Plant Physiology and Biochemistry 84 (2014) 78–86

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Phytotoxic action of naphthoquinone juglone demonstrated on lettuce seedling roots

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ARTICLE INFO

Article history:
Received 20 July 2014
Accepted 20 August 2014
Available online 11 September 2014

Keywords:
Juglone
Lettuce
Meristematic activity
Programmed cell death
Reactive oxygen species
Root

ABSTRACT

Juglone, 5-hydroxy-1,4-naphthoquinone, is the plant secondary metabolite with allelopathic properties, which was isolated especially from the plant species belonging to family *Juglandaceae* A. Rich. ex Kunth (walnut family). The mechanism of phytotoxic action of juglone was investigated on lettuce seedlings *Lactuca sativa* L. var. *capitata* L. cv. Merkurion by determining its effect at different levels. We have found that juglone inhibits mitosis (mitotic index 8.5 ± 0.6% for control versus 2.2 ± 0.9% for 250 μM juglone), changes mitotic phase index with accumulation of the cells in prophase (56.5 ± 2.6% for control versus 85.3 ± 5.0% for 250 μM juglone), and decreases meristematic activity in lettuce root tips (51.07 ± 3.62% for control versus 5.27 ± 2.29% for 250 μM juglone). In addition, juglone induced creation of reactive oxygen species and changed levels of reactive nitrogen species. Amount of malondialdehyde, a product of lipid peroxidation, increased from 24.0 ± 4.0 ng g^{-1} FW for control to 55.5 ± 5.4 ng g^{-1} FW for 250 μM juglone. We observed also changes in cellular structure, especially changes in the morphology of endoplasmic reticulum. Reactive oxygen species induced damage of plasma membrane. All these changes resulted in the disruption of the mitochondrial membrane potential, increase in free intracellular calcium ions, and DNA fragmentation and programmed cell death that was revealed by two methods, TUNEL test and DNA electrophoresis. The portion of TUNEL-positive cells increase from 0.96 ± 0.5% for control to 7.66 ± 1.5% for 250 μM juglone. Results of the study indicate complex mechanism of phytotoxic effect of juglone in lettuce root tips and may indicate mechanism of allelopathic activity of this compound.

1. Introduction

Naphthoquinones, a class of compounds derived from naphthalene, represent a group of plant secondary metabolites that have been found in some families of flowering plants, such as *Droseraceae* Salisb., *Juglandaceae* A. Rich. ex Kunth, and *Plumbaginaceae* Juss. (Babula et al., 2009, 2006). Juglone represents one of the best known members of this group. It was isolated from species of genus *Juglans* L. (e.g. *Juglans regia* L., *J. nigra* L., and *Juglans mandshurica* Maxim.) (Sun et al., 2013; Cosmulescu et al., 2014), *Pterocarya* Kunth (*Pterocarya fraxinifolia* Lam. ex Poir.) (Hadjmohammadi and Kamel, 2006), and *Caesalpinia* L. (*Caesalpinia sappan* L.) (Lee and Lee, 2006). Juglone is released from plants and plant tissues – it was detected in alley soils under black walnut trees (von Kiparski et al., 2007). In the light of this fact, juglone is an important allelochemical. Its allelopathic potential has been shown on several plant species. One of the most recent works published on this topic describes inhibitory effect of juglone on growth and changes in stress parameters of *Cucumis sativus* L. cv. Beith Alpha, *Cucumis melo* L. cv. Ananas, and *Cucumis melo* L. cv. Kis Kavunu based on the ability of juglone to generate reactive oxygen species; however, a different response between species and cultivars was observed

Abbreviations: AO, acridine orange; CDPK, calcium-dependent protein kinase; EB, ethidium bromide; FW, fresh weight; MAPK, 6 mitogen-activated protein kinase; MDA, malondialdehyde; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide; POD, peroxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; TTC, 1,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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http://dx.doi.org/10.1016/j.plaphy.2014.08.027
0981-9428/

(Altikat et al., 2013). The ability of juglone and other naphthoquinones to generate reactive oxygen species has been confirmed in many studies (Hao et al., 2012; El Hadrami et al., 2005). This fact is in agreement with conclusions of the work of Sytykiewicz, who observed that treatment of maize plants with juglone stimulated significantly the transcriptional activity of glutathione transferase gene (*GstI*) compared to control plants; glutathione transferases represent an important group of cytoprotective enzymes participating in detoxification of xenobiotics and limiting oxidative damages of cellular macromolecules (Sytykiewicz, 2011). Bohm et al. observed significant affecting of growth of roots connected with significant increase in PAL activity and lignification and decrease in soluble and cell wall-bound POD activities in the roots of soybean (*Glycine max* (L.) Merrill) (Bohm et al., 2010). Exposure of rice plants to juglone triggered changes in transcript levels of genes related to cell growth, cell wall formation, chemical detoxification, and abiotic stress response (Chi et al., 2011). Authors also showed upregulation of the expression of two calcium-dependent protein kinases (*CDPKs*), 6 mitogen-activated protein kinase (*MAPK*) genes and *1 MAPK* gene, and markedly increased activities of a CDPK-like kinase and MAPKs. Upregulated signaling and biosynthesis of abscisic acid and jasmonic acid as well as an inactivation of gibberellic acid observed by the authors bring connection with the works of Terzi et al., who observed alleviation of juglone-induced stress by plant growth regulators, namely gibberellic acid and cytokinin kinetin, in both dicot and monocot plants (Terzi and Kocacaliskan, 2009, 2010). MAPKs are mediators of both biotic and abiotic stress, especially osmotic and oxidative stress, in plants (Danquah et al., 2014; Sun et al., 2014). It has been also shown that MAPKs are involved in the processes of programmed cell death in plants (Hashimoto et al., 2012; Li et al., 2011). Ability of two naphthoquinones, juglone and plumbagin, to induce programmed cell death and its connection with oxidative stress in tobacco BY-2 cells has been demonstrated by Babula et al. (Babula et al., 2009). The study used BY-2 cells that are suitable for cytological studies and observations; however, they are not able to substitute intact plants in some studies. Due to this fact, we evaluated mechanism of phytotoxic action of naphthoquinone juglone (5-hydroxy-1,4-naphthoquinone) on experimental plants represented by lettuce seedlings (*Lactuca sativa* L. var. *capitata* L. cv. Merkurion) with accentuated attention to ability of juglone to induce changes in meristematic activity and processes of programmed cell death in root tips.

2. Material and methods

2.1. Chemicals

All of the chemicals used were obtained from Sigma–Aldrich, USA, unless otherwise noted. All of the fluorescence probes were purchased from Life Technologies, USA. They were stored in accordance with the manufacturer's recommendations. Working solutions were prepared immediately before use according to manufacturer's instructions. All fluorescence probes were used in accordance with manufacturer's instructions.

2.2. Experimental design

For experiments, two days old seedlings of lettuce (*L. sativa* L. var. *capitata* L. cv. Merkurion) of the same stage of ontological development, morphological appearance, and weight were used. Lettuce seeds were surface-sterilized with sodium hypochlorite (1%, aqueous solution), rinsed three times with distilled water and germinated in the dark at 20 °C. Pre-germinated seeds were placed onto filtration paper in each Petri dish and pre-cultivated (cultivation box: temperature 25 ± 1 °C (light)/18 °C ± 1 °C (dark), photoperiod 16 h (6–22 h), photon flux density 40 μmol m^{-2} s^{-1}, relative humidity 80%). Two days-old seedlings were transferred onto filter paper in each Petri dish with juglone (Sigma–Aldrich, USA). Juglone was added into the Petri dishes in the form of a stock solution in acetone (concentration 1 mg mL^{-1}) to create final concentrations of 25 μM, 75 μM, 150 μM, and 250 μM in water (pH 6.8). A control was represented by untreated lettuce seedlings cultivated only in the water, because the first stages of the seedling growth are completely sustained by seed reserves. Cultivation conditions were as follows: temperature 25 ± 1 °C (light)/18 °C ± 1 °C (dark), photoperiod 16 h (6–22 h), photon flux density 40 μmol m^{-2} s^{-1}, and relative humidity 80%. Plants were sampled 48 h after treatment, carefully rinsed three times with distilled water and used for further evaluation. The experiment was carried out in triplicates; at least sixty plants were used.

2.3. Determination of mitotic index and meristematic activity

In order to determine mitotic index, fixed roots were used (formaldehyde-acetic acid-ethanol-distilled water – FAA, 1:2:10:7, *v*/*v*/*v*/*v*, all concentrated, Sigma–Aldrich, USA). 1 cm long root tip segment was collected, fixed, and after 24 h transferred into 70% (*v*/*v*) ethanol. Then, the segments were washed three times with distilled water and softened in 2% cellulase and 2% pectinase (*w*/*v*, both Sigma–Aldrich, USA) at pH 4.5 for 4 h at room temperature, carefully washed with distilled water, stained using Hoechst 33258 reagent (5.0 μg mL^{-1}, 15 min, room temperature, Sigma–Aldrich, USA), and observed (Carl Zeiss Axioscop 40, Zeiss, Germany). Mitotic index was expressed as a number of dividing cells to the number of total cells; mitotic phase index was expressed as a number of cells in each mitotic phase to all mitotic cells. To evaluate activity of meristem, Click-iT® Plus EdU Alexa Fluor® 488 Imaging Kit (Life Technologies, USA) that uses modified thymidine analogue 5-ethynyl-2'-deoxyuridine, a nucleoside analogue of thymidine, which is efficiently incorporated into newly synthesized DNA, was used. The seedlings were incubated for 1 h in EdU solution and processed in accordance with manufacturer's instructions. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, Sigma–Aldrich, USA) was used to counterstain nuclei, the labelling index was evaluated as the percentage of EdU-labelled nuclei in relation to the DAPI-stained nuclei. At least 1000 cells were evaluated. The test was repeated three times for each treatment.

2.4. Observations with the microscope

For microscopic observations, different parts of roots were evaluated: peripheral cells of root cap, zone of cell division, and the youngest cortical cells of distance about 800 μm from the root tip). Following combinations of probes were used: acridine orange/ethidium bromide (nuclear architecture and programmed cell death), Cell ROX Deep Red reagent/$DiOC_6(3)$ (reactive oxygen species and mitochondria and endoplasmic reticulum), Amplex UltraRed/Fluo5F-AM (hydrogen peroxide and free intracellular calcium ions), DAF-FM DA/ER Tracker Red (reactive nitrogen species and endoplasmic reticulum), and JC-1 (changes in mitochondrial potential). Characteristic changes are shown; the bar represents 2.5 μm.

2.5. Visualization of cellular changes

Changes in the structure and stress of endoplasmic reticulum were observed using two fluorescence probes, ER Tracker Red and $DiOC_6(3)$. ER Tracker Red (587/615 nm) is a cell-permeant, live-cell stain that is highly selective for the endoplasmic reticulum (ER). This stain consists of the green-fluorescent BODIPY® TR dye and glibenclamide. Glibenclamide (glyburide) binds to the