

Article

Absorption and Elimination of the Allelochemical MBOA by Weeds during Seedling Growth

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Abstract: 6-Methoxy-2-benzoxazolinone (MBOA) is an allelochemical that is found in Poaceae and is generally associated with monocotyledon species. This compound is formed from the glycosylated form of 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (Gly-DIMBOA) by a two-stage degradation process. The MBOA detoxification capacity of two weed species, namely *Echinochloa crus-galli* and *Lolium rigidum*, and a resistant biotype of *Lolium rigidum* (SLR31) was studied both qualitatively and quantitatively. The product of metabolism is similar for both weed species. This finding indicates that these weeds probably metabolize xenobiotics by an identical route, since the product detected was the same in both cases. Kinetic studies on the absorption and translocation to the shoot showed differences in these processes depending on the species. The analysis of treated plants, which were subsequently transplanted to a growth medium without xenobiotic compound, showed that the weeds studied are capable of transmitting the previously absorbed compound to the medium by root exudation. The results show that this process is another defense mechanism of plants facing external threats.

Keywords: 6-Methoxy-2-benzoxazolinone; absorption; weed; elimination; allelochemical



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1. Introduction

Cyclic hydroxamic acids and related benzoxazolinone compounds are an important group of allelochemicals in Gramineous plants. They are specialized metabolites and an important element of the defense mechanism against biotic and abiotic stresses in plant species predominantly belonging to the Poaceae [1,2]. These compounds are found in corn, rye and wheat but not in rice, barley or oats [3]. The maximum recorded level of hydroxamic acids in cultivated wheat is 11 mmol/kg fresh weight [4]. Root exudation of these compounds has also been observed [5]. In the plant, the hydroxamic acids 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) and 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA) are sequestered and stabilized as 2-β-D-glucosides. The more toxic aglycones are produced in response to tissue damage or pathogen attack. After cells are damaged in plants—both in aqueous solution and in soil—cyclic hydroxamic acids decompose rapidly to form the respective ring-contracted compounds 6-methoxy-benzoxazolin-2(3H)-one (MBOA) and benzoxazolin-2(3H)-one (BOA) [6]. The degradation products BOA and MBOA are produced by means of spontaneous degradation in aqueous solution [7], as well as by biological processes [8,9]. A number of interesting studies have been carried out on the biosynthesis, bioactivity and mode of action of these compounds [10–16]. The chemical transformations outlined above depend on chemical and biological conditions and some of the transformation products are more biologically active than the original compounds [17]. The secretion to the soil environment of hydroxamic acids from living plants has been reported from rye [1,18]. However, Wu showed that selected varieties of

wheat were also able to exude hydroxamic acids [19]. Important research on the structure of the hydroxamic acids, the role of the compounds in the plants, and their biosynthesis has been published [20]. It has been claimed that this degradation product is responsible for the phytotoxic activity observed [21]. However, very few studies have concerned the quantification of the interactions between allelochemicals and plants at the molecular level. Regardless of this fundamental lack of understanding, interest in allelopathy in agricultural and natural ecosystems continues to increase. However, there is still some 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 related degradation products should be absorbed and translocated into the target plant before inducing physiological disturbances [22]. Although benzoxazolinone allelochemicals and their degradation products in soil have been extensively studied in terms of their phytotoxic activity against different *Standard Target Species* (STS), little is known about the characteristics of absorption, translocation and detoxification phenomena. In the few studies found, BOA is the derivative that has been most studied and its absorption and translocation in 8-day seedlings of *Raphanus sativus* L. has been demonstrated [21]. Very little has been reported on the last step of such allelochemical interferences, specifically the absorption of allelochemicals from the soil into the target plant. This crucial step in the assessment of phytotoxic interactions between plants in agrosystems must be clarified in order to identify the active chemical species. Regarding the phenomenon of detoxification, BOA has again attracted the greatest interest and the products generated in these biotransformation reactions have been determined. Studies carried out on different plant families, which include plants such as *Avena fatua* L. and *A. sativa*, show that the detoxification reactions involve the introduction of a hydroxyl group at position 6 of BOA, to generate BOA-6-OH, and glycosylation reactions on this group to generate BOA-6-O-glucoside, or reaction directly on the nitrogen atom to generate BOA-N-glucosides [23]. Moreover, in the case of MBOA there are no data on the dynamics of absorption and translocation, and only recently have the products of their detoxification reactions been published. Since numerous weeds are associated naturally with the hydroxamic acid-producing cereals rye and wheat, the question arises as to how these species and species that occur in others communities cope with benzoxazolinone. The study reported here addresses the ability of weeds associated with rye and wheat to metabolize MBOA. The results are compared with those obtained for some other associated species. The aim of this study was to build up a dynamic picture of absorption, translocation and elimination of MBOA allelochemicals for two weed species: *Echinochloa crus-galli* and *Lolium rigidum*. A resistant biotype of *Lolium rigidum* (SLR31) was also included in the study as this has developed the ability to metabolize imidazolinone and sulfonylurea herbicides [24].

2. Materials and Methods

2.1. Allelochemicals

6-Methoxy-2-benzoxazolinone (MBOA) was purchased from Lancaster Synthesis (Haverhill, MA, USA) and was used as received.

2.2. Seeds

Seeds of the target species (*Lolium rigidum* L., *Lolium rigidum* SRL31 and *Echinochloa crus-galli* (L.) P. Beauv.) were purchased from Herbiseed Co. (Twyford, UK). The seeds of the three weeds were initially washed with liquid detergent and this was followed by surface sterilization with aqueous calcium hypochlorite (10% *w/v*) with three drops of Tween 20 (Aldrich, San Luis, MO, USA) for 30 min. The seeds were then washed with sterile distilled water (120 °C, 1 atm, 25 min) × 4.

Regarding the growth medium for the seeds, it was used granulated agar free of microbial inhibitors (Merck, Germany) prepared with basal nutrient solution (Hoaglands No.2, Sigma) at pH 5.5. The medium was prepared by dissolving granulated agar (0.7 g) in basal solution (100 mL). The medium was autoclaved (1 atm, 120 °C, 20 min) and

discharged before solidification into Petri dishes. Pregermination of the seeds was carried out in glass Petri dishes (90 mm Ø, 25 seeds per plate, 100 plates) previously sterilized (120 °C, 1 atm, 25 min) using these medium as support for germination. After addition of the seeds, the Petri dishes were sealed with Parafilm®. Seeds were incubated at 25 °C in a Memmert ICE 700 controlled-environment growth chamber, with a photoperiod of 16 h of light/8 h of darkness.

2.3. Bioassay Design

2.3.1. Absorption Studies for Seedlings

For the growth of seedlings in MBOA inoculation studies, 10 seedlings were transferred (radicle length > 3 mm) to autoclavable, transparent polycarbonate and polypropylene growth vessels, specially marketed for plant culture, with a capacity of 350 mL (Mangenta Vassel GA-7. 6 × 6 × 10 cm), with 10 mL of semisolid agar medium containing MBOA at a concentration of 3 mM. Controls are transplanted into the same growth vessels, but without MBOA in the semisolid medium. At the end of the treatment time (2, 4, 6, 8 and 10 days), the samples and controls were removed from the growth medium. The roots were carefully washed with water and then dried with filter paper. Shoots and roots were extracted with MeOH (1% *v/v* acetic acid) by ultrasound (×3) for subsequent HPLC analysis.

2.3.2. Absorption and Elimination of MBOA by Weed Seedlings

Ten seedlings incubated in a glass Petri dish were cultured in a hydroponic system using the same type of growth solution as used in translocation studies in agar, with 3 mm-diameter glass perlite used as the culture medium in this case. The seedlings were transferred (radicle length > 3 mm) to the same growth vessels (Mangenta Vassel GA-7. 6 × 6 × 10 cm) and were treated with 10 mL of basal growth medium (Sigma-Hoaglands No. 2) at pH 5.7 with MBOA at a concentration of 3 mM. The control samples were cultured in the same solution but without MBOA. The seedlings were submitted to different treatment times (Tt). After the appropriate time had passed, the plants were removed from the containers and the roots were carefully washed with distilled water and sterilized (2 min each). The seedlings were then transplanted to another growth container with the same characteristics with 10 mL of nutrient solution and allowed to grow for another three days (Tst). Thus, in this assay, we have two controls. The first, which is analyzed after the treatment days (Tt); the second, which is transplanted and grown for three more days (Tst).

Both controls and treated plants were processed at the end of Tt for subsequent HPLC analysis. At the end of each growth time (treatment times: 1, 2, 3 and 6 days; time without treatment: 3 days) the weed seedlings were washed three times in ethanol. For the extraction, a fresh sample of shoots and/or roots from 10 seedlings was used. This sample was extracted with 50 mL of solvent by maceration. The resulting extract was placed in an ultrasonic bath for 15 min at 25 °C and then filtered (<11 µm). This procedure was repeated twice more with the solid material residue. The resulting solutions were collected, centrifuged, filtered (<0.22 µm) and concentrated to a volume of 3 mL for analysis by liquid chromatography after pre-purification in solid phase (Sec-Pak® C18, 400 mg MERCK).

2.4. Quantitation by HPLC Analysis

High Pressure Liquid Chromatographic analysis was performed on a Merck HITACHI HPLC system equipped with a LaChrom L-7100 quaternary gradient pump, an L-7455 LaChrom diode array detector and an L-7200 LaChrom autoinjector. Data were collected and processed using an HPLC Merck HITACHI D7000 data system. Instrument conditions for separation were a Lichrospher 100 RP-18 (250 cm, 4.0 mm, 5 µm) reversed-phase column at 25 °C. Mobile phases were water:1% AcOH (A) and methanol:1% AcOH (B) at a flow rate of 1 mL min⁻¹. The injection volume was 50 µL. All analytical procedures were validated by means of an inter-laboratory calibration study [19].

2.5. MBOA in Solutions

To determine the MBOA released by the seedlings after the treatment time (Tt), a sample of the culture solution in transplanted plants was analyzed by HPLC. The culture solution and the root washings were centrifuged, filtered (<0.22 µm), concentrated and dissolved in 1 mL of methanol with 1% acetic acid for analysis by High-Pressure Liquid Chromatography (HPLC) on a Merck HITACHI HPLC equipped with a LaChrom L-7100 quaternary gradient pump, an L-7455 LaChrom diode array detector and an L-7200 LaChrom autoinjector. Data were collected and processed by using an HPLC Merck HITACHI D7000 data system. Instrument conditions for separation were a Lichrospher 100 RP-18 (250 cm, 4.0 mm, 5 µm) reversed-phase column at 25 °C. Mobile phases were water: 1% AcOH (A) and methanol:1% AcOH (B) at a flow rate of 1 mL min. The injection volume was 50 µL. All analytical procedures were validated by means of an inter-laboratory calibration study [25].

2.6. Statistical Analysis

Data for the lengths of root and shoot were statistically analyzed using Welch's test, with significance fixed at 0.01 and 0.05. The fresh weights of seedlings were determined in triplicate, and the mean and standard deviation were calculated. A similar procedure was followed for endogenous (root and shoot content) and exogenous (exudate content) contents of the MBOA. The processing and statistical analysis of the data were carried out using the software Statgraphics Centurion 19 (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results

3.1. Occurrence of MBOA in the Control Treatments

MBOA was not detected in the untreated culture medium, in root organelles or in seedlings during the incubation process. This finding confirmed that MBOA was not naturally synthesized by the weeds under investigation. Therefore, the MBOA quantities detected in target seedlings can be attributed solely to MBOA absorption. Moreover, MBOA was not detected in the analysis of the solution, thus confirming that the weeds do not secrete MBOA through root exudates.

3.2. Absorption of MBOA by Weeds

The chromatographic analysis conducted on the shoots and roots of the weeds (transferred to growth vessels, with 10 mL of semisolid agar medium containing MBOA at a concentration of 3 mM) showed that MBOA was detected in the seedlings. In the case of *E. crus-galli* it was found that MBOA was located in the root and that the concentration increased during seedling development. Upon addition of MBOA, another peak appeared in the chromatographic analysis due to a compound of unknown structure. This fact is especially interesting in the case of *L. rigidum* L., where this peak was detected for both shoots and roots, albeit greater in the latter case (Figure 1).

Comparison of the UV-VIS spectra of MBOA and this new chromatographic signal, denoted as "transformed MBOA" (MBOA-tr), shows very similar maximum and minimum absorptions (Figure 2), which is consistent with a degree of structural similarity between the two compounds. It is feasible that the two compounds have the same basic structure but there are modifications due to detoxification reactions. This unknown signal also appeared in the analysis of shoot and root extracts of the *Lolium* SLR31 resistant biotype. In the species studied there is a similarity in the elimination of MBOA compounds and this may be indicative of a similar elimination mechanism for all three seeds samples.

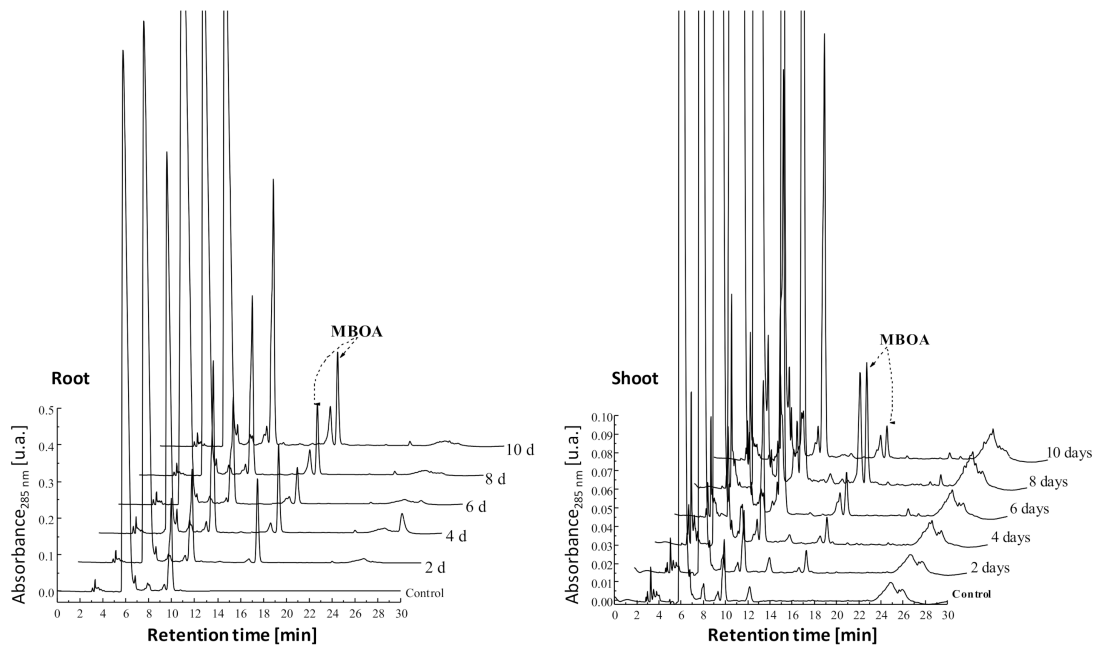


Figure 1. Chromatographic analysis of root and shoot in *Lolium rigidum* L. seedlings at different incubation times with 3 mM 6-Methoxy-2-benzoxazolinone (MBOA).

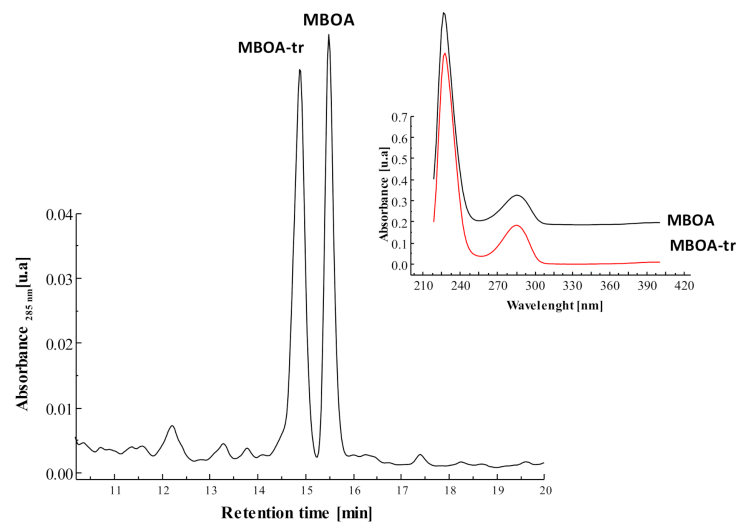


Figure 2. Chromatographic analysis of root extract of *L. rigidum* L. and comparative analysis of the UV-Vis spectra of MBOA and MBOA-tr. Conditions: 8 days of growth, 3 mM MBOA.

3.3. MBOA Detoxification

The dynamics of the accumulation of MBOA-tr were studied in order to determine the differential ability for MBOA transformation. This comparative study was carried out by considering the peak area for the MBOA/MBOA-tr ratio as the unit of measurement and this was normalized to the fresh weight of shoot or root. The relationship between the levels of MBOA-tr and MBOA in the three weed samples is shown in Figure 3.

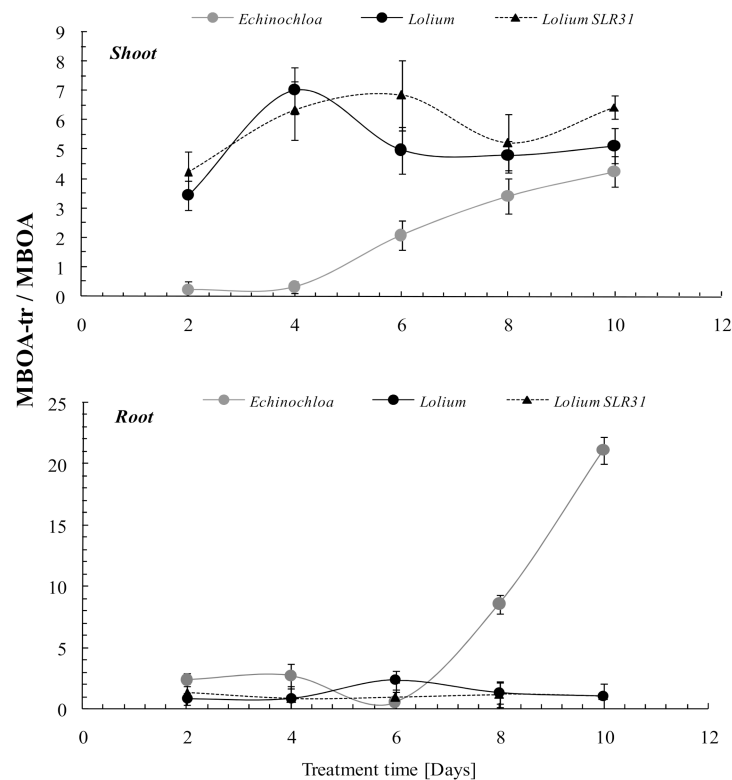


Figure 3. [MBOA-tr]/[MBOA] ratio in root extract of *Echinochloa crus-galli*, *Lolium rigidum* L and *Lolium rigidum* SLR31 at various treatment times. [MBOA] = 3 mM. Error bars represent standard deviation for $n = 3$.

The MBOA-tr and MBOA levels showed similar behavior for both wild and resistant (SLR31) *L. rigidum*, while for *E. crus-galli* an increase in the MBOA-tr/MBOA ratio from the sixth day of growth was observed. This finding verified the increased detoxification of MBOA with the growth of the plant, with this being the species that showed the greatest detoxification capability. This result can be explained by considering that *E. crus-galli* belongs to the Poaceae family, which is the main benzoxazinone-producing families. This plant could therefore have a detoxification and/or inactivation metabolism for this compound and this could be similar to those presented by the plants under investigation here.

3.4. MBOA Translocation

The capacity for the absorption and translocation of MBOA from the culture medium to a shoot is difficult to estimate since there are many variables associated with this process, including the concentrations of the compounds that are eliminated. However, the concentration of the compound in its free state is a good indicator to estimate the translocation ability. This balance was studied for the MBOA content in the shoot/root of the three samples at different treatment times. The results are summarized in Figure 4. An estimated capacity for translocation can be obtained from the levels of allelochemicals in shoot and root. It can be seen that all of the values for this ratio are less than one, which indicates higher concentrations of the compound in the roots than in shoots. In both of the *Lolium* sp biotypes, this value increases with treatment time, with higher values in the wild biotype, while for *E. crus-galli* this ratio remains relatively constant, except for one day of treatment.

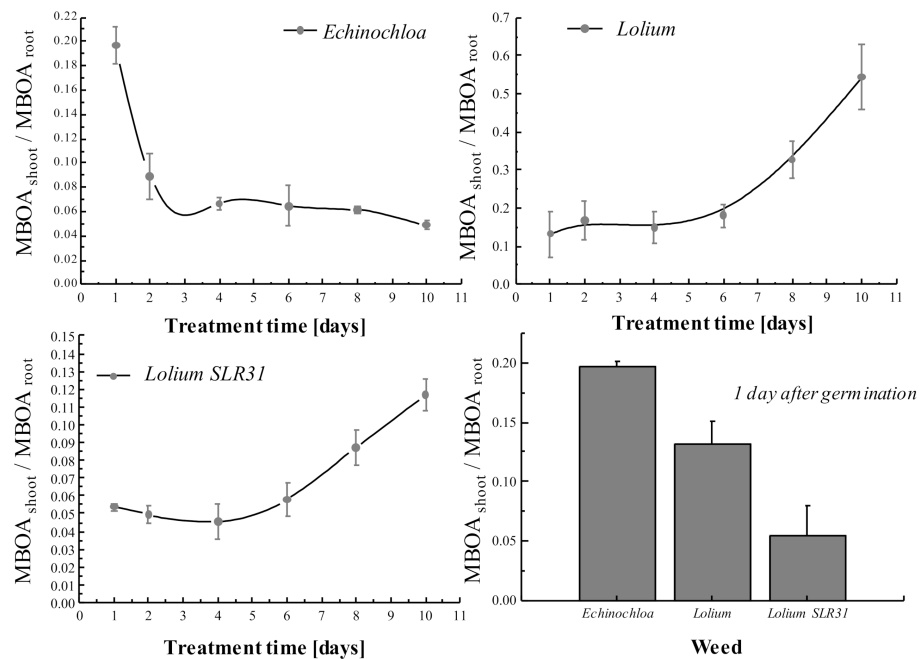


Figure 4. Relationships for the [MBOA]_{shoot}/[MBOA]_{root} ratio for different treatment times with 3 mM MBOA and comparative levels for one day after germination (Bar Graph). Error bars represent standard deviation for $n = 3$.

3.5. Uptake and Elimination of MBOA

An experimental design was carried out with the aim of estimating the capacity for the transformation of MBOA into MBOA-tr in the presence and in the absence of the allelochemical. The design (Figure 5) included the treatment of seedlings for a given time (treatment time, T_t), after which the treatment was suspended by transplanting (or not) the plants into a growth medium without the allelochemicals. The plants were subsequently analyzed after the second period of growth (time without treatment, T_{wt}). In this way, two types of controls and treatments are considered: one that had undergone the transplant procedure (CA and TA) and others that were not disturbed during the trial (CB and TB). Thus, by varying T_t at constant T_{wt} it is possible to determine the phytotoxic activity, resistance of the plant with a suspended treatment, and the amount of MBOA that had been detoxified.

The amount of MBOA metabolized can be calculated using the concentration levels measured during the growth of the plant at times T_t and T_{wt} , according to:

$$[MBOA]_{metabolized} = [MBOA]_{T_t} - [MBOA]_{T_{wt}} \quad (1)$$

where $[MBOA]_{metabolized}$ = Concentration of endogenous MBOA metabolized by the plant during time T_{wt} ; $[MBOA]_{T_t}$ = Concentration of endogenous MBOA in the plant at treatment time T_t ; $[MBOA]_{T_{wt}}$ = Concentration of endogenous MBOA in the plant at time without treatment T_{wt} .

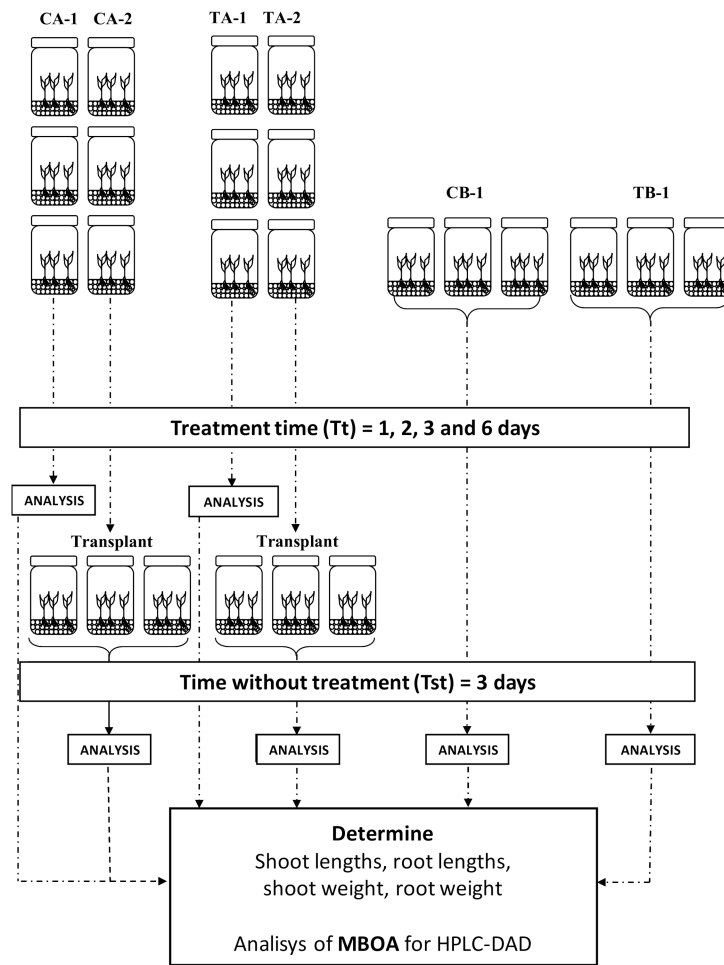


Figure 5. Experimental design for the analysis of the endogenous elimination capacity of the absorbed allelochemical. C = Controls, T = Treatment.

The results are represented in Figure 6. The estimated amount of MBOA metabolized varied depending on the treatment time (Tt). In two species there was an increase in this value up to Tt = 3 days and thereafter the level remained relatively constant for *E. crus-galli* and *L. rigidum* SLR31 but decreased for *L. rigidum* wild biotype after six days of treatment.

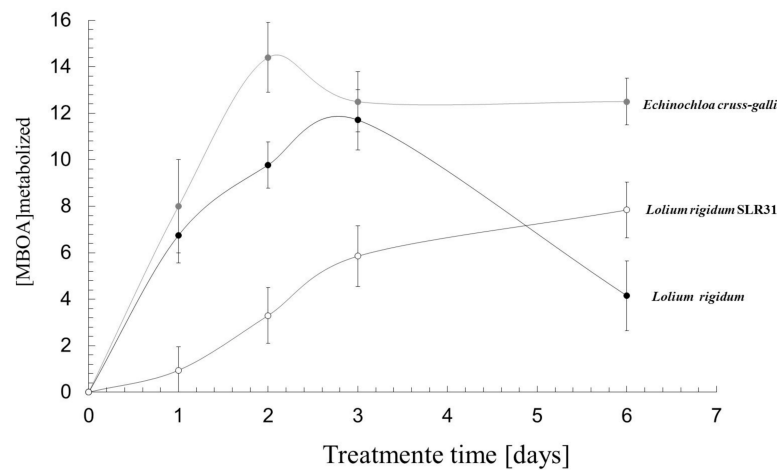


Figure 6. Metabolized MBOA levels calculated by eq.1 for *Echinochloa crus-galli* and *Lolium rigidum* (wild and SLR31), according to time of treatment with a concentration of 3 mM MBOA. The error bars represent standard deviation for $n = 3$.

According to the calculated levels, *E. crus-galli* can provide an enzymatic mechanism that leads to a much higher tolerance for MBOA. For the two biotypes of *Lolium*, it was found that the wild species showed higher initial values of eliminated MBOA but after 6 days of treatment, it had the lowest recorded capacity to metabolize this compound. In this case, there is a strong dependence on the detoxified compound concentration levels with treatment duration, and thus with the endogenous MBOA concentration after transplant and the degree of affectation to the plant. In general, a slower rate of growth and development is characteristic of resistant biotypes [26]. This means that required nutritional levels are lower and therefore the amount absorbed is smaller. The degree of impact is also lower in these cases and the seedlings maintain greater vitality. Thus, the resistant biotype of *Lolium* has a higher MBOA elimination capacity at longer treatment times (Tt = 6 days). This reasoning can also be applied to the case of *E. crus-galli*: this species has the slowest growth rate of all three weeds in question. For example, shoot weight values after a three-day growth period were 144.2 ± 2.3 , 121.3 ± 3.2 and 91.8 ± 2.5 mg ($n = 10$) for *Lolium*, *Lolium* SLR31 and *E. crus-galli*, respectively. This slower rate of growth, together with the characteristic ability of this species to detoxify MBOA, may explain its high detoxification capacity.

4. Discussion

A series of bioassays were conducted aimed at determining the capacity for the absorption and translocation of allelochemicals in weeds. The aim was to determine whether significant differences in this property existed between species and to evaluate the degree of impact that any difference had depending on the duration of treatment. Although studies on the metabolism of BOA in weed species have been published, the detoxification capability of MBOA has not been studied previously. Phytotoxicity studies show a moderate to low activity of MBOA against *E. crus-galli* and *L. rigidum*, with inhibition values on root and shoot of around 30% at a concentration of 1 mM for *Echinochloa crus-galli* [17] and 70% and 60% for shoot and root, respectively, in *L. rigidum* at the same concentration [27]. However, it should be noted that these values decrease markedly with dilution. Levels of MBOA absorbed during root and shoot growth were measured and the behavior of the levels measured was related to the root/shoot ratio in order to determine the capacity for MBOA translocation in these species.

The detoxification of allelochemicals is a mechanism that operates in order to reduce or completely inactivate phytotoxic action. This detoxification ability is primarily concerned with benzoxazolinones and is characteristic of these species towards the phenomenon of self-toxicity. Thus, the dynamics of the transformation of MBOA in *E. crus-galli* and the two biotypes of *Lolium* were studied. In this respect, the active absorption of compounds from the culture medium was estimated as a pre-requisite for the maximum expression of detoxification. Two general characteristics were observed in the detoxification process of MBOA (Figure 3). Firstly, MBOA-tr levels were higher than those of free MBOA in all species and organs. Secondly, the behavior over time was similar for both compounds. This similarity in the levels of the two compounds over time may be due to the capacity of the plant to absorb and transform the compound in question. Clearly, the constant concentration of MBOA, except in the root of *Lolium*, may be a consequence of rapid MBOA absorption from the culture medium. Although significant amounts of MBOA-Tr are present, this rate of absorption of MBOA allows its concentration to remain constant. Therefore, it can be concluded that the levels of MBOA and MBOA-tr are a function of the absorption rate and the rate of the detoxification reaction.

Interesting results were obtained for the levels of both compounds in the roots of the two biotypes of *L. rigidum* L. In the wild biotype, comparable levels of both compounds were observed, while for the resistant biotype (SLR31) there was a marked difference between the levels of the two compounds, with MBOA-tr being present at a much higher content. This finding may be a consequence of the resistance acquired by this plant. However, the resistant biotype contained lower levels of MBOA than the wild biotype. *E.*

crus-galli is also an interesting case because differences in levels of the two compounds were not found at two days of growth. In contrast, the process of MBOA detoxification increased with the growth of the seedling. This result may indicate that, although the mechanism of detoxification is similar in two species, in the case of *E. crus-galli* the reaction has slower kinetics and that the rate increases with seedling development.

In the study of MBOA translocation, there was evidence of behavior usually found in the dynamics of absorption of xenobiotics by plants [28]. There is an increase in concentration to relatively constant levels in the root and a gradually increasing concentration in the shoot, although this behavior may vary depending on the species studied and the compound being absorbed. *E. crus-galli*, which is the species with the greatest potential to detoxify BOA [29], showed the lowest concentration levels of these compounds in both shoot and root (Figure 6). However, the concentration levels translocated to the shoot are interesting, although it is possible that the amounts absorbed and translocated could be correlated. The variations in the concentration of allelochemicals in shoot and root could be due to several factors, but generally, the capacity to absorb nutrients is an intrinsic property of the plant. However, at different treatment times, the phytotoxic effects of the absorbed agent could modify the measured values of free MBOA. Thus, the species of weed that shows the highest degree of affectation by MBOA will have a lower detoxification capability for the compound, thus increasing the concentration of free MBOA. The allelochemical concentrations measured in the root and the shoot represent a time-dependent phytotoxic effect. It is generally considered that the maximum rates of absorption and detoxification are measured during the early stages of development of the plant [28]. Given this fact, the values of the root/shoot concentration ratio for different weeds for a treatment time of one day (Figure 4, bar graph) were compared. According to the values obtained, *E. crus-galli* shows the highest ratio [MBOA] shoot/[MBOA] root and this indicates that this species has a greater capacity for translocation of MBOA from the root to the shoot.

5. Conclusions

The results indicate that the seedlings show signs of recovery if they are transplanted into a growth medium without a test compound. This is an important result because it is a necessary condition for estimating the ability to remove the endogenous levels of the compound by the seedling. The seedlings are viable after treatment, which is a requirement for the study of dynamic detoxification processes. *E. crus-galli* and two biotypes of *L. rigidum* were evaluated for their absorption of DBOA, their translocation to shoot, and their ability to eliminate the absorbed compound. The results show that two species can absorb and translocate to the shoot MBOA occurring in the growth medium. However, there are differences in the ability to translocate and metabolize the absorbed compound depending on the species, as observed previously for the detoxification ability. Of the two species tested, *E. crus-galli* had the highest capacity for the translocation and removal of MBOA, and this difference was dependent on the treatment time. HPLC analysis showed the presence of a product formed by structural changes in the MBOA compound, with the UV spectra suggesting a structural similarity between these compounds. The analysis showed that the seedlings, especially those of *Echinochloa crus-galli*, have the ability to transmit to the environment, through root exudation, the compound previously absorbed. This represents another route for the elimination of xenobiotics in addition to detoxification. Experimental designs are currently being developed with the aim of structurally characterizing the products arising from the transformation of MBOA.

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