Allelochemical stress inhibits growth, leaf water relations, PSII photochemistry, non-photochemical fluorescence quenching, and heat energy dissipation in three C3 perennial species

M. Iftikhar Hussain* and Manuel J. Reigosa

Department of Plant Biology and Soil Science, University of Vigo, Campus Lagoas-Marcosende, E-36310, Vigo, España

* To whom correspondence should be addressed. E-mail: mih76@uvigo.es

Abstract

In this study, the effect of two allelochemicals, benzoxazolin-2(3H)-one (BOA) and cinnamic acid (CA), on different physiological and morphological characteristics of 1-month-old C3 plant species (Dactylis glomerata, Lolium perenne, and Rumex acetosa) was analysed. BOA inhibited the shoot length of D. glomerata, L. perenne, and R. acetosa by 49%, 19%, and 19% of the control. The root length of D. glomerata, L. perenne, and R. acetosa growing in the presence of 1.5 mM BOA and CA was decreased compared with the control. Both allelochemicals (BOA, CA) inhibited leaf osmotic potential (LOP) in L. perenne and D. glomerata. In L. perenne, Fv/Fm decreased after treatment with BOA (1.5 mM) while CA (1.5 mM) also significantly reduced Fv/Fm in L. perenne. Both allelochemicals decreased ΦPSII in D. glomerata and L. perenne within 24 h of treatment, while in R. acetosa, ΦPSII levels decreased by 72 h following treatment with BOA and CA. There was a decrease in qP and NPQ on the first, fourth, fifth, and sixth days after treatment with BOA in D. glomerata, while both allelochemicals reduced the qP level in R. acetosa. There was a gradual decrease in the fraction of light absorbed by PSII allocated to PSII photochemistry (P) in R. acetosa treated with BOA and CA. The P values in D. glomerata were reduced by both allelochemicals and the portion of absorbed photon energy that was thermally dissipated (D) in D. glomerata and L. perenne was decreased by BOA and CA. Photon energy absorbed by PSII antennae and trapped by ‘closed’ PSII reaction centres (E) was decreased after CA exposure in D. glomerata. BOA and CA (1.5 mM concentration) decreased the leaf protein contents in all three perennial species. This study provides new understanding of the physiological and biochemical mechanisms of action of BOA and CA in one perennial dicotyledon and two perennial grasses. The acquisition of such knowledge may ultimately provide a rational and scientific basis for the design of safe and effective herbicides.

Key words: CA, BOA, Dactylis glomerata, Lolium perenne, phenolics, physiological growth, Rumex acetosa.

Introduction

Although plant secondary metabolites are generally associated with plant defence responses against herbivores and pathogens, these unique compounds can be involved in a broad array of ecological functions (Bertin et al., 2003). There is considerable evidence to suggest that plant-derived chemical compounds are also involved in direct communication between plants (Weir et al., 2004). While this phenomenon was first documented in approximately
300 BC by Greek and Roman writers, it was not until 1937 that Hans Molisch coined the term ‘allelopathy’ to describe such plant-plant interactions (Rice, 1984; Willis, 2004). In the current literature, the term allelopathy can describe any direct or indirect effect of plant chemical compounds on another plant or microbe, although it is most often used to refer to chemical-mediated negative interference between plants (Willis, 2004).

The phenylpropanoid pathway is involved in the synthesis of a wide range of secondary products in plants, such as phenolic acids, flavonoids, tannins, coumarins (Kováčik et al., 2007), and lignin (Boerjan et al., 2003). Among them, benzoxazinoids are major secondary metabolites found in the Poaceae, including major agricultural crops, such as wheat, maize, and rye, but also in the dicotyledons, Acanthaceae, Lamiales, Ranunculaceae, and Scrophulariaceae (Sicker et al., 2002, 2004). Due to their multifunctional toxicity they are regarded as natural pesticides against pathogens, microorganisms, fungi, insects, and weeds (Silva et al., 2006; Soltoft et al., 2008). This study focused on 2-benzoxazolinone (BOA), which is associated with strong phytotoxic properties of rye residues, especially in *Avena fatua* (Perez and Ormeno-Nunez, 1991). Benzoxazolin-2(3H)-one (BOA) is an allelochemical formed from the O-glucoside of 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one by a two-step degradation process. BOA is considered to be a potential herbicide for weed suppression with its principal mode of action being on seed germination and on seedling development. This has been demonstrated with various test plants such as *Lactuca sativa* (Kato-Noguchi and Macias, 2005; Sánchez-Moreiras et al., 2008a), *Lepidium sativum* (Barnes et al., 1987), *Cucumis sativus* (Chase et al., 1991), and *Phaseolus aureus* (Singh et al., 2005; Batish et al., 2006). According to Burgos and Talbert (2000), BOA could be used as a natural herbicide, especially for some small weeds (e.g. *Echinochloa crus-galli*). BOA probably interferes with auxins (Anai et al., 1996) or by disrupting membrane integrity (Friebe et al., 1997). Recently, Singh et al. (2005), reported that BOA inhibits germination, early seedling growth, and rhizogenesis in the hypocotyls of mung bean (*Phaseolus aureus*). BOA occurs naturally in grass species and has been associated with dose-dependent germination inhibition and growth reductions in crop and weed species (Hussain et al., 2008). Baerson et al. (2005), explained in detail the detoxification and transcriptional responses of *Arabidopsis thaliana* to BOA, providing novel insights into xenobiotic detoxification mechanisms in plants.

Furthermore, a model of action related to oxidative damage has been proposed for BOA on plant metabolism (Sánchez-Moreiras et al., 2005; Batish et al., 2006). These studies concluded that BOA induces the generation of oxidative stress with high levels of lipid peroxidation and concomitant cell damage. Recently, Sánchez-Moreiras et al. (2008b) described the antagonistic effect (BOA alone) and synergistic effect of BOA (with salt) on the cell cycle of root tip meristem cells of lettuce (*Lactuca sativa*) by using flow cytometry with cell synchronization. Degradation of BOA was recently studied in great detail (Macias et al., 2006). One of the degradation products of BOA is APO (2-aminophenoxazin-3-one), which is very stable and more phytotoxic than BOA. The present experiments were conceived as ecophysiological experiments, so the treatments aimed to mimic natural processes (i.e. BOA was added to the soil, as with allelochemicals in nature, so that the observed effects could be due to BOA itself and or its decomposed products).

Cinnamic acid (CA) is a widespread phenolic acid released into soil by root exudates, leaf leachates, and decomposed plant tissues of different plants, for example, cucumber (Yu and Matsui, 1994) and alfalfa (Chon et al., 2002). Baziramakenga et al. (1997), concluded that benzoic acid and CA were responsible for negative allelopathic effects of quack grass on soybean by inhibiting root growth, reducing the root and shoot dry mass, by altering ion uptake and transport, and by reducing chlorophyll content. Cinnamic acid is the principal autotoxin in root exudates of cucumber and the model allelochemical used in many studies (Politycka, 1996; Yu and Matsui, 1997; Ye et al., 2004; Blum, 2005).

These previous studies demonstrate clear effects of BOA and CA treatment. However, the relation between allelochemicals and interference with the components of the photosynthetic apparatus in perennial species has not been the subject of much research. Chiapusio et al. (2004) described the translocation and accumulation of radio-labelled BOA in radish (*Raphanus sativus*) seeds and seedlings. They concluded that cotyledons were the sink organ for this allelochemical. However, these results were obtained in BOA-germinating seedlings, which makes it difficult to predict at which precise moment BOA reaches the leaf and to what extent this presence can be correlated with the observed phytotoxicity. Although BOA shows stronger effects on germinating seedlings, its impact on adult plant development is also important due to its continuous presence in the field when the BOA-containing crops are used as cover crops, mulch, smother crops, green manure, or grown in rotational sequences (Dhima et al., 2006). The ecological relevance of BOA is a small but constant effect, which can be highly effective in plant to plant interactions.

Although research on the biological control of weeds has been carried out for many years, interest in biological control has increased in response to greater concerns about herbicide residues in foods and the environment, and the evolution of herbicide resistance in weeds (Quimby et al., 2002). Biological control is being encouraged and supported by community and government action promoting sustainability and reduced dependence on non-renewable petrochemicals (Quimby et al., 2003). Allelochemicals that are biodegradable, exhibit structural diversity and complexity, and rarely contain halogenated atoms constitute one such class of chemicals (Dayan et al., 1999; Duke et al., 2000).

Natural phytotoxins represent a diverse array of chemical structures that are unlikely to be designed in herbicide discovery efforts based on synthetic chemistry. Previously, the inhibitory effect of BOA and CA in germination
bioassays (Reigosa and Pazos-Malvido, 2007; Hussain et al., 2008) has been reported, but the mechanisms involved, however, have not been extensively investigated regarding their interference with different biochemical and physiological processes in plants. In this work, physiological activity of four concentrations of BOA and CA (0.1, 0.5, 1.0, and 1.5 mM) was assessed to evaluate and compare the interference in PSII efficiency, components of photosystem II photochemistry, chlorophyll fluorescence quenching coefficients, and heat energy dissipation in three C3 perennial species having different sensitivities to allelochemical exposure. Finally, an attempt was made to demonstrate correlation between various concentrations of allelochemicals and their possible interference in different physiological processes when the plants were under stress. These results may help to determine if BOA or CA is involved in dissipating light energy as a consequence of increased oxidative stress due to allelochemical exposure. This information may provide the clues and biochemical modes of action of these chemicals that can be exploited by industry-led discovery programmes (Dayan et al., 2009; 1999, 2000; Duke et al., 2000). Previously, it was reported that root exudates of cucumber or watermelon (cinnamic acid) showed high phytotoxicity to both plants themselves, but not to other species such as figleaf gourd (Yu et al., 2000). Therefore, the three C3 perennial species (Dactylis glomerata, Lolium perenne, and Rumex acetosa) were selected because of the possible coexistence of these species with other allelopathic species (e.g. wheat, Avena fatua, rye, alfalfa, and cucumber) that produce BOA or CA.

Materials and methods

Plant culture and allelochemical treatments

The seeds of three plant species (i) cocksfoot: Dactylis glomerata L. cv. Amba (Poaceae); (ii) perennial ryegrass: Lolium perenne L. cv. Belida (Poaceae); (iii) common sorrel (Rumex acetosa L. cv. Belleville) (Polygonaceae), were purchased from Semillas Fito (Barcelona, Spain). Seeds were sown individually in plastic trays (32×20×6 cm) filled with 5 cm of perlite (500 g tray-1), placed in a growth chamber and irrigated on alternate days with tap water until germination. The greenhouse was ventilated with outside air to ensure steady CO2. Every second day the pots were well watered with tap water through an automatic irrigation system.

The phenolic compounds BOA and CA were obtained from Sigma Chemical Company (St Louis, MO, USA). Stock solutions of allelochemicals were made in methanol:water (20:80 v/v). The control was prepared with distilled water and methanol. The methanol was evaporated in a rotary vaporizer and the solution was adjusted to a concentration of 3 mM. These stock solutions were diluted to 1.5, 1.0, 0.5, and 0.1 mM. The pH of all these chemical solutions was adjusted to 6.0 with NaOH (Martin et al., 2002). One week after the transfer of plants to the greenhouse, treatment solutions (100 ml pot-1) were applied three times (days 1, 3, and 5) and measurements were taken.

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured on fully expanded leaves (one leaf per plant) using a pulse-modulated instrument fluorescence monitoring system (FMS) (Hansatech, Norfolk, England). The parameters of chlorophyll fluorescence: initial fluorescence (F0), maximum fluorescence (Fm), variable fluorescence (Fv = Fm-Fn), were measured after 20 min in the dark. The intensity of saturation pulses to determine the maximum fluorescence emission in the presence (Fm) and absence (Fm) of quenching was 1800 μmol m-2 s-1 for 3 s. The steady-state fluorescence (F0), basic fluorescence after light induction (Fv), maximum chlorophyll fluorescence (Fm), variable fluorescence from light-adapted leaves (Fv) were also measured. The quantum efficiency of open PSII reaction centres in the light-adapted state (ΦPSII) was determined from Fm and Fv values and also the quantum efficiency of excitation energy trapping of PSII (ΦP) was calculated (Genty et al., 1989). After turning off the actinic light, the leaves were illuminated with far-red light (7 μmol m-2 s−1) to oxidize the PQ pool to determine the minimum fluorescence level of the light-adapted state (F0). The fraction of open PSII reaction centres as qP = (Fm-Fn/Fm-Fn) were calculated using the ‘lack’ model according to Kramer et al. (2004), and non-photochemical quenching NPQ = (Fm-Fn)/Fm was calculated according to Bilger et al. (1995). The fraction of photon energy absorbed by PSII antennae that was trapped by open PSII reaction centres (centres with Qx in the oxidized state) and utilized in PSII photochemistry was estimated as P = (Fv/Fm)×ΦP.

The portion of absorbed photon energy that was thermally dissipated was calculated as the difference between the maximal theoretical and actual levels of PSII efficiency (D = 1 – (ΦP/Fm)). The fraction of absorbed radiation not attributed to P or D (denoted as ‘excess’, E) and representing the photon energy absorbed by PSII antennae and trapped by ‘closed’ PSII reaction centres was estimated using E = 1–(D + P) = (Fv/Fm)×(1–qP) (Demming-Adams et al., 1996).

Measurement of growth and leaf water status

The root and shoot lengths were measured with a ruler. Three measurements were taken from four concentrations from each treatment and averaged. For osmolarity measurements (milliosmol kg-1) one leaf per replicate was transferred into 10 ml syringes and kept frozen at −80 °C until further analysis (González, 2001). In analysis, syringes were thawed until the sample reached room temperature and osmotic potential was measured on the second drop from collected sap by using a calibrated vapour pressure osmometer (Automatic Cryoscopic Osmometer, Osmomat–030, GmbH, Gontec, Berlin, Germany) according to manufacturer’s instructions.

Measurement of leaf protein contents

Total protein was quantified by using the Bradford (1976) spectrophotometric assay. Leaves of target species (200 mg fresh weight) from three replicates per treatment were crushed in liquid nitrogen in cooled mortar with 0.05 g PVPP, and the powder was resuspended in 1 ml of TRIS buffer containing 0.05 M TRIS base, 0.1% (w/v) ascorbic acid, 0.1% (w/v) cysteine hydrochloride, 1% (w/v) PEG 4000, 0.15% (w/v) citric acid, and 0.008% (w/v)
2-mercaptoethanol (Arulsekar and Parfitt, 1986). Samples were centrifuged at 19,000 g, and 4 °C for 20 min after resuspension. 0.1 ml supernatant was mixed for protein dye-binding reaction with 3 ml Bradford reagent containing 0.01% Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol 95%, and 8.5% (v/v) phosphoric acid 85%. Absorbance was recorded at 595 nm after 5 min for quantification of the protein content. Commercial bovine serum albumin (BSA) was used as standard. Values were expressed per gram of dry weight.

Experimental design and statistical analysis
The experiment was laid out in a Randomized Complete Block Design (RCBD). There were three replicates of each concentration for each species. The analysis was performed separately for the combination of the allelochemicals/species by using one-way analysis of variance (ANOVA) with the statistical package SPSS® (version 15.00) for Windows® (SPSS Inc., Chicago, IL, USA). Differences between concentration, treatments, and species means were compared by the Dunnett test at the 0.05 probability level (when variance was homogeneous) or the Kruskal–Wallis test (when heterogeneous).

Results
Effect of allelochemicals on shoot and root length
The phenolic compounds (BOA, CA) evaluated for their phytotoxicity on D. glomerata, L. perenne, and R. acetosa exhibited various degrees of inhibition of root and shoot length. BOA, a promising phenolic compound, markedly inhibited the shoot and root length of D. glomerata, L. perenne, and R. acetosa in a concentration-dependent manner. Shoot length decreased as the concentrations of allelochemicals increased and the greatest inhibition was observed at the 1.5 mM concentration (Table 1). Application of BOA solution at a concentration of 1.5 mM decreased the shoot length of D. glomerata, L. perenne, and R. acetosa by 50%, 15%, and 35% with respect to the control. The shoot length of plants treated with CA (1.5 mM) had similar patterns of variation, with decreases of 17% in L. perenne, 35% in R. acetosa, and 43% in D. glomerata compared with the control.

The effects of BOA on root length were evident after one week of exposure. The root length of L. perenne growing in the presence of 1.5 mM BOA was substantially smaller compared with the control. Root length in D. glomerata showed almost similar patterns of variation, being the lowest in seedlings treated with 1.5 mM BOA followed by those receiving BOA at the 1.0, 0.5, and 0.1 mM levels. The maximum root length recorded in D. glomerata seedlings treated with water (control) was 52 cm. The root length in R. acetosa decreased as the concentration of BOA increased from 0.1 mM to 1.5 mM. Similar results were obtained in R. acetosa due to CA treatment with the most inhibition occurring at the 1.5 mM concentration (Table 2). Similarly, there was a reduction in root length of D. glomerata of 64% compared with the control at the 1.5 mM concentration. Application of CA at a concentration of 1.5 mM decreased the root length of L. perenne and R. acetosa by 26% and 29%, with respect to the control. Similarly, root length differed significantly (P < 0.05) among the treatments. It was greater in control seedlings compared with the seedlings receiving either 1.5 mM BOA or CA treatments.

Changes in leaf water status
The leaf osmotic potential (LOP) (milliosmol kg⁻¹) of L. perenne growing in the presence of 1.5 mM BOA was substantially lower compared with the control. LOP in D. glomerata showed a similar pattern of variation, and was least in leaves treated with the 1.5 mM BOA concentration followed by those receiving BOA at the 1.0, 0.5, and 0.1 mM levels. The maximum LOP was recorded in D. glomerata seedlings treated with water. Applying BOA (1.5, 1.0, 0.5, and 0.1 mM) concentrations decreased the LOP in R. acetosa (Fig. 1). Similarly, leaf osmotic potential decreased as the concentrations of allelochemicals increased.

Table 1. Effects of benzoxazolin-2(3H)-one (BOA) and cinnamic acid (CA) on shoot length (cm) of three C₃ perennials (L. perenne, D. glomerata, and R. acetosa) at four (0.1, 0.5, 1.0, and 1.5 mM) concentrations

| Concentration (mM) | L. perenne | D. glomerata | R. acetosa |
|-------------------|------------|--------------|------------|
| BOA               |            |              |            |
| 0                 | 42.53±1.34 | 70.88±2.60   | 19.08±0.70 |
| 0.1               | 40.17±0.72 | 58.33±4.41*  | 15.83±1.42*|
| 0.5               | 38.33±2.60*| 44.83±3.65*  | 13.93±1.98*|
| 1.0               | 38.67±3.11 | 41.33±6.74*  | 13.33±0.92*|
| 1.5               | 35.89±1.33 | 35.00±0.57*  | 12.50±2.78*|
| CA                |            |              |            |
| 0                 | 42.53±1.34 | 70.88±2.60   | 19.08±0.70 |
| 0.1               | 40.67±1.20 | 48.1±1.95*   | 18.43±2.30 |
| 0.5               | 39.00±2.64 | 48.17±6.69*  | 14.80±0.35*|
| 1.0               | 38.26±5.81*| 42.83±4.04*  | 13.67±0.92*|
| 1.5               | 35.33±1.32*| 40.4±7.55*   | 12.40±3.01*|

Each value represents the mean (±SE) of three replicates.
*Significant differences compared with the control for P < 0.05 according to the Dunnett test.

Table 2. Effects of benzoxazolin-2(3H)-one (BOA) and cinnamic acid (CA) on root length (cm) of three C₃ perennials (L. perenne, D. glomerata, and R. acetosa) at four (0.1, 0.5, 1.0, and 1.5 mM) concentrations

| Concentration (mM) | L. perenne | D. glomerata | R. acetosa |
|-------------------|------------|--------------|------------|
| BOA               |            |              |            |
| 0                 | 33.00±0.27 | 52.32±3.18   | 20.38±0.95 |
| 0.1               | 31.00±2.30*| 47.58±1.48*  | 18.00±1.73*|
| 0.5               | 26.33±2.33*| 47.50±4.92*  | 17.67±1.20*|
| 1.0               | 22.33±1.45*| 43.33±1.85*  | 16.00±0.93*|
| 1.5               | 22.67±1.45*| 42.33±1.45*  | 15.50±1.32*|
| CA                |            |              |            |
| 0                 | 33.00±0.27 | 52.32±3.18   | 20.38±0.95 |
| 0.1               | 27.10±0.49*| 43.57±2.82*  | 16.38±0.95*|
| 0.5               | 26.33±1.33 | 40.66±3.71*  | 15.67±1.20*|
| 1.0               | 25.00±1.76*| 37.33±2.40*  | 15.33±0.88*|
| 1.5               | 24.60±1.20*| 33.67±1.85*  | 14.33±1.76*|

Each value represents the mean (±SE) of three replicates.
*Significant differences compared with the control for P < 0.05 according to the Dunnett test.
and the greatest inhibition was observed at the 1.5 mM concentration. Meanwhile, CA decreased the LOP in *L. perenne*, *R. acetosa*, and *D. glomerata* plants at the 1.5, 1.0, and 0.5 mM concentrations (Fig. 1).

**Chlorophyll fluorescence attributes**

Damage to the thylakoid membrane caused by BOA and CA was evaluated on the basis of 6 d of treatment by analysing several fluorescence parameters determined under dark-adapted and steady-state conditions. In *L. perenne*, *D. glomerata*, and *R. acetosa*, the quantum efficiency of open PSII reaction centres in the dark-adapted state ($F_v/F_m$) decreased after treatment with BOA and CA for all of the six days (Fig. 2). The application of chemicals tended to reduce $F_v/F_m$, indicating damage to the photosynthetic apparatus. Both allelochemicals also inhibited quantum efficiency of open PSII reaction centres in the light state ($F'_v/F'_m$) in all three target species (Fig. 2).

Allelochemicals BOA and CA decreased the level of quantum yield ($\Phi_{PSII}$) of photosystem II in *D. glomerata*, *R. acetosa*, and *L. perenne* for all the days (Fig. 3). The reduction in $\Phi_{PSII}$ might be due to a decrease in the efficiency of excitation energy trapping of the PSII reaction centres. A significant reduction was observed in qP in *R. acetosa*, *L. perenne*, and *D. glomerata* after treatment with BOA or CA for all the days (Fig. 4). The reduction in the qP level might indicate that the balance between excitation rate and electron transfer rate has changed, leading to a more reduced state of the PSII reaction centres. A significant decrease in non-photochemical fluorescence quenching (NPQ) was observed in *D. glomerata*, *R. acetosa*, and *L. perenne* after treatment with BOA and CA (Fig. 4).

The fractions of light absorbed by PSII allocated to PSII photochemistry ($P$) values in *L. perenne*, *D. glomerata*, and *R. acetosa* was decreased by both allelochemicals (BOA, CA; Fig. 5). The portion of absorbed photon energy that was thermally dissipated (the difference between the

![Fig. 1](image-url). Osmotic potential (milliosmol kg$^{-1}$) in leaves of *Lolium perenne*, *Dactylis glomerata*, and *Rumex acetosa* one week after exposure to four concentrations (0.1, 0.5, 1.0, 1.5 mM) of benzoxazolin-2(3H)-one (BOA) and cinnamic acid (CA). Every column in each graph represents the mean ($\pm$ SE) of three replicates. Asterisks indicate significant differences at the 0.05 level compared with the control.
maximal theoretical and actual levels of PSII efficiency, $D$), values in $L. perenne$, $D. glomerata$, and $R. acetosa$ was decreased by both allelochemicals (BOA, CA) for all the days. The photon energy absorbed by PSII antennae and trapped by ‘closed’ PSII reaction centres ($E$), decreased after BOA and CA exposure in $L. perenne$ (Fig. 5). $E$ values were not significantly affected by the treatment with allelochemicals in $D. glomerata$ and $R. acetosa$.

Leaf protein contents

There was a reduction in leaf protein contents due to BOA and CA treatment in all three target species ($L. perenne$, $D. glomerata$, and $R. acetosa$) (Fig. 6), while CA was more phytotoxic than BOA and $D. glomerata$ was more sensitive to allelochemicals than the other two species.

Discussion

BOA is common in both cultivated and wild Gramineae plants (Sánchez-Moreiras et al., 2004). The results of this study indicate that BOA is a substance with multiple physiological targets and that its effects are generally stimulatory at low concentrations and inhibitory at high concentrations (Hussain et al., 2008; Sánchez-Moreiras et al., 2008a; b). In the present study, BOA reduced the shoot and root length. Root length decreased as the concentrations of BOA increased and the greatest inhibition was observed at the 1.5 mM concentration (Table 2). Root growth is characterized by high metabolic rates and, for this reason, roots are highly susceptible to environmental stresses such as allelochemicals in soils (Cruz-Ortega et al., 1998). Root swelling observed above the apex, after treatment with 1.5 mM BOA (MI Hussain, MJ Reigosa, personal observation), could result from a BOA-dependent increase in the number of radial cell divisions in the sub-apical root zone (Svensson, 1971). Similarly, Baerson et al. (2005) reported that BOA impaired root system development, resulting in reduced root lengths and a complete absence of lateral root formation in 10-d-old $A. thaliana$ seedlings by 50% ($I_{50}$) and 80% ($I_{80}$) at 540 μM and 1 μM concentration, respectively.

Allelochemical, CA, decreased root length at all concentrations and the greatest reduction was observed at the...
1.5 mM concentration. In a previous study, coumarin-treated alfalfa (*Medicago sativa*) roots revealed an increase in diameter due to an expansion of the vascular cylinder and cortical cell layers. Moreover, this allelochemical inhibited root elongation and cell division, indicating that the thickness of roots was enlarged due to the inhibition of root longitudinal growth (Chon *et al.*, 2002). Root length inhibition by these ubiquitous organic acids has been widely reported in the literature (Iqbal *et al.*, 2004; Hiradate *et al.*, 2005; Rudrappa *et al.*, 2007).

BOA also decreased the root length of *D. glomerata* and *R. acetosa* at all the concentrations tested (Table 2). Phytotoxic effects of BOA have been observed on root ultrastructure, where packed multiple columns of cells failed to lengthen at the root tip of cucumber plants (Burgos *et al.*, 2004). BOA inhibits plasma membrane bound H⁺ ATPases in roots of *Avena fatua* (Friebe *et al.*, 1997), causes a number of ultrastructural changes in the roots of *C. sativus* (Burgos and Talbert, 2000), and inhibits α-amylase activity in roots of *L. sativa* (Kato-Noguchi and Macías, 2005). Similarly, CA also reduced root length of three grass species at all concentration and the degree of inhibition was concentration dependent.

Allelopathy in natural and agricultural ecosystems is receiving increasing attention because allelochemicals significantly reduce the growth and yields of plants (Inderjit and Duke, 2003). Perhaps because of the diverse chemical structures within a group, the phytoxicity often varies by an order of magnitude when comparisons are made among known allelochemicals within a category. In the present study, both allelochemicals lowered relative water contents in leaves of all target species. Apparently, the three target species responded differently to the phytotoxic compounds, thereby contributing to species selectivity. Root membranes are a primary site of action for phenolic acids. The contact of phenolic acids with the root cell membrane leads to depolarization, an efflux of ions, and a reduction of hydraulic conductivity, water uptake and net nutrient uptake (Baziramakenga *et al.*, 1995; Lehman and Blum, 1999). Subsequent resulting changes in plant water relations and mineral nutrition lead to a cascade of secondary effects. Some other phenolic acids such as *p*-coumaric, caffeic, ferulic, and salicylic acids also cause water stress in plants (Einhellig, 1995; Barkosky and Einhellig, 2003). Sánchez-Moreiras and Reigosa (2005) reported that a 10% decrease in relative water content was correlated with a decline in leaf water potential in BOA-exposed lettuce plants.

Allelochemicals appear to alter a variety of physiological processes and it is difficult to separate the primary from the secondary effects. Several action modes have been suggested, including direct inhibition of PSII components and ion uptake, interruption of dark respiration and ATP synthesis, and ROS-mediated allelopathic mechanisms (Inderjit and Duke, 2003). PSII is a multisubunit integral membrane protein complex used by higher plants and cyanobacteria to catalyse water oxidation and plastoquinone reduction (Boekema *et al.*, 2000). It is a light-dependent water–plastoquinone oxido-reductase enzyme that uses light energy to oxidize water and is mainly located in the appressed grana stacks (Allakhverdiev *et al.*, 2008). Allelochemicals can affect the performance of the three main processes of photosynthesis: stomatal control of CO₂ supply, thylakoid electron transport (light reaction), and the carbon reduction cycle.
In our experiment, it was found that both allelochemicals (BOA, CA) decreased the efficiency of open photosystem II photochemistry ($F_v/F_m$) and photochemical fluorescence yield ($U_{PSII}$) in *L. perenne*, *D. glomerata*, and *R. acetosa* leaves (Figs 2, 3). Similarly, Ye et al. (2004) reported a reduction in $F_v/F_m$ and $U_{PSII}$ after CA exposure in *Cucumis sativus*. $(F_v/F_m)$, the efficiency of the excitation energy captured by open PSII reaction centres, was reduced by both allelochemicals in the three target species, reflecting that the efficiency of open reaction centres in the light was highly affected.

The quantum yield of electron transfer at PSII is the product of the efficiency of the open reaction centres $(F_v/F_m)$ and photochemical quenching (qP) (Lu and Zhang, 1999; Sinsawat et al., 2004). BOA and CA decreased the level of quantum yield ($\Phi_{PSII}$) of photosystem II in *D. glomerata*, *R. acetosa*, and *L. perenne* for all the days (Fig. 3). The reduction in $\Phi_{PSII}$ might be due to a decrease in the efficiency of excitation energy trapping of PSII reaction centres. In *R. acetosa*, $\Phi_{PSII}$ levels were also reduced by BOA and CA (Fig. 3). However, Huang and Bie (2009) reported that the 0.1 mM CA treatment decreased photosynthetic rate ($P_n$) and Rubisco activity, but it did not affect the maximal photochemical efficiency of PSII $(F_v/F_m)$, the actual photochemical efficiency of PSII ($\Phi_{PSII}$) or the relative chlorophyll content. From our results, it is clear that decreases in the relative water content of leaves in these plant species initially induce stomatal closure, imposing a decrease in the supply of CO$_2$ to the mesophyll cells and, consequently, photosynthesis could be lowered.

![Fig. 4. Changes in chlorophyll fluorescence quenching (qP) and non-photochemical fluorescence quenching (NPQ) in leaves of *L. perenne*, *D. glomerata*, and *R. acetosa* from days 1 to 6 following exposure to BOA and CA. Every column in each graph represents the mean ($\pm$SE) of three replicates. Asterisks indicate significant differences at the 0.05 level compared with the control.](image-url)
flow (Schreiber et al., 1994). Photochemical quenching indicates the oxidation-reduction state of the primary acceptor ($Q_A$) for PSII (Baker, 2008). In the present study, it was observed that both allelochemicals decreased the qP in all three target species. A decrease in qP induced by allelochemicals indicates that higher proportion of PSII reaction centres are closed (Genty et al., 1989), which probably generates a decrease in the proportion of available excitation energy used for photochemistry. In fact, in $R. acetosa$, qP has no significant changes even at the highest BOA or CA concentration (1.5 mM) during the first and second days. It was as low as in the controls (Fig. 4). Thus, $R. acetosa$ appears to be equipped with an efficient defence system, independently of qP, which prevents damage to the photosynthetic mechanism under allelochemical stress conditions. Similarly, Hajlaoui et al. (2009) reported similar results in maize under salt stress. Continuous application of allelochemicals (BOA, CA) decreased the qP in $R. acetosa$ during the third, fourth, fifth, and sixth days. A decrease in qP values also indicates an increase in the fraction of reduced $Q_A$ ($Q_A^*$) of PSII, which means there is an increase in susceptibility to photoinhibition (Lu and Lu, 2004).

In the absence of efficient detoxification of allelochemicals by leaf cells, their concentration in the leaf apoplast may reach excessive values, leading to leaf cell dehydration, stomata closure, and inhibition of photosynthesis (Meloni et al., 2003). In fact, BOA and CA were accumulated at high concentrations in leaves of three plant species, while this accumulation appears to be more prominent in the leaves of $L. perenne$ and $D. glomerata$ than in $R. acetosa$. In general, there was a decreasing trend in the growth and physiological characteristics of the three plant species with increasing concentration of allelochemicals. The ability of the cells to metabolize, accumulate, and extrude the natural compounds increases the probability that the toxicity measured after allelochemical application is due to some derivative compound and not necessarily to the ‘original’ allelochemical (Macías et al., 2006). Recently, Sánchez-Moreiras et al. (2010) reported that BOA-treated $L. sativa$ plants showed a reduced photosynthetic rate 6 h after the beginning of the treatment, and the efficiency of photosystem II started to be affected 10 h after treatment. These results were correlated with an increasing concentration of BOA in leaves that started 6 h after treatment and showed a maximum at 96 h.

Non-photochemical fluorescence quenching (NPQ) declined in $D. glomerata, R. acetosa$, and $L. perenne$ after treatment with BOA and CA (Fig. 4). The significant changes in PSII photochemistry in the light-adapted leaves, such as the decreased qP and $(F_v/F_m)$, as well as the
increased NPQ, can be seen as the regulatory response to down-regulate the quantum yield of PSII electron transport (ΦPSII) that would match with the decrease in CO₂ assimilation rate. The stomatal limitations imposed on photosynthesis will be accompanied by a decrease in the rate of consumption of ATP and NADPH for CO₂ assimilation which could result in decreases in the rate of linear electron transport and, consequently, in ΦPSII. By contrast, some researchers have reported that, for the operation of the water–water cycle (Mehler-peroxidase reaction) in C₃ plants, an increase in photorespiration under stress conditions may maintain rates of electron transport similar to those observed in non-stressed leaves despite the decrease in CO₂ assimilation rate (Flexas et al., 2002; Noctor et al., 2002). However, under severe stress conditions, decreases in the rate of utilization of ATP and NADPH in photosynthetic metabolism will not be compensated for by increases in water–water cycling and photorespiration, or other electron sinks, and, consequently, decreases in ΦPSII will occur (Fracheboud and Leipner, 2003). In this study, both allelochemicals (BOA, CA) decreased the P and D values in L. perenne, D. glomerata, and R. acetosa while E values were reduced only in L. perenne after treatment with BOA and CA. However, no significant effect of BOA or CA on E values in D. glomerata and R. acetosa was observed.

Leaf protein contents in all three species (L. perenne, D. glomerata, and R. acetosa) was reduced due to BOA and CA treatment, while CA was more phytotoxic than BOA and D. glomerata was more sensitive to allelochemicals than the other species. Baziramakenga et al. (1997) reported that many phenolic acids reduced the incorporation of certain amino acid into proteins and thus reduced the rate of protein synthesis. Mersie and Singh (1993) demonstrated that ferulic acid inhibited protein synthesis and reduced the incorporation of [¹⁴C] leucine by 50% (at~1.0 µM and 60 min of incubation). They also concluded that the maximum inhibition of protein synthesis by chlorogenic acid was 19% and by vanillic acid was 28% at 100 µM concentrations in velvetleaf (Abutilon theophrasti Medik). However, chlorogenic, vanillic, and p-coumaric acids at 0.1 µM caused increased protein synthesis over the untreated control. Recently, Hussain et al. (2010) reported that ferulic acid and p-hydroxybenzoic acid at the 1.5 mM concentration significantly reduced the leaf protein contents of L. sativa compared with the control leaves.

### Conclusion

Natural compounds are usually thought of as more environmentally and toxicologically safe than synthetic compounds. Our results confirmed that BOA and CA are phytotoxic to three C₃ perennial plant species, and R. acetosa was less sensitive than two other grass species. By using two promising allelochemicals (BOA and CA), it has been demonstrated that grass species showed different responses to allelochemical stress and that this varied between two perennial grasses and a perennial dicotyledonous species. From these results it has been concluded that BOA led to a greater inhibition of root/shoot growth, leaf water potential, quantum yield of photosystem II, photochemical fluorescence quenching, heat energy dissipation, and protein contents. Meanwhile, allelochemicals have shown a clear phytotoxic effect on the photosynthetic apparatus in mature plants of L. perenne, D. glomerata, and R. acetosa. Further field studies of the interactions between allelochemicals with microorganisms, soil particles, and movement in soil could provide useful clues to an understanding of the allelopathic phenomenon in natural environment.

### Acknowledgements

We thank Dr Aldo Barreiro, Carlos Bolaño, Maite Ricart, Alfredo Justo, and Paula Lorenzo with field and laboratory assistance. We also thank Dr Nuria Pedrol for her valuable help in leaf osmotic potential measurement and in statistical analysis.

### References

Alaakhiriev SI, Klimov VV, Nagata T, Nixon P, Shen J-R. 2008. Special issue: recent perspectives of photosystem II: structure, function and dynamics. Photosynthesis Research 98, 1–700.

Anai T, Aizawa H, Ohtake N, Kosemura S, Yamamura S, Hasegawa K. 1996. A new auxin inhibiting substance, 4-Cl-6, 7-dimethoxy-2-benzoxazolinone, from light-grown maize shoots. Phytochemistry 42, 273–275.

Arulsekar S, Parfitt DE. 1986. Isozyme analysis procedures for stone fruits, almond, grape, walnut, pistachio, and fig. HortScience 21, 928–933.

Allakhverdiev SI, Klimov VV, Nagata T, Nixon P, Shen J-R. 2008. Special issue: recent perspectives of photosystem II: structure, function and dynamics. Photosynthesis Research 98, 1–700.

Anai T, Aizawa H, Ohtake N, Kosemura S, Yamamura S, Hasegawa K. 1996. A new auxin inhibiting substance, 4-Cl-6, 7-dimethoxy-2-benzoxazolinone, from light-grown maize shoots. Phytochemistry 42, 273–275.

Arulsekar S, Parfitt DE. 1986. Isozyme analysis procedures for stone fruits, almond, grape, walnut, pistachio, and fig. HortScience 21, 928–933.
Physiological responses of three perennials towards allelochemicals

Baerson SR, Sánchez-Moreiras AM, Pedrol-Bonjoch N, Schulz M, Kagan IA, Agarwal AK, Reigosa MJ, Duke SO. 2005. Detoxification and transcriptome response in Arabidopsis seedlings exposed to the allelochemical benzoaxazolinol-2(3H)-one. Journal of Biological Chemistry 280, 21867–21881.

Baker NR. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annual Review of Plant Biology 59, 89–113.

Barkosky RR, Einhellig FA. 2003. Allelopathic interference of plant–water relationships by para-hydroxybenzoic acid. Botanical Bulletin of Academia Sinica 44, 53–58.

Barnes JP, Putnam AR, Burke BA, Aasen AJ. 1987. Isolation and characterization of allelochemicals in rye herbage. Phytochemistry 26, 1385–1390.

Batish DR, Singh HP, Setia N, Kaur S, Kohli RK. 2006. 2-Benzoxazolinone (BOA) induced oxidative stress, lipid peroxidation and changes in some antioxidant enzyme activities in mung bean (Phaseolus aureus). Plant Physiology and Biochemistry 44, 819–827.

Baziramakenga R, Leroux GD, Simard RR. 1995. Effects of benzoic and cinnamic acids on membrane permeability of soybean roots. Journal of Chemical Ecology 21, 1271–1285.

Baziramakenga R, Leroux GD, Simard RR, Nadeau P. 1997. Allelopathic effects of phenolic acids on nucleic acid and protein levels in soybean seedlings. Canadian Journal of Botany 75, 445–450.

Bertin C, Yang X, Weston LA. 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant and Soil 256, 67–83.

Bilger W, Schreiber U, Bock M. 1995. Determination of the quantum efficiency of photosystem II and of non-photochemical quenching of chlorophyll fluorescence in the field. Oecologia 102, 425–432.

Blum U. 2005. Relationships between phenolic acid concentrations, transpiration, water utilization, leaf area expansion, and uptake of phenolic acids: nutrient culture studies. Journal of Chemical Ecology 31, 1907–1932.

Boekema EJ, van Breemen JF, Roon L, Van H, Dekker JP. 2000. Conformational changes in PSII supercomplexes upon removal of extrinsic subunits. Biochemistry 39, 12907–12915.

Boerjan W, Ralph J, Baucher M. 2005. Detoxification and transcriptome response in Arabidopsis seedlings exposed to the allelochemical benzoaxazolinol-2(3H)-one. Journal of Biological Chemistry 280, 21867–21881.

Burgos NR, Talbert RE, Kim KS, Kuk YI. 2004. Growth inhibition and root ultra structure of cucumber seedlings exposed to allelochemicals from rye (Secale cereale). Journal of Chemical Ecology 30, 671–689.

Burgos NR, Talbert RE. 2000. Differential activity of allelochemicals from Secale cereale in seedling bioassays. Weed Science 48, 302–310.

Chase WR, Nair MG, Putnam AR. 2002. 2,2’-Oxo-1,1’-azobenzene: selective toxicity of rye (Secale cereale L) allelochemicals to weeds and crop species. Journal of Chemical Ecology 17, 9–19.

Chiaipusio G, Pellissier F, Gallet C. 2004. Uptake and translocation of phytochemical 2-benzoxazolinone (BOA) in radish seeds and seedlings. Journal of Experimental Botany 55, 1587–1592.

Chon SU, Choi SK, Jung S, Jang HG, Pyo BS, Kim SM. 2002. Effects of alfalfa leaf extracts and phenolic allelochemicals on early seedling growth and root morphology of alfalfa and barnyard grass. Crop Protection 21, 1077–1082.

Cruz-Ortega R, Anaya AL, Hernández-Bautista BE, Laguna-Hernández G. 1998. Effects of allelochemical stress produced by Sicyos depepi on seedling root ultrastructure of Phaseolus vulgaris and Cucurbita ficifolia. Journal of Chemical Ecology 24, 2039–2057.

Dayan FE, Cantrell CL, Duke SO. 2009. Natural products in crop protection. Bioorganic and Medicinal Chemistry 17, 4022–4034.

Dayan FE, Romagni J, Tellez M, Rimando A, Duke S. 1999. Managing weeds with natural products. Pesticide Outlook 5, 185–188.

Dayan FE, Romagni JG, Duke SO. 2000. Investigating the mode of action of natural phytotoxins. Journal of Chemical Ecology 26, 2079–2094.

Demming-Adams B, Adams WW, Barker DH, Logan BA, Bowling DR, Verhoven AS. 1996. Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Plant Physiology 96, 253–264.

Dhima KV, Vasilakoglou IB, Elehterohorinos IG, Lithourgidis AS. 2006. Allelopathic potential of winter cereal cover crop mulches on grass weed suppression and sugar beet development. Crop Science 46, 1862–1891.

Duke SO, Dayan FE, Romagni JG, Rimando AM. 2000. Natural products as sources of herbicides: current status and future trends. Weed Research 40, 99–111.

Einhellig FA. 1995. Mechanism of action of allelochemicals in allelopathy. In: Inderjit, Einhellig FA, Dakshini KMM, eds. Allelopathy: organisms, processes, and applications. Washington, DC: American Chemical Society, 96–116.

Flexas J, Bota J, Escalona JM, Sampol B, Medrano H. 2002. Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. Functional Plant Biology 29, 461–471.

Fracheboud Y, Leipner J. 2003. The application of chlorophyll fluorescence to study light, temperature and drought stress. In: DeEll JR, Tiovonen PMA, eds. Practical applications of chlorophyll fluorescence in plant biology. Boston: Kluwer Academic Publishers, 125–150.

Friebe A, Roth U, Kuck P, Schnabl H, Schulz M. 1997. Effects of 2,4-dihydroxy-1,4-benzoxazin-3-ones on the activity of plasma membrane H+-ATPase. Phytochemistry 44, 979–983.

Gentry B, Briantais JM, Baker NR. 1989. The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta 990, 87–92.

González L. 2001. Determination of water potential in leaves. In: Reigosa M, ed. Handbook of plant ecophysiology techniques. Dordrecht: Kluwer Academic Publishers, 193–206.

Hajiaoui H, Denden M, El Ayeb N. 2009. Differential responses of two maize (Zea mays L.) varieties to salt stress: changes on polyphenols composition of foliage and oxidative damages. Industrial Crops Production 30, 144–151.
Allelopathy: chemistry and mode of action of allelochemicals. Boca Raton: CRC Press, 77–102.

Sicker D, Schulz M. 2002. Benzoxazinones in plants: occurrence, synthetic access and biological activity. In: Atta-ur-Rahman S, ed. Studies in natural products chemistry, Vol. 27. Amsterdam, The Netherlands: Elsevier, 185–232.

Silva H, Copaja SV, Bravo HR, Argandona VH. 2006. Relationship between grain yield, osmotic adjustment and benzoxazinone content in Triticum aestivum L. cultivars. Zeitschrift für Naturforschung C: Journal of Biosciences 61, 704–708.

Singh HP, Batish DR, Kaur S, Setia N, Kohli RK. 2005. Effects of 2-benzoxazolinone on the germination, early growth and morphogenetic response of mung bean (Phaseolus aureus). Annals of Applied Biology 147, 267–274.

Sinsawat V, Leipner J, Stamp P, Fracheboud Y. 2004. Effect of heat stress on the photosynthetic apparatus in maize (Zea mays L.) grown at control or high temperature. Environmental and Experimental Botany 52, 123–129.

Soltoft M, Joergensen LN, Svensmark B, Fomsgaard IS. 2008. Benzoxazinoid concentrations show correlation with Fusarium Head Blight resistance in Danish wheat varieties. Biochemical Systematics and Ecology 36, 245–259.

Svensson S. 1971. The effect of coumarin on root growth and root histology. Physiologia Plantarum 24, 446–470.

Weir TL, Park S, Vivanco JM. 2004. Biochemical and physiological mechanisms mediated by allelochemicals. Current Opinion in Plant Biology 7, 472–479.

Willis RJ. 2004. Justus Ludewig von Uslar, and the first book on allelopathy. Dordrecht, The Netherlands: Springer Publications.

Ye SF, Yu JQ, Peng YH, Zheng JH, Zou LY. 2004. Incidence of Fusarium wilt in Cucumis sativus L. is promoted by cinnamic acid, an autotoxin in root exudates. Plant and Soil 263, 143–150.

Yu JQ, Matsui Y. 1994. Phytotoxic substances in root exudates of cucumber (Cucumis sativus L.). Journal of Chemical Ecology 20, 21–31.

Yu JQ, Matsui Y. 1997. Effects of root exudates of cucumber (Cucumis sativus) and allelochemicals on uptake by cucumber seedlings. Journal of Chemical Ecology 23, 817–827.

Yu JQ, Shou SY, Qian YR, Hu WH. 2000. Autotoxic potential in cucurbit crops. Plant and Soil 223, 147–151.

Zhou YH, Yu JQ. 2006. Allelochemicals and photosynthesis. In: Reigosa MJ, Pedrol N, González L, eds. Allelopathy: a physiological process with ecological implications. Dordrecht, The Netherlands: Springer Publishers, 127–139.