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OPPOSITE EXTREMES IN ETHYLENE/NITRIC OXIDE RATIO INDUCE CELL DEATH IN SUSPENSION CULTURE AND ROOT APICES OF TOMATO EXPOSED TO SALT STRESS

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The plant hormone ethylene or the gaseous signalling molecule nitric oxide (NO) may enhance salt stress tolerance by maintaining ion homeostasis, first of all K^+/Na^+ ratio of tissues. Ethylene and NO accumulation increased in the root apices and suspension culture cells of tomato at sublethal salt stress caused by 100 mM NaCl, however, the induction phase of programmed cell death (PCD) was different at lethal salt concentration. The production of ethylene by root apices and the accumulation of NO in the cells of suspension culture did not increase during the initiation of PCD after 250 mM NaCl treatment. Moreover, cells in suspension culture accumulated higher amount of reactive oxygen species which, along with NO deficiency contributed to cell death induction. The absence of ethylene in the apical root segments and the absence of NO accumulation in the cell suspension resulted in similar ion disequilibrium, namely K^+/Na^+ ratio of 1.41 ± 0.1 and 1.68 ± 0.3 in intact plant tissues and suspension culture cells, respectively that was not tolerated by tomato.

Keywords: Ethylene – ionic homeostasis – programmed cell death – root apex and cell suspension – salt stress

INTRODUCTION

Tissue culture techniques, the aseptic culture of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions provided new tools for plant biotechnology [32], plant molecular biology and for studying physiological processes such as cell cycle, cell differentiation [12], organogenesis [14] or programmed cell death (PCD) [39] at cell level. Cell suspension cultures are especially suitable for the investigation of stress-induced PCD, because the initiation and the development of the process can be synchronized as well as the number of dead cells can easily be detected [29].

Excess of NaCl in the culture solution induces salt stress and depending on the salt tolerance of the investigated plant genotype, it may initiate PCD. PCD induced by 250 mM NaCl in tomato cell suspension was accompanied by high ethylene production, by the accumulation of reactive oxygen species (ROS) and a moderate generation of nitric oxide (NO). PCD-inducing salt concentrations resulted in cell shrinkage,

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chromatin condensation, DNA fragmentation, TUNEL positive nuclei and activation of cysteine proteases in this system [30]. Moreover, high salinity initiated cell death in the apical cells of roots due to ion disequilibrium caused by Na^+ uptake [16]. Na^+ uptake was also accompanied by a strong membrane depolarisation resulting in K^+ efflux and potassium deficiency [36], which activated cysteine proteases, the effectors of PCD [10, 33]. The activity but not the abundance of 20S proteasome also increased in the root tip of wheat plants under salt stress [34].

Ethylene has been recognized as a ubiquitous plant hormone which influences the growth and development of plants [23]. *In vitro* studies have indicated that ethylene accumulates in the headspace of vessels and can affect the growth of cells [21, 35], the regeneration of shoots [31], somatic embryogenesis in tissue cultures [3] and PCD [30]. Ethylene in higher plants is synthesized from S-adenosylmethionine which is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by various ACC synthase (ACS) isoenzymes. ACC, the immediate precursor of ethylene is then oxidized to ethylene, CO_2 and cyanide by ACC oxidases [40]. Ethylene receptors are transmembrane proteins bound to endoplasmic reticulum membranes and have structural similarity to bacterial two-component histidine kinases. In tomato there are eight ethylene receptors, LeETR1, -2, to -7, and Never ripe (NR), five of them have been shown to bind ethylene with high affinity [18]. They negatively regulate ethylene signalling and the suppression is cancelled upon ethylene binding. Decreasing the number of receptors increases ethylene sensitivity and with fewer ethylene receptors actively suppressing ethylene signalling less ethylene is needed to relieve the suppression [37]. The downstream components of the pathway have been reviewed by several authors (e.g. [24]) and among signalling intermediates two types of positive regulators, EIN2, a metal ion transporter-like protein and the transcription factors EIN3/EIL1 and ERFs seems to be key players in ethylene response.

Moreover, ethylene regulates the proteolytic activity and the expression of cysteine proteases in senescing organs [17] and abiotic stress-induced cell death can also be accelerated through ethylene-induced cysteine proteases [39].

Maintenance of ion homeostasis, in particular K^+/Na^+ ratio is of critical importance under salt stress. Exogenous ACC increased the K^+ content relative to Na^+ and the activity of plasmamembrane (PM) ATPase in wild type *Arabidopsis* plants under salt stress. Since H^+-ATPase activity is necessary for the extrusion of excess Na^+ from the cytoplasm, this suggests that ethylene at appropriate concentration may mitigate the ionic stress under high salinity [22]. Previous studies have also demonstrated that exogenous nitric oxide (NO), a gaseous free radical and signalling intermediate could attenuate the NaCl-induced increase in Na^+/K^+ ratio and stimulated PM H^+-ATPase activity [41]. NO also plays a role in protecting plant tissues from oxidative stress [30] and it can stimulate or inhibit ethylene production in plants exposed to abiotic stresses [11].

A tomato cell suspension culture was established in order to reveal the initiation mechanism of salt stress-induced PCD at cell level and to compare it with the most important events in the root tip of intact tomato plants. The question is whether this heterotrophic cell culture can be used as a model of meristematic tissues to investigate the molecular events of PCD under salt stress.

MATERIALS AND METHODS

Plant material

Tomato (*Solanum lycopersicum* L. cvar Rio Fuego) plants were grown hydroponically in controlled environment (under 300 μmol m^{-2} s^{-1} photon flux density with 12/12 light/dark period, 25 °C, and 55–60% relative humidity) as described by Poór et al. [28].

Establishment of suspension cultures

Stem segments of 2-week-old plants were surface sterilised for 1 min with 70% (v/v) ethanol and for 20 min with 5% (v/v) NaOCl and then rinsed four times with sterile distilled water. Then they were placed on callus inducing MS medium [25]. Media were supplemented with Gamborg B5 vitamins [13], 30 g l^{-1} sucrose, 8 g l^{-1} agar, 5 μM α-naphthyl acetic acid (NAA) in combination with 8.8 μM 6-benzyladenine (BA) [26]. The pH of the culture medium was adjusted to 5.8 with 0.1 N KOH and the cultures were incubated at 25 °C in darkness. Segments produced calli 2 months after the culture initiation and then they were subcultured every 3 weeks. In order to establish cell suspension, 2 g of calli was initially transferred into a 100 ml Erlenmeyer flask containing 20 ml of medium (MS + GB5 vitamins, 30 g l^{-1} sucrose, 5 mM NAA, 1 mM BA) as described by Yakimova et al. [39]. Suspensions were incubated on rotary shaker (100 rpm) at 25 °C in darkness and were subcultured every 7 days.

NaCl treatments

A 100 mM NaCl caused salt stress and induced acclimation of tomato plants. A 250 mM concentration of NaCl was chosen to provoke fast and significant induction of PCD within 24 h both in cell suspension and intact plants. The cells in cell suspension were treated with 100 or 250 mM NaCl (pH 5.8) four or five days after subculture. Six-week-old intact plants were exposed to salt stress through the root system. In order to investigate the induction phase of PCD, the samples were harvested 6 hours after salt exposure.

Determination of ethylene production

Ethylene production of the suspension cells and apical root segments was measured with a Hewlett-Packard 5890 Series II gas chromatograph equipped with flame ionization detector and a column packed with activated alumina as described by Csiszár et al. [9]. 0.5 g of the cells from suspension cultures and of 1 cm long root apical segments was incubated in closed tubes for 6 h. The ethylene emanated from the plant tissues was withdrawn by gas-tight syringe from the gas phase and after GC analysis it was quantified using a calibration with pure ethylene.

Determination of ROS and NO production

ROS was visualized with 10 μM 2',7'-dichlorofluorescein diacetate (H_2DCFDA) and NO with 10 μM 4,5-diaminofluorescein-diacetate (DAF-2 DA) fluorescent dyes. Staining occurred for 20 min in 10 mM MES-TRIS/KCl buffer (pH 5.8) in the dark at 37 °C and the plant cells were rinsed twice with 10 mM MES-TRIS/KCl buffer (pH 5.8) [15]. Fluorescence intensity was detected with Zeiss Axiovert 200M type fluorescent microscope (Carl Zeiss Inc., Jena, Germany). Digital photographs were taken from the samples with a high-resolution digital camera (Axiocam HR, HQ CCD camera; Carl Zeiss Inc., Jena, Germany). The fluorescence intensity was determined with AXIOVISION REL. 4.5 software (Carl Zeiss Inc., Munich, Germany) using a filter set 10 (exitation 450–495 nm, emission 515–565 nm).

Determination of cell viability by electrolyte leakage

Electrolyte leakage was determined as described earlier [29]. Briefly, 0.5 g of cells or root tissues was transferred to 20 ml double distilled water. After 2 h of incubation at 25 °C, the conductivity of the bathing solution was determined (C1) with conductivity meter (OK-102/1 Radelkis, Budapest, Hungary). The samples were then heated at 95 °C for 40 min and the total conductivity (C2) of the cooled samples was measured. Relative electrolyte leakage (EL) was expressed as a percentage of total conductivity: $EL(\%) = (C1/C2) \times 100$.

Determination of macroelement contents

Macroelements in plant material were determined by atomic absorption spectrometry (AAS) (Hitachi Z-8200, Japan) [30]. The dry samples were incubated in 6 ml of cc. HNO_3 (Reanal, Hungary) and 2 ml of 30% H_2O_2 (Reanal, Hungary) for 20 h. The samples were digested in microwave destructor (MarsXpress CEM, USA) at 200 °C for 3 h. Cooled samples were diluted with double distilled water and transferred to Packard glass tubes.

Statistical analysis

Data are average values ± SE of a representing experiment from at least three independent biological repetitions. Statistical analysis was carried out with Sigma plot 11.0 software (Systat Software Inc., Erkrath, Germany). After analysis of variance (ANOVA) Duncan's multiple comparisons were performed. Differences were considered significant if $P < 0.05$.

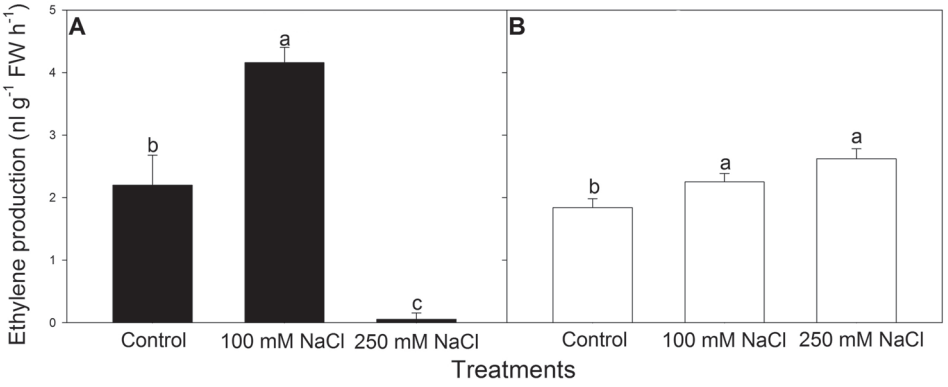

Fig. 1. Cumulative ethylene production of 1 cm-long apices of tomato roots (A) and tomato cell suspension cultures (B) were determined six hours after exposure to 100 or 250 mM NaCl. (Means ± SE, n = 5). Bars with different letters are significantly different at 0.05 level (Duncan's multiple range test)

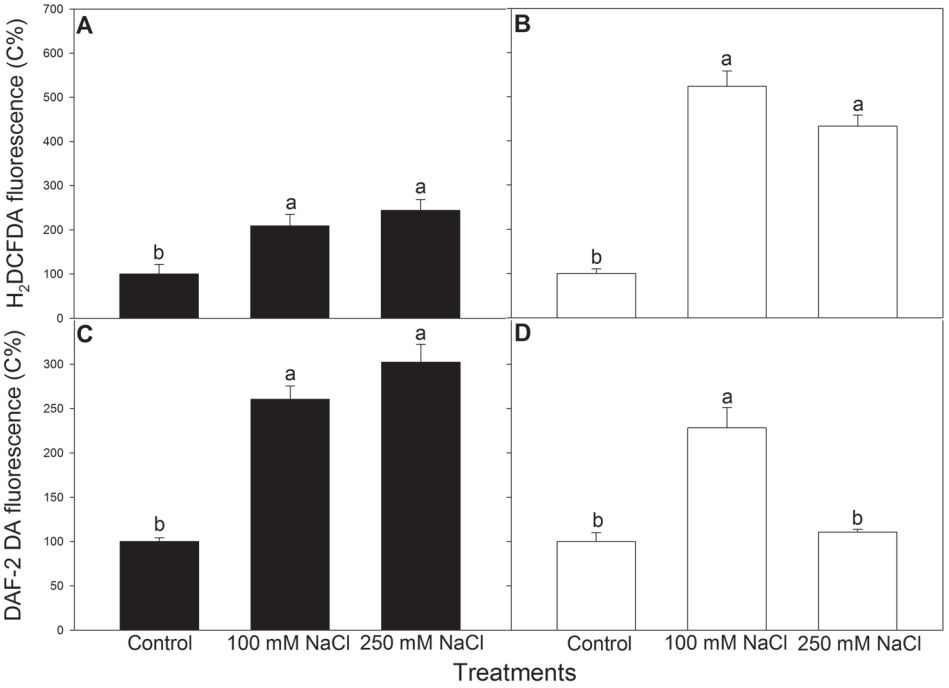

Fig. 2. Changes in ROS (A, B) and NO production (C, D) in 1 cm-long apices of tomato plants (A, C) and tomato cell suspension cultures (B, D) six hours after exposure to 100 or 250 mM NaCl. (Means ± SE, n = 5). Bars with different letters are significantly different at 0.05 level (Duncan's multiple range test). Root tissues and cell suspension were treated separately in the test

RESULTS

Ethylene production of 1 cm-long apical segments of tomato roots and tomato suspension cultures were measured 6 h after exposure to sublethal, 100 mM concentration of NaCl and to 250 mM NaCl, which induced PCD within 24 h both in the root apices and suspension culture cells. It was found that sublethal salt concentration resulted in significant increase in ethylene production of root tissues and cell suspension cultures, but the two experimental systems responded antagonistically to lethal salt stress. The ethylene production was inhibited in the root apices and increased in the cell cultures after exposure to 250 mM NaCl (Fig. 1A, B).

The oxidative and nitrosative stress elicited by high salinity was also different. In root apices the level of ROS was enhanced by ~100–150% and that of NO by about 160–200% compared to untreated controls at both salt concentrations (Fig. 2A, C). In cell cultures, however, the accumulation of ROS was similar at sublethal and lethal salt stress (Fig. 2B) but the production of NO increased at 100 mM NaCl and remained at control level at 250 mM in cell cultures six hours after salt exposure (Fig. 2D).

The electrolyte leakage in both cases was significantly increased, however, it was much higher at 250 mM NaCl (Fig. 3A, B).

In earlier experiments it was found that plant cells could be recovered after stress removal if the relative electrolyte leakage was not higher than ~30%, but the recovery was unsuccessful at higher membrane damage [30].

Although the intact root tissues accumulated more Na^+ than cell cultures on dry mass basis, the sodium uptake increased, and K^+ content of tissues decreased with increasing Na^+ concentration (Fig. 4A, B). Thus, the K^+/Na^+ ratio decreased more significantly at lethal salt stress. 250 mM NaCl also caused a significant reduction in Mg^{2+} content of apical root segments and the reduction was smaller in cell cultures (Fig. 4D). Total Ca^{2+} content has not been changed in the first 6 h of salt stress (Fig. 4C).

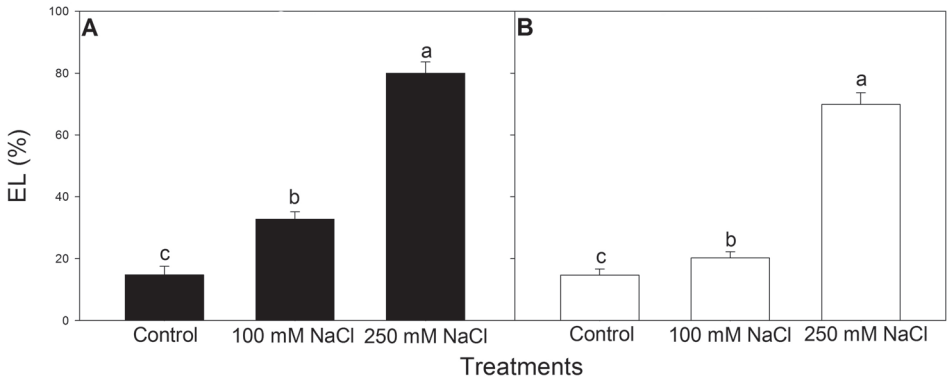

Fig. 3. Relative electrolyte leakage from 1 cm-long apices of tomato roots (A) and tomato cell suspension cultures (B) were determined six hours after exposure to 100 or 250 mM NaCl. (Means ± SE, n = 5). Bars with different letters are significantly different at 0.05 level (Duncan's multiple range test)

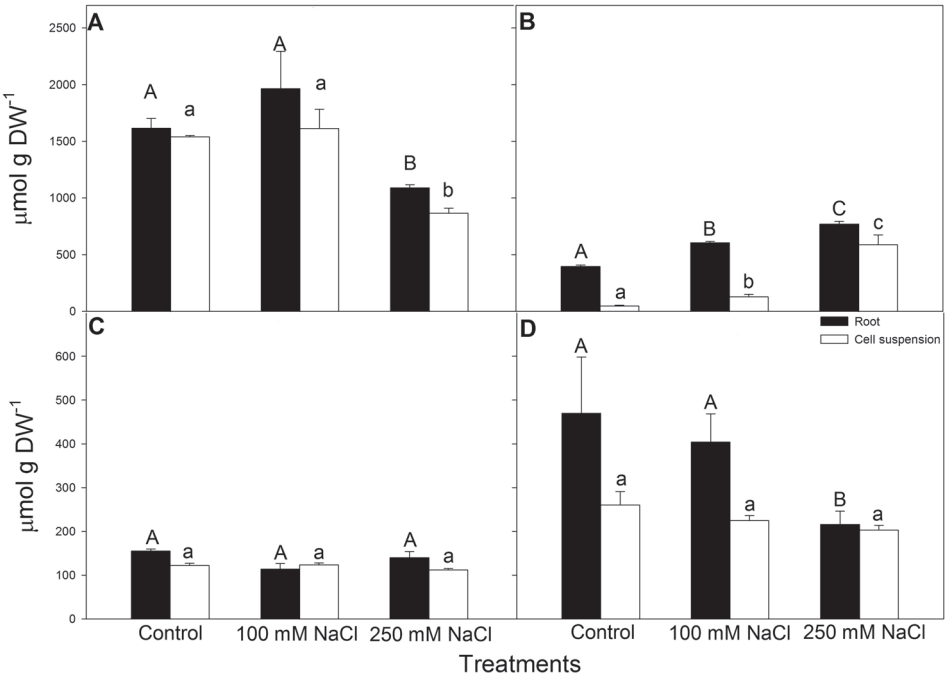

Fig. 4. Changes in K^+ (A), Na^+ (B), Ca^{2+} (C) and Mg^{2+} (D) content in 1 cm-long apices of tomato plants (black columns) and tomato cell suspension cultures (open columns) six hours after exposure to 100 or 250 mM NaCl. (Means±SE, n=5). Bars with different letters are significantly different at 0.05 level (Duncan's multiple range test). Root tissues and cell suspension were treated separately in the test.

DISCUSSION

The ethylene synthesis increased in both experimental systems at sublethal salt concentration.

Although a treatment of tomato cells with 250 mM NaCl initiated PCD both in root apices and suspension cultures within 24 hours, the signalling events were different. The apical segments of roots did not show increased ethylene production, but the ethylene synthesis was promoted in cell suspension cultures under lethal salt stress. The induction of ethylene production of suspension culture cells may be due to the components of the incubation solution. The concentrations of exogenous hormones were constant in this experimental system and it was not modified by the status of cells organized into tissues. The media which is generally used for callus cultures or shoot regeneration is supplemented with various auxins (e.g. 1-naphthaleneacetic acid, NAA) and cytokinins (e.g. 6-benzylaminopurine, BA) [6]. Stimulation of ethylene biosynthesis is common response of plants exposed to exogenous auxins [1]. Auxins have been shown to induce *de novo* synthesis of ACC by increased expression of specific ACS genes [19]. Cytokinins may also stimulate ethylene production by

enhancing the stability of ACS protein [7]. Increasing BA concentrations resulted in increased H_2O_2 accumulation, lipid peroxidation and reduction of biomass as well as activation of catalase in grape callus cultures [27]. Unexpectedly, BA at high concentration could induce PCD in carrot and *Arabidopsis thaliana* L. Heynh cell cultures within 24 h [5]. Thus, high ethylene production in tomato suspension cultures contributed to PCD initiation under high salinity.

Ethylene has also been shown to alleviate salt stress injury. In *Arabidopsis* the expression of ethylene receptor *ETR1* was downregulated by 24-hour salt stress both at transcript and protein levels which should cause increased ethylene sensitivity [42].

Moreover, a loss of function mutation in *EIN2*, a positive regulator of ethylene signalling led to salt sensitivity [4]. ERF1 an ethylene- and salt stress-induced transcription factor, which acts downstream of EIN2, is also related to salt stress response. Transgenic tomato [23a] and *Arabidopsis* [2, 8] overexpressing *ERF1* of tomato, *Arabidopsis* and wild radish, respectively, were more tolerant to high salinity. Ethylene can mitigate the ionic component of salt stress by increasing the extrusion of Na^+ from cytoplasm through the activation of PM ATPases. On the effect of 100 mM NaCl the ethylene production of tissues increased and the K^+/Na^+ ratio decreased to 51.6% and 42.9% of the respective controls in root apices and suspension culture cells, respectively. According to the relative electrolyte leakage from the cells these changes can be tolerated by tomato (Fig. 3).

PM ATPases in higher plants form a phosphorylated intermediate using Mg^{2+}-ATP as substrate, which increases the activity of the enzyme. The activation of PM-ATPase can be diminished in the absence of ethylene and disturbances in Mg^{2+} accumulation may also contribute to the decline in enzyme activity. Thus, the dramatic reduction in Mg^{2+} content observed in root apices can enhance the ionic stress at lethal salt concentration (Fig. 4).

Generation of ROS increased to the same extent at both salt concentrations, but it was more pronounced in suspension culture cells. There were, however, great differences in NO accumulation of the cells. The root tissues exhibited much higher NO accumulation compared to untreated control than the cells in suspension culture at lethal salt stress. NO application significantly enhanced the NaCl-induced osmotic stress tolerance via the accumulation of osmoprotectants such as glycine betaine, soluble sugars or proline [20]. The ionic effect of salt stress and the decrease in optimal K^+/Na^+ ratio can also be mitigated by the use of NO donors. Higher NO levels correlated with higher K^+/Na^+ ratios in *Arabidopsis thaliana* [38] and *Brassica juncea* [20] and the NO donor sodium nitroprusside enhanced the activities of PM and vacuolar H^+-ATPases as well as that of vacuolar H^+-pyrophosphatase in *Arabidopsis thaliana* calli [38]. Thus, the lack of NO accumulation may lead to cell death induction in cell suspension cultures under lethal salt stress. Moreover, NO can interplay with ROS in a variety of ways [30], thus it may control the antioxidant status of cells.

In the root tips, the salt stress-induced PCD is initiated independently of ethylene action. Under salt stress in the absence of ethylene or in the presence of the aminooxyacetic acid (AOA), an inhibitor of ethylene biosynthesis, the K^+/Na^+ ratio and the activity of PM H^+-ATPase decreased in roots of *Arabidopsis* plants leading to

enhanced ionic stress [22]. Thus, the absence of ethylene in the root tips or the absence of NO accumulation in cell suspension may cause similar severe ion disequilibrium, namely K^+/Na^+ ratio of 1.41 ± 0.1 and 1.68 ± 0.3 in intact plant tissues and suspension culture cells, respectively.

Cells in the suspension culture are exposed to an artificial environment containing constant concentration of hormones, sugars, vitamins and mineral elements. In organized tissues the surroundings of the cells at specific position varies from cell to cell and it is different from that of tissue culture, thus the initial events leading to PCD are also different in the two systems. The accumulating data suggest that a significant crosstalk occurs between various forms of reactive oxygen and nitrogen species. Moreover, NO and ROS can regulate each other's synthesis but the molecular mechanism of the interaction between ethylene and NO signalling and the possible role of NO-induced cGMP levels in this interaction needs further elucidation.

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