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# Optimization of Citric Acid and EDTA Levels Under Ni Stress Using Rapeseed *Brassica napus* L. for Phytoremediation

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**Abstract** Heavy metals like nickel, cadmium, and chromium found in industrial waste pose a serious threat to living organisms. Nickel is extremely poisonous and causes numerous human ailments when it is exceeded from the permissible limit. The most efficient and cost-effective method of removing nickel from contaminated soil is phytoremediation and chelating agents act as supporting material for phytoremediation. Consequently, the purpose of the current study was to enhance the phyto-extraction potential of *Brassica napus* by using chelating agents such as citric acid and ethylene diamine tetra acetic acid (EDTA). For phytoremediation, experimental treatments were comprised of different levels of citric acid, i.e., 10 mM and 20 mM and EDTA, i.e., 1.5 mM and 2.0 mM and the combinations of both (citric acid + EDTA), i.e., 10 mM + 1.5 mM, 20 mM + 1.5 mM, 10 mM + 2.0 mM, and 20 mM + 2.0 mM respectively under Ni toxicity. A control without citric acid and EDTA was kept for comparison. Different growth, physiological, and biochemical attributes were measured and analyzed statistically. Results revealed that the concentration of citric acid (10 mM) and EDTA (1.5 mM) separately and in combination EDTA (1.5 mM) + citric acid (10 mM) performed better for the purpose of phytoremediation and accelerate phytoextraction of Ni through hyper-accumulated *Brassica napus*. Highest Ni content (27.33 mg/kg) were observed in plants of Corn-II receiving EDTA followed by CA treatment (24.33 mg/pot) and combined EDTA + CA treatment (24.85 mg/pot). The ability of Rapeseed to bio-accumulate heavy metals can be used to reduce the level of contaminants in the soil making it suitable for the

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cultivation of other metals sensitive food crops. The current work demonstrates the effective application of chelating agents (CA and EDTA) to reduce Ni stress as well as to increase Ni accumulation, a requirement for phytoremediation. It is suggested that *B. napus* species can be utilized for phytoremediation as it is a good accumulator of Ni and other metals.

**Keywords** Rapeseed · Citric acid · EDTA · Nickel · Phytoremediation

## 1 Introduction

Anthropogenic activities have substantially increased the concentration of heavy metals in the environment in the form of industrial waste, metalloids, organic contaminants, mining and smelting of metals, production of batteries, sewage sludge, municipal waste, use of pesticides and fertilizers in agriculture, vehicle exhausts, and burning of fossil fuels (Gaur et al., 2014; Tak et al., 2013). Heavy metals contamination poses a serious threat to ecosystem due to their non-degradable nature (Azeem et al., 2022; Ren et al., 2020). Additionally, the health of living things is seriously threatened by the toxicity and susceptibility of heavy metals in the environment (Mani & Kumar, 2014; Wu et al., 2022). The higher concentration of heavy metals in the growth medium of plants can create oxidative stress by producing a reactive oxygen species. The equilibrium between pro-oxidant and antioxidant homeostasis is interrupted by oxidative stress (Xu et al., 2015). Among the heavy metals, Ni is widely present in soil, water, and air in different forms. Ni is an essential component for the activation of several enzymes like urease in plants and microorganisms which is required in small amounts for normal plant growth and development (Flora et al., 2008; Nawaz et al., 2022). Ni is lethal for plant respiration, photosynthesis, photoassimilates, and minerals nutrition when present in higher concentration (Shahzad et al., 2018). Ni can also become the source of oxidative stress by decreasing the efficiency of antioxidative system (Ahanger et al., 2016). An interaction of Ni with O-, N-, or S-ligands of enzymes restricted the enzyme activity due to inactivation of enzyme proteins (Xu et al., 2015). The higher concentration of Ni induces iron chlorosis (Nas & Ali, 2018) and reduces activities of heme enzymes (Pandey & Sharma, 2002; Singh & Bhatla, 2022). The most common symptoms of Ni toxicity in plants are growth inhibition, necrosis, wilting, and chlorosis (Gazewska & Sklodowska, 2007; Kumar et al., 2007; Rehman et al., 2021).

The current technologies, such as soil remediation, augmented chelate, fixation, mechanical, pyrometallurgical separation, and absorbent, are widely used to clean the environment. These techniques are beneficial and commercialized, but they are also expensive and come with some environmental and ecological risks (Hassan et al., 2023; Kopittke et al., 2007). Phytoremediation is the most effective biological approach since it is less expensive, more widely accepted, and environmentally sustainable (Wu et al., 2010; Zhang et al., 2012). The process of using green plants to release, transport, stabilize, or degrade pollutants from soil is known as phytoremediation (Elekes, 2014; Sinha et al., 2013). Many environmental companies have used phytoremediation as a result of the advantages it has shown in heavy metal-contaminated sites (Paz-Ferreiro et al., 2014). To enhance the heavy metals solubility and mobility in the soil, different chelating agents have been used. These chelating agents include synthetic chelates such as EDTA and organic chelates like citric acid have potential to mobilize heavy metals in soil and to increase the accumulation of heavy metals in plants (Chigbo & Batty, 2013; Sinhalet al., 2010).

The metal–EDTA is transported from root to shoot by the transpiration pull (Chen et al., 2011). Other studies have suggested that EDTA binds metal (metal-EDTA) in the nutrient solution, which is primarily taken up by plant roots through the apoplastic pathway (Vassil et al., 1998) and subsequently translocated to the shoots (Huang et al., 2021). Organic acid such as citric acid has higher degree of biodegradability and low leaching contamination as compared to the synthetic chelating agents (Xiao et al., 2022; Xu et al., 2009). Citric acid plays a significant role in phytoremediation and also involve for the uptake of other nutrients by plants (Bareen, 2012). The level of phytoremediation can be improved by adding the small amount of citric acid because high amount of citric acid is toxic (Freitas et al., 2013). The application of citric acid reduces the phytotoxicity and environmental problem (Bona et al., 2023; Turgut et al., 2004).

In this work, we looked at the different levels of EDTA and citric acid separately and together as they enhance phytoremediation in metal contaminated soils. Due to its inherent potential to hyper-accumulate metals, rapid growth, and high above-ground biomass, *Brassica napus* is frequently used for phytoremediation (Najeeb et al., 2009). The purpose of the current study is to use canola plants to optimize the levels of EDTA and citric acid for phytoremediation of Ni affected soil.

## 2 Materials and Methods

### 2.1 Experimental Layout

An experiment was conducted to determine the suitable levels of citric acid (CA) and ethylene-diamine-tetra-acetic acid (EDTA) as chelating agents for phytoremediation of Ni contaminated soil. Mature and healthy seeds of two cultivars of *Brassica napus*, Corn-II (tolerant) and Oscar (sensitive) were obtained from Ayub Agricultural Research Institute (AARI) Faisalabad, Punjab, Pakistan, selected after screening and exposed to 30 ppm nickel sulfate at the time of sowing. Before sowing, seeds were rinsed thoroughly with distilled water into plastic trays. After surface sterilization, seeds were sown in 7 kg growth medium containing 6 cm layers of sterilized soil, sand, and organic manure and incubated for 4 weeks at 22–25 °C. The soil texture was clay-loamy, sand, and organic manure were added in ratio of 3:1:1 (soil: sand: organic manure), respectively. For quality control, soil samples collected for this experiment were air dried, passed through a 5-mm sieve, and was water saturated twice before being used in pots. During incubation, soil moisture was adjusted and maintained 70% of its water holding capacity. The growth medium was continuously ventilated by an air pump and replaced every week. After 2 weeks of Ni application, two levels of EDTA (0, 1.5, and 2.0 mmol/kg soil) and citric acid (0, 10, and 20 mmol/kg soil) were applied to both normal and Ni contaminated soil through rooting medium. Six weeks after Ni, citric acid, and EDTA application, the plants were harvested and partitioned into different parts for further analysis. High purity reagents and double-distilled deionized water were used for all the experiments. The experiment was conducted in a completely randomized design in a factorial arrangement with three replications. In our earlier experiment, we tested citric acid and EDTA assisted phytoremediation of Ni through different cultivars of *B. napus* using the single dose of citric acid and EDTA, 10 and 1.5 mM, respectively (Nawaz et al., 2022). After 15 days of chelate application, vegetative samples were harvested to study the use of chelating agents not only to ameliorate Ni stress but also to enhance Ni accumulation. However, in this present investigation, we harvested plants after 40 days of chelate application and study the efficacy of supera-optimal levels of CA and EDTA.

### 2.2 Growth Attributes

To determine the effect of EDTA and citric acid separately and in combination under Ni stress, three plants were selected randomly from each treatment for the measurement of shoot and root length. Fresh weight was measured immediately in electronic balance with 0.0001 g accuracy. The plants were oven dried in an electric oven at 70 °C to calculate dry weight.

### 2.3 Leaf Water Relations

Three plants from each treatment were collected to measure the leaf water, osmotic, and turgor potential (Shackel, 1987). Leaf water potential was determined from the third leaf from top (fully expanded youngest leaf). The measurements were made during 08.00 to 10.00 a.m. with the help of Scholander type pressure chamber (Model 1000, PMS New York, USA). The same leaf was used to determine leaf osmotic potential. The frozen leaf material was thawed, and the cell sap was removed with the use of a disposable syringe after 7 days in a freezer at – 20 °C. With an osmometer, the sap was directly applied to quantify the osmotic potential (Wescor, Model-5500, USA). The difference between water potential ($\Psi_w$) and osmotic potential ($\Psi_s$) measurements was used to compute turgor potential.

### 2.4 Gas Exchange Attributes

The measurements were taken on each plant's fully developed upper and middle leaves between 10 a.m. and 2 a.m. under maximum daylight intensity. At least three plants were measured in one treatment, and three replicates were performed for each plant. The net $CO_2$ assimilation rate ($A$), stomatal conductance ($g_s$), and transpiration rate ($E$) were measured using a portable infrared gas analyzer (IRGA, Model Cl-340, Analytical Development Company, Hoddesdon, England).

## 2.5 Photosynthetic Pigments

Following the technique of Arnon (1949) and Berger (1953), chlorophyll and carotenoid content was determined from fresh leaves using 10 mL 80% acetone and maintained at 10 °C overnight. The sample was centrifuged at 14,000× for 5 min, and the absorbance of the supernatant was measured using a spectrophotometer (SPECORD® 200 PLUS, Analytikjena, Germany) at 645, 652, and 663 nm. The chlorophyll concentrations were estimated as total chlorophyll (a + b) (mg g$^{-1}$) = $(8.02 \times A_{663} + 20.20 \times A_{645}) \times V/1000 \times W$; where V = volume of extract (mL) and W = weight of the leaf tissue (g). Carotenoid content = $A_{480} + (0.114 \times A_{663} - 0.638 \times A_{645})$.

## 2.6 Osmolytes

### *2.6.1 Free Amino Acids*

Total amino acids were determined following protocol of Hamilton and Vinelike (1973). Briefly, fresh plant leaves were chopped and extracted in 4 mL phosphate buffer with pH 7 at room temperature. One 1 mL of extract was mixed with 1 mL of pyridine (10%) and 1 mL of 2% ninhydrin solution in a test tube. The mixture was heated at 100 °C in a water bath for 30 min. The final volume was raised up to 50 mL with autoclaved distilled water. Optical density was recorded at 570 nm using a spectrophotometer (IRMECO U2020). Total amino acid was calculated by using the formula.

Total amino acids = graph reading of the sample × dilution factor

Graph reading = comparison of the optical density of the sample with graph of known concentration

Dilution factors = dilution of samples with distilled water

## 2.7 Total Soluble Protein Contents

Total soluble proteins were determined using the method of Lowry et al. (1951). Solution A, B, C (Lowry solution for protein determination) and folin phenol reagent were prepared and used for protein estimation. About 0.5 g fresh leaf material was chopped into phosphate buffer and centrifuged at 5000×*g* for 5 min. Then, 1 mL of the leaf extract was taken in a test tube. In each test tube, solution C (1 mL) was added, and all reagents (Lowry solutions + sample) were mixed thoroughly. The solution was placed for 10 min at 25 °C. Afterwards, 0.5 mL Folin-Phenol reagent (1:1 diluted) was poured into each test tube and then mixed gently. This mixture was incubated for half an hour at 25 °C. Optical density was read at 620 nm using a spectrophotometer (Hitachi, 220, Japan).

## 2.8 Total Soluble Sugars Determination

Total soluble sugars (TSS) were determined by the method of Yemm and Willis (1954) and Riazi et al. (1985). Plant extracts were taken in test tubes, and anthrone reagent (6 mL) was added and heated in a boiling water bath (10 min). The test tubes were ice-cooled for 10 min and incubated for 20 min at 25 °C. Optical density (OD) of the plant extracts was recorded at 625 nm using a spectrophotometer (Hitachi, 220, Japan). The TSS concentration was calculated from the standard curve developed by using the above method.

## 2.9 Determination of Nickel (Ni) Concentration

Harvested plants were washed with tap-water, three times and distilled water two times to remove the dust and other foreign particles from plant surface. Their leaves, stems, and roots were separated and dried in hot air oven running at 65 °C. The plant material was ground through electrical grinder into fine particles of 2 mm size. This plant material was digested according to the method ascribed by Wolf (1982) using $H_2SO_4$ and $H_2O_2$. Dried ground plant material (0.5 g) was transferred into digesting tubes and 5 mL of con. $H_2SO_4$ was added to each tube. All the tubes were placed overnight at room temperature. Then 0.5 mL of $H_2O_2$ (37%) was poured along the sides of the digestion tubes and ported them in a digestion block at 300 °C for 30 min and then cooled. Afterwards, 0.5 mL of $H_2O_2$ was added and tubes were put back into the digestion block. The above process was repeated until solution become transparent and then diluted with distilled water to make final volume up to 25 mL. The final solution was then filtered, and filtrate was used for elemental analysis. Ni was analyzed by atomic absorption spectrometer (Aurora AI 1200, Canada).

### 2.10 Statistical Analysis

The data were subjected to three-way full factorial analysis of variance (ANOVA) and analysis was performed in Minitab 19 software. Least significant test (LSD) was used for comparison of means. The data was graphically illustrated in XLSTAT 18. The strength of association among different traits was performed through Pearson correlation and principal component analysis using *corrplot* and fviz_*pca_biplot* function respectively in R-studio (version, 4.5).

## 3 Results

### 3.1 Growth Attributes

Statistical analysis of the data (Fig. 1) revealed that shoot and root length significantly ($p<0.05$) differed at various levels of EDTA and citric acid. Both varieties of *Brassica napus* responded similarly when exposed to different levels of EDTA and citric acid. At all levels of EDTA and citric acid, shoot and root length increased under Ni stress (Fig. 1). In both varieties, the maximum shoot and root length was noticed at 1.5 mM EDTA and 10 mM citric acid, while the minimum shoot and root length was recorded at 2 mM EDTA and 20 mM citric acid (Fig. 1). Nevertheless,

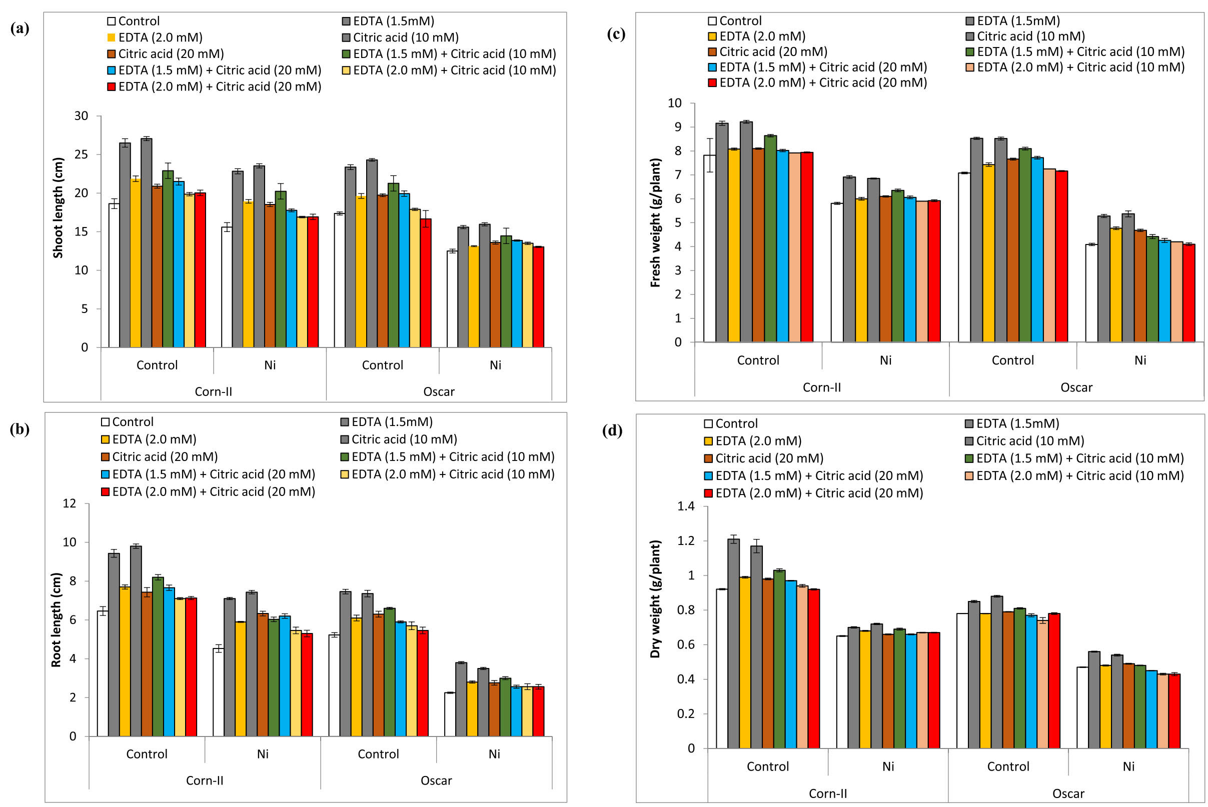


**Fig. 1** The role of EDTA and citric acid on growth attributes of Rapeseed (*Brassica napus* L.). Error bars are standard error of means. Bars with different letters are significant from each other at $p<0.05$

a decreasing trend in these parameters was observed when we used different combination of EDTA and citric acid but least decrease in shoot and root length was observed at EDTA (1.5 and 10 mM) combination as compared to other combination (Fig. 1). Although, the administration of Ni did not have an impact on shoot or root length during the early growth stages. But for longer Ni exposure time, the effect had become very strong. Variety Corn-II displayed smaller reduction in all growth parameters at various EDTA and citric acid concentrations (Fig. 1).

Analysis of variance (ANOVA) revealed that the fresh and dry weight was considerably reduced by Ni stress (Table 1). EDTA (1.5 and 2 mM) and citric acid (10 and 20 mM) application significantly increased fresh and dry weight in both varieties (Fig. 1). Maximum plant fresh and dry weight was recorded at EDTA level (1.5 mM) and citric acid level (10 mM) in both Oscar and Corn-II. Minimum fresh weight and dry weight was observed at EDTA (2 mM) and citric acid level (20 mM) (Fig. 1); however, plant fresh and dry weight decreased in both varieties of *Brassica napus* when EDTA and citric acid was applied in combination. The reduction in fresh and dry weight was less pronounced when varieties were exposed to the combination of 1.5 mM EDTA and 10 mM citric acid. Both varieties of rapeseed showed the same tendency with Ni treated and untreated plants.

### 3.2 Leaf Water Relations

Leaf water potential of Oscar and Corn-II was significantly affected in plants with Ni treatment (Fig. 2; Table 2). Individual application of EDTA and citric acid improved the water potential of both Oscar and Corn-II varieties but the combined application of EDTA and citric acid reduced the water potential of both Oscar and Corn-II; however, a slight reduction was observed at combination of 1.5 mM EDTA and 10 mM of citric acid (Fig. 2). The Ni stress significantly affected the osmotic potential of two cultivars Oscar and Corn-II. However, osmotic potential decreased with increase in EDTA and citric acid levels. The same trend of water potential was observed when EDTA and citric acid was applied together. Compared to non-Ni treated plants, leaf turgor potential deceased in both varieties under Ni stress. Data analysis also revealed that compared to control, turgor potential was significantly increased at all the level of EDTA and citric in Ni contaminated soil (Fig. 2; Table 2).

### 3.3 Gas Exchange Attributes

Under Ni stress, a substantial decrease in gas exchange parameters was recorded showing the toxic effect of nickel in growth medium in Oscar and Corn-II verities (Fig. 3). However, the addition of EDTA (1.5 + 2 mM), citric acid (10 + 20 mM), and EDTA + CA treatments significantly improved these gas exchange parameters in both varieties. The maximum increase was observed at EDTA level (1.5 mM) and citric acid level (10 mM) in both varieties as compared to other treatments. Compared to other treatments, combination (1.5 mM EDTA + 10 mM citric acid) showed better results in both varieties. In general, whether treated with nickel or not, both varieties exhibited the same pattern (Fig. 3; Table 3).

### 3.4 Photosynthetic Pigments

The concentration of photosynthetic pigments including chlorophyll *a*, *b*, total chlorophyll, and carotenoids were

**Table 1** Three-way full factorial (2×2×9) analysis of variance (ANOVA) showing the effect of citric acid and EDTA on morphological attributes of Rapeseed (*Brassica napus* L.) seedlings under nickel stress

| Source | DF | Shoot length | Root length | Fresh weight | Dry weight |
|---|---|---|---|---|---|
| Stress (S) | 1 | 567.187*** | 181.015*** | 186.835*** | 2.86652*** |
| Treatments (T) | 8 | 64.604*** | 7.457*** | 2.795*** | 0.03369*** |
| Varieties (V) | 1 | 348.841*** | 156.048*** | 34.015*** | 1.14969*** |
| S×T | 8 | 2.269*** | 0.306*** | 0.106*** | 0.00565*** |
| S×V | 1 | 58.521** | 15.580 ns | 7.120*** | 0.00319*** |
| T×V | 8 | 3.393** | 0.546*** | 0.053*** | 0.00233*** |
| S×T×V | 8 | 1.590** | 0.160 ns | 0.059*** | 0.00686*** |
| Error | 72 | 0.349 | 0.054 | 0.01 | 0.00012 |

*DF* degree of freedom, *ns* nonsignificant, ** significant at $p > 0.05$; *** highly significant at $p > 0.01$

significantly decreased in Ni-treated plants as compared to control (Fig. 4). However, the addition of EDTA (1.5 mM and 2 mM) and citric acid (10 mM, 20 mM) had a significant positive impact on chlorophyll *a*/*b*, carotenoids, and total chlorophyll in both varieties Oscar and Corn-II used in this experiment. Results revealed that chlorophyll *a*, *b*, and total chlorophyll were significantly increased at 1.5 mM EDTA level and 10 mM citric acid level in both varieties in Ni-treated and non-treated plants (Fig. 4). The concentration of chlorophyll *a*, *b*, and total chlorophyll decreased when EDTA and citric acid was used in combination. Application of EDTA (1.5 + 2 mM), citric acid (10 + 20 mM), and EDTA + CA increased the concentration of carotenoids. Nevertheless, in both varieties, combination of 1.5 mM EDTA + 10 mM citric acid improved chlorophyll content more than that of other three combinations both in control and Ni contaminated soil (Fig. 4; Table 3).

### 3.5 Organic Osmolytes

The concentration of various biomolecules including protein, amino acids, and sugar was significantly affected by the application of Ni stress (Table 4). The concentration of protein significantly decreased, indicating the toxic effect of Ni in growth medium of both varieties of Oscar and Corn-II (Fig. 5). The adverse effect Ni stress was also observed for amino acid in both varieties. However, the addition of EDTA (1.5 + 2 mM), citric acid (10 + 20 mM), and EDTA + CA treatment significantly improved the concentration of biochemical molecules (protein, sugar, and amino acid) in both varieties. The maximum increase in organic osmolytes was observed at EDTA level (1.5 mM) and citric acid level (10 mM) in both varieties of *Brassica napus*. The combination (1.5 mM EDTA + 10 mM citric acid) showed high concentration of all the biochemical molecules as compared to other combinations in both varieties (Fig. 5).

### 3.6 Nickel Content in Plants

The plants of Corn-II treated with EDTA had the highest Ni content (27.33 mg/kg), followed by CA (24.33 mg/kg), and those treated with both EDTA and CA (24.85 mg/kg). Oscar variety had much lower Ni accumulation in the above-ground parts than Corn-II. Combined EDTA and CA treatment showed significantly lower Ni contents in both cultivars compared to their individual treatments (Fig. 5; Table 4).

### 3.7 Multivariate Analysis

#### *3.7.1 Correlation Matrix*

Plant shoot and root length, fresh and dry weights, and protein content were positively correlated with photosynthetic pigments and gas exchange parameters, but negatively correlated with water relation parameters and nickel contents. Total free amino acids and sugar content showed no correlation with photosynthetic pigments. Osmotic potential, turgor potential, and water potential had strong positive correlation with organic osmotica and negative correlation with photosynthetic pigments and gas exchange parameters (Fig. 6a).

#### *3.7.2 Principal Component Analysis*

Principal component analysis demonstrated high variations in the dataset of both varieties under different treatments. The first principal component (PC1) accounted 34.9% variation along x-axis and variation explained by (PC2) was 26.9%. The total variation explained by first two PCs was 61.8%. PC1 and PC2 demonstrated a clear separation of the various parameters of both varieties Corn-II and Oscar in response to different levels of EDTA and citric acid during phytoremediation of Ni. The data revealed that morphological, photosynthetic, and gas exchange characteristics were the major contributors and had close association with each other and (Fig. 6b).

## 4 Discussion

Modern technology and the world's expanding population impose an enormous load on the environment by releasing toxic chemicals and hazardous waste into the ecosystem (Hassan et al., 2023;

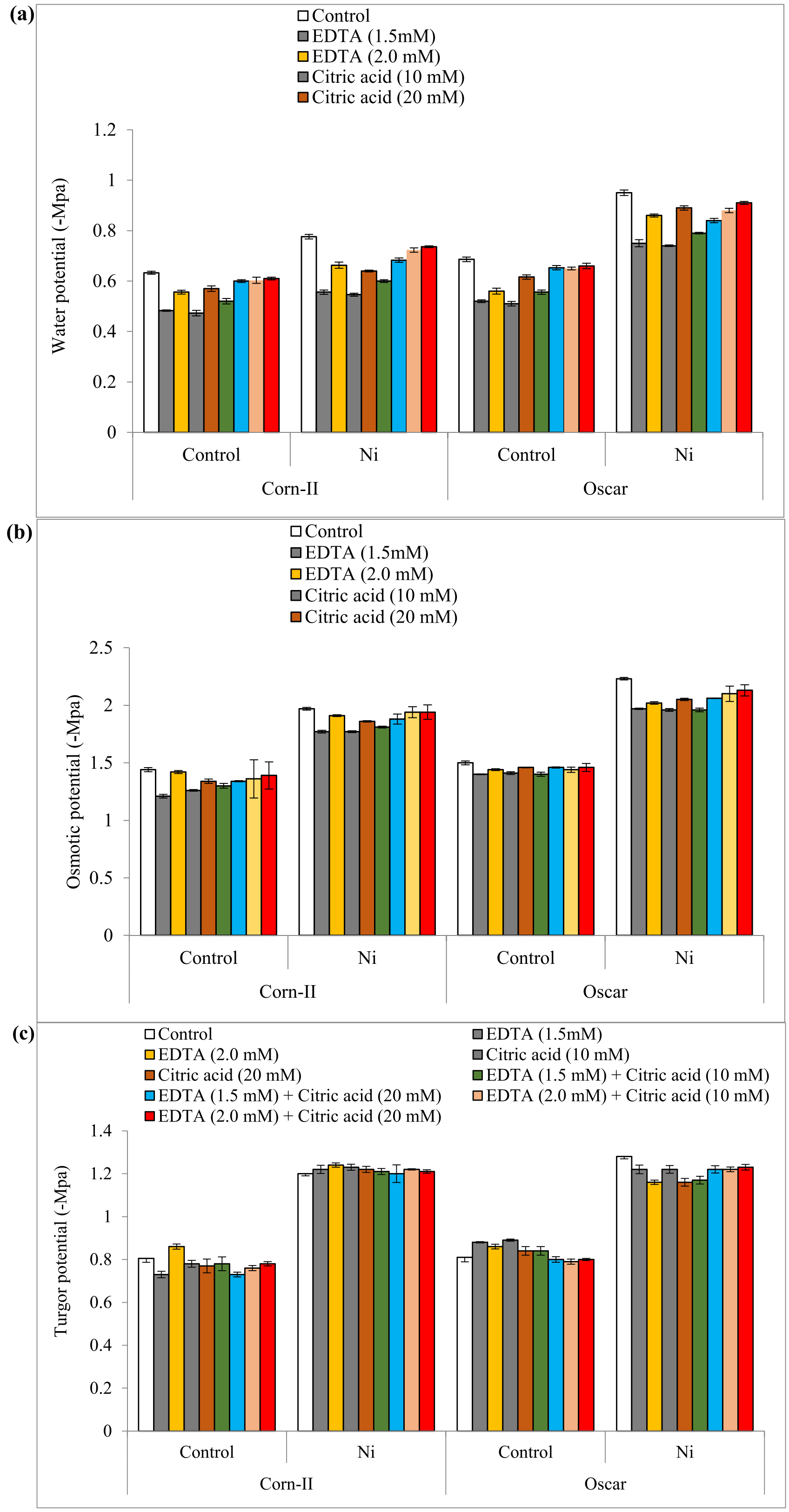


**Fig. 2** The role of EDTA and citric acid on water relation parameters of Rapeseed (*Brassica napus* L.) seedlings. Error bars are standard error of means. Bars with different letters are significant from each other at $p < 0.05$

**Table 2** Three-way full factorial (2×2×9) analysis of variance (ANOVA) showing the effect of citric acid and EDTA on water relations and gas exchange attributes of Rapeseed (Brassica napus L.) seedlings under nickel stress

| Source | DF | Water potential | Osmotic potential | Turgor pressure | Net $CO_2$ assimilation rate | Stomatal conductance | Transpiration rate |
|---|---|---|---|---|---|---|---|
| Stress (S) | 1 | 0.81141*** | 0.25110*** | 1.96528*** | 665.04*** | 16.10*** | 8.61*** |
| Treatments (T) | 8 | 0.08925*** | 0.02920*** | 1.74537*** | 4722.98*** | 92.77*** | 160.6*** |
| Varieties (V) | 1 | 1.27074*** | 0.00130 ns | 1.35344*** | 126.87*** | 2.59*** | 7.75*** |
| S×T | 8 | 0.00735*** | 0.01669*** | 0.35266*** | 27.81*** | 0.08 ns | 1.68*** |
| S×V | 1 | 0.04336*** | 0.00650*** | 0.08342*** | 2.79 ns | 0.16 ns | 0.64*** |
| T×V | 8 | 0.02231*** | 0.00256*** | 0.03724*** | 15.25*** | 0.64*** | 2.09*** |
| S×T×V | 8 | 0.00437*** | 0.00560*** | 0.01722*** | 13.53*** | 0.28** | 1.34*** |
| Error | 72 | 0.00029 | 0.00027 | 0.00046 | 3.61 | 0.13 | 0.12 |

*DF* degree of freedom, *ns* nonsignificant; ** significant at $p > 0.05$; *** highly significant at $p > 0.01$

Vasseghian et al., 2022). The increasing trend of heavy metals and metalloids in water and soil create a global health concern (Ahmad et al., 2023; Bilal et al., 2021), because these are non-degradable and remain persistent for long time (Shikha & Singh, 2021; Zhou et al., 2023). Majority of heavy metals are toxic at low concentration, enter the food chain (Xincheng et al., 2012), where they accumulate and cause different diseases in living organism by damaging their internal system (Mani & Kumar, 2014; Nivetha et al., 2022). As a result, it is important to remove and reduce the heavy metals contamination in order to protect the environment from the toxic hazardous consequences (Ndeddy & Babalola, 2016; Sall et al., 2020). The increasing awareness about the toxic effects of heavy metals has led researchers to look for new techniques to clean the environment for cultivating the land and preventing the contamination of food (Akcil et al., 2015; Oladoye et al., 2022).

In this study, we used different levels of citric acid and EDTA to accelerate Ni uptake and induced tolerance in the seedling of two cultivars of rapeseed plant (Oscar and Corn-II) under Ni stress. Such studies are helpful for understanding how Brassica species can successfully phytoremediate Ni-contaminated soils. Numerous studies have revealed the effectiveness of various chelating agents, including EDTA and organic acid, in removing hazardous heavy metals from contaminated soils while also promoting plant growth (Marchiol et al., 2004; Huang et al., 2021; Alsafran et al., 2022). Ni had a significant impact on plant growth by reducing biomass in both Rapeseed varieties. Our results revealed that Corn-II is more tolerant to Ni stress than Oscar. It has been reported in literature that toxic level of Ni in plants produce chlorosis, necrosis, wilting, and also inhibited growth depending upon the dose, time of exposure, and level of concentration (Rehman et al., 2021; Ruley et al., 2006).

In our investigation, biomass of plants (shoot, root length, fresh, and dry weights) slightly increased by adding citric acid and EDTA but higher biomass was observed at lower level of EDTA and citric acid (1.5 EDTA + 10 CA mM) in both varieties. Minimum biomass was observed at higher level (2 mM EDTA and 20 mM CA). However, a decreasing trend was observed when we used combination (1.5 EDTA + 10 CA mM). This is in accordance with results of Kopittke et al. (2007), who demonstrated that plants dry biomass of *Zea mays* decreased by adding more than 2 mmol/kg EDTA in the growth medium. The toxic effect of EDTA is mainly due to the increased concentration of heavy metals in soil solution (Chen et al., 2011; Saleem et al., 2020). EDTA is toxic at high concentration because it can disturb the mineral balance leading to disturbance in cell metabolism and disrupted biological membrane (Lambrechts et al., 2011; Marchiol et al., 2004). Photosynthetic pigments decreased under Ni stress. It is previously reported that elevated concentration of Ni affects the photosynthesis, respiration, photo assimilates, and mineral nutrition (Neugschwandtner et al., 2008; Rizwan et al., 2019).

The Ni stress had negative impact on all the attributes of water relations. However, the addition of EDTA and citric acid to the growth medium

**Fig. 3** Effect of EDTA and citric acid on gas exchange attributes of Rapeseed (*Brassica napus* L.) seedlings. Error bars are standard error of means. Bars with different letters are significant from each other at $p < 0.05$

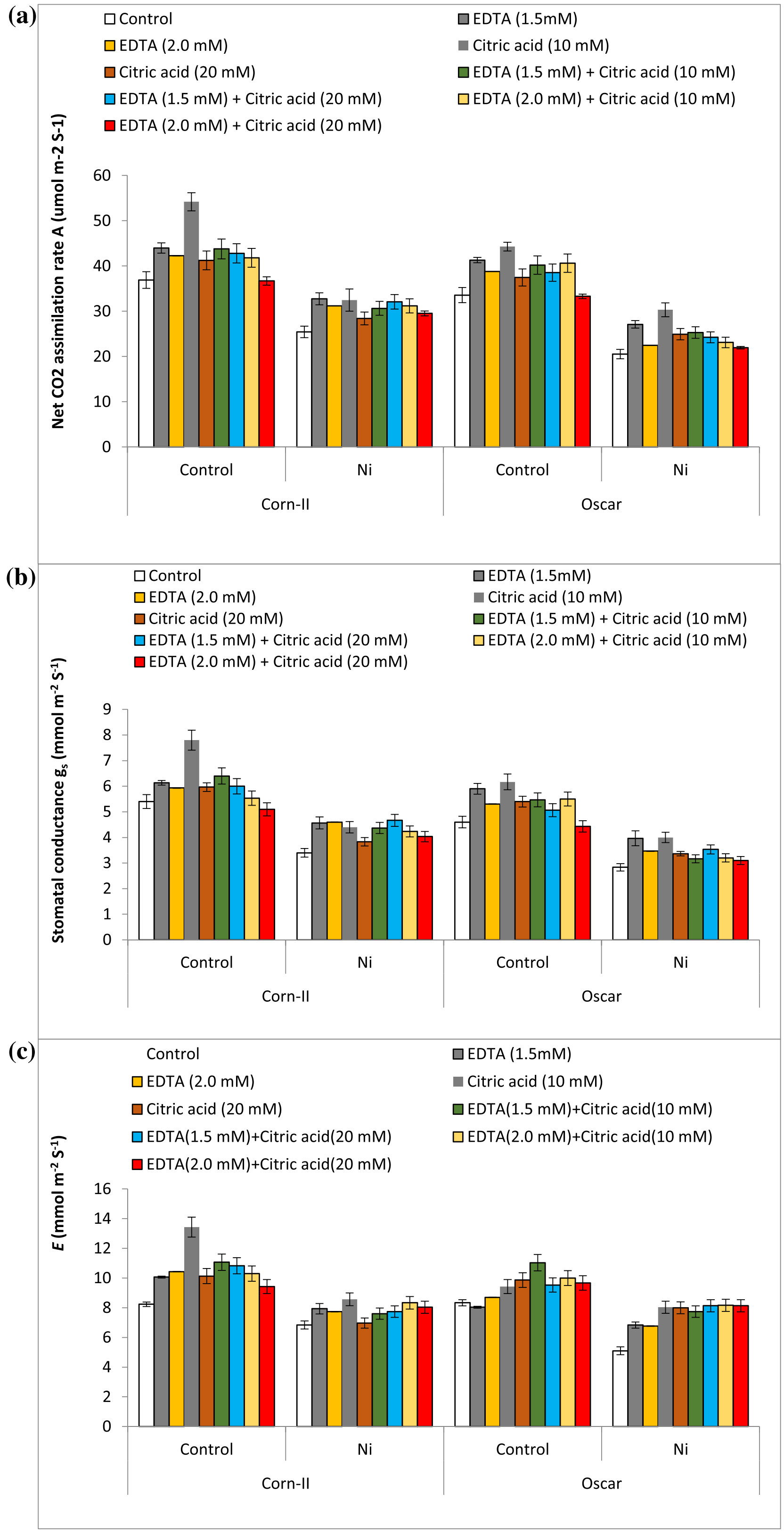

**Table 3** Three-way full factorial (2×2×9) analysis of variance (ANOVA) showing the effect of citric acid and EDTA on photosynthetic attributes of Rapeseed (*Brassica napus* L.) seedlings under nickel stress

| Source | DF | Chlorophyll *a* | Chlorophyll *b* | Carotenoid | Total chlorophyll |
|---|---|---|---|---|---|
| Stress (S) | 1 | 446.630*** | 42.4860*** | 45.5650*** | 0.80256*** |
| Treatments (T) | 8 | 7.819*** | 2.3169*** | 1.3709*** | 0.05400*** |
| Varieties (V) | 1 | 75.115*** | 9.7575*** | 8.1950*** | 0.35938*** |
| S×T | 8 | 1.470*** | 0.3668*** | 0.0988*** | 0.00163*** |
| S×V | 1 | 66.727*** | 4.6088*** | 0.6000*** | 0.14889*** |
| T×V | 8 | 0.299 ns | 0.3141*** | 0.0606** | 0.00056 ns |
| S×T×V | 8 | 0.484 ns | 0.2013*** | 0.0546 ns | 0.00087** |
| Error | 72 | 0.255 | 0.005 | 0.0191 | 0.00023 |

*DF* degree of freedom, *ns* nonsignificant; ** significant at $p > 0.05$; *** highly significant at $p > 0.01$

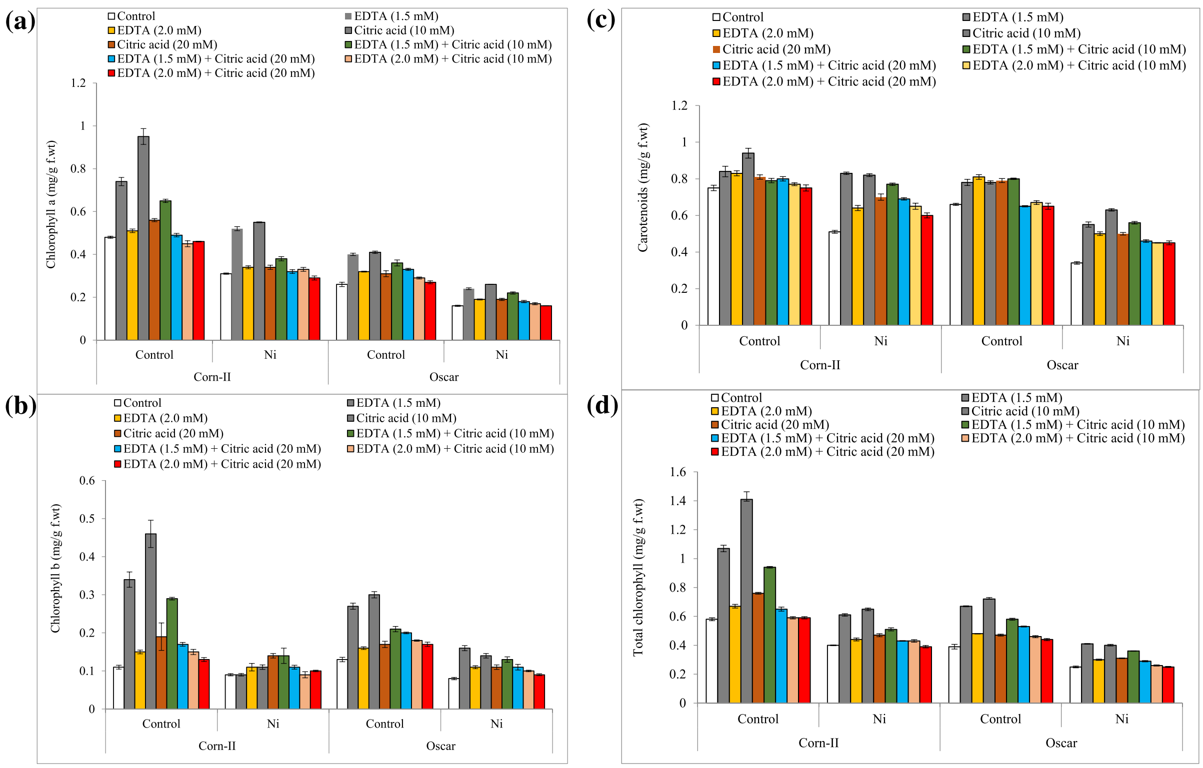


**Fig. 4** Effect of EDTA and citric acid on photosynthetic pigments of Rapeseed (*Brassica napus* L.) seedlings. Error bars are standard error of means. Bars with different letters are significant from each other at $p < 0.05$

caused a significant increase in water potential and osmotic potential. These chelating agents form a complex with Ni and increase the water potential. The decrease in osmotic and water potential might be related to a deposition of salt/osmolytes, which is helpful in alleviating the adverse effects of heavy metals (Kavi Kishor & Sreenivasulu, 2014). In this study, *B. napus* under treatment EDTA and citric acid separately and in combination has high water potential under nickel stress, which might have effectively maintained the photosynthetic rate to produce high biomass. Our results demonstrated that increasing

**Table 4** Three-way full factorial (2×2×9) analysis of variance (ANOVA) showing the effect of citric acid and EDTA on osmolytes and nickel contents of Rapeseed (*Brassica napus* L.) seedlings

| Source | DF | Amino acid | Sugar | Protein | Nickel content |
|---|---|---|---|---|---|
| Stress (S) | 1 | 4.37618*** | 0.86343*** | 8.92687*** | 94.70*** |
| Treatments (T) | 8 | 0.00352*** | 0.06257*** | 0.05066*** | 6573.6*** |
| Varieties (V) | 1 | 0.01893*** | 0.48900*** | 0.54329*** | 63.08*** |
| S×T | 8 | 0.00423*** | 0.00898*** | 0.00453*** | 106.4*** |
| S×V | 1 | 0.03413*** | 0.10670*** | 0.04045*** | 5.45*** |
| T×V | 8 | 0.00285** | 0.00411*** | 0.00364*** | 49.29*** |
| S×T×V | 8 | 0.00381** | 0.00305** | 0.00208** | 4.55*** |
| Error | 72 | 0.00076 | 0.00056 | 0.00055 | 0.41*** |

*DF* degree of freedom, *ns* nonsignificant; ** significant at $p > 0.05$; *** highly significant at $p > 0.01$

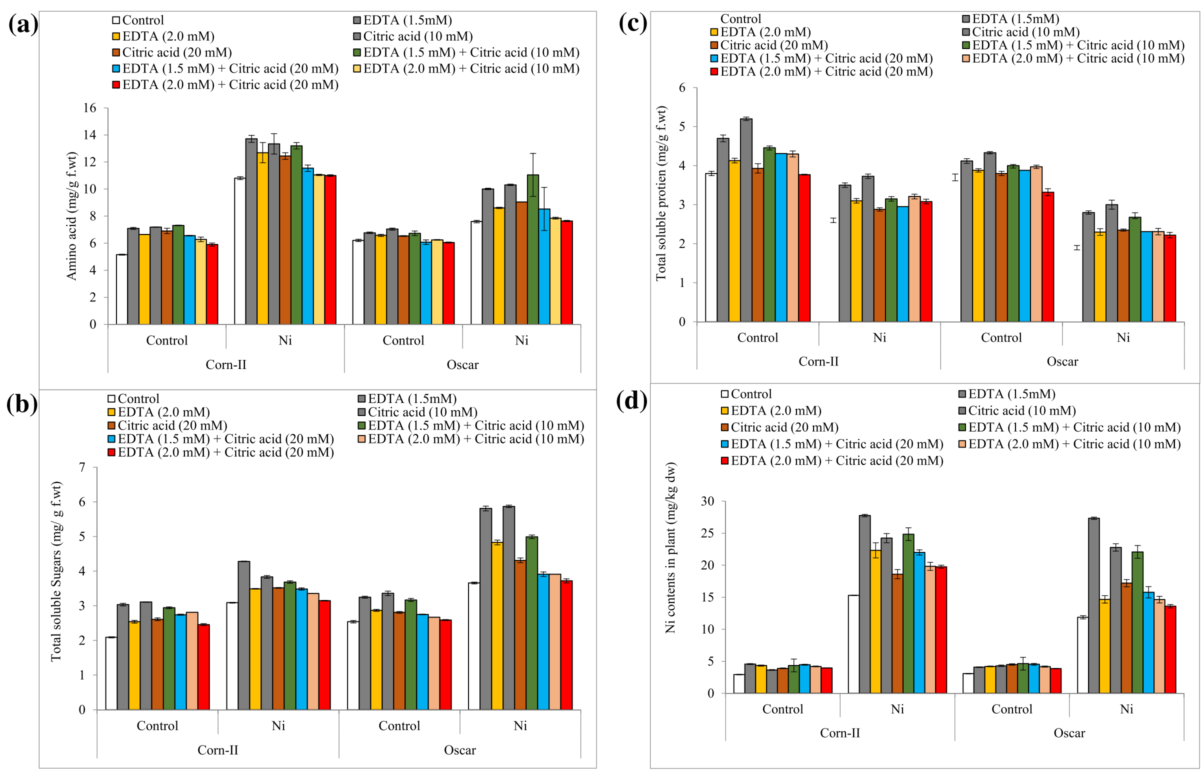


**Fig. 5** Effect of EDTA and citric acid on organic osmolytes and Ni contents of Rapeseed (*Brassica napus* L.) seedlings. Error bars are standard error of means. Bars with different letters are significant from each other at $p < 0.05$

the concentration of chelating agents can reduce plant biomass and also affect the ability of plant for accumulation of metals from soil. Thus, it is necessary to choose the suitable level and kind of chelating agents to allow optimal metal accumulation as well as optimal plant growth. In this perspective, EDTA level 1.5 mM and citric acid level 10 mM is more suitable for phytoremediation.

The present study further depicts that gas exchange parameters (net photosynthetic rate, transpiration rate, carbon dioxide concentration, and water use efficiency) progressively decreased under Ni stress as depicted in Fig. 3 and Fig. 4. Almost similar findings were reported by Ali et al. (2013), while studying the effect of Cd stress in Brassica napus. This reduction in gas exchange attributes may be due to

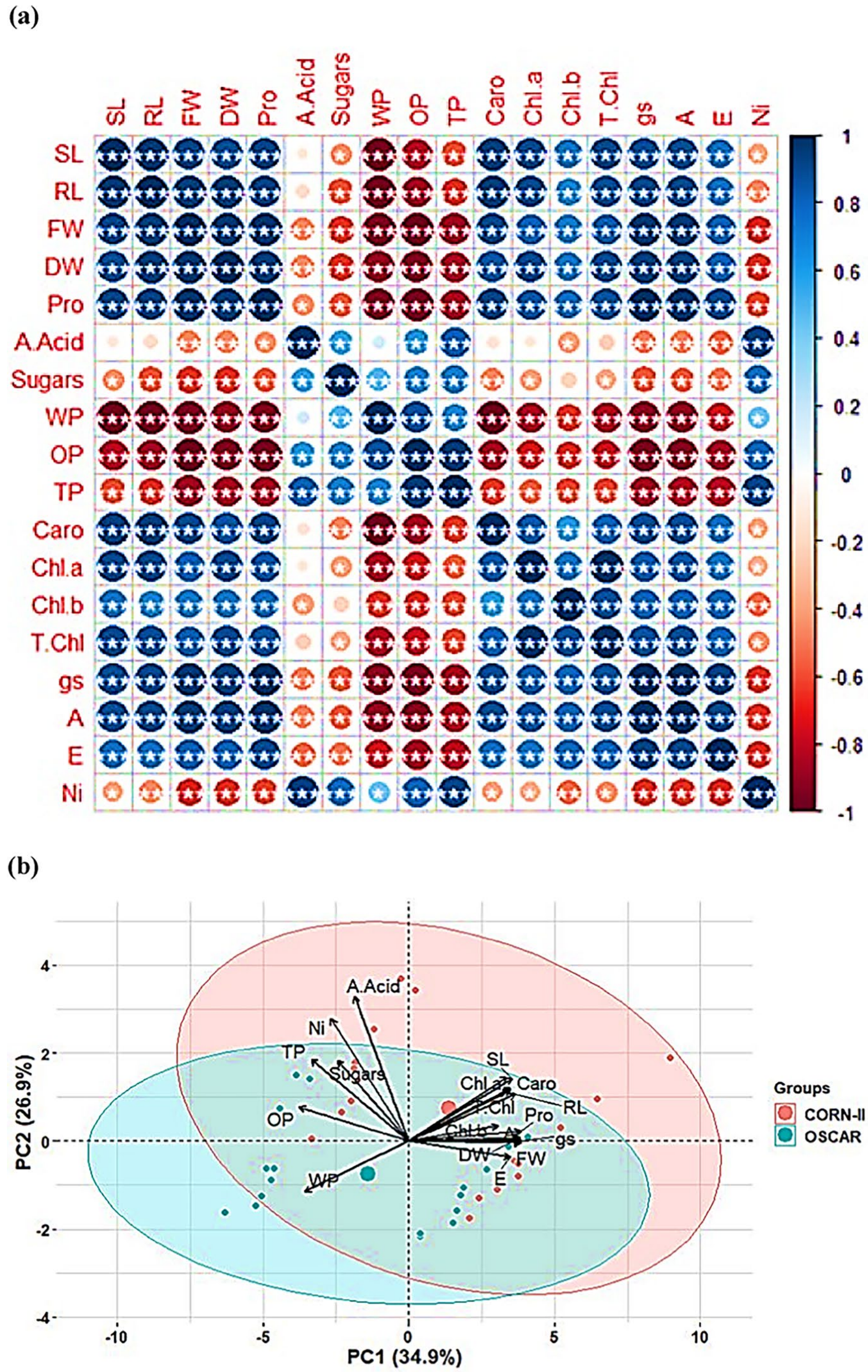


**Fig. 6** **a** Correlation studies between different growth, water relation, gas exchange, photosynthetic and organic osmolytes parameters. **b** PCA biplot showing the association in both varieties under different level of citric acid and EDTA. *SL* shoot length, *RL* root length, *FW* shoot fresh weight, *DW* shoot dry weight, *Pro* proline, *A acids* amino acids, *WP* water potential, *OP* osmotic potential, *TP* turgor potential, *Caro* carotenoids, *Chl a* chlorophyll a, *Chl b* chlorophyll b, *T.Chl* total chlorophyll, *Ni* nickel contents, $g_s$ stomatal conductance, *A* net $CO_2$ assimilation, *E* transpiration

direct effect of Ni on stomatal opening by altering potassium influx through guard cell membranes, thus reduced stomatal opening effect gaseous exchange and eventually reduced photosynthetic rate (Aggarwal et al., 1990; Vernay et al., 2007). Application of EDTA and CA to plants growing under nickel stress significantly improved growth, biomass, photosynthetic rate, and gas exchange parameters in both cultivars of Brassica napus showing their positive effect to combat Ni stress (Figs. 1–4). This increase biomass and improved efficiency of gas exchange parameters might be due to the formation of chelate with

Ni which reduced the toxic effect of Ni (Chen et al., 2001; Muhammad et al., 2009).

The reduction in chlorophyll and carotenoids may be due to the disruption of carotenoids and chloroplast ultrastructure (Nabi et al., 2021; Turgut et al., 2004). The rate of photosynthesis dropped due to a decrease in chlorophyll and carotenoids, which ultimately resulted in a reduction in plant growth (Chen et al., 2020; Parida et al., 2003). Under Ni stress, a significant reduction in chlorophyll concentration has already been reported in the leaves of plants (Aqeel et al., 2021; Liu et al., 2014). It is suggested that decrease in chlorophyll content inhibit chlorophyll synthesis due to Fe and Mg deficiency (Kazemi et al., 2010). Both chelators, i.e., EDTA and CA, helped in decreasing the detrimental effects of Ni on photosynthetic pigments. However, the lower level of EDTA and CA are effective than the higher level of EDTA and CA. The combination (1.5 mM EDTA + 10 mM CA) had provided more support in ameliorating the damaging effect of Ni as compared to other level both in Ni-treated and untreated cultivars of rapeseed. This may be due to organic acid which form compounds with heavy metals. They also make heavy metals desorption from the soil particles and increase the bioavailability of heavy metals to improve the efficiency of phytoremediation (Ashraf et al., 2019; Marchiol et al., 2004). Our result showed that the lower level of EDTA and CA are more effective than higher level when used alone or in combination. Another reason may be the direct action of EDTA and CA and increased bioavailability of heavy metals in soil (Seregin & Kozhevnikova, 2006; Guo et al., 2018).

A decrease in protein contents was observed in the under-study experiment. The less decrease was observed at citric acid 10 mM and EDTA 1.5 mM level as compared to the other levels of chelating agents both in Ni treated and non-treated plants. A decreasing trend in protein contents was observed when applied in combination of CA and EDTA. This decrease of protein may be due to the increasing concentration of heavy metals and chelating agents (Diarra et al., 2021; Seregin & Kozhevnikova, 2006). The higher level of Ni can delay the process of protein biosynthesis which is essential for plant growth. The two chelators CA and EDTA at lower level ameliorate the effects of Ni stress. Increase in protein and amino acid content at 1.5 mM EDTA and 10 mM CA level may be due to increased antioxidants activity and decreased $H_2O_2$ production and electrolytes leakage which protected plants under Ni stress.

Ni stress caused a significant increase in sugar and amino acid contents in *Brassica napus* cultivars. This is in accordance with the result of (Luo et al., 2006), who reported an increase in the contents of amino acid phenolics and glycosylates in *Brassica napus* under copper, iron, and manganese toxicity. This increase in amino acid and sugar are known to have a metal chelating effect which might be a response to metal toxicity (Aqeel et al., 2021). Some authors have reported that soluble sugar, proline, amino acid play an adoptive role in mediating osmotic adjustment and protecting the subcellular structure in stressed plants (Siddique et al., 2018). The level of amino acid and sugar further increase with the addition of EDTA and citric acid in growth medium in combination or by their separate application in both cultivars of canola, but this increase was more at 1.5 mM EDTA and citric acid 10 mM and combination level (1.5 + 10) as compared to the other levels of EDTA and CA. These levels had given more assistance in ameliorating the damaging effect of Ni.

## 5 Conclusion

Several Brassica species are capable of phytoextraction and are recognized as metal accumulators. Without exhibiting visible symptoms in their morphology, these plants can accumulate metals in their biomass. In this work, we found that EDTA and citric acid both increased *Brassica napus*' ability to tolerate nickel stress, and that EDTA at 1.5 mM and citric acid at 10 mM doses were better suited for accelerated phytoremediation. Future research on phytoremediation may explore genetic engineering, cellular, and molecular mechanisms to clarify how the process might be improved to remove metal contamination from soil. In order to extend the network of this green technology among academics, industrialists, and farmers, it is crucial to concentrate on the issues of human health concerns and environmental contamination while simultaneously highlighting the advantages of this technology.

**Author Contribution** Conceptualization, Ameer Ali; investigation, Humera Nawaz, Aamir Ali; formal analysis, Ansar

Mehmood, Naila Asghar, Amina Ameer, Ummar Iqbal, Fahim Nawaz; methodology, Khawaja Shafique Ahmad and Naila Asghar; resources, Ameer Khan; software, Muhammad Kaleem, Naila Asghar, Muhammad Sajjad Iqbal; writing—original draft, Humera Nawaz; writing— review and editing, Khawaja Shafique Ahmad, Muhammad Asif Sehzad, Ansar Mehmood, Fahim Nawaz, and Aamir Ali. All authors have read and agreed to the published version of the manuscript.

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**Data Availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of Interest** The authors declare no conflict of interest.

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