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# Multi-level approach to screen tomato inbred lines for resilience to Ni-enriched soils and water deficit

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ABSTRACT

Climate-driven increases in drought occurrence and the contamination of soils by heavy metals adversely affect tomato production both qualitatively and quantitatively. The aim of the present study was to investigate the responses of four inbred lines of *Solanum lycopersicum* to Ni toxicity and water deprivation. To evaluate the effects of Ni, we used two different growth approaches, in hydroponics and in soil, associating a morphological analysis of the lines with Ni and mineral nutrient quantification in both shoots and fruits. The effects of water stress were tested on germination capacity *in vitro* and on adult plants grown in soil. The responses of the different lines to water stress were assessed by physiological and phenotypic analyses, expression of drought-related genes and quantification of ABA. The multi-level approach allowed us to identify two lines, among the four investigated, as good candidates for future breeding programs due to their ability to accumulate less Ni and maintain fruit quality parameters and capacity to acclimate to repeated water stress.

## 1. Introduction

Global warming, climate change, and environmental pollution have increased the risk of failure of major crop yields (Rivero et al., 2022). In this context, due to the increased frequency and intensity of abiotic stress episodes such as drought and extreme temperatures, as well as plant nutrient deficiency and toxicity, significant losses of global crop yields have been estimated (Oshunsanya et al., 2019). Tomato (*Solanum lycopersicum*) holds high economic importance globally with about 189 million tons grown on 5.16 million hectares (Faostat, 2023). Tomato fruits and derived products are a source of nutrients such as minerals, vitamins, peptides and proteins, polyphenols, and carotenoids (Chaudhary et al., 2018; Collins et al., 2022). Several studies support the health-promoting effects associated with the regular consumption of tomato due to its anti-inflammatory, antiangiogenic, cardioprotective, and antioxidant properties (Hwang and Bowen, 2002; Etminan et al., 2004; Treggiari et al., 2017). However, tomato production faces nowadays several challenges, including high input costs for seeds, fertilizers, pesticides, watering, and post-harvest losses (Conti et al., 2023). Tomato cultivars are particularly sensitive to drought stress, which can cause stunted growth, reduced seed viability, flower drop, decreased fruit set and size, mineral nutrient deficiencies, and, in cases of prolonged drought, even plant death (Nuruddin et al., 2003; Sivakumar and Srividhya, 2016; Lamin-Samu et al., 2021; Sané et al., 2021). In general, how a plant responds to abiotic stresses depends on many factors, such as the severity and duration of stress, number of exposures, combination of stresses as well as plant characteristics such as genotype, developmental stage, and the organs impacted by the stress (Zandalinas et al., 2021; Zia et al., 2021). To withstand drought, plants have developed various strategies, such as shortening their life cycle, or modifying root structure for efficient water uptake (Kooyers, 2015; Zia et al., 2021). On the other hand, tolerant plants can withstand low internal water content while keeping the ability to grow and reproduce (Kooyers, 2015; Zia et al., 2021), for example, through osmotic adjustment (Chaves et al., 2003; Ramachandra Reddy et al., 2004). Depending on the degree of sensitivity, crops respond to drought by inducing a rapid accumulation of the stress hormone abscisic acid (ABA), activating antioxidative protective systems, which include non-enzymatic antioxidants such as phenolic compounds and antioxidant enzymes such as catalase (CAT), and inducing the expression of drought-responsive genes, as *Late*

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*Embryogenesis Abundant* (LEA), implicated in drought mitigation (Foyer and Noctor, 2005; Pandey et al., 2009; Liang et al., 2019; Amjad et al., 2020; Magray et al., 2023).

Crop yields are also challenged by the presence of pollutants in agricultural soils, such as heavy metals, which pose a health risk to humans and animals when they accumulate in edible plant tissues and are transferred into the food and feed chain (Correia et al., 2018). Nickel (Ni) contact is the most frequent cause of allergy worldwide; however, depending on the dose and time of exposure, it can produce other effects on human health, such as cardiovascular disease and respiratory cancer (Chen et al., 2019). Nickel is an essential micronutrient for plants as a metal cofactor of enzymes, but it becomes toxic when the level in the soil exceeds tolerable thresholds, generally Ni $>$ 75–100 mg/kg (Kumar et al., 2021; Roccotiello et al., 2022). For most crop species, the tolerable Ni concentration in shoots is between 1 and 10 mg/kg dry weight (DW), with a toxicity threshold of 10–100 mg/kg (Kabata-Pendias, 2010). Tolerant plants able to accumulate $>$1000 mg/kg DW of Ni in shoots are defined as Ni hyperaccumulators (Brooks et al., 1977). Nickel toxicity can result from industrial pollution or the application of high Ni sewage sludge and manure (Cakmak et al., 2023), and plants growing in soils with excess Ni show symptoms such as chlorosis, wilting, stunted growth, inhibited root elongation, and fruit yield depression (Sheoran et al., 1990; Gajewska and Skłodowska, 2007; Lesková et al., 2017). Nickel exerts its toxic action by interfering with the activity of enzymes, primarily by displacing essential metal cations at their active sites (Gajewska and Skłodowska 2005; Ghasemi et al., 2009; Pandey et al., 2009; Küpper and Andresen, 2016; Shahzad et al., 2018). In response to heavy metal stress, plants have evolved a range of mechanisms to avoid the cell accumulation of toxic concentrations, such as chelation with ligands, compartmentalization in the vacuole, and redistribution of transporters (Gajewska and Skłodowska, 2005; Sreekanth et al., 2013; Merlot, 2020). The bioavailability of Ni depends on the pH of the soil or growth medium, the presence of organic matter, and other ions in the soil or nutrient solution (Badawy et al. 2002; Chen et al., 2009). Nickel is rapidly taken up by plant roots through passive diffusion and active transport in the form of the divalent cation ($Ni^{2+}$), the most widespread in the environment. It is translocated as Ni-organic acid chelates to the shoot and then to sink organs, such as flowers and fruits. Having similar chemical properties to other essential divalent mineral nutrients like Mg, Mn, Fe, Cu, and Zn, Ni competes for root uptake, leading to deficiencies of these elements in the plant (Liu et al., 2008; Hassan et al., 2019). For example, a high level of Ni in soil and growth media affects Mn uptake (Palacios et al., 1998) and reduces Fe accumulation in shoots (Ghasemi et al., 2009; Lesková et al., 2017).

Currently, the goals of tomato breeding are to achieve sustainable production, adaptation to unfavourable environmental conditions, and improved nutritional quality (Mata-Nicolás et al., 2020). Considering that the regions where tomato cultivation is relevant could correspond to areas where soils can be subjected to drought or industrial Ni contamination, the selection of genotypes and cultivars with high-yield stability is of particular interest to ensure successful production from a food security and safety perspective (Conti et al., 2023).

The present study was planned to investigate the responses of four inbred tomato lines to Ni or water stress using a multi-level approach, combining morphological, physiological, mineral nutrient, and stress-related gene expression analyses. The rationale of our study was to identify the inbred line(s) with the best performance and reduced sensitivity to each stress separately to better orient the selection of lines for specific markets in Ni-contaminated environments or exposed to water deficit. In perspective, the two lines, identified as best-performing among the four assessed in our study, will be useful for the development of new hybrids valuable for adaptation to combined Ni and water stress.

## 2. Materials and methods

### 2.1. Plant material

The four lines under analysis, hereafter referred to as L1, L2, L3, and L4 (Fig. 1 and Table 1), are inbred lines used by ISI Sementi SpA (Fidenza, Parma, Italy) in breeding programs to produce high-quality F1 hybrids for various geographic markets. L1 and L2 are used to produce hybrids for the North African market, where there is a growing demand for large fruit; L3 to obtain hybrids for Eastern Europe, where the market requires pink tomatoes of intermediate size; and L4 is employed for markets in the Mediterranean basin. The fruits of the four lines have a round shape (Fig. 1 and Table 1) and are characterized by uniformity in green colour, which ensures homogeneous ripening. The flowers have a jointed pedicel, which allows the fruit to detach from the rachis along with the calyx and part of the peduncle, making the fruit more commercially attractive.

### 2.2. Ni treatments of tomato lines grown under hydroponic conditions

Tomato seeds were sterilized in 5 % diluted commercial bleach and *in vitro* germinated in Magenta vessels containing half-strength Murashige and Skoog (MS) agar medium supplemented with vitamins. Magenta vessels were placed for two days at 4 °C in the dark and then in a growth chamber at a constant temperature of 25 °C during a 10 h/14 h light/dark cycle, with an average irradiance of 1200 μmol/$m^2$s of Photosynthetic Photon Flux Density (PPFD). Ten days after sowing, seedlings were transferred into a hydroponic system under controlled conditions (i.e., 16 h/8 h light/dark cycle at 25 °C with 200 μmol/$m^2$s PPFD) in half-strength Hoagland solution (2.5 mM $KNO_3$; 2.5 mM $Ca(NO_3)_2 \bullet 4H_2O$; 1 mM $NH_4H_2PO_4$; 1 mM $MgSO_4 \bullet 7H_2O$; 23.1 μM $H_3BO_3$; 4.75 μM $MnCl_2 \bullet 4H_2O$; 0.35 μM $ZnSO_4 \bullet 7H_2O$; 0.1 μM $CuSO_4 \bullet 5H_2O$; 0.25 μM $Na_2MoO_4 \bullet 2H_2O$; 12.5 μM NaFeEDTA) or in half-strength Hoagland solution supplemented with 0.5 μM Ni (applied as $NiSO_4$). After 7 days, growth parameters were analysed (i.e. T0 of the trial) (Supplementary Figure 1), demonstrating that Ni supplemented at 0.5 μM improved shoot growth in all the lines. Then, seedlings were grown for five days in 2 L pots with fresh half-strength Hoagland solution containing 0.5 μM (control), 20 μM, and 85 μM Ni. The growth data (i.e. shoot length from cotyledonary leaves to apex, primary root length, and number of leaflets) were expressed as increments calculated by subtracting the values of the control (0.5 μM Ni) at T0 from the values of the same parameters after 5 days of Ni treatment. During the hydroponic growth, an air bubbler was applied to each pot to ensure root oxygenation and the pH of the medium was checked daily with a digital pH meter and kept between 5.5 and 6.5 with NaOH. The tolerance index was calculated as the ratio between the increment in growth in the presence of Ni to the increment in growth in control conditions and expressed as a percentage with respect to control.

### 2.3. Nickel treatment of tomato lines grown under pot cultivation conditions

Seeds were sown in 60-hole nursery plots containing peat and placed for 2 days in the dark at a constant humidity close to 86 %. Afterward, they were moved to a nursery and kept at temperatures ranging from 16-17C° to 25–30C°, with a humidity of 60 %. After one month, seedlings were transplanted into pots, transferred to a greenhouse, and grown for four months from April to July (2023) until fruiting. The greenhouse was equipped with a fogging system to maintain an optimal temperature of approximately 25 °C and 60 % humidity. To mimic field cultivation conditions, each pot was filled with 8 kg of soil collected from the surface layer (0–20 cm) of a cultivation field at the ISI Sementi. The physical and chemical properties of the soil were evaluated before the addition of $NiSO_4$. The Ni availability index was also assessed after the addition of Ni to the soil. The analyses were carried out by the ADESUD

**Fig. 1.** Representative phenotypes of L1, L2, L3, and L4 inbred lines at both vegetative and reproductive development under greenhouse cultivation conditions.

**Table 1**
Characteristics of ISI Sementi inbred lines.

| | Type[a] | Segment[b] | Fruit shape | Fruit weight (g) | Fruit color uniformity[c] | Pedicel characteristic[d] | Fruit color |
|---|---|---|---|---|---|---|---|
| L1 (22–4143-L) | MIR | BF | Round | 250 | UG | JD | Red |
| L2 (22–4101-L) | MIR | BF | Round | 240 | UG | JD | Red |
| L3 (22–4214-M) | MIR | MT | Round | 180 | UG | JD | Pink |
| L4 (22–4215-M) | MIR | MT | Round | 140 | UG | JD | Red |

[a] fresh-market indeterminate tomato (MIR); [b] beef (BF) and medium flower truss (MT); [c]uniformity of green color (UG); [d] jointed pedicel (JD).

laboratory (Battipaglia, Salerno, Italy), following the procedure defined by Ministerial Decree DM 13/09/99 SO n.185 GU n.248 21/10/99 of the Ministry of Agricultural and Forestry Policies of the Italian Government.

Five plants per line were used for the control (untreated soil) and for each of the three Ni concentrations tested (30, 120, and 300 mg Ni /kg soil), applied as $NiSO_4$ (Roccotiello et al., 2022). To spike the soil with Ni, 1 L of 4 mM Ni, 16 mM Ni, and 41 mM Ni solution was added to each pot to obtain 30, 120, and 300 mg/kg, respectively. The plants were grown following the standard cultivation practices employed by ISI Sementi, providing water in a controlled manner (~ 0.6 L per pot per day) to avoid Ni leaching into the saucers. Throughout the experiment, the following parameters were monitored: plant height, number of leaves before the first flower truss and between the first and second flower trusses, distance between the first and second flower truss, number of flowers in the first and second flower truss, number of flowers at anthesis, number and weight of fruits from the first and second flower trusses, as well as the SPAD index. The SPAD index was measured using a portable electronic chlorophyll meter (Konica Minolta SPAD 502) by quantifying the intensity of leaf green colour (2 leaves per plant, 3 measurements per leaf).

### 2.4. Inductively coupled plasma - Mass spectrometry (ICP-MS) analysis

For the ICP-MS analysis of roots and shoots, we used 3 biological replicates, each replicate was a pool of shoots or roots collected from 3 plants. After sampling, the roots were soaked for 5 min in ice-cold 50 mM Na-EDTA to remove the ions adsorbed to the plant walls (Nishida et al., 2020), then rinsed with ice-cold bi-distilled water. For fruit analysis, each of the five biological replicates consisted of one ripe fruit (collected 6–10 days after the turning stage) produced by the first flower truss of five independent plants.

Roots, shoots, and fruits were oven-dried at 65 °C. Dried tissues were homogenized, and about 20 mg of the homogenates were processed following a microscale high-throughput digestion method described by Hansen et al. (2009). Briefly, sample material was transferred into pre-cleaned 3-mL TFM vessels (Milestone) and mineralized with 350 μL of 69 % ultrapure $HNO_3$ (Romil) by incubation at 180 °C for 20 min in a Microwave Digestion System (Milestone). Three vessels were placed in a TFM 100-mL vessel with 11 mL of Milli-Q water and 1 mL of ultrapure-grade hydrogen peroxide (30 %, Romil). Digested solutions were diluted to 1 % $HNO_3$ with ultrapure-grade water (18.2 MΩ·cm at 25 °C). Selected elements (Ni, B, P, K, Cu, Zn, Mg, Mn, Fe, and Mo) were determined by iCAP RQ ICP-MS (ThermoFisher Scientific). Calibration curves were achieved by diluting a customized multielement standard (Romil). A standard reference material (NIST 1515 apple leaves, Merck KGaA) was employed to check for measurement accuracy and matrix effect.

### 2.5. *In vitro water stress induction using polyethylene glycol (PEG)*

Water stress was induced *in vitro* using 4 % and 8 % PEG 6000, as reported by George et al. (2013). After sterilization, 12 seeds from each line were placed in agar plates containing half-strength MS agar medium supplemented with vitamins in the presence or absence of PEG and maintained in a growth chamber under conditions reported in paragraph 2.2. The experiment was repeated three times. After ten days, seed germination capacity and root length were recorded. Seedlings with roots and emerged cotyledons were considered positively germinated. The root length was measured by means of the ImageJ program (Schneider et al., 2012).

### 2.6. *Water stress treatment of tomato lines in pots*

After *in vitro* growth in Magenta vessels under sterile conditions, 20-day-old seedlings of each line were transferred into pots (4 plants per pot) containing 700 g of a mixture of soil (GramoFlor substrate) and perlite (4:1) and watered regularly for 20 days. Then, all pots were kept at 100 % field capacity (FC) to obtain a homogeneous water supply condition. The 100 % FC was determined by the gravimetric method. Pots containing 700 g of soil/perlite were watered until saturation, and the weight was recorded once drainage stopped. The pots were divided into “control” and “treated” groups: control pots were watered every two days until saturation, while treated pots were completely deprived of water for 21 days (i.e., dry-down drought treatment). After a 5-day period of rehydration, a second water stress was imposed for 10 days only on plants that faced the first water stress. Subsequently, plants were rehydrated, and the trial was concluded 5 days later. During the experiment, stomatal conductance was measured using METER SC-1 Leaf Porometer (TMS) and shoot height and leaf number were assessed. For expression analysis and ABA quantification, fully-expanded non-senescing leaves were sampled at the end of the first severe water stress. Each of the 3 biological replicates was a pool of 4 leaves collected from plants grown in 3 independent pots.

### 2.7. *qRT-PCR analysis*

Total RNA extraction was performed using the "NucleoSpin RNA Plant" kit (Macherey-Nagel). cDNA was synthesized with the Improm-II reverse transcription system kit (Promega) from 1 µg of total RNA. Three cDNA samples derived from three biological replicates were prepared and then amplified on a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific) using Luna®Universal qPCR Master Mix (New England Biolabs). The mRNA expression levels of the housekeeping genes actin (NM_001321306) and beta-tubulin (NM_001247878.2) were used to normalize the expression of 9-cis-epoxycarotenoid dioxygenase (NCED-1; NM_001247526), catalase (CAT-1; NM_001247898), and Late Embryogenesis Abundant 3 (LEA3; Solyc01g095150.2) mRNAs. The following forward (F) and reverse (R) primers were employed: for actin F, 5′- TTCAAAGGGCGAGTACGACGAG-3′ and R, 5′- CAGCAGACCCGAGTTCACTTTT-3′; for beta-tubulin F, 5′-TGGGATTTGCCCCACTAACC-3′ and R, 5′-CAGGTAACGTCCATGACGGG −3′; for *NCED-1* F, 5′- TGTTGCAAACGCCGGTTTAGTCT-3′ and R, 5′-GCCGGTGGGTGTTACCTTTACAT-3′; for *CAT-1* F, 5′- ATGGTGCTATGAACATGACACA-3′ and R, 5′- ATGACACAATTTGTACGCCTTC-3′; for *LEA3* (Cao and Li, 2015) F, 5′-TATTCCGGTCATTGGCAACA-3′ and R, 5′-AGGAAGCTTATACTCGCCGCTAT-3′. Data analysis was conducted by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), employing the geometric mean of the two housekeeping genes.

### 2.8. *Lycopene determination in fruit extracts*

The extraction of carotenoids from dried tomatoes was performed following the method described by Dzakovich et al. (2019), with modifications. Briefly, 0.5 g of tomato sample was mixed with methanol (ratio 1:10) in the presence of BHT (0.1 % w/v), sonicated for 40 s, vortexed, and centrifuged at 4 °C for 5 min at 2000 x g. The supernatant was recovered and stored at 4 °C. The pellet was subjected to 3 successive extractions using a hexane/acetone mixture (ratio 1:10) in the presence of BHT (0.1 % w/v) and following the steps mentioned above. Obtained supernatants were combined, and 15 mL of water was added to induce phase separation. A 0.5 mL volume of the upper phase was taken, dried with a stream of nitrogen, and resuspended in hexane. Lycopene concentration was calculated using a Jasco V730 spectrophotometer (Jasco Europe Srl) at 503 nm, considering the specific extinction coefficient of lycopene in hexane, i.e., 3150 (100 mL/g cm). The results were expressed as µg lycopene/g of tomato (DW).

### 2.9. *Total phenols determination in fruit extracts by Folin-Ciocalteu assay*

Folin-Ciocalteu assay was performed to quantify the total polyphenols of the lower phase obtained after centrifugation, as mentioned above. The Folin-Ciocalteu assay was performed as described by De Marchi et al. (2024). Briefly, in a 96-well plate, 5 µL of the sample (diluted with water) was mixed with 150 µL of Folin-Ciocalteu reagent (previously diluted 1:15 with water). After 3 min, 40 µL of 20 % sodium carbonate solution was added and incubated for 30 min in the dark. The absorbance at 750 nm was measured with a Tecan Infinite 200 Pro microplate reader (Tecan Trading AG). A calibration curve was prepared with gallic acid (15.63 – 1000 mg/L), and the results were expressed as gallic acid equivalents (GAE) per gram of tomato (DW).

### 2.10. *ABA quantification in tomato leaves*

Extraction and quantification of ABA was performed at the Neuchâtel Platform of Analytical Chemistry, Université de Neuchâtel, Switzerland, following the protocol reported in Piskurewicz et al. (2023).

### 2.11. *Statistical analyses*

Statistical analyses were conducted using the GraphPad Prism or the software R (version 3.6.0), run under the free integrated development environment RStudio (version 1.0.153). Two-way ANOVA and post-hoc Tukey tests were performed using the R base functions. Data on ion concentrations were also resorted on generalized additive models (GAM), a class of linear models that can incorporate nonlinear forms of the features under study, to describe in the different lines the dependence of the measured concentrations of all ions on the different Ni doses used to treat the plants. Fits with GAM were carried out using the *gam()* function from the package mgcv (version 1.8–38; Wood, 2017) by assuming that the values of the response variables were normally distributed and by using an identity link function. In these models, "lines" was considered a factor with four levels (L1, L2, L3, and L4), and the "concentration" of Ni added to growth media of hydroponic cultures or the soil for plants grown in pots as a continuous real variable that incorporates a smooth function. The default spline basis was used as the smooth function. Pairwise comparisons of lineage factor levels in GAM objects were performed with the *glht()* function from the multcomp package (version 1.4–20; Bretz et al., 2010). To this purpose, the contrast matrix was set up as described (Hothorn et al., 2008; Bretz et al., 2010). PCA analysis was carried out using the functions from the multivariate exploratory data analysis and data mining package FactoMineR (Lê et al., 2008).

## 3. Results

### 3.1. *Effects of Nickel accumulation in tomato lines grown under hydroponic conditions*

Seventeen days after sowing, seedlings were grown for 5 days in hydroponic culture either under control conditions (0.5 μM Ni) or in the presence of 20 and 85 μM Ni (Fig. 2). To assess the response to Ni of the four lines, we calculated the increment with respect to the T0 control (Supplementary Figure 1) of the following growth parameters: shoot length from the cotyledonary leaves to apex, primary root length, and the number of leaflets. Treatments with 20 and 85 μM Ni caused in all the lines a statistically significant decline in shoot and root growth and a reduction in leaflet number compared to controls (Fig. 2C–E). Additionally, toxicity symptoms, such as leaf chlorosis, necrosis, and wilting, manifested in all lines, most severely at 85 μM (Fig. 2A and B). Considering the shoot length, L4 was able to maintain significantly better growth than the other lines at both 20 and 85 μM Ni, with a

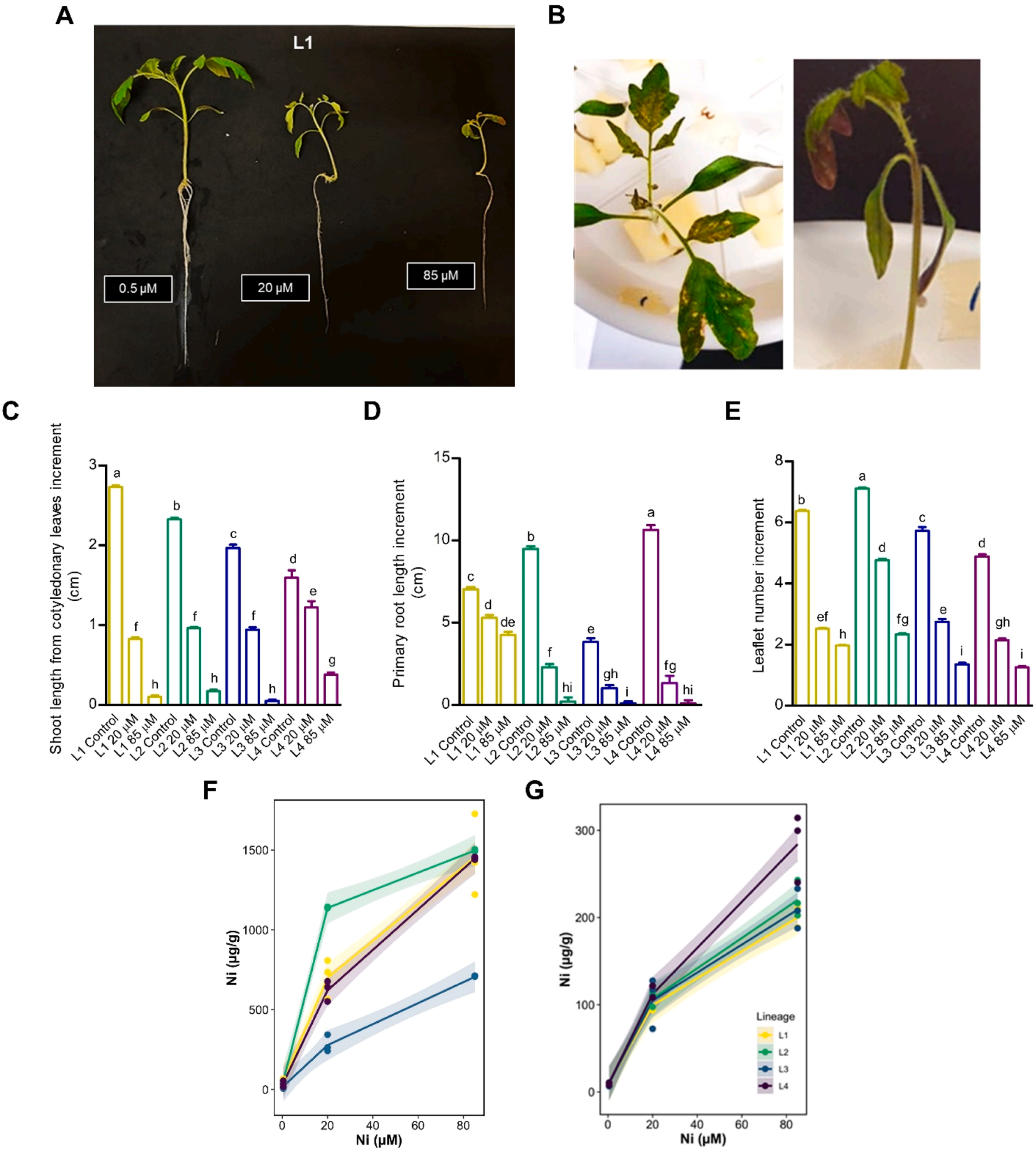

**Fig. 2.** Phenotypical response of tomato inbred lines to Ni treatments in a hydroponic system and Ni quantification in root and shoot. (A) Representative image of L1 seedlings after 5 days of Ni treatments; (B) Severe effects of 85 μM $NiSO_4$ on seedlings after 5 days of treatment; (C-E) Increments in shoot length from cotyledonary leaves to apex, primary root length, and number of leaflets. Values are mean ± SE ($n$ = 18–24), and the different letters on the top of the bars show statistically significant differences ($P < 0.05$) between lines as taken by two-way ANOVA followed byTukey's post-hoc test. (F, G) Fits of Ni concentration data with GAM in root and shoot, respectively. Shaded bands represent 95 % confidence intervals; statistical analyses (GAM and two-way ANOVA) are reported in Supplementary Table 2.

tolerance index of 77 % and 24 %, respectively, values higher than those recorded for the other three lines (Fig. 2C and Supplementary Table 1). Regarding the primary root, L1 displayed a very high capacity to cope with Ni compared to the other lines, as reflected by the tolerance index of 75 % and 61 % at 20 and 85 μM Ni, respectively. Indeed, the values of the tolerance index at 85 μM of the other three lines did not exceed 2 % (Fig. 2D and Supplementary Table 1). With respect to leaflet number, L2 showed a significantly better response to Ni than the other lines under both Ni doses, with tolerance index at 20 μM and 85 μM Ni of 67 % and 33 %, respectively (Fig. 2E and Supplementary Table 1). At 85 μM Ni, L3 and L4 showed the lowest increment in leaflet number (Fig. 2E and Supplementary Table 1). The evaluation of Ni concentration in roots and shoots revealed significant differences between lines (Fig. 2F and 2G and Supplementary Table 2). The post-hoc Tukey test and GAM analysis (Supplementary Table 2) showed that L2, compared to other lines, had overall the highest Ni accumulation capacity in the roots, whereas L3 exhibited the lowest Ni accumulation (Fig. 2F). Nonetheless, the increment in primary root growth of L3 at 85 μM Ni was not higher than that of L2 (Fig. 2D and 2F). L4 accumulated overall more Ni in the shoots than the other lines (Fig. 2G and Supplementary Table 2), though characterized by a higher Ni tolerance index on shoot growth compared to the other lines (Fig. 2C). The increment in leaflet number of L4 at 85 μM Ni was not statistically different from L3, although the concentration of Ni in L3 was lower than L4. Exposure to increasing Ni concentrations appeared to determine a general reduction in the accumulation of other essential elements (Fig. 3). Two-way ANOVA confirmed that different Ni concentrations significantly altered the levels of all ions measured in the shoots (Supplementary Table 2A). When stratified by line type, K, Mn, Fe, and Mo concentrations showed some differences across lines (Supplementary Table 2B and 2C). In particular, L4 had, as a whole, higher Mn concentrations than L1, L2, and L3, while L3 accumulated more Fe and L2 more Mo than the other lines (Fig. 3 and Supplementary Table 2B and 2C). Potassium concentration was significantly higher in both L1 and L3 than in L2 and L4 (Fig. 3 and Supplementary Table 2B and 2C).

### 3.2. *Effects of Nickel accumulation in plants grown under pot cultivation conditions*

To test the effects of different doses of Ni (30, 120, and 300 mg/kg), the plants were grown for four months after transplanting in pots containing soil collected from a field of ISI Sementi. The substrate was an alkaline silty clay loam soil with a medium level of organic matter and a pH of 8.1 (Supplementary Table 3A). The Ni availability index of the soil was measured before and after adding Ni (Supplementary Table 3B). Several vegetative and reproductive growth parameters and Ni and mineral nutrient concentrations in the fruit were measured. Overall, Ni treatments resulted in negligible effects on the plant height, the number of leaves before the first flower truss, the number of leaves between the first and second flower truss, and the SPAD index, as well as the number of flowers on the first and second flower truss and the number of fruits developed on the first two inflorescences (Supplementary Table 4). The values of the different growth parameters were used in a Principal Component Analysis (PCA) to explore the variability of the dataset (Fig. 4). The first two components explain 50.84 % of the variance observed in the data. The samples clustered mainly according to lines with respect to treatment. We conducted a multi-element analysis on fruits harvested from plants grown in the presence of 120 and 300 mg Ni/kg soil. The Ni concentration in the fruits increased as Ni in the soil rose (Fig. 5), with L4 showing the highest Ni concentration in fruits compared to the other lines (Fig. 5 and Supplementary Table 5). For all lines, the accumulation of Ni in the fruits affected neither their size nor their weight (data not shown).

The two-way ANOVA with post-hoc Tukey and GAM analyses applied to the multi-element concentrations in the fruits of Ni-treated plants are reported in Supplementary Table 5. Data modeling with GAM returned the same statistics of two-way ANOVA analysis about ion accumulation in the different lines, with only slight differences (Supplementary Table 5). Ni administered to the plants did not perturb the concentration of Zn, but significantly altered the concentrations of all other ions, although the variability of the data set does not allow to determine a general positive or negative effect of Ni in mineral ion accumulation in the fruit. Indeed, in some lines, we observed an increasing trend in P concentration, and the opposite was observed for Fe and Cu. The L4 line showed higher concentrations of B and Cu than the L1 (Supplementary Table 5). Tomatoes from the L2 plants contained more Mg, Mn, and Mo than those from the other lines, even under control conditions. To investigate if Ni treatment can affect fruit quality, we evaluated the concentrations of lycopene and total phenolic compounds in the fruits of lines L3 and L4, the latter being the line with the highest Ni concentration in the fruits (Fig. 6). The values were compared with those measured in fruits of plants grown in untreated soil. No significant change in lycopene concentration was measured in the L3 and L4 fruits (Fig. 6A). The concentration of total phenolic compounds in the fruits increased significantly in the L4 line compared to the control treatment (Fig. 6B), whereas no changes were observed in the L3 fruits.

### 3.3. *Response of tomato inbred lines to water stress*

#### 3.3.1. *In vitro response to PEG-induced water stress*

To test the effect of artificially simulated water stress on germination capacity and seedling growth, seeds of the four lines were sown in MS agar plates containing 4 % and 8 % polyethylene glycol (PEG-6000). The germination capacity of L1, L2, and L3 (at 4 % PEG only) was not statistically different from the respective control values (Fig. 7A), although L3 showed a low intrinsic germination capacity *in vitro* and PEG at 8 % completely inhibited L3 germination. PEG had a dose-dependent inhibitory effect on the germination of L4, suggesting greater sensitivity to water stress (Fig. 7A) However, L4 seedlings able to germinate on PEG showed hypocotyl (data not shown) and root lengths (Fig. 7B) comparable to those measured without PEG. No root and hypocotyl length differences were also observed for the other lines (Fig. 7B and data not shown).

#### 3.3.2. *Response of tomato lines to water deprivation*

We tested the capacity of the four inbred lines to tolerate water stress and respond to rewatering by growing the plants in the soil under greenhouse conditions and alternating periods of water deprivation and water re-supply. To monitor the effects of the water regimen on the plant water status, we measured stomatal conductance (Fig. 8A). At the beginning of the trial, the plants of each line displayed homogenous growth in terms of shoot height and number of leaves (data not shown). At the end of the first water deprivation period, stomatal conductance was strongly reduced in all lines (Fig. 8A and Supplementary Table 6). Plants of the different lines grown under water deficit displayed wilting and reduced shoot height and leaf number compared to the respective controls (Fig. 8B). Specifically, the shoot height of the stressed plants decreased compared to control plants by approximately 38, 35, 45, and 53 % for lines L1, L2, L3, and L4, respectively. The number of leaves decreased by about 16, 40, 27, and 30 % for L1, L2, L3 and L4, respectively. After five days of rehydration, the stomatal conductance increased in all lines without reaching the values of the unstressed control plants (Fig. 8A and Supplementary Table 6). To simulate intermittent drought, an event commonly occurring in the field, a second water stress was imposed on plants exposed to the first stress and rehydrated. A significant reduction in stomatal conductance was evident only in L1 (Supplementary Table 6), associated with a significant decrease in shoot growth (about 29 %) compared to the respective control (Fig. 8D). L4 is the only line showing diminished capacity to form new leaves after the second stress (Fig. 8D). After a second water supply for five days, the stomatal conductance of the control and stressed plants did not differ significantly (Fig. 8A and Supplementary Table 6), without reaching the values of the control plants at the

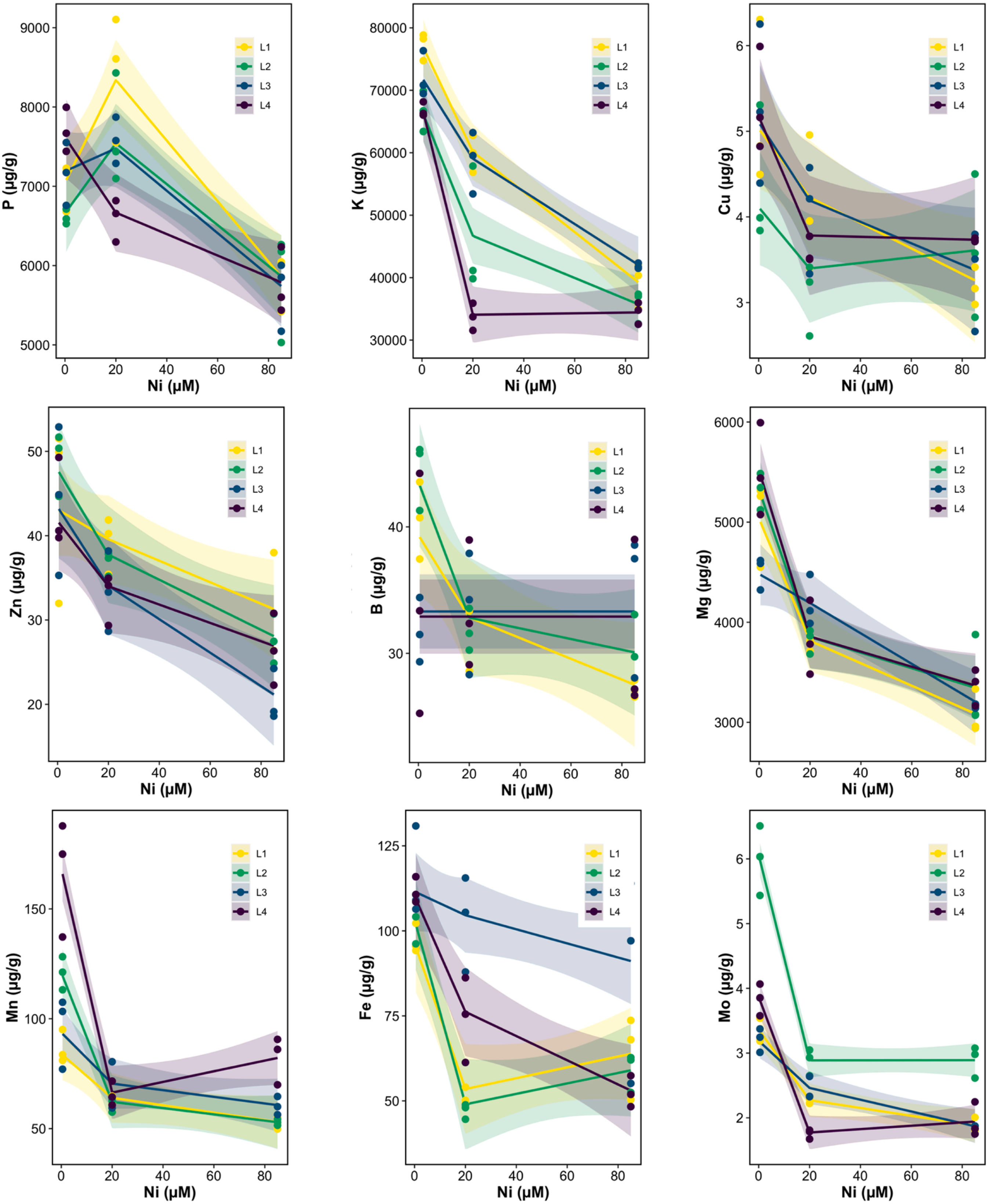

**Fig. 3.** Fits (lines) of ion concentration data (points) with GAM. The data were measured in the shoots of tomato plants grown under hydroponic conditions and treated with Ni. Shaded bands represent 95 % confidence intervals; statistical analyses (GAM and two-way ANOVA) are reported in Supplementary Table 2.

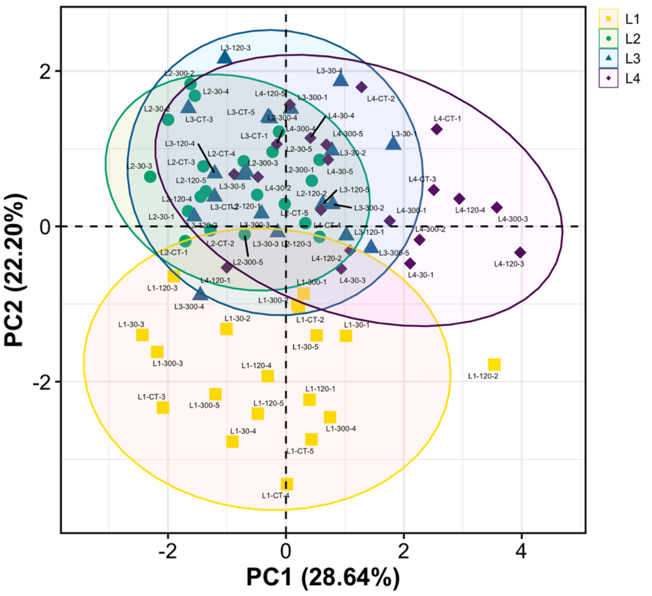

**Fig. 4.** PCA score plot in R. The following phenotypic parameters of plants of the four inbred lines (L1-L4) have been considered: plant height, number of leaves before the first flower truss, distance between the first two developed flower trusses, number of flowers of the first flower truss, number of fruits of the first and second flower truss, SPAD index. Plants were grown in pots in the absence (control, CT) or in the presence of different Ni concentrations (30, 120, and 300 mg/kg). Values given in parenthesis in axis labels represent the percentages of variation explained by the corresponding component. The first 2 components explained 50.84 % of the variance observed in the data. Confidence ellipses at 0.95 level from a multivariate t-distribution have also been drawn. The samples clustered mainly according to lines with respect to the treatment.

beginning of the trial. During drought, the increased expression of reactive oxygen species (ROS)-scavenging enzymes and ABA synthesis enzymes are important signals for regulating plant responses and overcoming stress. Other proteins, such as Late Embryogenesis Abundant (LEA) are highly responsive to drought and considered markers of abiotic stress (Cao and Li, 2015). We analyzed the expression of *catalase 1* (*SlCAT-1*) involved $H_2O_2$ detoxification, 9-cis-epoxycarotenoid dioxygenase *1* (*SlNCED-1*), a key enzyme of ABA biosynthetic pathway, and *SlLEA3* in leaves sampled at the end of the first water stress period, a condition that caused the most severe effects on the growth of all lines. Upon stress, the expression of *SlCAT-1* increased significantly in L4, *SlLEA3* in L2 and L3, and *NCED-1* in L1, L2, and L3 compared to the respective controls (Fig. 8C). ABA was quantified in L3 and L4 leaves at the end of the first stress period; ABA concentration increased in both L3 and L4, although *NCED-1* expression did not significantly change in L4 (Fig. 8C).

## 4. Discussion

The selection of crop genotypes based on yield and quality cannot prescind from their ability to preserve these characteristics even under stress conditions, such as drought, which are becoming increasingly frequent in the context of climate change. Another challenge to agricultural production is represented by contaminated soils, particularly heavy metals, such as Ni, an essential nutrient for plants at quite low levels but toxic at high levels, leading to crop pollution (Nagajyoti et al., 2010).

In this work, the responses of four inbred lines of fresh-market indeterminate tomato were evaluated for their ability to accumulate Ni in different organs and respond to Ni toxicity and drought stress.

The different abilities to respond to excess Ni exhibited by the four lines were deduced by combining the results obtained from hydroponic systems and pot trials with soil.

### 4.1. *Effect of Nickel on growth and mineral nutrient accumulation in inbred lines grown in hydroponics*

The results obtained with hydroponic experiments revealed that it is impossible to directly match the Ni accumulation in roots and shoots with the degree of growth inhibition in the respective organs, suggesting a different response mechanism of the four lines to internal Ni concentrations. Statistical analysis showed, for instance, that in L1 the negative effects on root growth were less severe than those observed in the other lines, even if L1 is not the line with the lowest Ni accumulation capacity in roots. On the other hand, L3, which is characterized by the lowest capacity to accumulate Ni in the roots, displayed a high sensitivity to damages (tolerance index of 2 % at 85 μM). These observations are also confirmed by the behaviour of L4, which accumulated Ni at higher concentrations than the other lines in the shoot, but this was not reflected in more damage. A common effect related to Ni toxicity is an imbalance in the accumulation of other mineral elements due to competition for uptake and translocation, which generally leads to nutrient deficiency (Chen et al., 2009; Bhalerao et al., 2015; Mustafa et al., 2023). Previously published data have reported significant reductions in macronutrients (K, and Mg) and micronutrients (Fe, Mn, and Cu) in tomato leaves under conditions of severe Ni stress (Kumar et al., 2015). Furthermore, Palacios and collaborators (1998) reported that high Ni concentrations significantly decreased the uptake of other divalent cations, such as Mg, Fe, Mn, Cu, and Zn. Our results agree with a general decline in the concentration of micro- and macronutrients in the shoots of all lines as Ni concentration increased in the nutrient solution. In particular, the decrease of the macronutrients K and P can be related to delayed plant growth induced by Ni treatment (Correia et al., 2018). It is remarkable that the degree of inhibition in micro- and micronutrients accumulation differed among the individual lines. For example, L4, L3, and L2 showed a significantly lower decrease in Mn, Fe, and Mo, respectively, than the other lines. Overall, the experiment conducted under hydroponic conditions demonstrated some differences among the four lines in their response to the negative effects of Ni on growth and nutrient status, although it is difficult to identify one line with the best characteristics in terms of limited Ni accumulation and a good performance in all the growth parameters analysed. Treatments under hydroponic conditions may be useful for the initial screening of lines or cultivars to identify those with high Ni accumulation capacity, like L4 that should be used with caution in breeding programs for the production of hybrids intended for Ni-enriched soils.

### 4.2. *Effect of Nickel on growth and mineral element accumulation in inbred lines grown in soil pots*

The trial conducted by growing the plants in pots was aimed at evaluating the response to toxic Ni concentrations under conditions close to normal cultivation practices and monitoring the concentration of Ni in the edible part. The concentrations of bioavailable Ni in the substrate included values both below and above the threshold limit for Ni in the soil established by Council Directive 86/278/CEE (75 mg Ni/kg soil at pH 7.0) and above those typically found in non-acidic cultivated soils in Italian regions (Barbafieri et al., 1996). Morphological analysis revealed only subtle changes in the vegetative and reproductive growth of Ni-treated plants. Remarkably, the size and weight of the fruits did not differ between Ni-treated and untreated plants and the lycopene concentration also remained unchanged. Maintaining the lycopene concentration in the fruit is important from a nutritional point of view, considering the beneficial effects of lycopene on human health (Khan, et al., 2021). Regarding the mineral nutrient levels in the fruits of Ni-treated plants, we cannot delineate a conclusive picture of the effects of Ni on the concentration of the other elements due to the variability of the dataset, except for Zn, which was not affected, and an overall

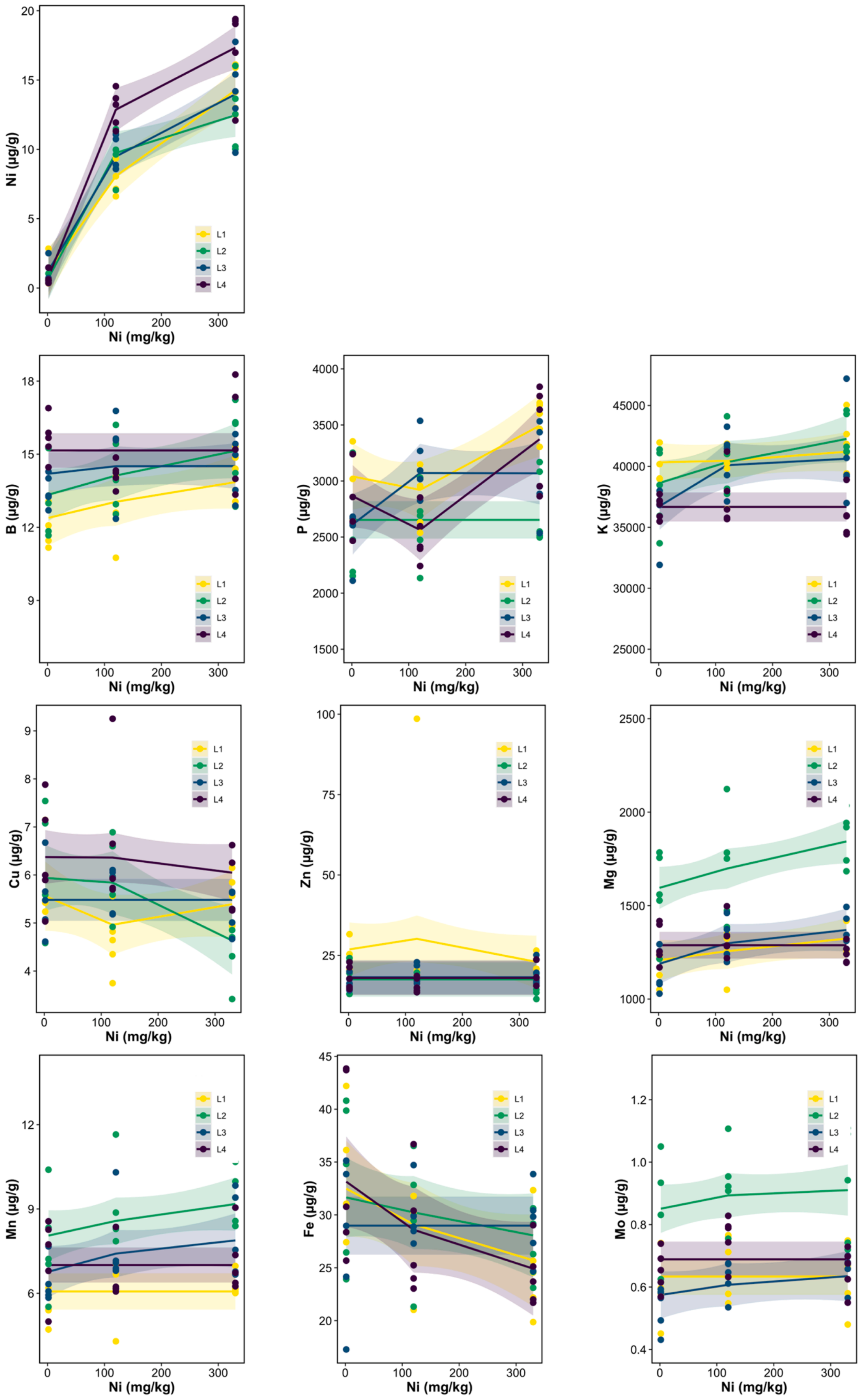

**Fig. 5.** Fits (lines) of ion concentration data (points) with GAM. The data were measured in tomatoes of plants grown in pots and treated with Ni. Shaded bands represent 95 % confidence intervals; statistical analyses (GAM and two-way ANOVA) are reported in Supplementary Table 5.

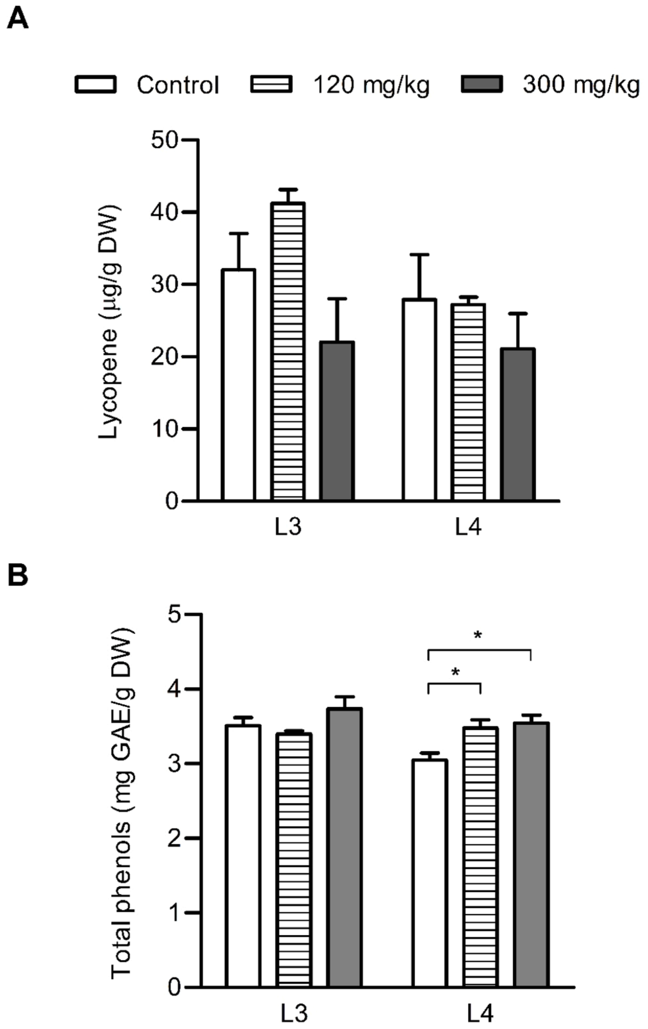

**Fig. 6.** Quantification of lycopene and phenolic compounds in fruits of lines L3 and L4 exposed to Ni treatments under pot cultivation conditions. (A) Lycopene and (B) total phenolic compounds determination in dried samples. Bars are mean ± SE ($n = 5$). Two-way ANOVA and Tukey's post-hoc test were used (*$P < 0.05$, versus the respective "Control" treatment).

reduction in Fe concentration. Fe and Zn are essential for a number of human metabolic processes, and their dietary deficiencies contribute to an increased disease risk (Kumar et al., 2019). On the other hand, we observed that some lines maintain higher concentrations of specific elements, for example, P in L1 and L4 and Mg, Mn, and Mo in L2. A relatively high concentration of Mg in fruits could represent a protective strategy used to counteract the negative effects of Ni, as Mg has been demonstrated to influence both fruit ripening and carotenoid accumulation (Jia et al., 2013; Liu et al., 2022; Liu et al., 2023). Overall, these experiments demonstrated that the analysis of morphological traits might not be predictive of tomato health status in terms of Ni concentration in fruits. Even L4, the line with the highest capacity to accumulate Ni in fruits (on the average ~16–17 μg/g DW at the soil treatment of 300 mg/kg) showed no significant alterations in growth. Limits for Ni concentration in tomato fruits have not been specifically defined, but recently, the maximum level of Ni in fruiting vegetables for consumption was set at 0,40 μg/g FW (Commission Regulation (EU), 2024/1987). The Ni concentration is expressed on a FW basis, thus not directly comparable with our values expressed on a DW basis. Considering that, on average, a tomato fruit can contain 90–95 % of water (Hou et al., 2020), 0,40 μg/g FW would correspond to 4–8 μg/g DW. Under our soil treatment conditions, the Ni concentration in the fruit would overcome these values in the absence of visible toxic effects on plant growth (Fig. 5 and Supplementary Table 4).

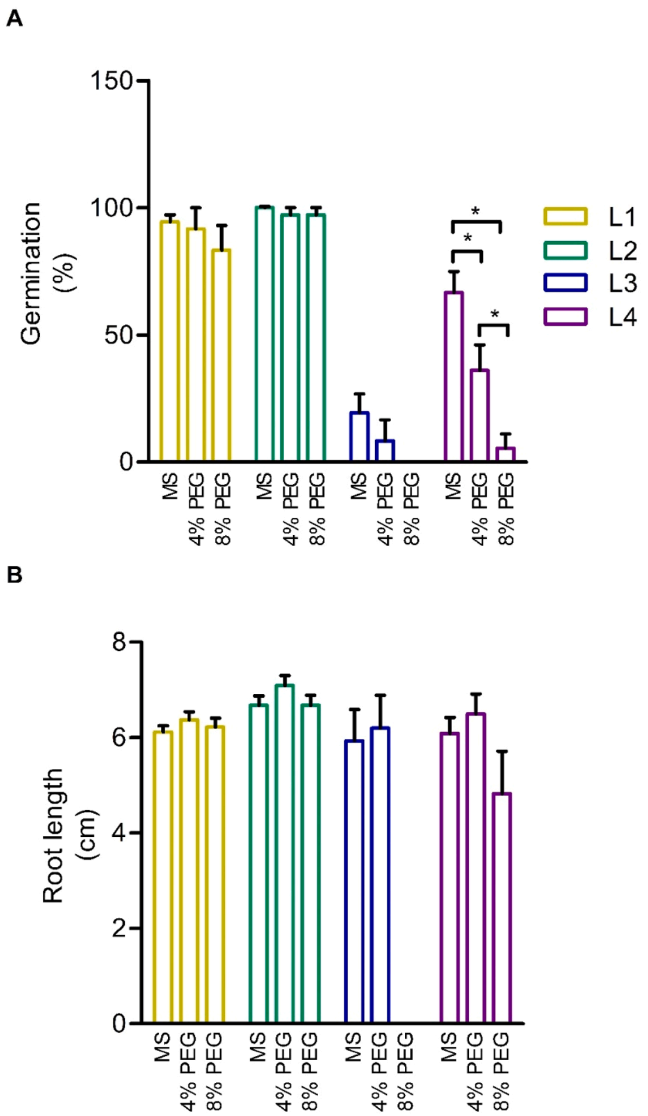

**Fig. 7.** Germination capacity and root length of germinated seedlings under PEG-induced water stress. (A) Percentage of germinated seedlings grown under control (MS) and stress conditions (4 % and 8 % PEG), and (B) root length of germinated seedlings. Data are the mean ± SE ($n = 3$ biological independent replicates, each of 12 seeds), and means were compared by two-way ANOVA followed by Tukey's post-hoc test. In panel A, only statistically significant results for the main effect "treatment" are shown (* $P < 0.05$).

### 4.3. *Effect of water stress in inbred lines grown in vitro and in pots*

The four inbred lines were also evaluated for their response to water stress in the first phase of the life cycle (germination and seedling growth) and at maturity by sequentially applying water deprivation and rehydration. The results suggest that if a water stress event occurred in the field at the seed germination stage, L1 and L2 would be more advantaged than L3 and L4. In the pot experiment, the behaviour of the adult plants of the four lines was quite homogeneous at the first water stress, both in terms of growth inhibition and induction of drought-responsive genes. Differences emerged after the second deprivation period for L1 and L4, which displayed greater sensitivity to water stress damage than the L2 and L3. On the whole, these analyses enabled us to identify L4 as the most sensitive line to water stress. The elevated ABA

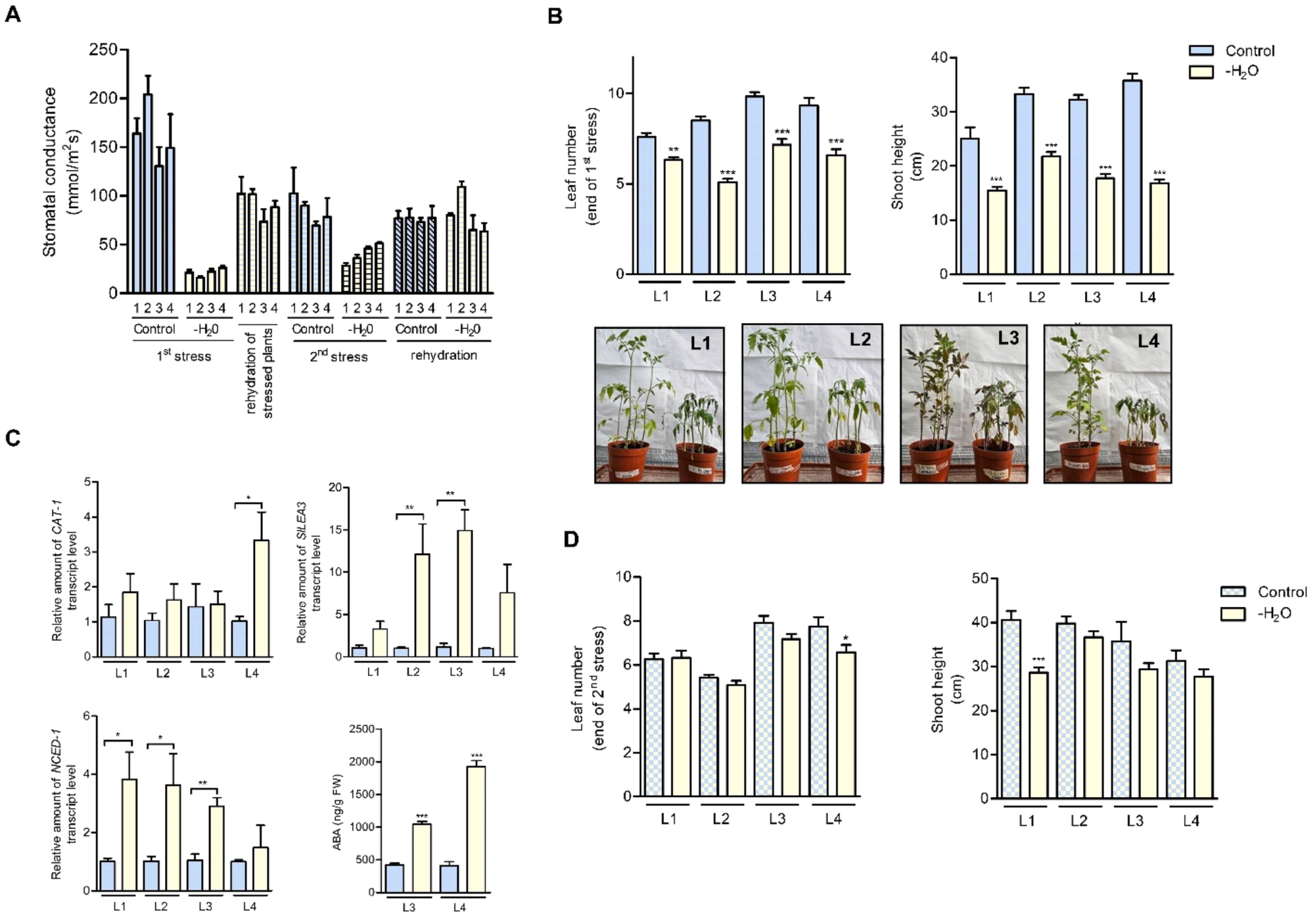

**Fig. 8.** Water stress response of inbred lines grown in pots under greenhouse conditions. (A) Stomatal conductance measured at different times of stress exposure and rehydration. Two-way ANOVA analysis followed Tukey's post-hoc test was conducted, and the results are reported in Supplementary Table 6. (B) Leaf number and shoot height measured at the end of the first stress; representative images of plants under control (on the left) and stress (on the right) conditions are reported below the graphs. (C) Expression analysis of *CAT-1, NCED-1*, and *LEA3* conducted by qRT-PCR and ABA quantification (lower on the right) on fully expanded leaves sampled at the end of the first water stress. (D) Leaf number and shoot height measured at the end of the second stress. The differences between treatments within each line were evaluated by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

concentration and low stomatal conductance of L4 at the end of the first stress, not accompanied by a significant induction of *NCED-1* observed in the other lines, could be explained by an earlier activation and shutdown of *NCED-1* expression than in the other lines, or by a different homeostatic regulation of ABA.

## 5. Conclusion

In conclusion, L4 is the line that accumulates the greatest amount of Ni in the fruit and is also the most sensitive to water stress, and L1, compared to the other lines, fails to acclimate to repeated water deprivation. On the other hand, L2 and L3 can be advantageous compared to the other lines, when grown in soils with relatively high levels of Ni because, besides a lower capacity to accumulate this metal than L4, L2 fruits accumulate more nutrients, and L3 fruits are not affected in the content of lycopene and total phenolic compounds. These fruit quality parameters, coupled with L2 and L3 lines' capacity to acclimate to repeated water stress, make these two lines the most suitable candidates for breeding programs. Our multi-level approach, combining phenotypic, biochemical, and molecular analyses derived from *in vitro* tests, hydroponics, and potted cultivations, has proven successful in discriminating the response of inbred lines to different stress conditions and could be useful in breeding programs to identify and develop multi-stress resilient varieties.

## CRediT authorship contribution statement

**Daniela Fortini:** Visualization, Investigation, Data curation. **Roberto Chignola:** Formal analysis. **Giulio Zanni:** Supervision, Resources, Methodology. **Alice Brunazzi:** Supervision, Resources, Methodology. **Anita Zamboni:** Writing – review & editing, Writing – original draft, Formal analysis. **Gianni Zoccatelli:** Writing – review & editing, Investigation. **Marco Ciulu:** Writing – review & editing, Investigation. **Diana Vanessa Santisteban Soto:** Investigation. **Tommaso Sanson:** Formal analysis. **Tiziana Pandolfini:** Writing – review & editing, Writing – original draft, Funding acquisition. **Barbara Molesini:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition.

## 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2025.100794.

## Data availability

Data will be made available on request.

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