Uptake and translocation of phytochemical 2-benzoxazolinone (BOA) in radish seeds and seedlings

Geneviève Chiapusio1,*, François Pellissier2 and Christiane Gallet2

1 University of Franche-Comté, Laboratoire de Biologie et Ecophysiologie, Pôle de Montbéliard, 4 place Tharradin, BP 71427, F-25 211 Montbéliard cedex, France
2 University of Savoie, Laboratoire d’Ecologie Alpine LECA-TDE, CISM, F-73376 Le Bourget-du-Lac cedex, France

Received 31 July 2003; Accepted 14 April 2004

Abstract
The molecular aspects of phytochemical interactions between plants, especially the process of phytochemical translocation by the target plant, remain challenging for those studying allelopathy. 2-Benzoxazolinone (BOA) is a natural chemical produced by rye (Secale cereale) and is known to have phytotoxic effects on weed seeds and seedlings. The translocation of BOA into target plants has been poorly investigated. Therefore, the total absorption of [ring U14C] BOA was estimated by oxidizing whole seedlings of Raphanus sativus cv. for 8 days and quantifying the radioactivity. Non-radiolabelled BOA in seedlings was also estimated by HPLC. BOA applied at $10^{-3}$ M was readily taken up by germinated radish at a rate of 1556 nmol g$^{-1}$ FW. At these same concentrations, BOA reduced radish germination by 50% and caused a delay in radicle elongation. Exogenous BOA was responsible for the observed germination inhibition. At a concentration of $10^{-5}$ M, BOA was taken up by germinated seeds (31 nmol g$^{-1}$ FW), but this quantity did not affect radish germination. Labelled BOA was not mineralized in the culture medium during seedling growth as no $^{14}$CO$_2$ was recovered. Both $10^{-3}$ and $10^{-5}$ M BOA were translocated into radish organs, mainly into roots and cotyledons. These organs were then identified as potential physiological target sites. Cotyledons remained the target sink (44% of the total radioactivity). The kinetics of BOA uptake at $10^{-3}$ and $10^{-5}$ M in radish seedlings was identical: BOA accumulation was proportional to its initial concentration. A comparison between radioactivity and HPLC quantification for $10^{-3}$ M BOA indicated that BOA (along with some metabolites) could effectively be recovered in radish organs using chromatography.

Key words: 2-Benzoxazolinone, HPLC, kinetic, phytotoxicity, radioactivity, Raphanus sativus, translocation, uptake.

Introduction
Few studies have effectively quantified the phytochemical interactions between plants at the molecular level. Regardless of this fundamental lack of understanding, an increased interest in allelopathy in agricultural and natural ecosystems continues to be observed. There is still difficulty in fully demonstrating such chemical interactions. One of the main mechanisms through which allelopathic potential is expressed is the exudation of active plant metabolites from the roots into the soil. These metabolites and/or their related products should be absorbed and translocated into the target plant before inducing physiological disturbances. For instance, Sorghum bicolor reduces the yield of succeeding crops such as Arachis hypogaea by producing phenolic compounds (Sène et al., 2000). Empetrum hermaphroditum, a shrub that forms dense cover in boreal forest is thought to release batatasin-III into the soil resulting in a strong negative effect on the establishment and growth of tree seedlings (Nilsson et al., 1998). Little has been reported on the last step of such allelochemical interferences, specifically the absorption of phytochemicals from the soil into the target plant. This crucial step in the assessment of phytotoxic interactions between plants in agrosystems has to be clarified for the commercial development of natural pesticides (Davies and Caseley, 1999).
Hydroxamic acids are natural pesticides found in numerous Gramineae genera such as Secale, Sorghum, Triticum, and Zea (Niemeyer, 1988) as well as in the Scrophulariaceae and Acanthaceae (Pratt et al., 1995). They serve as an important factor in the host plant’s resistance against microbial diseases (Bravo et al., 1997; Miché et al., 2003) and insects (Bravo and Copaja, 2002), and as allelochemicals (Sicker et al., 2000). 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). BOA is released by the decomposition of the cyclic hydroxamic acid DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one). This transformation occurs in soil during the decomposition of rye tissues or root exudates. BOA is considered to be a potential herbicide for weed suppression with its principal mode of action being on seed germination and on seedling growth. Moreover, this species is not known to produce benzoxazinones naturally.

**BOA effect on radish germination**

**Chemical solutions:** BOA was purchased from Sigma Chemicals Ltd (St Louis, MO, USA). Aqueous solutions were prepared at 10^{-3} and 10^{-5} M with demineralized water and buffered to pH 5.5 with HCl, the same pH as the control (demineralized water).

**Germination tests:** Twenty radish seeds (*Raphanus sativus* L., cv. 18 days) were placed into a glass jar (7 cm diameter and 9 cm high, closed with a cap), filled with 5 ml of a chemical solution or demineralized water (control). The glass jars were stored in a thermostatic growth chamber (20 °C, photoperiod 8/16 h light/dark) for a maximum of 4 d. The number of germinated seeds (characterized by the rupture of the seed coats and the emergence of the radicle) was counted every 24 h for 4 d. Treatments were replicated six times.

**BOA quantification by radiochemical analysis**

**Chemical solutions:** [*Ring-1^14C*] BOA was purchased from NEN™ (radiochemical purity >99%, specific activity 1.73×10^2 Bq mol^{-1}). An aqueous solution of BOA was prepared at 10^{-3} and 10^{-5} M with demineralized water and buffered to pH 5.5 with HCl. [*1^4C*] BOA was dissolved in 2 ml of 99.9% ethanol (reaching a concentration of 0.01 mmol ml^{-1}). Four microlitres of this radiolabelled BOA solution were added to the non-labelled BOA solution to obtain ~1.37×10^6 Bq mol^{-1} in the final solution. These two (BOA and [*1^4C*-BOA]) solutions at 10^{-3} and 10^{-5} M were used immediately in radish culture.

**Radish culture:** The radish culture conditions were the same as those for the germination experiment except that seedlings were grown for up to a maximum of 5 d. In order to trap potential [*1^3CO_2*], a Petri dish containing 5 ml of 10 N NaOH was placed at the top of each container. Four replicates per concentration and per incubation time were performed.

**BOA extraction by oxidizing:** At the end of each incubation time (24, 48, 72, 96, and 120 h), the radish seedlings of an individual glass jar were rinsed with deionized water and buffered to pH 5.5 with HCl. Radioactivity in the rupture of the seed coats and the emergence of the radicle was counted every 24 h for 4 d. Treatments were replicated six times. The frozen samples were wrapped in paper (Germaflor) for oven-drying at 80 °C for 48 h. Each sample was then combusted in a biological oxidizer (Ox-500 EG&G instruments) at 900 °C for 3 min. The released [*1^4CO_2*] was trapped in a vial containing a liquid scintillation cocktail (Oxysolve C400, Zinsser Analytic Co.). Radioactivity within samples was measured for 10 min using a liquid scintillation counter (1500 TriCab, Packard). For every sample, the total disintegrations min^{-1} (DPM) counted in one glass jar (seedlings + remaining culture medium + rinsing + [*1^4CO_2*]) was between 85 and 100% of the initial radioactive solution added to the original culture medium.

**Expression of results:** BOA levels in radish seedlings were either expressed as a concentration (nmol g^{-1} FW) or as a quantity (% of applied [*1^4C*-BOA]) for 20 seedlings (equivalent to one glass jar) as described by Chiapusio and Pellissier (2001). No dry weight was determined by chemical methods.

**Materials and methods**

**General procedure**

The radiochemical methodology used was based on previous work (Chiapusio and Pellissier, 2001). This technique determined the total amount of [*1^4C*-labelled BOA in radish seedlings: the non-extractable BOA forms associated with plant cell wall constituents plus the BOA extractable forms. Because the [*1^4C*]-BOA in order to other degradation products containing at least one of the six carbons of the BOA aromatic ring, HPLC analyses were undertaken to differentiate extractable BOA from the other related compounds. The differences observed between the radioactivity and
available due to the extraction techniques made with fresh material (i.e., HPLC experiment).

The radish seeds exposed to 10⁻³ M BOA showed a delay of ~24 h before germination compared with those treated with 10⁻⁵ M BOA or the control. Consequently, the results are presented according to the different physiological stages instead of as time after treatment. Stage 1, germinated seeds; stage 2, to seedling radicle growth; stage 3, to seedling hypocotyl elongation and to the beginning of photosynthesis; and stage 4, fully physiologically active seedlings.

**BOA quantification by HPLC analysis**

Seedling growth: The seedling growth was conducted with BOA 10⁻³ M treatment or demineralized water (control). No HPLC quantification was done with 10⁻⁵ M BOA due to the low concentration of BOA remaining in seedlings. The radish culture conditions were the same as those for the germination experiment. It proved necessary to bulk vegetative material to get one sample for BOA 10⁻³ M treatments (36 glass jars in total), but not for the control (9 glass jars in total). Three replicates per treatment were performed.

**BOA extraction:** The BOA sample preparation was the same as that for radiochemical analysis. The samples (~200 mg FW corresponded to 20 seedlings) were ground in a mortar and extracted with 3 ml of a 70% ethanolic solution. Homogenates were centrifuged (3000 rpm) for 3 min at 20 °C. The supernatant was kept in stock whereas the residue was sequentially extracted three times. Supernatants were bulked and used directly for HPLC analysis.

**High Pressure Liquid Chromatography (HPLC):** Chromatographic separation was performed using a Waters 600 Controller, equipped with the diode array detector Waters 996 and with Millennium software. A 250×4.6 mm column filled with µBondapak C₁₈, 10 µm was used for this procedure. Solvent A was made up of 0.5% acetic acid in distilled water and solvent B of 0.5% acetic acid in acetonitrile. Injection volumes were 20 µL. BOA was separated by using a linear gradient at a flow rate of 1.5 ml min⁻¹, from 0 to 20% of B in 45 min, with 15 min of re-equilibration between samples. BOA was further identified by comparison of UV spectra and retention times with the standard. Detection was performed at 270 nm. Some radish metabolites were detected in seeds and cotyledons which made BOA quantification difficult. Separation was then improved by using a Discovery C₁₈ column (250×4.6 mm, 5 µm). The retention time of BOA was 9.8 min, using an isocratic flow of 18% of B in A, at a flow rate of 1.5 ml min⁻¹.

**Statistical analysis**

All data were tested to determine if they met the assumptions of parametric statistical analysis. Since most data were found to lack homogeneity of variance and were found to lack a normal distribution, the non-parametric Mann-Whitney U test (P<0.05) was used to determine mean separation. Two categories of tests were performed to compare: (i) BOA content (concentration and quantity) in different organs within the same physiological stage and (ii) differences in BOA concentration from one physiological stage to the next, for the same organ. Finally, differences between concentrations of BOA recovered in radish seedlings by HPLC and radioactivity methods were compared using the Mann-Whitney U test (P<0.05).

**Results**

**BOA effects on radish germination**

The 10⁻⁵ M BOA had no effect on radish germination compared with the control (Fig. 1). By contrast, 10⁻³ M BOA drastically inhibited radish germination. For these treated seeds, the T₅₀ value (time when 50% of the germination occurred) was never reached during the four days of the experiment. A major consequence of this germination inhibition was a delay in physiological seedling development for ~24 h between control seedlings and those treated with BOA.

**Radioactivity results**

Removal of BOA in culture medium and released ¹⁴CO₂:

From its initial concentration of 10⁻³ M, BOA exhibited a regular decrease in concentration in the culture medium to 0.45, 0.44, 0.41, 0.32, and 0.28 10⁻³ M at, respectively, 1, 2, 3, 4, and 5 d after the start of the incubation process (data not shown). When applied at 10⁻⁵ M, BOA also disappeared rapidly from the culture medium and its concentration after 4 d of incubation reached 10⁻⁷ M. No detectable ¹⁴CO₂ was observed in the CO₂ trap for either of the BOA concentration treatments.

**BOA treatments on germinated radish seedlings:** BOA was taken up by germinating seeds, at both experimental concentrations. BOA was recovered in germinated seeds at a concentration of 1556 and 31 nmol g⁻¹ FW for the 10⁻⁵ and 10⁻³ M treatments, respectively.

The 10⁻⁻³ M BOA treatment on radish seedlings: At stage 2, BOA accumulated equally in all seedling parts (at ~1600 nmol g⁻¹ FW), whereas it accumulated mainly in roots and cotyledons (at ~2100 nmol g⁻¹ FW) at stage 3, and mostly in roots (2700 nmol g⁻¹ FW) at stage 4 (Table 1). Focusing on BOA kinetics, BOA concentrations only increased significantly in cotyledons from stage 2 to stage 4. Cotyledons always contained the highest quantity (%) of BOA compared with other organs. This recovery corresponded to 73% and up to 80% of the total BOA seedling content for each stage.

**BOA treatments on stage 4 radish seedlings:** The highest accumulations of BOA were recovered in cotyledons (41 nmol g⁻¹ FW) at stage 2, and in roots and cotyledons at stages 3 and 4 (24 and 27 nmol g⁻¹ FW, respectively) (Table 2). A significant decrease in BOA in cotyledons was...
observed from stage 2 to stage 4. Cotyledons always contained the highest quantity expressed as a percentage of BOA compared with other organs.

**HPLC analysis**

**BOA occurrence in control:** No BOA was detected in the untreated culture medium, in radish organelles, or in radish seeds during the incubation process. This confirmed that BOA is not naturally synthesized by radish organs. Therefore, the BOA quantities detected in target seeds and seedlings could be attributed completely to BOA absorption.

**BOA metabolism in treatments:** Exogenous $10^{-3}$ M BOA was translocated from the culture medium into germinated seeds (1028 nmol g$^{-1}$ FW). In stage 2 seedlings, the highest accumulation of BOA was observed in roots and hypocotyls (2900 nmol g$^{-1}$ FW), whereas in stage 3 seedlings it was detected in cotyledons (740 nmol g$^{-1}$ FW) (Table 3).

**Discussion**

**BOA uptake and effect during radish germination**

Radish seeds absorbed BOA at both concentrations applied ($10^{-3}$ and $10^{-5}$ M) from the onset of the experiment, but the phytotoxic effect of BOA on radish germination was dose-dependent. At $10^{-5}$ M, no effect of BOA on radish germination was observed, whereas at $10^{-3}$ M, BOA drastically reduced radish germination by ~50%. The effect on seedling germination is a well-known phenomenon in allelopathy. For example, biological differences between high and low BOA concentrations in germination were previously observed in *L. sativa* (Chiapusio et al., 1997), but not explained.

This work outlines the fact that BOA concentrations detected in germinated seeds (stage 1), as measured by the uptake of radiolabelled BOA (1556 nmol g$^{-1}$ FW) and HPLC (1028 nmol g$^{-1}$ FW), did not differ significantly. The $10^{-3}$ M BOA acted alone as an inhibitor of radish germination in its extractable form. BOA at $10^{-5}$ M was also absorbed by germinated seeds (31 nmol g$^{-1}$ FW), but this amount was not sufficient to cause a delay in germination.

**BOA translocation in radish seedlings**

After germination, BOA was translocated into the organelles of radish seedlings at both concentrations. The presence of any microbial contamination in the experiments did not induce BOA total mineralization. Other allelochemicals such as p-hydroxybenzoic acid have been found to be

### Table 1. Concentration of BOA, and fraction of applied $10^{-3}$ M BOA recovered in different radish organs

<table>
<thead>
<tr>
<th>BOA concentration (nmol g$^{-1}$ FW)</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinated seeds</td>
<td>1556±259</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant organs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots+Hypocotyls</td>
<td>1321±142</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td>1954±493</td>
<td>2700±573*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypocotyls</td>
<td>1604±205*</td>
<td>1723±379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotyledons</td>
<td>1954±100</td>
<td>2276±37</td>
<td>2367±35</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1556±259</td>
<td>3274±238</td>
<td>5834±504</td>
<td>6374±527</td>
</tr>
</tbody>
</table>

### Table 2. Concentration of BOA, and fraction of applied $10^{-5}$ M BOA recovered in different radish organs

<table>
<thead>
<tr>
<th>BOA concentration (nmol g$^{-1}$ FW)</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinated seeds</td>
<td>31±4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant organs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots+Hypocotyls</td>
<td>22±3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td></td>
<td>26±11</td>
<td>21±3</td>
<td>8±0.4</td>
</tr>
<tr>
<td>Hypocotyls</td>
<td>18±0.8*</td>
<td>11±0.8*</td>
<td></td>
<td>7±1</td>
</tr>
<tr>
<td>Cotyledons</td>
<td>41±4*</td>
<td>31±3</td>
<td>23±2</td>
<td>5±0.2</td>
</tr>
<tr>
<td>Total</td>
<td>31±4</td>
<td>63±7</td>
<td>75±13</td>
<td>55±6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BOA applied (%)</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>10±3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td></td>
<td>8±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 3</td>
<td></td>
<td>6±1</td>
<td>8±3</td>
<td></td>
</tr>
<tr>
<td>Stage 4</td>
<td></td>
<td>6±0.4</td>
<td>6±1</td>
<td></td>
</tr>
</tbody>
</table>
mineralized by micro-organisms under similar conditions (Chiapusio and Pellissier, 2001). Micro-organisms did not mineralize BOA into \( \text{CO}_2 \), but may have degraded BOA into other metabolites. For example, Friebe et al. (1996) demonstrated that bacteria converted BOA to 2-amino-\( \text{H} \)-phenoxazin-3-one and 2-acetylamino-\( \text{H} \)-phenoxazin-3-one during oat and rye growth.

When applied at \( 10^{-3} \) M, BOA was mainly concentrated in roots and/or cotyledons. These organs accumulated greater levels of BOA from stage 2 to stage 4. BOA was probably absorbed in the xylem and uptake followed the transpiration flow during the early growth of radish. Being lipophilic, BOA could have crossed through the root endodermic cell membrane (octanol-water partitioning coefficient: \( \log K_{\text{ow}}=1.8 \)) to reach the xylem. BOA could first disturb root cells as those constitute the first seedling barrier. Necrosis was observed in radish roots as well as on other species such as \( L. \text{sativa} \) (Barnes et al., 1987), \( A. \text{fatua} \) (Perez and Ormeno-Nunez, 1991), and \( V. \text{faba} \) (Wieland et al., 1999), respectively, for 1.05 \( \times 10^{-3} \), 0.25 \( \times 10^{-3} \), and 500 \( \times 10^{-6} \) M applied BOA. Cotyledons were clearly the target sink for BOA at the lowest concentration applied (37% at stage 4 compared with the total amount recoverd in plants).

Comparing exogenous \( 10^{-3} \) and \( 10^{-5} \) M BOA accumulation, the results showed a similar percentage recovery for each organ at each stage. It appeared that BOA recovery in seedlings was proportional to the initial concentration applied in the culture medium. This is similar to results observed after the application of certain herbicides (such as amitrole) to \( P. \text{vulgaris} \) roots (Lichner, 1983).

**Conclusion**

The results of this study demonstrated that the application of \( 10^{-3} \) M BOA to radish seeds resulted in a drastic inhibition of radish germination (\(-50\%\)) following penetration and absorption in germinating seeds (10% of the applied \( ^{14} \text{C} \) BOA corresponding to 1556 nmol g \(-1\) FW). Cotyledons of radish were apparently the key target for \( 10^{-3} \) and \( 10^{-5} \) M BOA (respectively, 44 and 37% of the applied \( ^{14} \text{BOA} \) corresponding to 2367 and 23 nmol g \(-1\) FW at stage 4). The use of radioactivity in parallel with HPLC techniques provided complementary findings regarding BOA uptake and metabolism in germinating seeds. However, further experiments need to be conducted to

---

**Table 3. Concentration of BOA, and fraction of applied \( 10^{-3} \) M BOA recovered in different radish organs**

<table>
<thead>
<tr>
<th>Plant organs</th>
<th>Stage 1 (nmol g (-1) FW)</th>
<th>Stage 2 (nmol g (-1) FW)</th>
<th>Stage 3 (nmol g (-1) FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinated seeds</td>
<td>1028±55</td>
<td>1989±2284</td>
<td>422±319</td>
</tr>
<tr>
<td>Roots+Hypocotyls</td>
<td>2899±3512</td>
<td>171±23</td>
<td>372±8</td>
</tr>
<tr>
<td>Roots</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypocotyls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotyledons</td>
<td>1078±13*</td>
<td>740±420*</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1028±55</td>
<td>1989±2284</td>
<td>422±319</td>
</tr>
</tbody>
</table>
identify and determine the phytotoxicity of associated BOA metabolites.

Acknowledgements

The authors would like to thank Dr Christian Gauvrit and his team (INRA, Dijon, France) for welcoming us in their laboratory in order to perform oxidative combustion experiments. They also thank Dr Thomas DeLuca (University of Montana, United States) for his edits, the two anonymous referees and the Associated Editor of the journal for their valuable review of this work.

References


