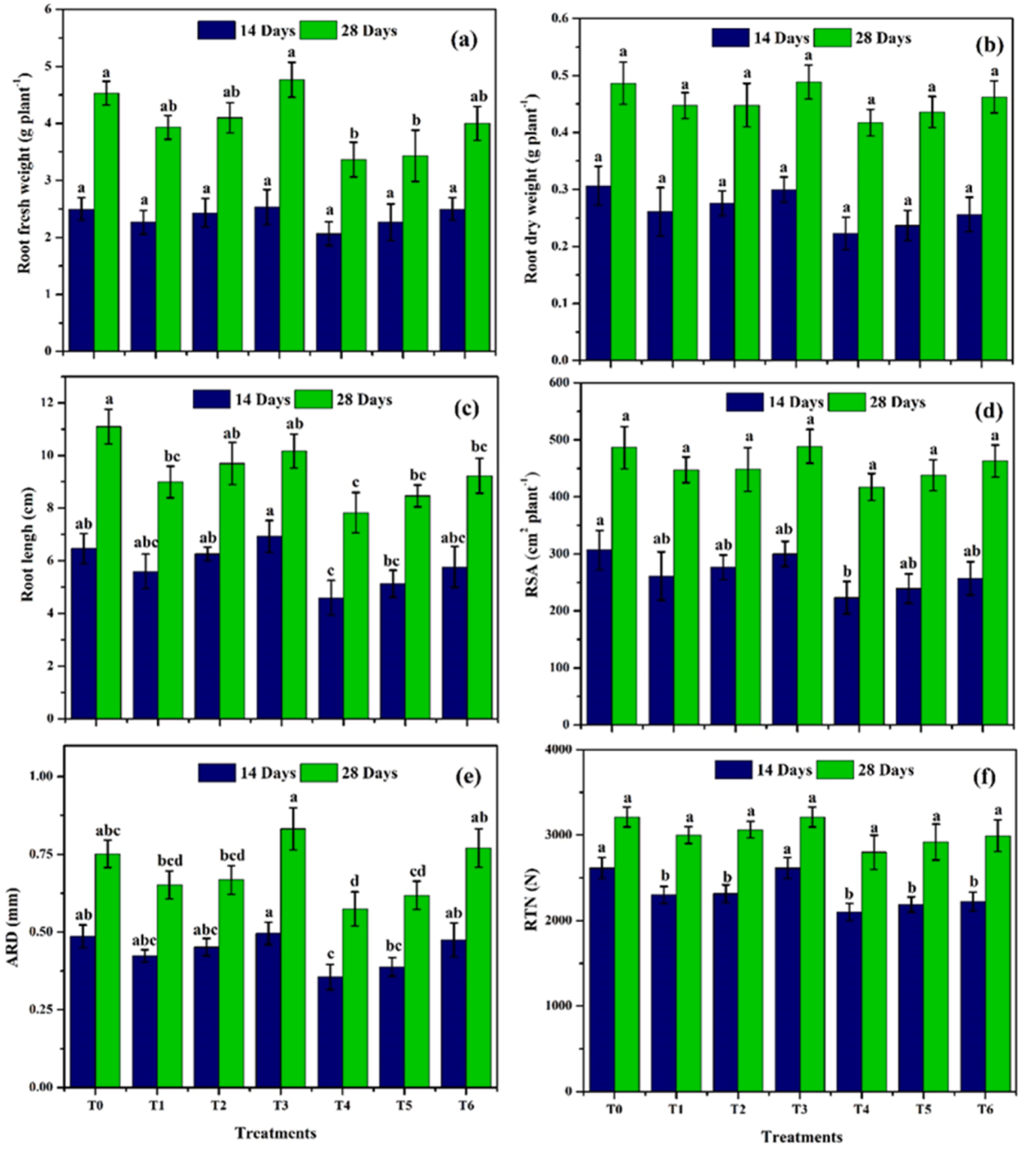

Fig. 3. Effects of Cd and Folic acid (FA) on morphological characteristics of *S. nigrum* root in hydroponics. A: root fresh weight (RFW), B: specific root length (SRL), C: specific root surface area (SRA), D: specific root volume (SRV), E: average root diameter (ARD), F: root tip number (RTN). Different treatments: T0 = (control); T1 = 100 μmol/L of Cd; T2 = 100 Cd+25FA μmol/L; T3 = 100 Cd+50FA μmol/L; T4 = 200 Cdμmol/L; T5 = 200 Cd+25FA μmol/L; T6 = 200 Cd+50FA μmol/L.

significantly inhibited the seed germination of *S. nigrum* (Fig. 1). This negative impact of Cd, however, was alleviated in the presence of FA that stimulated the germination (Fig. 1b-d). The application of 25 μM FA slightly increased the seed germination (83%) compared to control (80%). On the other hand, 15% germination was achieved at 300 μM Cd+ 25μM FA compared to 10% germination without FA. No germination could be witnessed when treated 25 μM FA at the highest Cd levels (Fig. 1b).

At 50 μM FA, significantly higher germination ($P < 0.05$) occurred from lower to higher Cd levels. At lower level of Cd (10 μM), germination was up to 95% compared to 80% (control). A 5% germination was still achieved at the highest Cd (400 μM) concentration

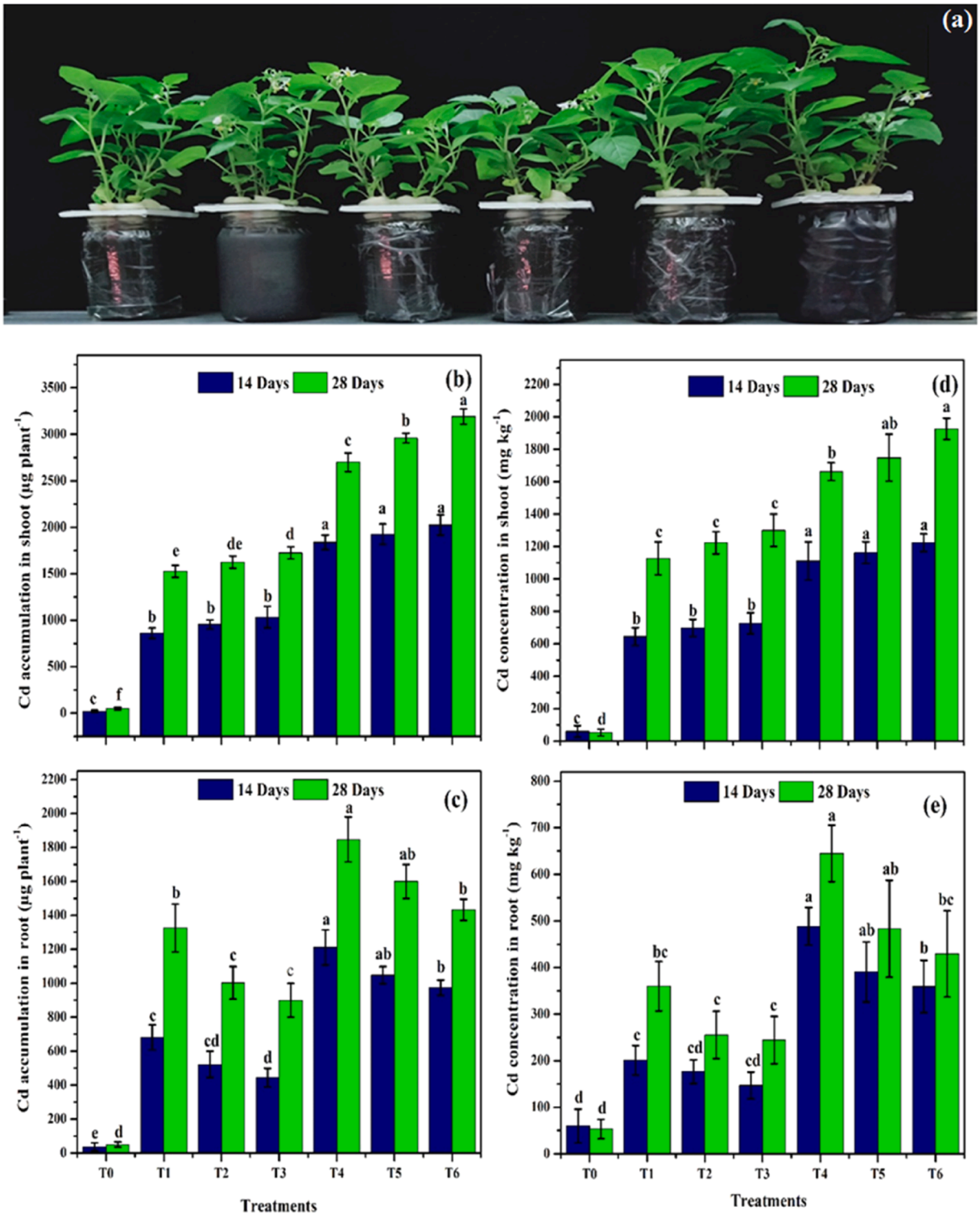

Fig. 4. Effects of Cd and Folic acid (FA) applications on cadmium uptake per plant and Cd accumulation mg/kg biomass of Solanum nigrum in hydroponics. A: Phenotypes of Solanum nigrum-hyperaccumulating ecotype under different treatments. (B) Cadmium uptake per plant (C) Cadmium accumulation mg/kg of plant biomass. Data are means ± SE (n=5). Different treatments: T0 = (control); T1 = 100 µmol/L of Cd; T2 = 100 Cd+25FA µmol/L; T3 = 100 Cd+50FA µmol/L; T4 = 200 Cdµmol/L; T5 = 200 Cd+25FA µmol/L; T6 = 200 Cd+50FA µmol/L.

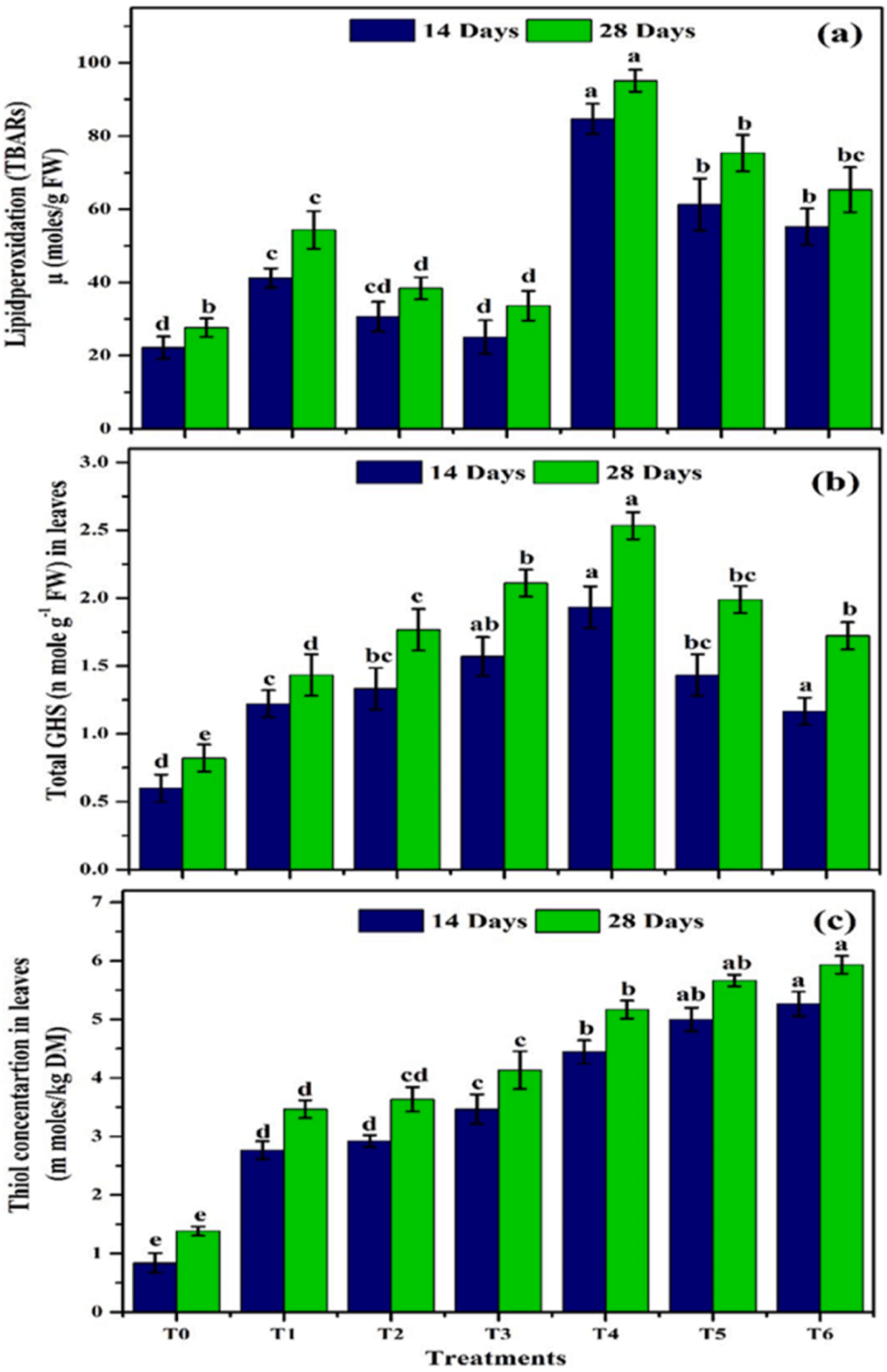

Fig. 5. Effects of Cd and Folic acid (FA) applications on lipidperoxidation (TBARs), Total GSH and Thiol concentrations in hydroponics. Data are means ± SE (n=5). Different letters indicate significant differences at $P < 0.05$. Different treatments: T0 = (control); T1 = 100 μmol/L of Cd; T2 = 100 Cd+25FA μmol/L; T3 = 100 Cd+50FA μmol/L; T4 = 200 Cdμmol/L; T5 = 200 Cd+25FA μmol/L; T6 = 200 Cd+50FA μmol/L.

at 50 μM FA compared to no germination without FA (Fig. 1c). On the contrary, the highest (100 μM) concentration of FA exhibited a non-significant ($P > 0.05$) influence on seed germination at all Cd levels (Fig. 1d).

3.2. Shoot biomass

A significant ($P < 0.01$) proliferation in shoot growth was found at low Cd levels under various FA concentrations at 28 d exposure (Fig. 2). A decline in Sl was found at the highest (200 μM) Cd level (5 cm 14 d: 10 cm 28 d) as compared to control (7.5 cm 14 d: 12.9 cm 28 d).

Application of FA substantially alleviated the metal stress even at 200 μM Cd, whereas, at T3 (50 μmol/L FA) a slight increase in the Sl (12.9 cm) was observed as compared to T0 (12 cm) after 28 d (Fig. 2b). On the flip side, at the highest Cd level (200 μM Cd), Sl was low under both FA concentration (T5: 25 μmol/L, 10.9 cm) and (T6: 50 μmol/L, 11.2 cm) then control. A similar trend was evident for the SFW where an increase in SFW (17 g/ plant) was also found in the T3 (100 μM Cd + 50 μmol/L FA) which was equal to the control treatment (Fig. 2c). The trend of increase in plant biomass was variable. Cd decreased the shoot biomass in general but with increasing time exposure, a greater biomass was produced. SDW after 28 d exposure increased from 0.9 g/plant to 1.2 g/plant at T1 (100 μM Cd). Highest shoot biomass (1.41 g/plant) was found, however in T3 (100 μM Cd + 50 μmol/L FA), whereas, high Cd level showed non-significant ($P < 0.05$) effect on shoot biomass even at both FA concentrations (T5 and T6) (Fig. 2d).

3.3. Root biomass of S. nigrum

There was a decreasing trend in the root morphological characteristics with the increase in Cd levels, however, addition of low FA concentration (T3: 50 μmol/L) increased root biomass after 28 d exposure (Fig. 3). We observed the highest RFW (4.5 g/plant) and RDW (0.48 g/plant) in T3 (100 μM Cd + 50 μmol/L FA) which was also equal to the control (Fig. 3a & 3b). On the other hand, a non-significant ($P < 0.05$) difference in RL was found in plants treated with 50 μmol/L FA under 100 (T3, 100 μmol Cd + 50 μmol/L FA: 9.5 cm) or 200 μmol Cd (T6, 200 μmol Cd + 50 μmol/L FA: 8.5 cm) concentrations compared to control (T0: 11.2 cm) (Fig. 3c). The RSA and RTN were the same (480 cm^2/plant: RSA, 31000: RTN) in both T0 and T3 treatments (Fig. 3d & 3 f). Maximum ARD (0.8 mm) was found in T3 (100 μmol Cd + 50 μmol/L FA) compared to 0.75 mm in control. In addition, both FA concentrations (25μmol/L and 50 μmol/L) inhibited the ARD at all Cd levels (Fig. 3e).

3.4. Cadmium concentration in plant parts

Increasing Cd concentration and exposure time resulted in metal accumulation both in shoot and root where its concentration was higher in shoot than root in terms of total plants (Fig. 4b). A 20–40 fold increase in shoot Cd concentration was witnessed at 14 d and 28 d intervals. Addition of high FA concentration at low Cd level (T3: 100 μmol Cd + 50 μmol/L FA) just increase 32- fold Cd concentration (men value: 1550 μg/plant) in shoot, but, highest Cd concentration (3200 μg/plant) was found in T6 (200 μmol Cd + 50 μmol/L FA) with 64-fold difference (mean value: 3150 μg/plant) from control (50 μg/plant). On the contrary, root Cd concentration was highest (18,200 μg/plant) at T4 (200 μmol/L Cd). The application of FA non-significantly affected the Cd concentration with a 13-fold (mean value: 16,800 μg/plant) reduction in root Cd concentration was exhibited in T6 (200 μmol Cd + 50 μmol/L FA).

There were significant differences ($P < 0.05$) in the accumulation of Cd at all FA concentrations, with their contents being generally higher in shoot than root in terms of mg/kg of biomass (Fg. 4c). Highest Cd accumulation (1700 mg/kg) was found in T6 (200 μmol Cd + 50 μmol/L FA) where there was 170-fold difference (mean value: 1690 mg/kg) from control (10 mg/kg). An erratic pattern of Cd accumulation was also evidenced in roots of *S. nigrum* where addition of FA concentrations substantially decreased the Cd accumulation at all Cd levels.

3.5. Lipid peroxidation

We observed that increasing Cd concentrations increased TBARs, total GSH and thiol levels in shoots of *S. nigrum* (Fig. 5). Regarding the ameliorative effect of FA, it can be observed that increasing FA concentration significantly ($P < 0.05$) increased Cd uptake, while TBARs level in leaves of *S. nigrum* was found to be low (Fig. 5a). As for GSH, it was observed that increase in exposure time caused a 2-fold increase in GSH level (1.9 nmol/g FW) in leaves at T3 (100 μmol/L Cd + 50 μmol/L FA) when compared to control (0.8 nmol/g FW) (mean value: 1.1 nmol/g FW). Though, increase in Cd and FA concentration (T4-T6) significantly decreased the GSH level in leaves of *S. nigrum* (Fig. 5b).

Thiol contents under different Cd and exogenously supplied FA are presented in Fig. 5c. In leaves, thiols significantly ($P < 0.05$) increased with increasing Cd concentrations, with exposure time. Addition of high FA concentration (in T6: 200 μmol/L Cd + 50 μmol/ L FA), a 4-fold increase (5.4 mmol/kg DW) in the thiol levels was evidenced as compared to control (1.4 mmol/kg DW) (mean value: 4 mmol/ kg DW).

3.6. Correlation between different growth factors of S. nigrum in response to Cd and exogenously applied FA

Correlation analyses indicated that in general the morphological traits of shoots (Sl, SFW, SDW) and roots (Rl, RFW, RDW, ASD, RTN and RSA) were in strong positive correlation after both exposures (viz. 14 d and 28 d) where the correlation co-efficient (r^2) was as high as > 0.8 in majority of comparisons (Fig. 6, Supplementary Table 1). Likewise, the physiological traits (Cd conc. in roots and

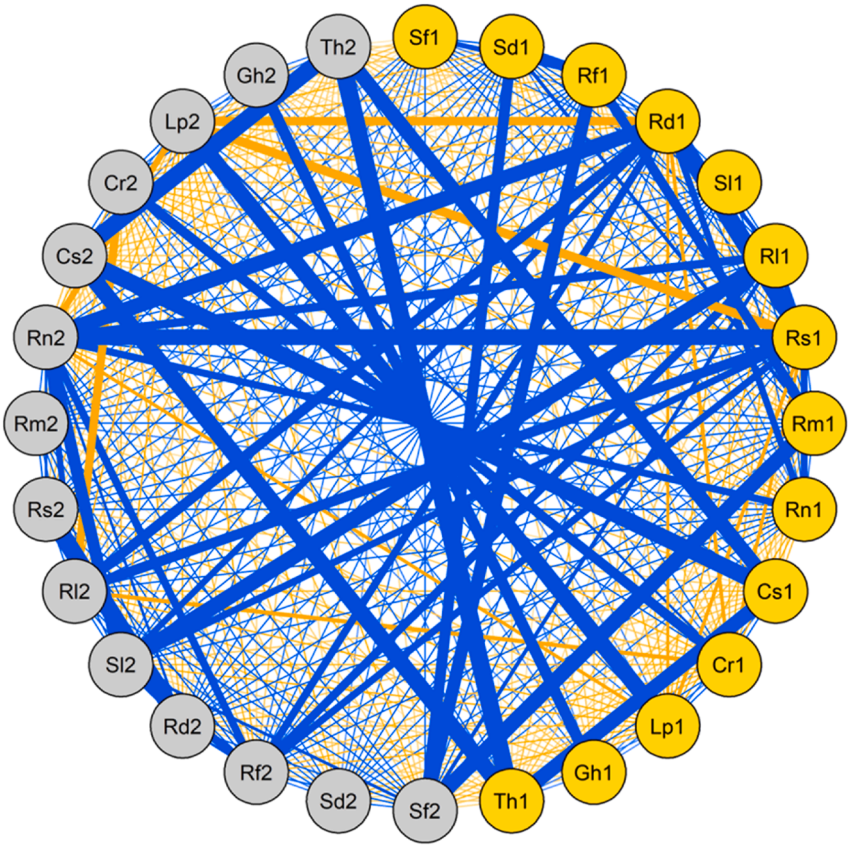

Abb	Trait
Sf1	Shoot fresh weight after 14 days
Sd1	Shoot dry weight after 14 days
Rf1	Root fresh weight after 14 days
Rd1	Root dry weight after 14 days
Sl1	Shoot length after 14 days
Rl1	Root length after 14 days
Rs1	Root surface area after 14 days
Rm1	Root diameter after 14 days
Rn1	Root tip number after 14 days
Cs1	Cadmium conc in shoots after 14 days
Cr1	Cadmium conc in roots after 14 days
Lp1	Lipid peroxidation after 14 days
Gh1	Glutatione conentation after 14 days
Th1	Thiol concetration after 14 days
Sf2	Shoot fresh weight after 28 days
Sd2	Shoot dry weight after 28 days
Rf2	Root fresh weight after 28 days
Rd2	Root dry weight after 28 days
Sl2	Shoot length after 28 days
Rl2	Root length after 28 days
Rs2	Root surface area after 28 days
Rm2	Root diameter after 28 days
Rn2	Root tip number after 28 days
Cs2	Cadmium conc in shoots after 28 days
Cr2	Cadmium conc in roots after 28 days
Lp2	Lipid peroxidation after 28 days
Gh2	Glutatione conentation after 28 days
Th2	Thiol concetration after 28 days

Fig. 6. Correlation network between different traits after 14 days (traits in yellow circles) and after 28 days (traits in grey circles). Blue connecting lines indicate positive correlation and orange connecting lines indicate negative correlations where the thickness of the line corresponds to the strength of the correlation. The table on the right explains the abbreviations for various traits. For details, see Supplementary Table 1.

shoots, lipid peroxidation, reduced GSH and total thiol contents) were in positive correlation with each other after 14 d and 28 d. However, this correlation was stronger ($r^2 > 0.8$) between Cd concentrations in root and shoots and total thiol contents than with other traits.

On the other hand, variable negative correlations existed between morphological root and shoot traits with the physiological changes. The highest negative correlation ($r^2 > 0.8$), however, was between lipid peroxidation and all the morphological traits except SFW. On the same grounds, Cd in roots and shoots were in strong negative correlation ($r^2 > 0.75$) with RDW, RSA and RTN after 14 d exposure. A relatively similar trend was observed after 28 d exposure for the same traits.

Across the two exposure times (14d and 28d), like within the exposure time individually, the traits' correlation was almost identical to those within the exposure time where morphological traits were in very strong positive correlation between each other. In addition, a negative correlation was observable between the Cd concentrations, lipid peroxidation, GSH levels and total thiols. Moreover, the physiological traits were also in strong positive correlation with each other.

4. Discussion

Cd is a toxic element linked to severe health complications in humans and animals (Zhai et al., 2014; Genchi et al., 2020). Currently, few technologies exist to remove Cd from the environment (Klimmek et al., 2001). One promising technology mainly rely on hyperaccumulating plant species like *Thlaspi caerulescens* and *Arabidopsis halleri, Sedum alfredii* and *Solanum nigrum* for effective phytoremediation (Ji et al., 2011; Yang et al., 2004; Zhao et al., 2003). These plant species have ability to uptake and accumulate higher concentrations of Cd in their tissue and reduce its concentrations in the soil. However, these hyperaccumulating plant species face challenges in phytoremediation due to slow growth and limited biomass production (Ozturk et al., 2003; Souza et al., 2013). Therefore, search for new strategies and technologies to improve efficiency and increase the growth rate of these plant species is underway (Guo et al., 2020; Hansda et al., 2014; Liu et al., 2015). Cd is also toxic to plants and adversely affects seed germination, growth, photosynthesis, and nutrient uptake (Dias et al., 2013; El Rasafi et al., 2022). Exposure to Cd also causes oxidative stress, damaging cellular structures and impairing overall plant health (Cuypers et al., 2010; Patra et al., 2011). To mitigate these adverse effects, the effects of Cd on plants is being investigate and strategies are developed to address the issues (Huang et al., 2017; Lin et al.,

2018). Cd induces oxidative stress in plants by generating reactive oxygen species (ROS) (Verma et al., 2013). ROS affects metabolic transport and damages cellular components (Van Breusegem and Dat, 2006). Multiple studies reported that biostimulant supplementation improves nutrient uptake, optimizes photosynthetic efficiency, promotes faster growth, increases biomass production and enhances metal uptake efficiency (Bulak et al., 2014; Cabello-Conejo et al., 2013; Srivastava et al., 2009). In this study, the link between FA, a biostimulant and Cd on seed germination, growth, and metal uptake efficiency in the hyper-accumulating *Solanum nigrum* (*S. nigrum*) mining ecotype was examined. Higher concentrations of Cd reduced the germination (%), affected growth performance and imposed oxidative stress and the effect was subsequently mitigated by the FA supplementation (Fig. 1 and Fig. 2). According to Fagioni (2009), Cd causes oxidative damage to photosynthetic machinery in thylakoids, disrupting electron transport chains and reducing ATP and NADPH production. This impairment affects photosynthesis causing stunting plant growth and reduced biomass production. To maintain these biochemical activities and optimal photosynthesis functioning, strategies should focus on minimizing oxidative stress. Furthermore, Cd can also interfere with the uptake and utilization of essential nutrients such as magnesium, calcium, and iron, which are necessary for proper chloroplast function (Yang et al., 1996; Baryla et al., 2001; Guo et al., 2016). The accumulation of Cd in plant tissues can also have toxic effects on other metabolic processes such as respiration and protein synthesis (Bavi et al., 2011). Overall, exposure to Cd can harm plant growth and development, as well as crop yield and quality. Therefore, it is important to develop strategies for minimizing cadmium contamination in agricultural soils and reducing plant uptake. Hyperaccumulating plant species have developed a number of defense mechanisms to protect themselves from the toxic effects of Cd i.e. production of metal-binding proteins that can sequester Cd ions (Choppala et al., 2014; Shahid et al., 2017). Sequestration of Cd ions into particular cellular compartments, i.e. cell walls and vacuoles, to minimize their interaction with vital cellular components is one such strategy (Parrotta et al., 2015; Krzesłowska, 2011). Another mechanism is the synthesis of phytochelatins, small peptides that bind to Cd ions and prevent them from damaging plant tissues (DalCorso et al., 2008; Pál et al., 2006). Additionally, some plants have evolved the ability to exclude Cd from their roots through a process known as exclusion (Verbruggen et al., 2009). This involves the selective uptake of essential nutrients while blocking the entry of toxic metals like Cd. Furthermore, certain plant species have been found to tolerate high levels of Cd by activating stress response pathways that help them cope with the toxic effects of this heavy metal. Overall, these defense mechanisms enable plants to survive in environments with high levels of cadmium and provide insights into how plants adapt to environmental stressors. Although the effect of folic acid (FA) have been studied in humans and animals, but its effects are still unclear in most of the plant species. Higher concentrations of Cd highly inhibited seed germination and emergence in *S.nigrum* seeds (Fig. 1). But the FA supplementation highly reduced the toxic effects and improved seed germination (Fig. 1d). Ibrahim et al. (2021) reported that folic acid, a protective agent and enhances the resilience of snap bean plants to water deficit conditions. The study found that folic acid increased antioxidant enzyme levels, higher photosynthetic rates, and reduced oxidative damage. Alsamadany et al. (2022) found that folic acid can help snap beans manage salt stress-induced oxidative damage by regulating growth, metabolites, antioxidant machinery, gene expression, increasing chlorophyll, proline, and soluble sugar levels.

The reduction of RFW, RDW, RL, RSA, RDA and RTN was observed with increasing doses of Cd concentrations (Fig. 3). Our results are in concurrence with Kohanová et al. (2018) who reported that availability of Cd in plants inhibited plant growth, decreased Rl and the number of lateral roots and root hairs per plant. Likewise, Sabella et al., (2022) also found morphological changes in roots (Rl and RTN) after Cd treatment.

The extent of Cd accumulation in the fresh and dry weight biomasses of shoot and root in *S. nigrum* in the hydroponics condition was found to be directly related with an increase dose of Cd integrated with FA dosage (Fig. 4). This evidenced that FA integrated with Cd supported the phytoextraction of Cd by *S. nigrum*. Similar results have been achieved for mustard plant which is reported to have great potential to accumulate Cd (Salt et al., 1995). To add to it, Rehman et al. (2017) has also reviewed the importance of *S. nigrum* in the phytoremediation of polluted sites owing to its ability to uptake toxic metals and tolerance. Thus, it has great potential to tolerate additional amount of toxic-metals through various mechanism, including antioxidant enzymes, metal deposition in non-active parts, root sequestration. The concentration of lipid peroxidation (LPO), glutathione (GHS) concentrations and thiol concentrations (TH) was reduced in *S. nigrum* plant under hydroponics condition with the application of Cd amended with FA (Fig. 5). It could be assumed that the FA had an ameliorating effect caused by Cd toxicity as evidenced by a decrease in the LPO, GHS, and TH concentration. This suggests that the presence of FA may protect the plant from oxidative stress and maintain cellular homeostasis. This fact is also supported by Deng et al. (2010) who found that that a 50 and 200 μM dosage of Cd application improved the level of TBARs and GSH levels in (S. *nigrum* L.) roots. However, further research is needed to understand the mechanisms behind this effect and determine the optimal levels of FA for promoting plant growth promotion and metal uptake in hydroponic systems and also in natural environments (field conditions).

5. Conclusion

To summarize, FA enhanced the *Solanum nigrum* L. plant growth and biomass under Cd stress through enhancing antioxidant enzyme activity, reducing oxidative damage and increased hyperaccumulating capacity for Cd. Seedlings exposed to 200 μM of Cd in combination with 50 μM of folic acid showed the highest growth rate, biomass accumulation and Cd uptake. On the other hand, those treated without folic acid showed the lowest growth rate, biomass production and Cd uptake. Folic acid supplementation can mitigate the negative effects of Cd toxicity on seedling growth and improve phytoextraction efficiency of hyperacumulator plant species in contaminated soil. However, our understanding of the dynamics of folic acid and the transport mechanism involving metal uptake and accumulation is still under infancy and further studies will shed more light on how FA improves the overall plant health under metal stress.

Table 1
Means of germination index (%), mean germination time, seedling vigor index, energy of germination and final germination percentage (%) as affected by Cadmium concentrations.

Treatments Cadmium ($\mu M\ L^{-1}$)	Germination index (%)	Mean germination time (d)	Seedling vigor index	Energy of germination	Final germination percentage (%)
0	99.46	3.55	3301.5	93.21	79.4
10	97.98	4.06	3137.7	88.12	76.3
25	94.35	4.45	2755.87	81.33	70.5
50	90.43	5.00	2257.34	74.66	58.2
100	83.76	5.40	1765.8	57.33	45.6
200	76.23	6.32	1101.33	34.66	29.3
300	62.76	6.52	7021.72	18.97	10.4
F-Test	**	**	**	**	**
$L.S.D_5$ %		0.28	132.8	5.44	2.8
$L.S.D_1$ %		0.35	179.4	7.21	3.7

*, ** mean significant at levels of probability 0.05 and 0.01.

Table 2
Germination rate and shoot length of germinating seedlings as affected by Cadmium concentrations.

Germination rate					Shoot length			
Cadmium	0	25 μM FA	50 μM FA	100 μM FA	0	25 μM FA	50 μM FA	100 μM FA
($\mu M\ L^{-1}$)						cm		
0	22.85	23.42	27.42	21.72	4.6	4.8	5.2	4.0
10	21.71	21.74	26.28	20.24	4.6	4.8	5.1	4.0
25	20.28	20.82	24.57	18.24	4.4	4.5	5.0	3.7
50	15.71	16.57	22.57	11.42	4.1	4.2	4.7	3.5
100	12.12	12.85	17.14	8.40	3.4	3.4	4.4	2.9
200	7.42	8.28	12.28	3.85	2.8	3.1	4.2	2.4
300	2.85	3.71	7.84	00	2.2	2.4	3.7	00
400	00	00	3.22	00	00	00	2.0	00

CRediT authorship contribution statement

Mai Mwaheb: Visualization, Validation, Investigation. **Song Yu:** Visualization, Formal analysis. **Afsheen Zehra:** Visualization, Validation, Software, Formal analysis. **Zulfiqar Ali Sahito:** Writing – original draft, Visualization, Project administration, Methodology, Formal analysis, Data curation. **Xiaoe Yang:** Supervision, Funding acquisition, Conceptualization. **Shaoning Chen:** Writing – review & editing. **Zhenli He:** Writing – review & editing, Conceptualization. **Altaf Hussain Lahori:** Visualization. **Syed Turab Raza:** Visualization, Validation. **Mian Abdur Rehman Arif:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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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.eti.2024.103600.

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