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Benzoxazinoids–cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae)

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Abstract

An improved method of sample preparation and simultaneous HPLC separation was developed that allowed the separation of 2,4-dihydroxy-1,4-benzoxazin-3(4*H*)-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3(4*H*)-one (DIMBOA), 2-hydroxy-1,4-benzoxazine-3(2*H*)-one (HBOA), 2-hydroxy-7-methoxy-1,4-benzoxazine-3(2*H*)-one (HMBOA) and their corresponding glucosides as well as the benzoxazolinones BOA and MBOA. The amount and distribution of these compounds was determined in the roots of *Aphelandra squarrosa* and *A. fuscopunctata* plants. There is a significant difference in the amount and distribution of this substance class in the two species analyzed. The results are discussed in relation to their function as defence compounds and allelochemicals.

Keywords: *Aphelandra*; Roots; Hydroxamic acids; HPLC separation; Allelopathy; Benzoxazinoids

1. Introduction

The first discovery of benzoxazolin-2(3*H*)-one (BOA) in plants was reported in 1955 in rye (*Secale cereale* L.) (Virtanen & Hietala, 1955). Shortly after, the methoxy derivative 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) was isolated from wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) (Virtanen, Hietala & Wahlroos, 1956). In 1959 it was shown that these compounds do not occur in planta but are degradation products of 2,4-dihydroxy-1,4-benzoxazin-3(4*H*)-one, its 7-methoxy derivative, and their corresponding glucosides (Wahlroos & Virtanen, 1959; Virtanen & Hietala, 1960).

Today a broad variety of hydroxamic acids, lactams and their glucosides are known. The main structures are shown in Scheme 1. Besides an intensive research on their functions as allelochemicals and chemical resistance factors against insects, fungi, bacteria and virus in many important crop plants of the family Gramineae (Niemeyer, 1988) also inhibitory effects on human cancer cell lines were reported (Zhang et al., 1995). The chemical synthesis and an overview treating the chemistry and the structural requirements for the biological activity was published recently (Sicker, Hartenstein & Kluge, 1997; Hashimoto & Shudo, 1996).

Despite of the intensive studies performed with monocot crop plants there are few studies on distribution of benzoxazinoids in dicots. DIBOA, DIBOA-Glc and BOA were isolated from dried seeds of *Acanthus mollis* (Wolf, Spencer & Plattner, 1985), HBOA and HBOA-Glc from seeds of *Blepharis edulis* (Chatterjee, Sharma, Banerji & Basa, 1990), both Acanthaceae species. In *Consolida orientalis* flowers (Ranunculaceae) DIBOA, DIBOA-Glc and BOA were reported (Oezden, Oezden, Attila, Kücükislamoglu & Okatan, 1992) and in *Scoparia dulcis* (Scrophulariaceae) MBOA was isolated from roots (Chen & Chen, 1976). Recently Pratt, Kumar and Chilton (1995) reported the occurrence of the cyclic hydroxamic acids DIBOA and DIMBOA in six of 34

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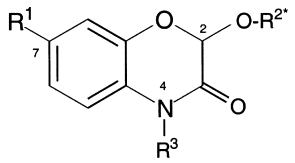
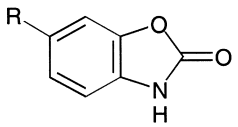


* all glucosides are present in the (2R)-configuration

1	$R^1 = H$	$R^2 = Glc$	$R^3 = OH$	DIBOA-Glc
2	$R^1 = OCH_3$	$R^2 = Glc$	$R^3 = OH$	DIMBOA-Glc
3	$R^1 = H$	$R^2 = H$	$R^3 = OH$	DIBOA
4	$R^1 = OCH_3$	$R^2 = H$	$R^3 = OH$	DIMBOA
7	$R^1 = OCH_3$	$R^2 = Glc$	$R^3 = OCH_3$	HDMBOA-Glc
8	$R^1 = OCH_3$	$R^2 = H$	$R^3 = OCH_3$	HDMBOA
9	$R^1 = H$	$R^2 = Glc$	$R^3 = H$	HBOA-Glc
10	$R^1 = OCH_3$	$R^2 = Glc$	$R^3 = H$	HMBOA-Glc
11	$R^1 = H$	$R^2 = H$	$R^3 = H$	HBOA
12	$R^1 = OCH_3$	$R^2 = H$	$R^3 = H$	HMBOA



5	R = H	BOA
6	$R = OCH_3$	MBOA

Scheme 1.

analyzed species of the family Acanthaceae, two *Acanthus*, two *Crossandra* and two *Aphelandra* species, namely *A. aurantiaca* and *A. squarrosa*.

During our research on polyamine alkaloids in *Aphelandra* (Acanthaceae) we detected BOA in the macerated root extracts of *A. tetragona* (Werner, Hedberg, Lorenzi-Riatsch & Hesse, 1993). Further analysis showed that the species *A. tetragona*, *A. squarrosa*, *A. chamissoniana* and *A. fuscopunctata* contain in their roots the hydroxamic acids, their glucosides as well as the corresponding lactams (Hedberg, 1994; Todorova, Werner & Hesse, 1994).

In order to obtain a more complete picture on distribution and exact composition of all benzoxazinoids in *Aphelandra* species, we were searching for a valuable method for their separation and quantification. During the last 15 years, several methods were published on this subject. Many agricultural research papers describe the decomposition of the hydroxamic acids by enzymatic and/or thermal degradation prior to the extraction. The analysis of the derivatives, mainly BOA or MBOA, is then carried out by HPLC, GC and spectroscopic methods (UV, IR, fluorometry) (Niemeyer, 1988). This does not allow any differentiation between glucosides and aglucones.

When the sample preparation is performed carefully the direct separation of the hydroxamic acids and their glucosides by HPLC should allow an insight into the distribution of the benzoxazinoids in planta. But as the separation of all compounds of this substance class is a tricky task, quantitative analysis were often reduced to a limited number of one or two structure types (Copaja, Barria & Niemeyer, 1991; Mayoral, Gutierrez, Ruiz & Castanera, 1994; Oezden et al., 1992).

In this paper, we describe an HPLC method which allows the separation of nine different benzoxazinoids including hydroxamic acids, lactams and their corresponding glucosides. The content of benzoxazinoids in roots of *A. fuscopunctata* and *A. squarrosa* during one vegetation period and the amount in different parts of the roots of these two species are presented.

2. Results and discussion

While the hydroxamic acid glucosides are reported in the differentiated tissues of the plants the aglucones are found in the meristem and in the callus (Zuniga & Massardo, 1991). The biosynthetic precursors, the corresponding lactams and their glucosides are rarely reported. In dicots detailed studies on this substance class are lacking. Recently a screening study on benzoxazinoids in Acanthaceae was published and their presence was reported for six species including *A. aurantica* and *A. squarrosa* (Pratt et al., 1995). There was no indication concerning the relationship between aglucones and glucosides in these plants as the working up procedure allowed the decomposition of the glucosides to the aglucones.

In a preliminary study, we analyzed four *Aphelandra* species (*A. tetragona*, *A. fuscopunctata*, *A. squarrosa* and *A. chamissoniana*) for their content in benzoxazinoids. These first results showed that in all species the glucosides as well as the hydroxamic acid aglucones are present in the roots, while in the aerial plant parts only traces of the glucosides were detected. In *A. fuscopunctata*, however, aglucones and glucosides were present in all parts of the plant (Hedberg, 1994).

As analytical method of choice High Performance Liquid Chromatography (HPLC) with Diode Array Detection (DAD) was used in the present study.

2.1. Isolation of the reference substances

As none of the hydroxamic acids or lactams are available commercially it was necessary to isolate all reference substances from natural sources.

The methoxylated benzoxazinoids DIMBOA-Glc (**2**), HDMBOA-Glc (**7**), HMBOA-Glc (**10**), DIMBOA (**4**) and HMBOA (**12**) were isolated from young corn (*Zea mays*) plants (Wahlroos & Virtanen, 1959), while DIBOA-Glc (**1**), DIBOA (**3**) and HBOA (**11**) was

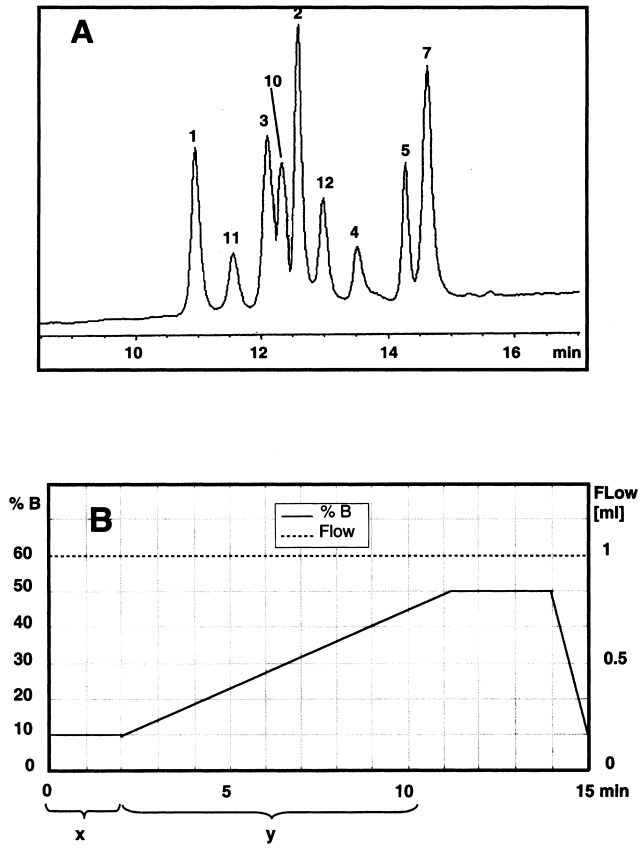


Fig. 1. HPLC separation of hydroxamic acids and their biogenetic precursors. Solvent system (A = H_2O; B = MeOH/*iso*-PrOH 95:5 + 0.025% HOAc). (A) HPLC-chromatogram (compound numbering see Scheme 1). (B) Gradient system used for the separation of the hydroxamic acids.

obtained from *Aphelandra tetragona* roots. All compounds were identified by spectroscopic methods.

2.2. HPLC separation

As basis for the development of the method we used the work of Xie, Atkinson, Arnason, Morand and Philogène (1991), Bücker and Grambow (1990), and Lyons, Hipskind, Wood and Nicholson (1988). The latter reported the separation of DIMBOA and DIBOA-Glc with a reversed phase Ultrasphere ODS 5μ-column (C_{18}). With the Nucleosil 100-7 C_{18} and Nucleosil 100-5 C_{18} AB columns these results were not reproducible. We developed an improved method in three independent steps, each consisting of a different separation problem: (A) Separation of DIMBOA and HMBOA, (B) Separation of DIBOA and DIMBOA-Glc, and (C) Separation of DIBOA and HBOA. The combination of three steps resulted in a good separation of the hydroxamic acids DIBOA and DIMBOA, their glucosides DIBOA-Glc and DIMBOA-Glc, their lactam precursors HBOA and HMBOA, HMBOA-Glc and the benzoxazolinones BOA and MBOA (Fig. 1). The solvent system which gave the best results was H_2O as solvent A and MeOH/*iso*-PrOH (95/5) + 0.025% HOAc as solvent B with a two-step gradient consisting of an isocratic and a linear part (Fig. 1(B)).

The main differences from published methods is the replacement of methanol by a higher alcohol in solvent B; the addition of the acid to the alcoholic solvent B (not in the aqueous solvent A) and a gradient consisting of an isocratic and a linear part. In the course of optimization we observed that: Higher alcohols improved the separation of DIMBOA (**4**) and HMBOA (**12**) but they decreased the separation of DIBOA (**3**) and DIMBOA-Glc (**2**) in shifting the retention time of **3** to shorter and **2** to longer retention times.

Increasing the acidity improved the separation of DIBOA (**3**) and DIMBOA-Glc (**2**) but prevented a separation of DIMBOA (**4**) and HMBOA (**12**).

Furthermore, the separation of DIBOA (**3**) and DIMBOA-Glc (**2**) could be monitored by the duration of the isocratic time x in retaining the retention times of all other components.

2.3. Sample preparation for quantification: extraction and pre-purification

As the lactams and hydroxamic acid glucosides are very sensitive to enzymatic deglucosylation followed by chemical degradation, the careful sample preparation is of major importance.

Several authors who reported the quantification of aglucones or benzoxazolinones after maceration of the plant material (Mayoral et al., 1994; Xie et al., 1991) neglected this fact. More sophisticated methods were published by Lyons et al. (1988) and Bücker and Grambow (1990). In the first case, the plant material was extracted with MeOH under reflux for 15 min in order to avoid the enzymatic degradation of the glucosides and the hydroxamic acids DIBOA-Glc, DIBOA, DIMBOA-Glc, DIMBOA and the benzoxazolines BOA, MBOA were quantified; in the second case, the plant material was macerated after freezing in liquid nitrogen and this careful working up procedure resulted in the detection of the hydroxamic acid and lactam glucosides. In this case no aglucones were reported. It can be concluded that the type of detected benzoxazinoids depends strongly on the working up procedure of the plant material.

We avoided these problems by freezing the samples immediately after harvesting under liquid nitrogen and performing all further manipulations (storing, maceration, weighting) at 0°C. Under these conditions benzoxazolin-2(3*H*)-one and 6-methoxy-benzoxazolin-2(3*H*)-one (BOA and MBOA) were not detectable confirming that they are not occurring naturally in plants.

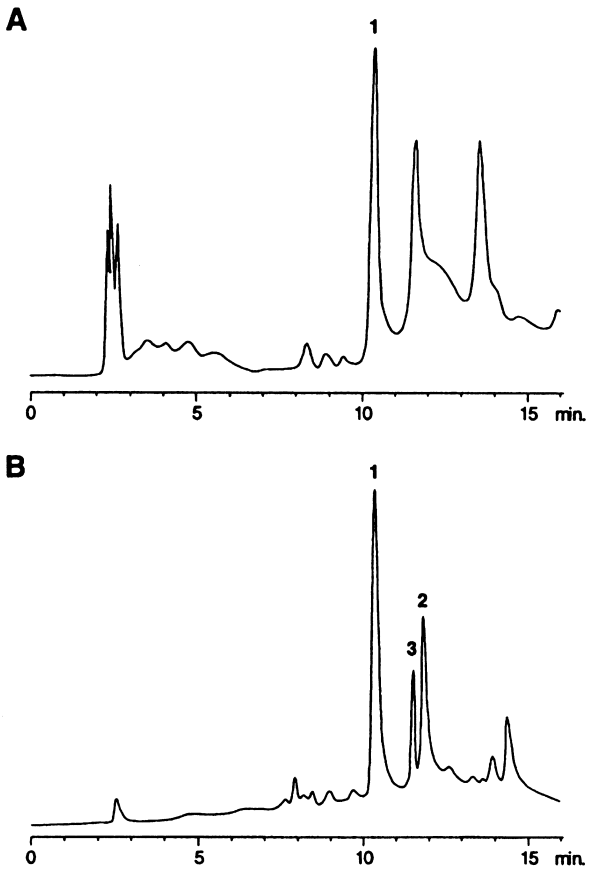


Fig. 2. Example of a HPLC chromatogram of extracts from roots of *Aphelandra squarrosa* for the quantification of hydroxamic acids. (A) Extract before purification on Sep-Pac® C_{18}, column. (B) Extract after purification on Sep-Pac® C_{18} column.

Another problem is the pre-purification of the samples prior to HPLC analysis. This sample preparation was neglected in all cited publications where the raw extracts were injected directly. Only the work of Lyons et al. (1988) reports a pre-purification, but the proposed method is not useful for measurements of large series of samples.

In the raw extracts of plants, the broad variety of substances (salts, lipids, glycosides, phosphates, peptides, macromolecules, chlorophyll) and their seasonal variation can influence HPLC quantifications. In addition, secondary compounds with identical retention times might interfere. During our analysis of samples of *Aphelandra* we observed that some peaks (according to their UV spectra probably flavonoids or flavonoid glycosides) eluted with identical retention times as the benzoxazinoids.

While using a Sep-pak® C_{18} cartridge for solid-phase extraction (SPE) we could eliminate these compounds by a simple pre-purification procedure.

The improvement of the HPLC chromatograms can be seen in Fig. 2. The chromatogram of the raw extract of *A. squarrosa* before and after purification on Sep-pac® are shown (Fig. 2(A) and (B)). Compound **3** is overlapping with a compound which has disappeared after pre-purification on Sep-pac®. After purification the peaks showed the pure UV spectra of the benzoxazinoids while earlier overlapping spectra indicated a mixture (data not shown). Without the possibility of a DAD-detector where UV-spectra of each peak are registered they would have been interpreted as benzoxazinoids.

2.4. Content of the hydroxamic acids and their glucosides in different root segments of A. squarrosa and A. fuscopunctata

Root segments of five plants (25 weeks after shoot propagation) of *A. squarrosa* and *A. fuscopunctata* were analyzed for their content in benzoxazinoids.

In both species the highest amount of benzoxazinoids was found in the root tip. In *A. fuscopunctata* the total amount is four times higher than in *A. squarrosa* (Fig. 3). While in *A. squarrosa* the root tip contains only glucosides, more than 50% of the benzoxazinoids are present as aglucones in *A. fuscopunctata*. Only the side roots of *A. squarrosa* contain glucosides and aglucones.

In Gramineae, by contrast, the aglucones appear only in the meristematic tissue in very small amounts. Differentiated tissue contains only glucosides (Zuniga & Massardo, 1991). In the roots of *A. fuscopunctata* also elder parts contain aglucones. Considering that these compounds may have an allelopathic function (Schulz, Friebe, Kuck, Seipel & Schnabl, 1994) it is interesting that the most exposed root parts, the root tips and the growing side roots, contain higher amount of the toxic aglucones. In *A. squarrosa* only the side roots contain aglucones. The lactams HBOA and HMBOA were only detected in traces in different root segments indicating that they are metabolic intermediates which are not accumulated. The corresponding glucosides were not detected.

2.5. Time course of benzoxazinoid content in A. squarrosa and A. fuscopunctata roots

The content of benzoxazinoids was monitored during 22 weeks in the roots of *A. squarrosa* and *A. fuscopunctata* (Fig. 4).

In *A. sqarrosa* the highest amount of benzoxazinoids was found in 5–7 week old roots. The main compound detected was DIBOA glucoside which reached 3 μmol/g fr.wt. at week 5. The level decreased till the 9th week and then fluctuated between 0.5 and 1 μmol. During the interval between week 5 and 10 the decrease of DIBOA-Glc was paralleled with a strong increase of DIBOA which decreased later to a constant level of 0.2 μmol/g fr.wt. DIMBOA-Glc and DIMBOA were

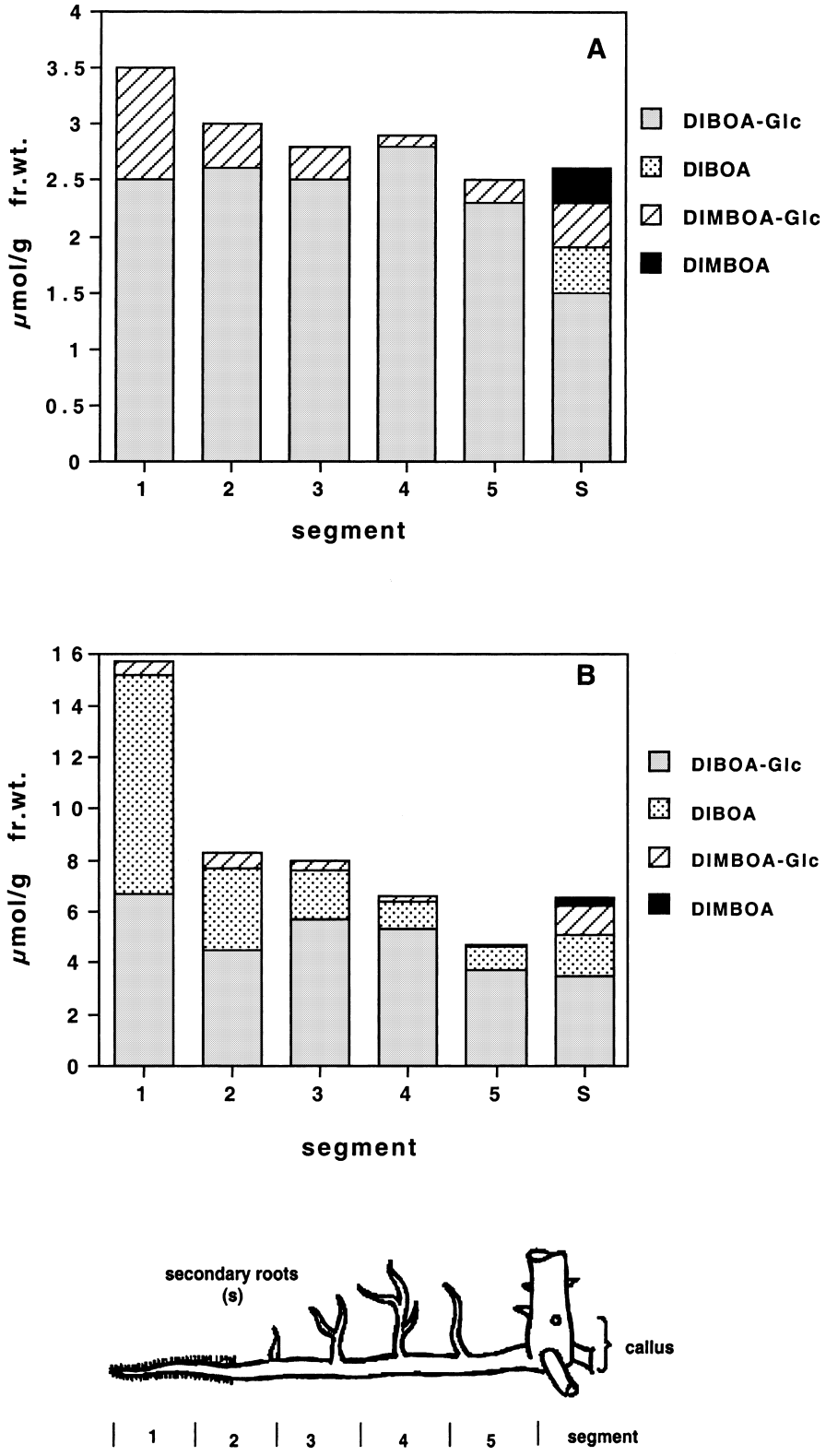


Fig. 3. Hydroxamic acid content in different root segments of *Aphelandra*. (A) *Aphelandra squarrosa*. (B) *Aphelandra fuscopunctata*.

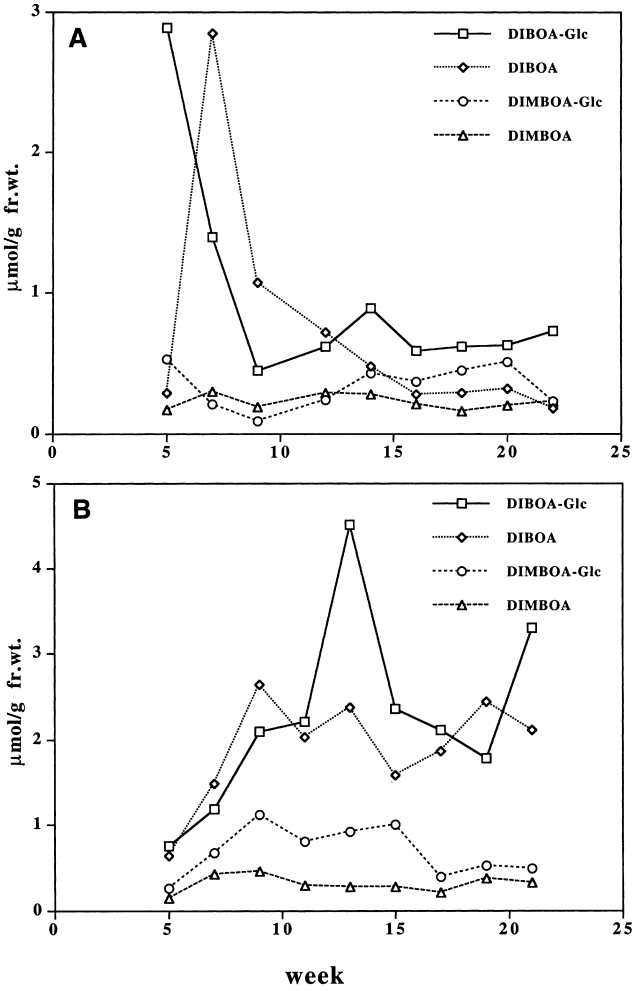


Fig. 4. Time course of hydroxamic acid content in roots of *Aphelandra*. (A) *Aphelandra squarrosa*. (B) *Aphelandra fuscopunctata*.

present in the roots at a constant level of 0.1–0.2 µmol/g.fr.wt. The lactams HMBOA, HBOA and HMBOA-Glc were present in all samples in amounts beyond the limit of calculation. The benzoxazolinones BOA and MBOA were not detectedable.

From these results it can be seen that the biosynthetic activity channelled into the secondary pathways is already very high in young growing roots. The polyamine alkaloid aphelandrine was also found in high amounts in the developing rootlets of *A. tetragona* after shoot propagation (Werner et al., 1993).

In seedlings of cereals the maximum level of benzoxazinoids was reached during the first 4–6 days after germination and then decreased progressively (Argandona, Niemeyer & Corcuera, 1981). A correlation between the aphid population and the content of benzoxazinoids could be shown in this case. Whether the accumulation of the hydroxamic acids in the young roots of *A. squarrosa* has a protective role against soil pests is unclear.

In the roots of *A. fuscopunctata* the situation was completely different. During the first 15 weeks there was a strong and parallel increase of DIBOA and DIBOA-Glc. The total amount and ratio between DIBOA and DIBOA-Glc were much higher than in *A. squarrosa*. This is true for roots as well as for the upper plant parts which was observed in the preliminary study (Hedberg, 1994). DIMBOA was only present in very small amount. DIMBOA-Glc reached about 10% of the amount of DIBOA-Glc. As in the case of *A. squarrosa* the lactams HMBOA, HBOA and HMBOA-Glc were detected in traces but their amount was beyond the limit of calculation. Again, in no part the benzoxazolinones BOA and MBOA were detected.

In general, it can be concluded from these analyses that in contrary to published results of Gramineae in *Aphelandra* roots the benzoxazolinones are present as aglucones and glucosides in the differentiated tissues during the whole period analyzed. In *Aphelandra* roots the aglucones are, therefore, not reduced to the meristematic tissue as was proposed for wheat (Zuniga & Massardo, 1991).

The composition of benzoxazinoids in the root tip of *A. fuscopunctata* and in the secondary roots of both species could indicate an allelopathic significance for these compounds. Their importance in allelopathy has been described in the case of rye (*Secale cereale* L.) and quackgrass (*Agropyron repens* L.). These results show that the degradation product of DIBOA the benzoxazolinone BOA has strong allelopathic activity (Barnes, Putman, Burke & Aasen, 1987; Schulz et al., 1994).

An interpretation of the differences of the two species analyzed can only be a speculative one. A possible reason may result from the differences in the morphological properties of the roots of the two species: the roots of *A. fuscopunctata* are widely dispersed and the root-system, therefore, contains considerably more meristematic tissue. Another observation is that under greenhouse conditions *A. fuscopunctata* is much more resistant against all kind of pests than *A. squarrosa*. Whether this indicates that the benzoxazinoids play a role in the resistance against insects in *Aphelandra* species has to be proven.

3. Experimental

3.1. Plant material

Aphelandra tetragona (Vahl) Nees, *Aphelandra fuscopunctata* Markgraf, and *Aphelandra squarrosa* Nees plants were cultivated in the greenhouse of our institute by shoot propagation. *Zea mays* Hybrid DK250 seeds were obtained from the Agricultural Research Station Reckenholz, Switzerland.

3.2. Chemicals

Solvents: All solvents for extractions were distilled prior to their use. The solvents for HPLC were from Scharlau (Barcelona, Spain). Standard reagents were from Fluka (Buchs, Switzerland), Silicagel from Merck (Darmstadt, Germany), Cellulose from Whatman, Sephadex G-10 from Pharmacia (Dübendorf, Switzerland), and Sep-Pak®-columns from Waters Corp. (Milford, MA, USA).

3.3. Analytic

Melting points (mp): Mettler FP-5.
IR-spectra (ν_{max} in cm^{-1}, in KBr): Perkin-Elmer 1600 Series FTIR.
UV-spectra (λ_{max} in nm, MeOH, ε): Perkin-Elmer 555 spectrophotometer.
^{1}H-NMR spectra at 300 MHz: Bruker AC 300 or Bruker ARX-300; chemical shifts δ in ppm, coupling constants J in Hz, using TMS ($\delta = 0$ ppm) as reference.
^{13}C-NMR at 75.6 MHz in MeOD: Bruker ARX-300; δ in ppm relative to MeOD.
CI-MS with NH_3 as reactant gas, and EI-MS: Varian MAT-112S.
ESI-MS: Finnigan TSQ 700 mass spectrometer.
HPLC-separation: Hewlett Packard 1090; DAD detection, for quantification LDC/Milton Roy Spectra Monitor 3000 UV detection at 280 nm.
Analytical columns: Nucleosil 100-7 C_{18} and 100-5 C_{18} AB, Macherey Nagel GmbH. Oensingen, Switzerland.

3.4. Isolation of the reference substances

DIBOA-Glc (**1**), DIBOA (**3**) and HBOA (**11**) were isolated from *A. tetragona* roots. 80 g of lyophilized roots were extracted three times for 24 h, each time with 400 ml of MeOH/H_2O (9:1) containing 3% of HOAc. The combined extracts were reduced to a volume of 100 ml in vacuo. The aqueous phase so obtained was diluted to 300 ml with H_2O and adjusted to pH 3 with 1 N HCl.

The aglucones were obtained by extracting the water phase three times with EtOAc. After drying the organic phase over Na_2SO_4 and concentration in vacuo two fractions were eluted by column chromatography on $Silicagel_{60}$ (40–60 μm) with $CHCl_3$–MeOH–Hexane–HOAc (8:1:1.5:0.2). Fr. 1 (35 mg orange crystals) contained mainly DIBOA and fr. 2 (white powder) mainly HBOA. Further purification with prep. HPLC (see analytical gradient) led to 10 mg DIBOA (**3**), 5 mg HBOA (**11**) and 2 mg HMBOA (**11**).

The glucosides were obtained by extracting the water phase five times with *n*-BuOH. After evaporation of the organic phase and dilution of the residue with 20 ml H_2O the extract was purified on a Sephadex G10-column with H_2O as solvent. The fraction containing the hydroxamic acid glucosides was Iyophilized (181 mg light brown powder). After further column chromatography on cellulose (Whatman CF1) with toluene : *n*-BuOH (1:1) 171 mg of a beige powder was obtained containing mainly DIBOA-Glc (**1**).

DIMBOA-Glc (**2**), HDMBOA-Glc (**7**), HMBOA-Glc (**10**), DIMBOA (**4**) and HMBOA (**12**) were isolated from *Zea mays* Hybrid DK250: 400 g of 4-week old corn plantlets were placed into boiling water for 3 min prior to maceration in liquid N_2. The plant powder was then extracted three times with 400 ml MeOH–H_2O (9:1 + 3% HOAc). Each extraction was stirred for 24 h. The MeOH from the combined extracts was evaporated and the remaining 100 ml of water phase was adjusted to pH 3 with 1 N HCl. The precipitated chlorophyll was discarded by filtration over Celite. Aglucones were extracted by EtOAc and Glucosides by *n*-BuOH as described earlier.

3.5. 2,4-Dihydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA, **3**)

Light orange crystals, mp 198–201°C (lit. 152°C, Virtanen & Hietala, 1960). IR ν_{max}: 3270, 1653, 1490, 1093, 1039. ^{1}H-NMR (300 MHz, MeOD): 7.38 (*m*, 1H); 7.05 (*m*, 3H); 5.70 (*s*, 1H). ^{13}C-NMR (50.4 MHz, MeOD): 160.5 (C-3), 142.8 (C-8a), 130.0 (C-8), 125.9 (C-5), 124.1 (C-6), 118.8 (C-7), 114.7 (C-4a), 94.0 (C-2). El-MS: 181 (30, $[M]^+$), 165 (9, $[M-OH]^+$), 152 (11, $[M-CHO]^+$), 136 (40), 135 (100, $[M-HCOOH]^+$), 108 (19), 79 (53).

3.6. 2-Hydroxy-2H-1,4-benzoxazin-3(4H)-one (HBOA; **11**)

White powder, mp 198–201°C (lit. 206–207°C, Sicker & Hartenstein, 1993). IR ν_{max}: 3250, 1620, 1475, 1180, 1104, 1057, 1028. ^{1}H-NMR (300 MHz, MeOD): 7.42 (*d*, J = 7.1, 1H); 7.01 (*m*, 4H); 5.53 (*s*, 1H). ESI-MS: 353 ($[2M + Na]^+$), 188 ($[M + Na]^+$).

3.7. (2R)-2-O-β-D-Glucopyranosyl-4-hydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA-Glc, **1**)

Beige needles, mp 183–185°C (lit. 256–257°C, Hartenstein & Sicker, 1994). UV/Vis (MeOH) λ_{max}: 287 (ε 9551), 259 sh (ε 7300), 202 (ε 24,681). CD (MeOH): 310 (0), 281 (−0.16), 250 (0), 227 (+0.62), 217 (0), 213 (−0.53). $[\alpha]_D^{20}$ (MeOH): +75.8° (+106° (H_2O), Nagao, Otsuka, Kohda, Sato & Yamasaki, 1985). IR ν_{max}: 3402, 1683, 1494, 1100–1000 (br). ^{1}H-NMR (300 MHz, MeOD): 7.33 (*m*, 1H); 7.04 (*m*, 3H); 5.87 (*s*, 1H); 3.80 (*d*, J = 11.4, 1H); 3.62 (*dd*, J = 11.4,

$J = 3.5$, 1H); 3.25 (*m*, 4H); 3.13 (*t*, $J = 8.1$, 1H). ESI-MS: 709 ($[2M + Na]^+$), 366 ($[M + Na]^+$).

3.8. 2,4-Dibydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA, **4**)

Colorless crystals, mp 138°C (lit. 168–169°C, Hartenstein, Lippmann & Sicker, 1992). IR ν_{max}: 3270, 1653, 1490, 1039. ^{1}H-NMR (300 MHz, MeOD): 7.89 (*d*, $J = 8.7$, 1H); 6.86 (*d*, $J = 8.4$, 1H); 6.63 (*m*, 2*H*); 5.51 (*s*, 1H); 3.76 (*s*, 3H). El-MS: 211 (7, $[M]^+$), 165 (100, $[M - HCOOH]^+$), 150 (41), 106 (17),

3.9. 2-Hydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (HMBOA, **12**)

White powder, mp 179–181°C (lit. 196–199°C, Nagao et al., 1985). IR ν_{max}: 3210, 1620, 1539, 1475, 1104, 1057, 1028. ^{1}H-NMR (300 MHz, MeOD): 7.38 (*m*, 1H); 7.05 (*m*, 3H); 5.70 (*s*, 1H). ^{13}C-NMR (50.4 MHz, MeOD): 160.5 (C-3), 142.8 (C-8a), 130.0 (C-8), 125.9 (C-5), 124.1 (C-6), 118.8 (C-7), 114.7 (C-4a), 94.0 (C-2). El-MS: 195 (45, $[M]^+$), 166 (100, $[M - CHO]^+$), 138 (20), 124 (27), 110 (28), 95 (9). ESI-MS: 412 ($[2M + Na]^+$), 218 ($[M + Na]^+$).

3.10. (2R)-2-O-β-D-Glucopyranosyl-4-hydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA-Glc, **2**)

White, fine needles, mp 249°C, decomp. (lit. 170–172°C, Hartenstein, Klein & Sicker, 1993). UV/Vis (MeOH) λ_{max} 287 (ε 7994), 278 sh (ε 7660), 205 (ε 15,500). CD (MeOH): 320 (0), 287 (−1.89), 249 (0), 226 (+4.77), 215 (0), 207 (−3.84). $[\alpha]_D^{20}$ (MeOH): +39.3° (−59.2° (pyridine), Nagao et al., 1985) IR (KBr) ν_{max}: 3396, 1683, 1507, 1018, 818. ^{1}H-NMR (300 MHz, MeOD): 7.23 (*d*, $J = 8.7$, 1H); 6.69 (*d*, $J = 2.4$, 1H); 6.64 (*dd*, $J = 8.6$, $J = 2.5$, 1H); 5.84 (*s*, 1H); 4.62 (*d*, $J = 8.0$, 1H); 3.81 (*m*, 2H); 3.73 (*s*, 3H); 3.65 (*dd*, $J = 12$, $J = 4.7$, 1H); 3.26 (*m*, 7H); 3.13 (*m*, 1H). ESI-MS: 396 ($[M + Na]^+$).

3.11. (2R)-2-O-β-D-Glucopyranosyl-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one (HDMBOA-Glc, **7**)

White, fine needles, mp 105–108°C (lit. 138–140°C, Kluge, Grambow & Sicker, 1997). UV/Vis (MeOH) λ_{max} 283 sh (ε 6976), 261 (ε 9127), 203 (ε 11,795). CD (MeOH): 307 (0), 290 (−0.021), 260 (−0.30), 247 (0), 233 (+0.72), 215 (0), 213 (−0.23). $[\alpha]_D^{20}$ (MeOH): +32.5° (−25.0 (pyridine), Nagao et al., 1985). IR ν_{max}: 3410, 1694, 1507, 1100–1000 (br). ^{1}H-NMR (300 MHz, MeOD): 7.39 (*d*, $J = 8.9$, 1H); 6.99 (*d*, $J = 2.6$, 1H); 6.93 (*dd*, $J = 8.9$, $J = 2.5$, 1H); 6.09 (*s*, 1H); 4.89 (*d*, $J = 8.0$, 1H); 4.15 (*s*, 3H); 4.08 (*dd*, $J = 12.0$, $J = 1.2$, 1H); 4.00 (*s*, 3H); 3.86 (*dd*, $J = 4.4$, 1H); 3.76 (*t*, $J = 6.6$, 1H); 3.52 (*m*, 5H). ^{13}C-NMR (50.4 MHz, MeOD): 158.1, 155.9, 142.5, 119.7, 113.5, 109.1, 104.4, 103.1, 97.5, 77.6, 77.0, 73.8, 70.1, 62.5, 61.6, 55.3. ESI-MS: 797 ($[2M + Na]^+$), 410 ($[M + Na]^+$).

3.12. (2R)-2-O-β-D-Glucopyranosyl-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (HMBOA-Glc, **10**)

White fine needles, mp 230–235°C (lit. 250–251°C, Nagao et al., 1985). UV/Vis (MeOH) λ_{max} 282 sh (ε 7100), 259 (ε 9141), 205 (ε 12,340). CD (MeOH): 302 (0), 288 (−0.027), 253 (0), 232 (+0.202), 220 (0), 207 (−0.304). $[\alpha]_D^{20}$ (MeOH): +38.4° (+31.4, pyridine, Nagao et al., 1985). IR (KBr) ν_{max}: 3300, 1680, 1530, 1160, 1080, 1060, 1020. ^{1}H-NMR (300 MHz, MeOD): 7.32 (*d*, $J = 8.9$, 1H); 6.87 (*m*, 1H); 6.65 (*dd*, $J = 8.2$, $J = 2.1$, 1H); 5.77 (*s*, 1H); 4.73 (*d*, $J = 7.6$, 1H); 3.80 (*m*, 7H); 3.52 (*m*, 9H). ^{13}C-NMR (50.4 MHz, MeOD): 156.0, 140.7, 118.2, 115.1, 107.8, 102.7, 101.8, 94.5, 76.3, 75.7, 72.7, 68.9, 60.4, 53.9. ESI-MS: 737 ($[2M + Na]^+$), 380 ($[M + Na]^+$).

3.13. Extraction of the roots from A. squarrosa and A. fuscopunctata for the quantification of lactams, hydroxamic acids, and their glucosides

A. fuscopunctata and *A. squarrosa* plants were cultivated by shoot propagation. The time is calculated from the day of planting. For the analysis of the root segments the main root was cut into five segments, the lateral roots were taken together. For the time course experiment the roots of three different plants were taken. The roots were cut, immediately frozen in liquid N_2 and stored at −20°C.

The samples of 0.5 g frozen powder were placed into 10 ml centrifuge vials and extracted three times with 5 ml MeOH at 20°C, the first two times for 4 h, the last time for 16 h. During the maceration process and the weighing of the samples, it is necessary to keep the temperature below 0°C to avoid the hydrolysis of the glucosides followed by degradation of the free hydroxamic acids. The combined extracts were partly evaporated at 40°C in vacuo (until 1 ml) and diluted with water to a final volume of 3 ml.

3.14. Pre-purification of the raw extracts on Sep-pak® C18

The extracts were purified on Sep-pak® C18 cartridges prior to HPLC analysis. For conditioning the cartridges were first washed with 5 ml MeOH followed by 5 ml H_2O. The sample was applied and the cartridge washed two times with 4 ml H_2O. The elution of the hydroxamic acid was achieved in two steps. First, with 4 ml H_2O/EtOH (9:1) and second also with

4 ml H_2O/EtOH (8.5:1.5). Both fractions were combined and evaporated to dryness in vacuo. For regeneration the cartridge was first washed with 5 ml MeOH, then with 5 ml H_2O in backwards flow direction. The cartridge can be used up to five times.

3.15. HPLC-quantification

For the HPLC analysis the dried samples were dissolved in an aliquot of MeOH. The quantification was achieved by the external standard method. Every compound was calculated individually with the help of its calibration curve. The standard deviations were in all measurements below 10%.

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