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Research article

Mechanism of artemisinin phytotoxicity action: Induction of reactive oxygen species and cell death in lettuce seedlings

Zhi-Qiang Yan [a, 1], Dan-Dan Wang [b, 1], Lan Ding [b], Hai-Yan Cui [a], Hui Jin [a, c], Xiao-Yan Yang [a], Jian-She Yang [b], Bo Qin [a, *]

[a] Key Laboratory of Chemistry of Northwestern Plant Resources of CAS and Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China
[b] College of Life Science, Northwest Normal University, Lanzhou 730070, China
[c] Key Laboratory of Tobacco Diseases and Insect Pests Monitoring Controlling and Integrated Management, Institute of Tobacco Research, Chinese Academy of Agricultural Sciences, Qingdao 266101, China

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ABSTRACT

Artemisinin has been recognized as an allelochemical that inhibits growth of several plant species. However, its mode of action is not well clarified. In this study, the mechanism of artemisinin phytotoxicity on lettuce seedlings was investigated. Root and shoot elongation of lettuce seedlings were inhibited by artemisinin in a concentration-dependent manner. The compound effectively arrested cell division and caused loss of cell viability in root tips of lettuce. Overproduction of reactive oxygen species (ROS) was induced by artemisinin. Lipid peroxidation, proline overproduction and reduction of chlorophyll content in lettuce seedlings were found after treatments. These results suggested that artemisinin could induce ROS overproduction, which caused membrane lipids peroxidation and cell death, and impacted mitosis and physiological processes, resulting in growth inhibition of receptor plants.

1. Introduction

Higher plants biosynthesize a group of secondary metabolites named allelochemicals to mediate direct plant–plant ecophysiological interactions. Allelochemicals are released into the environment from one plant to regulate growth and development of neighboring plants. Characterization of the mode of action of allelochemicals in receptor species is essential for a full understanding of this plant–plant interaction. Previous studies demonstrated that allelochemicals could have multiple phytotoxic effects to influence almost every aspect of plant growth and development such as gene expression, phytohormone activity, ion uptake and photosynthetic function (Babula et al., 2009). Therefore, phytotoxic mechanisms of allelochemicals will undoubtedly be complex.

Artemisinin, a sesquiterpene endoperoxide lactone isolated from the leaf tissues of *Artemisia annua* (China Cooperative Research Group, 1982), is commonly used as an antimalarial agent (Klayman, 1985). In addition, artemisinin also plays important roles in ecological interactions against insect pests and fungal pathogens, as well as in allelopathy (Jessing et al., 2014). Relatively high levels of artemisinin have been detected in the soils from both *A. annua* field and culture pots (Jessing et al., 2009, 2011, 2013; Herrmann et al., 2013). Once released into the surroundings, artemisinin could inhibit the growth of other plants (Lydon et al., 1997; Delabays et al., 2008; Jessing et al., 2009) and affect the soil microorganism (Herrmann et al., 2013). Additionally, bioassays showed that artemisinin affects growth and/or seed germination in crops, weeds and aquatic plants, as well as *A. annua* itself (Duke et al., 1987; Lydon et al., 1997; Dayan et al., 1999; Panamanik et al., 2008). In spite of many studies on its allelopathic potential, the detailed mode of action for the phytotoxicity of artemisinin still remains unclear. In the present study, the importance of ROS for artemisinin-induced cell death and the changes of cell division and several physiological indicators in artemisinin-treated lettuce seedlings were investigated. To our knowledge, this is the first

Abbreviations: Chl, chlorophyll; DHE, dihydroethidium; FDA, fluorescein diacetate; FW, fresh weight; MDA, malonyldialdehyde; MI, mitosis index; PCD, program cell death; PI, propidium iodide; ROS, reactive oxygen species.

* Corresponding author. Key Laboratory of Chemistry of Northwestern Plant Resources and Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, 18# Tianshuizhong Rd., Lanzhou 730000, China.
E-mail address: bqin@licp.cas.cn (B. Qin).
[1] These authors contributed equally to the work.

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report that ROS may be relatively important for the phytotoxicity of artemisinin to a receptor plant species.

2. Materials and methods

2.1. Plant culture

Filter paper (Qualitative, Whatman–Xinhua, Hangzhou, China) was placed into a Petri dish ($\Phi = 9$ cm) and then soaked in 2 mL distilled H_2O. Lettuce (*Lactuca sativa*) seeds 'Tai Yuan Sun', purchased from Gansu Academy of Agriculture Science, were transferred to the Petri dish and germinated in a growth chamber at 25 ± 2 °C in the dark. After 24 h, seedlings with similar size were used for all experiments.

2.2. Chemical treatment

Artemisinin (Meryer, Shanghai, China) was dissolved in DMSO (Solarbio, Beijing, China) at concentrations that were 1000-fold higher than the final treated concentrations. In all experiments, 1 μl of the stock solution was added per 1 ml of distilled H_2O in each well of a 6-well plate (NUNC, Shanghai, China), and for the control, 1 μl of DMSO was added per 1 ml of distilled H_2O. Lettuce seedlings were transferred into these wells with four seedlings per well and six wells in each treated group. The plates were then incubated at 25 ± 2 °C in the dark. After 48 h, seedlings were harvested and their root and shoot lengths were measured.

2.3. Mitotic index (MI)

Lettuce seedlings were treated as described above. Roots were collected and immediately fixed in Carnoy's fluid (ethanol/acetic acid, 3/1, v/v) for 24 h, then hydrolyzed in 5% HCl for 5 min. Schiff's reagent (Leagene, Beijing, China) was used to stain the chromosomes for 30 min. Root tips (about 2 mm) were excised and squashed, and then observed using an inverted microscope (Shanghai, Shanghai, China) equipped with a digital camera (Panasonic, Osaka, Japan). At least 1000 cells per treatment with three repeats were analyzed. The MI was calculated as the ratio between the number of cells in mitosis and the total number of cells observed. The proportions of cells in prophase, metaphase, anaphase and telophase were also calculated.

2.4. Cell viability

Double staining with fluorescein diacetate (FDA) and propidium iodide (PI) was used to evaluate cell viability (Pan et al., 2001). Red and green fluorescence reflects dead or viable cells, respectively. After treatment with artemisinin, root segments were excised from lettuce seedlings and stained with a mixture of 12.5 μg/mL FDA (MP Biomedicals, CA, USA) and 5.0 μg/mL PI (MP Biomedicals, CA, USA) for 10 min. Then, the stained roots were washed with distilled H_2O and observed using a fluorescence microscope (Leica DMI4000B equipped with an Ar ion laser, excitation 488 nm and emission > 510 nm).

Root cell viability was also evaluated by staining with Evans blue (Tamás et al., 2004). After treatment, root segments (about 1 cm from the tip) of lettuce seedlings were cut and stained with 0.25% (w/v) Evans blue (Solarbio, Beijing, China) for 1 h at room temperature. After a washing with distilled H_2O, the stained roots were soaked in 1 mL N,N-dimethylformamide for 24 h at 25 °C in the dark to extract the Evans blue that had been absorbed into the lettuce root segments. Absorbance of the released Evans blue was measured at 600 nm by a spectrophotometer (Shimadzu Corp., Kyoto, Japan). The relative Evans blue uptake was calculated as the ratio between the OD values of the treated group and the control.

2.5. ROS production

ROS production was estimated according to the procedure of Yamamoto et al. (2002) with minor modification. Dihydroethidium (DHE, Solarbio, Beijing, China) was used to determinate superoxide anion radical ($O_2^{\cdot-}$) levels. Roots were excised from lettuce seedlings and washed with distilled H_2O, then stained with the dye solution (10 mM DHE, 0.01% acetone, 100 mM $CaCl_2$, pH 4.75) by shaking gently for 10 min at room temperature in the dark. After soaking in 100 mM $CaCl_2$ for 20 min to remove the residual dye, the fluorescence photographs were observed using a fluorescence microscope (Leica DMI4000B equipped with an Ar ion laser, excitation 488 nm and emission > 510 nm).

2.6. Lipid peroxidation

Lipid peroxidation was determined by measurement of malonyldialdehyde (MDA) content according to Hodges et al. (1999). Lettuce roots and shoots (~100 mg, 20-30 seedlings) were separated and homogenized in 5 mL trichloroacetic acid (TCA, 10%, w/v), followed by centrifugation at 4000 g for 10 min. Then, 1.0 mL of the supernatant was added to 2.0 mL of thiobarbituric acid (TBA, 0.6%, w/v) in 10% TCA. The mixture was incubated in boiling water for 15 min and then dipped in an ice bath for 10 min. After centrifugation at 9000 *g* for 5 min, the absorbance of the supernatant was measured at 440, 532 and 600 nm, and the levels of MDA were calculated in the following manner:

$$[\mathrm{MDA}] = [6.452(A_{532} - A_{600}) - 0.56A_{450}] \cdot V_T/(V \cdot W)$$

[MDA] represents the concentration of MDA expressed in $\mu M\ g^{-1}$. A_{450}, A_{532} and A_{600} are the absorbance values at 450 nm, 532 nm and 600 nm respectively. V_T and V represent total volume of the extracting solution and the volume used in measurement, respectively. W is the fresh weight of the lettuce tissues used.

2.7. Free proline

Free proline was determined by a described literature method (Bates et al., 1973). After treatment with artemisinin, about 100 mg samples (lettuce roots and shoots form 20-30 seedlings) were homogenized in 3 mL sulfosalicylic acid (3%, w/v) and centrifuged at 2000 *g* for 10 min. The supernatant (1.0 mL) was mixed with acid ninhydrin and glacial acetic acid at a ratio of 1:1:1, and incubated in boiling water for 1 h, and then in an ice bath for 10 min. Toluene (4.0 mL) was added to the mixture, and after the organic and inorganic phases were separated, the organic phase was monitored at 520 nm spectrophotometrically. Proline content was read from a calibration line constructed with pure proline (Alfa Aesar, Shanghai, China) standards.

2.8. Chlorophyll content

After treatment with artemisinin under a 16 h/8 h day/night photoperiod for 48 h, fresh lettuce leaves (~200 mg, 50-60 seedlings) were collected and homogenized in aqueous acetone (80%, 4.0 mL) and then centrifuged at 2000 *g* for 5 min. The absorbance of the supernatant at 645 and 663 nm was recorded, and the contents of chl *a* and *b* were calculated according to Wellburn (1994).

2.9. Statistical analyses

All experiments were repeated at least three times and the