DOI: 10.1007/s11099-016-0656-1 PHOTOSYNTHETICA 55 (2): 386-390, 2017

BRIEF COMMUNICATION

Benzoxazolin-2-(3*H*)-one reduces photosynthetic activity and chlorophyll fluorescence in soybean

A.V. PARIZOTTO, R. MARCHIOSI, G.A. BUBNA, J.M. BEVILAQUA, A.P. FERRO, M.L.L. FERRARESE, and O. FERRARESE-FILHO+

Laboratory of Plant Biochemistry, Department of Biochemistry, University of Maringá, Av. Colombo, 5790, 87020-900, Maringá, PR, Brazil

Abstract

Benzoxazolin-2-(*3H*)-one (BOA) has been tested in many plants species, but not in soybean (*Glycine max*). Thus, a hydroponic experiment was conducted to assess the effects of BOA on soybean photosynthesis. BOA reduced net photosynthetic rate, stomatal conductance, and effective quantum yield of PSII photochemistry without affecting intercellular CO_2 concentration or maximal quantum yield of PSII photochemistry. Results revealed that the reduced stomatal conductance restricted entry of CO_2 into substomatal spaces, thus limiting CO_2 assimilation. No change found in intercellular CO_2 concentration and reduced effective quantum yield of PSII photochemistry revealed that CO_2 was not efficiently consumed by the plants. Our data indicated that the effects of BOA on soybean photosynthesis occurred due to the reduced stomatal conductance and decreased efficiency of carbon assimilation. The accumulation of BOA in soybean leaves reinforced these findings.

Additional key words: allelochemical; benzoxazolinone; gas exchange; nonstomatal limitation; stomatal limitation.

Benzoxazolinones are allelochemicals with a strong phytotoxic activity that act not only against microorganisms and herbivores, but also affect the growth and development of neighboring plants (Sanchez-Moreiras *et al*. 2011). These compounds are found mainly in Poaceae, such as *Secale cereale*, *Zea mays*, and *Triticum aestivum* (Batish *et al*. 2006), and its use as bioherbicide agents has been proposed (Macías *et al*. 2009, Sánchez-Moreiras *et al*. 2011). Among benzoxazolinones, the natural compound, benzoxazolin-2-(3*H*)-one (BOA), a stable product of hydrolysis of 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3(4*H*)-one (DIBOA), has been studied for its phytotoxic effects and herbicidal activity (Macías *et al*. 2005, 2007).

Several factors influence the concentration of BOA in soil, including the amount of straw residue on its surface and the microbial activity (Batish *et al*. 2006). For example, residues of some cultivars of *S. cereale* release about 1.17 mg(BOA) kg^{-1}(soil) (Burgos *et al*. 1999). Moreover, the total concentration of benzoxazolinones in the top 10 cm of two soil types (*i.e*., *S. cereale* residue incorporated or left on the soil surface) reaches 80–130 $\mu g\,kg^{-1}$ (Rice *et al*. 2012).

After its uptake by plants (Chiapusio *et al*. 2004), BOA affects many physiological processes, especially, seed germination and growth in monocot and dicot weeds and crops. Different modes of action for BOA toxicity have been suggested, including inhibition of antioxidant systems followed by accumulation of reactive oxygen species (ROS), protein denaturation, lipid peroxidation, and inhibition of ATPase activity (Sánchez-Moreiras and Reigosa 2005). Based on studies with *Arabidopsis* seedlings, this same research group has suggested that the primary phytotoxic action of BOA could be the induction of premature senescence followed by oxidative stress as a

Received 6 April 2016, *accepted* 15 July 2016, *published as online-first* 6 September 2016.
+Corresponding author, e-mail: osferrarese@gmail.com.
Abbreviations: BOA – benzoxazolin-2-(*3H*)-one; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DAC – days of cultivation; E – transpiration rate; F_0 – minimal fluorescence yield of the light-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_v – variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; g_s – stomatal conductance; P_N – net photosynthetic rate; ROS – reactive oxygen species; Φ_{PSII} – effective quantum yield of PSII photochemistry.
Acknowledgments: This work was supported by grants from The Brazilian Council for Scientific and Technological Development - CNPq (no. 470705/2011-6). M.L.L. Ferrarese and O. Ferrarese-Filho are research fellows of CNPq. A.V. Parizotto is the recipient of a CAPES fellowship. The authors are grateful to A.M.D. Ramos for technical assistance.

secondary effect (Sánchez-Moreiras *et al*. 2011).

Although BOA has significant effects on seed germination and seedling growth, its impacts on the whole plant, especially on photosynthesis, are also important. This is due to the presence of this allelochemical in the field when plant species that produce it are used as cover or during crop rotations (Dhima *et al*. 2006). Regarding photosynthesis, Sánchez-Moreiras and Reigosa (2005) noted that BOA reduced the development of *Lactuca sativa* by affecting its metabolic processes, transpiration, and water relations. BOA also affected the net photosynthetic rate (P_N) and the maximal quantum yield of PSII photochemistry (F_v/F_m) of *L. sativa* plants (Sánchez-Moreiras *et al.* 2010). In *Lolium perenne*, *Dactylis glomerata,* and *Rumex acetosa*, BOA reduced F_v/F_m and the effective quantum yield of PSII photochemistry (Φ_{PSII}) (Hussain and Reigosa 2011a). Although these studies were carried out in different plant species, no such a study has been performed in soybean, one of the most important crops worldwide. For all of these reasons, our current study focused on the understanding of how BOA affects photosynthesis, which was done by evaluating its effects on gas exchange and chlorophyll (Chl) fluorescence in soybean. In order to confirm this possible effect on photosynthesis, we also investigated whether soybean plants absorb and/or accumulate BOA.

Soybean (*Glycine max* L. Merr. cv. BRS-232) seeds were surface-sterilized with 2% sodium hypochlorite and rinsed with deionized water. Seeds were dark-germinated at 25°C on three sheets of moistened filter paper. After three days of germination, seedlings were selected for uniformity, supported by an adjustable Styrofoam plate, and dipped into 8 × 15-cm acrylic containers filled with 350 ml of a 1/6-strength nutrient solution, pH 6.0 (Dong *et al.* 2006). Every container contained one seedling. The containers were kept in a growth room [25°C, cool white fluorescent light/dark photoperiod of 14/10 h, irradiance of 400 μmol(photon) m^{-2} s^{-1}] for 15 d. After 4 days of cultivation (DAC), the solution was replaced by a 1/3-strength nutrient solution (pH 6.0), and after 8 DAC, by a half-strength solution (pH 6.0). On 10, 12, and 14 DAC, and in order to prevent nutritional deficiency, the solution was replaced by nutrient solution with or without 0.1 to 0.4 mM BOA. Seedlings were collected for analysis on the 11, 13, and 15 DAC. BOA was purchased from *Sigma-Aldrich* (St. Louis, MO, USA) and all other reagents used were of the purest grade available.

Gas exchange characteristics, such as P_N, stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were measured every other day from the 11 DAC onward using the first fully expanded trifoliate leaf. Measurements were carried out at 25°C under a PPFD of 1,200 μmol m^{-2} s^{-1} and a constant air flow of 200 μmol s^{-1}, from 7:00 to 11:30 h, using a portable photosynthesis system (*LcPro+*, *ADC BioScientific Ltd.*, Hertfordshire, UK).

Chl fluorescence was measured with a portable pulse amplitude modulation fluorimeter (*OS1-FL*, *Opti-Sciences Inc*., Hudson, USA) according to the method described by Hussain and Reigosa (2011a). Measurements were determined every other day from 11 DAC, under two conditions: plants adapted to light (Φ_{PSII}) and plants adapted to dark (F_v/F_m). The Φ_{PSII} and the gas exchange parameters were recorded simultaneously. In order to determine F_v/F_m, plants were dark-adapted for 20 min with the aid of a dark clip adapter. Minimal Chl fluorescence (F_0) and maximum Chl fluorescence (F_m) were measured in the first fully expanded trifoliate leaf. The F_v/F_m was calculated using the equation $F_v/F_m = (F_m - F_0)/F_m$, where $F_v = F_m - F$.

Depletion experiments were performed to determine BOA from the initial nutrient solution and thus to evaluate whether BOA accumulated in soybean organs. Experiments were conducted with 0.4 mM BOA, which was added to the nutrient solution on 10, 12, and 14 DAC. On the 11, 13, and 15 DAC, samples of the nutrient solution were filtered through a 0.45 μm disposable syringe filter (*Hamilton Co*., Nevada, USA). Sample injection (20 μl) and analysis were accomplished by a high performance liquid chromatography (*LC-20 Prominence*, *Shimadzu*, Kyoto, Japan). A reversed-phase *Shimpack*® CLC-ODS column (250 × 4.6 mm, 5 μm), protected with an equivalent precolumn (10 × 4.6 mm), was used at 30°C. The mobile phase consisted of a mixture of acetonitrile/acetic acid 1% in water (40/60, v/v) with a flow rate of 0.8 ml min^{-1} for an isocratic run of 30 min, and UV was carried out at 271 nm. BOA was identified by comparing its retention time with a standard compound. BOA was also extracted from roots, stem, and first trifoliate leaf on the same days of cultivation. Fresh tissues (0.5 g) were ground in 5 ml of 70% ethanol and homogenates were centrifuged (2,200 × g for 5 min), and the supernatants were separated for analyses (Chiapusio *et al*. 2004). Samples (20 μl) were filtered through a 0.45-μm disposable syringe filter and analyzed by HPLC, as described earlier.

One-way analysis of variance (*ANOVA*) was performed to test the significance of the observed differences using the *GraphPad Prism* package (*GraphPad Software Inc*., La Jolla, CA, USA). The differences between the parameters were evaluated by *Dunnett*'s multiple range test at $\alpha = 0.05$. Data were expressed as means ± SE.

Compared with the respective controls, BOA reduced P_N, g_s , and E of soybean plants, but C_i was not clearly changed (Fig. 1*A*,*B*). The inhibitory effect of BOA on P_N was related to its concentration and exposure time. The mean P_N decreased by 16% (11 DAC), 20% (13 DAC), and 20% (15 DAC). A similar trend was observed for g_s from 11 to 15 DAC for all concentrations, decreasing by 34% (11 DAC), 23% (13 DAC), and 24% (15 DAC). BOA also reduced E by 15% for both 0.1 and 0.4 mM treatments, after 11 and 13 DAC, respectively. After

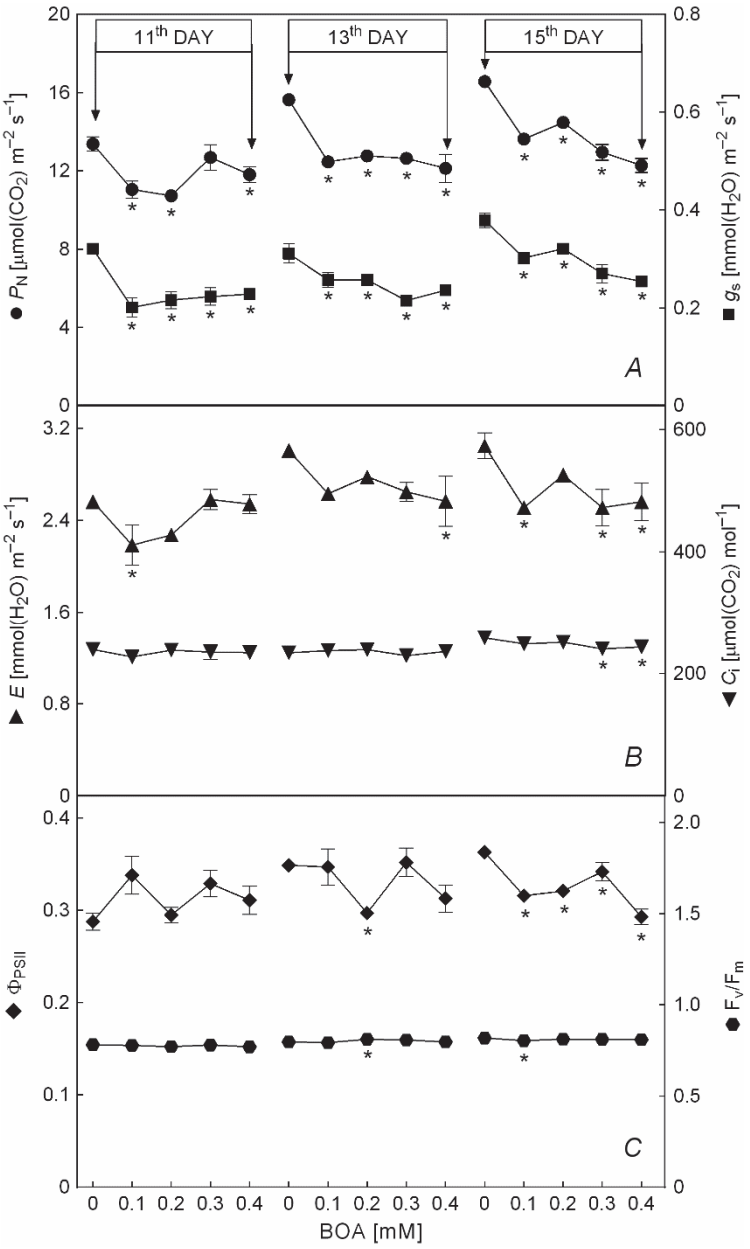

Fig. 1. Effects of BOA on (*A*) net photosynthetic rate (P_N) and stomatal conductance (g_s), (*B*) transpiration rate (E) and intercellular CO_2 concentration (C_i), (*C*) effective quantum yield of PSII photochemistry (Φ_{PSII}) and maximal quantum yield of PSII photochemistry (F_v/F_m) in soybean plants. Data are the means ± SE (n = 10). *Significant at P<0.05 level as compared with the control plants.

15 DAC, BOA reduced E by 18% (at 0.1 mM and 0.3 mM) and 16% (at 0.4 mM). The C_i was practically unaltered by BOA, except for slight decreases of 4% (at 0.3 mM) and 6% (at 0.4 mM), after 15 DAC.

The Φ_{PSII} of soybean subjected to 0.1 to 0.4 mM BOA was not altered after 11 DAC, but it was reduced by 15% with 0.2 mM after 13 DAC (Fig. 1*C*). However, BOA reduced Φ_{PSII}, from 13% to 20% for all concentrations, after 15 DAC. The F_v/F_m was not affected by BOA after 11 DAC. Slight changes were noticed on other days, such as an increase of 2% (0.2 mM BOA) and a similar decrease (0.1 mM BOA) after 13 and 15 DAC, respectively.

The main observation revealed herein was that BOA also reduced soybean photosynthesis, *i.e.*, gas exchange (P_N, g_s, and E) and Chl fluorescence in plants adapted to light (Φ_{PSII}). Overall, BOA did not affect the C_i or Chl fluorescence of plants adapted to dark (F_v/F_m). In the same way, BOA reduced both P_N and Φ_{PSII} in *L. sativa* (Sánchez-Moreiras *et al.* 2010); P_N after 6 h or Φ_{PSII} after 10 h of treatment. Reductions in P_N could be due to reduced g_s and/or interference with reactions of CO_2 assimilation.

Similar to P_N, BOA also reduced g_s (Fig. 1*A*). It is known that a reduced value of g_s is implicated in lowered P_N and E (Centritto *et al*. 2003), and both parameters were reduced by BOA (Fig. 1*A*,*B*). Thus, a decline in photosynthesis could be due to the limited water availability that closed stomata, as a primary response to BOA, followed by a decreased supply of CO_2 to mesophyll cells. A similar trend has been noted in three C_3 perennial species, *D. glomerata*, *L. perenne,* and *R. acetosa* (Hussain and

Reigosa 2011a).

From the results obtained herein, g_s seems to partly limit P_N because, under normal CO_2 assimilation conditions, a decrease in g_s reduces C_i. A decline in photosynthesis can also be due to decreased Rubisco activity under stress conditions (Ashraf and Harris 2013). Decreased activity and expression of Rubisco, associated with a reduction in P_N but no alteration in C_i, was observed in soybean grown under saline stress (Lu *et al*. 2009). It is known that stomatal limitation reduces g_s and C_i (Zhou and Yu 2006), while nonstomatal limitation reduces g_s and increases C_i (Farquhar and Sharkey 1982). As shown here, BOA reduced g_s and E, but had little effect on C_i; therefore, it cannot be a limiting factor for photosynthesis. At least in part, a nonstomatal limitation to photosynthesis (interference with reactions of CO_2 assimilation, for example) is possible.

Changes in Chl fluorescence reflect changes in photochemical efficiency and heat dissipation. The F_v/F_m ratio, a measure of the structural integrity of PSII (Lu *et al*. 2009), was not affected by BOA (Fig. 1*C*). Other studies have shown that F_v/F_m and Φ_{PSII} were reduced in *Cucumis sativus* (Ye *et al*. 2004), *D. glomerata*, *L. perenne, R. acetosa,* and *L. sativa* (Hussain and Reigosa 2011a,b) under stress of cinnamic acid, and in *L. sativa* after BOA exposure (Hussain *et al*. 2011). Damaged thylakoid membranes, especially those of PSII, can inhibit energy transfer from molecule antennae to the reaction centers and decrease F_v/F_m (Krause 1984).

Under light conditions, Φ_{PSII} measures the proportion of absorbed energy used in photochemical reactions (Sánchez-Moreiras and Reigosa 2010). A reduction of Φ_{PSII} is implicated in the low efficiency of the PSII reaction center and, by consequence, changes in the electron transport rate. This suggests a reduction in the proportion of photons absorbed by PSII, which are used by photochemistry (Hall and Rao 1999). Thus, analysis of emission of Chl fluorescence indicates the photochemical efficiency of PSII in the complexes (Demmig-Adams *et al*. 1996). As noted herein, the Φ_{PSII} values were markedly reduced by BOA after 15 DAC (Fig. 1*C*), suggesting a cumulative effect of the compound. The reduction of Φ_{PSII} is related to the decrease in P_N and to the limitations in carbon metabolism (Loreto *et al*. 2003). This is because the operating efficiency of PSII (Φ_{PSII}) is directly proportional to the quantum operating efficiency of CO_2 assimilation, given that a constant proportion of reducing equivalents from the linear flux of electrons is used for CO_2 assimilation (Genty *et al*. 1989, Baker *et al*. 2007). Furthermore, the lack of a relevant reduction in C_i (Fig. 1*C*) reflected inefficient use of CO_2 by soybean plants exposed to BOA. In agreement, Lu *et al*. (2009) noted decreased photosynthesis in soybean plants subjected to salt stress, associated with a decrease in Rubisco activity, an indicator that this enzyme can limit CO_2 fixation under stress conditions.

A relation between BOA phytotoxicity and its accumulation in *L. sativa* leaves has been reported by Sánchez-Moreiras *et al*. (2010). They found that a reduced P_N was correlated with an increase in BOA in the leaves, suggesting that the phytotoxicity was due to the activity of this compound and not due to some derivative or other degradation products. High accumulation of BOA in leaves was observed after 96-h treatment, although a significant accumulation also occurred after 24 h. As shown here, soybean plants significantly absorbed BOA (Fig. 2*A*), which accumulated in leaves after 24-h exposure (Fig. 2*B*), corroborating the results of Sánchez-Moreiras *et al*. (2010). Because BOA itself was detected in soybean leaves, its accumulation in this organ may be responsible for the detrimental effects on photosynthesis. In addition, the low amount of BOA quantified in roots and stems of soybean suggested that it was readily taken on by the roots and transported toward the leaves. We also noted that BOA caused chlorosis in soybean leaves; this symptom started appearing from the margins of foliar limb, and it was related to the BOA concentration (data not shown). This fact can be associated with the BOA accumulation in leaves. Chlorosis and low contents of Chl and carotenoids were observed in *Arabidopsis thaliana* exposed to BOA

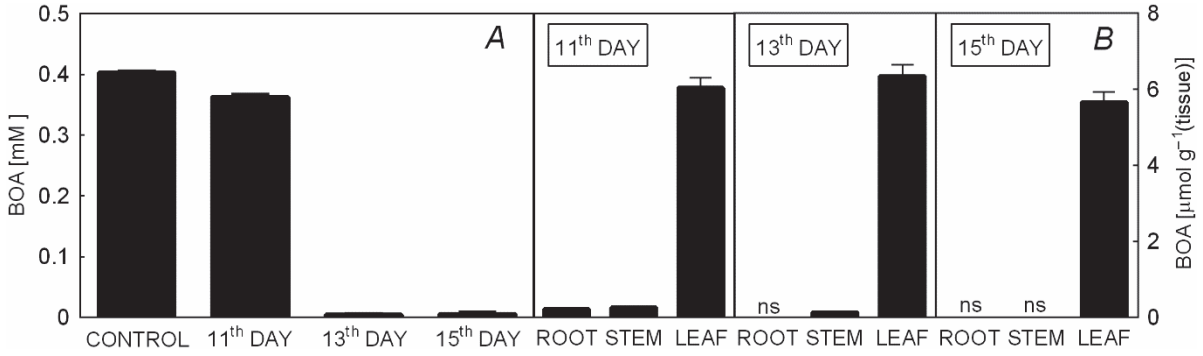

Fig. 2. Depletion of BOA from the nutrient solution (*A*) and its quantification (*B*) in roots, stems, and leaves of soybean. In *A*, control represents a nutrient solution containing 0.4 mM BOA, which was supplemented on the 10th, 12th, and 14th days of cultivation. Data are the means ± SE (n = 3). *Significant at P<0.05 level as compared with the control plants. ns – not significant.

(Sánchez-Moreiras *et al*. 2011). According to the authors, BOA induced an early senescence process in leaves, which was related to the reduction of nitrogen content and proteins, especially, of Rubisco.

Our results with soybean confirmed that, in fact, BOA affected the photosynthetic process. The reduced g_s restricted the entry of CO_2 into substomatal spaces, which could limit its assimilation. However, no change in C_i and a reduction of Φ_{PSII} indicated that CO_2 was not being consumed efficiently by plants subjected to BOA. In brief, our data indicated that the effects of BOA on soybean photosynthesis occurred mainly due to reduced g_s and decreased efficiency of carbon assimilation. In addition, the accumulation of BOA in soybean leaves reinforced these findings.

References

Ashraf M., Harris P.J.C.: Photosynthesis under stressful environments: An overview. – Photosynthetica **51**: 163-190, 2013.

Baker N.R., Harbinson J., Kramer D.M.: Determining the limitations and regulation of photosynthetic energy transduction in leaves. – Plant Cell Environ. **30**: 1107-1125, 2007.

Batish D.R., Singh H.P., Setia N. *et al*.: 2-Benzoxazolinone (BOA) induced oxidative stress, lipid peroxidation and changes in some antioxidant enzymes activities in mung bean (*Phaseolus aureus*). – Plant Physiol. Bioch. **44**: 819-827, 2006.

Burgos N.R., Talbert R.E., Mattice J.D.: Cultivar and age differences in the production of allelochemicals by *Secale cereale*. – Weed Sci. **47**: 481-485, 1999.

Centritto M., Loreto F., Chartzoulakis K.: The use of low [CO_2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. – Plant Cell Environ. **26**: 585-594, 2003.

Demmig-Adams B., Adams W.W., Barker D.H. *et al*.: Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. – Plant Physiol. **98**: 253-264, 1996.

Dhima K., Vasilakoglou I.B., Eleftherohorinos I.G. *et al*.: Allelopathic potencial of winter cereal cover crop mulches on grass weed suppression and sugarbeet development. – Crop Sci. **46**: 1682-1691, 2006.

Dong J., Wu F., Zhang G.: Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). – Chemosphere **64**: 1659-1666, 2006.

Farquhar G.D., Sharkey T.D.: Stomatal conductance and photosynthesis. – Annu. Rev. Plant Physiol. **33**: 317-345, 1982.

Genty B., Briantais J.M., Baker N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim. Biophys. Acta **990**: 87-92,1989.

Hall D.O., Rao K.K.: Photosynthesis, 6th ed. Pp. 160-174. Cambridge University Press, Cambridge 1999.

Hussain M.I., González L., Chiapusio G. *et al*.: Benzoxazolin-2(3H)-one (BOA) induced changes in leaf water relations, photosynthesis and carbon isotope discrimination in *Lactuca sativa*. – Plant Physiol. Bioch. **49**: 825-834, 2011.

Hussain M.I., Reigosa M.J.: A chlorophyll fluorescence analysis of photosynthetic efficiency, quantum yield and photon energy dissipation in PSII antennae of *Lactuca sativa* L. leaves exposed to cinnamic acid. – Plant Physiol. Biochem. **49**: 1290-1298, 2011b.

Hussain M.I., Reigosa M.J.: Allelochemical stress inhibits growth, leaf water relations, PSII photochemistry, non-photochemical fluorescence quenching, and heat energy dissipation in three C3 perennial species. – J. Exp. Bot. **62**: 4533-4545, 2011a.

Chiapusio G., Pellissier F., Gallet C.: Uptake and translocation of phytochemical 2-benzoxazolinone (BOA) in radish seeds and seedlings. – J. Exp. Bot. **55**: 1587-1592, 2004.

Krause G.H., Wies E.: Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. – Photosynth. Res. **5**: 139-157, 1984.

Loreto F., Centritto M., Chartzoulakis K.: Photosynthetic limitations in olive cultivars with different sensitivity to salt stress. – Plant Cell Environ. **26**: 595-601, 2003.

Lu K.X., Cao B.H., Feng X.P. *et al*.: Photosynthetic response of salt-tolerant and sensitive soybean varieties. – Photosynthetica **47**: 381-387, 2009.

Macías F.A., Marín D., Oliveros-Bastidas A. *et al*.: Structure-activity relationships (SAR) studies of benzoxazinones, their degradation products and analogues. Phytotoxicity on Standard Target Species (STS). – J. Agric. Food Chem. **53**: 538-548, 2005.

Macías F.A., Marín D., Oliveros-Batidas A. *et al*.: Rediscovering the bioactivity and ecological role of 1,4-benzoxazinones. – Nat. Prod. Rep. **26**: 478-489, 2009.

Macías F.A., Molinillo J.M.G., Varela R.M. *et al*.: Allelopathy - a natural alternative for weed control. – Pest Manage. Sci. **63**: 327-348, 2007.

Rice C.P., Cai G., Teasdale J.R.: Concentration and allelopathic effect of benzoxazinoid compounds in soil treated with rye (*Secale cereale*) cover crop. – J. Agric. Food Chem. **60**: 4471-4479, 2012.

Sánchez-Moreiras A.M., Martínez-Peñalver A., Reigosa M.J.: Early senescence induced by 2-*3H*-benzoxazolinone (BOA) in *Arabidopsis thaliana*. – J. Plant Physiol. **168**: 863-870, 2011.

Sánchez-Moreiras A.M., Oliveros-Bastidas A., Reigosa M.J.: Reduced photosynthetic activity is directly correlated with 2-(*3H*)-benzoxazolinone accumulation in lettuce leaves. – J. Chem. Ecol. **36**: 205-209, 2010.

Sánchez-Moreiras A.M., Reigosa M.J.: Whole plant response of lettuce after root exposure to BOA (2(*3H*)-benzoxazolinone). – J. Chem. Ecol. **31**: 2689-2703, 2005.

Ye S.F., Yu J.Q., Peng Y.H. *et al*.: Incidence of *Fusarium* wilt in *Cucumis sativus* L. is promoted by cinnamic acid, an autotoxin in root exudates. – Plant Soil **263**: 143-150, 2004.

Zhou Y.H., Yu J.Q.: Allelochemicals and photosynthesis. – In: Reigosa M.J., Pedrol N., González L. (ed.): Allelopathy: A Physiological Process with Ecological Implications. Pp. 127-139. Springer, Dordrecht 2006.