Plant Physiology and Biochemistry 143 (2019) 286–298

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Seed priming with H_2S and Ca^{2+} trigger signal memory that induces cross-adaptation against nickel stress in zucchini seedlings

Maryam Valivand[a,*], Rayhaneh Amooaghaie[a,b], Alimohammad Ahadi[c]

[a] Plant Science Department, Science Faculty, Shahrekord University, Shahrekord, Iran
[b] Biotechnology Research Institute, Shahrekord University, Shahrekord, Iran
[c] Genetic Department, Science Faculty, Shahrekord University, Shahrekord, Iran

ARTICLE INFO

Keywords:
Ascorbate
Cross-adaptation
Glutathione
Ni toxicity
Redox homeostasis
Seed priming `

ABSTRACT

In this study, the effect of seed priming with sodium hydro sulfide (NaHS) and $CaCl_2$ as well as the possible relationship between them in inducing post-germinative cross-adaptation in zucchini seedlings (cv Courgette d'Italie) were investigated. Results showed that Ni toxicity reduced plant growth and photosynthetic pigments, decreased the content of ascorbate (AsA) and total thiols, increased hydrogen peroxide (H_2O_2) content and electrolyte leakage (EL), up-regulated the transcription levels of Ca^{2+}-dependent protein kinase (*CDPK*) and phytochelatin (*PCs*) genes and elevated H_2S content in leaves of zucchini seedlings. Individual or combined seed priming with Ca^{2+} and NaHS improved the content of photosynthetic pigments and seedling biomass, reduced H_2O_2 content and EL, increased the content of AsA and total thiols, decreased ascorbate peroxidase activity and enhanced glutathione reductase activity in leaves. These findings suggest the last time effect of seed priming with Ca^{2+} and NaHS on inducing cross-adaptation in seedlings under Ni stress. H_2S accumulation and other responses induced with Ca^{2+} in leaves were weakened with hypotaurine (HT as H_2S scavenger), denoting seed priming with Ca^{2+} established cross-adaptation in a H_2S-dependent manner. Seed priming with NaHS amplified *CDPK* transcripts in leaves of seedlings and seed priming with ethylene glycol tetraacetic acid (as Ca^{2+} chelator), lanthanum chloride and verapamil (as plasma membrane channel blockers) reduced transcript levels of *CDPK* and *PCs* genes and reversed impacts of seed priming with NaHS. These results indicated that the cross-adaptation induced with NaHS is mediated through Ca^{2+} signaling. Overall our findings suggest that two-side cross-talk between Ca^{2+} and H_2S is involved in the acquisition of a signal memory in seed embryo cells which can be employed upon a later Ni-exposure and more strongly enhance AsA-GSH cycle, redox homeostasis and phytochelatin transcripts in leaves of zucchini seedlings grown from primed seeds.

1. Introduction

Nickel as an essential microelement is needed for normal plant growth and development and minor amounts of Ni (0.01–5 μg/g dry weight) are found in plants; but in most of the plant species, Ni beyond the critical limit is toxic and acts as a heavy metal (Yusulf et al., 2011). In Iran, agricultural soils nearby to the industrial establishments have been contaminated with Ni. A report by Mohammadpour et al. (2016) indicated that the mean concentration of Ni is up 60 $mg\,kg^{-1}$ in the soil of some zones that is higher than the background value of Ni concentration, that is, 35.2 $mg\,kg^{-1}$ (Rizwan et al., 2017).

Previous studies exhibited that Ni as a heavy metal reduces seed germination and plant yield (Aziz et al., 2015; Asrar et al., 2014; Siddiqui et al., 2011). The toxic level of Ni evokes reactive oxygen species (ROS) generation and oxidative stress and causes symptoms such as retardation of growth, leaf chlorosis and necrosis in plants (Rizwan et al., 2017; Aziz et al., 2015; Asrar et al., 2014; Siddiqui et al., 2011). Moreover, a high dose of Ni disrupts photosynthesis and prevents the activity of many enzymes and metabolic process in plants including enzymes involved in N assimilation (Yusulf et al., 2011; Rizwan et al., 2019).

To protect themselves against heavy metals such as Ni, plants employ many defense responses including: activation of ascorbate-glutathione (AsA-GSH) cycle, modulating the content of AsA and GSH that have central roles in the stability of the redox homeostasis (Asrar et al., 2014; Siddiqui et al., 2011) and generation of metal chelators such as metallothioneins and phytochelatins that are closely linked with the improvement of metal tolerance in plants (Singh et al., 2016).

It has been demonstrated that, when plants experience moderate stress, they become more tolerant to higher levels of the same stress via

---

* Corresponding author.
E-mail address: Valivand84@yahoo.com (M. Valivand).

https://doi.org/10.1016/j.plaphy.2019.09.016
Received 3 June 2019; Received in revised form 11 September 2019; Accepted 11 September 2019

retaining a stress memory, a response referred to as priming or cross-adaptation (Crisp et al., 2016). It is known that a complex signaling system containing many signal mediators such as nitric oxide (NO), calcium ions (Ca^{2+}), hydrogen peroxide (H_2O_2) and hydrogen sulfide (H_2S) as well as their crosstalk is involved in triggering cross-adaptation (Khodabakhsh et al., 2014; Amooaghaie et al., 2017; Amooaghaie and Nikzad, 2013; Amooaghaie and Tabatabaie, 2017; Amooaghaie et al., 2015; Li et al., 2016; Li et al., 2012; Savvides et al., 2016). Therefore, Ca^{2+}, H_2O_2, NO and H_2S or their donors can be considered as promising signal molecules that priming of the plant with them initiates a stress memory. This signal imprint enables the plant to produce stronger and faster responses (e.g. enhancing antioxidant activities, nutrition balance, osmolyte accumulation and redox state) in cells under subsequent exposure to abiotic stresses.

Hydrogen sulfide (H_2S) is known as an endogenous gaseous transmitter in plants which is involved in modulating developmental processes such as seed germination, root organogenesis and stomata movement (Li et al., 2012; Guo et al., 2016). Moreover, there is increasing evidence showing that the role of H_2S in triggering anti-oxidative responses against various abiotic stresses in plants (Guo et al., 2016). For instance, it has been reported that pretreatment of seedlings or mature plant with NaHS, as a H_2S donor, can increase plant tolerance upon subsequent exposure with Pb (Amooaghaie et al., 2017), Al (Zhu et al., 2018) and Ni (Rizwan et al., 2019). Singh et al. (2015) also reported that pre-exposure of pea seedlings with NaHS induces cross-adaptation to arsenic stress via decreasing the arsenic content and increasing the activity of AsA-GSH cycle in tissues. Moreover, pretreatment with NaHS attenuated aluminium-induced growth retardation and the dysfunction of organelles in leaf mesophyll and root tips cells and enhanced antioxidant enzyme activities (APX, CAT, APX, SOD, POD, and GR) in oilseed rape (Qian et al., 2013).

Numerous studies have also shown that pre-treatment of seedlings or mature plant with Ca^{2+} enhance plant tolerance against heavy metals such as Cu and Cd (Ahmad et al., 2015; Gonzalez et al., 2012). Min et al. (2013) reported pre-subjection with Ca^{2+} enhanced Cu tolerance by increasing the non-protein thiols, reduced glutathione and total antioxidant capacity in *Elodea canadensis* plants. Aziz et al. (2015) stated that application of Ca^{2+}reduced the adverse effects of Ni stress on chlorophyll content, the photosynthetic rate, shoot and root dry weights, transpiration rate and stomatal conductance in rice plants. It is well known that different environmental stimuli cause a transient Ca^{2+} burst in the cytosol, called “calcium signature”. Calcium signature is recognized by calcium sensors such as Ca^{2+}-dependent protein kinases (CDPKs), calmodulin (CaM), and calcineurin B-like (CBL) proteins and is translated to downstream defense responses (Wilkins et al., 2016; Zeng et al., 2015a). For example, it has been reported that the expression of *CDPK* genes was induced by Cr (Huang et al., 2014) and arsenic (Huang et al., 2012) in the rice seedlings. Vivek et al. (2013) revealed that over-expression of ginger *CDPK1* gene in tobacco conferred tolerance to salinity and drought stress as reflected by the high percentage of seed germination, higher relative water content (RWC), expression of stress-responsive genes, higher leaf chlorophyll content, increased photosynthetic efficiency and other photosynthetic parameters. Based on the dynamic changes in Ca^{2+} concentration, the specific CDPK isoforms are activated, which the extent and duration of their activity are varied and are differentially expressed inside the plant (Shi et al., 2018) and consequently regulate downstream responses through phosphorylation of certain substrates (Shi et al., 2018; Asano et al., 2012).

Although many studies have assessed the positive effect of pre-treatment of seedlings or mature plants with H_2S and Ca^{2+} on enhancing plant tolerance, few studies have employed H_2S and Ca^{2+} as agents for seed priming. Sun and Luo (2013) revealed that treatment of pumpkin seeds with exogenous NaHS significantly resulted in the alleviation of adverse effects salinity on germination rate, hypocotyl and radicle lengths, and soluble sugar concentrations, and enhanced amylase, SOD and POD activities. Zanganeh et al. (2018) found that seed priming with H_2S reduced proline, arginine and methionine accumulation and increased nitric oxide and glycine betaine contents under lead stress. Kaczmarek et al. (2017) reported that seed priming with $CaCl_2$ improved RWC, gas exchange parameters, and photosynthetic pigments concentration in stress-tolerant as well as drought-susceptible barley cultivars under drought stress. Talukdar (2012) also demonstrated that seed priming with calcium enables *Lens culinaris* seedlings to endure the adverse impacts of Cd treatment by modulating the antioxidant enzyme activity and reducing the Cd accumulation. In spite of the fact that seed priming is a simpler, cheaper and more practical method than priming of seedlings or mature plants, little attention has been paid yet to the application of signal molecules in seed priming.

In more studies, seed priming with various compounds such as water, hormones, osmolytes, and inorganic salts has been done and the beneficial effects of it on seed germination and plant growth has been documented not only in normal condition but also under abiotic stresses (Khodabakhsh et al., 2014; Amooaghaie and Nikzad, 2013; Amooaghaie and Tabatabaie, 2017; Alcantara et al., 2015; Batista et al., 2016). The formation of a ‘molecular stress memory’ in cells for enhancing acclimation responses and the acquisition of cross-adaptation is suggested as the superficial mechanism of seed priming (Abid et al., 2018; Chen and Arora, 2013). The mechanism of action of seed priming for the acquisition of stress memory has remained unclear. However, previous studies due to tomato (Amooaghaie and Nikzad, 2013) and wheat (Amooaghaie and Tabatabaie, 2017) seed priming, demonstrated the role of NO and H_2O_2 in the effects of seed priming on tolerance of cold and salinity stress respectively. Therefore it is possible that Ca^{2+} and H_2S also support the establishment of stress memory during seed priming in plants.

Zucchini (*Cucurbita pepo* L.) is one of the most economical members of the Cucurbitaceae family and is an important vegetable food source for many of the world's population (Knapp and Osborne, 2017). However, heavy metals such as Ni may restrict the production of this crop. Thus, finding efficient approaches are important for increasing heavy metal tolerance in this plant. To date, a few literatures are available on the harmful effects of Ni in zucchini. Furthermore, there is no report about the effect of seed priming with H_2S and Ca^{2+} on Ni tolerance in zucchini. In addition, to the best of our knowledge, effect of combined seed priming with H_2S and Ca^{2+} hasn't been investigated in plant species and there is no convincing information about whether seed priming with H_2S and Ca^{2+} changes the level of CDPKs transcripts, upon subsequent exposure of seedlings with abiotic stresses. Thus, the aim of our study was to investigate the significance of the interplay between Ca^{2+} and H_2S in the initiation of a signal memory during seed priming and the role of CDPKs for the acquisition of cross-adaptation in *C. pepo* seedlings under subsequent exposure with Ni stress.

2. Materials and methods

2.1. Plants treatments

2.1.1. Effects of Ni on seed germination and seedling growth

First, the effect of various concentrations of $Ni(NO_3)_2$ (0, 25, 50, 100, 150, 200 and 250 ppm) was evaluated on seed germination and seedling growth. Germination tests were conducted using 25 healthy and sterilized zucchini seeds (*Cucurbita pepo* L. var. *pepo*, cv Courgette d'Italie) in 15 cm Petri dishes (four replicates). Petri dishes were placed in a culture room with 65% relative humidity, under a thermo period 28/23 °C and photoperiod 14/10 h in day/night (a light intensity of $150\,\mu mol\,m^{-2}\,s^{-1}$) for ten days. Seed germination was counted daily for 10 days and the final germination percentages were calculated. After 10 days, relative seed germination (RSG), root tolerance index (RTI) and germination tolerance index (GTI) were calculated as described by Amooaghaie et al. (2015):

RSG (%) = (Seeds germinated in treatment/Seeds germinated in control) × 100
RTI (%) = (Mean root length in treatment/Mean root length in control) × 100
GTI (%) = (RSG × RTI)/100

Toxicity level (%) in roots was calculated using the following formula:

Toxicity level (%) = (Root length of control - Root length of treatment/Root length of control) × 100.

2.1.2. Effects of seed priming with NaHS or $CaCl_2$ on seed germination and seedling growth

During the preliminary experiments, seeds were primed with various concentrations of NaHS (0, 50, 100, 200 and 400 μM) and $CaCl_2$ (0, 10, 15, 20 and 25 mM) for 24 h. Then seeds were washed and finally, seeds from each treatment were divided into 2 groups and exposed to $Ni(NO_3)_2$ solution (selected concentration 50 ppm) and distilled water. Afterwards, laboratory seed germination tests were performed under the same condition mentioned above and final germination tolerance index was determined for each treatment after 10 days.

After selecting the best concentration of NaHS and $CaCl_2$, the effects of seed priming with 100 μM NaHS and 200 μM hypotaurine (HT), as a H_2S scavenger and also impacts of seed priming with 15 mM $CaCl_2$ and 5 mM ethylene glycol tetra-acetic acid (EGTA) as a Ca^{2+} chelator, 50 μM verapamil (VPL) and 100 μM $LaCl_3$ (La^{3+}) as Ca^{2+} channel blockers, were investigated on seed germination and growth parameters of *C. pepo* seedlings. For this purpose, the surface sterilized seeds were soaked separately for 24 h in darkness in the following solutions: (1) distilled water; (2) NaHS; (3) NaHS + HT; (4) $CaCl_2$; (5) EGTA + $CaCl_2$; (6) $LaCl_3$ + $CaCl_2$; (7) VPL + $CaCl_2$. Seeds from each treatment were divided into 2 groups and exposed to 50 ppm $Ni(NO_3)_2$ solution and distilled water. The final seed germination percentage was calculated after 10 days. In addition, length and mean dry weight of ten seedlings were measured per each treatment. Lipid peroxidation in leaves was measured as is described in the following sections and seed vigour index has been calculated through the formula given by Abdulbaki and Anderson (1973).

Seed vigour index (%) = Percentage germination × Mean dry weight.

2.1.3. Effect of seed priming with NaHS and $CaCl_2$ on biochemical parameters

In the main experiment, we examined the effect of cross-talk between calcium (Ca^{2+}) and hydrogen sulfide (H_2S) during seed priming on subsequent tolerance of zucchini seedlings against Ni stress. The surface sterilized seeds were primed for 24 h in darkness according to the following treatments:

(1) 0 (control); (2) 100 μM NaHS; (3) 15 mM $CaCl_2$; (4) 15 mM $CaCl_2$ + 100 μM NaHS; (5) 5 mM (EGTA) + 100 μM NaHS; (6) 100 μM $LaCl_3$ + 100 μM NaHS; (7) 50 μM VPL + 100 μM NaHS; (8) 200 μM HT + 15 mM $CaCl_2$.

After 24 h, seeds were washed in de-ionized water and germinated in petri dishes containing 20 ml distilled water for 10 days. Then seedlings were transferred to quarter-strength Hoagland's medium (Li and Cheng, 2014) with pH 5.8. After 3 days of acclimatization, when seedlings were 13-days-old, nickel at 50 ppm $Ni(NO_3)_2$ was added to Hoagland's medium. The grown seedlings in quarter-strength Hoagland's solutions without nickel were used as the control group.

Leaf samples were collected at 12 and 24 h after Ni exposure, and immediately were transferred to −80 °C freezer for gene expression analysis. The biomass of seedlings per plant was determined after laying the samples in an air oven for 48 h at 80 °C. Biochemical traits were measured on the true leaves of seedlings after 3 days of Ni exposure.

2.2. Determination of lipid peroxidation

The malondialdehyde (MDA) content was evaluated by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). The leaf samples (0.25 g) were extracted in 2 ml of 0.5% trichloroacetic acid (TCA). After centrifugation at 12000 rpm for 10 min, 1 ml supernatant was mixed with 5 ml of 0.5% TBA dissolved in 20% TCA. The mixture was boiled for 30 min and then was cooled. After centrifugation at 10,000 rpm for 5 min, the MDA content was computed by determining the optical density of the supernatant at 532 nm and 600 nm and through the following formula it was expressed as nmol/g.FW.

$$MDA = (A532 - A600) / 155 \times 1000$$

2.3. Determination of pigments photosynthetic

Fresh leaf (0.25 g) samples were extracted with 10 ml acetone (80%, v/v). The filtrates were prepared and absorbance values at 663, 645 and 470 nm were read, to determine the contents of chlorophyll (Chl) and carotenoids (Car) in the extracts using the method of Lichtenthaler (1987).

2.4. H_2O_2 concentration and electrolyte leakage assay

The hydrogen peroxide (H_2O_2) concentration was extracted and determined after reaction with potassium iodide (KI) following the method of Amooaghaie et al. (2017)

Electrolyte leakage (EL) was evaluated according to methods described by Lutts et al. (1995) using small pieces from leaves dipped in 15 ml of deionized water and was determined as:

$$E.L\ (\%) = EC_1 / EC_2 \times 100$$

where EC_1 and EC_2 were the electrical conductivity of the solution after standing at room for 24 h and after heating at 75 °C respectively.

2.5. Evaluation of GR and APX activities

The antioxidant enzyme extract was prepared by homogenizing 0.25 g of leaf tissue in 750 μl extraction buffer containing 1 mM ethylene diamine tetra-acetic acid (EDTA) and 1% polyvinyl pyrrolidone (PVP) in 50 mM potassium phosphate buffer (pH 7.0) The homogenate was centrifuged at 10000 × *g* at 4 °C for 20 min. The supernatant was used for the enzymatic assays.

The APX (EC 1.11.1.11) activity was measured following the method of Nakano and Asada (1981). The reaction mixture contained 50 μl enzyme extract and 2.95 ml 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM ascorbate, 0.1 mM hydrogen peroxide and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm and was quantified using the extinction coefficient of 2.8 $mM^{-1} cm^{-1}$ at 25 °C.

The GR (EC: 1.6.4.2) activity was measured according to the method of Foyer and Halliwell (1976). The reaction mixture contained 100 μl enzyme extract and 900 μl 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM GSSG and 0.2 mM NADPH. The decrease in absorbance at 340 nm was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 $mM^{-1} cm^{-1}$ at 25 °C.

2.6. Determination of H_2S production and total thiols content

The H_2S content in leaves was measured spectrophotometrically at 670 nm using N,N- dimethyl-pphenylenediamine dihydrochloride (DMPD) reagent, as described by Li (2015). The assay mixture contained 1 ml leaf extract supernatant in extraction buffer (20 mM Tris-HCl buffer (pH 8.0) containing 20 mM $Zn(OAc)_2$ and 10 mM EDTA), 1 ml of 30 mM $FeCl_3$ dissolved in 1.2 M HCl and 1 ml of 20 mM DMPD dissolved in 7.2 M HCl mix respectively. The amount of H_2S was determined using an extinction coefficient of $15 \times 10^6 M^{-1} cm^{-1}$ and expressed as nmol/g.FW.

The total thiols concentration in leaves was determined through the DTNB reagent (Moron et al., 1979). The frozen leaf samples (0.5 g) were homogenized in 2.5 ml of 5% TCA. After centrifugation at 1000 rpm for 10 min, 0.1 ml supernatant was added to 0.9 ml sodium phosphate buffer (0.2 M, pH 8.0) and 2 ml of freshly prepared 0.6 mM DTNB solution in 0.2 M phosphate buffer. The absorbance of this mixture was determined at 412 nm and the total thiols content was calculated via standard curve prepared with known concentrations of GSH.

2.7. Determination of ascorbate and dehydro ascorbat concentrations

The measurement of total AsA (AsA + DHA) and AsA concentrations was done by the spectrophotometric method described by Roe and Kuether (1943) with some modifications. Leaf sample (0.5 g) was homogenized with 10 ml of 5% metaphosphoric acid-acetic acid solution and centrifuged at 8000 × *g* at 4 °C for 20 min. To determine the total AsA (AsA + DHA) concentration, 0.5 ml of 3 mM 2, 6-dichlorophenolindophenol (DCIP) was added to 4 ml supernatant and shaken and filtrated. The mixture of 1.5 ml of the filtrated solution, 1 ml of 1% thiourea solution and 1 ml of 10 mM 2, 4-dinitrophenylhydrazine (DNPH) solution were kept in a thermostatic bath at 50 °C temperature for 1 h. After cooling, chilled H_2SO_4 was added and shaken. The absorbance was read at 520 nm. To determine AsA concentrations, the chemicals and procedure were the same as above, except that the DCIP was replaced by distilled water. Total AsA and AsA concentrations were estimated from the standard curve of 0–100 ppm L-AsA. Concentrations of DHA were calculated by the subtraction of AsA from total AsA.

2.8. Total RNA extraction and qRT-PCR

Total RNA isolation was done using the LiCl protocol by the method of Channuntapipat et al. (2001). One microgram of the total RNA was used for reverse transcription using the ViVantis, 2 steps RT-PCR kit-RTPL12 (cat. no. 0201010048) following the manufacturer's instructions. The gene-specific primers for qRT-PCR are shown in Table 1 and Actin gene was used as an internal standard. The qRT-PCR assay was performed via an applied Biosystems (The StepOne Real-Time PCR system). PCR products were amplified using the SYBR Premix Ex TaqΠ (TaKaRa, cat. no. RR820Q). The PCR conditions followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 95 °C for 3 s and extension at 60 °C for 30 s. The quantification of mRNA levels was calculated according to the $2^{-\Delta\Delta Ct}$ methods.

2.9. Statistical analysis

The first experiment was conducted in a completely randomized design with 4 replicates. The next experiments were performed as factorial with a completely randomized design with 3 replicates. After ANOVA analysis, the comparison between the control and treatment's means was carried out by Duncan's Multiple Range test at $p < 0.05$ significance level using SPSS 19 software.

Table 1
Characteristics of primers for Real-time PCR.

Gene	Primer sequence	Size
Actin	F: 5′- ACGGATATCTCGGCTCTCGC -3′ R: 5′- GACGTACCCTCGGCCAGAAG -3′	**131 bp**
CDPK	F: 5′- TTGGAGTTATGCACCGTGACC -3′ R: 5′- AAGACTTCAGGGGCAACATAG -3′	**106 bp**
PCS1	F: 5′- GTTTCAAGTATCCTCCTCATTG -3′ R: 5′- GTCTAGATATGAGCATGAACCC -3′	**108 bp**
PCS2	F: 5′- AATATCCTCCTCACTGGGTTCC -3′ R: 5′- GCAATCCATGTGATGAACTCTCTC-3′	**112 bp**

3. Results

3.1. Effects of various concentrations of Ni on seed germination and seedling growth

Results related to the first experiment showed that there was no significant difference between control and various concentrations of Ni $(NO_3)_2$ on parameters of seed germination and RSG. However, the exposure of zucchini seedlings to $Ni(NO_3)_2$ at concentrations of > 25 ppm for 10 days markedly inhibited root elongation (Fig. 1) and significantly reduced root tolerance index (RTI), germination tolerance index (GTI) of roots and increased toxicity level of roots compared with the control (Table 2).

3.2. Selection of the best concentration of NaHS and $CaCl_2$ for seed priming

As shown in Fig. 2 seed priming with lower dose of NaHS (50 μM and 100 μM) significantly increased germination tolerance index whereas, higher concentrations (200 and 400 μM) reduced germination tolerance index under both control and Ni stress conditions. Thus, 100 μM NaHS was the best concentration, which was then applied in later experiments. Similarly, seed priming with Ca^{2+} improved germination tolerance index in lower dose, and 15 mM Ca^{2+} was the best concentration which was then used in subsequent tests. However, high concentrations (20 and 25 mM Ca^{2+}) reduced germination tolerance index under both Ni stress and control conditions.

3.3. Effects of seed priming with NaHS and $CaCl_2$ on seed germination and seedling growth

As shown in Table 3 seed priming with NaHS and Ca^{2+} significantly enhanced seed germination compared with the control in normal and stress conditions. Nickel stress remarkably decreased the seedling length and the seed vigour index and increased the MDA content in leaves in comparison to control. Seed priming with Ca^{2+} significantly improved all attributes except the seed vigour index and the MDA content at normal condition. Seed priming with NaHS also significantly improved all parameters except the MDA content at normal condition (Table 3).

Under Ni stress, NaHS priming increased all parameters especially the seed vigour index and lowered the MDA content in leaves when was compared to the treatment of Ni stress alone. Seed priming with Ca^{2+} also significantly increased germination, length of seedling, seedling vigour index in Ni-stressed seedlings and lowered the MDA content in leaves when was compared to the treatment of Ni stress alone (Table 3).

The effects conferred by seed priming with Ca^{2+} on above mentioned parameters was repressed by EGTA, La^{3+} and VPL. The responses induced by seed priming with NaHS were also reversed by the addition of HT as a scavenger of H_2S under Ni stress and normal conditions (Table 3).

3.4. Effects of seed priming with NaHS and $CaCl_2$ on biomass and photosynthetic pigments

Nickel stress decreased the content of chlorophyll *a*, chlorophyll *b*, carotenoids and biomass of seedlings 26.38%, 34.09%, 29.50% and 25.34% respectively (Fig. 4). Individual or combined seed priming with Ca^{2+} or NaHS restored Ni -induced inhibition of the content of chlorophyll *a*, chlorophyll *b*, carotenoids and biomass of seedlings. Existence of HT during seed priming inhibited the positive effect of seed priming with Ca^{2+} on photosynthetic pigments and biomass. The recovery of pigments and seedling biomass induced by seed priming with NaHS

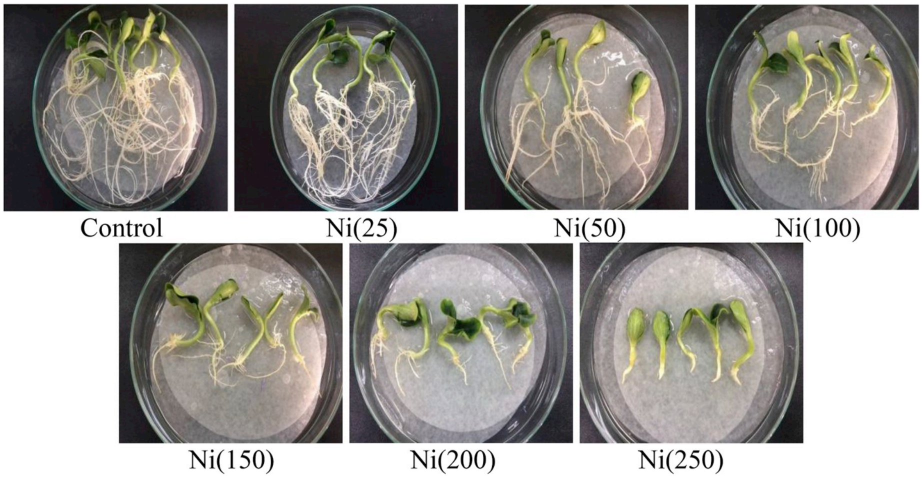

Fig. 1. The effects of various concentrations of $Ni(NO_3)_2$ (0, 25, 50, 100, 150, 200 and 250 ppm) on ten-day-old zucchini seedlings.

Table 2
The effects of various concentrations of $Ni(NO_3)_2$ (0, 25, 50, 100, 150, 200 and 250 ppm) on germination, relative seed germination (RSG), root tolerance index (RTI), germination tolerance index (GTI) and toxicity level in roots of 10 day-old zucchini seedlings.

Treatments	Germination (%)	RSG (%)	RTI (%)	GTI (%)	Toxicity level (%)
Control	70.0 ± 2.9 ^{ns}	100 ± 0 ^{ns}	100 ± 0 ^{a}	100 ± 0 ^{a}	0 ± 0 ^{d}
25	67.8 ± 5.0 ^{ns}	97.3 ± 9.5 ^{ns}	97.4 ± 1.0 ^{a}	94.9 ± 9.7 ^{a}	2.6 ± 1.0 ^{d}
50	66.7 ± 3.5 ^{ns}	95.8 ± 8.0 ^{ns}	32.9 ± 3.2 ^{b}	31.0 ± 0.2 ^{b}	67.1 ± 3.2 ^{c}
100	66.1 ± 6. 6 ^{ns}	95.5 ± 13.0 ^{ns}	30.9 ± 2.9 ^{b}	28.9 ± 2.0 ^{b}	69.1 ± 2.9 ^{c}
150	63.9 ± 3.9 ^{ns}	91.4 ± 5.6 ^{ns}	26.5 ± 2.6 ^{b}	24.3 ± 3.1 ^{bc}	73.5 ± 2.6 ^{c}
200	58.9 ± 11.5 ^{ns}	84.6 ± 16.8 ^{ns}	16.4 ± 1.0 ^{c}	13.5 ± 1.8 ^{cd}	83.6 ± 1.0 ^{b}
250	55.5 ± 4.0 ^{ns}	79.3 ± 4.0 ^{ns}	8.8 ± 1.3 ^{d}	7.1 ± 1.4 ^{d}	91.2 ± 1.3 ^{a}

Data in the table represent the mean ± SE (n = 4) and same letters per each column indicate.
Non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

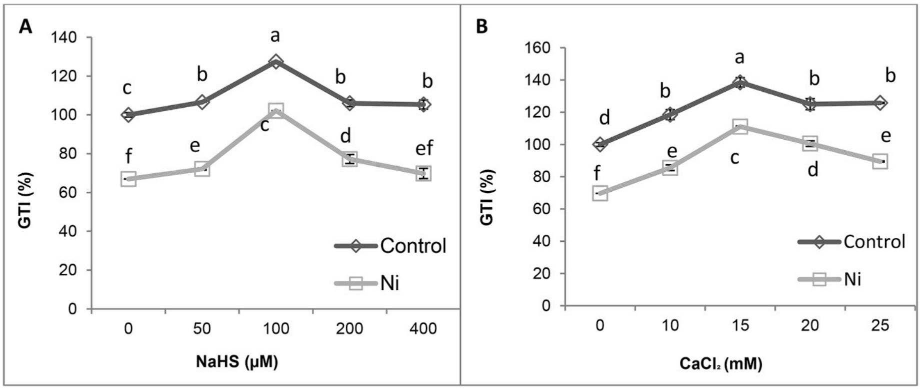

Fig. 2. The impacts of seed priming with NaHS (0, 50, 100, 200 and 400 µM) (A) and $CaCl_2$ (0, 10, 15, 20 and 25 mM) (B) on germination tolerance index (GTI) under control condition and 50 ppm Ni $(NO_3)_2$.
Data in the figure represent the mean ± SE and same letters on bars indicate non-significant differences. at $p < 0.05$ based on Duncan's multiple range tests.

were also inverted by seed priming in the presence of EGTA, La^{3+} and VPL (Fig. 4).

3.5. Effects of seed priming with NaHS and $CaCl_2$ on electrolyte leakage and H_2O_2 accumulation

Under Ni stress, a drastic rise was observed in the H_2O_2 content and electrolyte leakage level in leaves as compared with the control (Fig. 5A and B). Individual seed priming with NaHS and Ca^{2+} significantly lowered the electrolyte leakage level and H_2O_2 content in leaves of Ni-stressed zucchini plants. Interestingly, simultaneous application of H_2S and Ca^{2+} further reduced the damages induced by Ni on the membrane integrity. On the other hand, when seed priming with NaHS was done in the presence of EGTA and La^{3+} or VPL, the inhibitory impact of H_2S on the electrolyte leakage and H_2O_2 accumulation in leaves was weakened. Similarly, concurrent seed priming with HT and Ca^{2+} could reverse the alleviating effect of Ca^{2+} on the electrolyte leakage level and H_2O_2 contents in the leaves of seedlings (Fig. 5A and B).

3.6. Effects of seed priming with NaHS and $CaCl_2$ on APX and GR enzymes activity

Ni-exposure significantly increased the activities of APX and GR by 58.06% and 17.75% respectively in zucchini leaves compared to the control (Fig. 6). Individual and combined seed priming with Ca^{2+} or NaHS significantly decreased the APX activity but enhanced the GR activity under Ni stress.

However, H_2S-induced impacts on the activities of APX and GR were reversed in the leaves of seedlings grown from seeds primed with EGTA, La^{3+} and VPL together with NaHS. The effect of seed priming with Ca^{2+} on the activities of APX and GR was also reversed in the leaves of seedlings grown from seeds primed with HT + $CaCl_2$ (Fig. 6).

Table 3
The effects of NaHS (100 μM) singly or in combination with 200 μM hypotaurine (HT), $CaCl_2$ (15 mM) alone or together with 5 mM EGTA, 100 μM $LaCl_3$ and 50 μM verapamil (VPL) on germination, length of seedling, seedling vigour index and malondialdehyde (MDA) content in leaves of zucchini seedlings under both normal and Ni stress conditions.

Treatments	Germination (%)	Length of seedling (cm)	Seed vigour index (%)	MDA (nmol/g.FW)
Control	65.3 ± 2.4^{bc}	19.0 ± 0.6^{d}	6046.7 ± 240.9^{bc}	4.6 ± 0.5^{h}
NaHS	78.7 ± 1.3^{a}	24.3 ± 0.3^{ab}	7273.3 ± 488.5^{a}	5.5 ± 0.3^{gh}
NaHS + HT	57.3 ± 1.3^{de}	19.3 ± 0.9^{d}	4200.0 ± 78.8^{ef}	10.7 ± 0.3^{ef}
Ca	74.7 ± 1.3^{a}	25.3 ± 1.7^{a}	6805.3 ± 392.6^{ab}	4.3 ± 0.4^{h}
Ca + EGTA	60.0 ± 2.3^{cd}	22.3 ± 0.7^{bc}	4760.0 ± 117.8^{de}	8.1 ± 0.4^{fg}
Ca + $LaCl_3$	58.7 ± 0.7^{de}	22.3 ± 0.3^{bc}	4524.0 ± 103.6^{e}	8.3 ± 0.2^{fg}
Ca + VPL	57.3 ± 1.8^{de}	21.7 ± 0.7^{c}	3985.3 ± 100.8^{efg}	9.3 ± 0.4^{f}
Ni	60 ± 3.5^{cd}	14.7 ± 0.7^{e}	3392.0 ± 98.9^{fg}	28 ± 2^{a}
Ni + NaHS	66.7 ± 1.7^{b}	19.3 ± 0.3^{d}	5657.3 ± 271.9^{c}	13.5 ± 0.3^{e}
Ni + NaHS + HT	53.3 ± 1.8^{e}	14.7 ± 0.3^{e}	3650.7 ± 329.3^{fg}	21.7 ± 0.9^{c}
Ni + Ca	66.7 ± 0.7^{b}	18.7 ± 0.7^{d}	5489.3 ± 515.5^{dc}	12.5 ± 0.3^{e}
Ni + Ca + EGTA	54 ± 1.2^{de}	16.3 ± 0.3^{e}	3220.0 ± 131.1^{g}	17.7 ± 0.9^{d}
Ni + Ca + $LaCl_3$	60.0 ± 2.3^{cd}	15.7 ± 0.3^{e}	3385.3 ± 261.1^{fg}	25 ± 0.6^{b}
Ni + Ca + VPL	57.3 ± 1.8^{de}	15.3 ± 0.3^{e}	3490.7 ± 5.8^{fg}	25.2 ± 1.2^{b}

Data in the table represent the mean ± SE and same letters per each column indicate non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

3.7. *Effects of seed priming with NaHS and $CaCl_2$ on content of H_2S and total thiols*

Our finding indicated that under Ni stress, H_2S level increased 42.49% in the leaves of zucchini seedlings (Fig. 7A). Individual seed priming with Ca^{2+} and NaHS elevated the H_2S content 50.33% and 98.43% in leaves respectively compared with the control. Seed priming with NaHS + Ca^{2+} also enhanced the endogenous level of H_2S 87.66% in the leaves. In contrast, the accumulation of H_2S was significantly weakened in the leaves of seedlings grown from seeds primed with EGTA, La^{3+} and VPL together with NaHS. In addition, the presence of HT during seed priming decreased the stimulatory impact of seed

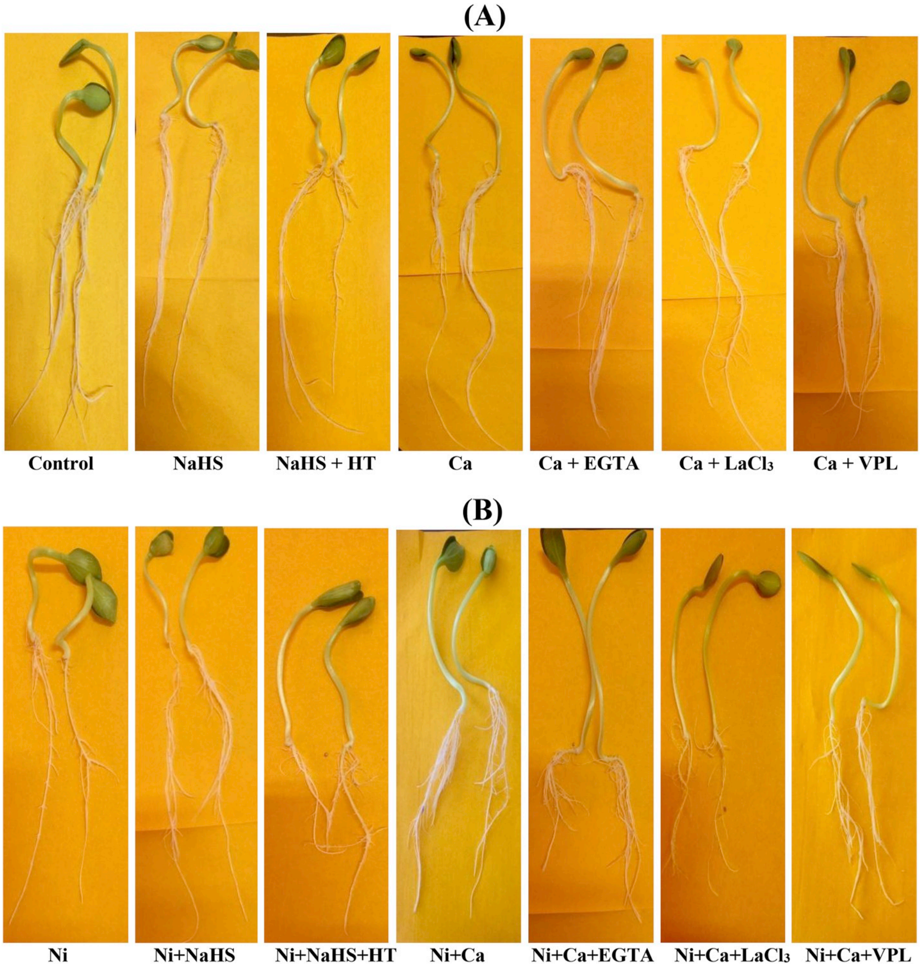

Fig. 3. The impacts of seed priming with NaHS (100 μM) and $CaCl_2$ (15 mM) and antagonists of Ca^{2+} and H_2S on ten-day-old zucchini seedlings under control condition (A) and 50 ppm $Ni(NO_3)_2$ (B). Hypotaurine (HT), as a H_2S scavenger, 5 mM ethylene glycol tetraacetic acid (EGTA) as a Ca^{2+} chelator, 50 μM verapamil (VPL) and 100 μM $LaCl_3$ as a known Ca^{2+}channel blockers were used during seed priming.

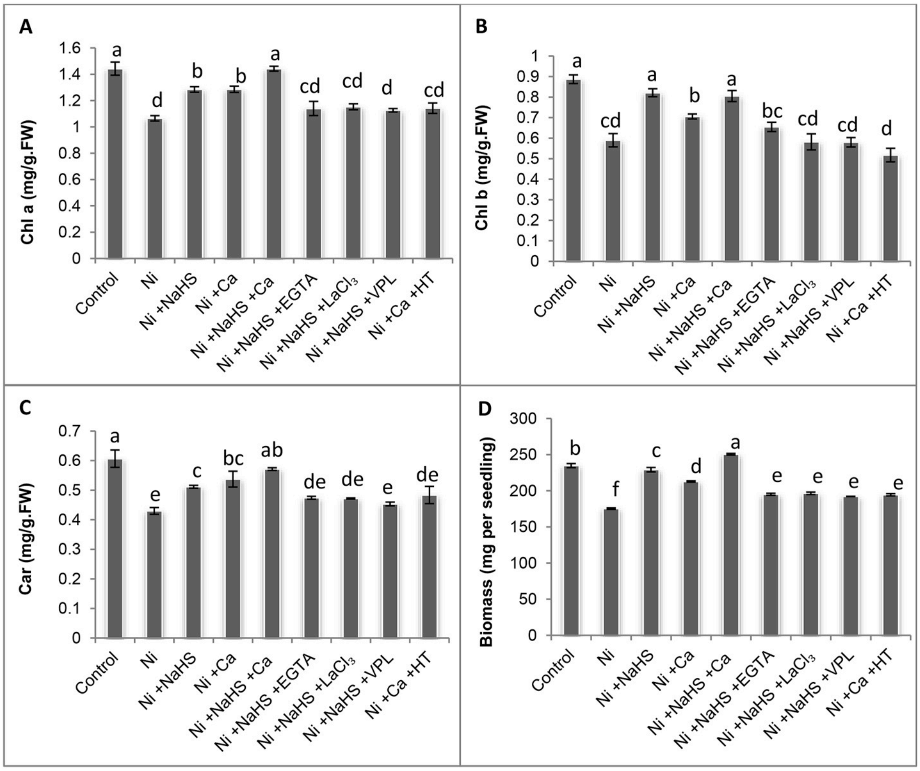

Fig. 4. The impacts of seed priming with NaHS (100 µM) and $CaCl_2$ (15 mM) and antagonists of Ca^{2+} and H_2S on chlorophyll *a*, *b* (A, B), carotenoids (C) in leaves and biomass of zucchini seedlings (D). exposed to 50 ppm $Ni(NO_3)_2$.
Hypotaurine (HT), as a H_2S scavenger, 5 mM ethylene glycol tetraacetic acid (EGTA) as a Ca^{2+} chelator, 50 µM verapamil (VPL) and 100 µM $LaCl_3$ as a known Ca^{2+} channel blockers were used during seed priming. Data in the figure represent the mean ± SE and same letters on bars indicate non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

priming with Ca^{2+} on the H_2S level in the leaves of Ni-exposed seedlings (Fig. 7A).

The results denoted that under Ni stress, the total thiols content in the leaves decreased 40.35% in comparison with the untreated control. Seed priming with Ca^{2+} and NaHS alone or in combination could enhance the total thiols content in the leaves in comparison with Ni treatment alone. However, total thiols content declined in leaves of seedlings grown from seeds primed with EGTA, La^{3+} and VPL together with NaHS and in the leaves of seedlings which rose from seeds primed with HT together with $CaCl_2$ (Fig. 7B).

3.8. Effects of seed priming with NaHS and $CaCl_2$ on AsA and DHA concentrations

The results related to the amounts of AsA, DHA and the ratio of AsA/DHA are shown in Table 4. The lowest value for the AsA content was recorded under Ni stress while the highest values for the AsA content were obtained in the leaves of seedlings grown from seed primed with NaHS and Ca^{2+} alone or together. Moreover, Ni stress increased the DHA content and reduced the AsA/DHA ratio. Seed priming with Ca^{2+} and NaHS alone or in combination had no significant effect on the DHA content but considerably increased the AsA/DHA ratio. In the leaves of seedlings grown from seed primed with EGTA, La^{3+} and VPL together with NaHS and in the leaves of seedlings which rose from seed primed with HT together with $CaCl_2$, the DHA content increased but the AsA content and the ratio of the AsA/DHA reduced when compared to the treatments of Ni + NaHS and Ni + Ca^{2+} respectively. These results suggest that the positive effects of seed priming with NaHS and Ca^{2+} on the content of AsA and DHA and the ratio of AsA/DHA were reversed by these antagonists (Table 4).

3.9. Effects of seed priming with NaHS and $CaCl_2$ on CDPK and PCS genes expression

The level of *CDPK* transcripts in leaves was up-regulated at 12 h and then decreased at 24 h after the Ni exposure. Seed priming with Ca^{2+} or NaHS individually or in combination significantly increased *CDPK* gene expression after 12 h and 24 h of the Ni exposure. The use of HT, EGTA, La^{3+} and VPL during seed priming inhibited the stimulatory effect of seed priming with Ca^{2+} and NaHS on *CDPK* transcripts in the leaves under the Ni stress (Fig. 8).

The transcript levels of *PCS1 and PCS2* genes were significantly

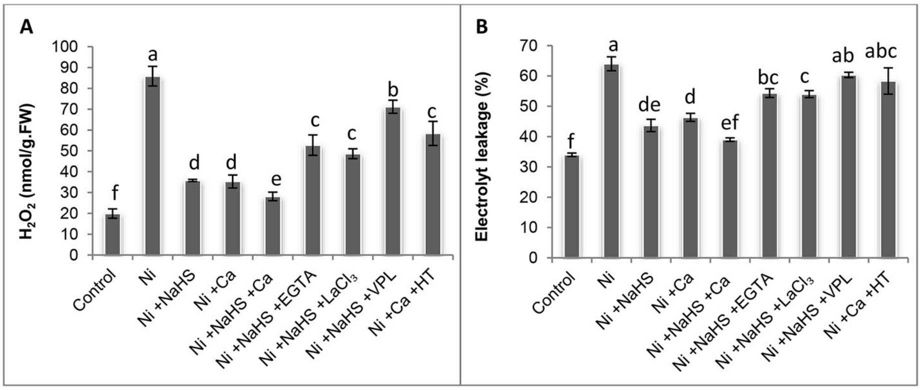

Fig. 5. The impacts of seed priming with NaHS (100 µM) and $CaCl_2$ (15 mM) and antagonists of Ca^{2+} and H_2S on H_2O_2 accumulation (A) and electrolyte leakage (B) in leaves of zucchini seedlings exposed to 50 ppm $Ni(NO_3)_2$.
Hypotaurine (HT), as a H_2S scavenger, 5 mM ethylene glycol tetraacetic acid (EGTA) as a Ca^{2+} chelator, 50 µM verapamil (VPL) and 100 µM $LaCl_3$ as a known Ca^{2+} channel blockers were used during seed priming. Data in the figure represent the mean ± SE and same letters on bars indicate non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

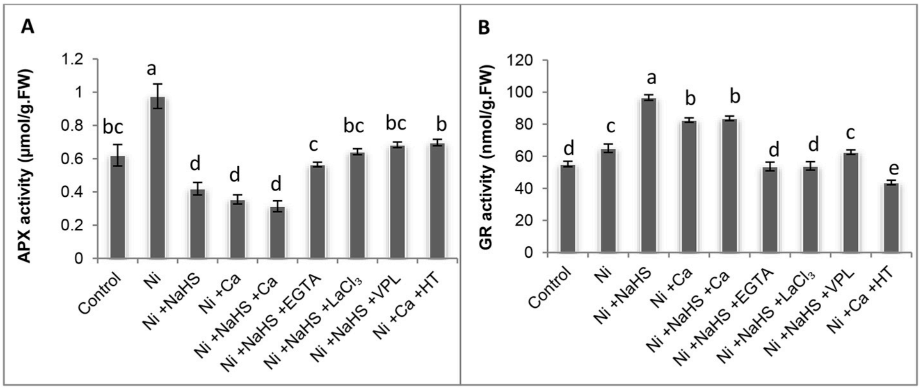

Fig. 6. The impacts of seed priming with NaHS (100 µM) and $CaCl_2$ (15 mM) and antagonists of $Ca^{2+}$and H_2S on the activities of APX (A) and GR (B) in leaves of zucchini seedlings exposed to 50 ppm $Ni(NO_3)_2$.
Hypotaurine (HT), as a H_2S scavenger, 5 mM ethylene glycol tetraacetic acid (EGTA) as a Ca^{2+} chelator, 50 µM verapamil (VPL) and 100 µM $LaCl_3$ as a known Ca^{2+}channel blockers were used during seed priming. Data in the figure represent the mean ± SE and same letters on bars indicate non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

increased at 12h the after exposure to Ni stress (Fig. 8C, E). The transcript level of *PCS2* gene had no significant difference with its level in the control at 24 h after the Ni exposure (Fig. 8F). Seed priming with Ca^{2+} or NaHS alone or in combination also significantly increased the transcript level of *PCS1* gene in the leaves of Ni-stressed seedlings at 12 and 24 h after the Ni exposure; while the transcript level of *PCS2* gene was significantly increased only at 24h after being subjected to the Ni stress (Fig. 8F). The application of HT and EGTA, La^{3+} and VPL often reversed the effect of Ca^{2+} and H_2S on the expression of *PCS1* and *PCS2* genes at 12 and 24 h after the Ni exposure (Fig. 8C–F).

4. Discussion

In the present study, increasing Ni concentration had no significant impact on seed germination and relative seed germination (RSG) but severely reduced root elongation and root tolerance index (RTI) (Fig. 1, Table 2). It is likely because of this seed coat protected seeds against Ni toxicity but roots were directly exposed to Ni stress (Fig. 1). Germination tolerance index (GTI) combines root growth and seed germination thus, providing a more perfect quantity of toxicity (Amooaghaie et al., 2015). Hence, it was better to use GTI as an indicator of phytotoxicity.

Results of the next two experiments clearly confirmed that individual seed priming with low levels of H_2S and Ca^{2+} conferred a protective effect on GTI while high concentrations caused a detrimental effect (Fig. 2).These results support reports of other authors about a dual role of H_2S and Ca^{2+}, as a toxic molecule at high doses and a signal messenger at lower doses under biotic and abiotic stress conditions. Baudouin et al. (2016) stated that NaHS had stimulatory and inhibitory effects on Arabidopsis seed germination at low and high concentrations respectively. The phytotoxicity of H_2S can be attributed to its high affinity to the Fe^{2+}- containing proteins such as cytochrome oxidase and hemoglobin (Guo et al., 2016). White and Broadley (2003) also pointed to the dual role of calcium, at optimal concentrations improved growth and at excessive concentrations in the rhizosphere solution acted as an inhibitory factor that reduced plant growth rates and prevented the germination of seeds. Calcium can simply interfere with membranes and macromolecules because of its high tendency to create various bonds. For this reason, calcium can be considered as a toxic cell component at higher concentrations since it can form insoluble compounds with phosphate derivatives, accumulate nucleic acids and proteins, and can disrupt the integrity of the lipid membrane (Zeng et al., 2015b).

However, the present study confirmed that seed priming with appropriate doses of Ca^{2+} and H_2S increased seed germination percentage, seedling length, seed vigour index and reduced malondialdehyde (MDA) under Ni stress (Table 3). In agreement to our finding, it has been demonstrated that seed priming with NaHS exaggerated radicle and hypocotyl lengths and sustained lower MDA levels in sodium bicarbonate-treated *Cucumis sativus* seeds (Sun and Luo, 2014) and in Pb-stressed cauliflower seedlings (Chen et al., 2018). Seed priming with Ca^{2+} had also positive effects on seedling growth of faba bean plants under cadmium stress (Nouairi et al., 2019) and *Brachiaria brizantha* under high humidity and temperature (Batista et al., 2016). The addition of the H_2S scavenger HT, during seed priming completely abrogated the effect of H_2S on seed germination, seedling length, seed vigour index and MDA content. The stimulatory effect conferred by seed priming with Ca^{2+} on the above parameters was also reversed by EGTA, La^{3+} and VPL (Table 3, Fig. 3). These results suggest that seed priming with H_2S or Ca^{2+} probably caused a memory in embryo cells of seeds that in turn enhanced Ni tolerance in seedlings; as seed priming with antagonists of H_2S or Ca^{2+} negated this memory and the effects of H_2S or Ca^{2+} during the subsequent seedling growth under Ni stress.

As Asrar et al. (2014) reported on tomato, Ni stress reduced the content of chlorophylls and carotenoids in zucchini leaves (Fig. 4). Carotenoids play a vital role in the photoprotection of chlorophylls against photo-oxidative damage by quenching ROS and reacting with excited chlorophyll molecules (Ramel et al., 2012). Thus a decrease in the carotenoids content of Ni-exposed zucchini leaves, led to the overproduction of H_2O_2 content and increased the electrolyte leakage

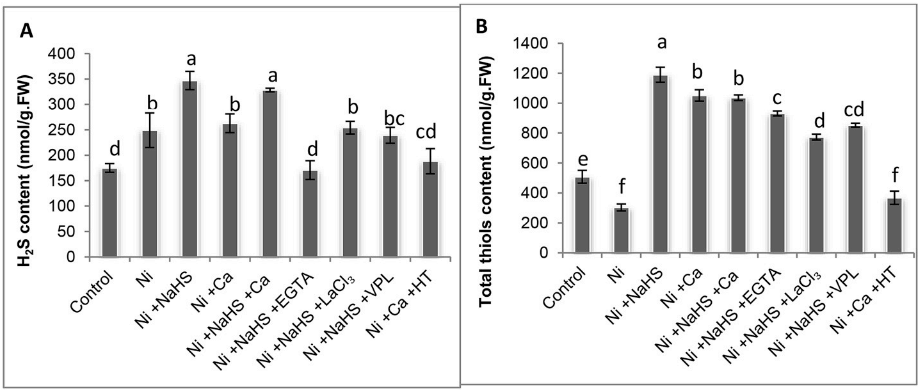

Fig. 7. The impacts of seed priming with NaHS (100 µM) and $CaCl_2$ (15 mM) and antagonists of Ca^{2+} and H_2S on H_2S (A) and total thiols (B) contents in leaves of zucchini seedlings exposed to 50 ppm $Ni(NO_3)_2$.
Hypotaurine (HT), as a H_2S scavenger, 5 mM ethylene glycol tetraacetic acid (EGTA) as a Ca^{2+} chelator, 50 µM verapamil (VPL) and 100 µM $LaCl_3$ as a known Ca^{2+}channel blockers were used during seed priming. Data in the figure represent the mean ± SE and same letters on bars indicate non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

Table 4

The impacts of seed priming with NaHS (100 μM) and $CaCl_2$ (15 mM) and antagonists of Ca and H_2S on the AsA and DHA concentrations and the ratio of AsA/DHA in the leaves of zucchini seedlings exposed to 50

ppm $Ni(NO_3)_2$. Hypotaurine (HT), as a H_2S scavenger, 5 mM ethylene glycol tetraacetic acid (EGTA) as a Ca^{2+} chelator, 50 μM verapamil (VPL) and 100 μM $LaCl_3$ as a known Ca^{2+} channel blockers were used during seed priming.

Treatments	AsA (nmol/g.FW)	DHA (nmol/g.FW)	AsA/DHA (nmol/g.FW)
Control	2329.35 ± 45.89 ^{c}	1004.35 ± 140.22 ^{d}	2.41 ± 0.32 ^{bc}
Ni	1163.35 ± 115.51 ^{d}	1401.85 ± 115.15 ^{c}	0.83 ± 0.06 ^{d}
Ni + NaHS	6913.85 ± 591.45 ^{a}	1083.85 ± 132.5 ^{d}	6.41 ± 0.25 ^{a}
Ni + Ca	5509.35 ± 596.13 ^{b}	871.85 ± 26.5 ^{d}	6.29 ± 1.02 ^{a}
Ni + NaHS + Ca	6251.35 ± 260.99 ^{b}	898.35 ± 45.89 ^{d}	7 ± 0.54 ^{a}
Ni + NaHS + EGTA	6198.35 ± 159.75 ^{bc}	1852.35 ± 159 ^{b}	3.44 ± 0.27 ^{b}
Ni + NaHS + $LaCl_3$	5853.85 ± 260.99 ^{c}	2620.85 ± 338.32 ^{a}	2.28 ± 0.09 ^{bc}
Ni + NaHS + VPL	4422.85 ± 413.94 ^{d}	2408.85 ± 183.59 ^{a}	1.87 ± 0.28 ^{c}
Ni + Ca + HT	4184.35 ± 606.03 ^{d}	2302.85 ± 226.41 ^{ab}	1.86 ± 0.33 ^{c}

Data in the table represent the mean ± SE and same letters per each column indicate non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

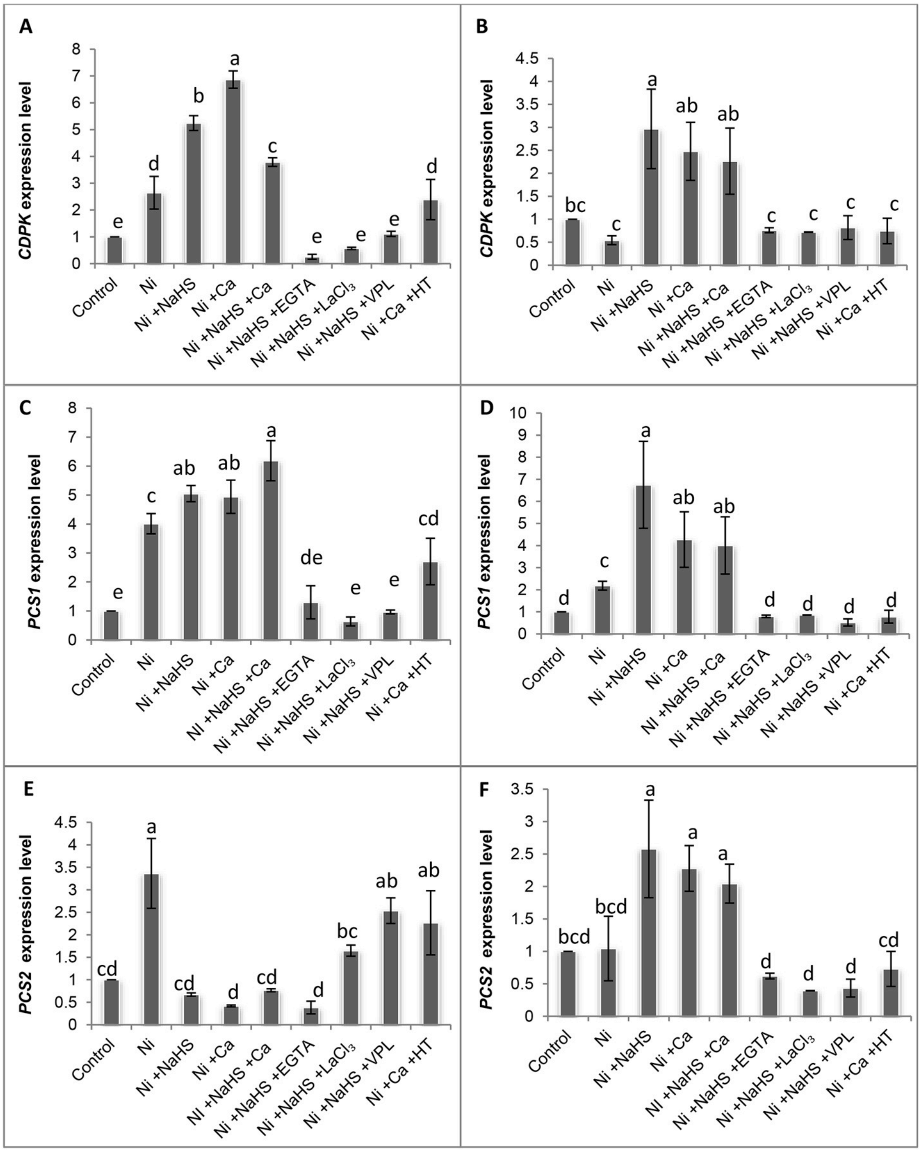

Fig. 8. The impacts of seed priming with NaHS (100 μM) and $CaCl_2$ (15 mM) and antagonists of Ca and H_2S on *CDPK and PCs* genes expression in leaves after 12 h (A, C, E) and 24 h (B, D, F) in leaves of zucchini seedlings exposed to 50 ppm $Ni(NO_3)_2$.

Hypotaurine (HT), as a H_2S scavenger, 5 mM ethylene glycol tetraacetic acid (EGTA) as a Ca^{2+} chelator, 50 μM verapamil (VPL) and 100 μM $LaCl_3$ as a known Ca^{2+} channel blockers were used during seed priming. Data in the figure represent the mean ± SE and same letters on bars indicate non-significant differences at $p < 0.05$ based on Duncan's multiple range tests.

level in the leaves (Fig. 5) that further disturbed photosynthetic apparatus and consequently inhibited plant growth. Seed priming with Ca^{2+} and H_2S improved the content of photosynthetic pigments in Ni-stressed zucchini seedlings (Fig. 4) and likely allowed for maintaining a greater carbon gain thereby, increasing the biomass of seedlings under Ni stress. Similarly, it has been found that the content of chlorophyll and carotenoids was enhanced by NaHS in *Spinacia oleracea* leaves (Chen et al., 2011) and in Ni-challenged rice (Rizwan et al., 2019) and by Ca^{2+} in Cr-stressed rice (Mukta et al., 2019). It has been reported that seed priming with Ca^{2+} increased the chlorophyll content in sunflower plants under drought stress (Madany and Khalil, 2017). Ca^{2+} protects the chloroplasts membrane and helps the maintenance of sufficient photochemical efficiency of PSII because enhances H_2O oxidation and electron flow (Huang et al., 2017).

Ni stress increased the H_2O_2 content and electrolyte leakage level in zucchini leaves (Fig. 5). These results are in line with findings Rizwan et al. (2017) who reported Ni induced oxidative stress and increased the content of H_2O_2 and MDA in roots and shoots of rice. Our results showed that seed priming with H_2S and Ca^{2+} singly or in combination decreased the H_2O_2 content and electrolyte leakage level in zucchini leaves (Fig. 5). Ben Massoud et al. (2017) also showed that seed priming with Ca^{2+} alleviated growth inhibition and oxidative stress injury and reduced contents of carbonyl groups, H_2O_2 and MDA in pea under Cu stress. Ca^{2+} ion reduces electrolyte leakage and provides structural integrity to cellular membranes by binding to phospholipids that stabilize the structure and function of lipid bilayers (Siddiqui et al., 2011).

Our results depicted that Ni stress altered the redox status of the cells because the content of total thiols, AsA and AsA/DHA ratio dramatically decreased in Ni-stressed zucchini seedlings (Fig. 7, Table 4). Seed priming with H_2S and Ca^{2+} alleviated Ni-caused oxidative stress in the leaves that were closely associated with increasing the GR activity (Fig. 6). This in turn, resulted in increase in the content of total thiols (Fig. 7) such as glutathione and decrease in the activity of APX (Fig. 6) in zucchini seedlings under Ni stress that ultimately led to maintaining the ASA content. These impacts enhanced the redox potential in primed seedlings as compared to non-primed seedlings under Ni stress. Shan et al. (2012) reported that pretreatment with sodium hydrosulfide (NaHS), enhanced the activities of GR and APX in the AsA-GSH cycle and restored oxidative damage by modulating the levels of glutathione and ascorbate metabolism in leaves of wheat seedlings under Cu stress. Asrar et al. (2014) stated that Ni increased EL in tomato leaves and decreased the leaf GSH and DHA content, the GR activity and application of Ca^{2+} ameliorated these effects and raised the GSH and DHA content and the GR activity in tomato leaves.

When zucchini seedlings were upon Ni exposure, the total thiols content was substantially dropped; whereas the expression of *PCS1* and *PCS2* increased in leaves, likely due to the fact that thiols such as glutathione are consumed by *C. pepo* for the synthesis of phytochelatins (Fig. 8). Glutathione maintains the redox status in the cells and phytochelatins (PCs) play a major role in metal sequestration (Lopez-Climent et al., 2014). Seed priming with Ca^{2+} or H_2S enhanced the total thiols content in zucchini leaves (Fig. 7). Similarly, increasing the GSH level has earlier been reported by Ca^{2+} treatment in citrus plants under Cd stress conditions (Lopez-Climent et al., 2014) and by NaHS supplementation in pea seedlings (Singh et al., 2015).

Seed priming with Ca^{2+} or H_2S amplified the expression of *PCS1* and *PCS2* in the leaves 24 h after the Ni exposure (Fig. 8D, F), that likely leading to the generation of metal chelators such as phytochelatins in plant cells and avoiding from heavy metal toxicity. Further, the application of Ca^{2+} alleviated Cd accumulation and growth retardation in *Lactuca sativa* by increasing the expression of *PC* synthase gene (He et al., 2005). Mukta et al. (2019) denoted that Ca^{2+} treatment enhanced the accumulation of phytochelatin and glutathione and increased activity of catalase, peroxidase, and glutathione reductase in rice roots under Cr stress. Fang et al. (2014) also pointed to the alleviatory effect of H_2S and Ca^{2+} on Cr toxicity in *Setaria italica* by the reduction of the Cr uptake and enhancing the *PC* expression and the activity of antioxidant enzymes.

In this study, Ni stress increased the H_2S content in the leaves (Fig. 7) and increased the *CDPK* gene expression in zucchini leaves (Fig. 8). These results suggest that H_2S and Ca^{2+} signaling are involved in triggering Ni tolerance in zucchini seedlings. Likewise, the elevation of the endogenous H_2S has been reported in rice under Cd (Mostofa et al., 2015) and Ni (Rizwan et al., 2019) stresses and in sesame under Pb stress (Amooaghaie et al., 2017). Upregulation of *CDPKs* gene expression has also been reported in *Ulva compressa* under copper (Gonzalez et al., 2012) and in foxtail millet seedlings under Cr^{6+} stress (Fang et al., 2014).

Interestingly seed priming with NaHS increased the *CDPK* gene expression and the application of Ca^{2+} chelator, (EGTA) and channel blockers (lanthanum chloride and verapamil) down-regulated the *CDPK* gene expression in zucchini leaves (Fig. 8). Furthermore, seed priming with NaHS, enhanced components of the AsA-GSH cycle (Fig. 7, Table 4), redox homeostasis (Fig. 7), and *PC* transcripts (Fig. 8) in leaves under Ni stress and seed priming with EGTA, lanthanum chloride and verapamil reversed these impacts. These findings indirectly proposed that seed priming with H_2S may trigger increases in cytosolic free Ca^{2+} ($[Ca^{2+}]$cyt) in embryo cells through the entry of extracellular Ca^{2+} into cells via the plasma membrane and this Ca^{2+} wave might share a pathway that led to forming stress memory in cells. It is possible that during subsequent seedling growth, this memory be activated and provoked Ca^{2+} signaling via accelerating the *CDPK* gene expression in leaves; therefore primed seedlings up-regulate defense reactions more efficiently than unprimed seedlings under later exposure with Ni stress. Kolupaev et al. (2017) also found that 100 μM NaHS increased the heat tolerance of wheat coleoptile cells in a Ca^{2+}-dependent manner; since the treatment with EGTA as calcium chelator suppressed the impacts of H_2S on the heat tolerance of wheat. However, by virtue of technical restrictions, we did not directly display raising ($[Ca^{2+}]$cyt) in seeds or leaves and next studies should assess it to address this hypothesis.

On the other hand, seed priming with Ca^{2+} elevated the endogenous H_2S level in leaves and this effect was weakened by seed priming in the presence of HT, as H_2S scavenger. Although we did not address the source of H_2S generation, it is conceivable that Ca^{2+} be up-regulated enzymes or genes implicated in H_2S biosynthesis. Fang et al. (2014) have also stated that in foxtail millet seedlings, the Cr^{6+} stress elevated the internal H_2S synthesis via inducing gene expression of L-DES, which was enhanced by the exogenous Ca^{2+} and suppressed by the Ca^{2+} chelator, EGTA. In addition, seed priming with Ca^{2+} improved growth and defense responses and seed priming with Ca + HT substantially reversed the effects induced by seed priming with Ca^{2+} on the biomass and Chl content (Fig. 4), the activities of APX and GR (Fig. 6), the content of total thiols and AsA (Fig. 7, Table 4) and the *PC* transcripts (Fig. 8) in the leaves. These findings indicated that performances of H_2S are downstream of Ca^{2+} for the establishment of stress memory during seed priming and the initiation of cross-adaptation in zucchini seedlings under Ni toxicity.

As shown in Fig. 9, on the basis our findings in this study, it could be concluded that seed priming with Ca^{2+} and H_2S likely trigger Ca^{2+} signature and internal H_2S biosynthesis in seeds and two-side crosstalk between H_2S and Ca^{2+} during seed priming may expand a signal imprint in embryo cells. This signal memory could induce cross-adaptation against Ni stress during the post-germinative growth stages, as the seedlings grown from primed seeds exhibited stronger Ca^{2+} signature *(CDPK* gene expression) and greater H_2S content than the seedlings raised from unprimed seeds. These more efficient signaling cascades could induce further adaptation to Ni stress through enhancing the AsA-GSH cycle activity, the redox homeostasis and the expression of *PCs* genes in the seedlings grown from primed seeds. However, by virtue of technical restrictions, we could not directly display raising ($[Ca^{2+}]$cyt) in seeds or leaves. In addition, we did not assay the enzymatic activities

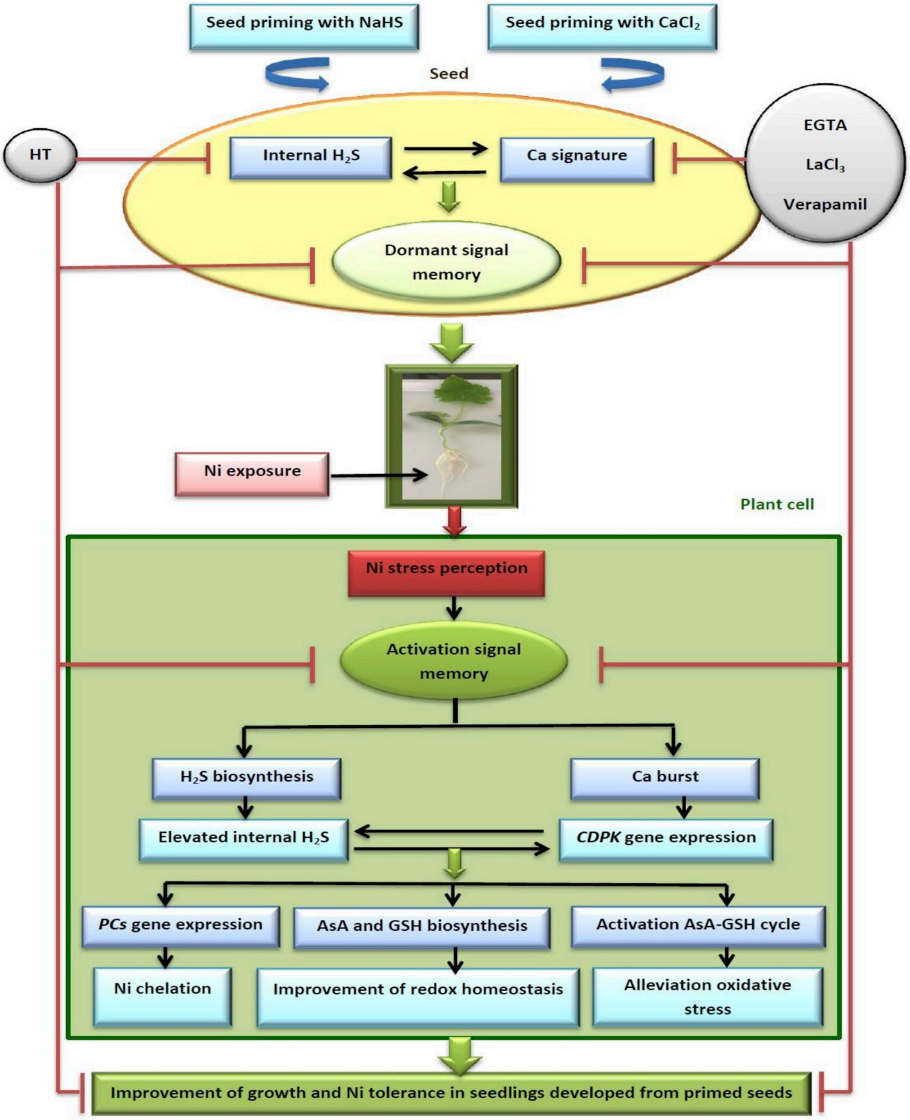

Fig. 9. Seed priming with H_2S and Ca^{2+} trigger signaling pathways which resulting in the systemic accumulation of dormant stress memory in embryo cells in seeds. Upon subsequent exposure to Ni stress, stress memory is activated and primed plants enables to show enhanced tolerance-related responses e.g., enhancing the AsA-GSH cycle activity, the redox homeostasis and the expression of *PCs* genes.

or gene expression levels involved in H_2S biosynthesis in primed seeds and in leaves of Ni-exposed seedlings. Furthermore, tolerance-related responses are at least partly regulated by different molecular mechanisms (e.g., transcriptional regulation, post-translational modifications) that should investigate in next studies. Further studies using progressive techniques, mutant analyses and molecular approaches are essential for better understanding the detailed mechanisms of stress memory/imprint acquisition by seed priming with H_2S and Ca^{2+} and its activation under subsequent exposure with abiotic stresses such as Ni toxicity.

In sum, our results help to confirm of the theory of stress memory/imprint acquisition by seed priming and offer the strategies to enhance heavy metal tolerance in plants.

Credit author statement

Maryam Valivand: Methodology, Visualization, Investigation, Data curation, Writing-Reviewing and Editing, formal analysis.

Rayhaneh Amooaghaie: Conceptualization, Methodology, Visualization, Supervision, Writing- Original draft preparation.

Ali-Mohammad Ahadi: Methodology, Visualization, Supervision, Writing- Reviewing and Editing.

Acknowledgment

This work was supported by the Plant Science Department of Shahrekord University, Iran. The authors thank editors and reviewers for reading the manuscript, their valuable comments and their final approval. No potential conflict of interest was reported by the authors.

References

Abdulbaki, A.A., Anderson, J.D., 1973. Vigour determination in soybean seed by multiple criteria. Crop Sci. 13, 630–633.

Abid, M., Hakeem, A., Shao, Y., Liu, Y., Zahoor, R., Fan, Y., Suyu, J., Ata-Ul-Karim, S.T., Tian, Z., Jiang, D., Snider, J.L., Dai, T., 2018. Seed osmopriming invokes stress memory against post-germinative drought stress in wheat *Triticum aestivum* L. Environ. Exp. Bot. 145, 12–20.

Ahmad, P., Sarwat, M., Bhat, N.A., Wani, M.R., Kazi, A.G., Tran, L.S., Zhang, J.S., 2015. Alleviation of cadmium toxicity in *Brassica juncea* (L.) by calcium application involves various physiological and biochemical strategies. PLoS One 10, e0114571.

Alcantara, B.K., Machemer-Noonan, K., Francides Gomes Silva Junior, F.G.S., Azevedo, R.A., 2015. Dry priming of maize seeds reduces aluminum stress. PLoS One 12, e0145742.

Amooaghaie, R., Nikzad, K., 2013. The role of nitric oxide in priming induced low temperature tolerance in two genotypes of tomato. Seed Sci. Res. 23, 123–131.

Amooaghaie, R., Tabatabaie, F., 2017. Osmopriming-induced salt tolerance during seed germination of alfalfa most likely mediates through H_2O_2 signaling and upregulation of heme oxygenase. Protoplasma 254, 1791–1803.

Amooaghaie, R., Tabatabaei, F., Ahadi, A.M., 2015. Role of hematin and sodium nitroprusside in regulating *Brassica nigra* seed germination under nanosilver and silver nitrate stresses. Ecotoxicol. Environ. Saf. 113, 259–270.

Amooaghaie, R., Zangene-Madar, F., Enteshari, S.H., 2017. Role of two-sided crosstalk between NO and H_2S on improvement of mineral homeostasis and antioxidative defense in *Sesamum indicum* under lead stress. Ecotoxicol. Environ. Saf. 139, 210–218.

Asano, T., Hayashi, N., Kikuchi, S., Ohsugi, R., 2012. CDPK-mediated abiotic stress signaling. Plant Signal. Behav. 7, 817–821.

Asrar, Z., Mozafari, H., Rezanejad, F., Pourseyedi, S., Yaghoobi, M.M., 2014. Calcium and L-histidine effects on ascorbate-glutathione cycle components under nickel-induced oxidative stress in tomato plants. Biol. Plant. 58, 709–716.

Aziz, H., Sabir, M., Ahmad, H.R., Aziz, T., Zia-ur-Rehman, M., Hakeem, K.R., Ozturk, M., 2015. Alleviating effect of calcium on nickel toxicity in rice. Clean. - Soil, Air, Water 43, 787–966.

Batista, T.B., Cardoso, E.D., Binotti, F.F.S., Costa, E., Sa, M.E., 2016. Priming and stress under high humidity and temperature on the physiological quality of *Brachiaria brizantha* cv. MG-5 seeds. Acta Sci. 38 1237–127.

Baudouin, E., Poilevey, A., Hewage, N.I., Cochet, F., Puyaubert, J., Bailly, C., 2016. The significance of hydrogen sulfide for Arabidopsis seed germination. Front. Plant Sci. 7, 1–8.

Ben Massoud, M., Karmous, I., El Ferjani, E., Singh, A.C.A., Sagar, S., Dipul Kumar Biswas, D.K., 2017. Calcium dependent protein kinase, a versatile player in plant stress management and development. CRC Crit. Rev. Plant Sci. 36, 336–352.

Channuntapipat, C., Sedgley, M., Collins, G., 2001. Sequences of the cDNAs and genomic DNAs encoding the S1, S7, S8, and Sf alleles from almond, *Prunus dulcis*. Theor. Appl. Genet. 103, 1115–1122.

Chen, K., Arora, R., 2013. Priming memory invokes seed stress-tolerance. Environ. Exp. Bot. 94, 33–45.

Chen, J., Wu, F.H., Wang, W.H., Zheng, C.J., Lin, G.H., Dong, X.J., He, J.X., Pei, Z.M., Hai-Lei Zheng, H.L., 2011. Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in *Spinacia oleracea* seedlings. J. Exp. Bot. 62, 4481–4493.

Chen, Z., Yang, B., Hao, Z., Zhu, J., Zhang, Y., Xu, T., 2018. Exogenous hydrogen sulfide ameliorates seed germination and seedling growth of cauliflower under lead stress and its antioxidant role. J. Plant Growth Regul. 37, 5–15.

Crisp, P.A., Ganguly, D., Eichten, S.R., Borevitz, J.O., Pogson, B.J., 2016. Reconsidering plant memory: intersections between stress recovery, RNA turnover, and epigenetics. Sci. Adv. 2, e1501340.

Fang, H.H., Jing, T., Liu, Z.Q., Zhang, L., Jin, Z., Pei, Y., 2014. Hydrogen sulfide interacts with calcium signaling to enhance the chromium tolerance in *Setaria italica*. Cell Calcium 56, 472–481.

Foyer, C.H., Halliwell, B., 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133, 21–25.

Gonzalez, A., Cabrera M de, L., Henriquez, M.J., Contreras, R.A., Morales, B., Moenne, A., 2012. Cross talk among calcium, hydrogen peroxide, and nitric oxide and activation of gene expression involving calmodulins and calcium-dependent protein kinases in *Ulva compressa* exposed to copper excess. Plant Physiol. 158, 1451–1462.

Guo, H., Xiao, T., Zhou, H., Xie, Y., Shen, W., 2016. Hydrogen sulfide: a versatile regulator of environmental stress in plants. Acta Physiol. Plant. https://doi.org/10.1007/s11738-015-2038-x.

He, Z., Li, J., Zhang, H., Ma, M., 2005. Different effects of calcium and lanthanum on the expression of phytochelatin synthase gene and cadmium absorption in *Lactuca sativa*. Plant Sci. 168, 309–318.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.

Huang, D., Gong, X., Liu, Y., Zeng, G., Lai, C., Bashir, H., Zhou, L., Wang, D., Xu, P., Cheng, M., Wan, J., 2017. Effects of calcium at toxic concentrations of cadmium in plants. Planta 245, 863–873.

Huang, T.L., Nguyen, Q.T., Fu, S.F., Lin, C.Y., Chen, Y.C., Huang, H.J., 2012. Transcriptomic changes and signalling pathways induced by arsenic stress in rice roots. Plant Mol. Biol. 80, 587–608.

Huang, T.L., Huang, L.Y., Fu, S.F., Trinh, N.N., Huang, H.J., 2014. Genomic profiling of rice roots with short-and long-term chromium stress. Plant Mol. Biol. 86, 157–170.

Kaczmarek, M., Fedorowicz-Stronska, O., Głowacka, K., Waskiewicz, A., Sadowski, J., 2017. $CaCl_2$ treatment improves drought stress tolerance in barley *Hordeum vulgare* L. Acta Physiol Plant 39, 41.

Khodabakhsh, F., Amooaghaie, R., Mostajeran, A., 2014. Effect of hydro- and osmo-priming on membrane deterioration, proline accumulation and H_2O_2 scavenging enzymes in two salt stressed chickpea cultivars. Environ. Eng. Manage. J. 13, 619–626.

Knapp, J.L., Osborne, J.L., 2017. Courgette production: pollination demand, supply, and value. J. Econ. Entomol. 110, 1973–1979.

Kolupaev, Y.E., Firsova, E.N., Yastreb, T.O., Lugovaya, A.A., 2017. The participation of calcium ions and reactive oxygen species in the induction of antioxidant enzymes and heat resistance in plant cells by hydrogen sulfide donor. Appl. Biochem. Microbiol. 53, 573–579.

Li, Z.G., 2015. Quantification of hydrogen sulfide concentration using methylene blue and 5, 5-dithiobis (2-NitrobenzoicAcid) methods in plants. Methods Enzymol. 554, 101–110.

Li, H., Cheng, Z., 2014. Hoagland nutrient solution promotes the growth of cucumber seedlings under light-emitting diode light. Acta Agric. Scand. Sect. B Soil Plant Sci 65, 74–82.

Li, Z.G., Gong, M., Liu, P., 2012. Hydrogen sulfide is a mediator in H_2O_2-induced seed germination in *Jatropha curcas*. Acta Physiol. Plant. 34, 2207–2213.

Li, Z.G., Min, X., Zhou, Z.H., 2016. Hydrogen sulfide: a signal molecule in plant cross-adaptation. Front. Plant Sci. 7, 1621.

Lichtenthaler, H., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.

Lopez-Climent, M.F., Arbona, V., Perez-Clemente, R.M., Zandalinas, S.I., Gomez-Cadenas, A., 2014. Effect of cadmium and calcium treatments on phytochelatin and glutathione levels in citrus plants. Plant Biol. 16, 79–87.

Lutts, S., Kinet, J.M., Bouharmont, J., 1995. Changes in plant response to NaCl during development of rice *Oryza sativa* L. varieties differing in salinity resistance. J. Exp. Bot. 46, 1843–1852.

Madany, M.M.Y., Khalil, R.R., 2017. Seed priming with ascorbic acid or calcium chloride mitigates the adverse effects of drought stress in sunflower *Helianthus annuus* L. seedlings, Egypt. J. Exp. Biol. 13, 119–133.

Min, H.L., Cai, S.J., Rui, Z., Sha, S., Xie, K.B., Xu, Q.S., 2013. Calcium-mediated enhancement of copper tolerance in *Elodea canadensis*. Biol. Plant. 57, 365–369.

Mohammadpour, G.H., Karbassi, A., Baghvand, A., 2016. Pollution intensity of nickel in agricultural soil of Hamedan region. Casp. J. Environ. Sci. 14, 15–24.

Moron, M.S., Depierre, J.W., Mannervik, B., 1979. Levels of glutathione, glutathione reductase and glutathione- S-transferase activities in rat lung and liver. Biochem. Biophys. Acta 582, 67–78.

Mostofa, M.G., Rahman, A., Ansary, M.M., Watanabe, A., Fujita, M., Tran, L.S.P., 2015. Hydrogen sulfide modulates cadmium-induced physiological and biochemical responses to alleviate cadmium toxicity in rice. Sci. Rep. 5, 14078.

Mukta, R.H., Khatun, M.R., Nazmul Huda, A.K.M., 2019. Calcium induces phytochelatin accumulation to cope with chromium toxicity in rice *Oryza sativa* L. J. Plant Interact. 14, 295–302.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.

Nouairi, I., Jalali, K., Zribi, F., Barhoumi, F., Zribi, K., Mhadhbi, H., 2019. Seed priming with calcium chloride improves the photosynthesis performance of faba bean plants subjected to cadmium stress. Photosynthetica 57, 438–445.

Qian, P., Sun, R., Ali, B., Gill, R., Xu, L., Zhou, W., 2013. Effects of hydrogen sulfide on growth, antioxidative capacity, and ultrastructural changes in oilseed rape seedlings under aluminum toxicity. J. Plant Growth Regul. 33, 526–538.

Ramel, F., Mialoundamd, A.S., Havaux, M., 2012. Nonenzymic carotenoid oxidation and photooxidative stress signaling in plants. J. Exp. Bot. 64, 799–805.

Rizwan, M., Imtiaz, M., Dai, Z., Mehmood, S., Adeel, M., Liu, J., Tu, S., 2017. Nickel stressed responses of rice in Ni subcellular distribution, antioxidant production, and osmolyte accumulation. Environ. Sci. Pollut. Res. 24 20587–20598.

Rizwan, M., Mostofa, M.G., Ahmad, M.Z., Zhou, Y., Adeel, M., Mehmood, S., Ahmad, M.A., Javed, R., Imtiaz, M., Aziz, O., Ikram, M., Tu, S., Liu, Y., 2019. Hydrogen sulfide enhances rice tolerance to nickel through the prevention of chloroplast damage and the improvement of nitrogen metabolism under excessive nickel. Plant Physiol. Biochem. 138, 100–111.

Roe, J.H., Kuether, C.A., 1943. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem. 147, 399.

Savvides, A., Ali, S., Tester, M., Fotopoulos, V., 2016. Chemical priming of plants against multiple abiotic stresses: mission possible? Trends Plant Sci. 21, 329–340.

Shan, C., Dai, H., Sun, Y., 2012. Hydrogen sulfide protects wheat seedlings against copper stress by regulating the ascorbate and glutathione metabolism in leaves. AJCS 6, 248–254.

Shi, S., Li, S., Mao, M. Asim J., Xu, D., Ullah, Z., Liu, G., Wang, Q., Liu, H., 2018. The Arabidopsis calcium-dependent protein kinases (CDPKs) and their roles in plant growth regulation and abiotic stress responses. Int. J. Mol. Sci. 19, 1900.

Siddiqui, M.H., Al-Whaibi, M.H., Basalah, M.O., 2011. Interactive effect of calcium and gibberellin on nickel tolerance in relation to antioxidant systems in *Triticum aestivum* L. Protoplasma 248, 503–511.

Singh, V.P., Singh, S., Kumar, J., Prasad, S.M., 2015. Hydrogen sulfide alleviates toxic effects of arsenate in pea seedlings through up-regulation of the ascorbate-glutathione cycle: possible involvement of nitric oxide. J. Plant Physiol. https://doi.org/10.1016/j.jplph.2015.03.015.

Singh, S., Parihar, P., Singh, R., Singh, V.P., Prasad, S.M., 2016. Heavy metal tolerance in plants: role of transcriptomics, proteomics, metabolomics and Ionomics. Front. Plant Sci. 6, 1143.

Sun, Y., Luo, W., 2013. Effects of exogenous hydrogen sulphide on the seed germination of pumpkin under NaCl stress. J. Food Agric. Environ. 11, 1097–1100.

Sun, Y.D., Luo, W.R., 2014. Effects of exogenous hydrogen sulfide on seed germination and seedling growth of cucumber *Cucumis sativus* under sodium bicarbonate stress. Seed Sci. Technol. 42, 126–131.

Talukdar, D., 2012. Exogenous calcium alleviates the impact of cadmium-induced oxidative stress in *Lens culinaris* Medic. seedlings through modulation of antioxidant enzyme activities. J. Crop Sci. Biotech. 15, 325–334.

Vivek, P.J., Tuteja, N., Soniya, E.V., 2013. CDPK1 from ginger promotes salinity and drought stress tolerance without yield penalty by improving growth and photosynthesis in *Nicotiana tabacum*. PLoS One 8, e76392.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511.

Wilkins, K.A., Matthus, E., Swarbreck, S.M., Davies, J.M., 2016. Calcium-mediated abiotic stress signaling in roots. Front. Plant Sci. 7, 1296.

Yusulf, M., Fariduddin, Q., Hayat, S., Ahmad, A., 2011. Nickel: an overview of uptake, essentiality and toxicity in plants. Bull. Environ. Contam. Toxicol. 86, 1–17.

Zanganeh, R., Jamei, R., Rahmani, F., 2018. Impacts of seed priming with salicylic acid and sodium hydrosulfide on possible metabolic pathway of two amino acids in maize plant under lead stress. Mol. Biol. Res. Commun. 7, 83–88.

Zeng, H., Xu, L., Singh, A., Wang, H., Du, L., Poovaiah, B.W., 2015a. Involvement of calmodulin and calmodulin- like proteins in plant responses to abiotic stresses. Front. Plant Sci. 6, 600.

Zeng, H., Xu, L., Singh, A., Wang, H., Du, L., Poovaiah, B.W., 2015b. Involvement of calmodulin and calmodulin- like proteins in plant responses to abiotic stresses. Front. Plant Sci. 6, 600.

Zhu, C.Q., Zhang, J.H., Sun, L.M., Zhu, L.F., Abliz, B., Hu, W.J., Zhong, C., Bai, Z.G., Sajid, H., Cao, X.C., Jin, Q.Y., 2018. Hydrogen sulfide alleviates aluminum toxicity via decreasing apoplast and symplast Al contents in rice. Front. Plant Sci. 9, 294. https://doi.org/10.3389/fpls.2018.00294.