

# Inhibition of root growth by narciclasine is caused by DNA damage-induced cell cycle arrest in lettuce seedlings

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**Abstract** Narciclasine (NCS) is an Amaryllidaceae alkaloid isolated from *Narcissus tazetta* bulbs. Its phytotoxic effects on plant growth were examined in lettuce (*Lactuca sativa* L.) seedlings. Results showed that high concentrations (0.5–5  $\mu\text{M}$ ) of NCS restricted the growth of lettuce roots in a dose-dependent manner. In NCS-treated lettuce seedlings, the following changes were detected: reduction of mitotic cells and cell elongation in the mature region, inhibition of proliferation of meristematic cells, and cell cycle. Moreover, comet assay and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay indicated that higher levels NCS (0.5–5  $\mu\text{M}$ ) induced DNA damage in root cells of lettuce. The decrease in meristematic cells and increase in DNA damage signals in lettuce roots in responses to NCS are in a dose-dependent manner. NCS-induced reactive oxygen species accumulation may explain an increase in DNA damage in lettuce roots. Thus, the restraint of root growth is due to cell cycle arrest which is caused by NCS-induced DNA damage. In addition, it was also found that NCS (0.5–5  $\mu\text{M}$ ) inhibited the root hair development of lettuce seedlings. Further investigations on the underlying mechanism revealed that both auxin and ethylene signaling pathways are involved in the response of root hairs to NCS.

**Keywords** DNA damage · Mitotic index · Reactive oxygen species · Root hair · TUNEL assay

## Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
DAPI	4',6-diamidino-2-phenylindole
DCFH-DA	2,7-dichlorofluorescein diacetate
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HU	Hydroxyurea
IAA	Indole-3-acetic acid
LMP	Low-melting-point
NCS	Narciclasine
NMP	Normal-melting-point
OTM	Olive tail moment
O <sup>2-</sup>	Superoxide ion
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

## Introduction

Plants in the Amaryllidaceae family are a group of herbaceous perennials including ca. 85 genera and 1,100 species that are widely distributed over the tropical and temperate regions in the world. For many centuries, they have not only been cultivated as ornamental plants for their colorful flowers and the production of volatile oils but also are extensively used as herbal medicines against various diseases in many countries as their pharmacological effects are frequently associated with several typical alkaloids they synthesize (Kornienko and Evidente 2008; Evidente and Kornienko 2009). A large number of natural products, including alkaloids, phenolic compounds, flavonoids, and glycosides, are produced in plants of

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this family. As the primary constituents, Amaryllidaceae alkaloids are frequently associated with significant pharmacological effects and are found to possess several important biological activities such as acetylcholinesterase inhibitory activity, cytotoxicity, and antitumor activity (Bastida et al. 2006; Berkov et al. 2008; Zupkó et al. 2009). The two prominent compounds are galanthamine, which is used in the treatment of Alzheimer's disease (Pearson 2001), and pancratistatin, which possesses a cytostatic effect (Kekre et al. 2005). Because of their potential use in medicine, these compounds are extensively studied in medicine, chemistry, and biology (Kornienko and Evidente 2008).

Narciclasine (NCS) is an Amaryllidaceae alkaloid isolated from *Narcissus tazetta* bulbs and also exists in the genera *Galanthus*, *Haemanthus*, *Leucojum*, *Pancratium*, *Sprekelia*, *Sternbergia*, and *Vallota* (Piozzi et al. 1969). Previous studies showed that NCS possesses antimetabolic (Ceriotti 1967) and antiviral functions (Gabrielsen et al. 1992), inhibits protein synthesis in rabbit reticulocyte and yeast cell-free systems (Carrasco et al. 1975), induces marked apoptosis-mediated cytotoxicity in certain human cancer cells but not in normal fibroblasts (Dumont et al. 2007), and increases the GTPase RhoA activity as well as induces actin stress fiber formation in glioblastoma multiform cells (Lefranc et al. 2009). These studies on NCS mainly focus on animal systems and their cytotoxicity, but show little interest in their function in plants. Our preliminary studies demonstrated that NCS exhibits a wide range of inhibitory effects on plant growth including seed germination and seedling growth in rice, Chinese cabbage, and *Arabidopsis* (Bi et al. 1998; Na et al. 2011) and on chloroplast development of excised radish cotyledons (Bi et al. 2003). In particular, in NCS-treated *Arabidopsis* seedlings, the inhibition of root growth correlates with a decrease in root meristem cell division by targeting mitotic cell cycle specific/cyclin complexes (Na et al. 2011). Our current knowledge of the physiological and biochemical effects of NCS suggests that it may have multiple potential metabolic targets in plants. However, little is known about primary phytotoxic effects of NCS on plant growth.

Although NCS is a plant growth inhibitor, it is interesting that NCS does not exhibit significant inhibitory effects on *N. tazetta* growth even at high concentrations (data not shown). This reminds us of the allelopathy as phenomenon occurring in natural ecosystems. Allelopathy is defined as plant to plant, plant to microorganism, and microorganism to microorganism interaction by chemicals belonging to secondary metabolites (allelochemicals). Allelochemicals secreted by plants are released into the environment and usually elicit either detrimental or beneficial effects on themselves and acceptor organisms (Inderjit and Callaway 2003; Gniazdowska and Bogatek 2005). Allelochemicals may directly affect many physiological and biochemical

reactions and, thereby, regulate the growth and development of plant organs (Weir et al. 2004). In past decades, big efforts have been made to elucidate physiological effects of allelochemicals in acceptor plants. Many allelochemicals, produced by numerous species, are already known but in most cases, mechanisms of their action remain unknown. Because of the special cultivation of Amaryllidaceae family as ornamental plants, whether NCS mediates interactions as an allelochemical in the natural surroundings is still unknown.

Plant growth is ultimately driven by the process of cell division coupled with the subsequent expansion and differentiation of resulting cells (Jakoby and Schnittger 2004). The growth-limiting effects of various biotic and abiotic stresses to which seedlings may be subject have been associated with the regulation of elongation and cell division (Peres et al. 2007). In plants, excess reactive oxygen species (ROS) reduces growth and delays development, and a role for oxidative stresses in triggering DNA damage in various tissues and organs during plant development has been well established (Roldán-Arjona and Ariza 2009; Vanderauwera et al. 2011). Moreover, it has been confirmed that oxidative stresses restrained root growth via DNA damage-induced cell cycle arrest and cell division reduction (Den Boer and Murray 2000; Hefner et al. 2006). Previous studies have shown that a variety of natural products act as inhibitors of cell division in root tips (Ding et al. 2008, 2010; Sánchez-Moreiras et al. 2008; Soltys et al. 2011; Teerarak et al. 2012). There are only a few papers describing the relationship between oxidative stresses induced-DNA damage and cell cycle retardation in plant cells after treatment with natural products.

In the work reported here, we performed conventional cytological observations of root tip cells that allowed us to analyze modifications of cell division and investigated the cell cycle activity of root tip meristem cells by flow cytometry in NCS-treated lettuce seedlings. The extent of DNA damage in root tip cells was examined by comet assay and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to elucidate the mechanism of NCS phytotoxic effects on lettuce seedlings. Oxidative damage caused by NCS was determined by monitoring hydrogen peroxide ( $H_2O_2$ ) free radical and superoxide ion ( $O_2^-$ ) accumulation. Because root hairs can increase the effective surface area available for nutrient and water uptake, we also evaluated the phytotoxic influence of NCS on root hair development. Based on our experimental findings, we established a link between an easily observable phytotoxic effect of NCS and a cellular-based explanation. It demonstrated that inhibition of lettuce root growth by NCS is mainly due to cell cycle arrest caused by oxidative stresses induced-DNA damage in root tips as well as inhibition of root hair development.

## Methods and materials

### Plant materials and culture conditions

Lettuce (*Lactuca sativa* L., Taiyuan) was used as the material in the present work. Lettuce seeds were surface-sterilized in 15 % bleach for 8 min and extensively rinsed with autoclaved water. Seeds were then placed on two sheets of filter paper soaked with distilled water in Petri dishes (9 cm diameter) and allowed to germinate for 48 h at 22 °C under a 16h-light/8h-dark photoperiod and photosynthetic photon flux density of 100–120  $\mu\text{M m}^{-2} \text{s}^{-1}$ . Two-day-old plants in uniform size were selected and transferred into Petri dishes containing various concentrations of NCS treatment solutions or distilled water (control) for 48 h.

NCS was isolated and purified previously in our laboratory from *N. tazetta* bulbs according to Bi et al. (1998). NCS was dissolved in DMSO and diluted to the desired concentrations with distilled water. After 48 h treatment, root elongation of lettuce seedlings was determined to evaluate the influence of NCS on root growth. The seminal root lengths of the seedlings in each treatment were measured before and after treatments, and root elongation was calculated as follows: final length–initial length.

### Measurement of cell size

For the measurement of cell size, root tips (8 mm long) excised from 2-day-old seedlings after 48 h of NCS treatment were fixed for 6 h at room temperature in an ethanol/acetic acid (9:1) solution and then sequentially rinsed with a series of ethanol solution of 90, 70, 50, and 30 %. Root tips were cleared with a chloral hydrate/glycerol/water solution (8:1:2, w/v/v) as described by Yadegari et al. (1994). Root tips were mounted in 50 % glycerol on microscopic glass slides and were photographed under the Leica CME compound microscope (Leica Microsystems, Wetzlar, Germany). Cells in the root hair-forming region of the roots were chosen to determine the mature cell size. The length and width of 40 mature cells were measured from each root, with six roots per treatment. All measurements were repeated for three times.

### Mitotic index

Mitotic index and changes in chromosome morphology were determined using the conventional acetocarmine squash method as follows. After 48 h treatment with NCS, distal fragments of primary roots (1 cm long) were cut off and fixed in freshly prepared glacial acetic acid/ethanol (3:1, v/v) for 24 h. The root segments were washed three times for 15 min each in 70 % ethanol and then macerated for 25 min with 1 N HCl at 60 °C. Root tips (1 mm) were excised and stained for 10 min with Schiff's reagent, after which the root tips were immersed in a

little acetic acid, and then the meristems were squashed under a coverslip to separate their cells. For each treatment, ten root tips from three biological replicates were analyzed, and at least 1,000 cells were randomly examined under a microscope to determine the mitotic index, which was calculated as the number of dividing cells per 1,000 observed cells.

### Measurements of root hairs

For evaluation of root hairs, a 1-cm root segment was excised from the primary root tip after NCS treatment, fixed in 70 % ethanol for 2 h, and then placed onto a microscope slide for microscopic observation. The length and the density of root hairs present in a 500- $\mu\text{m}$  region were measured from the ends and the middle of root segments with an ocular micrometer. Measurements for each treatment were determined from ten root segments. Each experiment was repeated at least three times and data from representative, individual experiments were presented.

### Cell cycle analysis

Samples were prepared as previously described (Sánchez-Moreiras et al. 2008). Briefly, seedlings grown in dark for 24 h on moistened filter paper at 22 °C were transferred to Petri dishes containing filter paper moistened with 2.5 mM hydroxyurea (HU) (pH 6.0). After 6 h incubation in dark at 22 °C, seedlings were washed three times in distilled water (pH 6.0) to remove the inhibitor HU. Seedlings were then transferred to Petri dishes containing filter papers moistened with distilled water (pH 6.0, controls) or 5  $\mu\text{M}$  NCS solution and incubated for indicated times in dark. Samples were taken every 3 h.

The cell cycle was analyzed according to the method of Sánchez-Moreiras et al. (2008) with a few modifications. The apical root meristem (2 mm long) of each seedling was removed and chopped with a razor blade on clean Petri dishes containing 700  $\mu\text{l}$  Galbraith's nuclear buffer (45 mM  $\text{MgCl}_2$ , 30 mM sodium citrate, 20 mM MOPS, 0.1 % Triton X-100; pH 7.0) supplemented with 100 % Tween-20 (8  $\mu\text{l}$  in 3 ml buffer) and 100 %  $\beta$ -mercaptoethanol (7  $\mu\text{l}$  in 1 ml buffer). The obtained suspension was filtered twice through 30  $\mu\text{m}$  mesh nylon cell filters, and the filtrate (400  $\mu\text{l}$ ) was collected in an Eppendorf tubes, treated with 5  $\mu\text{l}$  of 1 % RNase A solution. Immediately, the nuclei suspension was stained with propidium iodide (10  $\mu\text{g/ml}$ ) for 15 min at room temperature before cell DNA contents were evaluated by Epics XL-4 flow cytometer (Beckman-Coulter, USA) using a Coulter Elite apparatus with a 488-nm excitation laser. For each sample, at least 10,000 nuclei were analyzed. Histograms were analyzed using a DPAC v.2.2 computer program (Partec). Analyses were performed on five biological replicates.

### Measurement of ROS production

NCS-induced  $H_2O_2$  accumulation in root tips was monitored using the fluorescent probe DCFH-DA (Sigma). Two-day-old lettuce seedlings were treated with NCS for 48 h, the treated roots were incubated with 10  $\mu$ M DCFH-DA in phosphate-buffered saline (PBS) buffer for 30 min at 37 °C, washed three times in PBS buffer, and then viewed with an Olympus BX53 fluorescence microscope. At least 20 root tips were scored for each sample in each experiment.

$O_2^-$  levels were monitored by staining for 20 min in a solution of 2 mM NBT in 20 mM PBS (pH 6.1). The reaction was stopped by transferring the seedlings into distilled water.  $O_2^-$  content was quantified using the method of Ramel et al. (2009). The NBT-stained plantlets were ground in liquid nitrogen; the obtained powder was solubilized in 2 M KOH-dimethyl sulfoxide (1:1.16, v/v) and then centrifuged for 10 min at 12,000 g. The  $A_{630}$  was immediately measured and compared with a standard curve obtained from known amounts of NBT in the KOH-dimethyl sulfoxide mixture.

### Comet assay

Isolation of nuclei from root tips and comet assay were performed following the methods of Gichner et al. (2008) and Ding et al. (2010) with slight modifications. After incubating for 48 h, 80 seedlings of each treatment were used for the nuclei isolation and comet assay. The apical 2-mm long root tip of each seedling was removed and placed in a Petri dish kept on ice and spread with 250  $\mu$ l of cold 400 mM Tris buffer (pH 7.5). Using a fresh razor blade, the root tips were gently sliced. The plate was kept tilted in the ice so that the isolated root nuclei would be collected in the buffer. Immediately, the obtained suspension was filtered twice through 30  $\mu$ m mesh nylon cell filters, and the filtrate (200  $\mu$ l) was collected in Eppendorf tubes. All operations were conducted under dim or yellow light.

Frosted microscope slides were coated with 0.5 % normal-melting-point (NMP) agarose prepared with water at 50 °C, dried overnight at room temperature, and kept dry in slide boxes until use. The nuclear suspension (50  $\mu$ l) and 1 % low-melting-point (LMP) agarose (50  $\mu$ l) prepared with PBS were added onto each slide at 40 °C. The nuclei and the LMP agarose were gently mixed by repeated pipetting using a cut micropipette tip, and a coverslip was placed on the mixture. The slides were kept at 4 °C for 15 min for agarose to solidify. After removing the cover glass, the slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM  $Na_2EDTA$  and 300 mM NaOH, pH >13). The nuclei were incubated for 15 min to allow the DNA to unwind prior to electrophoresis at 0.74 V/cm (25 V, 300 mA) for 25 min at 4 °C. After electrophoresis, the slides were rinsed three times with

400 mM Tris buffer (pH 7.5), stained with 50  $\mu$ l EtBr (20  $\mu$ g/ml) for 5 min, dipped in ice-cold water to remove the excess stain, and covered with a coverslip. The nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm.

Digital images were captured and analyzed using CASP image-analysis program (Końca et al. 2003). Thirty randomly chosen nuclei were analyzed for each slide. Three slides were evaluated per treatment, and each treatment was repeated three times. From the repeated experiments, the average median percentage of tail DNA (% tail DNA) and Olive tail moment [OTM, the product of the distance between the gravity centers of the DNA head and the DNA tail ( $LX_{Gravity}$ ) and percent tail DNA ( $Tail_{DNA}$ ),  $OTM=LX_{Gravity} \times Tail_{DNA}$ ] (Liu et al. 2004) were used to measure DNA damage for each treatment group.

### TUNEL assay

To detect the DNA damage in the NCS-treated roots, an in situ Cell Death Detection Kit (DeadEnd colorimetric TUNEL system, Promega) was used. The TUNEL assay was performed according to the manufacturer's instructions with a few modifications. In brief, the roots were fixed in 4 % paraformaldehyde in PBS (pH 7.4) at 20 °C for 1 h. After washing three times in PBS buffer for 10 min, the samples were incubated in permeabilization solution (0.1 % Triton X-100, 0.1 % sodium citrate) for 30 min (4 °C), followed by three washes with PBS buffer for 10 min. After washing, the roots were incubated at 37 °C for 90 min with TUNEL reaction mixture in a humidified atmosphere in the dark. Finally, the roots were examined with a fluorescence microscope (excitation/emission maxima ~475/540 nm) (Olympus FV1000). Experiments were carried out in three replicates; at least 20 root tips were scored for each sample in each experiment.

### Statistical analysis

Each experiment was repeated at least three times. Values were expressed as mean  $\pm$  SD. For all experiments, the overall data were statistically analyzed in the SPSS version 17.0 (SPSS). All comparisons were performed using one-way analysis of variance (ANOVA) and Duncan's multiple range tests for independent samples. In all cases, the confidence coefficient was set at  $p < 0.05$ .

## Results

### Effects of NCS on root growth in lettuce seedlings

The use of pre-germinated seedlings in growth bioassays was a great advance to detect effects of phytotoxic compounds on growth processes avoiding previous resistance (Sánchez-



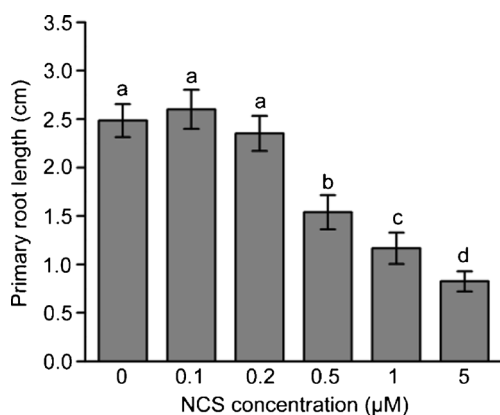
Moreiras et al. 2008). In this study, 2-day-old pre-germinated lettuce seedlings were used to evaluate phytotoxic effects of NCS on root growth. As shown in Fig. 1, the root growth of lettuce seedlings responded to NCS in a dose-dependent manner in 48 h treatment. No significant differences in root length were observed in roots treated with 0.1 and 0.2  $\mu\text{M}$  NCS. However, high concentration of NCS (0.5–5  $\mu\text{M}$ ) resulted in a marked reduction of the radicle length. The inhibition rates ranged from 20.69 to 59.43 %, as compared to the control (Fig. 1).

#### NCS modifies the size of root cells

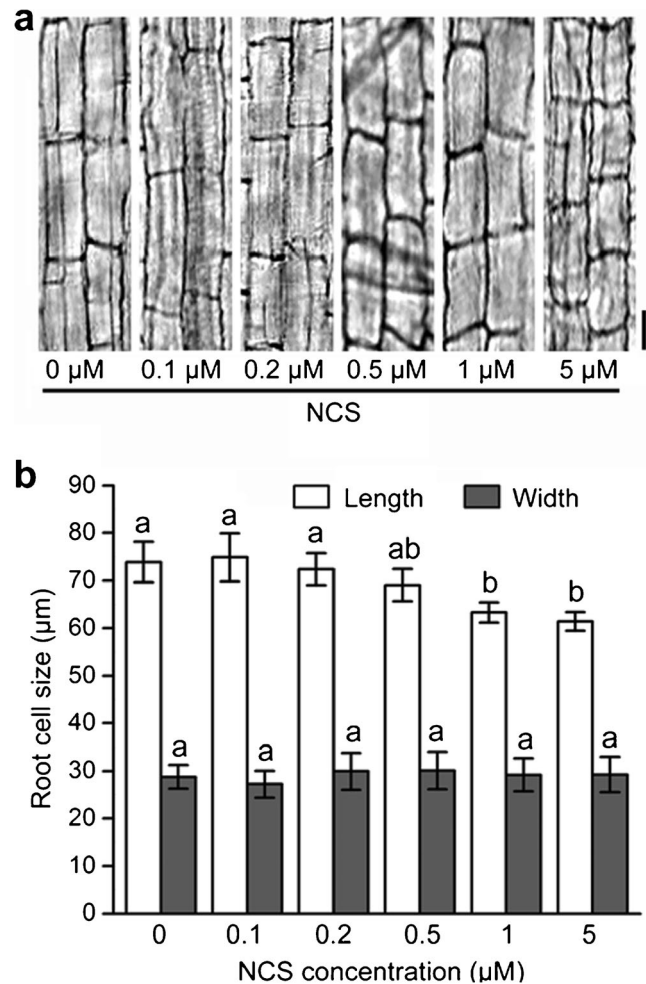
To examine whether NCS affects root cell elongation, the sizes (length and width) of mature cells were determined in the root-hair-forming regions of lettuce roots (Fig. 2). Cell images showed that the cell length, but not the width, of mature cells was significantly affected by NCS (Fig. 2a). The effects of NCS on cell length were consistent with the pattern of root elongation inhibition. Compared to the control, the cell length was significantly reduced by high concentration of NCS (1–5  $\mu\text{M}$ ) treatments (Fig. 2b).

#### Effects of NCS on the mitotic activity of root tips

In order to test whether the inhibitory effects of NCS on lettuce root growth involve alterations in cell division of root tips, we determined the mitotic activity of the root apical meristem. Mitotic observation of root tip cells in lettuce seedlings showed mitotic depression and alteration in proportion of mitotic phase index, as illustrated in Fig. 3. The mitotic index of root tip cells treated with high concentrations (0.5–5  $\mu\text{M}$ ) of NCS exhibited marked decreases compared to the controls (Fig. 3a). The cell numbers in prophase (Fig. 4a), metaphase (Fig. 4b), anaphase (Fig. 4c), and telophase (Fig. 4d) were also counted. Results indicated that NCS



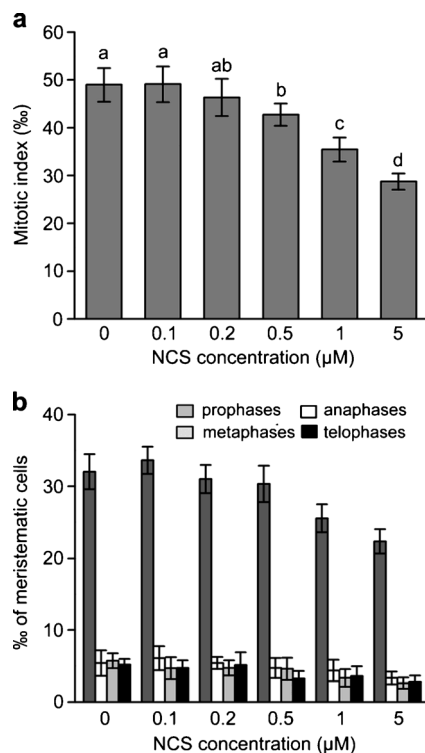
**Fig. 1** Effects of NCS on the root growth of lettuce seedlings at 48 h of treatment. Values are mean $\pm$ SD of three independent experiments. Bars with different letters indicate statistical differences at  $p < 0.05$



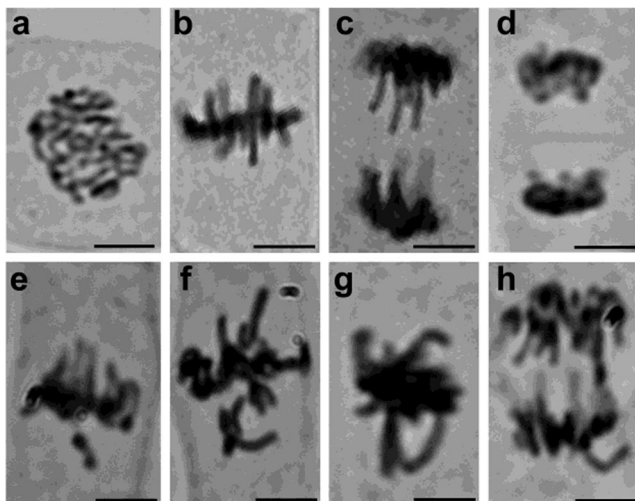
**Fig. 2** Effects of NCS on the size of matured cells in lettuce roots at 48 h treatment. **a** Image represents the length and the width of mature cells in water-treated roots (control) or NCS-treated roots. Bar=25  $\mu\text{m}$ . **b** The length and width of matured cells. Values are mean $\pm$ SD of three independent experiments. Bars with different letters indicate statistical differences at  $p < 0.05$

modified the progressions of various phases of mitosis in meristematic cells with different stages showing different sensitivity to NCS (Fig. 3b). The strongest effect was observed in prophase, in which the change in the number of meristematic cells was similar to the mitotic index, whereas the percentage of the remaining phases did not change significantly, except for 5  $\mu\text{M}$  NCS which resulted in the reduction in the percentages of all phases compared to the control (Fig. 3b).

Moreover, NCS also induced a low incidence of mitotic abnormalities compared to the control in root tips, as shown in Fig. 5. In control root tips, every phase of mitosis and appropriate mitotic figures were observed (Fig. 4a, b, c, d). However, the occurrences of incorrect metaphasal plate and chromosome fragment (Fig. 4e), non-oriented chromosome at metaphase (Fig. 4f), sticky metaphase (Fig. 4g), and anaphase bridge (Fig. 4h) were observed in seedlings treated with NCS.



**Fig. 3** Mitotic activity in root apical meristem cells of lettuce seedling after 48 h of NCS treatment. **a** Mitotic index in root meristem cells of control (non-treated) and NCS-treated lettuce seedling. **b** Percentage of different mitotic phases relative to the total number of meristematic cells. Data are expressed as percentage of 1,000 meristem cells. Values are mean $\pm$ SD of three independent experiments. Bars with different letters indicate statistical differences at  $p < 0.05$



**Fig. 4** Representative photomicrographs showing phases of mitosis in control and NCS-treated root tip meristem cells. Control stages of mitosis: normal prophase (**a**), normal metaphase (**b**), normal anaphase (**c**), and normal telophase (**d**). Chromosome aberrations: chromosome fragment (**e**), non-oriented chromosome at metaphase (**f**), sticky metaphase (**g**), and anaphase bridge (**h**). Scale bar=20  $\mu$ m

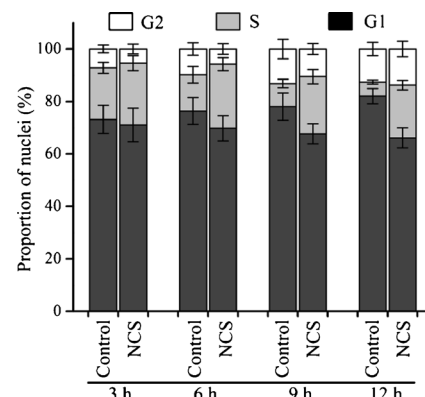
### Effects of NCS on cell cycle progression

To further study the effects of NCS on particular cell cycle progression, cell cycle phase distribution of the root tip cells were measured by flow cytometry. First, cells in root meristems were synchronized using the inhibitor hydroxyurea (HU), which reversibly halts the cell cycle in the G1 and early S phases by inhibiting the ribonucleotide reductase, and have been extensively used for cell cycle studies in plants (Doležel et al. 1999). There was no obvious difference between the control and NCS-treated cells in 3 h after HU removal, suggesting that NCS did not affect cells entry into S phase (Fig. 5). Six hours after depletion of HU, 14.7 % of control cells and 23.2 % of NCS-treated cells were in S phase (Fig. 5), whereas the control cells in G2 phase doubled that of NCS-treated cells. After NCS treatment for 9 and 12 h, more cells remained at S phase compared to the control, indicating that the cell cycle progression was slowed down by NCS treatment, and fewer cells could pass S phase into G2 phase under this condition (Fig. 5).

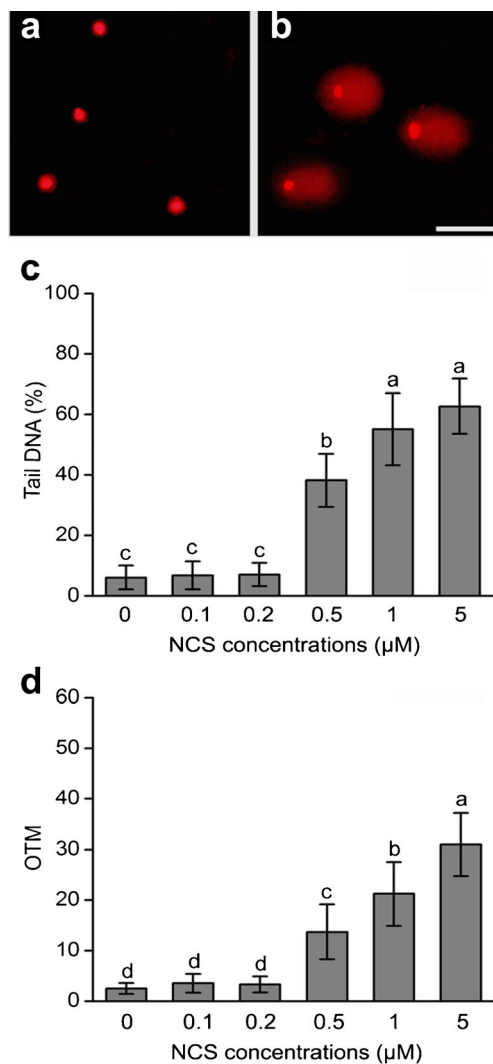
### NCS induced DNA damage

To elucidate whether the cell cycle arrest of root tips involved DNA damage, DNA strand breaks were assessed by comet assay (Fig. 6a). Results indicated a dose-dependent increase in DNA damage in the form of % tail DNA values and average median Olive tail moment (OTM) values by 0.5–5  $\mu$ M NCS. After treatment with NCS (0.5–5  $\mu$ M) for 48 h, comet assay data clearly demonstrated a significant increase in average median % tail DNA values from  $38.24 \pm 8.75$  to  $62.70 \pm 9.07$  % and, in average, median OTM values from  $13.67 \pm 5.42$  pixels to  $30.98 \pm 6.26$  pixels (Fig. 6b, c). There was no significant difference in either % tail DNA or OTM at the lower levels of NCS (0.1–0.2  $\mu$ M).

We used TUNEL assay to further examine whether NCS treatment causes DNA damage in root tips of lettuce



**Fig. 5** Flow cytometric analysis of time course of the cell cycle distribution in root tip cells after 48 h of 5  $\mu$ M NCS treatment. Data shown are mean $\pm$ SD of three independent experiments

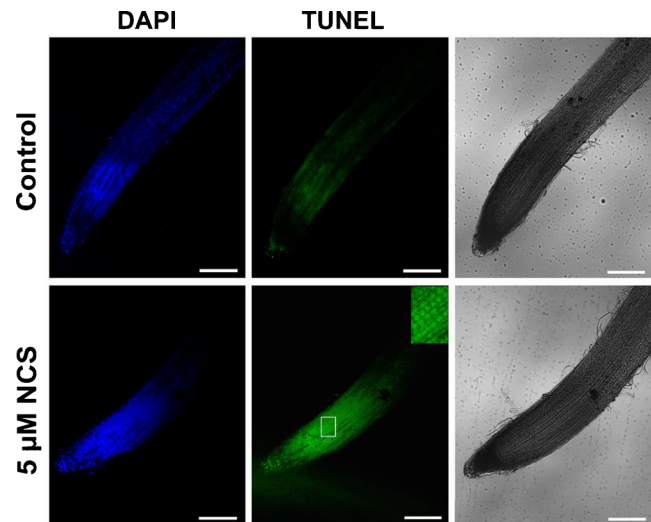


**Fig. 6** Effect of NCS on the DNA damage of lettuce roots through comet assay. Comet character showing tails of different lengths induced by NCS in nuclei of lettuce root cells for a period of 48 h in order to determine the dose-response for DNA damage: control (a) and treated with 5  $\mu\text{M}$  NCS (b). c The average median % tail DNA. d The average median Olive tail moment (OTM). Different letters indicate significant differences at  $p < 0.05$ . Values are mean  $\pm$  SD of three independent experiments. Scale bar = 50  $\mu\text{m}$

seedlings. As shown in Fig. 7, no TUNEL-positive nuclei were present in untreated roots (control), but TUNEL-positive nuclei (green-fluorescent) were observed in NCS-treated lettuce roots, suggesting that nuclear DNA degradation became early evident as a consequence of NCS treatment.

#### NCS induces oxidative stress in roots of lettuce seedlings

In order to investigate the level of oxidative stress induced by NCS treatment in lettuce roots, we examined the production of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  after root treatment with NCS. The 2,7-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe has been successfully used for  $\text{H}_2\text{O}_2$  detection in plants



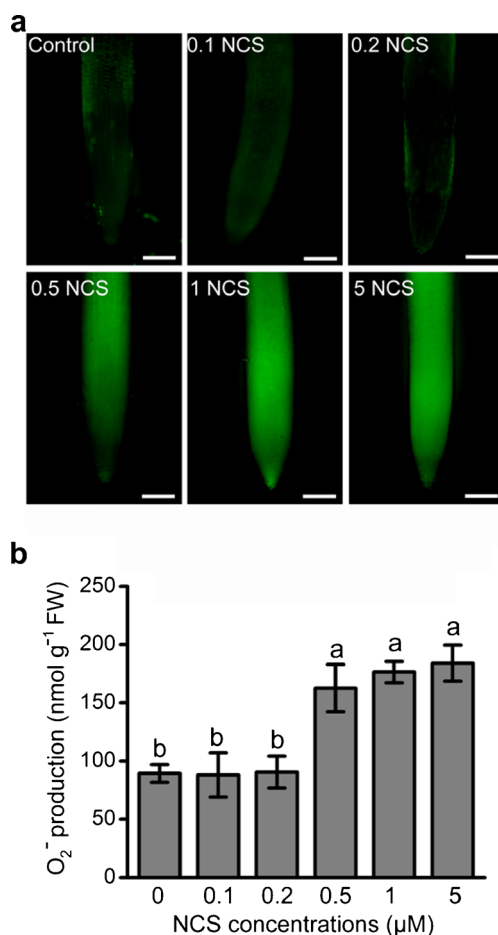
**Fig. 7** NCS-caused DNA damage. 4',6-diamidino-2-phenylindole (DAPI) and TUNEL staining of lettuce roots at 48 h of 5  $\mu\text{M}$  NCS treatment. DAPI staining of nuclei (blue). TUNEL positive nuclei control (green). No TUNEL-positive nuclei are visible in the control, while TUNEL-positive nuclei are evident after treatment with NCS. Scale bars = 50  $\mu\text{m}$

(Ramel et al. 2009). As shown in Fig. 8, high concentrations of NCS (0.5–5  $\mu\text{M}$ ) treatments significantly induced the accumulation of  $\text{H}_2\text{O}_2$  in roots of lettuce seedlings compared with the control (Fig. 8a). Identical results were obtained from  $\text{O}_2^-$  levels monitored by using the method of Ramel et al. (2009). Lower NCS concentrations (0.1 and 0.2  $\mu\text{M}$ ) did not significantly affect the  $\text{O}_2^-$  production, while 0.5  $\mu\text{M}$  or higher NCS concentrations resulted in marked increase in  $\text{O}_2^-$  concentration in roots treated for 2 days (Fig. 8b). These results indicate that oxidative damage is probably the primary effect of NCS in lettuce roots.

#### Effects of NCS on root hair development

In addition to phytotoxic effects of NCS on the root growth, inhibition of root hair development was also observed in NCS-treated lettuce seedlings (Fig. 9). After 48 h of treatment, NCS decreased the average root hair length and root hair density in a dose-dependent manner at concentrations between 0.5 and 5  $\mu\text{M}$  (Fig. 9b). However, low levels of NCS (<0.5  $\mu\text{M}$ ) did not affect root hair growth in terms of density and length compared to controls (data not shown).

Auxin and ethylene have important roles in specifying initiation and growth of root hairs (Pitts et al. 1998). We investigated whether NCS effects on root hair development are involved in auxin and ethylene signaling. Two-day-old lettuce seedlings were transferred to Petri dishes containing NCS alone or together with indole-3-acetic acid (IAA) or 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor of ethylene) for 48 h. In the study, exogenous IAA or ACC alone significantly promoted root hair development, whereas NCS



**Fig. 8** NCS induced ROS production in roots of lettuce seedlings. **a** 2-day-old lettuce seedlings were treated with different concentration of NCS for 48 h, then examined for H<sub>2</sub>O<sub>2</sub> by fluorescence microscopy in roots stained with DCFH-DA. Scale bars=50 μm. **b** O<sub>2</sub><sup>-</sup> production in roots of control (non-treated) or NCS-treated lettuce seedlings was quantified as described in “Methods and materials”. Data represent the mean±SD of three independent experiments. Within each set of experiments, bars with different letters are significantly different at the level of  $p < 0.05$

treatments dramatically inhibited IAA- or ACC- stimulated elongation of root hairs (Fig. 10). These results indicated that NCS also disturbs the responses of root hair development to auxin and ethylene.

## Discussion

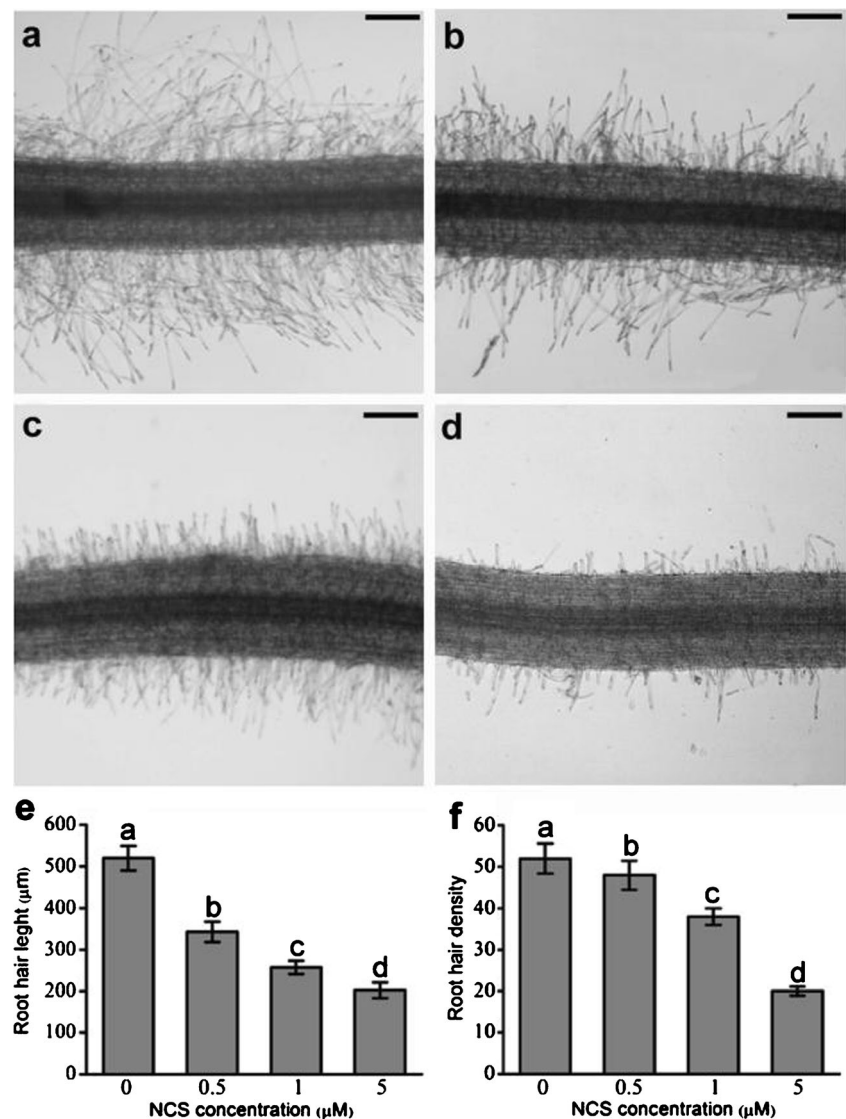
In the present study, we examined the phytotoxic effects of NCS on root growth and root hair development in lettuce seedlings. We previously reported that NCS exhibits significant inhibition on seed germination in rice and Chinese cabbage (Bi et al. 1998). In order to reduce possible complications of NCS on germination, we used pre-germinated lettuce seedlings for treatments. Our results indicated that the inhibitory effect of NCS on root growth is dose-dependent (Fig. 1). This is in line with the observation of Bi et al. (1998) showing that

NCS inhibits the growth of rice radicle by 28.72 % at the concentration of 0.5 μM and by 62.76 % at 1 μM (Bi et al. 1998). Na et al. (2011) reported that 0.1 μM NCS caused 33.40 % inhibition of *Arabidopsis* root growth after 2 days treatment. The comparison of above data suggests that the NCS effect on plant growth depends not only on NCS concentration but also on plant susceptibility. A similar restriction in plant growth after treatment with some particular allelochemicals was also found by many researchers (Ding et al. 2008; Soltys et al. 2011). Although a dual stimulatory and inhibitory effect on plant growth after treatment with specific allelochemicals was detected (Kong et al. 2002; Ding et al. 2010), the stimulatory effect of NCS on plant root growth was not observed in other plants.

The visible growth-limiting effects of various stresses may result from their effects on the regulation of cell elongation and division. We provided evidence that the inhibition of root growth by NCS at 0.5–5 μM strongly correlates with both the decrease of cell sizes in mature regions and the reduction of mitotic index of root meristematic cells (Fig. 2). The mitotic index, which is defined as the number of cells in mitotic phases, can be used as an indicator of cell proliferation. The reduction in the mitotic index reveals that the inhibition of root growth by NCS may be due to the suppressed mitotic activity (Fig. 3a). Similar results have been reported in plants treated with allelochemicals leukamenin E (Ding et al. 2008), xanthoxyline (Charoenying et al. 2010), rhodopsin B (Ding et al. 2010), and cyanamide (Soltys et al. 2011). In addition, our results also demonstrated that NCS causes the alteration in the percentage of cells in the different phases of mitosis (Fig. 3b). The reduction of dividing cells at prophase indicated that NCS could interfere with the sequence of mitotic division and lead to the reduction in the number of cells entering mitotic division. Previous studies have shown that some plant secondary metabolites induce mitotic abnormalities resulting from its action on chromatin organization and the mitotic spindles which are known to block cell cycle progression (Decordier et al. 2008; Teerarak et al. 2012; Soltys et al. 2011). For example, in lettuce seedlings treated with leukamenin E, chromosome stickiness, chromosome bridges, multipolar divisions or C-colchicine metaphase were induced (Ding et al. 2008). As a result of the toxic effect on destruction or incomplete development of mitotic spindles, the appearance of spindle disturbance at late prophase, non-oriented chromosomes at metaphase, delayed anaphase, anaphase bridge, and diagonal at anaphase group occurred in the meristematic cells of *Allium cepa* L. roots treated with ethyl acetate fraction from *Aglaia odorata* (Teerarak et al. 2012). In our experiments, a low incidence of chromosomal aberrations (e.g., chromosome bridges in anaphase) was observed following NCS treatments (Fig. 4), suggesting that this compound might influence the mitotic spindle organization. Those authors also indicated that the allelochemical impact on



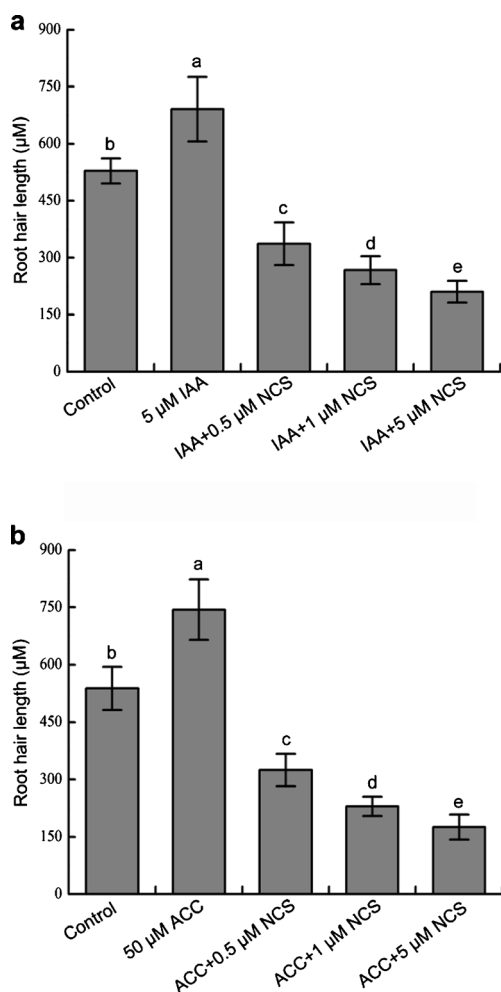
**Fig. 9** Effects of different concentrations of NCS on the root hair growth of lettuce seedlings after 48 h of treatment. **a** Control. **b–e** Treatments with 0.5, 1, and 5  $\mu\text{M}$  NCS, respectively. Effects of NCS on the root hair development of lettuce seedlings were evaluated through root hair length (**f**) and root hair density (**g**). Root hair density was measured as the number of root hairs per 500  $\mu\text{m}$  root segment. Values are mean $\pm$ SD of three independent experiments. Bar=250  $\mu\text{m}$ . Bars with different letters indicate statistical differences at  $p<0.05$



mitosis could be the effect of depolymerization of microtubules which blocks the formation of the mitotic spindle. It is possible that NCS disturbs the structure of microtubules, which could explain abnormal mitosis and also confirm our other cytological observations. However, according to Sánchez-Moreiras et al. (2008), not all allelochemicals result in chromosomal abnormalities or such observations are rare to date. Sánchez-Moreiras et al. (2008) found that treatment of lettuce seedling cultures with 1 mM BOA (2(3H)-benzoxazolinone) reduced the number of cells in mitosis but did not affect the chromosomal structure and orientation.

Induction of oxidative stress by some allelochemicals seems to be a general phenomenon reported previously for many plants (Oracz et al. 2007; Sánchez-Moreiras et al. 2011; Soltys et al. 2011). Recently, it was proved that alteration in ROS production or accumulation in different root zones of *Arabidopsis* might severely influence root growth (Tsukagoshi et al. 2010). Our data also confirmed such a

tendency. NCS treatment resulted in over accumulation of ROS both  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  (Fig. 8). It is possible that NCS-mediated inhibition of lettuce root growth may be due to a rise in  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  levels in the root meristems. ROS-derived DNA oxidation can lead to altered bases and damaged sugar residues, resulting in DNA single- and double-strand breaks (Roldán-Arjona and Ariza 2009). Strand breaks trigger a DNA damage response. Induction of DNA damage by oxidative stresses could provoke cell cycle retardation at G1/S and/or G2/M phases and contribute to the reduction in mitotic index (Den Boer and Murray 2000). Moreover, Hefner et al. (2006) reported that DNA damage-induced cell cycle checkpoints could be initiated in cells of meristematic organs (root and shoot tip) of irradiated *Arabidopsis* seedlings. Therefore, we hypothesized that NCS-mediated inhibition of lettuce roots depends on the general mechanism responsible for the mitotic cycle arrest in cells with damaged DNA (Fusconi et al. 2007). To test this hypothesis directly, the comet assay and TUNEL



**Fig. 10** Effects of NCS on IAA- or ACC-induced root hair promotion. Two-day-old lettuce seedlings were treated with or without IAA (5 µM) and ACC (50 µM) in the presence of different concentrations of NCS for 48 h. **a** Effect of NCS on IAA-induced root hair promotion. **b** Effect of NCS on ACC-induced root hair development. Root hair length in the differentiated zone of the primary roots from 10 seedlings was analyzed. Data represent the mean±SD of three independent experiments. Within each set of experiments, bars with different letters are significantly different at the level of  $p < 0.05$

assay were used to determine whether NCS induces DNA damage in root tips of lettuce seedlings and to elucidate the mechanism of the cell cycle arrest. It was seen clearly in Fig. 6 that higher concentrations of NCS (0.5–5 µM) significantly induced a dose-dependent increase in DNA damage in the form of % tail DNA and OTM values compared to controls. In addition, our results also showed that the nuclear DNA degradation became evident as a consequence of NCS treatment, as demonstrated by the presence of green-fluorescent TUNEL-positive nuclei (Fig. 7). These data are consistent with the supposition that DNA damage of lettuce root tips induced by NCS could result in a decrease in the mitotic activity. When DNA damage takes place in meristems, plants apparently have evolved DNA stress checkpoint mechanisms of arresting the cell cycle and activating the DNA repair

machinery to preserve the genome (Cools and De Veylder 2008).

Root hairs, one of the smallest root structures and projections from root epidermal cells that increase the effective surface area available for nutrient and water uptake, can be used to evaluate the phytotoxic influence of natural compounds on root development. In this study, we found that NCS exhibits strong inhibitory effects on root hair development in a dose-dependent manner (Fig. 9). This is consistent with previous observations that some diterpenoids might act as a potential ethylene action antagonist in the inhibition of lettuce root hair development (Ding et al. 2008, 2010). Auxin and ethylene are both positive regulators of root hair development. They exert their influences on root hair development after the TTG/GL2 pathway has established early differentiation events (Masucci and Schiefelbein 1996). Furthermore, auxin-resistant mutant *axr1*, *axr2* and ethylene-insensitive mutant *etr1*, *ein2* were all found to have abnormalities in root hair elongation (Lincoln et al. 1990; Masucci and Schiefelbein 1996; Pitts et al. 1998). These observations suggested that the auxin and ethylene signaling pathways are required for root hair development. We investigated whether NCS affects the responses of lettuce root hair development to auxin and ethylene. Our results showed that exogenous application of IAA or ACC promotes root hair development, whereas NCS (0.5–5 µM) treatments significantly inhibits the IAA- or ACC-induced root hair growth (Fig. 10), suggesting that both auxin and ethylene signaling pathways might be involved in the response of root hair to NCS. Further studies are required to confirm its action on lettuce root hair development.

In conclusion, our findings in this study showed the potential phytotoxic effects of NCS on root growth and root hair development in lettuce seedlings and provided a plausible evidence for the mechanism of this action. It includes induction of oxidative stress and DNA damage, reduction of the number of mitotic cells, inhibition of proliferation of meristematic cells, and root hair development. Specially, we postulate that DNA damage induced by higher levels of NCS (0.5–5 µM) significantly correlates with a decrease of mitotic activity of root meristematic cells, resulting in NCS-induced lettuce root growth inhibition. However, all data presented suggest that the effect of NCS on lettuce root growth is a complex phenomenon. To get more insights into the mechanism, further investigation is necessary.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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