Chemoecology 17: 1–12 (2007)
0937-7409/07/010001-12

DOI 10.1007/s00049-006-0350-z

CHEMOECOLOGY

# How *Doratomyces stemonitis* copes with Benzoxazolin-2(*3H*)-one (BOA), its derivatives and detoxification products

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**Summary.** *Doratomyces stemonitis* (Hyphomycetales, Dematiaceae) is a saprotrophic fungus belonging to the mycobiota of the cereal rhizosphere. The fungus is able to metabolize benzoxazolin-2-(3*H*)-one and a variety of its derivatives including higher plant detoxification products, microbial degradation products and the chemically rather stable 2-amino-(3*H)*-phenoxazin-3-one. *D. stemonitis* can use all of these compounds as sole C-sources but their utilization, especially that of microbial degradation products and 2-amino-(3*H)*-phenoxazin-3-one, seems to be highly energy consuming, resulting in slow mycelium growth and a change of colony morphology. Benzoxazolin-2-(3*H*)-one-derived compounds induce the synthesis of different isoforms of a glycosylated protein with sequence homologies to the endo-1,3-β-glucanase Asp f2, an allergen from *Aspergillus fumigatus* and other Asp f2-like proteins e.g., from *Verticillium dahliae* or PRA1 antigen from *Candida albicans.* The induction of the protein is regarded as a stress response.

**Key words.** allelopathy – *Doratomyces stemonitis* – benzoxazolin-2(3*H*)-one – glucoside carbamate – 2-amino-3*H*-phenoxazin-3-one metabolization – allergen Asp f 2 similar protein – endo-1,3-β-glucanase

## Introduction

Benzoxazinone glucosides are secondary products of several Poaceae and of a few species belonging to dicotyledoneous families (Alipieva *et al*. 2003; Gierl & Frey 2001, Sicker *et al*. 2000; Sicker & Schulz 2002). They can be released into the environment by plant rotting or by root exudation. The aglycones are compounds with biocidice properties and are therefore of interest for the development of ecologically oriented pest control in agriculture (Bravo & Lazo 1996; Macias *et al*. 2005). The aglycones are not stable in aqueous solution but are converted via ring contraction and release of formic acid to benzoxazolin-2-(3*H*)-ones. These products are still bioactive molecules. For example, benzoxazolin-2-(3*H*)-one (BOA) is thought to be the major phytotoxic component of rye mulch (Barnes & Putnam 1987). Benzoxazolin-2-(3*H*)-ones are more stable than their precursors 2,4-dihydroxy-substitued 2*H*-1,4-benzoxazin-3(*4H*)-ones, with 2,4-dihydroxy-2*H*-1,4-benzoxazin-3 (*4H*)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-2*H*-1, 4-benzoxazin-3(*4H*)-one (DIMBOA) as main representatives (fig. 1). Nevertheless, they are degraded by microorganisms able to cleave the heterocycle. One of the resulting products, 2-aminophenol, undergoes oxidative dimerization to 2-amino-3*H*-phenoxazin-3-one which has been isolated from soil samples (Gagliardo & Chilton 1992). Recent investigations showed that the compound was persistent in wheat crop soils over a period of three months (Macias *et al*. 2005).

Several bacteria and fungi have been described which are able to degrade benzoxazolin-2-(3*H*)-one (Gagliardo & Chilton 1992; Friebe *et al*. 1996). A number of fungal plant pathogens are sensitive to benzoxazinones and benzoxazolinones whereas others, including several *Fusarium* species, were found to be less affected since they are able to degrade these allelochemicals (Morrissey & Osbourn 1999).

An endophytic fungus from *Aphelandra tetragona* (this plant species is one of the rare dicots containing benzoxazinones as secondary compounds) was found to produce a number of hydroxylated 2-amino-*3H*-phenoxazin-3-one derivatives from 2-hydroxy-*2H*-1,4-benzoxazin-3(*4H*)-one (HBOA) and BOA (Baumeler *et al*. 2000; Zikmundova *et al*. 2002a; Zikmundova *et al*. 2002b). The maize endophytic fungus *Fusarium verticillioides (F. moniliforme)* and several wheat-associated fungi transform BOA and the 6-methoxybenzoxazolin-2-(*3H*)-one (MBOA) into N-(2-hydroxyphenyl)-and N-(2-hydroxy-4-methoxyphenyl)-malonamic acid (HPMA and HMPMA, respectively). (Glenn *et al*. 2003; Yue *et al*. 1998; Friebe *et al*. 1998). In *Fusarium verticillioides* two genes have been identified, *FDB1* and *FDB2,* that are responsible for BOA and MBOA detoxification. The *FDB1* encoded enzyme is proposed to hydrolyze the oxazolinone ring, which in combination with

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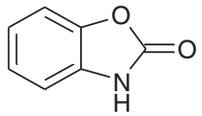
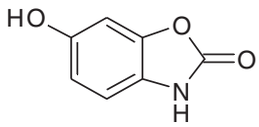
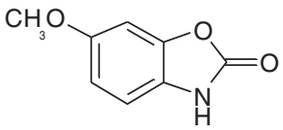
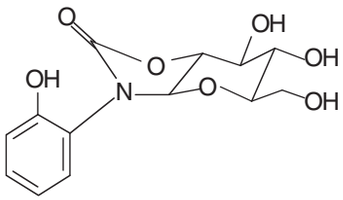
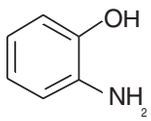
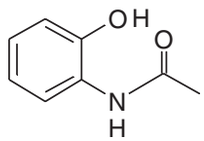
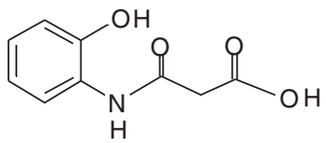
| *Structure* | *Acronym* | *Compound No.* | *Name* |
|---|---|---|---|
|  | BOA | 1 | benzoxazolin-2-(3*H*)-one |
|  | BOA-6-OH | 2 | 6-hydroxybenzoxazolin-2(*3H*)-one |
|  | MBOA | 3 | 6-methoxybenzoxazolin-2-(*3H*)-one |
|  | | 4 | 1-(-2-hydroxyphenylamino)-1-deoxy-ß-D-glucoside-1,2-carbamate (glucoside carbamate) |
|  | | 5 | 2-aminophenol |
|  | AAP | 6 | 2-acetamidophenol |
|  | oHPMA | 7 | N-(2-hydroxyphenyl) malonamic acid |

*Fig 1.* (*continued*)

decarboxylation results in 2-aminophenol. The enzyme encoded by *FDB2* is thought to use 2-aminophenol as a substrate for HPMA synthesis since in *FDB1/fdb2* mutants, 2-acetamidophenol is produced instead of HPMA. This implies that BOA and probably numerous other allelochemicals too, induces alterations in fungal gene expression followed by *de novo* syntheses of proteins, which may be, for instance, involved in BOA degradation pathways. At present, no study is available where the influence of benzoxazinoids on fungal protein pattern has been investigated.

Many plants also have the potential to diminish harmful effects of BOA by detoxification, especially cereals and other grasses, as well as European weeds that co-exist with benzoxazinones-containing crops like rye and wheat (Schulz & Wieland, 1999; Sicker *et al*. 2001). In plants, two detoxification pathways for BOA are known at present, the first one leads to the accumulation of BOA-6-O-glucoside via 6-hydroxybenzoxazolin-2(*3H*)-one (BOA-6-OH), an intermediate that is toxic to higher plant radicle growth. The second detoxification pathway results in 1-(-2-hydroxyphenylamino)-1-deoxy-β-D-glucoside-1,2-carbamate (glucoside carbamate) production. This pathway appears to be the more effective one since in bioassays, glucoside carbamate was found to be not toxic for higher plants at

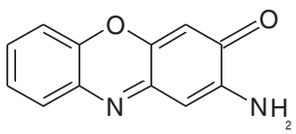
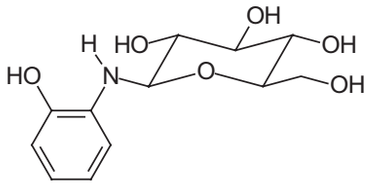
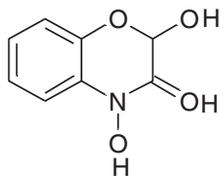
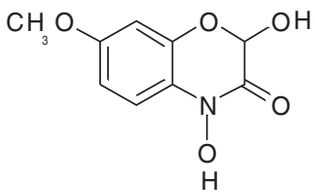
| *Structure* | *Acronym* | *Compound No.* | *Name* |
|---|---|---|---|
|  | | 8 | 2-amino-3*H*-phenoxazin-3-one |
|  | H1 | 9 | 2-aminophenol-N-ß-D-glucopyranoside |
|  | DIBOA | - | 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(*4H*)-one |
|  | DIMBOA | - | 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(*4H*)-one |

**Fig. 1** Structures, acronyms and names of DIBOA, DIMBOA and the compounds used for incubations

concentrations up to 1 mM. While detoxification via BOA-6-O- glucoside was found in all species tested, glucoside carbamate production was found to be the major detoxification pathway in Poaceae. In *Zea mays* glucoside carbamate can be further glucosylated yielding gentiobioside carbamate or malonylated to malonyl glucoside carbamate (Hofmann *et al*. 2006). At least a portion of these detoxification products does not stay within the root, the most important plant organ for BOA detoxification, but is exuded. Thus, not only BOA from benzoxazinoid - producing species but also BOA detoxification products are released into the soil. This raises the question whether or not any soil- or root-colonizing micro-organisms exist that are able to metabolize or convert the degradation products released by the plants, including highly toxic BOA-6-OH, and whether the compounds influence the protein pattern of the micro-organisms.

Promising organisms in this respect are fungi belonging to the mycobiota of the cereal rhizosphere. In this study we tested one member of this group, *Doratomyces stemonitis* (Hyphomycetales, Dematiaceae), a saprotrophic fungus living on plant material in soils of Europe and North America, for its ability to cope with MBOA, BOA, BOA detoxification products from higher plants and additional BOA derived compounds. We were particularly interested in alterations of fungal protein patterns in response to allelochemical treatment.

## Material and Methods

*Doratomyces stemonitis* was obtained from the collection of D.K. Zabolotny, Institute of Microbiology and Virology, National Academy of Sciences of the Ukraine. Mycelial plugs from agar plates (discs 1 cm in diameter with 0.5 mg mycelium) were transferred into 250 ml flasks with 100 ml sterilized Czapak medium. For growth tests (series A), the incubations were performed in the dark with complete medium and medium lacking sucrose at 25 °C (controls), and with addition of either BOA (**1**), BOA-6-OH (**2**), MBOA (**3**), glucoside carbamate (**4**), 2-aminophenol (**5**), 2-acetamidophenol (AAP, **6**), N-(2-hydroxyphenyl)-malonamic acid (oHPMA, **7**), 2-amino3*H*-phenoxazin-3-one (**8**), or 2-aminophenol-N-β-D-glucopyranoside (H1, **9**), (fig. 1). The compounds were found to be stable at 25 °C in the dark and under sterile conditions over a period of two months. Each compound was tested individually. The concentration of each tested compound was 10 µmol, the one of 2-amino-3*H*-phenoxazin-3-one was 1 µmol. For metabolism studies the incubations were performed in the same manner, but with medium lacking sucrose using mycelia previously cultivated in presence of sucrose (series B: BOA and higher plant detoxification products **1**, **2**, **3**, **4**; series C: microbial compounds **6, 7, 8**, and the synthetic compound **9**). For 2-dimensional electrophoresis mycelia were incubated

without sucrose. BOA and 2-acetamidophenol were from Aldrich, MBOA, BOA-6-OH, N-(2-hydroxyphenyl) malonamic acid and 1-(-2-hydroxyphenylamino)-1-deoxy-β-D-glucoside 1,2-carbamate (glucoside carbamate) were synthesized as previously described (Sicker, 1989; Wieland *et al*. 1999; Friebe *et al*. 1998; Wieland *et al*. 1998; Sicker *et al*. 2001). Hitherto undescribed 2-aminophenol-N-β-D-glucopyranoside was obtained by the following method: 2-Aminophenol (1.9 g, 10 mmoles) and D-glucose (1.8 g, 10 mmoles) were heated in ethanol (30 ml) under reflux and $N_2$-atmosphere for 12 h. The volume of the mixture was reduced to 10 ml in vacuo and the product allowed to stand at 4 °C overnight resulting in white crystals (2.1 g, yield 77%), mp. 157-159 °C (EtOH). All spectroscopic analyses were in accordance with the structure and will be published elsewhere.

The cultures were analyzed after 3 weeks of cultivation, unless otherwise stated. Mycelia were separated from the liquid by filtration through 100µm nylon nets, dried between paper sheets, weighed and extracted with 70% methanol. The medium was extracted with ethyl acetate. The organic and aqueous phases were evaporated to dryness and the residues dissolved in 70 % methanol. The resulting solutions were analyzed by HPLC.

*HPLC analysis*

HPLC was performed with a Beckman model 126 chromatograph equipped with a diode array detector 168 and an Ultrasphere ODS RP 18 column. Compounds were eluted by the following linear gradient: 0-1 min solvent A, 1- 21 min 20 % methanol in solvent A, 21-40 min 80 % methanol, 41-43 min 100 % methanol; A: 0.1 % TFA in $H_2O$. The detection wavelengths were 280 and 227 or 410 nm. The identity of known compounds was verified by co-chromatography with synthetic and natural reference substances and by the UV-scans. The phenoxazinone references (2-acetylamino-3*H*-phenoxazin-3-one, 2-(2-hydroxyacetyl)-amino-3*H*-phenoxazin-3-one,2-amino-8-hydroxy-3*H*-phenoxazin-3-one, 2-amino-8-hydroxy-3*H*-phenoxazin-3-one, 2-acetylamino-8-hydroxy-3*H*-phenoxazin-3-one,2-acetylamino-8-methoxy-3*H*-phenoxazin-3-one) were a gift from Dr. C. Werner, University of Zürich.

The major compound appearing in glucoside carbamate medium was isolated by HPLC using the column and the gradient described above. With 18 analytical HPLC runs 0.8 mg of the compound could be obtained. The solution was evaporated by vacuum centrifugation to dryness and the residue analyzed by 1H-NMR and EI-HR-MS.

*Two dimensional Electrophoresis*

Two dimensional electrophoresis was performed with the BioRad Protean IEF Cell and the BioRad Mini Protean II Cell, using Ready Strip ™ IPG strips pH 4-7 (BioRad). The mycelia were collected by filtration (100µm nylon net), washed with distilled water, dried between paper sheets and weighed. They were vigorously mortared with quartz sand and the 6 - fold (w/v) amount of extraction buffer (9.5 M urea with 400 mg Chaps, 150 mg dithiothreitol, 80 µl 50% n-hexyl-β-D-glucopyranoside, 40 µl 10% n-decyl-ß-D-maltopyranoside, 20 µl 10% SDS, 200 µl ampholyte Pharmacia TM 3-10 for IEF, all per 10 ml buffer). The homogenates were centrifuged at 20,000 g for 15 min at room temperature. 500 µl of the supernatants were used for protein precipitation with ice-cold methanol (1,5 ml / 500 µl). The precipitates were centrifuged for 10 min (20,000g, 4 °C) and the pellets washed twice with 200 µl ice-cold acetone. The pellets were dried to remove residual acetone and dissolved in 200 µl extraction buffer. 50 µl of the protein solutions were used for IEF, which was run with 50 µA/gel strip and finished after 20,000 vh. The strips were previously equilibrated in equilibration buffer (6 M urea, 30% w/v glycerol, 2 % SDS in 0.05M Tris-HCl pH 8.8) with 100 mg DTT / 10 ml for 10 min, followed by a 10 min equilibration in presence of iodoacetamide (400 mg/ 10 ml). Per strip, 2 ml of the buffers were used. The equilibrated strips were rinsed with distilled water for 2 seconds and used for SDS-PAGE (17.5 % gels) as the second dimension. The gels were stained with Coomassie Blue, destained and scanned. Major spots were excised for protein identification.

*Digest protocol*

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.

*In-gel Deglycosylation protocol*

A second round of samples underwent in-gel deglycosylation with PNGase F (Sigma P 7367) according to the manufacturer's instructions prior to tryptic digestion. In short, following destaining, excised spots were dried via vacuum centrifugation and then rehydrated with a PNGase F solution and incubated 30 minutes at 37 °C, water was added to cover the gel pieces and samples were incubated overnight at 37 °C. The gel pieces were then repeatedly rinsed with water to remove residual PNGase F, and tryptic digests were performed as described above.

*MS - Methods*

LC MS/MS data were collected using a Q-ToF II tandem mass spectrometer coupled to a CapLC system fitted with a 75um NanoEase Atlantis dC18 analytical column and a

**Table 1** Series A: weight of mycelium after 6 weeks of incubations with compounds 1, 2, 3, 4, and 3 weeks of incubation with compounds 5, 6, 7, 8, 9. Weight of control 0.441 ± 0.04 g in average, data present average values and the standard deviations (n = 4).

| Compound | Weight of mycelium (g) cultured without sucrose | Weight of mycelium (g) cultured with sucrose |
|---|---|---|
| BOA (**1**) | 0.143 ± 0.029 | 0.256 ± 0.012 |
| BOA-6-OH (**2**) | 0.145 ± 0.021 | 0.272 ± 0.08 |
| MBOA (**3**) | 0.117 ± 0.05 | 0.25 ± 0.09 |
| glucoside carbamate (**4**) | 0.136 ± 0.03 | 0.193 ± 0.01 |
| 2-aminophenol (**5**) | 0.129 ± 0.017 | 0.11 ± 0.03 |
| 2-acetamidophenol, AAP (**6**) | 0.146 ± 0.055 | 0.293 ± 0.05 |
| N-(2-hydroxyphenyl)malonamic acid, oHPMA(**7**) | No growth | 0.272 ± 0.07 |
| 2-amino-3*H*-phenoxazin-3-one (**8**) | no growth | no growth |
| N-(2-hydroxyphenyl)-β-D-glucopyranosylamine, (**9**) | 0.113 ± 0.014 | 0.11 ± 0.02 |

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% acetronitrile 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 4-5x10(-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 LutefiskXP 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).

## Results

*Growth studies*

When the fungus was cultured in the complete medium (with sucrose) containing BOA (**1**), BOA-6-OH (**2**) or MBOA (**3**) at concentrations up to 10 µmol growth was inhibited by about 40% in comparison to the control (table 1, series A). In the presence of the compounds growth started after a lag phase of one to two weeks. Without sucrose, mycelial growth was more strongly inhibited by the three compounds **1**,**2**, and **3** (up to 70 %).

Glucoside carbamate (**4**) displayed slightly stronger inhibition (up to 55 % with sucrose, 70 % without sucrose), and a longer lag phase was observed (up to three weeks).

The growth inhibition by 2-aminophenol (**5**), compound (**9**) and 2-amino-3*H*-phenoxazin-3-one (**8**) was independent from the presence of sucrose. Mycelial growth was reduced to 25% of the control by (**5**) and (**9**). The strongest reduction (more than 90 % inhibition) was found with 2-amino-3*H*-phenoxazin-3-one (**8**). 2-Acetamidophenol (AAP, **6**) and N-(2-hydroxyphenyl) malonamic acid (oHPMA, **7**) inhibited growth to a similar extent as observed with BOA (**1**) or BOA-6-OH (**2**) (both about 70% without sucrose, 40-35% with sucrose). The growth data are presented in table 1.

Longer lag phases (2 up to 3 weeks) were accompanied by changes in the colony morphology: many small pellets were observed rather than the few large, fluffy colonies seen in the controls. Changes in colony morphology together with a propensity to generate conidia and particular chlamidospores were observed especially with MBOA, BOA-6-OH and 2-amino-3*H*-phenoxazin-3-one. An alteration in the morphology of the hyphae was also observed (fig. 2). *D. stemonitis* formed discolored, swollen and twisted hyphae with numerous big, round chlamidospores as a reaction to unfavorable conditions (fig. 2 b) The presence of 2-amino-3*H*-phenoxazin-3-one, for example, caused also a simplification of sporulation structures. Formation of conidiophores was found on single hyphae and not in coremia (fig. 2 c and d).

Accumulation of the compounds **1-4** within the mycelium was negligible, with concentrations in the pmol range (table 2), but it is likely that these low amounts act as priming agents, since shorter lag phases (less than one week) were observed in subsequent incubations when material from these cultures was used.

*Metabolism of compounds*

*Benzoxazolinones and higher plant detoxification products*

For studying compound metabolism, a series of incubations were carried out in the absence of sucrose with mycelia previously cultured in complete medium or without sucrose for depletion of C-stores within the hyphae and metabolite levels are presented in table 2. BOA (**1**), BOA-6-OH (**2**), MBOA (**3**) and glucoside carbamate (**4**) were degraded. In the absence of sucrose, the final concentration of glucoside carbamate (**4**), MBOA (**3**) and BOA-6-OH (**2**) concentration was lower. The latter compound was completely degraded,

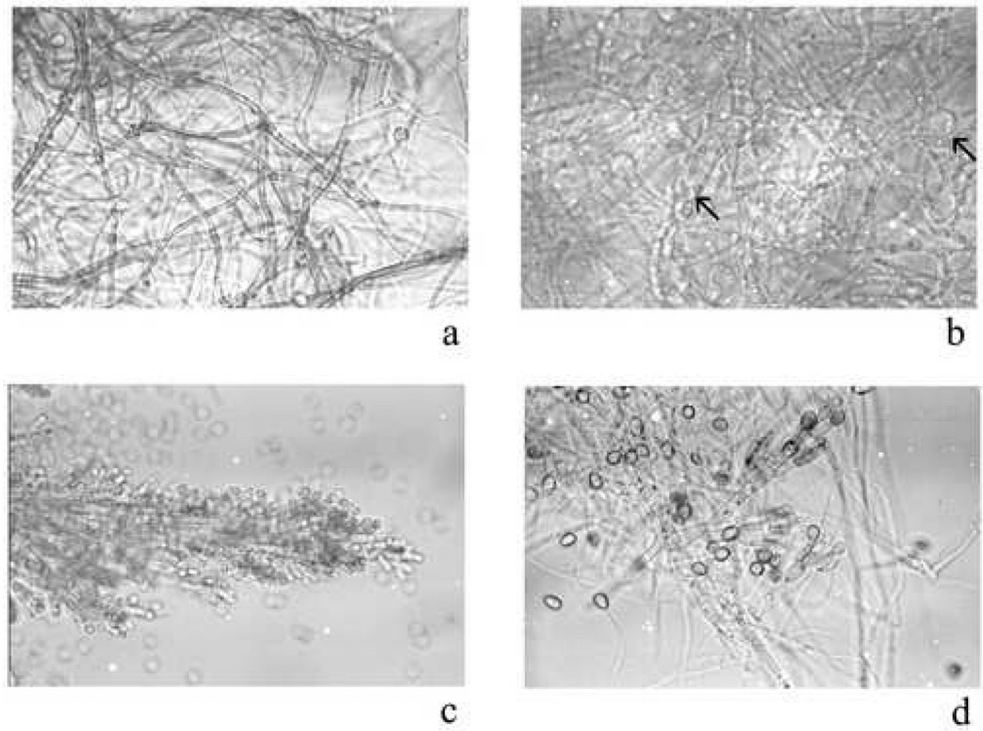



**Fig. 2** Morphological changes of *Doratomyces stemonitis* mycelium in presence of phenoxazinone. A: mycelium, control (x 198); b: mycelium, grown in presence of phenoxazinone, with swollen hyphae and formation of chlamidospores (arrows); c: conidiophore on coremia, control (x 132); d: conidiophore formed without coremia in presence of phenoxazinone (x 198). All incubations were performed in complete medium (with sucrose)

**Table 2** Series B: Metabolism of BOA and higher plant detoxification products. Amount of compound left in the medium (µmol), (n = 4). Incubation time: 6 weeks. Data present average values and standard deviations (n = 4).

| Culture In presence of 10 µmol | Highest Accumulation pmol/mg mycelium (incubation without sucrose) | Highest Accumulation pmol/mg mycelium (incubation with sucrose) | Incubation in presence of sucrose, Mycelium from culture with sucrose | Incubation without sucrose, Mycelium from culture with sucros | Incubation without sucrose, Mycelium from culture without sucrose |
|---|---|---|---|---|---|
| BOA (**1**) | 83,5 ± 6 | 46 ± 7,6 | 4,12 ± 0,22 | 3,45 ± 0.37 | 2,37 ± 0.48 |
| BOA-6-OH (**2**) | 100,5 ± 9,7 | 16,6 ± 3,4 | 3,75 ± 1,28 | traces | traces |
| MBOA (**3**) | traces | n.d. | 5,45 ± 0,65 | 2,27 ± 0,64 | 2,3 ± 0,96 |
| glucoside carbamate(**4**) | 36 ± 5,4 | 11,2 ± 2,2 | 7,15 ± 1,05 (BOA: traces) | 1,97 ± 1,61 (3,4 ± 1,32 BOA) | 0,95 ± 0,64 ( BOA: traces) |

with only trace amounts remaining. From these experiments we conclude that *D. stemonitis* can use most of the compounds as a C-source (table 2, series B).

BOA-6-OH (**2**) was degraded without considerable accumulation of new compounds. Only traces of 2-acetylamino-7-hydroxy-3*H*-phenoxazin-3-one were present. Interestingly, the largest amount of BOA (**1**) was metabolized via BOA-6-OH (**2**), which was found in the medium together with traces of 2-amino-3*H*-phenoxazin-3-one (**8**). MBOA (**3**) was diminished in the incubation medium without accumulation of any additional product. Neither BOA-6-OH (**2**) nor BOA (**1**) nor 3*H*-phenoxazin-3-ones could be detected. Apparently, there are two different routes of BOA degradation, one via BOA-6-OH (**2**) and another one by direct cleavage of the heterocycle. As accumulation of 2-amino-3*H*-phenoxazin-3-ones was generally low and not adequate to explain the disappearance of BOA-6-OH (**2**) or BOA (**1**), accumulation of high levels of 2-aminophenol (**5**) amounts seems to be avoided.

Glucoside carbamate (**4**) was not metabolized directly (fig. 3, tab. 2). Instead, a compound with an UV spectrum and a retention time identical to BOA (**1**) appeared and could be isolated from the medium. Chemical analysis of this compound verified it to be BOA (**1**). Thus BOA represents an intermediate of glucoside carbamate degradation, which is further degraded to 2-aminophenol (**5**), a fraction

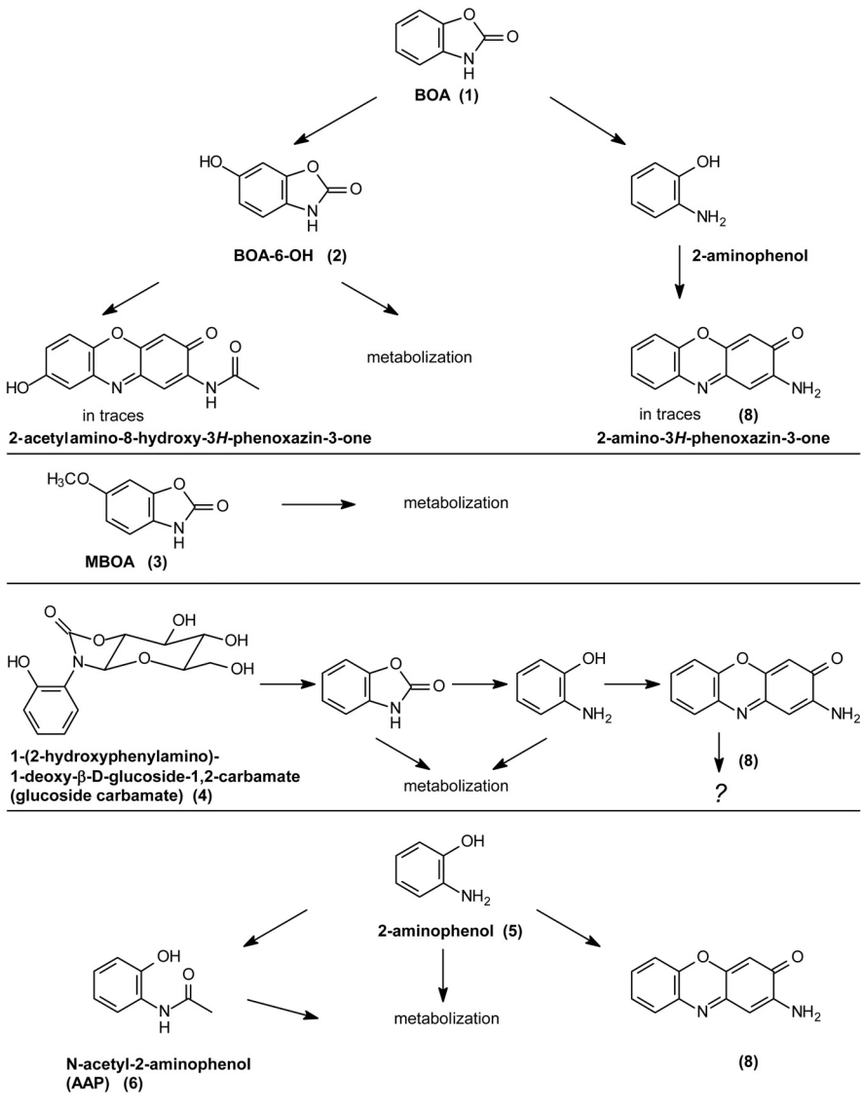



**Fig. 3** Metabolization of different BOA derivatives, higher plant and microbial detoxification products.

of which is detoxified by acetylation yielding 2-acetamidophenol (**6**), while a considerable amount is converted to 2-amino-3*H*-phenoxazin-3-one (**8**). BOA-6-OH (**2**) was detectable only in traces in the medium. It is not clear why the hydroxylation of BOA (**1**) to BOA-6-OH (**2**) observed with BOA incubations does not occur prior to its further degradation in this pathway.

*Microbial degradation products*

In all incubations supplemented with 2-aminophenol (**5**), the compounds 2-acetamidophenol (AAP,**6**), 2-amino-3*H*-phenoxazin-3-one (**8**) and 2-acetylamino-3*H*-phenoxazin-3-one could be found in high concentrations, indicating that a large amount of 2-aminophenol (**5**) was not directly degraded and only a part was detoxified via 2-acetamidophenol (**6**) production. Since 2-aminophenol (**5**) starts to dimerize to 2-amino-3*H*-phenoxazin-3-one (**8**) immediately after application to the media, incubation conditions were very soon comparable with incubations that were directly started with 2-amino-3*H*-phenoxazin-3-one (**8**). Therefore it was not possible to determine residual 2-aminophenol (**5**) concentrations at the end of the incubation period.

After three weeks of incubation with 2-aminophenol-N-β-D-glucopyranoside ( **9**) this synthetic compound could not be detected in the medium any more and only traces of 2-amino-3*H*-phenoxazinone (**8**) were present (table 3, series C). In incubations with 2-acetamidophenol (AAP,**6**), 40 to 50% of it was degraded within the same incubation period. The fungus was able to hydrolyze malonamic acid from N-(2-hydroxyphenyl) malonamic (oHPMA, **7**) acid resulting in traces of 2-amino-3*H*-phenoxazin-3-one (**8**). When 1 µmol 2-amino-3*H*-phenoxazin-3-one (**8**) was directly applied to the medium, most of it was obviously metabolized without considerable accumulation of UV-active intermediates. Figure 3 illustrates the degradation of the different compounds.

*Influence on major proteins*

Two dimensional electrophoresis with protein extracts from the mycelia that used the compounds as a C-source revealed alterations of the protein pattern (fig. 4). Several of the

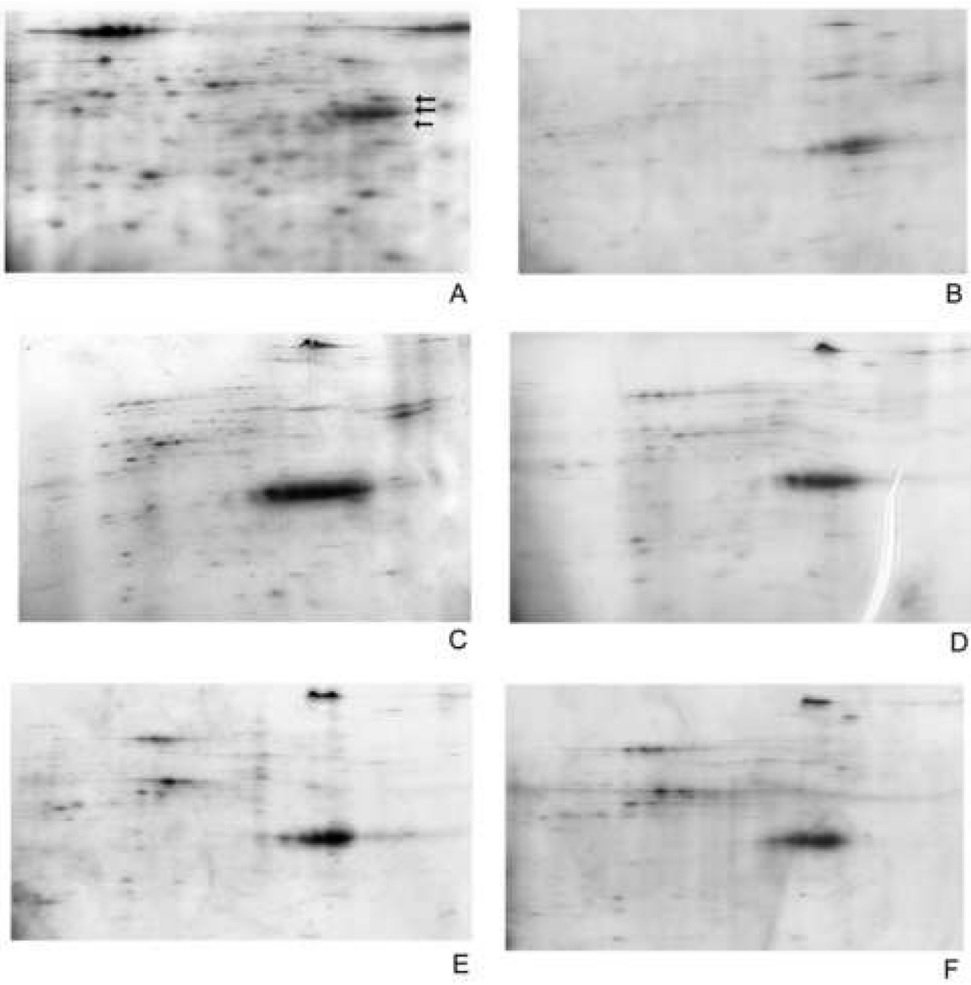



**Fig. 4** Two-dimensional electrophoresis of D. stemonitis mycelia. A: control; after incubation with B: BOA; C: MBOA; D: BOA-6-OH, E: 2-acetaminophenol, similar to 2-aminophenol and phenoxazinone incubation (data not shown); F: glucoside carbamate. The arrows (A) point to a group of proteins which were highly effected by the compounds and to minor spots adjacent to this row

**Table 3** Series C: Metabolism of microbial and one synthetic BOA derived compounds, µmol presents amount of compound left in the medium. Incubation time: 3 weeks. Data presents average values and standard deviations (n = 4).

| Culture in presence of 10 µmol | Incubation without sucrose, Mycelium from culture without sucrose | Detectable degradation products |
|---|---|---|
| 2-acetamido-phenol, AAP **(6)** | 5,7 ± 0,25 | Phenoxazinones (traces) |
| N-(2-hydroxyphenyl)malonamic acid, oHPMA **(7)** | 6,3 ± 0,58 | Phenoxazinones (traces) |
| 2-amino-phenoxazinone **(1 µmol) (8)** | 2,9 ± 0,54 | Low amounts of unknown products |
| N-(2-hydroxyphenyl)β-D-glucopyranosylamine **(9)** | Not detectable | Phenoxazinones (traces) |

major proteins of the control samples were strongly diminished whereas others were new or increased. A group of major proteins in the pH range of 6 and a molecular weight range of 40-45 kD was especially affected. In the control samples this group consists of at least 9 spots, five of which (spot 3-7) are arranged in a row. Several small spots were adjacent to this row of proteins. The major spots were of special interest because they increased dramatically in abundance when the fungus was grown in presence of MBOA (**3**) and BOA detoxification products. The strongest increase was observed with MBOA (**3**) and to a lower extent with BOA-6-OH (**2**). Glucoside carbamate (**4**) and 2-aminophenol (**5**) (2-amino-3*H*-phenoxazin-3-one, **8**) also caused some increase in abundance of the proteins while abundance of others decreased. The same was found with 2-acetamidophenol or H1 (**9**) (not shown).

**Table 4** Analyzed strongest de novo peptide sequences

| | | |
|---|---|---|
| LAATAHATA[YR] | 1144.59 | +2 |
| RAMPADALEETVVLAR | 1740.84 | +2 |
| [158.08]DRGDMLFR | 1166.55 | +2 |
| [158.09]AQYG[509.09]TQ NDWAGHWR | 2355.94 | +3 |
| WDATAETVICDASFR | 1740.79 | +2 |
| [184.12]NSSLCGGGYTV VESPLNTYWAVDLLHR | 3191.62 | +3 |
| [260.15]ENPDYFAEVFAK | 1688.82 | +2 |

To elucidate the identity of major protein sensitive to BOA treatment, selected protein spots were excised and subjected to LC/MS/MS analysis. Though spots were strong, the initial sample digests resulted in very few fragmentation spectra. The strongest and most frequently observed precursor yielded *de Novo* sequences containing the subsequence NSSLCGGGY TVVESPLNTYWAVDLLHR, which hit fungal proteins during MSBLAST searching (Shevchenko *et al*. 2001). Searching with BLAST (Altschul *et al*. 1997) the following hits were obtained: Allergen Asp f2-like protein from *Verticillium dahliae*, EAA73422.1 hypothetical protein from *Gibberella zeae*, EAA51681 hypothetical protein from *Magnaporthe grisea*. The repeated observation of this peptide mass in multiple samples indicates that other spots in the row are isoforms of one protein.

The first SwissProt entry among the BLAST hits for this peptide is the pH-regulated antigen PRA1 precursor from *Candida albicans* (P87020) a secreted glycoprotein. The *de novo* peptide observed aligns to a region between proposed glycosylation sites on PRA1. We therefore performed an in-gel deglycosylation reaction prior to digestion and analysis of subsequent samples. The strongest *de novo* peptide sequences from this analysis were used as queries for MSBLAST and sequences matching Trypsin and PNGase F were removed (table 4). The resulting MSBLAST matched six new peptides to Allergen Asp f2-like proteins from various fungi. A multiple alignment of the six best protein hits and the fit of the seven *de novo* sequences to the alignment is shown in figure 5. The observed peptides constitute roughly 30% of the likely protein sequence. The first aspartate in the peptide sequence WDATAETVICDASFR aligns with a conserved asparagine which is annotated as an N-glycosylated site in the annotated proteins. This conflict is consistent with the deamidation reaction that occurs when PNGase F removes the N-glycosylation.

In the course of analysis, spots adjacent to the main row were also digested and analysed and a *de novo* peptide matching Aspartate protease was found.

## Discussion

Detoxification of BOA (**1**) and MBOA (**3**), described for several fungi e.g. *Fusarium verticillioides, Fusarium subglutinans* and *Gaeumannomyces graminis* (Glenn *et al*. 2003, 2002, 2001; Yue *et al*. 1998; Friebe *et al*. 1998, results in the major detoxification products N-(2-hydroxyphenyl) malonamic acid (oHPMA, **7**) and N-(2-hydroxy-4 methoxyphenyl)-malonamic acid. 2-Acetamidophenol (AAP, **6**) is thought to represent a branch product synthesized by *F. verticillioides*. *Doratomyces stemonitis* does not produce oHPMA (**7**), but 2-acetamidophenol (AAP, **6**) is detectable under the growth conditions used when media were supplemented with 2-aminophenol (**5**) or glucoside carbamate (**4**). Thus, *D. stemonitis* does not seem to possess the same detoxification capacity as, for example, *Fusarium verticillioides.* However, *D. stemonitis* can use the compounds BOA (**1**), MBOA (**3**), and BOA-6-OH (**2**) a C-source at concentrations of up to 10 µmol.

Degradation of the chemically relatively stable 2-amino-3*H*-phenoxazin-3-one (**8**) seems to be a challenge for *D. stemonitis,* as indicated by the extremely slow growth, independent of the presence or absence of sucrose. All the compounds tested, except for MBOA, finally led to the accumulation of 2-aminophenol (**5**), which underwent easily dimerization to 2-amino-3*H*-phenoxazin-3-one (**8**), some of which was further converted. From N-(2-hydroxyphenyl) malonamic acid (oHPMA, **7**) and from 2-acetamidophenol (AAP,**6**) the acetyl group and the malonyl group, respectively, can be hydrolyzed prior to further metabolization. Clearly, *D. stemonitis* has the capacity to degrade 2-amino-3*H*-phenoxazin-3-one (**8**) when applied at low concentrations, but the activated metabolic machinery results in severe growth inhibition and a reduced biomass.

In contrast to *D. stemonitis*, *F. verticillioides* is able to grow in the presence of significantly higher concentrations of 2-aminophenol (**5**), and its detoxification appears to be completed within 3 days ( Glenn *et al*. 2003, 2002, 2002; Yue *et al*. 1998). This indicates tremendous differences in the ability of fungi to cope with allelopathic compounds, such as BOA (**1**).

Detoxification of the benzoxazolinones is thought to enhance the ecological fitness of fungi that get in contact with plants containing benzoxazinoids (Glenn *et al*. 2003). The same should be true for higher plants. As already mentioned, a portion of the higher plant detoxification products is exuded by the roots. *D. stemonitis* has the capacity to cleave the glucose moiety from glucoside carbamate (**4**), a more complex enzymatic reaction than the simple hydrolysis catalyzed by a common β-glucosidase. BOA degradation may start at the moment when the glucoside carbamate is no longer available. In fact, the BOA concentration might even increase in the rhizosphere due to the activity of fungi, which may regenerate the original phytotoxic compound from the non-toxic metabolite glucoside carbamate (**4**). As a result the allelopathic impact that BOA may exert on the plants via the rhizosphere may temporarily increase.

With regard to the fungus living in the rhizosphere, BOA (**1**) can be reabsorbed by the roots of a higher plant that is able to synthesize glucoside carbamate (**4**) and to exude it again. When re-absorption stops, BOA regenerated from glucoside carbamate may be directly degraded to 2-aminophenol (**5**) and phenoxazinones. On the one hand, glucoside carbamate (**4**), which is not toxic to higher plants at concentrations up to 1 mM, has the potential to inhibit growth of *D. stemonitis* as a result of 2-amino-3*H*-phenoxazin-3-one formation. On the other hand, the intermediate 2-aminophenol (**5**) can be converted to 2-acetamidophenol (AAP,**6**) or N-(2-hydroxyphenyl) malonamic acid (oHPMA,

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CLUSTAL W (1.82) multiple sequence alignment


sp|P79017|ALL2_ASPFU            ----MAALLRLAVLLPLAAPLVATLPTSP-------VPIAARATPHEPVF 39
sp|Q00746|ANG1_EMENI            -------MQLLALTLALCAS-IAALPTQQ-------TPLPLEDPIKSP-- 33
tr|Q8J0P2|Q8J0P2_COCPO          ------MQLYLVSLLAVVSPLLAAPLAGR-------NSYPGNDLPAWD-- 35
tr|AACU01000690|Magnaprthe      ---MKTSSIILAASLAATAVALPAGGGEPDRVVTITVPAPPTAATAAPTW 47
tr|Q6QUX8|Q6QUX8_VERDA          -MLSLQTAALLLFPLVAASPVARAAETSVT------VTVDTAPAGPTSST 43
tr|AACM01000168|                MMFKSTTAAMLLFGAATATPLFGRAEASQT------KSASQSSKTSESSS 44
                                          *      :                .
peptides                                  LAATAHATA


sp|P79017|ALL2_ASPFU            FSWDAGAVTSFPIHSSCNATQRRQIEAGLNEAVELARHAKAHILRWGNES 89
sp|Q00746|ANG1_EMENI            ----------FPIHHSCNATEQRQLATALQETVTLAEHAKDHILRWGNES 73
tr|Q8J0P2|Q8J0P2_COCPO          ----HGAVTQYPIHDSCNVTERRLIERGLQDAITLANHAKKHVLRFSNSS 81
tr|AACU01000690|Magnaprthe      N-WREGAVDSYPIHSSCNGTERLQLARALDETVSLARQARDHILRFGKTS 96
tr|Q6QUX8|Q6QUX8_VERDA          YNWAEGWKANFPIHQSCNITLRTQLEAALAETMTIAAHARDHLL-HNPKS 92
tr|AACM01000168|                YNWSEGWTKDYPIHQSCNATLRHQLSSALDETVQLAQHAKDHILRHGHKS 94
                                          :*** *** * :  :  .* ::: :* :*: *:*  .  *
peptides                                  RAMPADALEETVVLAR


sp|P79017|ALL2_ASPFU            EIYRKYFGNRPTMEAVGAYDVIVNGDKANVLFRCDNPDGNCA-LEGWGGH 138
sp|Q00746|ANG1_EMENI            AIYRKYFGDRPSLTAIGAYDIIVNGNPDNILFRCDNPDGNCA-LEGWGGH 122
tr|Q8J0P2|Q8J0P2_COCPO          SIYRKYFGNAPSGEVIGNLDRIVNADRAKTLFRCDNPDGNCK-LPKWGGH 130
tr|AACU01000690|Magnaprthe      SLYSKYFGNASTAEPVGWYHKLVGGDKAGLLFRCDDIDGNCH-QEGWGGH 145
tr|Q6QUX8|Q6QUX8_VERDA          ELATKFFGNQSVAGPIGWYSKVVSTDKSEMLFRCDDPDRNCATQDGWAGH 142
tr|AACM01000168|                EFFTKYFGNASTSQPIGWYDRVVNADKTGVLFRCDDPDKNCATQDAWAGH 144
                                 :  *:**: .    :*    :*. :    *****: * **     *.**
peptides                                        DRGDMLFR         TQNDWAGHWR


sp|P79017|ALL2_ASPFU            WRGA<b>NAT</b>SETVICDRSYTTRRWLVSMCSQGYTVAGSETNTFWASDLMHRL 188
sp|Q00746|ANG1_EMENI            WRGE<b>NAS</b>DETVICELSYTTRRSLSTMCSQGYTISEWETNTFWAGDLLHRL 172
tr|Q8J0P2|Q8J0P2_COCPO          WRGS<b>NAT</b>GETVICPLSYTTRLFLEHFCTRGYTVAGSPLNTYFGTDLLHRL 180
tr|AACU01000690|Magnaprthe      WRGE<b>NAT</b>SETVICPLSYTTRKSLEGLCGFGYTVAAGKLNTFWAGDLMHRI 195
tr|Q6QUX8|Q6QUX8_VERDA          WRGS<b>NAT</b>QETVICDLSYEIRRPLAALCGGGYTVAESKLNTYWATDLLHRA 192
tr|AACM01000168|                WRGD<b>NAT</b>SETVICPLSFEIRRNLDSVCNLGYTVANSKLNTFWATDLLHRV 194
                                *** **: *****  *:  *  *  .*  ***::    **::. **:**
peptides                        WR W<b>DAT</b>AETVICDASFR    NSSLCGGGYTVVESPLNTYWAVDLLHR


sp|P79017|ALL2_ASPFU            YHVPAVGQGWVDHFADGYDEVIALAKSNGTESTHDSEAFEYFALEAYAFD 238
sp|Q00746|ANG1_EMENI            YHMPAIGQGLVEHYADGYEGVLELAEGNRTEAVHDSETLQYFALEVYAYD 222
tr|Q8J0P2|Q8J0P2_COCPO          YHLPAIGEGHIEHFSDSYEDVLELGAHNSSFAVRDSNALQYFAADVYGFD 230
tr|AACU01000690|Magnaprthe      FHLEPVGEGVLEHYADSHAECLELAKSDPAKAARNSHTLQYFALDVYAYD 245
tr|Q6QUX8|Q6QUX8_VERDA          FHLPGISDGIIDHYAEDYAEALKLAATEPELSIIDSDTLQYFAIEAYAYD 242
tr|AACM01000168|                LHVPIISEKTVDHFAENYTDAIALAKSDPSKSVIDSDALQYFAIDVWAYD 244
                                 *:  :.:  ::*:::.:   : *.  :   :  :*.:::*** :.:.:*
peptides                                                    DYFAEVFAK


sp|P79017|ALL2_ASPFU            IAAPGVGCAGESHGPDQ-GHDTGSASAPASTSTSSSSSGSGSGATTTPTD 287
sp|Q00746|ANG1_EMENI            VAVPGIGCVGGEEEND--GQGEEQTEEPAQDDQQDE-------------- 256
tr|Q8J0P2|Q8J0P2_COCPO          IAVPGIGCAG------------TPSPKPSTTSQPPA-------------- 254
tr|AACU01000690|Magnaprthe      IALPGEGCAGKSPSASSDGGHSTPSPIPASPPTQTS-------------- 281
tr|Q6QUX8|Q6QUX8_VERDA          IAIPGVGCPGEKPIIDTAAGTSTAAPTTTTASDASG-------------- 278
tr|AACM01000168|                IAAPGEGCTGE---VEDETEEEKPTATKSDSSKPSA-------------- 277
                                :* ** ** *             :   :
peptides


sp|P79017|ALL2_ASPFU            SPSATIDVPSNCHTHEG-GQLHCT---- 310
sp|Q00746|ANG1_EMENI            --AAEEEIPENCHTHEG-GELHCT---- 277
tr|Q8J0P2|Q8J0P2_COCPO          --TTTAAVPPGCHTHDG-GVIHCP---- 275
tr|AACU01000690|Magnaprthe      --AAPTLLRAWIIGHVGNGRTKARLMLG 307
tr|Q6QUX8|Q6QUX8_VERDA          ----TTTANASCHTHDD-GFVHCS---- 297
tr|AACM01000168|                ----TKEAPKECHTHDD-GVVHCS---- 296
                                          * . *  :.
```

**Fig. 5** Multiple alignment of the six best protein hits and the fit of the *de novo* sequences of analyzed peptides from *D. stemonitis* protein. The glycosylation site found in one *D. stemonitis* peptide is in boldface. ASPFU: *Aspergillus fumigatus*; EMENI: *Emericella nidulans* (*Aspergillus nidulans*); COCPO: *Coccidioides posadasii*; Magnaprthe: *Magnaporthe grisea*; VERDA: *Verticillium dahlia*; AACN01000168*: Gibberella zeae* (*Fusarium graminearum*)

**7**) by other species existing in the same environment. Thus, BOA, its derivatives and phenoxazinones can be finally degraded by microorganisms that act in concert. The ability or inability to metabolize benzoxazinoids could affect the diversity of the microbial community of the rhizosphere, in the worst case in favor of BOA-insensitive pathogens. Carter *et al*. (1999) assumed an influence of avenacin sensitive and insensitive cereal-root-colonizing fungi on the special microbial community. Similarly, benzoxazinoids and related compounds may cause shifts in the species composition of micro-organisms. The ability of micro-organisms to degrade the bioactive compounds contribute to their relatively short existence in the soil. The resulting soil decontamination is of ecological importance, since possible shifts in the species composition might be reversible in case of crop rotations.

Degradation of BOA, its derivatives and detoxification products is accompanied by a metabolic transition of the fungus as evident from the alterations in the abundance of major proteins on 2D gels. The strong accumulation of a group of proteins with similarity to Asp f2-like protein from *Verticillium dahliae* may be in fact a stress response of the fungus to the presence of the compounds in the culture medium (Banerjee *et al*. 1998; Wang *et al*. 2004). At present, it is unknown whether the protein from *Doratomyces stemonitis* has effects on plants or animals. The sequence similarity to Asp f2 (*Aspergillus fumigatus*) and Asp f2-like protein from *Verticillium dahliae* could imply a similar activity. The analyzed peptides from the *Doratomyces stemonitis* protein show also a high degree of sequence homology to ASPND1 (*Emericella nidulans*), to AY170477 protein from *Coccidioides posadasii,* to the *Fusarium graminearum* (*Gibberella zeae*) protein gi42550795, and to PRA1 antigen from *Candida albicans*. All these proteins are glycoproteins with several glycosylation sites. *Aspergillus fumigatus* is associated with diseases in humans and animals*, Emericella nidulans* is an agent of diverse infections in humans and *Coccidioides posadasii* a respiratory pathogen in humans. PRA 1 is an antigen from *Candida albicans,* which causes superficial infections of skin and mucosae, whereas *Verticillium dahliae, Magnaporthe grisea* (rice blast fungus*)* and *Fusarium graminearum* are plant pathogens. Asp f2 protein, a major allergen of *A. fumigatus,* presents an endo-1,3-β-glucanase (Fontaine *et al*. 1997). It is rather likely that all the proteins are glucanases explaining their presence in fungi that belong to quite different taxonomic groups. Glucanases are supposed to have fundamental functions in fungal metabolic activities, such as adaptation to stress conditions and/or in fungal development. The enzymes seem to be required for bud initiation, cell expansion, cell conjugation, and sporulation (Cenamor *et al*. 1987; Moy *et al*. 2002). 1,3-β-Glucan is a dominant polysaccharide in fungal cell walls. Glucanases are therefore essential for continuous rearrangements of 1,3 glucans accompanying cell wall degradation and reconstruction during the development and perhaps during the construction of chlamidospores. Surface located PRA1, a glycoprotein with motifs characteristic of secreted proteins, seems to have a role in morphogenesis as deletion of the gene resulted in temperature dependent defects of hyphae formation (Sentandreu *et al*. 1998). The observed changes in hyphae and colony morphology as well as the formation of chlamidospores when *D. stemonitis* is exposed to BOA derivatives, such as MBOA and BOA-6-OH or phenoxazinone may point to a glucanase function.

*D. stemonitis* is a saprotrophic fungus, but it is suspected to be the causal agent for speck rot on potatoes. Abiotic and biotic stress conditions, such as exposure to allelochemicals, starvation, mineral deficiency, and especially combinations of these conditions might enhance or generate pathogenicity of the fungus, which is also known as a potential pathogen to humans. Thus, the application of benzoxazolinones for weed control in agricultural systems might have unexpected effects.

## Acknowledgement

We thank Dr. C. Werner, University of Zürich for phenoxazinone reference substances. M.S. and A.G. thank the BMBF, Internationales Büro for financial support of the cooperation project between the University of Bonn, Germany and the National University of Agriculture, Kiew, Ukraine.

## References

Alipieva KI, Taskova RM, Evstatieva LN, Handjieva NV, Popov SS (2003) Benzoxazinoids and iridoid glucosides from four *Lamium* species. Phytochemistry 64:1413–1417

Altschul SF, Madden TL, Schäfer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Grapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402

Banerjee B, Greensberger PA, Fink JN, Kurup VP (1998) Immunological characterization of Asp f2, a major allergen from *Aspergillus fumigatus* associated with allergic bronchopulmonary asperillosis. IAI 66: 5175–5182

Barnes P, Putnam AR (1987) Role of benzoxazinones in allelopathy of rye (*Secale cereale* L.). J Chem Ecol 13: 889–906

Baumeler A, Hesse M, Werner C (2000) Benzoxazinoids-cyclic hydroxamic acids, lactams, and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae). Phytochemistry 53: 213–222

Bravo HR, Lazo W (1996) Antialgal and antifungal activity of natural hydroxamic acids and related compounds. J Agric Food Chem 44: 1569–1571

Carter JP, Spink J, Cannon PF, Daniels MJ, Osbourn AE (1999) Isolation, characterization, and avenacin sensitivity of a diverse collection of cereal-root-colonizing fungi. Appl Environ Microbiol 65: 3364–3372

Cenamor R, Molina M, Galdona J, Sanchez M, Nombela C (1987) Production and secretion of *Saccharomyces cerevisiae* ß-glucanases: differences between protoplast and periplasmic enzymes. J Gen Microbiol 133: 619–628

Fontaine T, Hartland RP, Beauvais A, Diaquin M, Latge J-P (1997) Purification and characterization of an endo-1,3-β-glucanase from *Aspergillus fumigatus*. Eur J Biochem 243:315–321

Friebe A, Vilich V, Hennig L, Kluge M, Sicker D (1998) Detoxification of benzoxazolinone allelochemicals from wheat by *Gaeumannomyces graminis* var. *tritici*, *G. graminis* var. *graminis*, *G. graminis* var. a*venae*, and *Fusarium culmorum*. Appl Environ Microbiol 64:2386–2391

Friebe A, Wieland I, Schulz M (1996) Tolerance of *Avena sativa* to the allelochemical benzoxazolinone. Degradation of BOA by root-colonizing bacteria. J Appl Bot 70:150–154

Gagliardo RW, Chilton, WS (1992) Soil transformation of 2 (3*H*)-benzoxazolinone of rye into phytotoxic 2-amino-3H-phenoxazinone-3-one. J Chem Ecol 18: 1683–1691

Gierl A. Frey, M (2001). Evolution of benzoxazinone biosynthesis and indole production in maize. Planta 213: 493–498

Glenn AS, Meredith FI, Morrison III WH, Bacon CW (2003) Identification of intermediate and branch metabolites resulting from biotransformation of 2-benzoxazolinone by *Fusarium verticillioides*. Appl Environ Microbiol 69:3165–3169

Glenn A E, Gold SE, Bacon CW (2002) *Fdb1* and *Fdb2, Fusarium verticillioides* loci necessary for detoxification of preformed antimicrobials from corn. Mol Plant-Microbe Interact 15:91–101

Glenn AE, Hinton DM, Yates IE, Bacon CW (2001). Detoxification of corn antimicrobial compounds as the basis for isolating *Fusarium verticillioides* and some other *Fusarium* species from corn. Appl Environ Microbiol 67:2973–2981

Hofmann D, Knop M, Hao H, Hennig L, Sicker D, Schulz M. (2006). Glucosides from MBOA and BOA detoxification by *Zea mays* and *Portulaca oleracea*. J Nat Prod 69:34–37

Macias FA, Oliveros-Bastidas A, Marin D, Castellano D, Simonet A.M., Molinillo JMG (2005) Degradation studies on benzoxazinoids. Soil degradation dynamics of (2R)-2-β-D-glucopyranosyl-4-hydroxy(2H)-1,4-phenoxazin-3-(4H)-one (DIBOA-Glc) and its degradation products, phytotoxic allelochemicals from Gramineae. J Agricult Food Chem 53: 538–548

Moy M, Li HM, Sullivan R, White Jr JF, Belanger F C (2002) Endophytic fungal β-1,6-glucanase expression in the infected host grass. Plant Physiol 130:1298–1308

Morrissey JP, Osbourn AE (1999) Fugal resistance to plant antibiotics as a mechanism of pathogenesis. MMBR 63: 708–724

Schulz M, Wieland I (1999) Variations in metabolism of BOA among species in various field communities - biochemical evidence for co-evolutionary processes in plant communities? Chemoecology 9:133–141

Sentandreu M, Lorza MV, Sentandreu R, Fonzi WA (1998) Cloning and Characterization of *PRA1*, a gene encoding a novel pH-regulated antigen of *Candida albicans*. J Bacteriology 180: 282–289

Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, Standing KG (2001)
Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. Anal Chem 73:1917–26

Sicker D (1989) A facile synthesis of 6-methoxy-2-oxo-2,3-dihydrobenzoxazole. Synthesis 875–876

Sicker D, Frey M, Schulz M, Gierl A (2000) Role of natural benzoxazinones in the survival strategies of plants. Pp 319-346 *in* Jeong KW (ed) International Review of Cytology - A Survey of Cell Biology Vol. 198. Academic Press

Sicker D, Schneider B, Hennig L, Knop M, Schulz M (2001) Glucoside carbamate from benzoxazolin-2(3*H*)-one detoxification in extracts and exudates of corn roots. Phytochemistry 58: 819–825

Sicker D, Schulz M (2002) Benzoxazinones in plants: occurrence, synthetic access and biological activity. Pp 185-232 *in* Atta-ur-Raman (ed) Studies in Natural Product Chemistry 27. Bioactive Natural Products Part H

Wang JY, Cai Y, Gou JY, Mao YB, Xu YH, Jiang WH, Chen XY (2004) VdNEP, an elicitor from *Verticillium dahliae*, induces cotton plant wilting. Appl Environ Microbiol 70: 4989–4995

Wieland I, Kluge M, Schneider B, Schmidt J, Sicker D, Schulz M (1998) 3-β-D-Glucopyranosyl-benzoxazolin-2(3*H*)-one - A detoxification product of benzoxazolin2(*3H*)-one in oat roots. Phytochemistry 48:719–722

Wieland I, Friebe A, Kluge M, Sicker D, Schulz M (1999) Detoxification of benzoxazolinone in higher plants. Pp 79-88 in Macias FA, Galindo JCG, Molinillo JMG, Cutler HG (eds) Recent Advances in Allelopathy - A Science for the Future Vol 1 Cadiz SAT

Yue Q, Bacon CW, Richardson DM (1998) Biotransformation of 2-benzoxazolinone and 6-methoxy-benzoxazolinone by *Fusarium moniliforme*. Phytochemistry 48:451–454

Zikmundova M, Drandarov K, Bigler L, Hesse M, Werner C (2002a) Biotransformation of 2-benzoxazolinone and 2-hydroxy-1,4-benzoxazin-3-one by endophytic fungi isolated from *Aphelandra tetragona*. Appl Environ Microbiol 68: 4863–4870

Zikmundova M, Drandarov K, Hesse M, Werner C (2002b) Hydroxylated 2-amino-3*H*-phenoxazin-3-one derivatives as products of 2-hydroxy-1,4-benzoxazin-3-one (HBOA) biotransformation by *Chaetosphaeria sp*., an endophytic fungus from *Aphelandra tetragona*. Z Naturforsch 57C:660–665

Received 28 April 2006; accepted 2 August 2006.
Published Online First 12 January 2007.