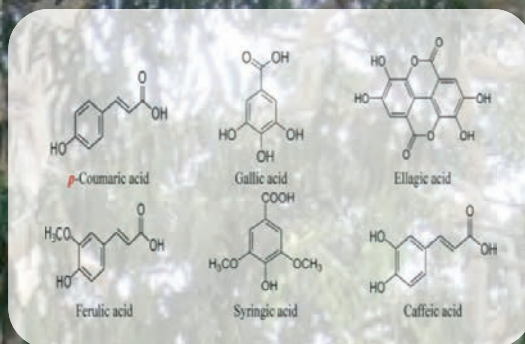
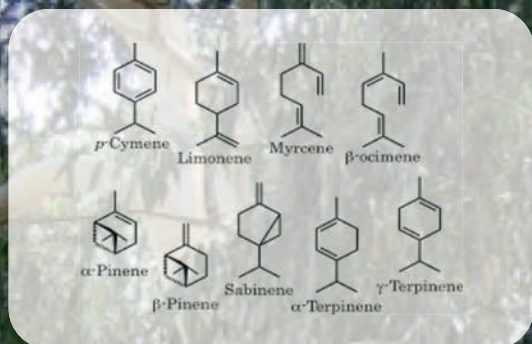


Journal of Allelochemical Interactions

January 2016 (1)

I.S.S.N: 2312-8666

INTERNATIONAL ALLELOPATHY SOCIETY



WEB PAGE: http://uvigo.es/J_Allel_Inter.html
Submission of Manuscripts: j_allel_in@uvigo.es

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I.S.S.N.: 2312-8666

I.S.S.N.: 2312-8682 (electronic version)

Dep. Leg.: VG 307-2014

Nº 2 (1) - 2016

Printed in Vigo. Spain

Cover Photograph:

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Journal of Allelochemical Interactions

VOLUME 2 (1)

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Reducing herbicide rate in combination with allelopathic sunflower residues for weed control in cowpea

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ABSTRACT

A field trial was conducted with the aim of utilizing sunflower residues to reduce the input of herbicide in cowpea field. Sunflower residues at rates of 3 and 6 t ha⁻¹ were incorporated alone or in combination with reduced (50% of the label rate) rate of trifluralin herbicide. Weedy check, weed free and label rate (2.4 L ha⁻¹) of herbicide were also included for comparison. Results indicated that Incorporation of sunflower residues in to field soil at 3 and 6 t ha⁻¹ significantly reduced weed population by 54 and 75% of control and weed dry weight biomass by 72 and 82% over control, respectively. However, combination of lower rate of trifluralin and sunflower residues at 6 t ha⁻¹ significantly reduced weed density and weed population by 79 and 90% over control, respectively while combination of lower rate of herbicide and residue rate at 3 t ha⁻¹ provide 68% higher weed biomass suppression over the control. Maximum seed yield (5.1 t ha⁻¹) and biological yield (19.64 t ha⁻¹) of cowpea was recorded by integration of reduced rate of herbicide with sunflower residues rate at 6 t ha⁻¹ which was greater than the label rate of herbicide. Chemical analysis on field soil amended with sunflower residues revealed the presence of phenolic compounds in soil which were started to release after two weeks of decomposition, reached its peak at 4-week and remained at higher concentration until 8-week then declined until reaching the lowest concentration at 10-week of decomposition. Bioassay of soil amended with sunflower residues at different periods of decomposition exhibited a significant suppression of seedling emergence of the test weeds and the higher suppression was highly correlated with the higher quantity of phenolics content of soil. The importance of this approach in reducing the reliance on synthetic herbicide is briefly discussed.

Keywords: Allelopathy, sunflower residues, reduced herbicide, weed management, cowpea.

Introduction

Weed management in field crops has been a serious problem for many world countries (Zhang, 2001). Potential yield reductions caused by uncontrolled weed growth throughout growing season have been estimated to be 45 to 95%, depending on crop species, weed species, weed densities, and ecological factors (Ampong-Nyarko and De Datta, 1991). Therefore, weed management is a key element of most agricultural systems. Various methods to weed control have been practiced including cultural, mechanical, chemical and biological methods. However, the application of herbicides has been a major method enabling the intensification of agriculture in past decades. Indeed, three million tones of herbicides per year are used in most agricultural systems (Stephenson, 2000). The indiscriminate use of herbicides and large amount of their input in agricultural ecosystems pose serious problems, such as environmental pollution, human health concerns, developing weed resistance, depletion of crop diversity, and reduction of crop productivity

has been raised (Khanh *et al.*, 2005). For these reasons, scientists has been searched to develop an alternative eco-friendly method to minimize rely on synthetic herbicides. Allelopathy is suggested to be a potential safer method for weed control compared to the use of synthetic herbicides (Batish *et al.*, 2001; Weston *et al.*, 2003). However, in most cases, the efficacy of allelopathy was below the efficacy of herbicide (Batish *et al.*, 2001; Fujii, 2001). Several researchers reported that combination of sorghum water extracts combined with lower doses of herbicides is more effective to achieve desired weed control as in case of label dose of herbicides in field crops (Khaliq *et al.*, 2002; Mushtaq *et al.*, 2010; Nawaz *et al.*, 2001). However, to employ this technology, large volumes of sprays are required for field application, and therefore appropriate concentrations for each crop should be determined for large scale field operations. In addition, the process is time consuming and cost effective. Due to these limitations, an alternative practical and feasible approach has been developed where the residues of allelopathic crops have been left to dry under

field conditions and then promptly incorporated into production sites for weed management (Lahmod, 2013). Low herbicide doses were applied along with residue incorporation. By applying this technique, it was found that application of sunflower or sorghum residues in combination with lower rate of herbicides provide weed suppression and crops yield similar to that achieved by the label rate of the tested herbicides (Alsaadawi and Al-Temimi, 2011; Alsaadawi *et al.*, 2013).

Cowpea is an important legume crop in Iraq following sunflower in crop rotation. The field of cowpea is infected by various weed species that may reduce the yield as much as 30-90% compared to weed free conditions (Aggarwal and Ouedraogo, 1989; Poehlman, 1991). The weeds are customarily managed by application of pre emergence trifluralin herbicide (Weston *et al.*, 2013). Information concerning the effect of combination of sunflower residues and lower rate of herbicide has not been explored for weed management in cowpea. The present study was, therefore, conducted to evaluate the possibility of using of allelopathic sunflower residues in combination with reduced (50% of label rate) trifluralin rate for weed control with the objective of getting costly effective and safe weed control practice in cowpea.

Materials and methods

Site description

The proposed study was conducted at Research station of State Board of Agricultural Extension, Ministry of Agriculture, Wasit Province, Iraq. The soil of experimental site was calcareous clay loam. Organic carbon, pH and EC were 0.8%, 7.7 and 7.4 dS m⁻¹, respectively. Average annual rainfall is less than 50 mm and day/night temperatures during the growing season were 30-40/15-30 °C.

Seeds and herbicide procurements

Seeds of sunflower cv. Asagri and cowpea cv. Biader were obtained from Department of Crop Production, College of Agriculture, Baghdad University. Trifluralin 48% EC (a,a,a-Trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine), a product of Shanghai Bosman Industrial Company was purchased from the local market.

Preparation of sunflower residues

Four field plots of 5 × 30 m area were plowed to the depth of 30 cm and received nitrogen as urea (46%N) at 240 kg ha⁻¹ (50% before planting and 50%

at flowering stage) and P as triple superphosphate (46% P₂O₅) at 240 kg ha⁻¹ at planting time. Seeds of sunflower were sown manually in the plots in rows with a distance of 25 between seeds and 50 cm between rows. All plots received recommended irrigation water during the entire course of study. At physiological maturity, heads were removed then each plot was divided in to two plots of 2.5 × 30 m area. Half of the whole sunflower plant parts were cut at approximately 50 cm, while plant parts of the other half were left without cutting in order to achieve two different residue rates. The plant parts were left for drying for several days in their plots. Field measurements revealed that the plant residues added to the soil before and after removal of half of sunflower plants were approximately equal to 3 and 6 t ha⁻¹. The plots were then tilled twice in July 20 by using a disc plough to incorporate sunflower residues in to the soil. Residue free weed control treatment was maintained on an intentionally un-cropped area of the same field.

Bioassay of sunflower residues in combination with reduced trifluralin rate

Each half of the plots of the previous experiment were divided into sub plots measuring 2 × 2.5 m. Fertilizers used were nitrogen as urea (46% N) at 80 kg ha⁻¹ and triple phosphate (46% P₂O₅) at 240 kg ha⁻¹. All phosphorus and half of nitrogen were applied with irrigation at sowing time, while the remaining amount of nitrogen was applied 4 weeks after sowing. Seeds of cowpea were manually sown at the mid of August in all plots in 40 cm spaced crop rows keeping plant to plant distance of 20 cm. All plots received recommended irrigation water during the entire course of study. Treatments were comprised of sunflower residue rates (3 and 6 t ha⁻¹) with and without half rate of trifluralin (1.2 L ha⁻¹). A weedy check without sunflower residue and herbicide application, trifluralin at label rate (2.4 L ha⁻¹) and a weed free were included for comparison. Weeds from weed free plots were manually removed every week by hand pulling throughout the crop's life span. The experiment was laid out in a randomized complete block design with three replications. Trifluralin was applied as pre-plant soil incorporation. Volume of spray (300 L ha⁻¹) was calibrated using water. Trifluralin was applied using a Knapsack hand sprayer fitted with T-Jet nozzle at a pressure of 207 k Pa.

At 60 days after sowing (DAS), Weed density and biomass were recorded from four randomly selected quadrates (50×50 cm) of each sub plot and then averaged. Dry weight of weeds was recorded after drying in an oven at 75°C for 72 h.

Cowpea plants were harvested at physiological maturity. Ten plants from each plot of cowpea were randomly selected. Dry weight biomass at 70°C for 48 h, number of pods per plant, number of seeds per pod, number, weight of 500 seeds and seeds yield/m² were recorded following standard procedure and then averaged.

The experiment was laid out in randomized complete block design (RCBD) with four replications. Data collected were analyzed by Fisher's analysis of variance technique. Least significance difference (LSD) test was applied at 0.05 probability level to compare treatment means (Steel, 1984).

Total phenolics dynamic in the field soil

Soil samples were taken from soil of plots of all treatments except weed free to a depth of 30 cm at 14, 28, 42 and 56 DAS. The soil of each sample was mixed thoroughly and allowed to dry at room temperature for 3 days (Nasyef *et al.*, 2014). Samples of 250 g dry soil were extracted separately in 250 ml of distilled water by shaking for 24 h at 200 rpm using electrical shaker (Ben-Hammouda *et al.*, 1995). Soil suspensions were filtered through Whatman No.2 filter paper under vacuum. Folin-Denis (0.5 ml) and Na₂CO₃ (one ml) were added to one ml of soil water extract and left to stand for 30 minutes. Absorbance was determined at 750 nm by aspectrophotometer (Blum *et al.*, 1991). Total phenolic content was determined by standard curve using different concentrations of ferulic acid.

Residues bioassay at different periods of decomposition

This assay was basically run to test if the phenolics released from decomposed sunflower residues are responsible for the inhibition of weeds in cowpea crop. Soil samples were taken biweekly from plots of all treatments of field trial experiment and

packed in plastic pots of 250 kg capacity. Twenty five seeds of *Portulaca oleracea* and *Echinochloa colonum* (L.) were sown separately in their respective plots and watered with appropriate amount of water. All plots were placed under greenhouse conditions and distributed in randomized complete block design with 3 replications. Ten days after sowing, seedling emergence was counted.

Results

Weed population and dry biomass

Weed flora dominated the experimental site comprised of *Echinochloa colonum* (L.) Link, *Echinochloa crus-galli* (L.) Beauv, *Cynodon dactylon* (L.) Pers, *Physalis angulata* (L.), *Malva parviflora* (L.), *Convolvulus arvensis* L., *Amaranthus retroflexus* L., *Portulaca oleracea* L. and *Beta vulgaris* L.

All treatments significantly averted weed density and dry biomass over control (Table 1). Incorporation of sunflower residues in to field soil at 3 and 6 t ha⁻¹ significantly reduced weed population by 72 and 82% of control and weed dry weight biomass by 54 and 75% over control, respectively. However, this reduction was further increased when the lower rate (50% of recommended rate) of trifluralin herbicide was applied to plots amended with the residues of sunflower. Combination of lower rate of trifluralin and sunflower residues at 6 t ha⁻¹ significantly reduced weed density and weed dry biomass by 92 and 90% over control, respectively while combination of lower rate of herbicide and residue rate at 3 t ha⁻¹ provide 68% higher weed biomass suppression than control. Interestingly, combination of sunflower residues at 3 and 6 t ha⁻¹ and reduced rate of trifluralin provides weed population and dry biomass suppression statistically similar to that achieved by the label rate of herbicide.

Table 1. Effects of reduced rate of trifluralin herbicide and different rates of sunflower residue cv. Asgria on population density and dry weight biomass of weeds in cowpea.

Treatments	Weed population (plants per m ²)	Weed dry biomass (g per m ²)
Weedy check (Control)	159.3	701.6
Residues at 3 t ha ⁻¹	44.7	319.5
Residues at 6 t ha ⁻¹	28.0	174.8
Residues at 3 t ha ⁻¹ +50% of label rate trifluralin	49.7	146.1
Residues at 6 t ha ⁻¹ +50% of label rate trifluralin	13.0	69.4
Label rate of trifluralin (2.4 L ha ⁻¹)	9.0	57.8
LSD ≤ 0.05	30.7	67.9

*Each number is an average of 4 replicates

Seed and biological yields

Both herbicide rates and residue application and their interaction significantly affected seed and biological yields over control (Table 2). Trifluralin at label rate and weed free treatment increased seeds yield by 21 and 15 % and biological yield by 38 and 20% over control. Incorporation of sunflower residues into field soil at 3 and 6 t ha⁻¹ plants increased seeds yield by 12 and 24 % of control and biological yield by 14 and 17 % of control, respectively. However, integration of sunflower residues at 6 t ha⁻¹ and reduced rate of herbicide produced maximum seeds yield (5.1t ha⁻¹) and biological yield (19.64 t ha⁻¹) which was 24and 12% higher than the label rate of herbicide, respectively. Meanwhile, the integration of sunflower residues at 3 t ha⁻¹ scored similar seed yield and more biological biomass as was observed by the label rate application of herbicide.

Yield components

Label rate of herbicide and weed free treatment scored higher yield components parameters over control (Table 3).Incorporation of sunflower residues in to the field soil significantly increased number of seeds per pod, number of pods per plant and 500-seed weight by 48, 68 and 10% over control, respectively. The aforementioned yield components were increased by 76, 25 and 34 % with respect to control, respectively by application of sunflower residues at rate of 6 t ha⁻¹. However, integration of reduced dose of herbicide with sunflower residues at 6 t ha⁻¹ scored superior number of seeds/pod, number of pods/plant and 500-seed weight which was 51, 47 and 18 % higher than sole application of label rate of herbicide.

Table 2. Effects of different rates of sunflower residues cv. Asgria in combination with reduced rate of trifluralin herbicide on seed and biological yields of cowpea.

Treatments*	Seed yield (t/ha)	Biological yield (t/ha)
Weedy check (Control)	3.4	12.64
Residues at 3 t ha ⁻¹	3.8	14.36
Residues at 6 t ha ⁻¹	4.2	14.77
Residues at 3 t ha ⁻¹ +50% of label rate of trifluralin	4.2	18.31
Residues at 6 t ha ⁻¹ +50% of label rate of trifluralin	5. 1	19.64
Label rate of trifluralin (2.4 L ha ⁻¹)	4.1	17.50
weed free	3.9	15.20
LSD ≤ 0.05	0.3	1.75

*Each number is an average of 4 replicates

Table 3. Effects of reduced rate of trifluralin herbicide and different rates of sunflower residue cv. Asgria on yield components of cowpea.

Treatments*	Yield components		
	Number of seeds per pod	Number of pods per plant	Weight of 500 seeds (g)
Weedy check (Control)	2.9	8.0	102.1
Residues at 3 t ha ⁻¹	4.3	13.4	112.6
Residues at 6 t ha ⁻¹	5.1	18.0	136.4
Residues at 3 t ha ⁻¹ +50% of label rate of trifluralin	4.6	22.0	130.5
Residues at 6 t ha ⁻¹ +50% of label rate of trifluralin	6.2	23.8	139.1
Label rate of trifluralin (2.4 L ha ⁻¹)	4.1	16.2	118.3
weed free	4.7	16.6	141.2
LSD ≤ 0.05	1.9	4.7	14.1

*Each number is an average of 4 replicates

Table 4. Total phenolics concentration released during different periods of decomposition of sunflower residues amended in field soil alone or in combination with reduced rate of trifluralin herbicide.

Treatments*	Total phenolics concentration **				
	Decomposition periods (week)				
	2	4	6	8	10
Weedy check (Control)	0.073	0.101	0.183	0.160	0.167
Residues at 3 t ha ⁻¹	0.080	0.167	0.565	0.415	0.142
Residues at 3t ha ⁻¹ +50% of label rate of trifluralin	0.074	0.193	0.652	0.400	0.167
Residues at 6 t ha ⁻¹	0.077	0.365	0.826	0.523	0.258
Residues at 6 t ha ⁻¹ +50% of label rate of trifluralin	0.091	0.380	0.848	0.602	0.299
Label rate of trifluralin (2.4 L ha ⁻¹)	0.088	0.094	0.088	0.143	0.163
LSD ≤ 0.05	NS	0.042	0.070	0.040	0.062

*Each number is an average of 4 replicates

**Total phenolic acid expressed in ferulic acid equivalents per gram soil

Total phenolics

All treatments including control showed no significant differences in total phenolics at the beginning of the experiment (Table 4). The release of total phenolics started after two weeks of decomposition, reached its peak at 4-week and remained at higher concentration until 8- week then declined until reach the lowest concentration at 10-week of decomposition. Soil from plots amended with sunflower residues alone or in combination with reduced rate of herbicide showed higher amount of total phenolics and the concentration increased with

increasing sunflower residue rate. Both weedy check and label rate of herbicide showed lower concentration of total phenolics at all decomposition periods.

Residues bioassay at different periods of decomposition

All treatments significantly suppressed seedling emergence of both weeds during all decomposition periods compared to the weedy check treatment (Table 5). Maximum suppression was recorded during the second and third decomposition periods then decreased but still below the weedy check treatment. However, the reduction was not significantly different

Table 5. Effects of residues of sunflower cv. Asgria decomposed at different periods in field soil on seedling emergence of *Portulaca oleracea* and *Echinochloa colonum*.

Treatments	Decomposition Periods (Weeks)				
	2*	4	6	8	10
	% Emergence				
	<i>Portulaca oleracea</i>				
Weedy check (Control)	70.0	66.7	63.3	80	76.7
Residues at 3 t ha ⁻¹	50.0	36.7	40	46.6	50.0
Residues at 3t ha ⁻¹ +50% of label rate of trifluralin	30.0	16.6	26.7	40	30.0
Residues at 6 t ha ⁻¹	36.7	20.0	26.6	40	36.7
Residues at 6 t ha ⁻¹ +50% of label rate of trifluralin	23.3	11.3	16.7	43.3	23.3
Label rate of trifluralin (2.4 L ha ⁻¹)	16.7	20.0	26.7	50.0	56.7
LSD ≤ 0.05	14.5	16.3	13.6	16.8	15.0
	<i>Echinochloa colonum.</i>				
Weedy check (Control)	93.3	90.0	90	86.7	83.3
Residues at 3 t ha ⁻¹	43.3	20.0	23.3	43.3	43.3
Residues at 3t ha ⁻¹ +50% of label rate of trifluralin	60.0	36.7	40.0	50.0	60.0
Residues at 6 t ha ⁻¹	36.7	20.0	30.3	53.3	36.7
Residues at 6 t ha ⁻¹ +50% of label rate of trifluralin	33.3	20.0	20.0	50.0	33.3
Label rate of trifluralin (2.4 L ha ⁻¹)	43.3	46.7	46.7	63.3	63.3
LSD < 0.05	14.7	16.8	10.9	18.7	14.0

Table 6. Regression and correlation coefficient between total phenolics and per cent of seedling emergence of test weeds grown in the field soil amended with different rates of sunflower residues.

Treatments	Regression (r)	Correlation Coefficient (R ²)
<i>Portulaca oleracea</i>		
Residues at 3 t ha ⁻¹	- 0.917	0.957*
Residues at 3t ha ⁻¹ +50% of label rate of trifluralin	-0.851	0.922*
Residues at 6 t ha ⁻¹	- 0.947	0.973*
Residues at 6 t ha ⁻¹ +50% of label rate of trifluralin	- 0.743	0.862*
<i>Echinochloa colonum</i>		
Residues at 3 t ha ⁻¹	- 0.726	0.852*
Residues at 3t ha ⁻¹ +50% of label rate of trifluralin	- 0.958	0.978*
Residues at 6 t ha ⁻¹	- 0.866	0.930*
Residues at 6 t ha ⁻¹ +50% of label rate of trifluralin	- 0.735	0.856*

* Significant at 0.05 probability level.

among the treatments. Label rate treatment showed maximum inhibition to seedling emergence of both weeds at the first three decomposition periods and declined thereafter.

Results showed that in all treatments in which sunflower residues amended in field soil alone or in combination with the trifluralin herbicide, the amounts of total phenolics released from the decomposed residues is highly correlated with the seedling emergence suppression of both weeds over all decomposition periods (Table 6).

Discussion

The result of present study revealed that sunflower residues inhibited weed growth and the inhibition magnitude achieved under field condition was residue rate dependent. This suggests that sunflower residues contain water-soluble phytotoxins which can imbibed by weed seeds and hamper their germination and subsequent seedling growth causing decline in the density, vigor and stand establishment of the overall weed community (Gallandt and Huggins, 1999). Greater weed inhibition was observed at higher residue incorporation rates. Khanh *et al.* (2005) and Khaliq *et al.* (2011) pointed out that suppression magnitude in allelopathic interactions is directly proportional to the applied dose of an allelopathic product. No attempt was made to separate and identify of the phytotoxins in sunflower residues; however, previous work revealed that sunflower residues contain several phytotoxins of phenolic in nature *viz* chlorogenic acid, isochlorogenic acid, caffeic acid, gallic acid, syringic acid, hydroxy benzoic acid, p- coumaric acid, ferulic acid, vanillic acid, α -naphthol, scopolin, annuionones and catechol (Alsaadawi and Al-Temimi, 2011;Alsaadawi *et al.*, 2011). Most of these compounds have been reported to interfere with several metabolic activities of the cells causing inhibition in growth

and development of plant (Prusty *et al.* 1994). The contribution of allelochemicals other than phenolics in the allelopathic mechanism of sunflower residues is not excluded (Macias *et al.*, 1993a).

Although considerable suppression of weed population and biomass was achieved by incorporation of sunflower residues in to field soil; yet the efficacy of the residues was lower than that achieved of label rate of herbicide. However, when sunflower residues were incorporated in to plots amended with herbicide at reduced (50% of the label) rate a similar or even greater weed suppression than sole application of herbicide was recorded (Table 1). This results confirmed hypothesis proposed by Bhowmik and Inderjit (2003) that lower rate of herbicide applied in combination with allelopathic conditions could have a complementary interaction and may help to reduce input of synthetic herbicides for weed management in field crops. Thus, it seems that a reduced level of herbicide may be feasible for providing satisfactory weed control when it works simultaneously with allelopathic conditions.

The promotion of yield and yield components of cowpea by sunflower residues was primarily due to reduce weed-crop competition for any of the growth factors which might have contributed to higher yields. In addition, the residues could also have a positive bearing on the soil physical and biological characteristics, and add nutrients to soil during microbial degradation. Improvement in crop yield and related components by integrating allelopathy with reduced herbicide dose is in line with the previous findings of several investigators (Alsaadawi *et al.*, 2013; Abdul Khaliq *et al.*, 2012a).

Chemical analysis provided additional evidence of residue mediated weed suppression by establishing quantitative presence of phenolics in sunflower. It is

imperative to mention that the periods indicating higher quantities of total phenolics was significantly correlated (Table 6) with the periods in which higher suppressive activity against seedling emergence of weeds dominated field condition (Tables 4 and 5). Sunflower phenolics are found to be released in higher quantities in field soil during the early stage of residue decomposition and thereby reducing greatly weed seedling emergence, while in the subsequent stages, the same residues may enhance growth and development of the crop due to microbial degradation and improve the physical and nutritional characteristics of soil (Alsaadawi *et al.*, 2013). Organic matter content, water holding capacity and bulk density of soil were greatly improved in plots receiving sunflower residues (un presented data). These results could explain the field observation in which weed emergence and growth was highly suppressed during the first two months and flourished thereafter; however, at this time cowpea plants become highly competitive to weeds.

In conclusion, Application of allelopathic crop residues along with the reduced rate of trifluralin herbicide is an effective approach in minimizing reliance upon synthetic herbicides in sustainable agriculture and conserves the environment. Further research may be carried out to investigate possible complementary interaction of allelopathic crop residues with other herbicides against weeds in field crops to further improve the efficacy of this technique.

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RECEIVED July, 6, 2015

ACCEPTED August 15, 2015



Phytotoxic potential of *Serjania lethalis* A. St.-Hil.

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ABSTRACT

Serjania lethalis A.St.-Hil is a species of liana from the Brazilian savanna (cerrado) that belongs to the family Sapindaceae. In this study, we evaluated the phytotoxic profile of young and mature leaves of *Serjania lethalis* by assaying inhibition of growth of wheat coleoptile (*Triticum aestivum* L.). The bioactive fractions were fractionated by medium pressure chromatography and the subfractions obtained from this process were assayed over again. Phytotoxic subfractions were analyzed by NMR and LC-MS techniques. Subfractions obtained from ethanolic extracts of young leaves exhibited a pronounced phytotoxic effect when compared to others, inhibiting the growth of wheat coleoptile. Spectroscopic analysis led to the identification of polymeric procyanidin, procyanidin oligomers (tannins) and a saponin.

Keywords: Phytotoxicity, Procyanidins, Saponin, Tannins.

Introduction

The family Sapindaceae includes approximately 1000 species in 120 genera widely distributed in the tropics (Reitz, 1980). The genus *Serjania* encompasses about 226 species with the greatest variety in southern Mexico, home to 48 species, followed by Brazil, which has 35 species (Acevedo-Rodríguez, 1990). *Serjania lethalis* A. St-Hil occurs in the Brazilian savanna (Cerrado), dry semi-deciduous forest, upland forest and Atlantic Forest hillside (Wanderley *et al.*, 2009). This liana species climbs over trees and shrubs and has a great potential for dispersal and colonization in the Cerrado and thus it can be considered a weed or harmful species because of its ability to affect native biodiversity and/or the ecosystem balance.

Some secondary metabolites produced by plants are often used in plant defense against pathogens, competitors and predators (Croteau *et al.*, 2000) such as microorganisms, insects or other plants. Due to their potential for inhibiting the growth of other plants, acting at the cellular level (Grisi *et al.*, 2013 a; Grisi *et al.*, 2015) and causing biochemical and physiological changes that adversely affect the growth and development of seedlings (Weir *et al.*, 2004), these compounds can be used as natural herbicides (Grisi

et al., 2014; Grisi *et al.*, 2013 a). In addition, natural products have advantages over synthetic herbicides because they do not damage the environment (Souza-Filho, *et al.*, 2006), do not contain halogen atoms and their half-lives are shorter (Duke *et al.*, 2000; Rimando and Duke *et al.*, 2006).

One way to assess the potential phytotoxic products of plant secondary metabolism is by testing their effect on growing wheat coleoptiles (*Triticum aestivum* L.) (Macías *et al.*, 2010; Arroyo *et al.*, 2015). This method was first proposed by Hancock *et al.* (1964), and nowadays it represents a very useful assay to verify the ability of a product to stimulate coleoptile growth when in contact with phytotoxic agents.

The accumulation of chemical components differs between the maturation stages of leaves; thus, young leaves suffer higher herbivory pressure and produce a greater variety of defensive substances (Lokvam and Kursar, 2005). It is known that many plant species produce secondary metabolites to defend themselves during the juvenile stages of development owing to the absence of mechanical protection (Bryant and Julkunen-Tiitto, 1995). So a higher concentration of allelochemicals is expected in young leaves than in mature leaves (Grisi *et al.*, 2013 b). During the leaf aging process, the nutrients are relocated to develop

organs or tissue reserves (Gan and Amasino, 1997), which may lead to low concentrations of phytotoxic compounds, resulting in a lower rate of inhibition caused by extracts of mature leaves. The aim of this study was to evaluate the phytotoxic activity of fractions and subfractions of young and mature leaves of *Serjania lethalis* and identify the majority compounds present in fractions and active subfractions. Since information about phytochemistry and phytotoxicity of this species remains lacking, studies such as this provide information regarding the phytotoxicity of native Brazilian plants little studied.

Material and Methods

Plant material

Young and mature leaves of *Serjania lethalis* were collected from at least ten different wild plants growing in a region of Brazilian savanna, of Federal University of São Carlos, São Carlos campus, São Paulo, Brazil (21 ° 58 'to 22 ° 00' S and 47 ° 51' to 47 ° 52' W). The morphological criteria used for leaf differentiation included the categorization of young leaves as those presenting a light green color and membranous texture, whereas mature leaves presented a dark green color and papyraceous texture (Grisi *et al.*, 2011). (Fig.1a and 1b, respectively). The plant material was dried in an air circulation oven at 40 °C for 72h, then pulverized in an electric mill and stored in sealed and evacuated plastic bags under cool and dry conditions. A voucher specimen (8340) was deposited in the herbarium of the Botany Department at UFSCar.

Extraction, isolation and identification of bioactive compounds

Alcoholic and aqueous extracts were obtained by steeping 100g of the powder of young and mature dry

leaves of *Serjania lethalis* in 1L of ethanol and distilled water, separately and respectively, during a 24 h period whilst kept in a refrigerator at 4 °C. The aqueous extracts were submitted to vacuum filtration, frozen at -70 °C and lyophilized, while ethanolic extracts were dried in a rotary evaporator under reduced pressure after filtration. Bioguided studies concerning ethanolic and aqueous extracts from young and mature *S. lethalis* leaves were carried out to identify the compounds presents in phytotoxic subfractions.

Ethanolic extracts of the two types of leaves were fractionated by normal phase chromatography (0.063-0.2 mm / 70-230 mesh). Initially 10g of dry extract (Fig. 2) was chromatographed with 450 mL of each eluent (v/v), respectively: hexane-acetone 7:3; hexane-acetone 3:7; acetone; acetone-methanol 7:3; acetone-methanol 3:7; methanol; methanol-water 9:1. Process described above was also performed to aqueous extracts, (Fig. 1). After chromatographic separation, fractions were analyzed by thin layer chromatography (TLC) and pooled according to their chemical similarity.

The fractions that showed phytotoxic potential on coleoptile growth of wheat (**Fye2**, **Fye3**, **Fye5**) were chromatographed in a medium-pressure chromatography system (TELEDYNE ISCO CombiFlash®Rf 200) using a C18 reversed phase column with isocratic elution composed of methanol: water (7:3) and 0.1% formic acid (v/v). Fraction **Fme1** was refractionated and eluted with hexane-ethyl acetate in the proportions: 9:1, 8:2, 7:3, 6:4, 1:1 v/v. (Fig.2). This procedure led to the isolation of four substances that were identified by uni- and bidimensional nuclear magnetic resonance (BRUKER DRX-400 NMR spectrometer operating at 9.4 Tesla) and mass spectrometry (QuattroLC-MICROMASS triple quadrupole mass spectrometer equipped with an *electrospray* ionization source) experiments.

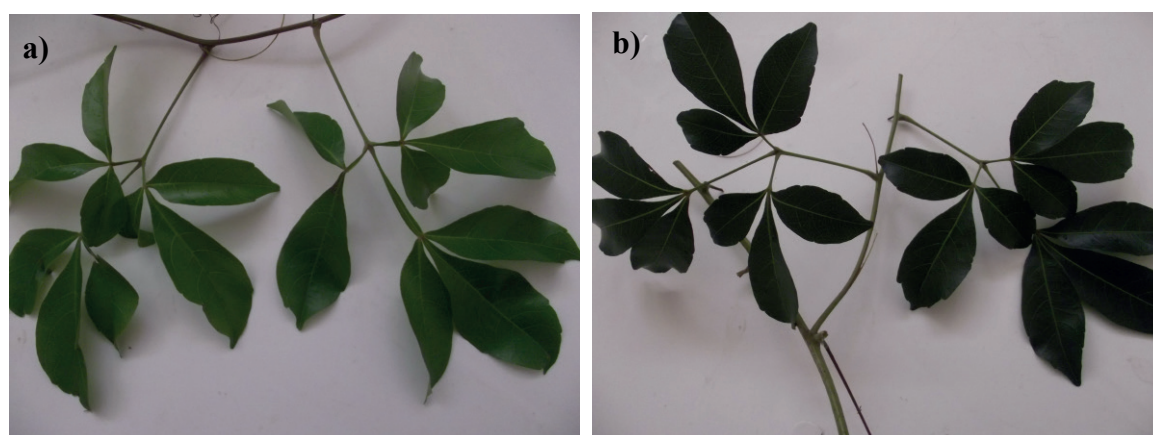


Figure 1. *Serjania lethalis* leaves (a) young leaves (b) mature leaves. (Pereira, V.C. 2015).

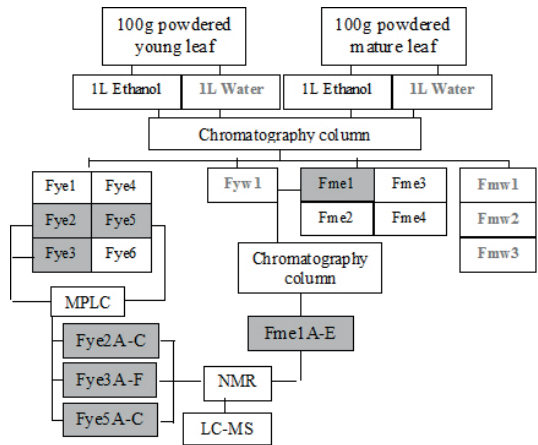


Figure 2. Scheme of the process of extraction and isolation of phytotoxic substances present in young and mature leaves of *Serjania lethalis*. Fractions (F) from young leaves extracted with ethanol (ye), extracted from mature leaves with ethanol (me), extracted from young leaves with water (yw) and mature leaves extracted with water (mw). Nuclear Magnetic Resonance.

Phytotoxic activity on coleoptiles of wheat (Triticum aestivum)

Phytotoxic activity was assessed by the coleoptile bioassay of wheat (*Triticum aestivum* L. variety Pizon). Firstly, wheat caryopses were germinated in distilled water and kept in a growth chamber at 25 °C for 4 days in the absence of light, as described by Hancock *et al.* (1964). Subsequently, coleoptiles were selected and cut with a Van der Weij guillotine (Macías *et al.*, 2010) under green light to avoid stagnation of cell growth (Nitsch and Nitsch., 1956). The apices of the coleoptiles were discarded, while the next 4 mm was cut and placed in test tubes containing a buffer solution (pH 5.6) composed of sucrose (20 g/L), citric acid monohydrate (1.05 g/L) dipotassium hydrogen (2.9 g/L) (Macías *et al.*, 2010). To each tube were added 2 mL of test solutions in DMSO at concentrations of 0.8, 0.4 or 0.2 mg.mL⁻¹ and five coleoptiles. The commercial herbicide GOAL® (Oxyfluorfen, 240 g.i.a L⁻¹) was used as a positive control at the same concentrations as the

fractions and buffer solution with DMSO as a negative control. These tubes were randomly divided and maintained at 25°C in the dark under a constant rotation of 6 rpm, with three replicates per treatment (Macías *et al.*, 2010). After 24 hours, the coleoptiles were removed from the tubes, photographed and measured with the aid of image scanning software ImageJ (free).

Data were evaluated by the percentage of inhibition or stimulation compared to the negative control, where 0 % is the length of the control, while positive values imply stimulation of growth and negative values imply inhibition (Novaes *et al.*, 2013).

Statistical analysis

All results were tested for normality and homogeneity by Shapiro-Wilk and Levene tests, respectively. Normal homogeneous data was analyzed by ANOVA, followed by the Tukey test (p < 0.05), using PAST version 2.5 software (Hammer *et al.*, 2001).

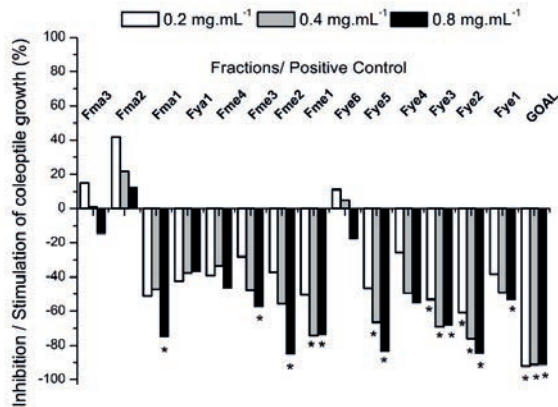


Figure 3. Effect of ethanolic fractions of young leaves (Fye1 to Fye6) and mature leaves (Fme1 to Fme4) and aqueous fractions of young leaves (Fya1) and mature leaves (Fma1 to Fma3) of *Serjania lethalis* on the coleoptile length of wheat (*Triticum aestivum*) (* indicates the fractions that showed significant differences from the negative control).

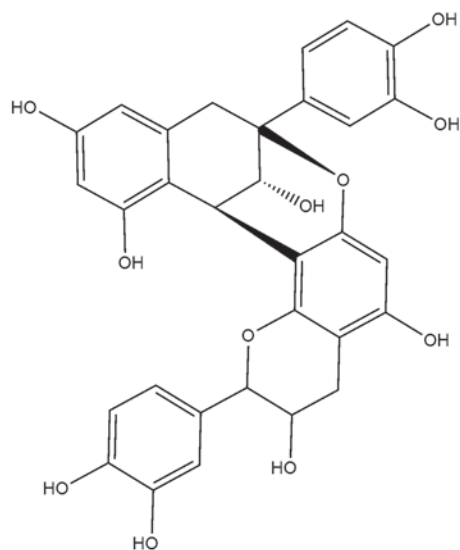


Figure 4. A-type procyanidin dimer present in subfraction Fye2A.

Results and Discussion

In this study, fractions were considered phytotoxic when showing a growth inhibition of wheat coleoptiles in, at least, two of the tested concentrations. Fractions **Fye2**, **Fye3**, **Fye5**, obtained from ethanolic extract of young leaves, and **Fme1**, obtained from ethanolic extract of mature leaves, proved to be bioactive against coleoptile growth assay (Fig. 2). Fraction **Fye2** exhibited an inhibition profile of coleoptiles growth of 84.7% at 0.8 mg.mL⁻¹; 76.1% at 0.4 mg.mL⁻¹ and 60.7% at 0.2 mg.mL⁻¹. The Fraction **Fye3** reduced the growth of coleoptiles at 0.8 (-67.9%), 0.4 (-69.0%) and 0.2 (-53.0%) mg.mL⁻¹. Fraction **Fye5** showed an inhibition of growth at 0.8 (-83.3%) and 0.4 mg.mL⁻¹ (-66.4%). Fraction **Fme1** also reduced the growth of

coleoptiles at 0.8 (-74.2%) and 0.4 mg.mL⁻¹ (-73.6%). The positive control, herbicide GOAL[®] (240 g.i.a L⁻¹), inhibited the growth of coleoptiles more than 90.0% (Fig. 3). Others fractions showed no phytotoxic activity. Most fractions from mature leaves showed no phytotoxicity. Thus, these fractions were subjected to further chromatographic separation to identify the compounds presents.

The ¹H NMR spectrum of subfraction **Fye2A** showed a series of multiplets for protons arranged on an aromatic ring in positions 1, 3 and 5 (δ 7.72, dd; δ 7.56, d; and δ 7.20, d) as observed in the catechin system, as well a set of three singlets around 6.7 ppm. LC-PDA-MS analysis indicated that this subfraction was composed by two epimers (Fig. 4). Their UV absorption spectra showed only a weak absorption at

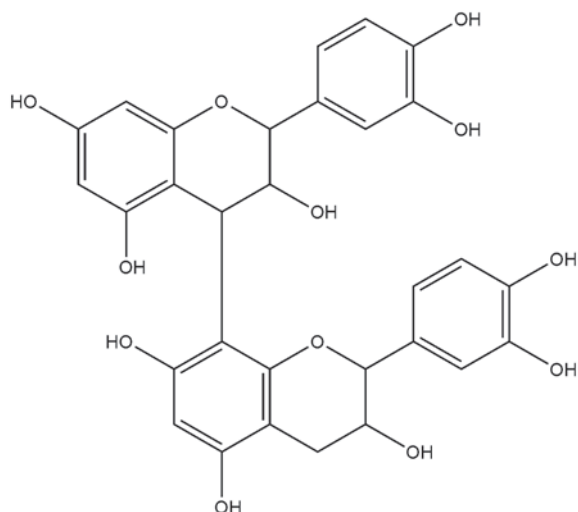


Figure 5. B-type procyanidin dimer present in subfraction Fye2B.

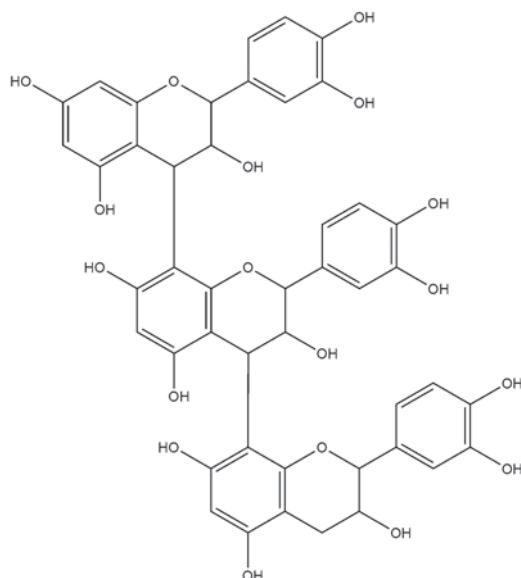


Figure 6. Oligomeric catechins (tannins) in subfraction Fye3A.

279 nm, an absorption characteristic of procyanidin A2. MS spectra of the two epimers are identical and contain a *quasi* molecular ion $[M-H]^-$ at m/z 575. MSMS experiments led to the production of ions with m/z 449 (40%), 423 (48%), 289 (68%) and 285 (100%). According to Poupard and colleagues (2011), product ions at m/z 449 and 423 are markers of procyanidin A2. They are formed by heterocyclic ring fission (loss of D ring) and retro Diels–Alder reactions (loss of B or E-ring), respectively. Thus, comparison of obtained NMR and MS data with literature, it was possible to conclude that both epimers belong to A-type procyanidin (Poupard *et al.*, 2011). This type of procyanidin A2 is formed as a result of one intramolecular addition following the oxidation of procyanidin B2. The mechanism proposed for the formation of procyanidin A2 is, firstly, a tautomeric rearrangement of theo-quinone formed on the upper unit (E-ring) of procyanidin B2 to quinone methide under alkaline conditions. The next step consists of the nucleophilic addition of the 7-hydroxyl group of

the lower unit (A-ring) to the carbon C-2 on the upper unit (F-ring) forming an ether-linkage (Burger *et al.*, 1990; Poupard *et al.*, 2011). This intramolecular linkage reaction can lead to the formation of two stereoisomers, which explains the presence of two epimers observed in LC-MS analysis. Due to their mechanistic formation, *quasi* molecular ions of A2 are also represented by $[(B2 + Ox)-H]^-$ (Poupard *et al.*, 2011).

1H spectrum of **Fye2B** showed characteristic signals of catechoic system in the B ring of the flavonoid unit (δ 7.72, dd; δ 7.56, d and δ 7.2, d) and also signals in the shielded region that are characteristic of the ABX system, typical for C proanthocyanidin rings (δ 2.1, dd; δ 2.6, dd; δ 2.84, dd; δ 3.82, d; δ 4.19, d; δ 4.36, d) (Fig. 5). LC-MS analysis revealed that this subfraction was enriched by the main component (B-type procyanidin dimer) due to the presence of $[M-H]^-$ product ions at m/z 451 and 425, markers of procyanidin B2, and a very small amount of A-type dimer identified in **Fye2A** (procyanidin A2) (Poupard *et al.*, 2011).

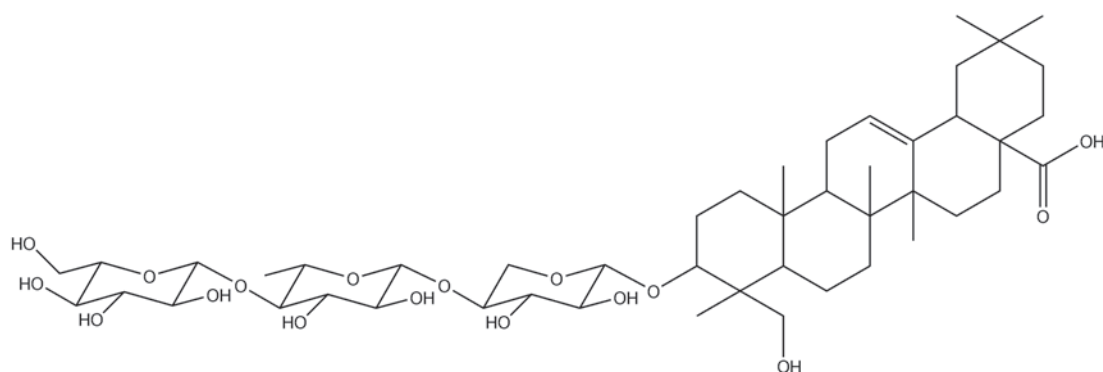


Figure 7. Saponin in fraction Fye5.

Fye3A was observed in LC-MS as a mixture of oligomeric catechins (tannins) (Fig. 6). LC-MS data of **Fye3A** led to the detection and identification of B-type procyanidins in polymeric form by detection of ions at m/z 579, 864 and 1153 as dimer ([procyanidin B - H]⁺ - Sun and Miller, 2003), hexamer and tetramer [(2procyanidinB2 + Ox)-H]⁺ (Poupard *et al.*, 2011) respectively. Studies performed by Hong *et al.* (2006) showed that ions at m/z 864 corresponds to hexamer polymeric form of B-type procyanidin with loss of two hydrogen, generating a pseudomolecular ion with double charge ($z=2$).

Fraction **Fye5** was found to be largely composed majoritarily by saponin. After the purification process by normal phase chromatography, saponin was identified by mass spectrometry and NMR as hederagenin glycoside. The aglicon hederagenin moiety was recognized by HMBC correlations of the CH₂-OH methylenic hydrogens (δ 3.45) with C-4 (δ 45.1), C-24 (δ 28.3) and C-3 (δ 71.9). Also, chemical shifts of unsaturated ¹³C (δ 121.3 and 145.4) are typical of C-12 and C-13 of oleanane skeleton. Moreover, the MSMS spectrum obtained for this saponin produced an abundant ion detected at m/z 471 (negative ion mode), which correspond to deprotonated hederagenin in aglycon after having lost the sugar-chain. Precursor ion experiments showed an abundant precursor ion at m/z 911 in the ESI (-) mass spectrum of this saponin. Fragmentation of ion at m/z 911 through MS/MS experiments produced the following fragments ions at m/z 749 (- gly), 603 (- Rha), 585, and 471 (- Xyl or Ara). Therefore, this substance was found to be a saponin, as showed in Figure 7.

Bioguided studies concerning *S. lethalis* phytotoxic activity led to the identification of some phytotoxic compounds such as saponin, tannin and procyanidin derivatives present in active fractions. Tannins and catechin derivatives found in this study were isolated from ethanolic fractions of young leaves that showed phytotoxicity to the wheat coleoptile, suggesting that these compounds are able to interfere negatively with the growth of plant species. Several studies have pointed out that the (-)-catechin moiety is responsible for the phytotoxic activity due to its ability to produce a reactive oxygen species (ROS) (Bais *et al.*, 2003). On the other hand, (+)-catechin showed antibacterial activity against pathogens and weed roots (Bais *et al.*, 2002). Studies performed by Rice *et al* (1984) listed fourteen categories of phytotoxic chemical compounds, among which can be found phenols and tannins. Saponins are also known as phytotoxic compounds (Faizal and Geelen, 2013). A possible mode of action of saponin is water absorption in the lipid bilayer (Scognamiglio *et al.*, 2012). This work supports this idea, since this class of compounds was present in a fraction phytotoxic to coleoptile growth of wheat.

This study showed that the young leaves of *Serjania lethalis* probably produce more active secondary metabolites than the mature leaves, according to the activity found in the extracts. Four compounds were isolated and identified from young leaves. The quantity of secondary metabolites produced can vary with the age, development stage and organ of the plant (Batish *et al.*, 2007 ; Gobbo-Neto and Lopes, 2007). In a study of *Paulownia tomentosa* (Thunb.) Steud. leaves, it was found that the flavonoid-containing trichome density of young leaves is higher than that of mature leaves (Kobayashi *et al.*, 2008). Others have reported a greater inhibitory activity in young leaves than in mature leaves in the species *Pouteria torta* (Nascimento *et al.*, 2007) and *Sapindus saponaria* (Grisi *et al.*, 2012).

In view of the lack of previous studies on this species, the compounds isolated and identified in this study represent a large contribution to chemotaxonomy and phytochemistry of the Sapindaceae family. These fractions could be tested as natural herbicides, owing to their phytotoxic activity, and other bioactivities can be investigated. Furthermore, the identification of phytotoxic compounds produced by this invasive species may contribute to studies on the strategies of invasion of *S. lethalis* in the Brazilian savanna and other natural environments.

Conclusions

Results obtained showed that the maturation of *S. lethalis* leaves interferes with the phytotoxic activity and suggest that the search for new bioactive substances involved in plant growth regulating properties are more promising if carried out on young tissues, in which the production of secondary metabolites is more intense. Bioactive secondary metabolites were identified in subfractions of ethanol extracts of young leaves of *Serjania lethalis*. Thus, we point towards this species as a promising source of natural herbicides.

Acknowledgments

This work was supported by CNPq (Conselho Nacional de Pesquisa), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) by the scholarship of Enzo Monte Canedo 2011/09580-4.

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RECEIVED October 14, 2015
ACCEPTED November 13, 2015



Zea mays Glucosyltransferase BX9- An Essential Enzyme for Benzoxazolinone Detoxification

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ABSTRACT

Purification of the benzoxazolinone (BOA)-glucosylating activity from maize roots by chromatographic methods and LC/MS/MS peptide analysis after 2d electrophoresis identified glucosyltransferase BX9 as the enzyme responsible for BOA-6-O-glucosylation. Immunoprecipitation and experiments with transgenic *Arabidopsis thaliana* lines corroborate the function of BX9 in detoxification of the intermediate BOA-6-OH. The BOA-6-O-glucosylation pathway is a “ready to go” mechanism, important to detoxify benzoxazolinone (BOA), before the detoxification pathway leading to glucoside carbamate synthesis is established. Oxidative modifications of BX9 after exposure to BOA seem to trigger the enzyme for acceptance of BOA-6-OH as a substrate. Consequently, reducing sulphur compounds inactivated BOA-6-OH glucosylation. BX9 has a higher affinity to BOA-6-OH than to DIMBOA when assayed at pH 8.0. For the substrate BOA-6-OH, K_m -values were determined to be 120.7 μ M, the K_m value for UDPG were 360 μ M. The intermediate BOA-6-OH is a linker between BOA and MBOA detoxification, when MBOA is demethylated. Glucoside carbamate, a major BOA detoxification product later in the process, is a potent inhibitor of BOA-6-O-glucosylation (K_i 84 μ M). BOA detoxification pathways are competitive to the formation of benzoxazolinone derived phenoxazinone, a highly toxic compound for many organisms including plants. Benzoxazolinone detoxification is therefore of great importance for the survival of plant seedlings.

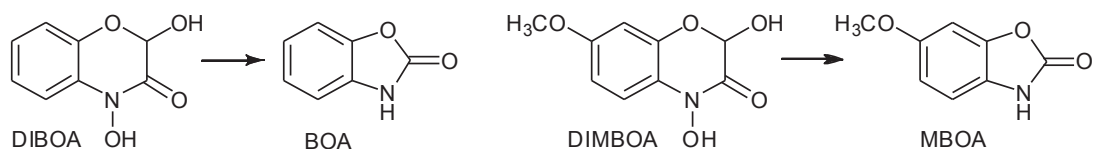
Keywords: Allelopathy, benzoxazolinone detoxification, BOA-6-O-glucoside, glucosyltransferase BX9, *Zea mays*.

Introduction

Benzoxazinone- β -D-glucosides are characteristic secondary compounds of several Poaceae including maize, wheat, and rye and some dicotyledonous species (Dick *et al.* 2012; Sicker and Schulz 2002). The aglycons possess antimicrobial, insecticidal and herbicidal activity and have therefore received attention. The biosynthesis of the compounds has been thoroughly investigated in maize (Dick *et al.* 2012). Following the synthesis of benzoxazinone DIBOA, the molecule is subsequently glucosylated at the 2-position by the glucosyltransferases BX8 and BX9. Glucosylated DIBOA is the precursor of DIMBOA-glucoside, the major benzoxazinone in maize (Frey *et al.* 1997; Glawischnig *et al.* 1999; von Rad *et al.* 2001).

The allelopathic potential of benzoxazinoids was demonstrated in many studies with rye and meanwhile, it is an important tool to suppress

certain weeds in environmentally friendly farming systems (Schulz *et al.* 2013). The allelopathic potential of maize is less studied. In solution, the aglycons DIBOA and DIMBOA are unstable. They undergo heterocyclic ring contraction and the release of formic acid lead to the benzoxazolinones BOA or MBOA (Niemeyer 2009). Benzoxazinoids are exuded by young roots or are released into the environment by plant rotting. The compounds are converted into further degradation products by microorganisms, the phenoxazinones, which are present in the soil for several months. The known degradation sequence starts with microbial opening of the heterocycle, decarboxylation and release of 2-aminophenol. Phytotoxic 2-aminophenoxazinones, bio-inactive acetamides and malonamic acids are subsequently occurring products (Macias *et al.* 2005). Specialized microorganisms, such as certain fungi are able to eliminate phenoxazinones by H_2O_2 release and Fenton reactions (Schulz *et al.* 2012).



Known Plant Detoxification Products of BOA and MBOA

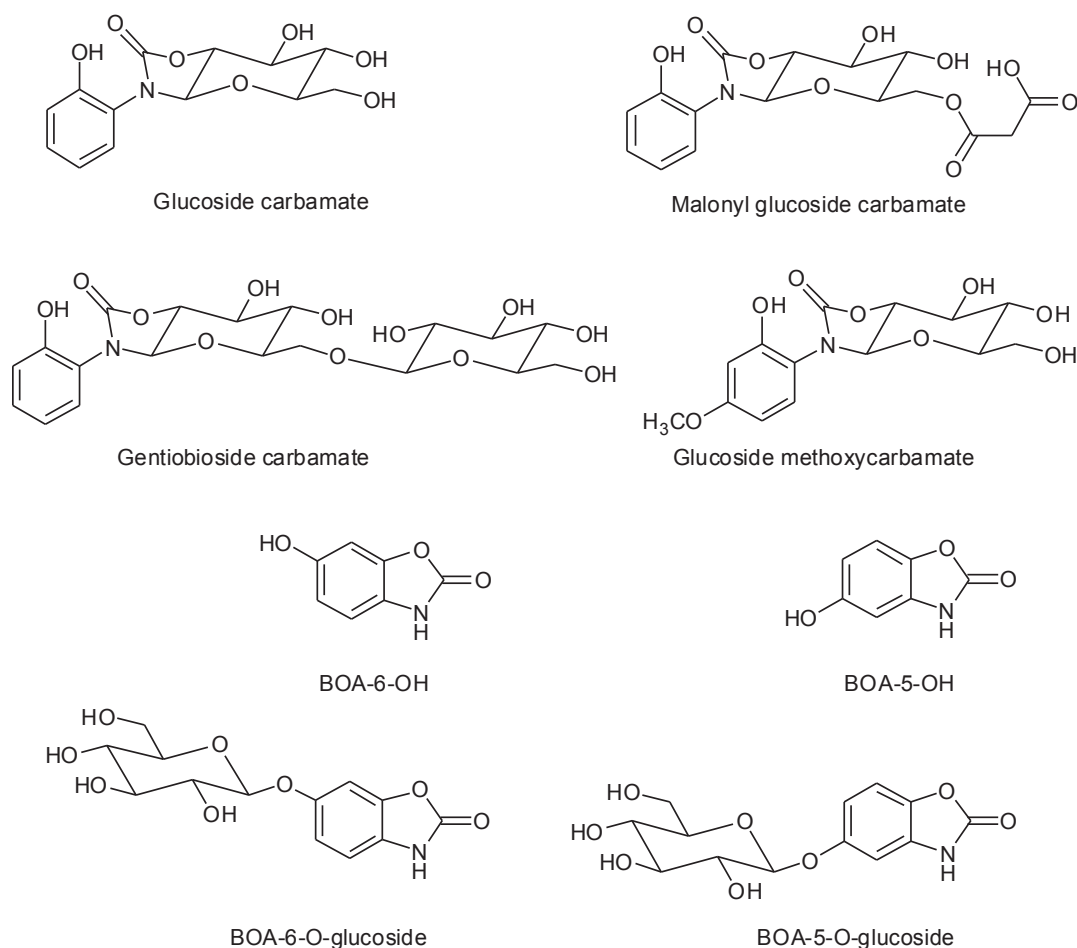


Figure 1. Benzoxazolinone detoxification products found in higher plants.

Although they are less toxic than the precursor molecules, benzoxazolinones can be deleterious to other plants, when they are absorbed in sufficient amounts. The generation of more toxic hydroxylated BOA molecules should be pivotal (Schulz *et al.* 2013) as is the generation of phenoxazinones by root associated microorganisms. ROS production (Reactive Oxygen Species) and oxidative stress are key events in the mode of action. Membrane damage, lipid peroxidation, protein oxidation, enzyme inactivation and other fatal events take place (Friebe *et al.*, 1997; Sanchez-Moreiras *et al.*, 2005; 2011; Burgos *et al.*, 2004; Singh *et al.*, 2005;

Batish *et al.*, 2006; Hussain *et al.*, 2008; Kato-Noguchi *et al.*, 2010; Hussain and Reigosa, 2011; Schulz *et al.*, 2012a, b, 2013). Senescence and death occurs, unless plants start rescue and defense programs (Baerson *et al.* 2005). When exposed to BOA, maize roots react immediately with an increase of malondialdehyde (MDA), a marker for oxidative stress and lipid peroxidation. The return to MDA control levels after 4-6 hours is a clue for the successful activation of defense mechanisms in the maize and the switch to highly effective detoxification by glucoside carbamate synthesis (Schulz *et al.* 2012a; Schulz *et al.* in press).

When confronted with BOA, plants react in a species- and dose-dependent manner. Generally, members of the Poaceae were found to be less sensitive to the compounds than most of the dicotyledonous species. One important reason for the different sensitivity is the better developed ability of grasses to reduce the toxicity of benzoxazolinone(s), in comparison to many dicot species. Roots are the predominant and primary organs for detoxification processes. Almost all investigated weed and crop seedlings detoxify benzoxazolinone (BOA) via 6- or 5- hydroxylation and subsequent O-glycosylation (Hofmann *et al.* 2006). In most cereals and wild grasses, glucoside carbamate is the major product (Fig. 1), (Wieland *et al.* 1998; Sicker *et al.* 2004; Schulz and Wieland 1999; Schulz *et al.* 2006). The highly complex, compartment spanning and redox-regulated glucoside carbamate synthesis was elucidated in maize (Schulz *et al.*, in press). However, the accumulation of glucoside carbamate starts relatively late, about 6h after exposure to BOA, whereas during the first hours BOA-6-O-glucoside is synthesized. The detoxification by BOA-6-O-glucoside formation in maize is therefore regarded as a constitutive mechanism, valuable to bridge over the time until glucoside carbamate synthesis is possible. The high importance of benzoxazinoid detoxification prior to the possible formation of phenoxazinones in larger amounts was underlined by the work of Venturelli *et al.* (2015), who described the inhibition of histone deacetylases by APO/AMPO and enhancement of the effect by locus-specific alterations of histone acetylation and gene expression. These actions are discussed as major reasons for phenoxazinone-caused plant growth reduction.

In this study, we focus on the identification of a maize glucosyltransferase and its biochemical properties enabling the enzyme for the BOA-6-OH glucosylation step during the early process of BOA detoxification until the switch to glucoside carbamate, requiring a massive shifting of enzymes into the extraplasmic space, is possible.

Material and Methods

Plant Material, incubation conditions and analyses of extracts

Seven-days-old, hydroponically grown maize seedlings (cultivars *Zea mays* L. "Nicco", "Pedro" and "Cassila" with similar BOA detoxification behavior, all gifts from KWS, Germany) were incubated for 24 hours in 25mM MES-KOH pH 5.8; 0.5mM KCl; 0.25mM CaSO₄; 0.1% (w/v) Na-ascorbate, containing 500μM BOA (Fluka) or in tap water containing 0.5 mM BOA. Controls contained no benzoxazolines. Maize seedlings were also incubated with 2 mM MBOA and with 0.5 mM MBOA without and in presence of

1 and 5 mM methionine. Growth conditions were as described in Sicker *et al.* (2001).

Arabidopsis thaliana Col-0 and the transgenes 35S::Bx8, 35S::Bx9 and 35S::Co-Bx8 were grown from seeds in a phytotron at 20°C (with 150 μE light intensity, a 16 h light period and 55% relative humidity) for four weeks in pots with a Vermiculite/soil mixture (1:3). Plants were harvested and soil particles were removed from the root system by repeated water baths. 12 plants were incubated with 250 ml incubation medium containing 0, 0.5, 1.0 and 2.0 mM BOA for 24 h. Plants were washed and separated in shoots and roots, which were either used for protein extraction or for methanolic extract preparation. Methanolic extracts of maize and *Arabidopsis* roots and shoots were prepared as described in Schulz *et al.* (2012b) with 50% methanol and analyzed by HPLC.

Maize UGT Purification

After incubation, the maize roots were harvested and powdered on ice with quartz in ice-cold extraction buffer (w/v:1/5) containing 100mM MES-KOH pH 6.0; 10% (v/v) glycerol; 2% (w/v) Na-ascorbate; 1mM DTT, 100μM Pefabloc / g roots and 0.1 g Polyclar / g roots). The homogenate was filtrated through Miracloth (Calbiochem) and centrifuged (20,000g, 20min, 4°C). The crude extract was precipitated with 30-60% ammonium sulfate and subsequently centrifuged (40.000 g, 1h, and 4°C). The pellet was resuspended in extraction buffer and applied to a Sephadex G-100 (Sigma) column, pre-equilibrated with extraction buffer. The elution was performed with the same buffer (flow rate 0.3 mL min⁻¹). Fractions with enzyme activity were pooled and concentrated by ultrafiltration (PBGC membrane, Millipore). Protein concentrations were determined with the Bradford method. The concentrate was further purified by FPLC (Biosys 2000, Beckman Coulter). For each run, 1mL of the concentrate was loaded onto a QHyperD 10 column (4.6x100mm, Beckman ProScale; flow rate 1mL min⁻¹) pre-equilibrated with buffer B (100mM MES-KOH pH 6.0; 10% (v/v) glycerol; 1mM DTT). After washing with buffer B (1 ml / min), the enzyme was eluted with a linear gradient of 0 – 0.5 M KCl in buffer B. Active fractions, eluting at 0.23 M KCl, were pooled and concentrated by ultra-filtration. The concentrate, supplemented with 5mM UDPG, was subjected to affinity chromatography on a HiTrap Blue column (0.7 x 2.5cm, Pharmacia) pre-equilibrated with buffer B. After washing with 5mL buffer B (0.5mL per run; flow rate 0.5mL min⁻¹), the enzyme was eluted with a linear gradient of 0 – 500 μM BOA-6-OH in 100 mM TRIS pH 8.4. The enzyme elutes at

pH 7.8 and a BOA-6-OH concentration of 400 - 420 μ M. Alternatively, the enzyme was eluted with 40 mM UDPG in 50 mM HEPES-KOH, pH 7.5 containing 5 mM DTT. Pre-runs with 5 mM ascorbate, malate or pyruvate were performed to reduce the amount of proteins co-purified by the previous steps. After elution with BOA-6-OH active fractions were pooled, concentrated and either used for 2d electrophoresis. For further enzyme characterizations elution with UDPG was preferred (see below).

UGT Assays

The standard reaction mixture contained 25 μ L 200 mM HEPES for adjustment to pH 8, 2.5 mM UDPG in extraction buffer, 200 μ M BOA-6-OH in ethanol and 68 μ L enzyme solution in a total volume of 100 μ L. The mixture was incubated at 30°C for 10 min, the reaction was stopped by boiling and centrifuged (10000g, 15min, 4°C). Other substrates (sugar acceptors) were 200 μ M DIMBOA, 6-hydroxyindolon, BOA-5-OH, DIBOA or 2,4 D. *O*-glucosylation was also assayed at pH 6.5 with all sugar acceptor substrates. Adjustment of the pH was done by addition of 5 μ L 200mM HEPES and 20 μ L extraction buffer. For K_m determinations the UDPG concentration ranged from 0.5 to 15 mM, the BOA-6-OH concentration from 20 to 500 μ M. Michaelis-Menten constants (K_m) were determined from Lineweaver-Burk plots. For K_i (glucoside carbamate) determination 0, 10, 25 and 50 μ M glucoside carbamate were used with the standard reaction mixture. The K_i value was determined from Hanes plots.

Assays were analyzed by HPLC (Schulz *et al.*, 2012), concentrations of produced BOA-6-*O*-glucoside were calculated on the basis of external standards (Wieland *et al.*, 1998; Sicker *et al.*, 2001). Concentrations of produced BOA-6-*O*-glucoside or -*N*-glucoside / carbamate were calculated on the basis of synthetic BOA-6-OH and BOA-*N*-glucoside / glucoside carbamate used as external standards (Wieland *et al.* 1998; Sicker *et al.* 2001). BOA-5-OH was synthesized as previously described (Hofmann *et al.* 2006), 6-hydroxyindolone was synthesized by Aurora, Wien. 2,4 D was from Sigma.

UGT activity determination in transgenic *Arabidopsis* lines

Arabidopsis roots and shoots were homogenized by mortaring on ice with sea sand, PVPP (20% of fresh weight), 5 μ L protease inhibitor cocktail (Sigma)/ 100 mg FW, and the extraction buffer used for maize UGT purification (1:3, FW:buffer volume). The homogenate was centrifuged for 20 min at 4°C and 20.000g. The supernatant was immediately used for UGT activity

determinations, performed as described with maize protein. Assays contained 50 μ g protein in average, according to the Bradford method. Analyses of the assays were done by HPLC.

Two-dimensional Electrophoresis

The pooled active fractions from the HiTrap Blue column were concentrated by ultra-filtration. The concentrated proteins were precipitated with ice-cold methanol (3:1). After centrifugation, the pellet was dissolved in 9.5M urea; 4% Chaps; 1.5% DTT; 2% Pharmalyte 3-10 for IEF (Pharmacia); 0.8% *n*-hexyl- β -D-glucopyranoside; 0.004% *n*-decyl- β -D-maltopyranoside and 0.002% SDS.

For isoelectric focusing (IEF) a Bio-Rad Protean IEF Cell and Ready Strip IPG strips pH 4-7 (Bio-Rad) were used. Focusing with 50 μ Am / gel was completed after 20.000Vh. The second dimension was performed on a 15% gel. Coomassie stained protein spots were excised and subjected to LC/MS/MS analysis.

Western blots were performed as described by von Rad *et al.* (2001) using polyclonal antibodies raised against recombinant BX9. Alkaline phosphatase coupled anti-rabbit antibodies (Sigma) were used for visualization.

Identification of the UGT by LC/MS/MS

Gel spots were repeatedly dehydrated and rehydrated prior to digestion by soaking them in dehydration (2:1 acetonitrile/25mM ammonium bicarbonate) and rehydration solutions (25mM ammonium bicarbonate) in turn. Spots were dehydrated four times. Following the fourth dehydration, the supernatant was removed and spots were dried in a vacuum centrifuge.

Spots were then resuspended in ~10ng/microliter solution of trypsin in 25mM ammonium bicarbonate with ~5mM calcium chloride. Small amounts of trypsin solution were added as the spots rehydrated. Once rehydrated, the spots were covered with a minimal volume of ammonium bicarbonate solution and incubated 4.5 hours at 37°C, and generated peptides were extracted by adding roughly twice the digest buffer volume of acetonitrile with 1%-2% formic acid. Samples were vortexed and then the peptide-containing supernatant was transferred to new tubes, which underwent vacuum centrifugation to remove the organic solvent.

MS - Methods

LC MS/MS data were collected using a Q-ToF II tandem mass spectrometer coupled to a CapLC

system fitted with a 75µm NanoEase Atlantis dC18 analytical column and a NanoEase Symmetry300 C18 Trap column in a forward flushing configuration (Waters Corporation). All chromatographic solutions contained 0.1% formic acid. Peptide mixtures were separated with a gradient of 5-50% acetonitrile in water (Aldrich HPLC grade) over a 15 minute period, followed by column cleaning and re-equilibration. Data were collected in survey mode using argon as a collision gas at a pressure of $4\text{--}5 \times 10^{-5}$ bar. Capillary voltage was 1.6 kV and the cone voltage was 45 V. The collision voltage was kept at 10V during MS-ToF mode collection.

Multiply-charged precursor ions of sufficient intensity were fragmented in MSMS mode with collision voltage between 20 and 70 volts, depending on precursor charge and mass. Multiple runs were performed with each sample. Peak files were generated by Masslynx3.5 (Waters Corp.) and de novo sequencing was performed both with LutfiskXP version 1.0 (Richard S. Johnson) and ProteinLynx/Global Server2.1 (Waters Corp). Resulting de novo sequences were visually checked and the highest scoring sequences were used to form queries for MSBLAST searches (Shevchenko *et al.*, 2001).

Modulation of glucose transferase activities

The active protein fraction from anion exchange chromatography (2ml) was incubated with 25 mM glutathione disulfide at 30°C (Barrett *et al.*, 1999). Aliquots (68µl) of the mixture were taken after 20, 40, 60, 80, and 90 min and assayed for glucosyltransferase activity using DIMBOA or BOA-6-OH as acceptor molecule. In a parallel analysis the incubation of

the protein with 25 mM GSSG for 90 min was followed by buffer exchange to extraction buffer without DTT by centrifugation (Centricon tubes). For reconstitution, the resulting solution was incubated in presence of 20 mM DTT at 30°C. Aliquots (68 µl) were taken every 10 min over a period of 120 min and assayed for DIMBOA or BOA-6-OH glucosylation. In another series of experiments, the NO-donor GSNO (S-nitrosoglutathione) was used (Lindermayer *et al.* 2005). The protein was preincubated for 15 min at 20°C before incubation with 250 µM GSNO and analysis. In a third series, the protein was assayed in presence of 200 µM H_2O_2 , and 10 or 20 mM DTT.

Immunoprecipitation of BX9 from maize roots

Maize root tips (3 cm, 2.8 g FW) were harvested and proteins extracted as described above. The crude extracts were used for immunoprecipitation with BX9 antibodies. Immunoprecipitation was done with the Pierce Direct IP Kit (Fisher Scientific) according to the instructions of the manufacturer. Elution of the bound protein was with Pierce elution buffer, pH 2.8 in a total volume of 100 µl. Crude protein extracts, flow throughs and eluates were assayed for UGT activity as described and aliquots of all fractions were subjected to western analysis after SDS-PAGE.

Results

Protein crude extracts from maize roots of control plants contain a constitutive glucosyltransferase activity able to glucosylate BOA-6-OH, but BOA-N-glucosylation for glucoside carbamate production could not be reproducibly measured in extracts prepared for BOA-OH glucosyltransferase measure-

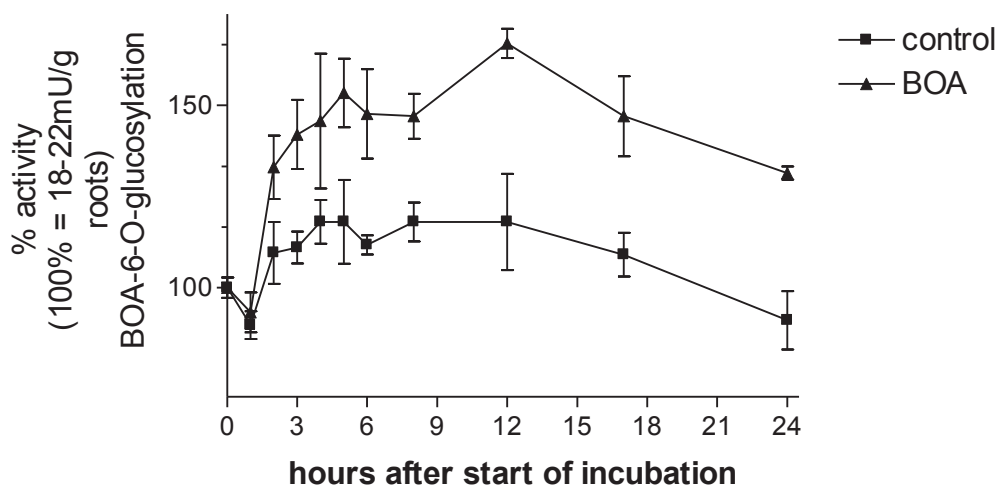


Figure 2. BOA-6-OH glucosylating activity during the course of BOA incubation in comparison to the control plants, which incubated with water. Values present the means $\pm$ standard deviations.

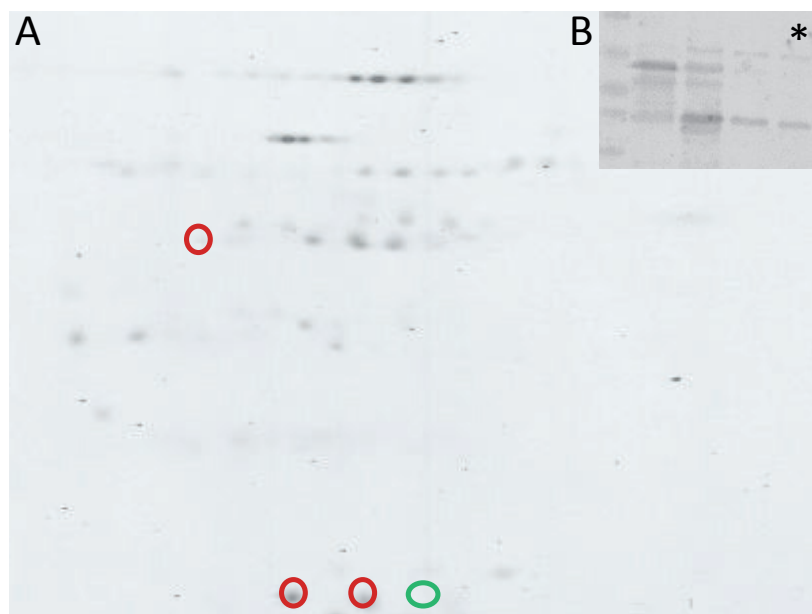


Figure 3. Coomassie stained gel after 2d-electrophoresis of the Hi Trap Blue fraction indicated the co-purification of different other proteins. The red markers encircle BX9 and its fragments, the green marker a BX8 fragment. B: The insert shows a Western blot of the different purification steps, indicating the loss of intact BX9. B-lines: marker proteins, crude extract, gel filtration, anion exchange chromatography, affinity chromatography (*).

ments. The BOA-6-OH glucosylating activity was enhanced after incubation with BOA. After 1 h, the activity increased up to 35% during the next hour and stayed about 45 % in average above the control levels during the whole course of monitoring with a maximum of activity after 6-12 hours of incubation, followed by a plateau and a slight decrease to the end of the incubation period after 24 h (Fig. 2).

Although BOA-6-O-glucoside does not accumulate anymore, the BOA-6-OH glucosylating activity was still above the control level after a 24h BOA exposure.

Addition of more than 1 mM DTT to the extraction buffer resulted in a 30-25% loss of the BOA-6-O-glucosylating activity. Following ammonium sulfate fractionation, the activity was enriched by chromatography on Sephadex G 100, anion exchange chromatography using a Q-Hyper D 10 column and chromatography on Hi Trap Blue. From all columns, the activity eluted in a single peak, but some activity was found in the wash fraction of the Q-Hyper D 10 column. The overall enrichment was 395 – fold (table 1), but the final yield was low (0.5 %). Elution from the Hi Trap Blue column was not only possible with BOA-6-OH and 40 mM UDPG but also with 20 mM homocysteine or 2 mM NADH. The use of homocysteine and NADH for elution led to a rapid loss of activity. With desalted $(\text{NH}_4)_2\text{SO}_4$ fractions, low BOA-N-glucosylation activity was measurable. However, during the further steps of the purification procedure, this activity was lost.

Identification of BX9 as a glucosyltransferase responsible for BOA-6-OH glucosylation

Two-d-electrophoresis and LC/MS/MS analysis of the Hi Trap Blue fraction revealed large amounts of a BX9 fragment in the range of 21 kDa whereas only traces of the complete protein were found (Table 2, Fig. 3). Several proteins were co-purified, mainly methionine synthase, malate dehydrogenase, mono-hydroascorbate reductase, enolase, four glutathione-S-transferases and traces of a BX8 but no other UGTs or their fragments. The BX9 fragment, which was already abundant in SDS-extracted proteins from roots, represents the C-terminal domain of the enzyme. Although only this fragment was mainly enriched, BX9 might be responsible for BOA-6-OH detoxification.

Further evidence for BX9 as a BOA-6-OH glucosylating enzyme was obtained by pull down experiments using maize crude protein extracts and BX9 antibodies. Incubation of maize crude extracts with the antibody led to a complete loss of activity and when the immuno-bound enzyme was eluted the BOA-6-OH-glucosylating activity was recovered (Fig. 4). The BX9 fragments are enriched in the flow through but no BOA-6-OH glucosylating activity was detected for this fraction. Hence the native BX9 is responsible for BOA-6-OH detoxification.

Table 1. Enrichment of BOA-6-*O*-glucosylating activity.

Purification step	Total activity (nkat)	Specific activity (nkat mg ⁻¹ protein)	Yield (%)	Purification factor
Crude extract	13.75	0.2	100	1
Ammonium sulphate precipitation	2,78	0.34	20.3	1.72
Gel filtration	1.84	1.41	13.4	7.16
Anion exchange chromatography	0.03	12.61	0.22	64.17
Affinity chromatography	0.07	78	0.50	395.3

BOA incubation accelerate BX9 cleavage

The increase of the 23/21 kD fragments could be caused by the allelochemical already during the incubation. Western blots with BX9 antibodies and SDS-extracted maize proteins clearly showed that BX9 undergoes fragmentation in presence of BOA. The fragments occur mainly in the root hair and maturation zones (Fig.5).

*Biochemical characteristics of BOA-*O*- and *N*- glucosylation*

To get more insights in biochemical characteristics that lead to the different BOA glucosylations, the Q-Hyper D10 column fraction was used for a characterization of the BOA-6-OH glucosylation and the desalted ammonium sulfate fraction for *N*-glucosylation (Tab. 3). The pH optimum of BOA-6-*O*-glucosylation was at pH 8 with half maxima at pH 7 and 9.3, the optimum of BOA-*N*-glucosylation was found between pH 6.5 and 7 with half maxima at pH 6 and 7.5. Thus, *O*-glucosylation is shifted to the more alkaline range in comparison to BOA-*N*-glucosylation. Substrate specificities differed

depending on pH. At pH 8, the highest activity was found with BOA-6-OH (150%), followed by the natural substrate DIMBOA (100%) and DIBOA (53.3%). A low activity was determined with BOA-5-OH and 2,4D, whereas BOA and 6-hydroxyindolone were not accepted. At pH 6.5 only 30% of the activity was found with BOA-6-OH but still 61.5% with DIMBOA. Glucosylation of DIBOA dropped to 8.6 %, but 6-hydroxyindolone was accepted (21%). BOA-5-OH and 2,4-D were not accepted as substrates at pH 6.5. BOA was *N*-glucosylated, but with very low efficiency (less than 5%). The low activity was not in agreement with the fact that BOA-*N*-glucosylation is the major detoxification reaction in the roots and presents a residual activity as BOA-*N*-glucosylation takes place in the extraplasmic space (Schulz *et al.* in press).

For the substrate BOA-6-OH, K_m -values were determined to be 120.7 μ M for BOA-6-OH. Lower BOA-6-OH concentrations led to sigmoid graphs, higher ones than 400 μ M to substrate inhibition. The K_m values for UDPG were 0.36 mM. Glucoside carbamate was an effective inhibitor of BOA-6-OH glucosylation (K_i 89.7 μ M) (Fig. 6).

Table 2. Sequences of identified BX9 Peptides and their position within the BX9 sequence.

AA Position	Peptide
15-31	RVVVFPPFQGHFNPVM
49-61	RGLAITVFHSGALDPADYPADY
64-73	VPVTVEADPK
194-207	RVDTSDLEEF AELLA
238-253	ALSVPVFAVAPLNK
323-338	RGFESGALPDGVEDEV
395-401	RYVCDVWK
428-436	EGEEIKER
446-459	GIGIGVDVDETTSP
460-471	RTDLTDLVDLIK

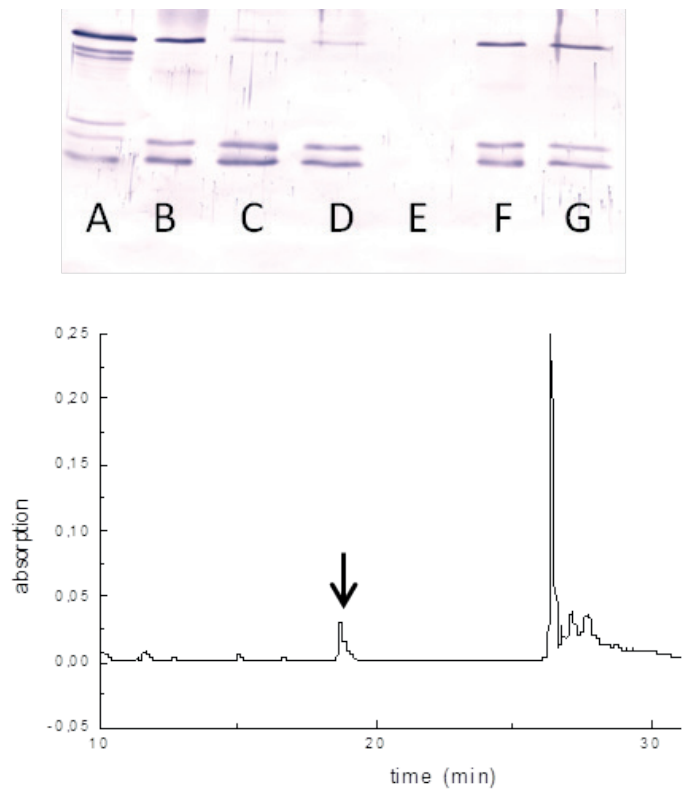


Figure 4. Western blot of the immunoprecipitation steps with BX9 antibodies. I: crude protein extract B: antibody treated crude extract, C: column flow through; D, E: 1. and 2. wash; F and G: 1st and 2nd elution. A: crude extract (aliquot of 500µl containing 4-6 mU enzyme activity); B: no activity; C: 0.04 nkat; D: 0.027 nkat; E no activity; F,G: 0.01-0.013 nkat The fractions obtained during the immunoprecipitation procedure contained co-eluting antibody, which hampered correct determination of the enzyme activity and BX9 protein concentration. Activity found in F and G could therefore considerably higher than the determined 10-20% of the one found in the crude extract. II: HPLC chromatogram using 50µl of eluate assay with BOA-6-OH and UDPG as substrates. Arrow points to product BOA-6-O-glucoside. 100µl Assays with 20 µl protein solution.

Modulation of BX 9 Activities by sulfur containing effector molecules

Several substances known to be involved in stress signal transduction and redox-regulations were tested for their influence on BOA-6-OH and DIMBOA-glucosylation (West *et al.* 2006; Lindermayer *et al.* 2005; Klatt and Lamas 2000), (Tab. 3). Short term treatments with S-nitrosoglutathione, a compound used for S-nitrosylation and NO-mediated protein

S-glutathionylation reduced the BOA-6-O-glucosylation to 20% and inhibited DIMBOA glucosylation up to 50%. The addition of 200 µM H₂O₂ had no effect on BOA-6-O-glucosid production, but decreased DIMBOA glucosylation to 60% and assaying in presence of 20 mM DTT inhibited BOA-6-OH glucosylation to 25 %. A strong inactivation by GSSG toward all sugar acceptors was observed. For DIMBOA, the activity could be reconstituted by DTT up to 60% within 2h, reconstitution of BOA-6-OH glucosylation with DTT failed.

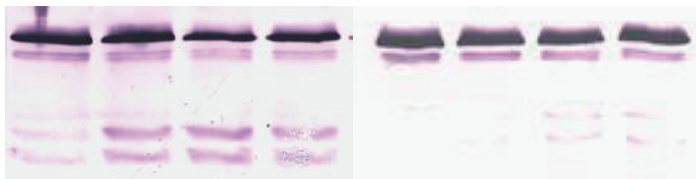


Figure 5. Western blot of proteins extracted from 1cm root segments (left to right: tip-1cm, 1-2cm, 2-3cm, 3-4cm) with BX9 antibodies. A: extracts from BOA-incubated (24h) B: extracts from control plants, which were incubated only with water. BX9 fragments are mainly in the 2-4 cm part of the root.

Table 3. Biochemical characteristics of the glucosylating activity. Substrate concentrations: 2.5 mM UDPG, glucose acceptor substrates: 200µM. 100% = 8.5 nkat/mg protein.

Activity Properties			
pH optimum	8.0, BOA-O-glucosylation		
Substrate specificity	at pH 8.0		at pH 6.5
	DIMBOA	100%	DIMBOA 61.5 %
	DIBOA	53%	DIBOA 53 %
	BOA-6-OH	150%	BOA-6-OH 30%
	BOA	0	BOA < 5%
	6-hydroxindolone	0	6-hydroxindolone 21%
Km values	BOA-6-OH 120.7 µM		
	UDPG 360 µM		
K _i value	glucoside carbamate 89.7 µM		

From this characterization we conclude, that the redox-status of the enzyme is important. Most likely, reduced or oxidized strategically important –SH groups of BX9 could be crucial for BOA-6-OH glucosylation. An oxidized form of the enzyme could be necessary for full activity when BOA-6-OH is the sugar acceptor molecule. This oxidized form seems to be unstable and undergoes cleavage during the course of the BOA incubation. The results agree with the inactivation of the BOA-6-OH competent enzyme by homocysteine and NADH.

BOA-detoxification in transgenic *Arabidopsis* lines

We used transgenic BX9, BX8 and Co-BX8 (which expresses the *Consolida orientalis* BX8 gene, Frey *et al.*) *Arabidopsis* lines to substantiate a role of BX9 in BOA-6-OH glucosylation (Fig. 7). BOA-6-O-glucoside accumulation was significantly higher in the BX9 line when the incubation was done with 0.5 mM

and 1mM BOA, whereas 2 mM led to a similar accumulation as found in WT and BX8 plants. The Co-BX line accumulated the highest amount of BOA-6-O-glucoside. With 2 mM BOA 100 to 200 % more of this compound compared to the BX9 line was found.

BOA-6-OH glucosylating UGT activity was measured with extracts of soluble proteins prepared from the transgenic *Arabidopsis* lines after 24h incubations with 0, 0.5, 1 and 2 mM BOA (Fig. 8). In roots of WT plants not exposed to BOA, the BOA-6-OH glucosylating activity was in the range of 10 mU / g FW (0.17nkat). The activity was 4-5 fold increased after 0.5 mM BOA incubation, whereas higher BOA concentration had no additional effect. It is known that several genes encoding UGTs are induced by BOA (Baerson *et al.* 2005). Their activities certainly interfere with the maize UGTs in the transgenic lines. The BX8 and the Co-BX8 lines exhibited both a higher activity in the control roots (2-3 fold:Co-BX8; 2-2.5 fold: BX8, compared to the WT.

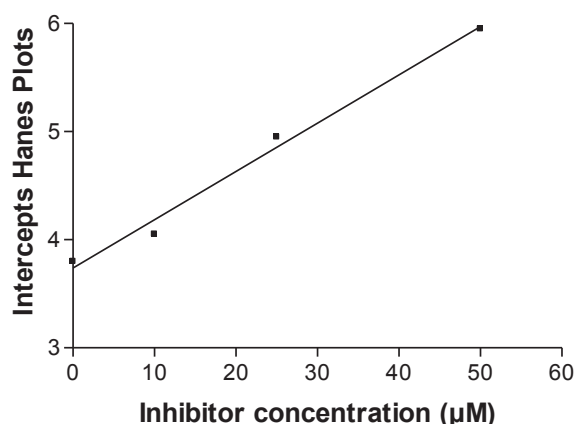
**Figure 6.** Glucoside carbamate is an inhibitor for BOA-6-OH glucosylation.

Table 4. Influence of effectors on the glucosylating activity at pH 8.0, substrate concentrations 200 µM, 100%=8.5 nkat/mg protein.

Effectors	% Activity O-glucosylation	
	DIMBOA	BOA-6-OH
20mM S-nitrosoglutathione	50	20
200 µM H ₂ O ₂	40	100
20 mM DTT	100	25
25 mM GSSG	0	0
Reconstitution with DTT	60 (2h)	no reconstitution

Incubation with 0.5 mM BOA led to an increase in the lines (Co-BX8: 50%, BX8: 25%), 1 mM BOA increased the activity in BX8 line 30% compared to the WT, the Co-BX8 line activity was similar to the WT. 2 mM BOA incubations decreased the activity in the roots of both lines, probably due to interferences with the induced endogenous detoxifying glucosyltransferase.

The BX9 line showed a different activity profile. The highest BOA-6-OH glucosylation was found in the control (5 fold compared to the WT), increasing BOA concentrations led to a loss of activity that could dropped below the average WT value when 1 or 2 mM BOA were used for the incubation. The UGT activity profiles coincide with the BOA-6-O-glucoside accumulation pattern in the roots. The higher accumulation of this detoxification product in BX9 plants has to be assigned to the high enzyme activity already before the start of the incubation. The decrease of BOA-6-OH glucosylation with higher BOA concentrations can be explained again by interference with the *Arabidopsis* UGT or the BOA-6-OH competent form of BX9 is no longer generated. BX9 cleavage, occurring also in *Arabidopsis*, cannot be the only reason, since the enzyme is constantly synthesized. The BX9 cleaving product can be demonstrated by Western blotting (Fig. 9). The Western blot also shows

that BX8 is heavily degraded under BOA influence.

Possible crosslinks of BOA and MBOA detoxification

Methylation and demethylation or transmethylation reactions may interlink the BOA/MBOA detoxification pathways. Transmethylation reaction could also be involved in cell wall incorporation of detoxification products. Therefore, the influence of methionine on the accumulation of detoxification products was tested. Methionine is, for instance, an essential methyl-group donor in pathways leading to the methylation of lignin precursors (van der Mijnsbrugge *et al.*, 2000; Ravel *et al.* 2004; Barrière *et al.* 2009).

Incubations with MBOA resulted in the accumulation of BOA-6-O-glucoside, but the presence of methionine shut down BOA-6-O-glucoside accumulation (Fig. 10). Thus, the accumulation of BOA-O-glucoside as a detoxification product of MBOA is influenced by the methyl-donor methionine and indicates trans-methylation reactions. This needs, however, further proof. The experiment demonstrates that BX9 could be important for detoxifying MBOA derived BOA-6-OH. The intermediate BOA-6-OH is therefore a linker between BOA and MBOA detoxification.

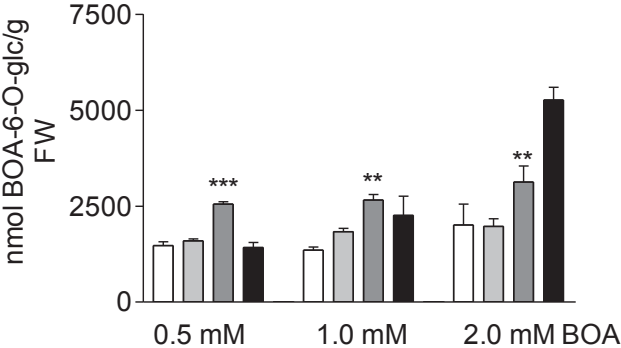


Figure 7. Accumulation of the two BOA-6-O-glucoside in transgenic *Arabidopsis* lines and the wild type Col-0, (Mean ± ES; N = 6). Asterisks indicate significant differences between BX9 line and the other lines/WT (P < 0.005).

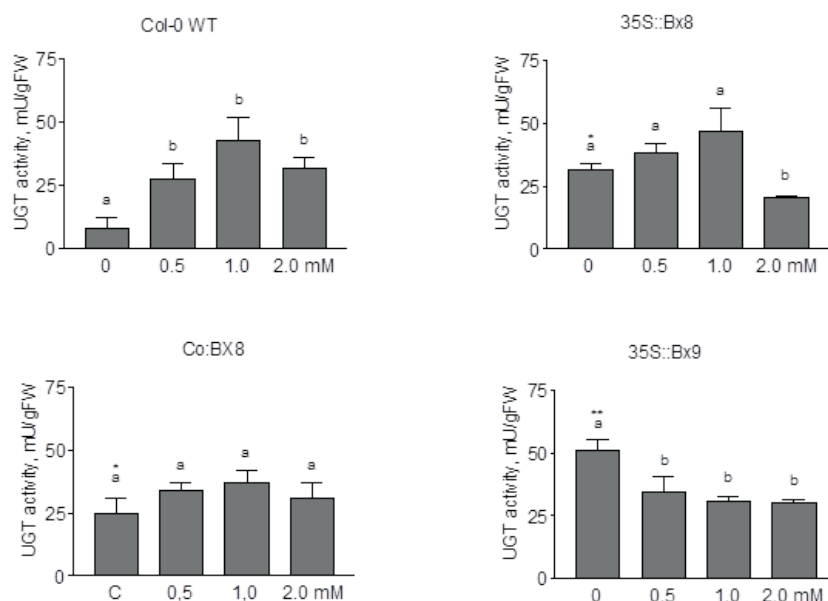


Figure 8. Root UGT activity profiles for BOA-6-O-glucosylation in the three transgenic lines and the WT after incubations no BOA and with different BOA concentrations. The BX9-line shows a different activity profile, compared to the wild type and the other transgenic lines (mean $\pm$ standard error; N = 6). Asterisks indicate significant differences between the WT and the different lines (P < 0.005). Means $\pm$ SE with different letters are significantly different within one line. Data sets of the different lines were analyzed by one-way ANOVA.

Discussion

The BOA-6-O-glucosylation pathway is a “ready to go” mechanism, important to detoxify BOA during the first hours of BOA exposure. It seems to be an important mechanism to avoid autotoxification when high amounts of MBOA are present in the root tip. Possibly the latter event is the major function of BX9 but not of BX8. BX9 was identified as a protein which can be nonconventional exuded and tightly bound to cell wall polymers (Schulz *et al.* in press). The latter behavior was not yet known when the purification of the BOA-6-OH glucosylating activity was started. Regarding the actual knowledge, the shifting from the cytosol to the extraplasmic space and concurrent breakdown of the cytosolic protein in two major fragments would explain first, the increasing occurrence of the cytosolic fragments during BOA incubations and secondly, the low enrichment of the enzyme during purification.

Since the addition of H₂O₂ had no influence on BOA-6-O-glucosylation, BOA-6-O-glucoside synthesis may be catalyzed by an already oxidized but unstable form of cytosolic BX9. Consequently, reducing sulfur compounds inactivated BOA-6-OH glucosylation as well as glutathionylation by GSSG. It is assumed that oxidation changes the enzyme

conformation and the sugar acceptor specificity, but finally destabilizes the enzyme and proteolytic fragmentation is easier possible.

BX9 is sensitive to oxidations eliciting substrate specificity alterations, and to nitrosylation and glutathionylation at -SH groups. As concluded from the 60% deactivation in presence of H₂O₂, the redox status is essential for acceptance of the substrate DIMBOA, which is corroborated by the experiments with GSNO and GSSG. At present, no redox-modulated glucosyltransferase with affinity to small sugar acceptor molecules is described. As a consequence of redox modulations the conformation of the enzyme might be changed and a number of structurally related molecules hitherto not accepted for substrates can be specifically used for glucosylation. Redox modulations of proteins are growingly recognized and are also assumed for apoplastic proteins (Go and Jones 2013; Cohen *et al.* 2014). Reversible glutathionylation was found to regulate actin polymerization and many other proteins (Wang *et al.* 2001). The modification could explain why recombinant BX9 failed in BOA-6-OH glucosylation. Another explanation for the failure could be a necessity of a tight interaction with a mono-oxygenase that introduces the hydroxyl group in position 6 of the BOA molecule. This would avoid the accumulation of toxic BOA-6-OH.

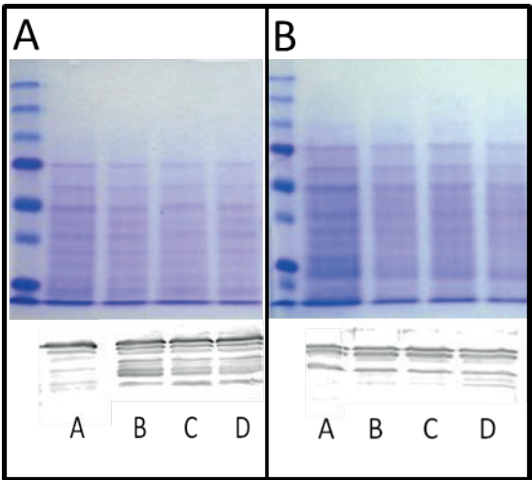


Figure 9. SDS-PAGE of root proteins from the transgenic BX8 (A) and BX9 (B) lines after 24h incubation with 0 (A), 0.5 (B), 1.0 (C) and 2.0 (D) mM BOA. The 0-control is next to the marker proteins. Lower line: Western blots of the BX8 (A) and BX9 (B) lines with polyclonal BX8- and BX9-antibodies. The latter react with the complete protein and degradation products of the proteins. Particularly BX8 shows massive degradation in BOA incubated roots.

Cleavage of cytosolic BX9 could be a key event in the switch to glucoside carbamate synthesis. Although the fragments have no catalytic function, the stability of the C-terminal fragment is surprising. This fragment contains the UDPG binding domain with the unusual feature of two neighbored cysteines. It can be speculated that the cysteines can serve as a redox sensor and the fragments have a redox reporter function during the detoxification process and are therefore not quickly degraded. Also reversible interactions with –SH groups of other proteins, including those of BX9, cannot be excluded. Moreover, vicinal thioles are thought to be involved in signal transduction (Cohen *et al.* 2014).

Concomitant glycosylation for detoxification of BOA-6-OH and performance of glycosylation of

DIBOA during the biosynthesis of DIMBOA-glc is clearly an advantage which was evolved by gene duplication in maize. In contrast, *Consolida regalis* has only one enzyme that has to fulfill both functions. It is postulated that during detoxification the biosynthesis of benzoxazinones is almost disrupted, but this needs further investigations.

The ability to glucosylate BOA-6-OH by induced or constitutive enzymes is rather wide-spread in plants. Corresponding glucosyltransferase activities were measured, for example, not only in protein extracts from *Arabidopsis thaliana*, but also with other species such as *Abutilon theophrasti* or *Avena sativa*. An increase of BOA-6-O- glucosylation activity after BOA incubation is also a common effect (Schulz *et al.* 2006; Haghi Kia *et al.* 2014). None of the above mentioned

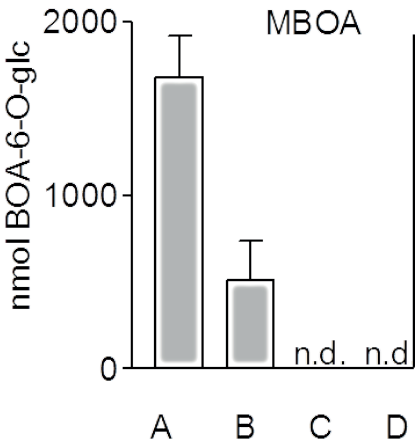


Figure 10. BOA-6-O-glucoside, one of the detoxification products of MBOA, is not produced in presence of methionine. The MBOA Incubations with 2.0 (A) and 0.5 mM MBOA (B,C,D); C + 1 mM methionine ; D +5 mM methionine.

species produces benzoxazinones, but all of them are able to perform BOA detoxification by BOA hydroxylation in position 6 and glucosylation of this intermediate, whereby *Hordeum vulgare*, *Lolium perenne* and *Avena sativa* switch later to glucoside carbamate production during the detoxification process.

Formation of N- and O-glucosides of xenobiotics is well known. For example, 3,4-dichloraniline detoxification via N- and O-glucosylation were found with glucosyltransferase GT72B1 from *Arabidopsis thaliana* suspension cultures and root cultures (Loutre *et al.* 2003). Sandermann *et al.* (1991) reported on N- and O-glucosylation of chlorinated anilines and phenols in soybeans due to distinct isozymes. Therefore, redox regulated substrate specificity might be not generally valid. On the other hand, in many studies, the natural substrates of the recombinant enzymes remain obscure. As stated by Bowles *et al.* (2005), the natural function of most of the enzymes studied by *in vitro* characterizations, often performed with recombinant proteins, remained extensively unknown. Redox modulations with pronounced effects on the substrate specificity and the mode of glucosylation may be one reason for the difficulties to pinpoint the natural function of recombinant glucosyltransferases. To our opinion, cytosolic UGTs are important when xenobiotics, including allelochemicals, have already passed the plasma membrane and entered the protoplast. An advanced detoxification strategy is to transfer enzymes needed for detoxification reactions into the extraplasmic space.

Acknowledgements

We thank Monika Frey, TU München, Lehrstuhl für Genetik, for providing the transgenic *Arabidopsis* lines and the BX8/ BX9 polyclonal antibodies.

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RECEIVED November 18, 2015

ACCEPTED December 16, 2015



Changes in polyamines, proline and protein contents in radish seedlings could serve as indicators of allelopathic stress induced by 2-benzoxazolinone and *p*-hydroxybenzoic acid

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ABSTRACT

Allelochemicals are secondary metabolites released by plants into the environment, generally acting as inhibitors of several physiological processes in the target plants, thus creating sometimes metabolic imbalances that can produce situations that have been found similar to abiotic stress. We call allelopathic stress to the set of challenges that the plant receiving allelochemicals has to face. Stress conditions usually induce an inhibition of protein synthesis (and then, a growth inhibition) and an accumulation of low molecular weight metabolites such as proline and polyamines. Within this framework, objectives were to test phytotoxicity of two known allelochemicals (*p*-hydroxybenzoic acid and 2-benzoxazolinone) on germination and growth of radish seedlings and identifying substances that could be used as reliable allelopathic stress markers.

The most powerful inhibitory allelochemical was 2-benzoxazolinone. Results revealed drastic germination (-50%) and root growth (-40% biomass) inhibitions due to 2-benzoxazolinone 10^{-3} M. Furthermore, root morphology was different than in control plants, without hairy zone and brown-coloured.

Allelopathic stress induced by *p*-hydroxybenzoic acid and 2-benzoxazolinone showed different results in stress markers in radish roots, hypocotyls and cotyledons; total protein content provided only general information about the growth state (enhanced or inhibited), polyamines (spermidine, spermine and putrescine) were ineffective whereas proline was found to be the most reliable allelopathic stress marker.

Keywords: 2-benzoxazolinone, allelopathy, *p*-hydroxybenzoic acid, *Raphanus sativus*, stress.

Abbreviations: 2-benzoxazolinone (BOA), *p*-hydroxybenzoic acid (POH), polyamines (Pas), Spermidine (Spd), Putrescine (Put), Spermine (Spm)

Introduction

Allelopathy, the “direct or indirect effect of one plant upon another through the production of chemical compounds released into the environment” (Rice, 1984), is often studied by testing the effects of allelochemicals on germination, growth and various physiological processes in target plants. Allelochemicals are secondary metabolites, and they are very different from the chemical point of view. Many researchers have studied the physiological and biochemical effects of phenolics that are usually released by most of plants (Al Harum *et al.*, 2015; Blum, 1996; He *et al.*, 2012; Hussain *et al.*, 2015; Inderjit, 1996; Mahmoud *et al.*, 2013; Martínez-Peñalver *et al.*, 2012). Recently there has been a great interest in the biological effects of cyclic hydroxamic acids and compounds related to benzoxazolinone in plants (Chiapusio *et al.*, 1997; Hussain *et al.*, 2011b;

Sánchez-Moreiras *et al.*, 2009, 2010, 2011; Schulz *et al.*, 2012; Lu *et al.*, 2012). In many cases, published studies in allelopathy described these chemical interactions as a stress factor for target species (Hussain *et al.*, 2011a; Kato-Noguchi, 2009; Singh and Singh, 2009; Terzi and Kocacaliskan, 2010). Abiotic stresses have been blamed of changes both in the production and release of allelochemicals (Fang *et al.*, 2010; Niakan *et al.*, 2008; Poulson-Ellestad *et al.*, 2014) and also in the effect on target plants (Jozwiak-Zurek *et al.*, 2011). Biotic stress, as competition, could also modify the effects of allelopathy (Graneli and Salomon, 2010; Hussain and Reigosa, 2011; Rivoal *et al.*, 2011). In some cases, allelopathic stress could induce oxidative damage or regulation of the oxidative cycle (Bogatek and Gniazdowska, 2007; Dmitrovic *et al.*, 2015; El-Shora and Abd El-Gawal, 2015; Hussain and Reigosa, 2014; Mushtaq *et al.*, 2013; Yang *et al.*, 2014).

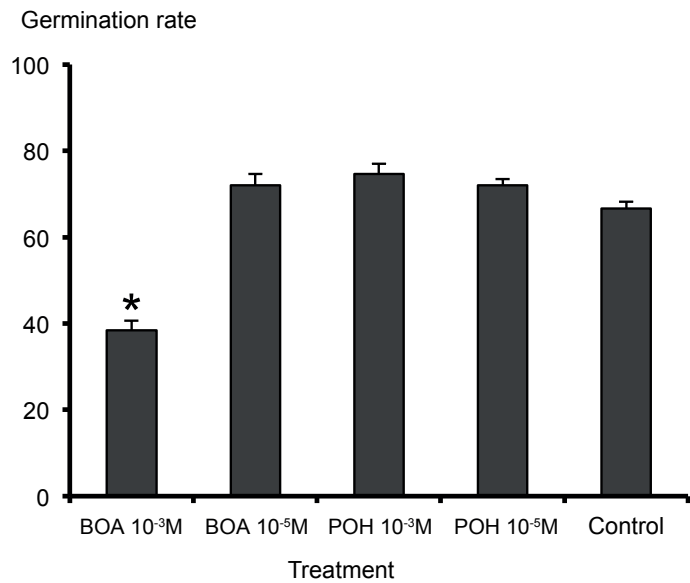


Figure 1. Phytotoxic effect of two concentrations of 2-benzoxazolinone and *p*-hydroxybenzoic acid on germination rate of radish.

* Significant difference, compared to control, for *P* < 0.05 according to Mann-Whitney *U* test

BOA = 2-benzoxazolinone and POH = *p*-hydroxybenzoic acid.

Sometimes plants have to perform in stressful environments such as drought, extreme temperatures, salinity, oxygen deficiency, pollution... In such cases, they develop adapted morphological, physiological and biochemical mechanisms to survive. McCue and Hanson (1990) showed a close relationship between stress conditions and the accumulation of low molecular weight metabolites, mostly involved in growth regulation, such as amino acids (e.g., proline) or polyamines (e.g., putrescine, spermidine, and spermine). Generally, stress induces a decrease of growth, implying an inhibition of protein synthesis. At the same time, free amino acids, such as proline, accumulate. The synthesis of this imino acid may be one of the earliest metabolic responses that link the perception of many environmental stresses to the elicitation of physiological response at the cellular level (Hare and Cress, 1997; You *et al.*, 2011).

Polyamines (PAs) are also well-known to be widespread low molecular weight metabolites that accumulate in stressed plant cells (Al-Harun *et al.*, 2015; Rajam, 1997). Many reports indicate that the plant PA content changes due to various stresses (Flores, 1990). For example, in most stress cases, putrescine accumulation is noticed (Galston and Kaur-Sawhney, 1995). However, the precise biological role of these amines (putrescine, spermidine, and spermine) still remains unclear. Considering that total proteins, proline and polyamines levels reveal a physiological disturbance in stressed plants, we hypothesised that these substances could act as allelopathic stress markers. Thus,

objectives of this work were: (i) to test phytotoxicity of *p*-hydroxybenzoic acid and 2-benzoxazolinone on germination, and early growth of radish (*Raphanus sativus*), (ii) to quantify total proteins, proline and polyamines in roots, hypocotyls and cotyledons of radish seedlings having germinated and grown in presence of the two allelochemicals and (iii) to identify one (or more) of these measured substances as reliable allelopathic stress markers.

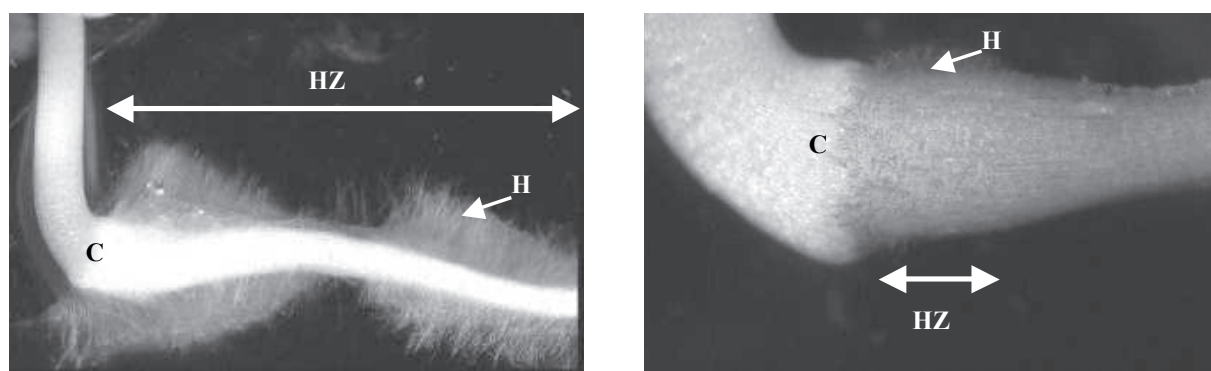
Materials and Methods

General procedure

The experimental procedure consisted in testing allelochemicals stress on radish germination and early growth and the efficiency of using total proteins, proline and the three polyamines (putrescine, spermidine and spermine) as allelopathic stress markers.

Chemical solutions

p-hydroxybenzoic acid and 2-benzoxazolinone (hereafter termed POH and BOA respectively) were selected due to their allelopathic effects previously described (Chiapusio *et al.*, 1997; Levi-Minzi *et al.*, 1994; Sánchez-Moreiras *et al.*, 2005, 2007, 2009, 2010, 2011; Schulz *et al.*, 2012). For each chemical (SIGMA Chemicals Co.), aqueous solutions were prepared at 10⁻³ and 10⁻⁵ M with distilled water (buffered to pH 6) as control.



Photograph 1. Root tips of control (top, x 7) and BOA 10^{-3} M treated (bottom, x20) radish seedlings after 4 days of incubation. HZ = hairy zone, H = hairs, C = Crown.

Allelopathic effects of BOA and POH on radish

Twenty radish seeds (*Raphanus sativus*, var. 18 jours) were placed into a glass (7 cm diameter and 9 cm high, closed with a Petri dish), and leached with a 4 ml chemical solution or distilled water (buffered to pH 6 as control). Glasses were placed for a maximum of 5 days in a thermostatic controlled growth chamber (25°C, photoperiod 8h light/16h dark).

Four days after the beginning of incubation (hereafter termed D4), when no further seeds would have germinated, germinated seeds (rupture of seed coats and emergence of radicle) were recorded.

Growth of seedlings was recorded after 3, 4 and 5 days of incubation (termed D3, D4, and D5, respectively), by means of biomass. Dry weights (after 48h in oven at 80°C) of cotyledons, hypocotyls and roots were measured separately. These data were also summed in order to obtain dry weight of total seedlings. Three sets of glasses (one per measuring day D3, D4 and D5) with at least triplicates per treatment (5 for BOA 10^{-3} and 4 for control) were prepared for the germination and growth experiments.

Allelopathic stress markers

Radish seedlings were obtained as described above. Roots, hypocotyls, and cotyledons were carefully removed from 3-, 4- and 5-days-old seedlings and immediately frozen in liquid nitrogen. They were stored at -20°C for proteins and proline extractions or at -50°C for polyamines extractions. There was enough material available to perform 4 to 5 replicates for proteins and proline analysis and triplicates for polyamines analysis.

For total proteins quantification, samples (100 mg FW) were ground in liquid nitrogen, with an extraction tampon (Arulsekar and Parfin, 1986) and polyvinylpyrrolidone. Homogenates were then

centrifugated (16000 rpm for 20 min, 2°C). The soluble protein content of the supernatant was determined by the Coomassie Blue method (Bradford, 1976), using serum albumin as standard.

Samples (250 mg FW) for proline quantification were ground in liquid nitrogen, then homogenised in 5 ml 3% (w/v) sulfosalicylic acid before assaying free proline by the acid ninhydrin method (Bates *et al.*, 1973).

Samples (200 mg FW) for polyamines quantification were ground in liquid nitrogen with perchloric acid. Homogenates were centrifuged at 12000g for 15 min at 4°C, and the supernatant fraction used directly for analysis of three polyamines (spermidine, spermine, and putrescine) as described in Reggiani *et al.* (1989). After dansylation, the polyamines were separated in TLC plates with ethyl acetate/cyclohexane (4:5, v/v). They were then placed under UV light, scraped from the plates, eluted and quantified with a fluorimeter (350 nm excitation, 495 nm emission).

Statistical analysis

Germination rate and growth data, as well as total proteins, proline and polyamines contents were compared to their respective controls using the Mann-Whitney U test ($p < 0.05$).

Results

Phytotoxic effects of BOA and POH on radish

Germination rate of seeds leached with BOA 10^{-3} M was significantly lower than control (almost -50%), whereas other treatments did not induce significant differences (Figure 1).

If total seedlings dry weight did not significantly differ from control for all treatments on any of the measured days (Figures 2a), there were noticeable

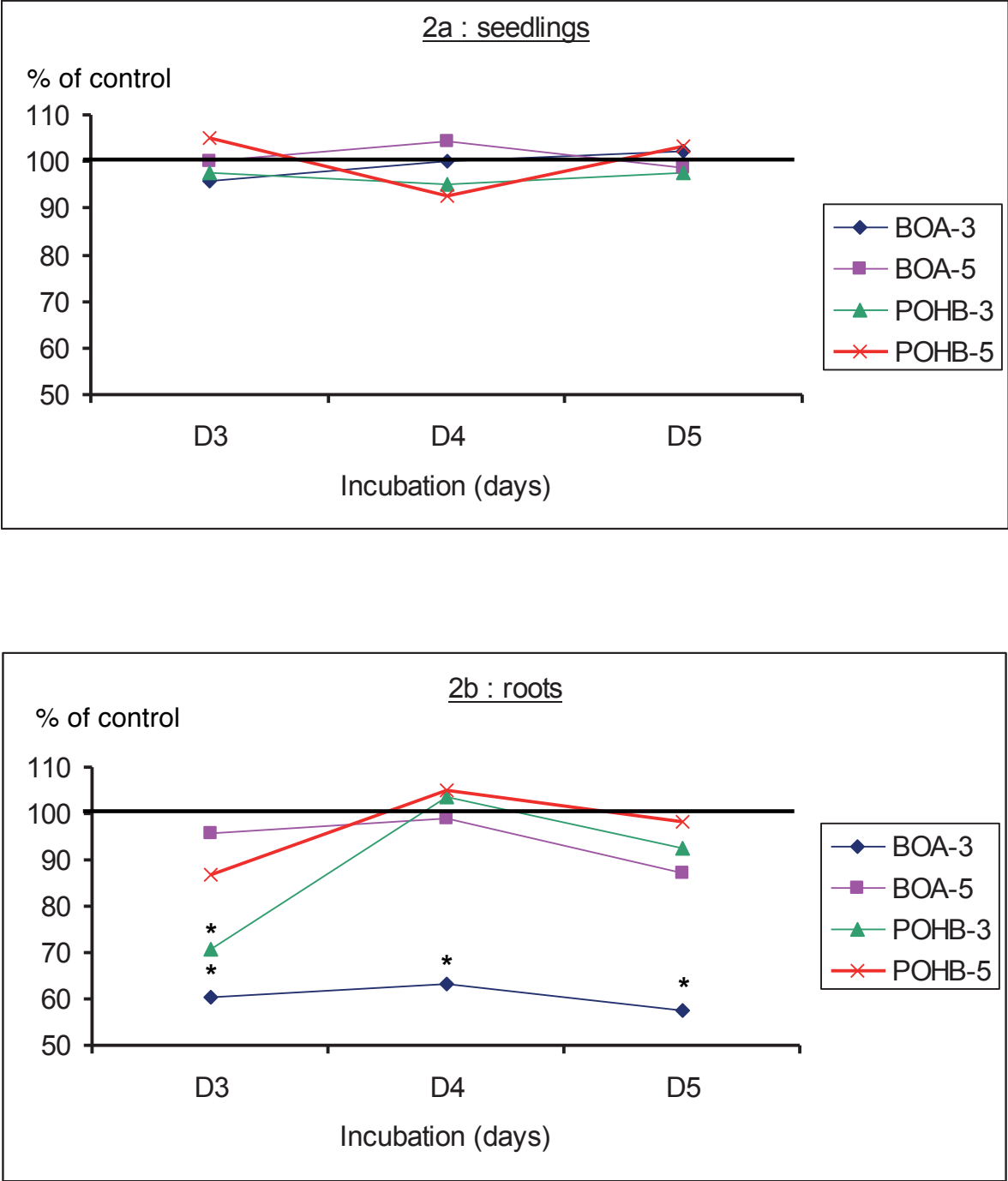


Figure 2.

Dry weight evolution of radish seedlings (2a) and roots (2b) after 3, 4 and 5 day incubations with two concentrations of 2-benzoxazolinone and *p*-hydroxybenzoic acid. Data are expressed as per cent of control (horizontal line, 100%). For the clarity of figures, standard error bars are not shown.

* Significant difference, compared to control, for $P < 0.05$ according to Mann-Whitney U test
BOA = 2-benzoxazolinone and POH = *p*-hydroxybenzoic acid.

Table 1. Proline content in roots, hypocotyls and cotyledons of radish seedlings after 3, 4 and 5 day incubation period with allelochemicals.

Proline content (μmol g ⁻¹ dry weight)					
	control	BOA 10 ⁻³ M	BOA 10 ⁻⁵ M	POH 10 ⁻³ M	POH 10 ⁻⁵ M
D3					
Roots	0.7±0.1	nd	0.6±0.1 *	0.7±0.1	0.9±0.2 *
Hypocotyls	3.4±0.2	nd	4.2±0.5 *	4.3±0.4 *	5.8±0.6 *
Cotyledons	5.5±0.3	6.6±0.6 *	3.0±0.3 *	4.5±1.1 *	3.6±0.3 *
D4					
Roots	0.4±0.1	0.7±0.05 *	0.4±0.02	0.4±0.1	0.2±0.01 *
Hypocotyls	1.5±0.3	1.6±0.2	1.6±0.2	1.1±0.1 *	1.0±0.1 *
Cotyledons	1.2±0.4	2.2±0.3 *	0.6±0.2 *	0.7±0.2 *	0.7±0.1 *
D5					
Roots	0.4±0.01	0.6±0.03 *	0.6±0.05 *	0.3±0.03	0.4±0.04
Hypocotyls	0.8±0.1	2.0±0.4 *	1.1±0.2 *	0.8±0.1	0.9±0.1
Cotyledons	0.6±0.1	1.3±0.3 *	0.8±0.2 *	0.6±0.1	0.7±0.1

* Significant difference, compared to control, for *P* < 0.05 according to Mann-Whitney *U* test
nd : not determined, due to insufficient material.

BOA = 2-benzoxazolinone and POH = *p*-hydroxybenzoic acid.

differences when focusing on seedling parts. The most drastic effect was observed in roots, especially with BOA 10⁻³ M, which induced a 40% reduced biomass (Figure 2b). Furthermore, morphological differences were also noticed. Roots in contact with BOA 10⁻³ M were not only 10 times smaller, but also brown with less hairs than control (Photograph 1). The highest concentration of POH also inhibited root growth of radish, but only after 3 days of incubation (Figure 2b). When focusing on cotyledons or hypocotyls, no significant differences were observed for both treatments and incubation times (data not shown). BOA was thus the most phytotoxic.

Allelopathic stress markers

In control seedlings, levels of free proline varied considerably among the three different radish organs (Table 1). In 3-day-old seedlings, proline content in cotyledons was almost 8 times greater than in roots and 1.6 greater than in hypocotyls. After this, contents decreased until the 5th day. In stress conditions, only BOA induced a proline accumulation in roots,

hypocotyls and cotyledons by the 5th day. The highest BOA concentration (10⁻³ M) was able to increase proline content up to the 5th day. As POH induced only proline accumulation in hypocotyls by the 3rd day, it appeared less stressful than BOA, confirming again that it was more phytotoxic.

Proteins content in hypocotyls and cotyledons of the control decreased up to 3 times from the third to the fifth day (Table 2). Proteins content in roots, on the other hand, remained stable. BOA 10⁻³ M induced an increase of proteins contents for 3 days running. Thereafter, POH 10⁻³ M and 10⁻⁵ M provoked an increase (3rd day), a decrease (4th day) and no effect the 5th day.

Spermine, one of the sought-after polyamines, was not detected at any time in the radish seedling growth (data not shown). Putrescine (Table 3) in controls was almost concentrated in cotyledons and decreased from the 4th to the 5th day; roots contained 3 times less whereas it disappeared in hypocotyls and remained stable in cotyledons. Allelochemicals supply led to heterogeneous results. Only POH 10⁻⁵ M and BOA 10⁻⁵ M during the 5th day induced higher putrescine levels

Table 2. Total proteins content in roots, hypocotyls and cotyledons of radish seedlings after 3, 4 and 5 day incubation periods with allelochemicals.

Total proteins content (mg g ⁻¹ dry weight)					
	control	BOA 10 ⁻³ M	BOA 10 ⁻⁵ M	POH 10 ⁻³ M	POH 10 ⁻⁵ M
D3					
Roots	0.3±0.02	0.9±0.1 *	0.3±0.03	0.4±0.03 *	0.4±0.04 *
Hypocotyls	1.0±0.1	2.6±0.2 *	1.3±0.2	1.7±0.2 *	1.9±0.3 *
Cotyledons	5.2±0.2	7.0±0.2 *	5.0±0.1	7.5±0.9	5.9±0.3 *
D4					
Roots	0.3±0.02	0.4±0.1 *	0.2±0.03 *	0.2±0.03 *	0.1±0.02 *
Hypocotyls	0.7±0.1	1.1±0.3 *	0.6±0.1	0.6±0.1	0.6±0.1
Cotyledons	2.8±0.3	4.1±0.2 *	2.0±0.3 *	2.7±0.3	2.1±0.3 *
D5					
Roots	0.2±0.03	0.2±0.04	0.2±0.02	0.2±0.02	0.2±0.03
Hypocotyls	0.3±0.02	0.6±0.05 *	0.2±0.03 *	0.4±0.1	0.3±0.01
Cotyledons	1.8±0.1	2.1±0.3	2.2±0.1 *	1.9±0.1	1.9±0.4

* Significant difference, compared to control, for *P* < 0.05 according to Mann-Whitney *U* test

BOA = 2-benzoxazolinone and POH = *p*-hydroxybenzoic acid.

than the control, so did POH 10⁻⁵ M the 4th day. Other statistically significant results outlined a decrease such as BOA 10⁻³ M in hypocotyls (4th day) and cotyledons (5th day), BOA 10⁻⁵ M and POH 10⁻³ M (4th day) in roots.

Like putrescine, spermidine (Table 4) rate in control seedlings decreased from the 4th to the 5th day (4.7 times less in roots, 4 in hypocotyls and 2 in cotyledons). Again, cotyledons were the radish organs containing the highest spermidine level. All allelochemicals induced a decrease in roots the 4th day and in cotyledons just in presence of BOA 10⁻⁵ M and POH 10⁻⁵ M. Increases were noticed with BOA 10⁻³ M and POH 10⁻⁵ M in cotyledons during the 4th day and during the 5th day with BOA 10⁻³ M in hypocotyls and with POH 10⁻³ M in roots.

Discussion

The present study aimed at identifying cellular signals of allelopathic stress. None of the tested chemicals, at any concentration, induced positive effects on germination or early growth of radish. The highest concentration (10⁻³ M) of BOA produced the

highest inhibition, decreasing by 50% the germination and by 40% the root biomass of radish. These results are consistent with previous observations on lettuce (Chiapusio *et al.*, 1997) and other species (Schulz *et al.*, 2012). The most dramatic inhibition was observed on roots biomass and morphology. The almost non-existing hairy zone of root tips suggests a decrease in the capacity of the plant to absorb minerals (Bergmark *et al.*, 1992).

Knowing the phytotoxic effects on radish makes possible the discussion about the reliability of various stress markers, proline, total proteins and polyamines. In the absence of stress, free proline levels varied in the different radish organs. This intrinsic variation has also been reported for other species such as *Arabidopsis thaliana* (Chiang and Dandekar, 1995).

Comparing allelochemicals effects, POH appeared to be less stressful than BOA because it did not induce proline increases in D4 or D5. This is in accordance with its low allelopathic potential observed in radish germination and growth. On the contrary, BOA induced proline accumulation which could be mediated by increased synthesis and reduced oxidation from the imino acid. Metabolic

Table 3. Putrescine content in roots, hypocotyls and cotyledons of radish seedlings after 4 and 5 day incubation periods with allelochemicals.

	Putrescine content (nmol g ⁻¹ dry weight)				
	control	BOA 10 ⁻³ M	BOA 10 ⁻⁵ M	POH 10 ⁻³ M	POH 10 ⁻⁵ M
D4					
Roots	12±7	nd	0 *	0 *	7±2
Hypocotyls	19±2	13±4 *	19±1	19±2	21±1
Cotyledons	69±18	71±29	48±11	40±11	54±2
D5					
Roots	3±1	nd	5±1 *	4±1	4±0 *
Hypocotyls	0	0	0	0	0
Cotyledons	59±26	25±2 *	47±11	39±8	95±47

* Significant difference, compared to control, for $P < 0.05$ according to Mann-Whitney U test

nd : not determined, due to insufficient material.

BOA = 2-benzoxazolinone and POH = *p*-hydroxybenzoic acid.

studies indicate that most of the proline accumulation in stressed plants is the result of enhanced de novo synthesis from glutamate (Delauney and Verma, 1993). This is the major pathway taking place in the cytoplasm, the other is from ornithine. It involves conversion of glutamate to proline via the intermediates γ -glutamyl phosphate, glutamic- γ -semialdehyde and P5C ($\rightarrow$ -pyroline-5-carboxylate). Thus, compared with most other amino acids, proline presents the metabolic advantage of being the terminal product of a relatively short and highly regulated pathway. Proline accumulation, therefore, affects fewer metabolic reactions than glutamate. If interconversions of proline and P5C involve transfers of redox, then changes in proline content may constitute a form of metabolism signalling (Hare and Cress, 1997). The drastic inhibition of roots growth observed with BOA 10⁻³ M was closely associated with the rapid accumulation of proline. This correlation has been also established in rice roots under salt stress (Lin and Cao, 1995, 1996). This proline accumulation may not inhibit any enzymatic activities but rather adjust osmotic pressure at the plant cell level (Lahrer *et al.*, 1996). Moreover, protein content increased at the same time as proline under BOA treatment, suggesting that proline could be more synthesised to accelerate protein synthesis. As

growth is considered an energy-requiring system, proline accumulation is a readily utilizable source of energy.

Considering total proteins, it has been reported that most stress situations induce proline increase whereas proteins decrease. For instance, this has been well-described under salt stress (Hurkam and Tanaka, 1987; Muthukumarasamy and Panneerselvam, 1997; Niakan *et al.*, 2008; Panneerselvam *et al.*, 1998). But proteins results are herein contradictory to the literature because proteins and proline levels increased or decreased concurrently in presence of any used allelochemicals. The observed protein increase (BOA 10⁻³M) could consequently be explained by proline synthesis. These results also lead us to consider that the method used to quantify proteins was not precise enough to establish what really occurred in radish seedlings. Kirtikara and Duncan (1996) pointed out that an oxidative stress due to an herbicide (paraquat) induced an increase or a decrease of different molecular weight polypeptides. Similar results have been found in allelopathic effects including ROS changes (Al-Harun *et al.*, 2015; Kamal, 2011; Sánchez Moreiras and Reigosa, 2005). Separating different kind of proteins should have given more information. Furthermore, the use of Stress Proteins (SP) as biomarkers appears also as another reliable technique (Siesko *et al.*, 1997).

Table 4. Spermidine content in roots, hypocotyls and cotyledons of radish seedlings after 4 and 5 day incubation periods with allelochemicals.

Spermidine content (nmol g ⁻¹ dry weight)					
	control	BOA 10 ⁻³ M	BOA 10 ⁻⁵ M	POH 10 ⁻³ M	POH 10 ⁻⁵ M
D4					
Roots	28±11	nd	5±2 *	10±1 *	15±2 *
Hypocotyls	36±4	35±16	34±7	31±2	33±5
Cotyledons	83±3	109±1 *	44±2 *	57±3 *	68±11 *
D5					
Roots	6±1	nd	7±0	9±1 *	11±4
Hypocotyls	9±1	32±8 *	8±2	9±1	7±1 *
Cotyledons	39±11	32±6	30±5	36±9	44±13

* Significant difference, compared to control, for *P* < 0.05 according to Mann-Whitney *U* test
nd : not determined, due to insufficient material.

BOA = 2-benzoxazolinone and POH = *p*-hydroxybenzoic acid.

The naturally occurring polyamines (PAs), spermidine (Spd) and spermine (Spm) and their obligatory precursor, putrescine (Put) are ubiquitous in plants cells (Rajam, 1997). Cells undergoing division contain high levels of free PAs whereas low levels are found in cells undergoing expansion (Galston and Kaur-Sawhney, 1997). It has also been well established that putrescine is synthesised from arginine or ornithine through arginine decarboxylase or ornithine decarboxylase, and then converted to Spd and Spm by the addition of a propylamine group from decarboxylated S-adenosylmethionine (Evans and Malmberg, 1989). In radish seedlings, this synthesis occurred mostly in cotyledons. Spermine was absent even in controls. This suggests that seedlings may be too young to elaborate spermine. In other words, the PAs biosynthetic pathway could be stopped at the spermidine synthesis. This is supported by the fact that spermine was not quantified in young seedlings (Felix and Harr, 1987). In contrast, Erdei *et al.* (1996) revealed that in maize, spermine absence indicated its fast turnover. Tiburcio *et al.* (1989) established that spermidine was the most effective PAs during vegetative growth whereas putrescine in triggering root formation. Concurrently, diamines putrescine and spermidine accumulate to rather high concentrations (from micromolar to millimolar) under many stress conditions such as mineral deficiency (Smith, 1984), low temperature, low pH, osmotic shock (Erdei *et al.*, 1990), chilling tolerance (Lee *et al.*,

1997), salt stress (Benavides *et al.*, 1997) and herbicide treatment (Zheleva *et al.*, 1993). In this study, the used allelochemicals had very little effect on putrescine and spermidine levels. Lin and Kao (1995) also drew such a conclusion, being unable to link PAs contents with growth inhibition of rice seedlings under NaCl stress. Putrescine has been reported to be increased in other allelochemical stress situations (Sánchez-Moreiras *et al.*, 2009).

To conclude, the most powerful germination and root growth inhibitor was BOA 10⁻³ M. Checking for proline, proteins and PAs quantities as allelopathic stress markers led to propose the former as the most sensitive one.

Acknowledgements

This research was supported by a EURODOC fellowship from the Région Rhône Alpes, granted to G. Chiapusio, permitting experimentation at the University of Vigo, Spain. We would like to thank Ángeles García Domínguez for her helpful technical support and also Kathleen Mottershead and Katy Duperrier for their reliable english corrections.

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RECEIVED November 17, 2015

ACCEPTED December 21, 2015



INTERNATIONAL ALLELOPATHY SOCIETY