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The combined effect of Cr(III) and NaCl determines changes in metal uptake, nutrient content, and gene expression in quinoa (*Chenopodium quinoa* Willd.)

Francesco Guarino[a,1], Karina B. Ruiz[b,c,1], Stefano Castiglione[a], Angela Cicatelli[a,*], Stefania Biondi[c]

[a] Dipartimento di Chimica e Biologia "A. Zambelli", Università di Salerno, Fisciano, Salerno, Italy
[b] Departamento Agricultura del Desierto, Universidad Arturo Prat (UNAP), Iquique, Chile
[c] Dipartimento di Science Biologiche, Geologiche e Ambientali, Università di Bologna, Bologna, Italy

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ABSTRACT

Many areas of the world are affected simultaneously by salinity and heavy metal pollution. Halophytes are considered as useful candidates in remediation of such soils due to their ability to withstand both osmotic stress and ion toxicity deriving from high salt concentrations. Quinoa (*Chenopodium quinoa* Willd) is a halophyte with a high resistance to abiotic stresses (drought, salinity, frost), but its capacity to cope with heavy metals has not yet been fully investigated. In this pot experiment, we investigated phytoextraction capacity, effects on nutrient levels (P and Fe), and changes in gene expression in response to application of Cr(III) in quinoa plants grown on saline or non-saline soil. Plants were exposed for three weeks to 500 mg kg^{-1} soil of $Cr(NO_3)_3 \cdot 9H_2O$ either in the presence or absence of 150 mM NaCl. Results show that plants were able tolerate this soil concentration of Cr (III); the metal was mainly accumulated in roots where it reached the highest concentration (*ca.* 2.6 mg g^{-1} DW) in the presence of NaCl. On saline soil, foliar Na concentration was significantly reduced by Cr(III). Phosphorus translocation to leaves was reduced in the presence of Cr(III), while Fe accumulation was enhanced by treatment with NaCl alone. A real-time RT-qPCR analysis was conducted on genes encoding for sulfate, iron, and phosphate transporters, a phytochelatin, a metallothionein, glutathione synthetase, a dehydrin, Hsp70, and enzymes responsible for the biosynthesis of proline (*P5CS*), glycine betaine (*BADH*), tocopherols (*TAT*), and phenolic compounds (*PAL*). Cr(III), and especially Cr(III) + NaCl, affected transcript levels of most of the investigated genes, indicating that tolerance to Cr is associated with changes in phosphorus and sulfur allocation, and activation of stress-protective molecules. Moderately saline conditions, in most cases, enhanced this response, suggesting that the halophytism of quinoa could contribute to prime the plants to respond to chromium stress.

1. Introduction

Chromium (Cr) has become the second most common inorganic contaminant, owing to industrial activities, agricultural practices, and waste disposal, so that pollution by this metal now constitutes a serious environmental problem and poses relevant health risks (Peralta-Videa et al., 2009; Farid et al., 2019). Cr has a complex electronic chemistry and several oxidation states, but Cr(III) and Cr(VI) are the most stable and common forms in the environment (Singh et al., 2013). These forms have different features and play diverse roles in living organisms. Thus, while Cr(III) is an essential trace element for humans and animals, it is not so for plants. Most studies on chromium have been dedicated to the hexavalent form, whose phytotoxicity derives from a variety of mechanisms, amongst which ROS accumulation, alteration in the enzymatic antioxidant system, DNA damage and genotoxicity, and effects on the photosynthetic machinery (Wakeel et al., 2020). Cr(III), however, also impacts growth and metabolism in plants (Shahid et al., 2017)., in some cases by causing oxidative stress (Scoccianti et al., 2006); under field conditions, it may have no effect due to its low bioavailability in contaminated soil (Cicatelli et al., 2017). To date, however, physiological and molecular responses to Cr(III) in plants remain less studied than those of Cr(VI).

Although plants do not have a specific Cr uptake mechanism, the metal is absorbed along with other elements. Therefore, Cr may interfere with plant mineral nutrition due to its similarity with essential ions and competition for the same carriers. In general, Cr is taken up by

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[*] Corresponding author.
E-mail address: acicatelli@unisa.it (A. Cicatelli).
[1] These authors contributed equally to this work.

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plants via phosphate or sulfate transporters, but both Cr(III) and Cr(VI) have been shown to interfere with the uptake of other macronutrients, such as nitrogen, potassium (K), and magnesium (Singh et al., 2013; Gomes et al., 2017 and references therein).

Soil salinization represents a major constraint on crop production since most crops are glycophytes. It has been estimated that salinity affects at least 33% of arable lands worldwide, as a result of natural or anthropogenic activities (salt discharge, irrigation with saline water, seawater intrusion in coastal areas, etc.) and more areas are expected to deteriorate in the near future due to climate change. In some areas, soils polluted with high concentrations of heavy metals (HMs) also have high concentrations of salts. Such situations may arise in semi-arid regions where mining contributes to the release of both HMs and soluble salts (Lutts and Lefèvre, 2015; Zaman et al., 2018). For example, in the salt-affected arid zones of northern Chile, arsenic (As) accumulates in the environment as a result of anthropogenic activities (Vromman et al., 2016). For phytoremediation of these areas, suitable plants must be identified that are able to tolerate HMs under saline conditions. Due to their ability to thrive in saline environments, where they accumulate and tolerate large amounts of toxic ions, halophytes are also able to cope with metal stress (Lutts and Lefevre, 2015; Nikalje and Suprasanna, 2018). They are, therefore, receiving increasing attention as candidates for the rehabilitation of these sites (Manousaki and Kalogerakis, 2011; Van Oosten and Maggio, 2015). From a practical viewpoint, it is important to check whether or not, and to what extent, soil salinity influences the plant's capacity to absorb and translocate a given HM. To this aim, several studies have been conducted using halophytic species, such as: *Suaeda maritima*, *Sesuvium portulacastrum*, *Atriplex halimus*, *Zygophyllum fabago*, and *Echinochloa stagnina,* showing that they are able to effectively remove salt and HMs from saline and polluted soils (Liang et al., 2017). In *Atriplex atacamensis,* a halophyte native of the Atacama Desert in N. Chile, salinity strongly increased As translocation from the root to the shoot (Vromman et al., 2016).

Both HMs and salt affect morphological, physiological, and metabolic pathways in plants, including water balance and nutrient uptake; these deleterious effects often lead to growth reduction and may be associated with oxidative stress. Adaptation to salinity and HMs relies on physiological mechanisms, such as controlled uptake and compartmentation of potentially toxic ions (Na^+, Cl^-) and HMs, activation of the antioxidant defence system, and synthesis of compatible organic solutes. Moreover, both salt and metal stress result in massive changes in gene expression profiles (Trivellini et al., 2016; Aprile et al., 2018). Studying the way in which salinity affects HM tolerance in halophytes will further our understanding of their stress-adaptive mechanisms.

Quinoa (*Chenopodium quinoa* Willd.) is native of the Andes and very tolerant to multiple types of abiotic stresses (Ruiz et al., 2016b; Hinojosa et al., 2018). Some ecotypes are well adapted to extremely saline and arid soils where few other species can develop (*e.g., altiplano* of the Andes). In particular, quinoa is one of the very few plants that can grow at the altitude and under the soil and climatic conditions of Lake Titicaca. This lake is heavily polluted with HMs originating from natural sources, mining activities, and urban sewer discharge (Monroy et al., 2014). Quinoa is also unique among halophytes in being a crop species whose seeds have exceptional nutritional properties (Vega-Gálvez et al., 2010). The importance of quinoa is such that it can be considered a model halophyte for investigating stress tolerance mechanisms at cellular, molecular, and metabolic levels (Ruiz et al., 2016b) and this is corroborated by the fact that its genome was recently sequenced (Jarvis et al., 2017). Genome-wide transcriptomic analyses have been conducted in quinoa under drought stress (Morales et al., 2017) and salt stress (Ruiz et al., 2019) and expression levels of target genes under salt stress were comparatively analysed in several Chilean landraces (Ruiz-Carrasco et al., 2011; Ruiz et al., 2017). Thus, a large amount of information is available with regard to quinoa's salt and drought tolerance mechanisms, but much less is known about its adaptability to metal pollution, although its phytoextraction potential has been assessed for some HMs (Bhargava et al., 2008; Thomas and Lavkulich, 2015; Haseeb et al., 2018). We have previously conducted a study on the short-term responses to Cr(III) in young quinoa plants under semi-hydroponic conditions (Scoccianti et al., 2016) but, to our knowledge, the interaction between Cr and salinity has not yet been investigated in any halophytic species. To further our knowledge on the long-term effects of Cr(III) and on the impact of moderately saline soil, in this work we performed a pot study in which mature quinoa plants were exposed to Cr(III) alone or combined with 150 mM NaCl. Given the lack of information regarding metal-induced changes at the molecular level in quinoa as well as in other halophytes, the expression of several genes involved in nutrient transport and in HM or other types of abiotic stresses was determined.

The aims of this work were to assess in quinoa plants exposed to Cr (III), under moderately saline or non-saline conditions, the following: (i) plant growth; (ii) Cr and Na uptake and organ distribution, (iii) tissue contents of phosphorus (P) and iron (Fe), and (iv) expression levels of genes coding for sulfate, Fe, and phosphate transporters, genes involved in HM stress, i.e., phytochelatins (PCs), and metallothioneins (MTs), and in cellular redox status (glutathione synthetase), other stress-related genes (dehydrins, heat-shock proteins), and, finally, genes responsible for the biosynthesis of compatible solutes (proline, betaine), and secondary metabolites with protective functions (tocopherols, phenolic compounds).

2. Materials and methods

2.1. Plant material and treatments

Quinoa (cv. Titicaca) seeds were sown in polystyrene alveoli containing peaty garden soil (Turco Silvestro, Albenga, Savona-Italy) and incubated in a growth chamber under the following conditions: 16 h light/8 h dark with a light intensity of 50 μmol m^{-2} s^{-1} at 25 °C ± 1 °C. Plantlets (5–10 cm high) were then transferred to pots (0.25-L) filled with the same garden soil and maintained in the growth chamber for two weeks. When the plants reached a height of *ca.* 20 cm (approximately 15 leaf pairs), they were transferred to plastic pots (3.5-L) containing 3.0 kg of garden soil (soil characteristics are reported in Table SM1) and kept in a greenhouse under artificial light (16 h light/8 h dark). Pots were watered regularly, but percolation was avoided. After 15 days of acclimation to greenhouse conditions, $Cr(NO_3)_3{\cdot}9H_2O$ (Merck Life Science s. r.l., Milano-Italy) was added to pots in three weekly doses in order to reach 500 mg kg^{-1} dry weight (DW) soil; in half of the pots, 150 mM NaCl was also added. The experimental design comprised four treatments: control (CNT), NaCl, Cr, Cr + NaCl, with seven pots per treatment (one plant per pot). Three weeks from the start of treatment (i.e., three months after sowing), plants were harvested, separated into roots, stems, and leaves, weighed and stored at −80 °C until use. For dry biomass and element determination, leaf, stem, and root samples were oven-dried at 75 °C up to constant weight. For RNA extraction, leaves and roots from each plant were harvested separately and processed in order to have three biological replicates. Soil was collected from pots at the start and at the end of the experiment (three weeks after Cr and/or NaCl addition) and oven-dried at 75 °C up to constant weight.

2.2. Element (Cr, Na, P, Fe) determinations

Dried soils were pulverized in a planetary ball mill (PM4, Germany), while plant organs were pulverized with liquid N_2 in a mortar (leaves, roots) or reduced to ashes (stems) by baking at 550 °C for 4 h. For each plant organ, three biological replicates were analysed. All materials (soils and plant organs) were digested with an acid mixture (HNO_3 65%: HF, 50%; Merck Life Science s. r.l., Milano-Italy) in a microwave oven (Ethos, Milestone). The available element concentrations were determined in soil extracts obtained from the dried granulometric fraction, according to Lindsay and Norwell (1978). Cr, Na, and nutrient (Fe, P) concentrations were evaluated by atomic absorption spectroscopy and inductively coupled plasma-optical emission spectrometry (ICP-OES Optima 7000DV, PerkinElmer). Standard reference material (1575a Pine Needles; NIST, 2004) was also analysed in order to verify the accuracy of the data. Standard solutions of each element were used to generate calibration curves to convert emission readings to concentrations.

Translocation Factors (TFs) were calculated to estimate the ability of plants to transfer elements from roots to leaves (TF_{leaf}) and from roots to stem (TF_{stem}). The Bioaccumulation Factor (BAF) for Cr was estimated as the root-to-soil concentration ratio; for Na it was calculated as the ratio between the concentration in each organ and that of the soil.

2.3. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

RNA extraction and qRT-PCR analyses were conducted as previously described (Ruiz et al., 2017). Sequences of genes belonging to several functional categories were obtained from the NCBI database and from the quinoa RNA-seq database (https://www.ncbi.nlm.nih.gov/bioproject/305752). Except for *CqDHN1* and *CqBADH*, primer sequences were designed from orthologous genes (Table SM2); these were transporter-related genes (*SULTR1;1, SULTR3;4, DMT1, PHT1;1*), phytochelatin genes (*PCS1/CAD1* and *PCS2*), a metallothionein gene *(MET2A)*, as well as several stress-related genes (*DHN2, GSH2, P5CS, PAL1*, and *TAT*). PCRs were carried out with StepOne Plus (RM) 7500 Fast (Applied Biosystems, Monza-Italy) for 30 s at 95 °C and then for 40 cycles as follows: 95 °C for 3 s, 60 °C for 30 s. *Elongation Factor1a* (*CqEF1a*) was used as reference gene to normalize and estimate up- or down-regulation of the target genes for all qRT-PCR analyses. Fold changes in RNA expression were estimated using threshold cycles and analysed by the comparative threshold cycle method, also known as the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data are the means ± standard errors of three biological replicates.

2.4. Statistical analysis

The data relative to element content and biomass were processed by statistical tests using the SigmaPlot 12.0 software package (Systat Software, Inc). Significant differences in biomass production, element soil content, and element concentration in plant organs were tested by the Kruskal-Wallis test. The analyses were followed by the Nemenyi *post-hoc* test ($\alpha = 0.05$). To determine the significance of differences in transcript level data, a one-way analysis of variance (one-way ANOVA) was used, followed by a *post-hoc* F-test with $P < 0.05$ as the significance cut-off.

3. Results

3.1. Plant biomass

Metal and/or salt treatments did not provoke any symptoms of toxicity (Fig. 1) nor a negative impact on growth; in fact, root and leaf DW increased *ca.* four-fold in plants under Cr treatment alone (Fig. 2). On saline soil, leaf DW increased significantly relative to controls both in the presence or absence of Cr(III) (Fig. 2).

3.2. Soil Cr and Na concentrations

At the beginning of the trial, background soil concentrations of Cr were below detection level (data not shown). At the end of the experiment, total concentration of Cr in spiked soils had increased to 0.35–0.4 mg kg^{-1} DW, irrespective of the presence or absence of NaCl; bioavailable Cr contents were, however, extremely low in all analysed soils (data not shown). Soil Na concentration prior to addition of NaCl was ca. 2.0 mg g^{-1} DW; when measured three weeks after the addition of NaCl, soil Na concentration was approximately three-fold relative to controls and reached *ca.* 7.0 mg g^{-1} DW (equivalent to an EC value of *ca.* 10 dS m^{-1}), both in the presence or absence of Cr(III) (data not shown).

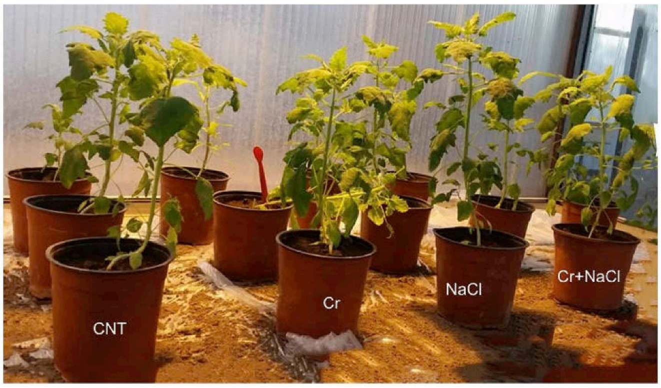


Fig. 1. *Chenopodium quinoa* (cv. Titicaca) plants exposed to Cr, NaCl or Cr + NaCl, and control (CNT) plants.

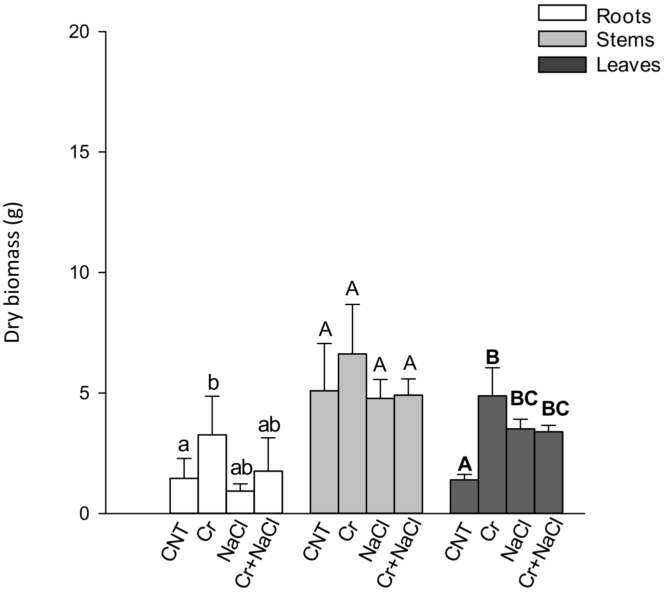


Fig. 2. Dry weight (g) of roots, stems, and leaves of control (CNT) and of plants collected three weeks after addition of Cr, NaCl or Cr + NaCl. Different letters (lowercase, uppercase, bold) indicate significantly different values for treatments with reference to the same organ ($p < 0.005$). Bars indicate standard deviations ($n = 5$).

3.3. Cr and Na accumulation in plants and organ allocation

Cr was detected only in plants grown on Cr(III)-spiked soils. Roots accumulated high amounts of Cr, reaching the highest concentration (*ca.* 2.6 mg g^{-1} DW) on saline soil (Fig. 3A). In leaves, where Cr concentration was very low (*ca.* 0.03 mg g^{-1} DW), the presence of NaCl had no effect; Cr levels were below detection limit or extremely low (Cr + NaCl) in stems (Fig. 3A).

On control and Cr-spiked soils, Na was present in very low amounts in all organs (ranging from 0.13 in roots to 3.83 mg g^{-1} DW in leaves; Fig. 3B). Treatment with Cr(III) significantly reduced root Na levels relative to controls on non-saline soil, but not its translocation to aboveground organs. On saline soils, Na accumulated in all organs, but

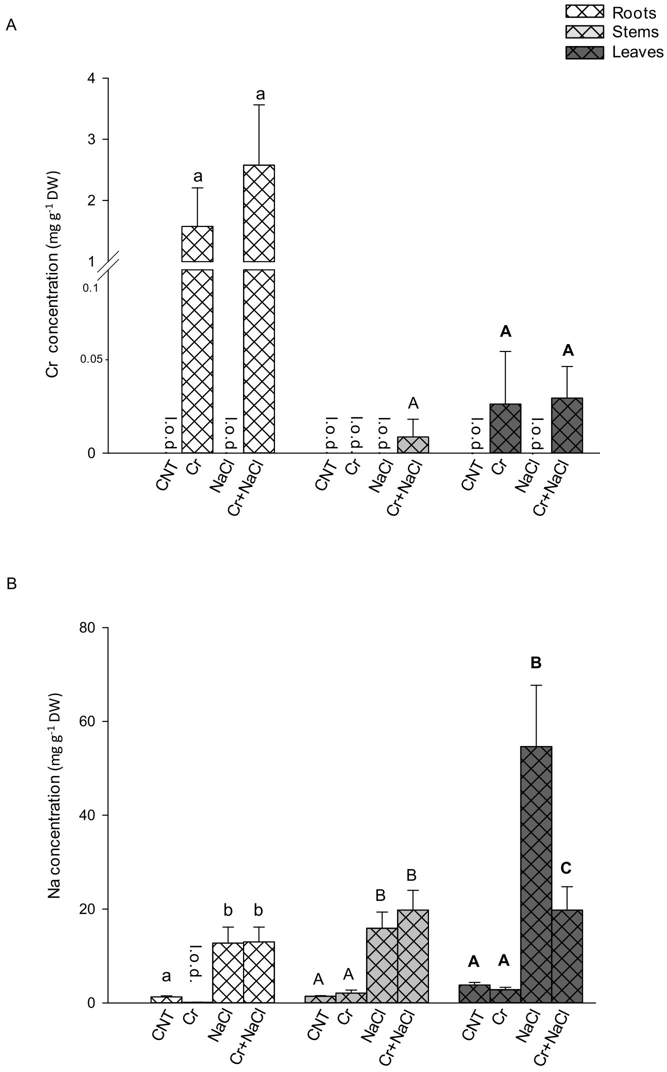


Fig. 3. Cr (A) and Na (B) concentrations in roots, stems, and leaves of plants grown for three weeks under the different treatments. CNT, control. Different letters (lowercase, uppercase, bold) indicate significantly different values for treatments with reference to the same organ ($p < 0.005$). Bars indicate standard deviations ($n = 3$).

mainly in leaves; leaf Na accumulation was significantly reduced in the presence of Cr(III) (Fig. 3B).

TF_{leaf} values for Cr were extremely low (0.017 and 0.011 in Cr and Cr + NaCl treatment respectively), thus confirming the low root-to-shoot translocation of this metal. Root accumulation of Cr relative to soil concentration of the metal (BAF_{root}) was relatively high for plants grown on Cr-spiked soils (4.50) and even higher when combined with NaCl (6.23).

TF values for Na, particularly those of TF_{leaf}, were high in controls (3.01) and especially in NaCl-treated plants (4.52); the TF_{leaf} value for Na decreased significantly in the Cr + NaCl treatment (1.52) as compared with NaCl alone (4.52).

On non-saline soil spiked with Cr(III), the BAF for Na in roots was not calculated because Na concentration was below the limit of detection, while it was close to 1 in the case of stems and leaves. On saline soil, Cr(III) did not dramatically affect Na BAFs in roots and stems (close to 2 in all treatments). BAF values in leaves for CNT and NaCl treatments were 1.54 and 7.46, respectively. Cr reduced the Na BAF in leaves both on non-saline (1.17) and, especially, on saline soil (3.04).

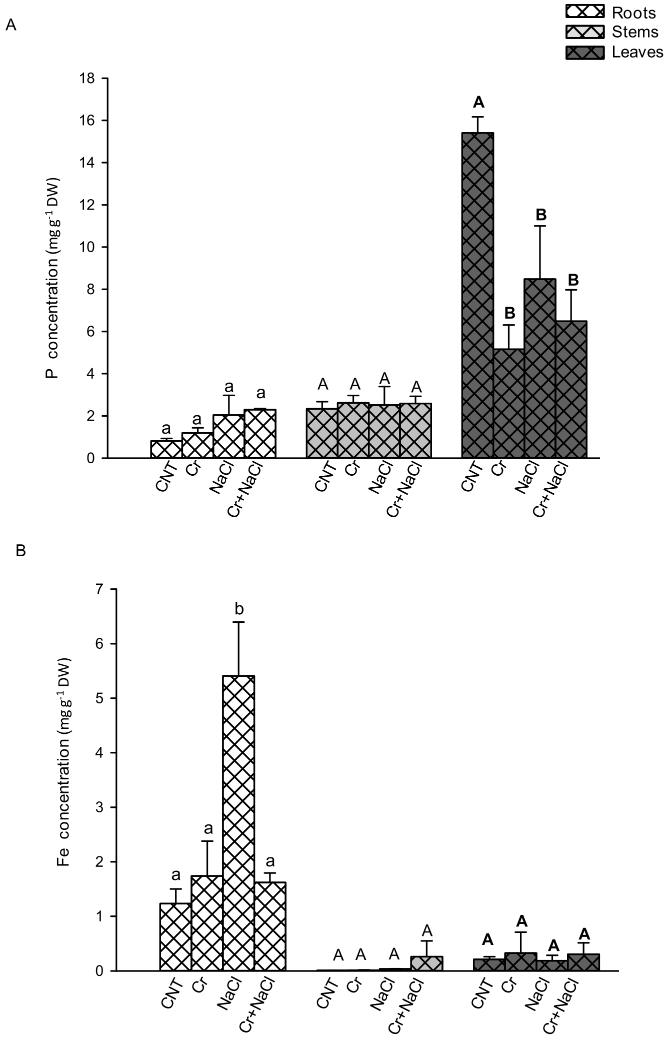


Fig. 4. P (A) and Fe (B) concentrations in roots, stems, and leaves of plants grown for three weeks under the different treatments. CNT, control. Different letters (lowercase, uppercase, bold) indicate significantly different values for treatments with reference to the same organ ($p < 0.005$). Bars indicate standard deviations ($n = 3$).

3.4. Nutrient concentrations

Uptake of P and Fe was differentially and significantly influenced by addition of Cr(III) and/or NaCl. Phosphorous was mainly translocated to the leaves, where its concentration declined in the presence of Cr(III) and/or NaCl, without significant differences between treatments (Fig. 4A). On the contrary, Fe concentration, which was highest in roots, was enhanced when NaCl was supplied alone (Fig. 4B).

3.5. Gene expression

The mean normalized expression of *SULTR1;1* and *SULTR3;4a* was much higher in leaves than in roots (data not shown). In leaves, *SULTR1;1* expression was enhanced several-fold relative to controls in the presence of Cr + NaCl, while it was unaffected by each of the two treatments alone (Fig. 5a).

Expression of *SULTR3;4a* (Fig. 5b) was also slightly (*ca.* 1.8-fold), but significantly, induced when Cr and salt were added together and not modified when they were supplied alone, while *PHT1;1* was strongly down-regulated in leaves by treatment with Cr(III) and/or NaCl (Fig. 5c). In roots, *SULTR1;1* expression was not affected by any of the treatments and *SULTR3;4a* was slightly up-regulated by Cr + NaCl

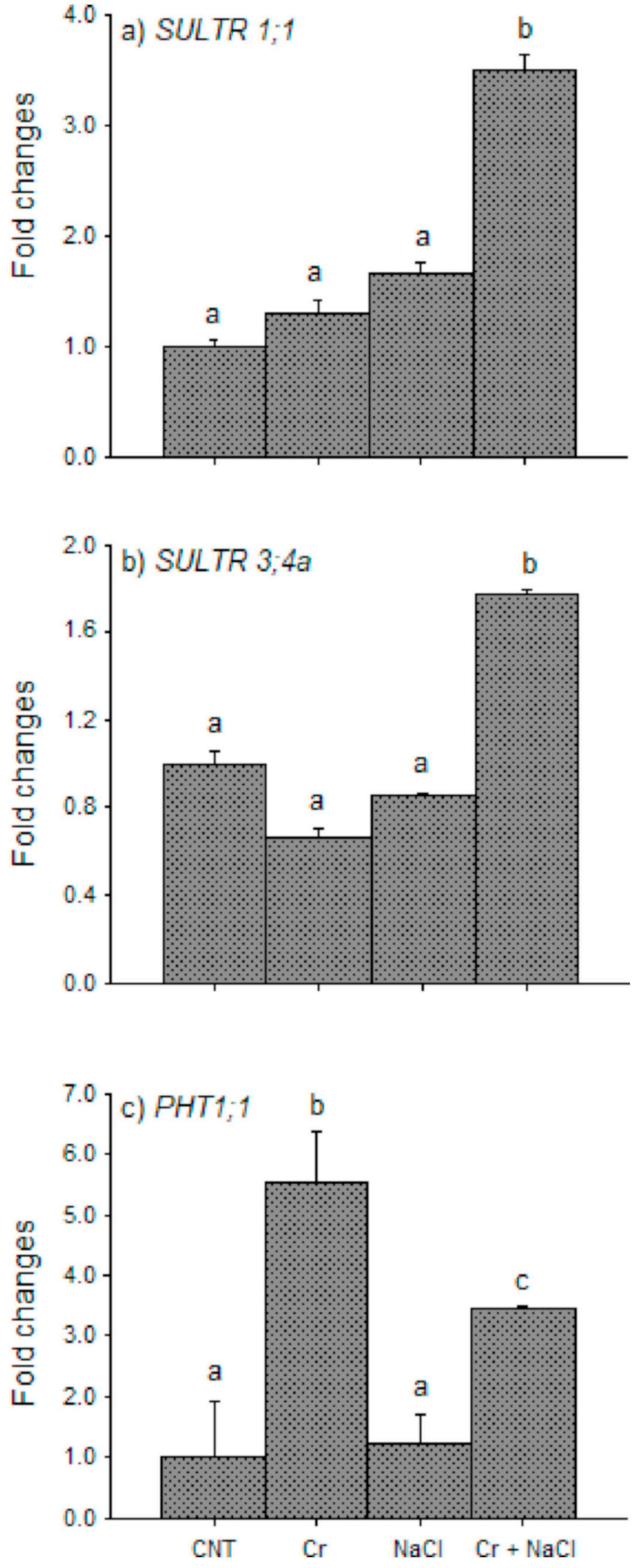


Fig. 5. Fold-changes, relative to controls, in the expression of ion transport-related genes, *SULTR1;1*, *SULTR3;4a*, and *PHT1;1*, in leaves of quinoa plants grown for three weeks in the presence of Cr and/or NaCl. CNT, control. Different letters indicate significantly different values for treatments.

(data not shown).

In leaves, *DHN2* was upregulated *ca.* three-fold relative to controls by Cr + NaCl while *CqDHN1* was slightly down-regulated by NaCl (Fig. 6a and b).

The proline biosynthetic gene, *P5CS*, was slightly (1.5-fold) up-regulated by Cr both under saline and non-saline conditions, but not by NaCl alone (Fig. 6c). Foliar transcript levels of *CqBADH1* also increased (ca. 2.5-fold) under the Cr + NaCl treatment, and, albeit to a lesser extent, with the other two treatments (Fig. 6d). Foliar mRNA levels of *HSP70*, *CAD1*, *MET2A,* and *TAT* increased above controls only in the presence of Cr(III) under saline conditions (Fig. 6e, f, g, i). Conversely, the gene encoding for phenylalanine ammonia lyase1 (*PAL1*), which was much more highly expressed in leaves than in roots (data not shown), was up-regulated *ca.* two-fold in leaves by all three treatments (Fig. 6h).

In roots, *SULTR3;4b* expression was, as expected, much higher than in leaves (data not shown) and the gene was up-regulated by Cr(III) both under non-saline and saline conditions (Fig. 7a). *PHT1;1* expression levels in roots were comparable to those of leaves (data not shown) and the gene was up-regulated by Cr(III) only in the absence of NaCl (Fig. 7b). Instead, *CqDHN1* in roots was up-regulated by Cr(III) only under saline conditions, whereas *TAT* was significantly upregulated by Cr(III) both under saline and non-saline conditions; by contrast, *PAL1* was down-regulated in roots by all three treatments (Fig. 7c). For *DHN2, P5CS*, *CqBADH1*, *CAD1*, and *MET2A*, no relevant effects were registered in roots.

Finally, transcript levels of *DMT1, PCS2*, and *GSH2* genes were similar in roots and leaves, and treatments had no effect (data not shown). An overview of the most relevant changes, relative to controls, in the expression of all analysed genes is shown in Fig. 8.

4. Discussion

4.1. Biomass

Our study revealed that quinoa plants were able to tolerate 500 mg kg^{-1} Cr(III) added to the soil and a root concentration of up to 2.6 mg g^{-1} DW since all plants survived, both under saline and non-saline conditions; no significant reduction in dry biomass was observed and no wilting or chlorosis. Indeed, root and leaf dry biomass increased in the presence of Cr(III), probably due to the fact that the metal was supplied as nitrate salt. Although Cr is generally toxic at concentrations of 0.5–5.0 mg l^{-1} in nutrient solution and 5–100 mg kg^{-1} of available Cr in soil, some studies have evidenced that, at very low concentrations Cr may stimulate plant growth (Ghosh and Singh, 2005; Gomes et al., 2017). In a previous work, we reported that when quinoa plants were exposed to Cr(III) in semi-hydroponic conditions, root and shoot DW was not significantly affected relative to controls even at the highest concentration tested (5 mM), even though fresh biomass was reduced (Scoccianti et al., 2016). In the presence of 150 mM NaCl, both with and without Cr(III), leaf dry biomass was higher than in controls. For quinoa, optimal plant growth, and seed yield have, in fact, been observed under moderately saline (10–20 dS m^{-1}) conditions (Hariadi et al., 2010). Moreover, Bankaji et al. (2016) showed that addition of NaCl improved plant growth of the halophyte *Suaeda fruticosa* treated with 600 μM Zn. They hypothesized that growth stimulation induced by moderate NaCl doses dilutes trace metal content or that NaCl induces stress tolerance mechanisms, mitigating the metal-induced stress.

4.2. Uptake and translocation of Cr and Na

The concentration of $Cr(NO_3)_3{\cdot}9H_2O$ added to the soil was similar to the one measured by us in a previous work aimed at analyzing the phytoextraction potential of plants growing on Cr-polluted soil (Cicatelli et al., 2017). In the present experiment, bioavailable Cr was extremely low in all analysed soils (three weeks after spiking),

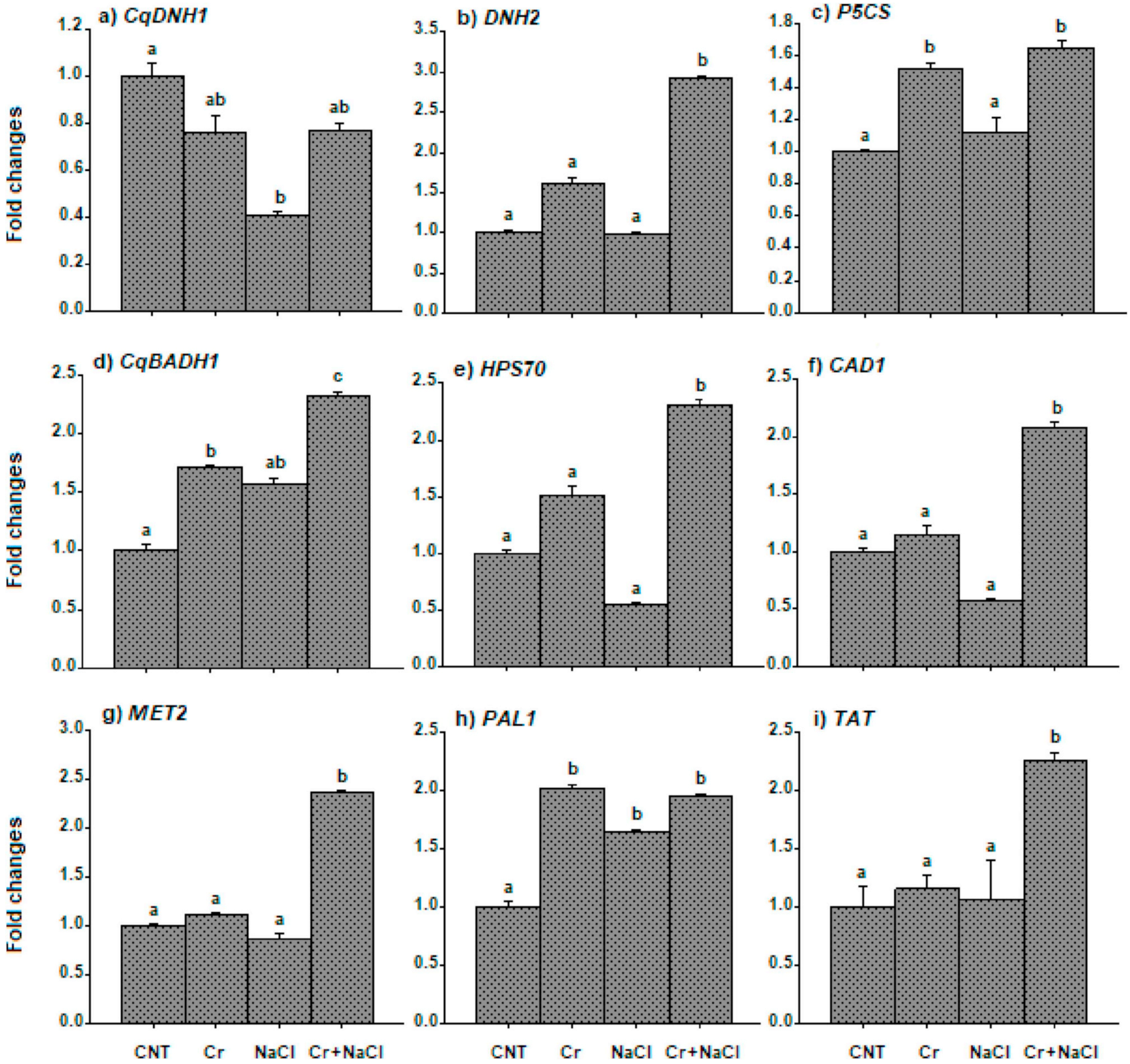


Fig. 6. Fold-changes, relative to controls, in the expression of stress-related genes in leaves of quinoa plants grown for three weeks in the presence of Cr and/or NaCl. CNT, control. Different letters indicate significantly different values for treatments.

indicating that the metal was rapidly bound to the soil matrix. Nonetheless, under our experimental conditions, quinoa reached a relatively high BAF for Cr (4.5–6) and the highest absolute concentration of Cr in plant organs was also relatively high. Most studies with Cr(III) have been performed with nutrient solution or with industrially-contaminated or sewage-irrigated soil, making it difficult to make comparisons with our results. In a similar pot experiment with sunflower grown on soil to which 0.4, 2.0 and 10 mmol $CrCl_3$ [Cr(III)] was added, root Cr concentrations (five weeks after treatment) reached *ca.* 36, 300, and 1000 μg g^{-1} DW (or mg kg^{-1}), respectively; soil Cr concentrations were *ca.* 13, 75, and 230 μg g^{-1} DW (Davies et al., 2001). In our experiment, soil concentrations were much lower (*ca.* 0.4 mg kg^{-1} DW) while the amount accumulated in roots was much higher (2.6 mg g^{-1} DW = 2600 mg kg^{-1}). Thus, quinoa cannot be regarded as a Cr excluder, but, given the low root-to-shoot translocation of the metal, may represent a suitable option for phytostabilisation of contaminants.

Halophytes can accumulate high concentrations of metals with a BAF far exceeding the critical value of 1, but always exhibit a TF value < 1 (Redondo-Gómez et al., 2011). As shown here and in our previous study with hydroponically grown quinoa, TF_{leaf} values for Cr are of the order of 0.01 (Scoccianti et al., 2016). This may represent a tolerance strategy whereby plants accumulate Cr in roots, where the metal is sequestered in the vacuole or bound to root cell walls, so that its translocation to photosynthetic tissues is restricted. It also suggests that Cr is unlikely to reach the edible parts (seeds). Thomas and Lavkulich (2015) investigated the potential human health risks associated with growing quinoa in a HM-contaminated soil. They showed that, given the levels of some HMs accumulated in seeds, these would not be suitable for human consumption. The TF_{leaf} values for Cr obtained in our study were, however, one order of magnitude lower than those reported by them for Zn (0.49–0.84), Pb (0.26–0.45), and Cu (0.18–0.23). Conversely, Haseeb et al. (2018) reported that lead was not translocated to quinoa seeds so that its concentration was within safety limits for human consumption.

Present results also indicate that Cr uptake was higher in quinoa plants grown on saline soil. Salt increases metal mobility due to

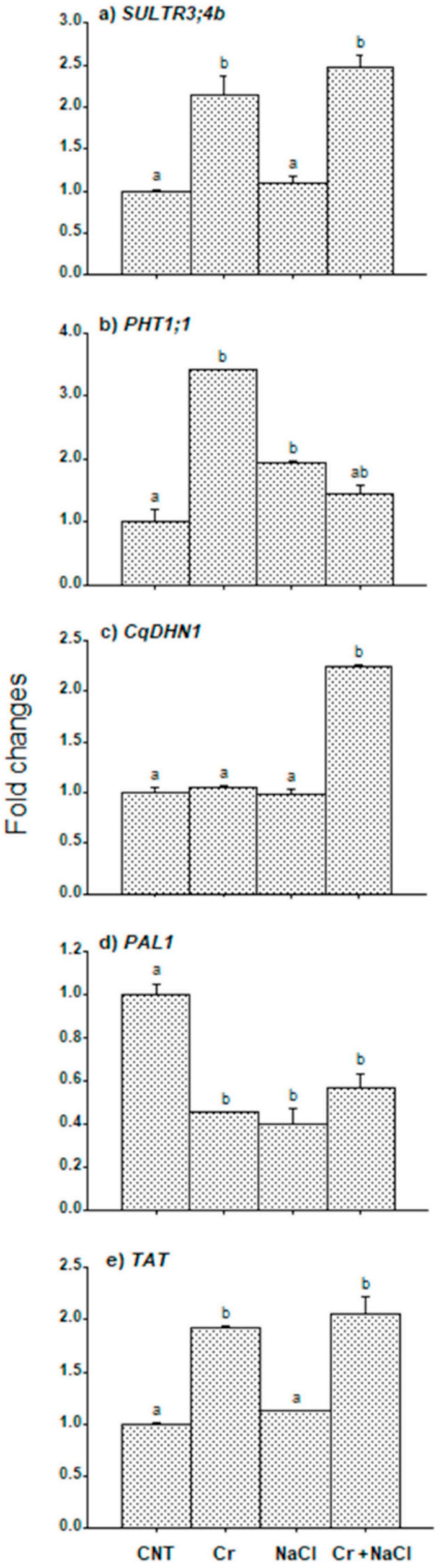


Fig. 7. Fold-changes, relative to controls, in the expression of ion transport- and stress-related genes in roots of quinoa plants grown for three weeks in the presence of Cr and/or NaCl. CNT, control. Different letters indicate significantly different values for treatments.

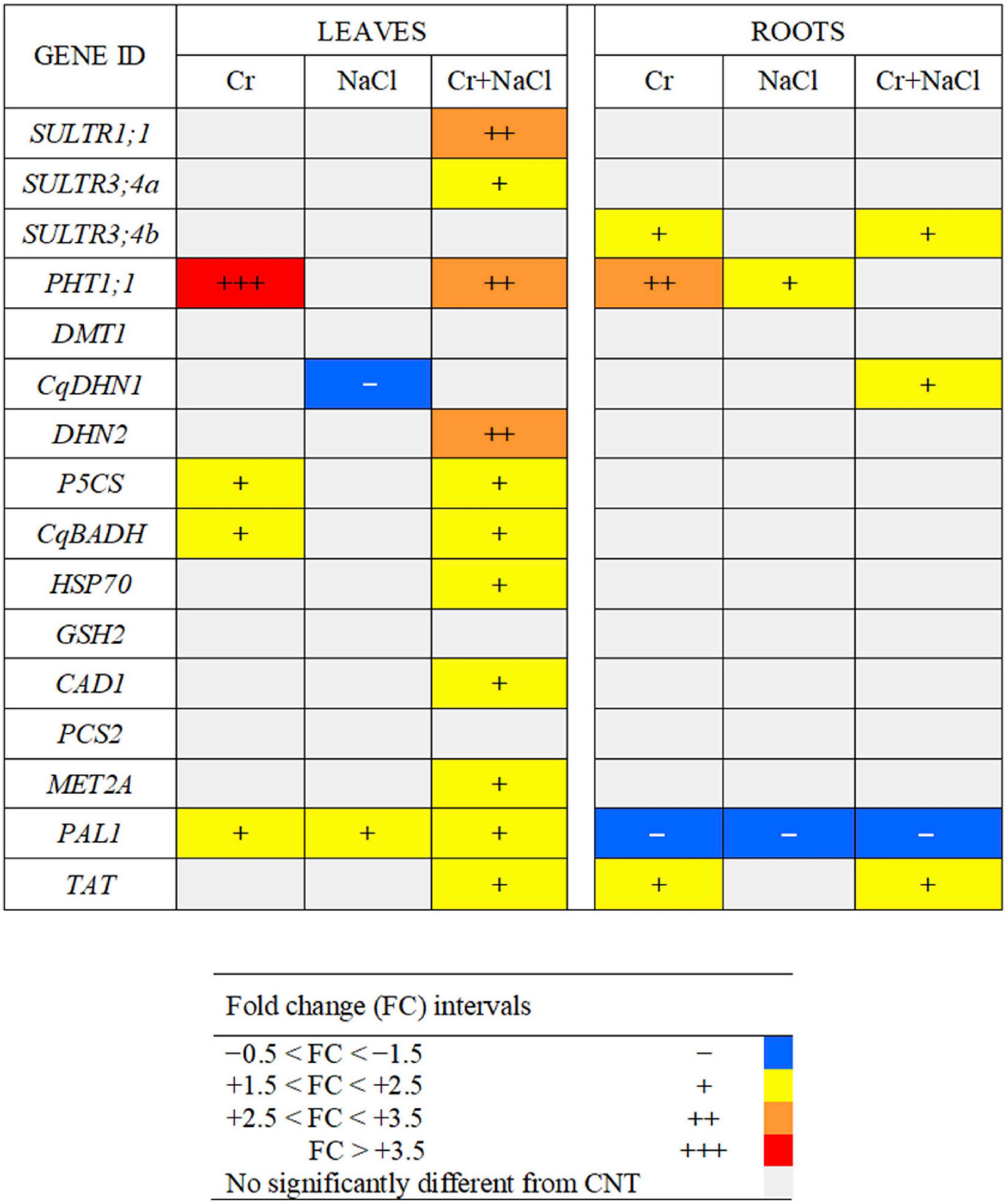
complexation of salt-derived anions with HMs and competition between salt-derived cations with HM ions for sorption sites on solid phases (Lutts and Lefèvre, 2015). Furthermore, the effect of NaCl on metal absorption may be independent of metal bioavailability, *e.g.*, via its effect on ion transporters. In *Kosteletzkya virginica*, a wetland halophyte, Han et al. (2013b) showed that 50 mM NaCl had a positive impact on the plant's response to Zn toxicity by modifying Zn distribution in the plant (*i.e.*, accumulation in older leaves), rather than reducing its uptake by roots. Similarly, in *Atriplex halimus* grown with either Cu or Cd in the presence of NaCl, a drastic reduction in metal uptake, improved plant growthas well as decreased oxidative damage was observed as compared with plants grown in the absence of salt Bankaji et al. (2016). This could explain why, in spite of higher root Cr concentrations, growth of quinoa plants exposed to Cr(III) under saline conditions was not negatively affected. As discussed below, the activation of stress-protective genes in leaves of quinoa plants exposed to the Cr + NaCl treatment could also account for this lack of growth inhibition.

In our study, Na was prevalently accumulated in leaves; this is a common feature of halophytes, which use inorganic ions, in addition to organic osmolytes, for osmotic adjustment (Hariadi et al., 2010). Interestingly, in the presence of Cr(III), Na accumulation in quinoa plants grown on saline soil was lower. In *Suaeda fruticosa*, Bankaji et al. (2016) reported that the combination Zn + NaCl in the medium increased plant accumulation of Na, whereas the combination Pb + NaCl decreased Na concentration in shoots. Thus, the effect is metal-specific; it is noteworthy that, like Cr, Pb is also retained in the roots. Other studies in salt-affected soils revealed an interaction between Ni uptake and other elements, resulting, for example, in enhanced K uptake and reduced Na uptake (Ain et al., 2016). The reduced availability of Na^+ as osmoticum may account for the enhanced expression of the proline and glycine betaine biosynthetic genes presently observed in leaves of Cr + NaCl-treated quinoa plants.

4.3. *Effects of Cr(III) on nutrient levels*

It is known that HMs can interact in various ways, synergistically or antagonistically, with macro- and micro-element uptake in plants (Kalavrouziotis and Koukoulakis, 2012). Due to its structural similarity with essential ions, Cr can compete with nutrient transporters, in particular those for phosphate and sulfate (Singh et al., 2013; Gomes et al., 2017). Present results indicate, however, that root and stem concentrations of P were not affected by Cr(III), but that translocation of this nutrient to the leaves was hampered. As discussed below, transcript levels of the phosphate transporter *PHT1;1* were up-regulated by Cr(III) in roots, but strongly down-regulated in leaves, suggesting that, in quinoa, this transporter is involved in translocation/allocation, rather than root uptake. Foliar levels of P also decreased in the presence of NaCl alone. Indeed, reduced P uptake/transport is a common response of plants to salinity. The interaction between salinity and P nutrition of halophytes is, however, very complex, since it depends on the plant species, developmental stage, level of salinity, and concentration of P in the substrate (Zribi et al., 2012). Maintenance and remobilization of internal P among organs or cellular compartments occurs via changes in metabolic pathways and, presumably, the activity of transporters. In fact, as with Cr(III), *PHT1;1* transcript abundance was strongly reduced by growing quinoa plants in the presence of NaCl.

Although Cr did not significantly affect the accumulation of Fe, NaCl alone enhanced root Fe concentration. In alkaline soils, Fe occurs mainly in the form of insoluble hydroxides and oxides that limit its bioavailability. *Puccinellia tenuiflora* and *Poa annua* differ in their tolerance to saline-alkaline stress. An RNA-sequencing analysis revealed that homologs of the genes involved in Fe acquisition were up-regulated in the former and down-regulated in the latter when exposed to $NaHCO_3$, suggesting that efficient Fe acquisition is an important trait in conferring tolerance to saline-alkaline stress (Kobayashi et al., 2015). Similarly, Li et al. (2016) reported that on saline-alkaline medium,



GENE ID	LEAVES				ROOTS		
	Cr	NaCl	Cr+NaCl		Cr	NaCl	Cr+NaCl
SULTR1;1			++				
SULTR3;4a			+				
SULTR3;4b					+		+
PHT1;1	+++		++		++	+	
DMT1							
CqDHN1		−					+
DHN2			++				
P5CS	+		+				
CqBADH	+		+				
HSP70			+				
GSH2							
CAD1			+				
PCS2							
MET2A			+				
PAL1	+	+	+		−	−	−
TAT			+		+		+

Fold change (FC) intervals		
−0.5 < FC < −1.5	−	🟦
+1.5 < FC < +2.5	+	🟨
+2.5 < FC < +3.5	++	🟧
FC > +3.5	+++	🟥
No significantly different from CNT		⬜

Fig. 8. Schematic representation of the most relevant changes, relative to controls, in the expression of all analysed genes in roots and leaves of quinoa plants three weeks after addition of Cr, NaCl or Cr + NaCl.

shoot Fe concentrations declined in a less salt-tolerant rice genotype while they remained stable in a more tolerant one. We were, however, unable to find a correlation between *DMT1* transcript levels and enhanced Fe acquisition/translocation in quinoa upon NaCl treatment. This lack of transcript level regulation may depend upon the fact that many transporters are activated at a posttranslational level.

4.4. Gene expression

Halophytes have been used as model plants to study the combined effect of moderate (non-stressful) salinity and potentially harmful doses of HMs (*e.g.*, Manousaki and Kalogerakis, 2009; Lefèvre et al., 2009; Han et al., 2013b; Bankaji et al., 2016; Nikalje and Suprasanna, 2018). These studies show that soil salinity not only reduces metal absorption and increases plant growth, but also improves tolerance to accumulated metals. Several factors have been shown to be involved in this enhanced tolerance, including synthesis of osmo/stress-protective compounds (Lefèvre et al., 2009), phytohormones, and a more efficient antioxidant response (Han et al., 2013a; Vromman et al., 2016; Zhou et al., 2018). Indeed, as pointed out by Lutts and Lefèvre (2015), the biochemical basis of HM tolerance in halophytes, as in other plants, essentially relies on (i) protection from oxidative stress, (ii) PCs and MTs, and (iii) osmoprotectants.

In Cr(III)-treated plants, moderate salinity activated the transcription of genes involved in ion transport and in the response to HMs and to abiotic stresses in general. Notably, treatments affected the expression of genes more often at the foliar level than in roots. Global expression profiling or expression analyses for various plant species indicate that leaves and roots differ considerably in their response to abiotic stresses. For example, a comparison of a salt-tolerant olive cultivar with a salt-sensitive one under NaCl treatment revealed 70 differentially expressed clusters in leaves compared to 24 in roots

(Bazakos et al., 2015). Similarly, in salt-treated rice, more genes were variably expressed in leaves than in roots (3687 and 695, respectively; Razzaque et al., 2019). These results suggest a greater responsiveness/plasticity in leaves, where metabolic activities that are crucial for plant growth and productivity, namely photosynthesis, take place.

In the present study, genes whose expression was modulated by treatment with Cr(III) fell into two categories: (1) genes whose up-regulation either in roots or leaves, or both, was induced under both saline and non-saline conditions, usually more strongly in the former than in the latter (*SULTR3;4b*, *PHT1;1*, *P5CS*, Cq*BADH1, TAT*, *PAL1*); (2) genes whose expression was up-regulated only under saline conditions (*SULTR1;1*, *SULTR3;4a, CqDHN1*, *DHN2*, *HSP70*, *CAD1*, *MET2A*). Interestingly, *PHT1;1* was the only gene whose expression was most strongly up-regulated by Cr(III) in the absence of salt, while in a few cases genes were also affected (up- or down-regulated) by NaCl alone (*PHT1;1, CqDHN1,* and *PAL1*).

Sulfur (S) is a key component in helping plants to cope with abiotic stresses, such as drought and salinity. Sulfur is needed for the synthesis of glutathione, which maintains the cellular redox balance and mitigates oxidative damage. In Arabidopsis, high-affinity sulfate transporters belonging to Group 1 are involved in sulfate uptake or distribution to sink organs (Gallardo et al., 2014 and references therein). Group 3 is the largest group, with multiple roles, such as facilitating sulfate transport to aerial parts or controlling cysteine levels that serves as a sulfur donour in ABA biosynthesis (Cao et al., 2014). Recently, Schmöckel et al. (2017) identified candidate salt tolerance genes in the quinoa genome and reported the up-regulation of *SULTR1;1*, and *SULTR3;4* in the presence of 300 mM NaCl. They also observed a differential response in the two organs, with *SULTR3;4b* up-regulated in roots and *SULTR3;4a* and *SULTR1;1* up-regulated in leaves. Our results confirm the fact that *SULTR3;4b* is root-specific and that 150 mM NaCl, unlike 300 mM NaCl, does not cause salt stress in quinoa since these genes were not significantly affected by the salt treatment. In the presence of HMs, there is an increased demand for reduced S-containing compounds, such as PCs. Thus, transcription of genes involved in uptake and reduction of sulfate are up-regulated under these conditions (Leustek et al., 2000; Lindblom et al., 2006). Present results indicate that Cr(III) caused a significant up-regulation of *SULTR1;1* and slightly induced *SULTR3;4a* in leaves, but only under saline conditions, possibly due to the relationship between sulfate and ABA and the above-mentioned need for S compounds under stress conditions. Cr + NaCl was, in fact, the treatment resulting in the highest accumulation of Cr in the roots. In roots, *SULTR3;4b* was also Cr-induced.

Results showed that P was mainly translocated to the leaves, where its concentration declined significantly in the presence of Cr(III) and/or NaCl, without significant differences between treatments. This is in accord with the foliar transcript levels of *PHT1;1*, which declined strongly under all treatments, indicating that this gene is involved in P translocation rather than uptake. Dehydrins (DHNs), or group 2 Late Embryogenesis Abundant (LEA) proteins, play a fundamental role in plant responses and adaptation to abiotic stresses. Though expressed at high levels to protect the embryo from dehydration in the maturing seed, they are induced in vegetative tissues upon exposure to stress factors, such as salinity, drought, and freezing. Dehydrin gene transcript levels can be induced by HMs and some dehydrins possess the ability to bind metals. Xu et al. (2008) reported that DHN genes from the hyperaccumulator *Brassica juncea* (*BjDHN2/BjDHN3*) exerted a protective role in transgenic tobacco plants exposed to Cd or Zn. Present results confirm that 150 mM NaCl does not represent a salt stress condition for quinoa, but that Cr, both under non-saline and saline conditions, induces HM stress-responsive reactions, such as up-regulation of *DHN* genes.

Heat-shock proteins (HSPs) are ubiquitous stress-related proteins that are induced by almost all types of biotic and abiotic stresses. The 70-kDa HSPs are a group of conserved chaperone proteins that have housekeeping functions in protein folding, and quality control. Recently, significant variation in expression of *CqHsp70*s in response to drought stress was reported in quinoa (Liu et al., 2018). The *Hsp70* gene analysed in the present work was not induced by the non-stressful NaCl treatment; instead, its expression was enhanced by the combined Cr + NaCl treatment.

Proline has been shown to accumulate under drought and salinity, but also in response to HM stress (Dar et al., 2016). In an earlier paper (Scoccianti et al., 2016), we showed that Cr(III) caused a significant increase in foliar levels of proline in hydroponically grown quinoa. Salinity (300 mM NaCl) also induced proline accumulation (Ruiz-Carrasco et al., 2011) and increased *P5CS* transcript levels in quinoa (Ruiz et al., 2017). In the present work, the relatively low salt concentration used did not induce *P5CS* expression in response to NaCl alone (nor was proline accumulated, data not shown). As with other stress-responsive genes, however, *P5CS* was up-regulated by the combined Cr + NaCl treatment.

Betaine aldehyde dehydrogenase (BADH) is a key enzyme in glycine betaine (GB) biosynthesis in higher plants. Paralogous genes encoding several BADH isozymes have been identified in several species, including quinoa. Expression profiling in quinoa seedlings exposed to 100 or 250 mM NaCl revealed that salt led to increased levels of *CqBADH1* mRNA accompanied by the accumulation of GB (Jiang et al., 2016). GB, like proline, is regarded as an osmolyte, but evidence points to the importance of GB in improving the ability of plants to adapt to abiotic stresses in general, partly through ROS detoxification (Giri, 2011). Indeed, several studies have shown that overexpression of *BADH* genes in transgenic plants improved tolerance to salt, low temperature, and oxidative stresses (Jiang et al., 2016 and references therein). The increased *CqBADH1* transcript abundance observed here in the presence of Cr suggests that GB may also play a protective role under HM stress, as previously reported in ryegrass and wheat (Ali et al., 2015; Lou et al., 2015).

Phenylalanine ammonia lyase (PAL) is the first committed enzyme in the pathway that diverts the flux of carbon from primary metabolism to the synthesis of phenolics. The role of phenolic compounds in protection from stress is due to their capacity to scavenge free radicals produced in stress-induced oxidative reactions. Halophytes accumulate phenolic compounds in response to salinity (*e.g.* Ksouri et al., 2007) and a more effective use of polyphenols and higher antioxidant activities in halophytes as compared with glycophytes has been suggested (Bose et al., 2014). In a comparative study on salt responses in different Chilean landraces of quinoa, the one most adapted to saline conditions (*salares* ecotype) was the only landrace to exhibit significantly enhanced foliar total polyphenol content and unaltered flavonoid concentrations as opposed to the other landraces, where both classes of compounds decreased in the presence of salt (Ruiz et al., 2016a). Ismail et al. (2016) reported that rutin levels increased by over 25-fold in quinoa leaves in response to salinity. In the present study, mRNA levels of *PAL1* increased to a similar extent in leaves of both NaCl- and/or Cr (III)-treated plants of quinoa, suggesting that this enzyme may contribute to the plant's tolerance to these compounds. The *PAL1* gene may be one of the factors conferring a “stressed-like” expression profile to quinoa plants under non-stressful conditions (*i.e.*, 150 mM NaCl), preparing them to react upon exposure to more adverse conditions (see Concluding Remarks).

Tocopherols and tocotrienols (vitamin E) are likewise part of the antioxidant machinery of plants. Hence, they play a protective role during abiotic/oxidative stresses (Sadiq et al., 2019). In an earlier work with quinoa, we showed that at low concentrations of Cr(III) no changes were observed in any of the growth and physiological parameters analysed, except for TAT activity, which was induced in a time- and concentration-dependent manner (Scoccianti et al., 2016). Present results confirm the fact that TAT is Cr-inducible, also at the transcriptional level. Although in leaves it was up-regulated only under saline conditions, in roots both Cr and Cr + NaCl enhanced *TAT* transcript levels, suggesting that this enzyme could be a useful marker of imposed stress.

Tolerance to HM stress in plants involves, among other mechanisms, metal-binding ligands, such as PCs (Liu et al., 2015) and MTs (Sharma et al., 2016). The enzyme catalyzing the biosynthesis of PCs from GSH, PC synthase, has been reported in a wide variety of plant species. In Arabidopsis, the *CAD1* gene encodes PC synthase (Ha et al., 1999). Type 1 MT genes are expressed more highly in roots than leaves, whereas, as presently observed, the reverse is true for type 2 MT genes. The effect of metals on the expression level of MT genes has been studied in different plant species, but no general conclusion is available. Differential regulation of MT genes by different metal ions (Huang and Wang, 2010 and references therein) as well as salt-induced expression of MT genes have been reported (Landouar-Arsivaud et al., 2011). There are a few studies on MTs in halophytes under HM stress, but none, to our knowledge, on HMs under saline conditions. Chaturvedi et al. (2012) reported that *SbMT-2*, a full-length type 2 MT from *Salicornia brachiata*, was up-regulated by Zn, Cu, salt, heat, and drought stress. *CAD1* and *MET2* are amongst those genes whose foliar transcript levels were enhanced, suggesting a role for them in abiotic stress tolerance also in quinoa. In fact, although little is known about quinoa's response or tolerance to HMs, it certainly displays an innate resilience to a variety of abiotic stresses; therefore, the *CAD1* and *MET2* genes analysed in this work may be potential components of its tolerance to HMs, especially under moderately saline conditions. A combination of stresses, commonly found in nature, does not necessarily exert a negative effect on plant metabolism and, in some cases, might alleviate the effects of individual stressors. The mechanisms underlying this co-tolerance include the activation of the antioxidant machinery and rearrangements in mineral status of specific organs. Another mechanism may involve the stress-related phytohormone ABA. Exogenous ABA has been reported to alleviate HM stress. A recent study by Song et al. (2019) indicates that ABA application in grapevine decreased the phytotoxic effect of Zn by inducing the expression of Zn transport- and detoxification-related genes. Salinity induces the ABA pathway in quinoa (Ruiz et al., 2017), consequently HM stress mitigation under saline conditions may derive from this factor.

5. Concluding remarks

Overall, our data confirm the notion that regulation of gene expression plays a pivotal role in the ability of quinoa to respond to environmental stressors, including HMs. It is noteworthy that, in many cases, treatments affected the expression of genes at the foliar level more than in roots, suggesting a greater responsiveness/plasticity in leaves, where metabolic activities that are crucial for plant growth and productivity, namely photosynthesis, take place. Present results also indicate that moderate soil salinity did not significantly affect the amount of Cr accumulated in the plants. Nonetheless, the combined Cr + NaCl treatment sometimes caused a transcriptional response that was absent when plants were exposed to either Cr or NaCl alone. It may, therefore, be assumed that, in response to a moderate salinity level, the plant activated tolerance mechanisms that amplified the response induced by Cr, which was not detectable when the metal was supplied alone. Moreover, it is now generally believed that constitutive expression of salt stress-responsive genes, which are salt-inducible in glycophytes, is a salt tolerance trait in halophytes. In a stress-tolerant plant such as quinoa, a "stressed-like" expression profile (Janiak et al., 2018) may be present even when it is exposed to optimal conditions or to non-stressful levels of salinity (150 mM NaCl). An evident surge in expression levels would occur only when plants are exposed to more seriously adverse environmental conditions (Cr + NaCl). Thus, one may suppose that, under saline conditions, quinoa would be better equipped to tolerate even higher, toxic concentrations of Cr.

CRediT authorship contribution statement

Francesco Guarino: Data curation, Investigation, Writing - original draft. **Karina B. Ruiz:** Data curation, Funding acquisition, Investigation, Writing - original draft. **Stefano Castiglione:** Conceptualization, Data curation, Supervision, Writing - original draft, Writing - review & editing. **Angela Cicatelli:** Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Writing - original draft, Writing - review & editing. **Stefania Biondi:** Conceptualization, Data curation, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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