South African Journal of Botany 112 (2017) 89–94

Contents lists available at ScienceDirect

South African Journal of Botany

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

2,4-Di-tert-butylphenol-induced leaf physiological and ultrastructural changes in chloroplasts of weedy plants

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ARTICLE INFO

Article history:
Received 8 January 2017
Received in revised form 12 March 2017
Accepted 11 May 2017
Available online 25 May 2017

Edited by AO Aremu

Keywords:
Allelochemicals
Oxidative stress
Lipid peroxidation
Membrane damage

ABSTRACT

2,4-Di-tert-butylphenol (2,4-DTBP), a natural compound from plants, causes severe chlorosis and necrosis on *Hedyotis verticillata* and *Leptochloa chinensis* leaf blades by inducing oxidative stress through the generation of reactive oxygen species, leading to lipid peroxidation and membrane damage in leaf and root tissues. However, its effects on the physiology of photosynthesis through ultrastructural alterations in chloroplasts remain unclear. Therefore, this study examined the phytotoxic effects of 2,4-DTBP on chloroplast ultrastructure and leaf physiology of *H. verticillata* and *L. chinensis* in a 3-day assay. The results revealed that treating *H. verticillata* and *L. chinensis* seedlings with 2,4-DTBP significantly decreased shoot fresh weight on day 3 after treatment. 2,4-DTBP increased electrolyte leakage in *H. verticillata*, but no notable electrolyte leakage was detected in *L. chinensis*. Ultrastructural damage to chloroplasts was evident in both species, with a disorganised thylakoid system and undulating membranes, coupled with the absence of starch grains and an increased number of plastoglobuli. Damage to the chloroplast ultrastructure subsequently reduced the leaf greenness, quantum yield and stomatal conductance values. These results suggest that 2,4-DTBP has potent herbicidal properties that can alter chloroplast ultrastructure, thereby reducing physiological activity of these weedy plants.

1. Introduction

2,4-Di-tert-butylphenol (2,4-DTBP) is a natural compound present in medicinal plants, such as *Gynura cusimbua* (Rana and Blazquez, 2007), *Pereskia bleo* (Malek et al., 2009), *Heliotropium indicum* (Oluwatoyin et al., 2011) and *Plumbago zeylanica* (Ajayi et al., 2011). This compound has been identified in culm and leaf extracts of *Pennisetum purpureum* and is reported to have herbicidal properties by preventing root growth of *Hedyotis verticillata* and *Leptochloa chinensis*, common broadleaf and grassy weeds found in oil palm plantations and rice fields, respectively (Chuah et al., 2015). 2,4-DTBP induces oxidative stress by generating reactive oxygen species (ROS); thus, leading to lipid peroxidation and membrane damage in chloroplast and leaf tissues (Chuah et al., 2015). Chuah et al. (2016) also demonstrated that *H. verticillata* and *L. chinensis* show symptoms of leaf blade wilting and necrosis when their roots were submerged in 2,4-DTBP solution 14 and 7 days after treatment, respectively.

Despite these findings, there is a lack of information regarding the effects of 2,4-DTBP on chloroplast ultrastructure. Chloroplasts play an essential role carrying out photosynthesis to synthesise food by converting light into chemical energy (Wang et al., 2015). Chloroplasts are important organelles in plant leaves where essential metabolic pathways, such as amino acid, vitamin, lipid and pigment synthesis occur (Seigneurin-Berny et al., 2008). Plant chloroplasts are bound by a double membrane called the chloroplast envelope, which consists of inner and outer envelope membranes. Besides the chloroplast envelope, chloroplasts also have an internal membrane system called the thylakoid membrane. This internal membrane is normally arranged in stacks called grana that form a network of closed hollow discs called thylakoids (Cooper, 2000). Although 2,4-DTBP causes chlorosis in *H. verticillata* and *L. chinensis* leaf blades (Chuah et al., 2016), the ultrastructural alterations to chloroplasts and its effect on the physiology of photosynthesis remain unknown. Thus, this study determined how 2,4-DTBP affects chloroplast ultrastructure and leaf physiology in *H. verticillata* and *L. chinensis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Hedyotis verticillata and *Leptochloa chinensis* seeds were collected from oil palm plantations in Kuala Berang, Terengganu and rice fields in Pasir Puteh, Kelantan, respectively. The seeds were sown in seedling trays and placed in a greenhouse at the University Malaysia, Terengganu

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http://dx.doi.org/10.1016/j.sajb.2017.05.022
0254-6299/

until they reached the eight-leaf stage based on the method of Chuah et al. (2014) with some modifications. Eight-leaf stage seedlings were transferred to glass vials filled with 1/8-strength Hoagland nutrient solution. The vials were placed in a controlled growth room with a 12 h/12 h light/dark regime 30/20 °C (day/night) temperature, photon flux density of 140–160 μmol m^{-2} s^{-1} with relative humidity of 78%–80%. The 2,4-DTBP (99% purity; Sigma Chemical Co., Kuala Lumpur, Malaysia) was dissolved in 1% acetone and added to the nutrient solution at a concentration of 75 mg/L. Plants placed in a mixture of Hoagland nutrient solution and 1% acetone served as the control treatment. The solution in the glass vial was maintained by adding Hoagland nutrient solution (1/8-strength) at 24 h intervals. On day 1 and 3 days after treatment, the second fully expanded leaves of untreated and treated seedlings were harvested.

2.2. Shoot fresh weight

H. verticillata and *L. chinensis* shoot fresh weight (SFW) loss was measured after the treatment was completed using an analytical balance. SFW loss values were obtained using Eq. (1). SFW loss was expressed as a percentage of the SFW loss observed in the control treatment using Eq. (2).

$$\text{SFW loss } (\%) = \frac{\text{Initial SFW} - \text{final SFW}}{\text{Initial SFW}} \times 100 \quad (1)$$

$$\text{SFW loss } (\%\text{ of control}) = \frac{\text{SFW loss in treated plants}}{\text{SFW loss in untreated plants}} \times 100 \quad (2)$$

2.3. Leaf greenness

Soil Plant Analysis Development (SPAD) measurements were made with a SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., USA) to measure leaf greenness based on optical responses when the leaf was exposed to light. The mean of three SPAD readings from the lamina of second fully expanded leaves of *H. verticillata* and *L. chinensis* was determined at the end of treatment. The SPAD values are expressed as a percentage of the SPAD reading observed in the control treatment.

2.4. Assessing the effect of the compound on ultrastructural changes in chloroplasts

These structural changes were evaluated based on the method of Djebali et al. (2005) with some modifications. The second fully expanded lamina leaves of *H. verticillata* and *L. chinensis* were grown for 1 or 3 days in either the control (0 mg/L) or 75 mg/L 2,4-DTBP solution and were excised and processed for transmission electron microscopy (TEM). The lamina of each species was hand cut with a razor blade into 1-mm pieces, prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (pH 7.4), washed in buffer and post-fixed in 2% OsO_4 in the same buffer. The samples were dehydrated through a 10%–70% alcohol series and embedded in Araldite resin. Semi-thin sections (0.5–1 μm) were cut from plastic-embedded tissue, stained with Toluidine Blue, examined and photographed with a light microscope (Leica Microsystems, Wentzler, Germany). Ultra-thin sections were cut serially using the semi-thin sections, collected on copper grids and examined under TEM after double staining with uranyl acetate and lead citrate.

2.5. 2,4-DTBP-induced physiological changes in leaves

2.5.1. Electrolyte leakage

Electrolyte leakage was assessed using a handheld electrical conductivity meter (Eutech Instruments Pte. Ltd., Singapore) based on the method of Galindo et al. (1999) with some modifications. The laminae of the second fully expanded *H. verticillata* and *L. chinensis* leaves were punched out with a cork borer to obtain a 6-mm diameter disc of tissue. Three discs from *H. verticillata* and *L. chinensis* were placed in 5 mL of deionised water in 50 × 10 mm Petri dishes and loaded in a 25 °C-growth chamber kept in the dark at room temperature. Electrical conductivity (EC1) of the bathing solution was measured at the end of the treatment. Maximum conductivity was measured by boiling three leaf discs in the bathing solution for 20 min at 95 °C and EC2 was measured after cooling. The electrolyte leakage value was expressed as a percentage of electrolyte leakage observed in the control treatment.

2.5.2. Quantum yield

Quantum yield was measured based on the method of Ishii-Iwamoto et al. (2006) with some modifications. The laminae of the second fully expanded *H. verticillata* and *L. chinensis* leaves were punched out with a cork borer to obtain 6-mm diameter discs. Three *H. verticillata* and *L. chinensis* discs each were placed on 5 mL deionised water in 50 × 10 mm Petri dishes and placed in a 25 °C-growth chamber in the dark at room temperature. Quantum yield was measured using a CI-340 hand-held photosynthesis system with an attached chlorophyll fluorescence meter (CID Bio-Science, Inc. USA) after 24 h incubation. The quantum yield value was expressed as a percentage of the quantum yield observed in the control treatment.

2.5.3. Stomatal conductance

H. verticillata and *L. chinensis* (abaxial and adaxial) stomatal conductance values were measured at the end of treatment using a leaf porometer (SC-1, Decagon Devices, USA). The stomatal conductance value is expressed as a percentage of stomatal conductances observed in the control treatment.

2.6. Statistical analyses

Each experiment was arranged in a randomised design with six replicates. The *t*-test was used to compare means between treatments for the leaf physiology and growth attribute parameters. Some of the percentage *L. chinensis* stomatal conductance (abaxial) data were analysed with the Mann–Whitney *U*-test. A p-value < 0.05 was considered significant.

3. Results

3.1. Shoot fresh weight and leaf greenness

SFW loss of *H. verticillata* and *L. chinensis* seedlings increased 1 day after the 2,4-DTBP treatment and then tended to increase 3 days after treatment. Notably, the 2,4-DTBP treatment significantly reduced the *H. verticillata* and *L. chinensis* SPAD readings by 43 and 51%, respectively, 3 days after treatment compared to that in the untreated plants (Fig. 1).

3.2. Effect of 2,4-DTBP on ultrastructural changes in chloroplasts

The TEM (Fig. 2A–H) observations of chloroplasts from plant leaves treated with 2,4-DTBP revealed distinct differences compared to those of untreated leaves. Chloroplasts from control plants were elongated and contained numerous, well compartmentalised grana stacks with starch grains (Fig. 2A, C, E, G). Untreated leaves contained large numbers of starch grains on day 1 (Fig. 2A, E), as compared to 2,4-DTBP-treated plants which had fewer starch grains for the same time period (Fig. 2B, F). Furthermore, treating plants with 2,4-DTBP led to internal disorganisation of plastids. The chloroplasts of 2,4-DTBP-treated plants were severely damaged on day 3 (Fig. 2D, H) compared to those in control plants (Fig. 2C, G). 2,4-DTBP-treated plants had disorganised thylakoid systems with undulating membranes and an increased number of plastoglobuli (Fig. 2D, H). The thylakoids of 2,4-DTBP-treated plants