

## BRIEF COMMUNICATION

**Benzoxazolin-2-(3H)-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<sup>†</sup>*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<sub>2</sub> concentration or maximal quantum yield of PSII photochemistry. Results revealed that the reduced stomatal conductance restricted entry of CO<sub>2</sub> into substomatal spaces, thus limiting CO<sub>2</sub> assimilation. No change found in intercellular CO<sub>2</sub> concentration and reduced effective quantum yield of PSII photochemistry revealed that CO<sub>2</sub> 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-(3H)-one (BOA), a stable product of hydrolysis of 2,4-dihydroxy-1,4(2H)-benzoxazin-3(4H)-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<sup>-1</sup>(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 µg kg<sup>-1</sup> (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

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*Abbreviations:* BOA – benzoxazolin-2-(3H)-one; Chl – chlorophyll; C<sub>i</sub> – intercellular CO<sub>2</sub> concentration; DAC – days of cultivation; E – transpiration rate; F<sub>0</sub> – minimal fluorescence yield of the light-adapted state; F<sub>m</sub> – maximal fluorescence yield of the dark-adapted state; F<sub>v</sub> – variable fluorescence; F<sub>v</sub>/F<sub>m</sub> – maximal quantum yield of PSII photochemistry; g<sub>s</sub> – stomatal conductance; P<sub>N</sub> – net photosynthetic rate; ROS – reactive oxygen species; Φ<sub>PSII</sub> – effective quantum yield of PSII photochemistry.

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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 ( $\Phi_{PSII}$ ) (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  $\mu\text{mol}(\text{photon})\text{m}^{-2}\text{s}^{-1}$ ] 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  $\text{CO}_2$  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  $\mu\text{mol}\text{m}^{-2}\text{s}^{-1}$  and a constant air flow of 200  $\mu\text{mol}\text{s}^{-1}$ , 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 (*OSI-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 ( $\Phi_{PSII}$ ) and plants adapted to dark ( $F_v/F_m$ ). The  $\Phi_{PSII}$  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  $F_v = F_m - F_0$ .

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  $\mu\text{m}$  disposable syringe filter (*Hamilton Co.*, Nevada, USA). Sample injection (20  $\mu\text{l}$ ) and analysis were accomplished by a high performance liquid chromatography (*LC-20 Prominence*, Shimadzu, Kyoto, Japan). A reversed-phase *Shimpack*<sup>®</sup> CLC-ODS column (250 × 4.6 mm, 5  $\mu\text{m}$ ), 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  $\text{ml}\text{min}^{-1}$  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  $\mu\text{l}$ ) were filtered through a 0.45- $\mu\text{m}$  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 *Dunnnett's* multiple range test at  $\alpha = 0.05$ . Data were expressed as means  $\pm$  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. 1A,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

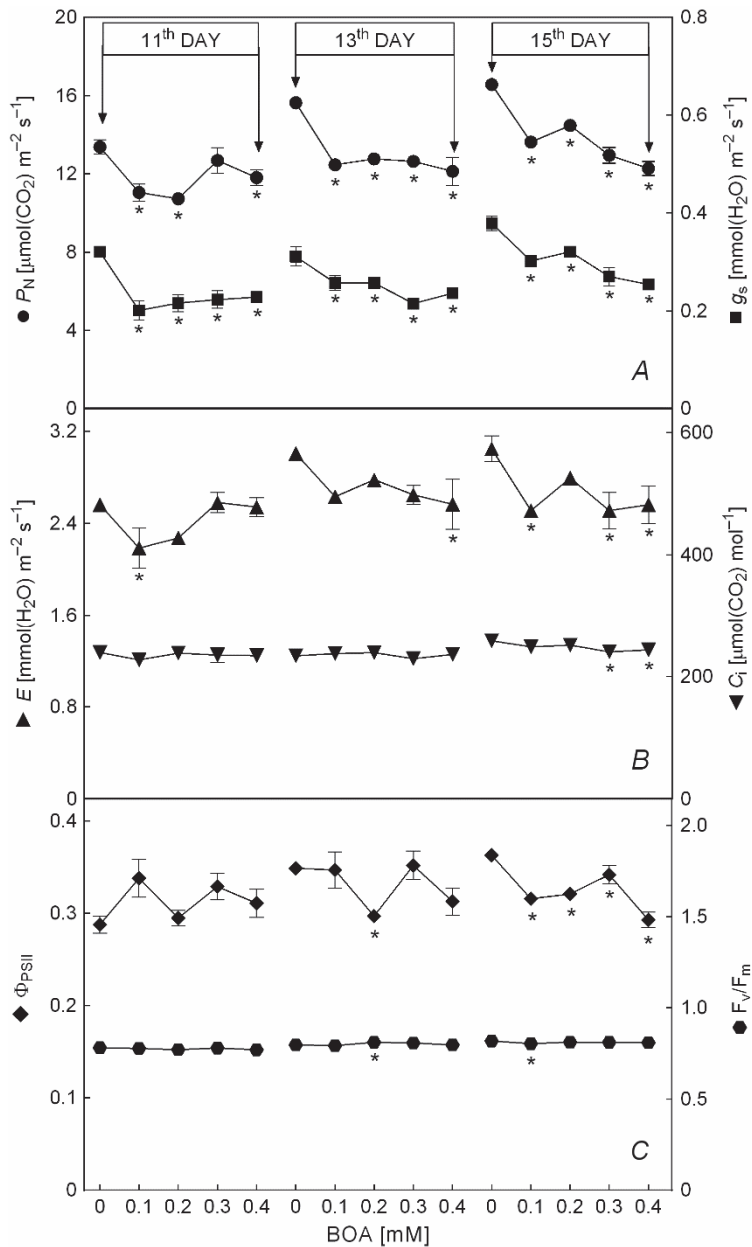


Fig. 1. Effects of BOA on (A) net photosynthetic rate ( $P_N$ ) and stomatal conductance ( $g_s$ ), (B) transpiration rate ( $E$ ) and intercellular  $CO_2$  concentration ( $C_i$ ), (C) effective quantum yield of PSII photochemistry ( $\Phi_{PSII}$ ) and maximal quantum yield of PSII photochemistry ( $F_v/F_m$ ) in soybean plants. Data are the means  $\pm$  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  $\Phi_{PSII}$  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. 1C). However, BOA reduced  $\Phi_{PSII}$ , 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 ( $\Phi_{PSII}$ ). 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  $\Phi_{PSII}$  in *L. sativa* (Sánchez-Moreiras *et al.* 2010);  $P_N$  after 6 h or  $\Phi_{PSII}$  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. 1A). 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. 1A,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. 1C). Other studies have shown that  $F_v/F_m$  and  $\Phi_{PSII}$  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,  $\Phi_{PSII}$  measures the proportion of absorbed energy used in photochemical reactions (Sánchez-Moreiras and Reigosa 2010). A reduction of  $\Phi_{PSII}$  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  $\Phi_{PSII}$  values were markedly reduced

by BOA after 15 DAC (Fig. 1C), suggesting a cumulative effect of the compound. The reduction of  $\Phi_{PSII}$  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 ( $\Phi_{PSII}$ ) 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. 1C) 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. 2A), which accumulated in leaves after 24-h exposure (Fig. 2B), 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

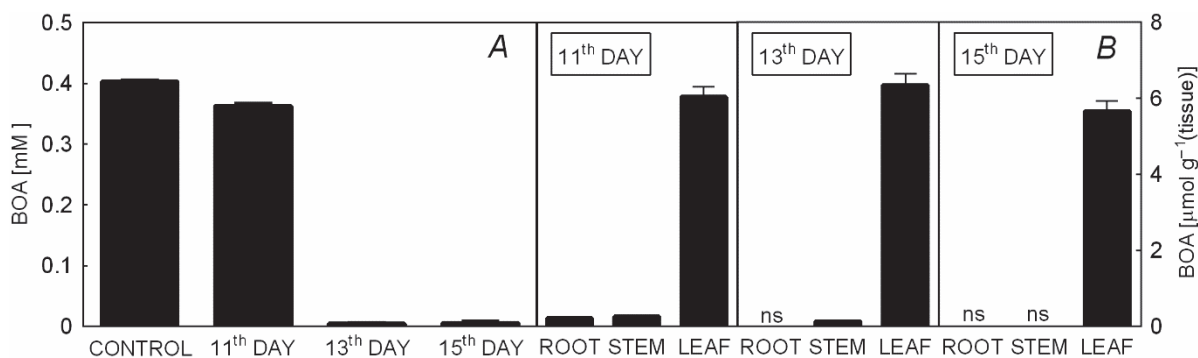


Fig. 2. Depletion of BOA from the nutrient solution (A) and its quantification (B) in roots, stems, and leaves of soybean. In A, control represents a nutrient solution containing 0.4 mM BOA, which was supplemented on the 10<sup>th</sup>, 12<sup>th</sup>, and 14<sup>th</sup> days of cultivation. Data are the means  $\pm$  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<sub>2</sub> into substomatal spaces, which could limit its assimilation. However, no change in C<sub>i</sub> and

a reduction of  $\Phi_{PSII}$  indicated that CO<sub>2</sub> 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.

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