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M. Iftikhar Hussain, Manuel J. Reigosa & Abdullah J. Al-Dakheel

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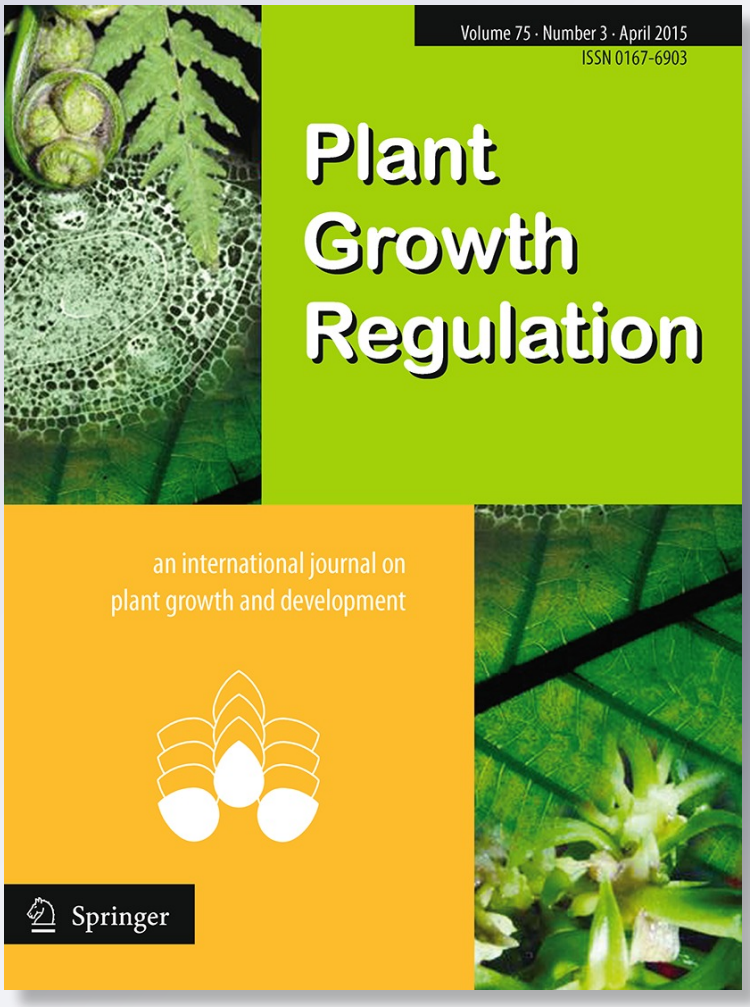

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ORIGINAL PAPER

Biochemical, physiological and isotopic responses to natural product *p*-hydroxybenzoic acid in Cocksfoot (*Dactylis glomerata* L.)

M. Iftikhar Hussain · Manuel J. Reigosa · Abdullah J. Al-Dakheel

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Abstract The present study was conducted to evaluate the role of natural compound *p*-hydroxybenzoic acid (pHBA) on physiological, biochemical and isotopic responses of *Dactylis glomerata* L. Application of pHBA in the range 0.5–1.5 mM reduced the leaf fresh/dry biomass, shoot/root length, relative water content and leaf osmotic potential of *D. glomerata*. pHBA decreased the photosynthetic efficiency and quantum yield of photosystem II photochemistry in *D. glomerata* seedlings following treatment at all concentrations. Photochemical and non-photochemical fluorescence quenching were reduced after treatment with 1.5 mM pHBA. Carbon isotope composition ratio in *D. glomerata* leaves was significantly less negative following treatment with pHBA than the control. Carbon isotope discrimination value was declined by pHBA. The leaf protein content was lower after treatment with 1.5 mM pHBA. Our results suggest that pHBA possesses allelopathic potential against *D. glomerata* and this study provide new insights into the physiological, biochemical and isotopic action mechanism of pHBA.

Keywords Allelochemical stress · Carbon isotope discrimination · *Dactylis glomerata* · Growth · Leaf water relations · *p*-hydroxybenzoic acid · Physiological responses

Introduction

Modern agricultural productivity relies heavily on the use of synthetic chemicals to control weeds, insects and pests. However, there is overwhelming evidence that most of the agrochemicals (pesticides, herbicides) pose serious risk to human health, environment and animals (Igbedioh 1991; Garbarino et al. 2002). Furthermore, continuous use of the same pesticides/herbicides is causing resistance in pests and weeds (Gressel and Baltazar 1996). This situation demands that efforts should be made to develop an alternative technology for weed control which is environment friendly, safe and also reduces the cost of production. Allelopathy may be an appropriate potential technology for this purpose. It is the influence of plants (directly or indirectly) on one another through the production of allelochemicals that escape into the environment (Rice 1984). Allelochemicals have been shown to be less toxic, environmentally safer, to conserve resources which, with their potential biodegradability, would nullify the problems raised by synthetic chemicals (Rizvi et al. 1992). Plant based natural products can act directly as bioherbicides or may provide lead structures for new herbicidal discovery programs (Dayan et al. 1999; Duke et al. 2000).

Phenolic acids, flavonoids, tannins, coumarins and lignin are an extensive group of natural compounds that derive from the phenylpropanoid pathway, (Kovacik et al. 2007; Boerjan et al. 2003) having multifunctional phytotoxicity against pathogens, microorganisms, fungi, insects, and weeds (Silva et al. 2006; Soltoft et al. 2008). This

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M. I. Hussain (✉) · M. J. Reigosa
Department of Plant Biology and Soil Science, University of Vigo, Campus Lagoas-Marcosende, 36310 Vigo, Spain
e-mail: mih76@uvigo.es

M. I. Hussain
Laboratoire Chrono-Environment UMR CNRS 6249, University of Franche Comté, BP 71427, 25311 Montbéliard Cedex, France

M. I. Hussain · A. J. Al-Dakheel
International Center for Biosaline Agriculture (ICBA), P.O. Box 14660, Dubai, UAE

includes common benzoic and cinnamic acid derivatives, polyphenols and quinones. The *p*-hydroxybenzoic acid (hereafter pHBA), is a widespread phenolic compound released into soil by leaf leachates, decomposed plant tissues and root exudates (Einhellig and Rasmussen 1979; Lehle and Putnam 1983; Dalton 1999). Other plants implicated in allelopathic release of pHBA include *Camelina alyssum* (Grummer and Beyer 1960), several members of the genus *Althaea* (Gude and Bieganowski 1990), grass *Imperata cylindrica* (Hussain and Abidi 1991). Perez and Ormeno-Nunez (1991) examined the release of pHBA from root exudates of wild oat (*Avena fatua* L.) and reported the inhibition of root and coleoptile growth of wheat seedlings.

Although several physiological effects of pHBA have been reported, however, its primary action affecting the plant growth is obscure. It was demonstrated that benzoic acid and cinnamic acid were responsible for negative allelopathic effects of quack grass on soybean; reducing root growth and dry biomass by altering ion uptake/transport and chlorophyll content (Baziramakenga et al. 1997). pHBA is considered to be a potent herbicide for grass and weed suppression with its principal mode of action on germination and seedling growth inhibition in *Deschampsia flexuosa*, *Scrophularia nodosa*, *Senecio sylvaticus*, and *Chamaenerion angustifolium* (Kuiters 1989), radish and grain sorghum (Einhellig and Rasmussen 1978), *Phaseolus mungo* L. (Ghafar et al. 2000) and Corn (Opoku et al. 1997). Chaves et al. (2001) reported the inhibitory effect of several allelochemicals including pHBA on germination, cotyledon emergence, root length, and cotyledon length of *Rumex crispus*. The short term application of pHBA on spring barley reduced the yield up to 20 % and was concentration dependent (Christon and Lovett 1993). Glass (1975) demonstrated the inhibitory action of pHBA on potassium and inorganic phosphate absorption of roots and membrane depolarization in barley. Allelochemicals may interfere with cell division, hormone biosynthesis, mineral uptake and transport (Rizvi et al. 1992), leaf water relations, respiration, protein metabolism, photosynthesis (Sánchez-Moreiras and Reigosa 2005; Sánchez-Moreiras et al. 2010; Hussain and Reigosa 2011), membrane perturbations, respiration, oxidative stress (Einhellig 2004; Weir et al. 2004; Gniazdowska and Bogatek 2005; Li et al. 2010) and programmed cell death (Díaz-Tielas et al. 2012).

The phytotoxic effects of allelochemicals are species specific and dependent on plant growth stage, conditions, including fate and persistence in soil and are selective like synthetic herbicides (Weston 1996). Cheema et al. (2000) evaluated sorghum crop mulch and aqueous extract against weeds in mungbean and found that the dry weight of *Cyperus rotundus*, *Convolvulus arvensis* and *Portulaca oleracea* were decreased while *Trianthema portulacastrum* remained unaffected following exposure to sorghum aqueous extract. Previously, we have reported that hydroxybenzoic acids were more toxic than the structurally similar hydroxycinnamic acids in germination and seedling growth bioassays in *Arabidopsis thaliana* (Reigosa and Pazos-Malvido 2007). We evaluated the phytotoxic potential of different phenolic compounds on ecophysiological constraints, leaf water relationships, growth and carbon isotope discrimination in general biotest specie, *Lactuca sativa* (Hussain et al. 2010, 2011). Allelopathy is a novel approach offering multiple solutions to conundrum of decreasing food availability under rising global population. With vast application in weed management, it can replace hazardous chemical and mechanical approaches being used in crop production. The *L. sativa* is a model horticultural crop used previously in allelopathic and phytotoxic studies from our group (Sánchez-Moreiras et al. 2010; Hussain et al. 2010). However, the goal of the present work was to evaluate the mode of action of the secondary metabolite pHBA, and to examine the phytotoxic impact of pHBA on leaf-water relationships, plant biomass, photosystem II photochemistry, fluorescence quenching coefficient, protein contents and correspondence of these actions with effects on plant growth in grass weed, *Dactylis glomerata*. The relative concentration of stable carbon isotope composition in leaf tissue was also analyzed in order to elucidate the mechanism of action of this phenolic compound.

Materials and methods

Chemicals

The phenolic compound *p*-hydroxybenzoic acid (C_7 H_6 O_3) (pHBA) was selected for the present study because its phytotoxicity has been previously described. Different concentrations of allelochemical pHBA were prepared as described in Hussain et al. (2010). Briefly, the pHBA (purity ≥99 %) was dissolved in water:methanol mixture (80:20) and different concentrations (0.1, 0.5, 1.0 and 1.5 mM) were prepared from stock solution (3 mM). The control was prepared with distilled water and methanol and pHs of all these solutions were adjusted at 6.0 by the addition of NaOH and solutions were stored in amber glass vials at 4 °C.

Plant material and growth conditions

Cocksfoot (*D. glomerata* L.) was selected as bioassay specie due to its fast germination and homogeneity (Hussain et al. 2008). The seeds of *D. glomerata* were purchased from Semillas Fito (Barcelona, Spain), surface

sterilized for 3 min in NaOCl (0.5 %) and washed in autoclaved water. The seeds were placed in plastic trays (32 × 20 × 6 cm) with a 5 cm deep layer of perlite (500 g/tray). The trays were irrigated on alternate days with tap water until the germination of seeds and thereafter, with 500 mL 1:1 Hoagland solution/tray twice per week. For seedling growth, the temperature was: 18/8 °C (day/night) and 12/12 h (light/darkness) photoperiod, 80 % relative humidity and 200 µmol m^{-2} s^{-1} irradiance. One-month-old seedlings (when the plants had 3-fully expanded leaves), were transferred to pots (10 cm diameter) containing perlite (70 g) to stimulate the development of the root system. The pots were placed in glass house having same growing conditions (as in growth chamber) and irrigated with nutrient solution of 100 ml/pot. One week after the plants were transferred, the treatment solutions (100 ml pot^{-1}) were applied three times (day 1, 3, and 5) and measurements were taken. The temperature in the glass house was maintained at 21 ± 2 °C with a relative humidity of 75 %.

Chlorophyll fluorescence measurement

The chlorophyll fluorescence was measured on randomly selected leaves of *D. glomerata* with a portable, pulse-modulated fluorescence monitoring system (FMS) (Hansatech, Norfolk, England) using the method of Weiss and Reigosa (2001) as described in Hussain et al. (2010). The chlorophyll fluorescence was measured by Pulse-modulated fluorescence meter every day up to 1 week continuously for the three replicates per treatment and three values per plant were recorded for each fluorescence measurements and averaged. The minimum chlorophyll fluorescence (F_o), maximum fluorescence (F_m), quantum efficiency (F_v/F_m) of open PSII reaction centers in dark-adapted plants, maximum fluorescence in light saturated state (F'_m), photochemical fluorescence quenching (qP) and non-photochemical quenching (NPQ) were recorded as documented previously (Genty and Briantais 1989; Kramer et al. 2004).

Carbon isotope discrimination and mass spectrometry analysis

Carbon isotope compositions were determined as reported by Hussain et al. (2010) from the dry leaf sample. Briefly, dry and ground plant material weighed (1,700–2,100 µg), filled into tin capsules (5 × 3.5 mm, Elemental Microanalysis Limited, UK) and entered automatically into combustion oven at 1,600–1,800 °C in the presence of oxygen and converted to CO_2. Subsequently carbon isotope ratios were determined in an Isotope Ratio Mass Spectrometer (Finnegan: Thermo Fisher Scientific, model MAT-253, Swerte, Germany) coupled with an Elemental Analyzer (Flash EA-1112, Swerte Germany). Carbon isotope compositions ratios ($\delta^{13}C$), carbon isotope discrimination ($\Delta^{13}C$) and ratio of leaf intercellular CO_2 concentrations to that in the atmosphere (*ci*/*ca*), was calculated as described (Hussain et al. 2010). Carbon isotope discrimination and mass spectrometry analysis was done from dry biomass at the end of experiment from three replicates per treatment and averaged. Carbon isotope measurements were performed in CACATI (Centro de Apoio Cientifico Tecnologico a la Investigacion), University of Vigo, Spain.

Plant growth bioassays

The plant height and root length were obtained with a ruler and values were expressed in cm. The fresh and dry weight of leaf/root were obtained by first independently weighing fresh leaves and roots, and then drying these samples in a circulatory air oven at 70 °C for 72 h. The samples were weighed again to get dry weight. The fresh and dry biomass was recorded after 7 days from each treatment application and averaged. The shoot/root length and fresh/dry weight of *D. glomerata* was determined as described (Hussain et al. 2010).

Relative water content and leaf osmotic potential

The leaf relative water content (RWC) was calculated by measuring fresh (W_f), saturated (W_t) and dry (W_d) weights of *D. glomerata* as described in Hussain et al. (2010). Leaf osmotic potential (LOP) was determined according to González (2001) in milliosmol kg^{-1} using a calibrated vapor pressure Osmometer (Automatic Cryoscopic Osmometer, Osmomat-030, GmbH, Gonatec, Berlin, Germany) as reported in Hussain et al. (2010).

Leaf protein content determination

Total protein content were determined from *D. glomerata* leaves at the end of experiment using spectrophotometer by Bradford's method as described by Pedrol and Ramos (2001) using bovine serum albumin (BSA) as standard.

Statistical analysis

Statistical analysis was performed based on the randomized complete block design with three replicates using SPSS® 15.00 (SPSS Inc., Chicago, IL, USA). Multiple comparisons of means were performed by LSD test at 0.05 significance level (when variance was homogeneous) or Kruskal–Wallis test (when heterogeneous).

Results

The understanding of how allelopathy actually works requires knowledge at molecular level of how allelochemicals exert their effects. Important developments and applications can be expected in a short time from the results of mode of action studies with allelopathic compounds. The development of accurate and case-related bioassays is required, as the allelopathic phenomenon is extremely specific in each situation. This information's are important because it is very hard to determine a definite mechanism due to the diverse nature and complicated responses of such chemicals under natural conditions. In the present work, the measured parameters are comprehensive and complete; giving a better explanation of the pHBA interference with physiological processes in *D. glomerata*.

Effect of pHBA on growth and seedling biomass

The phytotoxic effects were prominent in *D. glomerata* following 1 week pHBA treatment. Leaf fresh weight (LFW) was significantly suppressed by different concentrations of pHBA as compared to control. pHBA reduced LFW by 43 % at 1.0 mM, followed by 42 % at 1.5 mM, respectively (Table 1). The leaf dry weight of *D. glomerata* was significantly reduced following exposure to pHBA at 1.5 mM (63 %) and 1.0 mM (55 %) than control (Table 1). In *D. glomerata* the root fresh weight was decreased significantly at all pHBA concentrations and maximum reduction (50 %) was observed following treatment at 1.5 mM pHBA than control (Table 1). Root dry weight was reduced after pHBA treatment and the lowest value in root dry weight was recorded in *D. glomerata* seedlings at 1.5 mM pHBA concentrations followed by those receiving lower dose of pHBA.

In *D. glomerata* seedlings, shoot length (SL) was significantly suppressed by pHBA as compared to the control. The reduction in SL was 50 % (at 1.5 mM pHBA) followed by 46 % (at 0.1 mM) and 33 % (0.5 mM) respectively (Table 2). However, the maximum reduction (56 %) was found in plants treated with 1.0 mM pHBA than control. Moreover, pHBA, a promising phenolic compound, markedly inhibited the root length of *D. glomerata* at all tested concentrations. The results revealed that pHBA (1.0 mM) had the ability to suppress root length up to 41 % than the control (Table 2). The *D. glomerata* seedlings treated with 0.5 mM and 0.1 mM pHBA had similar patterns of variation, and caused reductions in root length of 34 and 31 % respectively, than control.

Effect of pHBA on leaf water status

The relative water content (RWC) express the water content in percent at a given time as related to the water content at full turgor. It is a useful measurement to check impact of any environmental stress on the plant growth. Allelochemical pHBA significantly decreased the relative water content (RWC) at all concentrations. Exposure of the plants to 7 days of pHBA stress led to a decrease in RWC from 5 to 33 % in the *D. glomerata* (Fig. 1). RWC decreased gradually as stress progressed, but particularly during the last 5 days of treatment and maximum reduction was observed after pHBA treatment at 1.5 mM concentration than control.

Water potential is the potential energy of water per unit volume relative to pure water in reference conditions. A leaf can increase its resistance to dehydration through a reduction in cellular osmotic potential by a net accumulation of cellular solutes. The key role of osmotic adjustment is in turgor maintenance during water deficits, which in turn is essential for maintenance of turgor-related

Table 1 Effect of *p*-hydroxybenzoic acid (pHBA) at 1.5, 1.0, 0.5 and 0.1 mM concentrations on leaf/root fresh and dry weight (g) of 1 month old *D. glomerata* L.

Growth characteristics	Treatments	1.5 mM	1.0 mM	0.5 mM	0.1 mM
Leaf fresh weight (g)	Control	10.58 ± 0.60	10.58 ± 0.60	10.58 ± 0.60	10.58 ± 0.60
	pHBA	6.13 ± 1.03*	6.00 ± 0.16*	7.53 ± 0.30*	9.19 ± 1.32
Leaf dry weight (g)	Control	1.63 ± 0.15	1.63 ± 0.15	1.63 ± 0.15	1.63 ± 0.15
	pHBA	0.60 ± 0.12*	0.72 ± 0.18*	1.03 ± 0.15	1.42 ± 0.12
Root fresh weight (g)	Control	6.31 ± 0.34	6.31 ± 0.34	6.31 ± 0.34	6.31 ± 0.34
	pHBA	3.13 ± 0.40*	4.01 ± 0.25*	4.17 ± 0.20*	4.33 ± 0.14*
Root dry weight (g)	Control	0.45 ± 0.042	0.45 ± 0.042	0.45 ± 0.042	0.45 ± 0.042
	pHBA	0.28 ± 0.65*	0.32 ± 0.02*	0.37 ± 0.38*	0.48 ± 0.12

Each value represents the mean (±SE) of three replicates

* Significant differences as compared to control for $p < 0.05$ according to LSD test

Table 2 Effect of *p*-hydroxybenzoic acid (pHBA) at 1.5, 1.0, 0.5, and 0.1 mM concentrations on shoot length (cm) and root length (cm) of *D. glomerata* L.

Growth characteristics	Treatments	1.5 mM	1.0 mM	0.5 mM	0.1 mM
Shoot length (cm)	Control	15.30 ± 0.23	15.30 ± 0.23	15.30 ± 0.23	15.30 ± 0.23
	pHBA	7.63 ± 0.33*	6.71 ± 0.28*	10.15 ± 0.10*	8.25 ± 0.19*
Root length (cm)	Control	27.77 ± 0.22	27.77 ± 0.22	27.77 ± 0.22	27.77 ± 0.22
	pHBA	21.12 ± 0.54*	16.32 ± 0.10*	18.10 ± 0.43*	19.07 ± 0.65*

Each value represents the mean (±SE) of three replicates

* Significant differences as compared to control for $p < 0.05$ according to LSD test

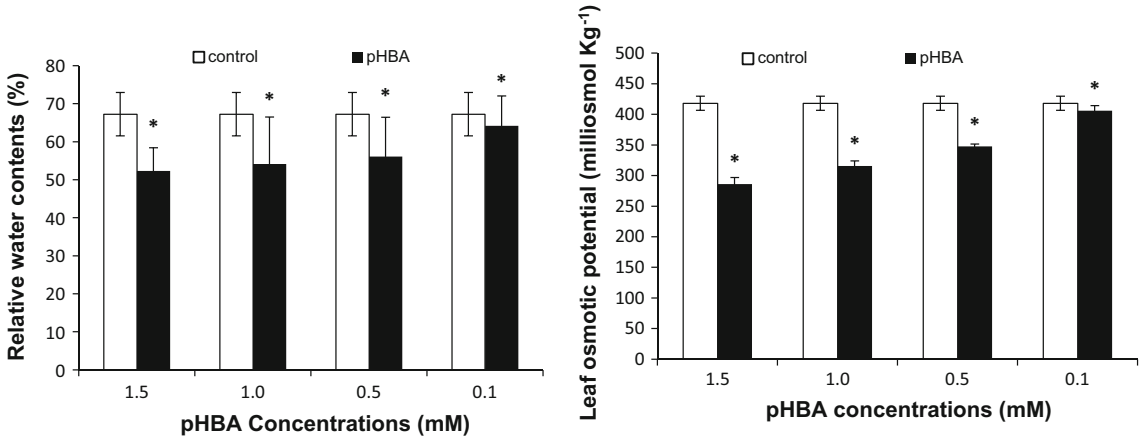


Fig. 1 Relative water contents (%) and leaf osmotic potential (milliosmol kg^{-1}) of *D. glomerata* L. following exposure to different concentration of *p*-hydroxybenzoic acid (1.5, 1.0, 0.5, 0.1 mM). Every column in each *graph* represents the mean (±SE) of three replicates. *Significant differences as compared to control for $p < 0.05$ according to LSD test

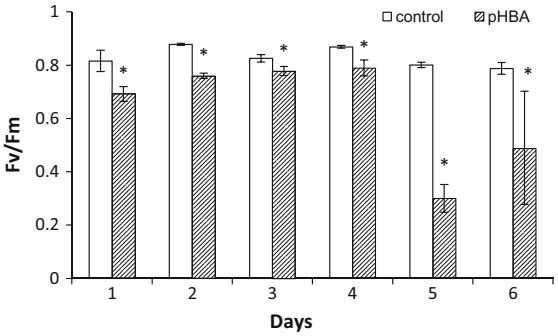
processes, especially stomatal regulation (Cram 1976). In this study, there was a significant reduction in leaf osmotic potential (LOP) due to pHBA treatment at all concentrations while pHBA at 1.5 mM reduced the LOP ranging from 3 to 32 % (Fig. 1).

Effect of pHBA on photosystem II photochemistry

Chlorophyll fluorescence and gas exchange measurements provide the experimental interface between the plant and environment including physiological and biochemical effects (McDermitt et al. 2000). Allelochemicals can affect the performance of the three main processes of photosynthesis: stomatal control of CO_2 supply, light reaction and dark reaction (Zhou and Yu 2006). pHBA significantly reduced the photosynthetic efficiency (F_v/F_m) of open PSII reaction centers in dark adapted states in *D. glomerata* during all days but the inhibition was more severe during days 5–6 ranging from 63 to 38 % respectively (Fig. 2). Effective quantum yield (ΦPSII) of photosystem II photochemistry level in *D. glomerata* leaves decreased during all days after pHBA treatment; reaching a maximum reduction in the plants after 3rd day of treatment (Fig. 2), coincident with a major decrease in the RWC. The levels of ΦPSII were lowest from 67 to 60 % during days 3–6. However, the maximum reduction in ΦPSII was observed during the day 5; that reached to 78 % decrease as compared to the control. Changes in the chlorophyll fluorescence quenching parameters are good indicators of photosynthetic electron flow (Schreiber et al. 1994). All pHBA treatments significantly affected the level of photochemical fluorescence quenching (qP) that was threefolds less during the day 5 as compared to control (Fig. 3). The level of qP was decreased progressively as the pHBA stress increased, but maximum reduction was observed at 1.5 mM pHBA from days 3 to 6. Non-photochemical fluorescence quenching (NPQ) level in *D. glomerata* decreased during all days after pHBA treatment while maximum inhibition was threefold from day 2 to 3 (Fig. 3).

Effect of pHBA on carbon isotope discrimination

pHBA significantly affected the leaf carbon % in *D. glomerata* following exposure to pHBA. The maximum reduction in carbon contents (C %) (33 %) was observed at 1.5 mM concentrations of pHBA followed by 1.0, 0.5 and



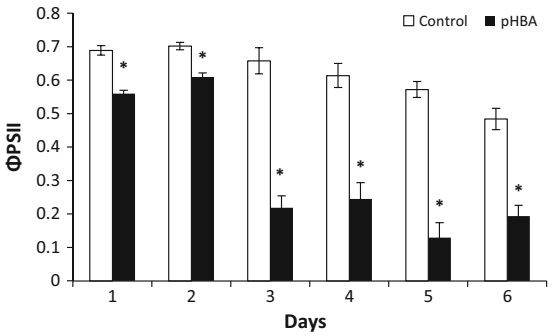
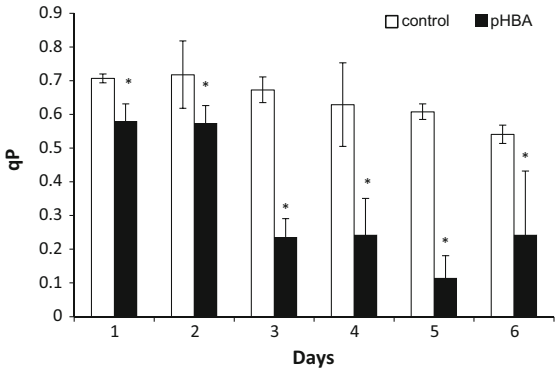


Fig. 2 Quantum efficiency of open PSII reaction centers in the dark-adapted state (F_v/F_m) and quantum yield of photosystem II (ΦPSII) in *D. glomerata* L. leaves from days 1 to 6 following exposure to *p*-hydroxybenzoic acid (pHBA) at 1.5 mM concentration and control. Every column in each *graph* represents the mean (±SE) of three replicates. *Significant differences as compared to control for $p < 0.05$ according to LSD test



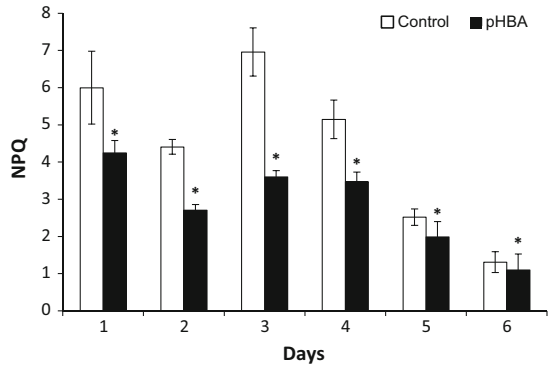
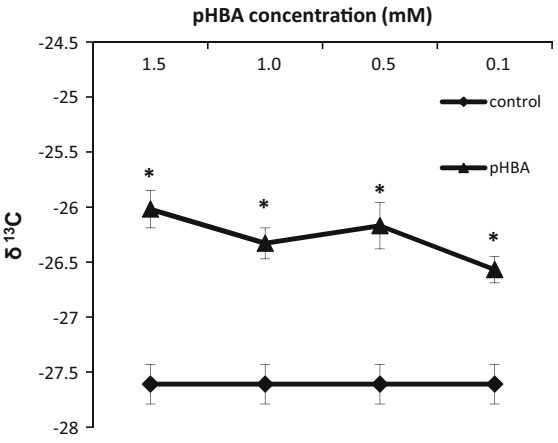


Fig. 3 Chlorophyll fluorescence quenching (qP) and non-photochemical fluorescence quenching (NPQ) in leaves of *D. glomerata* L. from days 1 to 6 following exposure to pHBA (1.5 mM) and control. Every column in each *graph* represents the mean (±SE) of three replicates. *Significant differences as compared to control for $p < 0.05$ according to LSD test



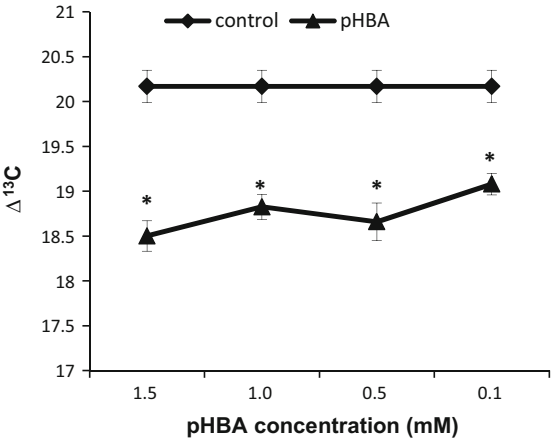


Fig. 4 Changes in carbon isotope ratios ($\delta^{13}C$) and carbon isotopes discrimination ($\Delta^{13}C$) in leaves of *D. glomerata* L. following exposure to *p*-hydroxybenzoic acid (pHBA) at 0 (control), 0.1, 0.5, 1.0, 1.5 mM concentration. Every column in each *graph* represents the mean (±SE) of three replicates. *Significant differences as compared to control for $p < 0.05$ according to LSD test

0.1 mM (Supplementary materials, Fig. S1). Carbon isotope composition ratio ($\delta^{13}C$) was significantly less negative (−26.02) than the control (−27.61) in *D. glomerata* plants treated with pHBA (1.5 mM) (Fig. 4). Carbon isotope discrimination ($\Delta^{13}C$) values were significantly less after treatment with pHBA at all concentrations (Fig. 4).

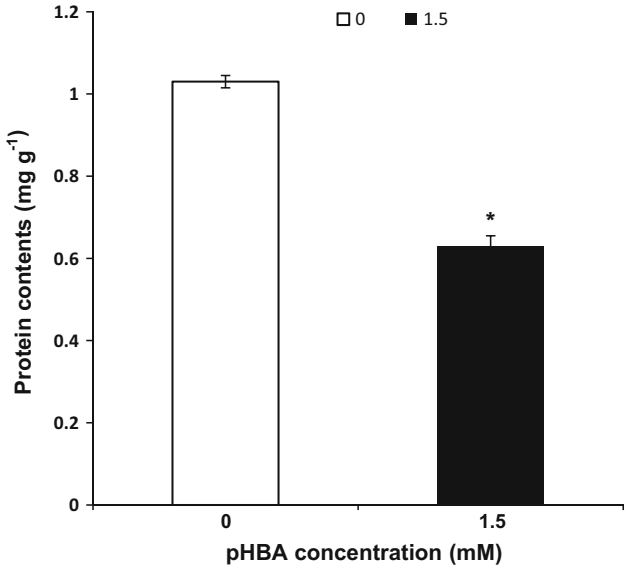


Fig. 5 Effect of *p*-hydroxybenzoic acid (pHBA) on leaf protein contents (mg g^{-1}) of *D. glomerata* L. at 0 (control) and 1.5 mM concentration. Every column in each *graph* represents the mean (±SE) of three replicates. *Significant differences as compared to control for $p < 0.05$ according to LSD test

The $\Delta^{13}C$ values were in the order of 18.50 > 18.83 > 18.66 > 18.08 following exposure to concentrations of 1.5 > 1.0 > 0.5 > 0.1 mM pHBA, respectively compared to the control (20.17).

Effect of pHBA on leaf protein contents

Analysis of total protein contents (TPC) in the leaf tissue showed that TPC was significantly decreased after treatment with pHBA (0.63 mg g^{-1}) compared to control leaves (1.03 mg g^{-1}) at 1.5 mM conc (Fig. 5). The reduction of total protein contents after pHBA application proved that pHBA has caused biochemical stress in metabolism of the *D. glomerata* leaves.

Discussion

Secondary metabolites produced and accumulated by plants can induce both inhibitory and stimulatory effects on organisms and may play roles in shaping plant and microbial communities (Pennacchio et al. 2005). The search for allelochemicals/phytotoxins is a growing research field, because these compounds have a great potential for controlling noxious weeds and could be used as natural bioherbicides in agriculture (Singh et al. 2003). In this study, we confirm that *p*-hydroxybenzoic acids (pHBA) reduced the root and shoot length, leaf and root fresh weight of *D. glomerata*. Other studies have documented that root growth of *Rumex crispus* was inhibited by *p*-hydroxybenzoic acid at 0.01 M concentrations (Reigosa et al. 1999) The root length and root fresh weight of soybean were decreased following treatment with pHBA at 0.5–1.0 mM (Doblinski et al. 2003). Secondary metabolities are reported to inhibit root growth and the modification of root morphology and histology (Kupidlowska et al. 1994). The changes in root structure and distribution are accompanied by changes in mineral nutrition (Clarkson 1996), so pHBA might affect the uptake of mineral nutrients. The potential role of allelochemicals as herbicides for their direct or bio-activated use against weeds, as well as the alternatives in the transgenic management of allelopathic plants to obtain a more available, active and stable concentration of allelochemicals in agroecosystems has been described previously (Duke et al. 2000; Dayan et al. 2009). Environmental factors can influence the physical process of evaporation, diffusion, stomatal aperture and closure on the leaf surface (Salisbury and Ross 1992). This study shows that pHBA reduced RWC and LOP at all concentrations tested in *D. glomerata* L (Fig. 1). Meanwhile, it was also reported that phenolic acids (*p*-coumaric, caffeic, ferulic, and salicylic acids), causes water stress in plants (Barkosky and Einhellig 2006; Barkosky et al. 2000).

During photosynthesis, radiation energy is transformed into chemical energy by several physical and chemical mechanisms. The process starts in the chlorophyll with light absorption by the antenna molecules. In the next step the radiation energy is transferred as excitation energy and either used in a reaction center for chemically useful work, or dissipated mainly as heat, redox energy and emitted radiation, or in other words, as in vivo chlorophyll fluorescence (Strasser et al. 2000). The best-characterized phytotoxic mechanisms induced by allelochemicals are the inhibition of photosynthesis and oxygen evolution through interactions with components of photosystem II (Rimando et al. 1998). Allelochemical pHBA reduced the quantum efficiency (F_v/F_m) of open PSII reaction centers in dark adapted states and effective quantum yield (ΦPSII) of photosystem II in *D. glomerata* leaves (Fig. 2). A sustained decrease of F_v/F_m may indicate the damage to photosystem II reaction centers (Maxwell and Johnson 2000). Phytochemical, sorgoleone is considered as an efficient bioherbicide because of its potential to inhibit electron transfer between Q_A and Q_B at the reducing site of photosystem II (Czarnota et al. 2001). When applied at a rate of 0.6 kg ai/ha, sorgoleone significantly inhibited growth of several broadleaf and grass species. Zhou and Yu (2006), demonstrated that allelochemicals can significantly affect the performance of three main processes of photosynthesis: stomatal control of CO_2 supply, thylakoid electron transport (light reaction) and carbon reduction cycle (dark

reaction). Similarly, Hussain and Reigosa (2011), stated that benzoxazolin-2(3*H*)-one and cinnamic acid (1.5 mM) reduced the F_v/F_m and ΦPSII in *Lolium perenne* within 24 h of treatment. Similarly, Sánchez-Moreiras et al. (2010) studied the 2-(3*H*)-Benzoxazolinone (BOA) phytotoxicity on photosynthesis of lettuce (*L. sativa* L.) and results were correlated with BOA quantities in the leaves. BOA-treated plants showed reduced rate of photosynthesis; 6 h after the beginning of the treatment, and the efficiency of photosystem II started to be declined 10 h after treatment. The photochemical fluorescence quenching (qP) was decreased after pHBA treatment (values were threefold less than the control), (Fig. 3) indicating that the balance between excitation rate and electron transfer rate has changed leading to a more reduced state of PSII reaction centers. The decrease in qP induced by the allelochemicals indicates a higher proportion of closed PSII reaction centers, (Hussain and Reigosa 2011) which probably decreases the proportion of available excitation energy used for photochemistry. Non-photochemical quenching (NPQ) was reduced in allelochemical treated plants (Fig. 3).

The variations in isotope ratios contain a potential wealth of information regarding ecological processes, as the stable isotope ratios of environmental substrates are determined by spatially and temporally dynamic biological and chemical processes (West et al. 2008). Plant photosynthesis discriminates against the stable ^{13}C isotope, (Farquhar et al. 1989) when atmospheric CO_2 passes through stomata and during CO_2 carboxylation in Rubisco. Our study indicates that the $\delta^{13}C$ was less negative than control following treated with pHBA (Fig. 4). Barkosky and Einhellig (2006) reported that carbon isotope ratio ($\delta^{13}C$) in leaf tissue of soybean at termination of the 28-day experiment showed significantly less discrimination (less negative $\delta^{13}C$) against ^{13}C in plants grown with 0.75 mM *p*-hydroxybenzoic acid. Moreover, Barkosky et al. (2000) concluded that *Euphorbia sula* leaf tissue treated with 0.25 mM caffeic acid had a less negative $\delta\ ^{13}C$ compared to controls, indicating less discrimination against ^{13}C in these plants. Carbon isotope discrimination ($\Delta^{13}C$) values in *D. glomerata* leaves were significantly less following treatment with pHBA at all concentrations. Carbon isotope discrimination decreases with a decrease in intercellular CO_2 concentration due to stomatal closure and consequently with water use efficiency. Similarly, Hussain et al. (2011) reported that in *L. sativa*, $\Delta^{13}C$ values were significantly less (19.45) as compared to control (20.17) at 1.0 mM BOA. In C_3 species, leaf level $\Delta^{13}C$ during photosynthetic gas exchange primarily reflects the balance between CO_2 supply by diffusion through stomata and CO_2 demand by biochemical reactions in chloroplasts, most importantly catalysis by Rubisco (Farquhar and Richards 1984). Both processes discriminate against the heavier isotope, but the fractionation occurs during carboxylation by Rubisco (Guy et al. 1986). The leaf protein contents of *D. glomerata* L. were reduced after treatment with pHBA at 1.5 mM concentration compared to control leaves. Similarly, Mersie and Singh (1993), reported reduction in total protein contents after *p*-hydroxybenzoic acid application.

Natural compounds are usually thought of environmental friendly and toxicologically safe than synthetic compounds. Our results confirmed that pHBA is phytotoxic and is a potent growth inhibitor of *D. glomerata*. It has been demonstrated that *D. glomerata* showed different responses to allelochemical (pHBA) stresses that it was dose-dependent. However, from these results it was concluded that pHBA led to a greater inhibition of root/shoot growth, leaf water potential, photosynthetic efficiency, quantum yield of photosystem II photochemistry and protein contents that coincide with reduced levels of carbon isotope ratios. Further field studies of the interactions between allelochemical with microorganisms, soil particles, and movement in soil could provide useful clues to understand the allelopathic phenomenon in the natural environment.

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