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Evaluation of the biochemical and physiological activity of the natural compound, 2,4-ditert-butylphenol on weeds

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Abstract. 2,4-Di-tert-butylphenol (2,4-DTBP) is a natural compounds present in medicinal plants. It is reported to have herbicidal properties. However, the mechanism of action is unknown for use in weed management. Measurements were made of lipid peroxidation, ion leakage, antioxidant enzymes, chlorophyll content, chlorophyll fluorescence and photosynthesis in the grassy weed *Leptochloa chinensis* (L.) Nees and the broadleaf weed *Hedyotis verticillata* (L.) Lam. at 7 and 14 days, respectively, after treatment with 2,4-DTBP. The 2,4-DTBP reduced the shoot fresh weight of *L. chinensis* and *H. verticillata* by 50% when applied at concentrations of 50 and $200 \, \mu g \, mL^{-1}$, respectively. Treatment with 2,4-DTBP significantly increased levels of malondialdehyde, caused excessive ion leakage and increased activities of antioxidant enzymes such as superoxide dismutase, peroxidase and catalase in leaf and root tissues of the two bioassay species. Most notably, 2,4-DTBP treatment caused great reduction in chlorophyll content, thereby decreasing chlorophyll fluorescence, transpiration and net photosynthetic rate in the leaf tissues. The results suggest that 2,4-DTBP induces oxidative stress through the generation of reactive oxygen species, which cause lipid peroxidation and membrane damage in root tissues and chloroplast in leaf tissues, thus leading to increased levels of antioxidant enzymes.

Additional keywords: allelochemical, *H. verticillata.*, *L. chinensis*, weed management.

Introduction

Plants synthesise an array of chemical compounds that are involved in a variety of plant–plant, plant–microbe and plant–herbivore interactions. These compounds exhibit great structural and functional diversity and are produced within plants through secondary metabolism (Hadacek 2002). Many phytotoxic allelochemicals have been isolated, identified and found to influence physiological functions in crops, for example, stomatal closure and plant water balance (Barkosky and Einhellig 2003), cell elongation (Nishida *et al.* 2005), membrane permeability (Galindo *et al.* 1999), nutrient uptake (Baar *et al.* 1994), photosynthesis (Baziramakenga *et al.* 1995), respiration (Norman *et al.* 2004), and many other metabolic processes.

Production of reactive oxygen species and the related oxidative stress have been proposed as a major mechanism of action of phytotoxins (Weir *et al.* 2004). Plants generate more molecules of reactive oxygen species under various stressful conditions such as suboptimal temperature (Farooq *et al.* 2009), high light and salinity, and pathogen attacks (Halliwell 1991; Yamamoto *et al.* 2003; Rhoads *et al.* 2006). The reaction centres of photosystem II and photosystem I in chloroplasts are

considered the major generation sites of reactive oxygen species because they possess an environment rich in oxygen, reductants and high-energy intermediates (Asada 2006). In the case of some photosynthesis-inhibiting herbicides, their primary toxic mechanism is to block the photosynthetic electron chain, which is followed by generation of reactive oxygen species, oxidative damage and cell death (Halliwell 1991). In an attempt to alleviate such damage, plants developed antioxidant systems to protect their cells (Ribera *et al.* 2013). Superoxide dismutase, which detoxifies singlet oxygen, is the first line of defence (Pompeu *et al.* 2008), while catalase and peroxidase scavenge toxic hydrogen peroxide (H_2O_2) and provide plant tolerance to biotic and abiotic stresses (Ünyayar *et al.* 2005).

2,4-Di-tert-butylphenol (2,4-DTBP) is an off-white–yellow crystalline solid that does not mix with water (EPA 2001). It is also one of natural compounds 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 reported to have medical properties such as antioxidant (Choi and Lee 2009; Kadoma *et al.* 2009), anticancer (Malek *et al.* 2009), antifungal (Zhou *et al.* 2011),

and antibacterial (Abdullah *et al.* 2011). In food processing, it has been proposed to prevent browning in fresh apple juices (Suh *et al.* 2011). Zhang *et al.* (2011) identified 2,4-DTBP in rhizosphere soil extracts of hops plants (*Humulus lupulus*); their results suggest that autotoxicity caused by this compound in the rhizosphere soil could be a reason for quality degradation in hops. In addition, 2,4-DTBP extracted from the rhizome of cogon grass (*Imperata cylindrica*) was found to have allelopathic effects on germination and seedling growth of weedy plants under soilless conditions; for instance, 2,4-DTBP at $100\,\mu g\,mL^{-1}$ completely inhibited the germination of cogon grass and showed 78–95% inhibition of root and shoot growth of beggar tick (*Bidens pilosa*), leucaena (*Leucaena leucocephala*) and barnyard grass (*Echinochloa crus-galli*) (Xuan *et al.* 2009). Recently, Chuah *et al.* (2014) identified 2,4-DTBP in culm plus leaf extracts of Napier grass (*Pennisetum purpureum*). They found that 2,4-DTBP exhibited potent herbicidal activity, whereby it completely prevented root growth of *L. chinensis* in soil at an application rate as low as $0.60\,kg\,a.i.\,ha^{-1}$.

Despite the above findings, little is known about the mode of action of 2,4-DTBP for use in weed management. Hence, the present study was conducted to elucidate the biochemical and physiological mechanisms of 2,4-DTBP on two selected weed species.

Materials and methods

Plant materials

Seeds of the bioassay species *Leptochloa chinensis* (L.) Nees (Chinese sprangletop), a grassy weed, and *Hedyotis verticillata* (L.) Lam. (woody borreria), a broadleaf weed, were collected from rice fields of Pasir Mas, Kelantan and oil palm plantations of Setiu, Terengganu, respectively. The seeds were sown in seedling trays (40 cm by 30 cm by 5 cm; two seeds per hole) filled with potting mixture. All trays were placed in a glasshouse with a 12-h photoperiod and photosynthetic photon flux density $800 \pm 200\,\mu mol\,m^{-2}\,s^{-1}$, temperatures ranging from 20°C to 35°C and relative humidity of 70–80%. The seedlings were watered daily with tapwater and they were grown until they reached the 6-leaf stage (5-week-old plants).

Dose-response tests

Dose-response tests were conducted, with the seedling plants of *L. chinensis* and *H. verticillata* to ascertain the suitable concentration of 2,4-DTBP to inhibit seedling growth by 50% relative to untreated seedlings. The seedlings at 6-leaf stage were transferred into a glass vial (2 cm in diameter, 7 cm high) filled with 1/8-strength Hoagland nutrient solution at pH 6.0 ± 0.2 and electrical conductivity (EC) at $1.2\,mS\,cm^{-1}$. The vials were placed in a controlled growth room with a light–dark regime of 12–12 h, $30°-20°C$ and photon flux density 140–160 $\mu mol\,m^{-2}\,s^{-1}$ and maintained at a relative humidity of 78–80%. Seedlings were allowed to acclimatise for 2 days in the Hoagland nutrient solution before treatments were applied. The 2,4-DTBP (99% purity; Sigma Chem. Co., Kuala Lumpur), was dissolved in 2% dimethyl sulfoxide (DMSO) and added to the nutrient solution at concentrations of 25, 50, 100 and 200 $\mu g\,mL^{-1}$ for *L. chinensis* and 50, 100, 200 and 400 $\mu g\,mL^{-1}$ for *H. verticillata*. Non-treated seedlings of both species were placed in a mixture of the Hoagland

nutrient and 2% DMSO and used as the control treatments. The solution in the glass vial was maintained by topping up with the 1/8-strength Hoagland nutrient solution at 24-h intervals. Shoot fresh weight was determined by harvesting and weighing the shoot tissues of non-treated and treated seedlings on days 3 and 7 (*L. chinensis*) or days 7 and 14 (*H. verticillata*). The data were expressed as percentage of the control.

The concentration of 2,4-DTBP that gave 50% inhibition of seedling growth was 50 $\mu g\,mL^{-1}$ for *L. chinensis* and 200 $\mu g\,mL^{-1}$ for *H. verticillata* at 7 and 14 days after treatment, respectively. These two concentrations were further utilised for subsequent experiments.

Biochemical action of 2,4-DTBP

Measurement of malondialdehyde

Lipid peroxidation was determined by measuring malondialdehyde accumulation via the method of Baziramakenga *et al.* (1995) with some modifications. Leaves or root samples (0.5 g) were homogenised in 5 mL of 0.1 M phosphate buffer and 0.1 g polyvinylpyrrolidone (PVP) and centrifuged at 6000 rpm for 15 min. Supernatant (0.75 mL) was added to 0.5% thiobarbituric acid in 20% trichloroacetic acid (3 mL). A blank contained 0.75 mL of supernatant and 3 mL of 20% trichloroacetic acid. The mixtures were placed in a water-bath at 95°C for 30 min and then quickly cooled in an ice-bath for 15 min. Samples were centrifuged at 6000 rpm 5 min, and then the absorbance of the supernatant was measured at 532 and 600 nm against the blank after subtracting the non-specific absorbance (600 nm). The malondialdehyde content was determined by using the molar extinction coefficient of $155\,mM^{-1}\,cm^{-1}$ and the results were expressed as nmol malondialdehyde g^{-1} fresh leaves or root weight by using the following formula:

$$((A532\,nm - A600\,nm)/155\,mm^{-1}\,cm^{-1})\,(10^6)$$
$$\times (V\,mL/1000\,mL) \times (1\,g/\text{leaves or root quantity})$$

where V is volume used in the spectrophotometric measurement, leaves or root quantity is (weight of sample (g) × amount of supernatant (mL)/amount of extraction buffer (mL)).

Electrolyte leakage

Membrane integrity is assessed in terms of electrolyte leakage (Galindo *et al.* 1999). Fresh leaves or root samples (0.1 g) were placed in a vial containing 10 mL of deionised water and allowed to stand in dark for 24 h at room temperature. The EC of the bathing solution (EC1) was measured at the end of incubation period. The tissue with bathing solution was then heated in water bath at 95°C for 20 min and the EC was measured again after cooling (EC2). Electrolyte leakage was calculated as percentage of EC1/EC2.

Preparation of crude enzyme extracts

Antioxidant enzymes of superoxide dismutase, peroxidase and catalase were extracted according to the method of Yu *et al.* (2003) with some modifications. Leave or root samples (0.4 g) were homogenised in 10 mL of 0.1 M phosphate buffer (pH 7.5) and 1% PVP by using pre-chilled mortar and pestle. The homogenates were kept for 1 h at 0°C before being centrifuged

at 6000 rpm for 15 min, and the supernatant was used for enzyme analysis. All assays were carried out at $2-4°C$.

Antioxidant enzyme activities

Superoxide dismutase

Superoxide dismutase activity was determined following the method of McCord and Fridovich (1969). Potassium phosphate buffer (216 mM, pH 7.8), 10.7 mM EDTA, 1.1 mM cytochrome C, 0.108 mM xanthine, titrated with 1 M KOH, and 0.05 unit mL^{-1} of xanthine oxidase in chilled distilled water were used. The cocktail was prepared by mixing the distilled water (23.0 mL), potassium phosphate buffer (25.0 mL), EDTA (1.0 mL), cytochrome C (1.0 mL) and xanthine (50.0 mL). The pH of mixtures was adjusted to 7.8 with 1 M HCl or 1 M KOH if needed. For the no inhibition test, 2.8 mL cocktail, 0.1 mL distilled water and 0.1 mL xanthine were added into a cuvette. For the inhibition test, 2.8 mL cocktail, 0.1 mL supernatant and 0.1 mL xanthine oxidase were added. The blank contained 2.8 mL cocktail and 0.2 mL distilled water. The increase of absorbance at 550 nm was determined for 5 min using a spectrophotometer (Model U-2000; Hitachi Ltd, Tokyo). One unit of superoxide dismutase activity is defined as the amount of enzyme activity that is able to inhibit the photo-reduction of cytochrome C by 50%.

Peroxidase

Peroxidase activity was measured following the method of Putter (1974). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0; 2.2 mL), supernatant (0.5 mL), 20.1 mM guaiacol (0.2 mL) and 246 mM hydrogen peroxide (0.1 mL). The blank contained 0.1 mL distilled water and 2.9 mL phosphate buffer at pH 7.0. When the absorbance has increased to 0.05, a stopwatch was activated to obtain the time required (min, Δt) to increase the absorbance to 0.1. The increase in absorbance was measured at 436 nm due to oxidation of guaiacol.

Catalase

For measurement of the catalase activity, the method of Aebi (1984) was used. Reaction mixtures contained 1.5 mL of 100 mM potassium phosphate buffer (pH 7), 0.5 mL of 75 mM hydrogen peroxide, 0.05 mL enzyme extract and distilled water to make the volume up to 3 mL. The reaction was started by adding hydrogen peroxide and the decrease in absorbance was recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of hydrogen peroxide decomposed.

Protein determination

Preparation of protein standards curve

Soluble protein was estimated by using the Coomassie Brilliant Blue G-250 reagent according to the method of Bradford (1976) with bovine serum albumin (BSA) as the standard. In order to measure and plot a standard curve of protein concentration *v.* absorbance at 595 nm, a series of dilutions of BSA concentrations ranging from 1.0 to 10.0 µg mL^{-1} was prepared. Dye reagent (0.2 mL) was added into each tube of 0.8 mL standard solution and vortexed. Test tubes were incubated at room temperature for at least 5 min. Absorbance (595 nm) increased over time; samples were incubated at room temperature for no more than 1 h. The blank

contained 0.2 mL dye reagent and 0.8 mL distilled water. The absorbance of the range of concentrations was plotted.

Determination of total protein concentration in sample

To determine the units of superoxide dismutase, peroxidase, and catalase activity in each mg protein, total protein concentration in the sample was measured. Dye reagent (0.2 mL) was added to 0.8 mL supernatant, and total protein concentration was determined as above, where the blank contains 0.2 mL dye reagent and 0.8 mL 0.1 M phosphate buffer.

Physiological mechanisms of 2 4-DTBP

Photosynthetic pigment content

The chlorophyll content of *L. chinensis* and *H. verticillata* treated with 50 and 200 µg mL^{-1} of 2,4-DTBP, respectively, was measured at the end of the incubation period based on the method of Ashraf *et al.* (1994). Photosynthetic pigments (chlorophyll *a* and *b*) were measured in fully expanded leaves. Prior to extraction, leaves of the bioassay species were cleaned with deionised water to remove surface contamination. The fresh leaves were cut into small pieces, and 0.5 g was homogenised with acetone (80% v/v), filtered and made up to a final volume of 5 mL. After centrifugation (5000 rpm) for 10 min at 4°C, the supernatant was withdrawn and absorbance was recorded at 663 nm (A663) and 645 nm (A645) with the Hitachi U-2000 spectrophotometer. The amount of chlorophyll extracted per g fresh weight (FW) was calculated using the following formulae:

$$\text{Chlorophyll } a \, (\text{mg g}^{-1} \, \text{FW}) = (12.7 \times (A663) - 2.69 \\ \times (A645)) \times 0.5$$

$$\text{Chlorophyll } b \, (\text{mg g}^{-1} \, \text{FW}) = (22.9 \times (A645) - 4.69 \\ \times (A663)) \times 0.5$$

$$\text{Chlorophyll } a + b \, (\text{mg g}^{-1} \, \text{FW}) = (20.2 \times (A645) + 8.02 \\ \times (A663)) \times 0.5$$

Photo-inhibition

Fluorescence (F_v/F_m, ratio of variable to maximal fluorescence) measurement was determined based on the method of Ishii-Iwamoto *et al.* (2006). The lamina of the second fully expanded leaf of the bioassay species was punched out with a cork borer to obtain a disc of 6 mm diameter. Five discs of *L. chinensis* and *H. verticillata* were placed in each of 1.5-cm-diameter test tubes containing 5 mL of 2,4-DTBP at 50 and 200 µg mL^{-1}, respectively. Deionised water was used for the control treatments. The test tubes were covered with aluminium foil to protect the leaf discs from light exposure during incubation at 25°C. After 2 h of incubation, the leaf discs were washed with distilled water and transferred to 9-cm-diameter Petri dishes that contained 5 mL deionised water and placed in the growth chamber at 25°C in darkness. Fluorescence was measured after 0, 3, 6, 12 and 24 h of incubation and after 60 s illumination time.

Photosynthetic activity

Net photosynthesis rate, stomatal conductance, transpiration rate and leaf internal CO_2 concentration for *L. chinensis* and

H. verticillata seedlings were measured at the end of incubation period with a CI-340 hand-held photosynthesis system (CID Bio-Science, Inc., Camas, WA, USA).

Statistical analyses

Each experiment was arranged in a completely randomised design with four replications. The data from the dose-response test and chlorophyll fluorescence were subjected to one-way analysis of variance (ANOVA). The *t*-test was used to compare the means among the treatments for biochemical and other physiological parameters. Differences were regarded as significant when $P < 0.05$. In certain cases, differences in mean values between groups were analysed with the Mann–Whitney U test, for example, the percentage data for electrolyte leakage, antioxidant enzymes (superoxide dismutase and peroxidase activities), chlorophyll content and chlorophyll fluorescence (3 h) (*H. verticillata*), and electrolyte leakage and chlorophyll fluorescence (24 h) (*L. chinensis*).

Results

Plant growth attributes

When evaluated for its phytotoxicity, 2,4-DTBP gave a similar pattern of shoot fresh weight inhibition in the two weed species. 2,4-DTBP significantly reduced the shoot fresh weight of *L. chinensis* and *H. verticillata*, and the effect was species- and concentration-dependent. Shoot fresh weight of both bioassay species decreased as the concentration of 2,4-DTBP in solution increased and the effect was evident after 1 and 2 weeks of exposure, respectively. The greatest inhibition in shoot fresh weight of *L. chinensis* and *H. verticillata* (80–85% reduction) was observed at 200 and 400 $\mu g\,mL^{-1}$, respectively, at the end of incubations (Fig. 1*a, b*). Application of 2,4-DTBP treatment at a low concentration of 50 $\mu g\,mL^{-1}$ decreased the shoot fresh weight of *L. chinensis* by 50% compared with the untreated seedlings. For a 50% reduction in shoot fresh weight of *H. verticillata*, a higher concentration of 200 $\mu g\,mL^{-1}$ was needed. The bioassay species growing in 50 or 200 $\mu g\,mL^{-1}$ of 2,4-DTBP were substantially smaller than the non-treated seedlings, with moderate symptoms of leaf wilting and necrosis as well as shorter root length.

Biochemical response to 2,4-DTBP

Treatment with 2,4-DTBP increased the amount of malondialdehyde, an indicator of the lipid peroxidation process, in *L. chinensis* and *H. verticillata* (Table 1). Malondialdehyde concentration and electrolyte leakage values in *L. chinensis* leaf and root tissues increased by ~300–1900% at 7 days after 2,4-DTBP treatment at 50 $\mu g\,mL^{-1}$. Exposure to 200 $\mu g\,mL^{-1}$ of 2,4-DTBP also resulted in an increase in the malondialdehyde content and electrolyte leakage in leaf and root tissues of *H. verticillata* by ~190–1200% at 14 days after treatment.

Treatment with 2,4-DTBP at 50 $\mu g\,mL^{-1}$ caused significant increases in superoxide dismutase, peroxidase and catalase enzyme activities in both leaf and root tissues of *L. chinensis* compared with untreated plants (Table 1). Peroxidase activity increased sharply (>700%) in leaves and to a lesser extent in roots
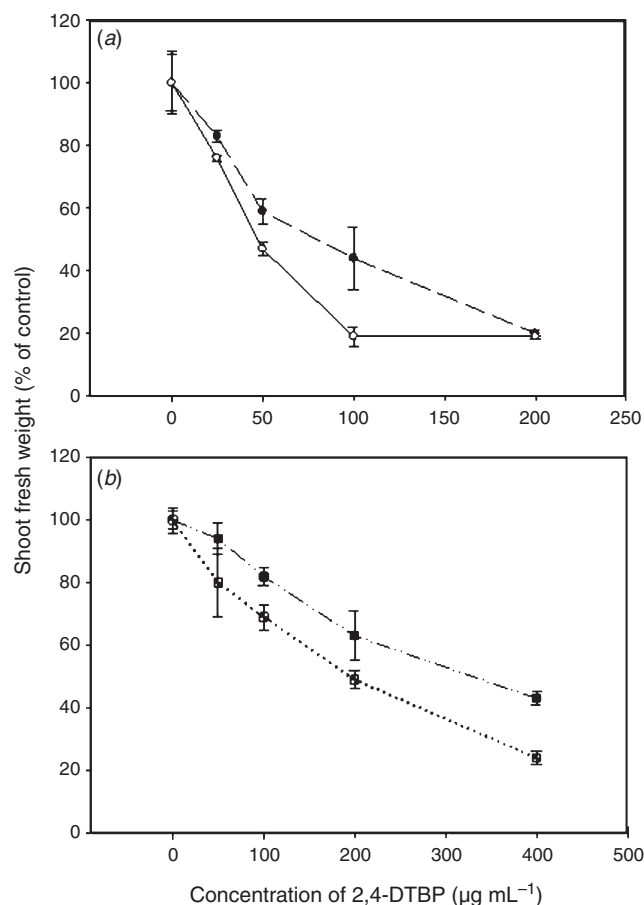
Fig. 1.   Shoot fresh weight of (*a*) *Leptochloa chinensis* at 3 days (●) and 7 days (○), and (*b*) *Hedyotis verticillata* at 7 days (■) and 14 days (□) after treatment with various concentrations of 2,4-DTBP. Values are means of four replicates ± standard deviation.

(256%). On the other hand, the increase in superoxide dismutase activity was slightly more pronounced in roots than leaves and vice versa for catalase activity after 2,4-DTBP treatment.

A significant increase was observed in all measured antioxidant enzymes in leaves and roots of *H. verticillata* at 14 days after 2,4-DTBP treatment (Table 1). Superoxide dismutase and peroxidase activities were increased sharply in leaves, by ~400–680%, relative to the control. Superoxide dismutase and peroxidase activities in roots were enhanced to a lesser extent, by ~170–300%. Catalase showed a lesser increase in activity in both plant tissues but still significantly higher than the control.

Physiological response to 2,4-DTBP

A decrease in chlorophyll content of *L. chinensis* and *H. verticillata* seedlings under 2,4-DTBP stress was observed (Table 2). There was a significant reduction in chlorophyll *a* and chlorophyll *b* contents of *L. chinensis* seedlings subjected to 2,4-DTBP at 50 $\mu g\,mL^{-1}$; contents of chlorophyll *a* and chlorophyll *b* were reduced by 54% and 38%, respectively. For *H. verticillata*, chlorophyll *a* and chlorophyll *b* contents were significantly reduced by 38% and 61%, respectively,

Table 1. Biochemical changes induced by exposure of *Leptochloa chinensis* and *Hedyotis verticillata* to 2,4-DTBP solution at 50 and 200 μg mL^{-1} , respectively

Assessment was done 7 and 14 days after 2,4-DTBP treatment for *L. chinensis* and *H. verticillata*, respectively. Values in parentheses are percentage of control. Data were analysed using *t*-tests or Mann–Whitney U test. Level of significance in 2,4-DTBP data with respect to control: **$P \leq 0.01$; ***$P \leq 0.001$

	Malondialdehyde (nmol g^{-1} FW)	Electrolyte leakage (%)	Enzyme activity (U min^{-1} mg^{-1} protein)		
			Superoxide dismutase	Peroxidase	Catalase
Leptochloa chinensis					
Leaves					
Control	3.30	17.38	19.10	7.49	16.20
2,4-DTBP	62.78	80.26	73.97	54.11	69.90
Per cent	(1902)	(462)	(387)	(722)	(432)
Significance	***	***	***	***	***
Roots					
Control	5.05	19.78	20.71	17.49	15.80
2,4-DTBP	49.23	68.81	80.50	44.76	65.40
Per cent	(975)	(348)	(389)	(256)	(414)
Significance	***	***	***	***	***
Hedyotis verticillata					
Leaves					
Control	5.01	7.57	19.0	4.48	32.2
2,4-DTBP	50.45	85.70	81.90	30.60	53.47
Per cent	(1007)	(1132)	(431)	(683)	(166)
Significance	***	***	***	***	**
Roots					
Control	9.93	27.2	36.6	7.33	33.6
2,4-DTBP	27.97	53.47	65.53	21.30	47.18
Per cent	(282)	(197)	(179)	(291)	(140)
Significance	***	***	***	***	***

Table 2. Leaf physiological changes induced by exposure of *Leptochloa chinensis* and *Hedyotis verticillata* to 2,4-DTBP solution at 50 and 200 μg mL^{-1}, respectively

Values in parentheses are percentage of control. Data were analysed with *t*-tests or Mann–Whitney U test. Level of significance in 2,4-DTBP data with respect to control: **$P \leq 0.01$; ***$P \leq 0.001$

	Chlorophyll content (mg g^{-1} FW)		Stomatal conductance (mmol m^{-2} s^{-1})	Intercellular CO$_2$ conc. (μmol CO$_2$ mol^{-1})	Transpiration rate (mmol H$_2$O m^{-2} s^{-1})	Net photosynthetic rate (μmol CO$_2$ m^{-2} s^{-1})
	a	*b*				
Leptochloa chinensis						
Control	6.04	2.01	1.10	126.00	5.43	46.08
2,4-DTBP	2.75	1.24	0.30	137.63	2.29	18.00
Per cent	(46)	(62)	(27)	(109)	(42)	(39)
Significance	***	***	***	***	**	***
Hedyotis verticillata						
Control	9.39	5.11	1.10	211.37	18.31	30.35
2,4-DTBP	5.85	1.99	0.26	247.22	15.80	13.61
Per cent	(62)	(39)	(24)	(117)	(86)	(45)
Significance	***	***	***	***	**	***

when seedlings were treated with 2,4-DTBP of 200 μg mL^{-1}. However, a reduction of ~45–50% was observed in the total chlorophyll content (*a* + *b*) for both bioassay species.

Application of 2,4-DTBP at 50 μg mL^{-1} directly to the leaf disc of *L. chinensis* resulted in significant inhibition of fluorescence (lower F_v/F_m ratio) at each time interval compared with the untreated leaf disc (Fig. 2*a*). A steady decrease in F_v/F_m was also observed for non-treated leaf discs with increasing time. However, *L. chinensis* treated leaf discs appeared to show a drastic decrease in the F_v/F_m ratio within 6–12 h after treatment. Application of 200 μg mL^{-1} of 2,4-DTBP significantly reduced the F_v/F_m ratio in *H. verticillata* within
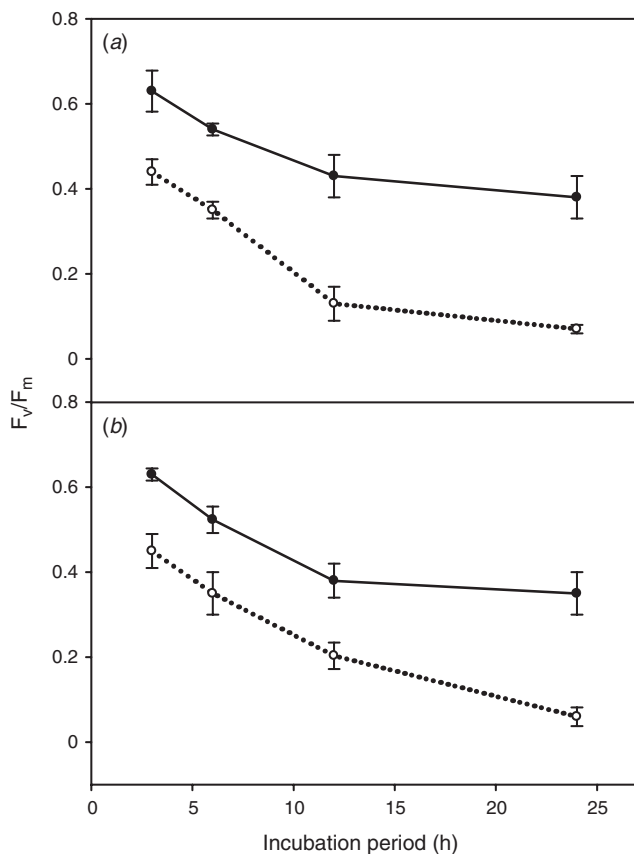
Fig. 2. Fluorescence in (*a*) *Leptochloa chinensis* and (*b*) *Hedyotis verticillata* grown in 1/8-strength Hoagland nutrient solution with (○) or without (●) 2,4-DTBP throughout the 24-h incubation period. *Leptochloa chinensis* and *H. verticillata* seedlings were subjected to 2,4-DTBP at 50 and 200 μg mL^{-1}, respectively. Values are means of four replicates ± standard deviation.

24 h of treatment (Fig. 2*b*). Non-treated leaf discs again exhibited a gradual decline in the F_v/F_m ratio as the time of incubation increased, and the treated leaf discs showed a small comparative reduction in F_v/F_m ratio within 6–12 h after treatment but a greater reduction at the end of the incubation time compared with untreated leaf discs.

The photosynthetic parameters of *L. chinensis* and *H. verticillata* seedlings were mostly negatively affected by 2,4-DTBP treatment, except for intercellular CO_2 concentration (Table 2). After 7 days of incubation, a large reduction in stomatal conductance was observed in the treated *L. chinensis* seedlings (>70% reduction). Net photosynthetic and transpiration rates of *L. chinensis* seedlings also decreased significantly, ranging from 50% to 60%, when grown in a 50 μg mL^{-1} solution of 2,4-DTBP. However, there was a slight increase in intercellular CO_2 concentration (9%). Similarly, after 14 days of incubation, a large reduction in stomatal conductance was observed in *H. verticillata* seedlings (>70% reduction) when treated with 200 μg mL^{-1} of 2,4-DTBP. In addition, a maximum reduction of 45% was observed for net photosynthetic rate in *H. verticillata*, and a reduction of only 14% for transpiration rate. Surprisingly, the intercellular CO_2 concentration was 17%

significantly higher than that in the untreated seedlings when 2,4-DTBP was applied.

Discussion

In the present study, there was significant reduction in the shoot fresh weight of *L. chinensis* and *H. verticillata* after the root parts were treated with 2,4-DTBP (Fig. 1). This suggests that 2,4-DTBP was most likely translocated to the shoot tissues and diminished the green colour of the leaf blades, thereby retarding plant growth and development. Uddin *et al.* (2012) showed that sorgoleone application at 200 μg mL^{-1} significantly reduced the growth of weedy plants *Digitaria sanguinalis* and *Amaranthus retroflexus* by 60% and 80%, respectively, after 2 weeks of treatment. Yang *et al.* (2002) reported that phenolics such as ferulic and *p*-coumaric acids at 100 μg mL^{-1} cause not only growth retardation in rice (*Oryza sativa*) seedlings, but also leaf dehydration, leaf shrinkage and a decrease in leaf width after 1 week of treatment. Batish *et al.* (2007) showed that ferulic acid and *p*-coumaric acid present in the aqueous extracts of *Chenopodium murale* reduced the overall growth of wheat (*Triticum aestivum*). These findings are in agreement with studies on phytotoxic effects of organic acids: benzoic, phenylacetic, cinnamic and *p*-hydroxybenzoic acids on lettuce (*Lactuca sativa*) (Lee *et al.* 2006), and 1,2-benzenedicarboxylic acid on maize (*Zea mays*) (Chai and Feng 2007). Growth inhibition caused by these allelochemicals may be due to its interference with the plant growth processes (Hassannejad *et al.* 2013).

The level of malondialdehyde produced during lipid peroxidation is a good indicator of oxidative damage within cells (Masia 2003). In the present study, enhanced malondialdehyde in both bioassay species (Table 1) suggests that 2,4-DTBP probably induced oxidative stress and, as a result, disrupted the cellular membrane structure and caused a loss of cellular integrity. Similar results have been reported by Ye *et al.* (2006), who showed that phenolic acid (cinnamic acid) induced oxidative stress in cucumber seedlings. Many studies have shown that increased malondialdehyde content is associated with increased superoxide radicals and hydrogen peroxide production (Forman *et al.* 2002; Lara-Nuñez *et al.* 2006). Likewise, 2,4-DTBP might have induced superoxide anion and hydrogen peroxide production in the leaves and roots of *L. chinensis* and *H. verticillata* (Table 1), which suggests that the 2,4-DTBP could have triggered generation of reactive oxygen species and induced oxidative stress in the tissues of both the bioassay species. This is consistent with results obtained with mung bean (*Phaseolus aureus*) treated with the allelochemical 2-benzoxazolinone (Batish *et al.* 2006). Production of reactive oxygen species induced by 2,4-DTBP also led to increased electrolyte leakage in both the leaves and roots of *L. chinensis* and *H. verticillata* (Table 1), and the increase was greater in leaf tissues. Increased electrolyte leakage is a result of increased membrane permeability by allelochemical reaction, among others via lipid peroxidation and generation of reactive oxygen species (Singh and Sunaina 2014). These activities consequently result in dysfunctions of ion channels and metabolic centres localised on membranes. In addition, the toxic effects of 2,4-DTBP most probably were translocated from the root to the shoot, since the leaf blades were also strongly dehydrated and this, in itself, leads to a loss of membrane integrity.

Recently, generation of reactive oxygen species and related oxidative stress have been proposed as one of the modes of action of plant growth inhibition by allelochemicals (Weir *et al.* 2004). To avoid cellular damage due to generation of reactive oxygen species, plants produce in response several antioxidant enzymes to provide secondary protection against oxidative stress (Apel and Hirt 2004; Mittler *et al.* 2004). The increases in superoxide dismutase, peroxidase and catalase activities (Table 1) indicate that excessive reactive oxygen species were triggered by the 2,4-DTBP treatment, and consequently, these antioxidant enzyme activities were regulated to mitigate the oxidative damage. Superoxide dismutase scavenges the highly reactive free radicals by converting them into hydrogen peroxide. Although hydrogen peroxide is equally toxic, hydrogen peroxide is further reduced to water by catalase in the peroxisomes and by peroxidase in the cell wall (Blokhina *et al.* 2003). The present data are in agreement with results of other studies where increased activities of superoxide dismutase and catalase were reported in other plants such as tomato (Romero-Romero *et al.* 2005) and mustard (Oracz *et al.* 2007) under different allelochemical stress. In the present study, increases in the activities of these antioxidant enzymes paralleled the accumulation of malondialdehyde in tissues of *L. chinensis* and *H. verticillata* after exposure to 2,4-DTBP (Table 1). Observations are consistent with those of Cruz-Ortega *et al.* (2002), who reported that allelochemical stress caused increased levels of free radicals and activity of antioxidant enzymes and suggest that increased induction of these enzymes was necessary to prevent lipid peroxidation (i.e. to counter the higher malondialdehyde in leaves and roots of *L. chinensis* and *H. verticillata*).

Theoretically, a decrease in chlorophyll *a* and chlorophyll *b* will reduce photosynthesis. Reduced chlorophyll content in allelochemical-treated plants has been reported (Singh *et al.* 2010). Chlorophyll content is decreased by phenolic acids in rice (Yang *et al.* 2004), by monoterpenes in *Cassia occidentalis* (Singh *et al.* 2002), and by secalonic acid in sorghum (*Sorghum bicolor*) (Zeng *et al.* 2001). In the present study, the accumulation of photosynthetic pigments in leaves of *L. chinensis* and *H. verticillata* was inhibited by 2,4-DTBP, and the decline in shoot fresh weight mentioned above might be ascribed to the decrease in the chlorophyll content of both species (Table 2). The reduction in chlorophyll content could be due to destruction of chloroplast membranes caused by lipid peroxidation, leading to photosynthesis failure, which would eventually inhibit weed growth. These results are in agreement with findings documented by Patterson (1981) whereby treatment of soybean plants (*Glycine max*) with phenolic acid (*p*-coumaric) and vanillic acids greatly decreased the biomass, associated with reduced chlorophyll content in the leaves.

Phytotoxic chemicals can reduce the capacity of the photosynthetic system to utilise incident light, leading to a photo-inhibition process. Photo-inhibition of photosynthesis is typically characterised as a reduction in the quantum of yield of the photosystem II photochemistry and a decrease in chlorophyll *a* fluorescence (Zhou and Yu 2006). A value of 0.8 is considered as a threshold F_v/F_m ratio for photo-inhibition (Lüttge *et al.* 1998). Chlorophyll fluorescence of most plant species can be measured. Values lower than the thresholds are observed when plants are subjected to stress, indicating the phenomenon of photo-inhibition, in particular. The F_v/F_m ratios of both *L. chinensis* (0.07 arbitrary units, a.u.) and *H. verticillata* (0.06 a.u.) plants treated with 2,4-DTBP were lower than those of the control plants (0.38 a.u.) and (0.35 a.u.), respectively, after 24 h of incubation (Fig. 2), indicating that 2,4-DTBP inhibited photosynthetic activity at the photosystem II level in both bioassay species. Results from the present study also showed that treated leaf discs of the broadleaf weed, *H. verticillata*, showed slow reduction in the F_v/F_m ratio within 6–12 h after 2,4-DTBP treatment at $200\,\mu g\,mL^{-1}$ (35–45% inhibition). By contrast, Uddin *et al.* (2012) reported that sorgoleone at $200\,\mu g\,mL^{-1}$ drastically reduced chlorophyll fluorescence and F_v/F_m values of broadleaf weed leaf discs (*Galium spurium*, *Aeschynomene indica* and *Rumex japonicus*) by 93%, 88% and 84%, respectively, after 6 h application.

According to Maxwell and Johnson (2000), chlorophyll fluorescence is light that has been re-emitted after being absorbed by the chlorophyll molecules in plant leaves. Therefore, the decrease in chlorophyll content in the present study might cause a decrease in chlorophyll fluorescence of the bioassay species treated with 2,4 DTBP. These results are similar to the findings of Yu *et al.* (2006), where 2,4 DTBP reduced chlorophyll content and chlorophyll fluorescence of eggplant (*Solanum melongena*) seedlings. Zeng *et al.* (2001) showed that the destruction of chloroplasts by secalonic acid F reduced photosystem II efficiency in higher plants. A few studies have also shown that the allelochemicals or phytochemicals xanthorrhizol (Gonzalez-Bernardo *et al.* 2003) and trachyoban-19-oic acid from *Iostephane heterophylla* (Hernández-Terrones *et al.* 2003), resorcinolic lipids (Rimando *et al.* 2003) from sorghum, and polyphenolic allelochemicals from the aquatic angiosperm *Myriophyllum spicatum* (Leu *et al.* 2002) significantly inhibited PSII.

In the process of photosynthesis, the movement of CO_2 from air to the photosynthetic section in the chloroplast is affected by many factors, including stomatal conductance, intercellular CO_2 concentration, transpiration rate and net photosynthesis rate (Hanba *et al.* 2003). In the present study, a decrease in stomatal conductance was more apparent than the other photosynthetic variables in both bioassay species (Table 2), implying that stomatal closure resulted in reduced net photosynthesis and transpiration rates. Stomata are triggered to open in light so that CO_2 is available for the light-dependent process of photosynthesis. However, very low levels of light can restrict the opening of the stomata, and consequently, they can access only small amounts of CO_2 for photosynthesis. When stomata are restricted or closed, transpiration rate also decreases (Tallman 2004). A decrease in stomatal conductance in the present study was similar to results reported by Halliwell (1991), where overproduction of reactive oxygen species disrupted chloroplast cell membranes, causing chlorophyll breakdown and restricted opening of stomata to absorb the light, leading to lower stomatal conductance. Similarly, Matsumoto *et al.* (2005) found that low leaf chlorophyll content imposed a restriction on the opening capacity of the stomata in bao li (*Quercus serrata*) trees.

A decrease in stomatal conductance under plant stress caused by phytotoxic compounds has been documented. Patterson (1981) reported that cinnamic, benzoic, and salicylic acids

inhibited the net photosynthesis rate and stomatal conductance of soybean leaves. Mersie and Singh (1993) reported that ferulic and vanillic acid caused a significant decline in the net photosynthesis rate of *Calathea leopardina* leaves, with a decrease in stomatal conductance. However, in the case of stomatal limitation, reduced stomatal conductance is generally accompanied by decreased intercellular CO_2 concentration. On the other hand, non-stomatal limitation is characterised by reduced stomatal conductance and increased intercellular CO_2 concentration (Farquhar and Sharkey 1982), as shown in the present study. Results also indicate that the decrease in stomatal conductance coincided with a decline in transpiration rate, suggesting that the decrease in net photosynthesis rate induced by 2,4-DTBP was at least partly due to stomatal closure. The non-stomatal limitation results are in agreement with the findings of Shao *et al.* (2013) that intercellular CO_2 concentration of wheat increased significantly whereas the stomatal conductance, net photosynthesis and transpiration rates decreased when treated with *Xanthium italicum* residues.

Although shoot fresh weight, chlorophyll content, chlorophyll fluorescence and photosynthetic activities (except intercellular CO_2 concentration) of both bioassay species were reduced, *L. chinensis* was considerably more sensitive to 2,4-DTBP than *H. verticillata*. At a shorter incubation period (7 days), the shoot fresh weight of *L. chinensis* decreased by 50% at the lower concentration ($50\,\mu g\,mL^{-1}$) of 2,4-DTBP (Fig. 1*a*). This may be due to great reduction of chlorophyll *a* (Table 2), coupled with a rapid reduction rate in chlorophyll fluorescence that occurred after 6–12 h incubation (Fig. 2). Furthermore, transpiration rate in *L. chinensis* decreased greatly (Table 2) and affected photosynthetic activity, thus inhibiting plant growth. Importantly, the present study also showed that treated seedlings of *L. chinensis* experienced a great increase in lipid peroxidation and maintained higher activity of superoxide dismutase, peroxidase and catalase (Table 1) than *H. verticillata*, which may be related to difference in the intrinsic capacity for scavenging of reactive oxygen species between the species. These results are in agreement with those of Darier and Tammam (2012), who reported a difference in the intrinsic scavenging capacity for reactive oxygen species in barley and broad bean treated with *Achillea santolina* aqueous shoot extract. However, this does not mean that *H. verticillata* was experiencing less oxidative stress, because it also showed elevated activities of antioxidant enzymes (Table 1). Furthermore, the greater membrane injury observed in the leaf blades than the roots in both species can be explained by the leaf blades possibly being more stressed (Table 1). The malondialdehyde content of the plant tissues after the 2,4-DTBP treatment increased by >90% in the leaves and 70–90% in the roots (Table 1), suggesting that the leaves appear to encounter more cell damage from free radicals, especially in important organelles such as chloroplasts and mitochondria (Zeng *et al.* 2001).

Conclusions

Exposure to 2,4-DTBP induced oxidative stress through the enhanced generation of reactive oxygen species, which was accompanied by enhanced lipid peroxidation levels, membrane damage, and activation of antioxidant enzyme systems. These membrane damages caused great reduction in chlorophyll content, thereby decreasing chlorophyll fluorescence, transpiration and net photosynthetic rate, with consequent retardation of growth and development of weedy plants *L. chinensis* and *H. verticillata*. Increased levels of scavenging enzymes indicate their induction as a secondary defence mechanism in response to 2,4-DTBP. However, this increase was not sufficient to eliminate all of the deleterious effects provoked by 2,4-DTBP, only to alleviate to the impact of the stress. These findings imply that 2,4-DTBP has potential to be used as a template for designing new, natural and environmental friendly herbicides for weed management.

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