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Benzoxazolinone Detoxification and Degradation – A Molecule´s Journey

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1. Introduction

Benzoxazinoids are important secondary products of maize, several other Poaceae and a few dicotyledonous species belonging to the Acanthaceae, Lamiaceae, Scrophulariaceae and Ranuculaceae. The synthesis which was investigated in maize by the group of Gierl and Frey starts with the conversion of indole-3-glycerol phosphate to indole. The following steps involve four cytochrome P450 dependent monooxygenases (*BX2-BX5*) that convert indole to benzoxazinone by incorporation of oxygen. Glucosylation at the 2-position of DIBOA results in DIBOA-glucoside, an intermediate of the final product DIMBOA-glucoside (Frey et al., 1997; Glawischnig et al., 1999; von Rad et al., 2001; Jonczyk et al., 2008; Schuhlehner et al., 2008). Whereas the benzoxazinoid acetal glucosides are stable under neutral conditions, the aglycones with the 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one skeleton underlay a degradation by ring contraction and release of formic acid which yields the benzoxazolinones BOA or MBOA (Sicker et al., 2000; Sicker & Schulz, 2002). These derivatives are more stable and can be detected in the soil of rye or wheat fields over a period of several weeks until they are absorbed by other plants or they are converted by microorganisms. The release of benzoxazinoids into the environment and their final degradation are cornerstones within the lifetime of these molecules. In between, a complex set of (re)-modulations and conversions take place due to the activities of a variety of organisms, such as higher plants, fungi and bacteria. Our contribution will give an impression of shuttles between those organisms that end up in the degradation of phenoxazinone(s) as the final conversion products with a limited life time but will also present several reactions of maize to the treatment with benzoxazolinone BOA.

Investigations of weed specific and of benzoxazinoid producing crops specific reactions, reactions of microorganisms, effects on the biodiversity of soil organisms and the elucidation of degradation processes are unequivocally necessary before bioherbicides can be used.

2. Functions of benzoxazinoids

Benzoxazinone glucosides are stored in the vacuole until the tissue is damaged, for example by herbivores, and hydrolysis of the sugar moiety by ß-glucosidases takes place. The highly bioactive aglycones can be released into the environment also by root exudation or by plant residue degradation (Barnes & Putnam, 1987). The mutagenic benzoxazinoids are electrophilic compounds that interact with proteins, intercalate with nucleic acids and are deleterious for many cellular structures and activities (Frey et al., 2009; Sicker & Schulz, 2002). In maize, DIMBOA may have an additional endogenous function. Recently Frebortova et al. (2010) discussed a possible role in cytokinin degradation. Oxidative cleavage of DIMBOA led to coniferron, an electron acceptor of cytokinin dehydrogenase. However, benzoxazinoids have first of all an outstanding role as chemical weapons against other organisms (Niemeyer 2009). Aside of their insecticidal, fungicidal and bactericidal properties, benzoxazinoids are phytotoxic to susceptible plants. Often observed reactions are an inhibited germination but particularly the reduction of seedlings growth. Therefore, the compounds could play an important role in sustainable agricultural systems for natural weed and pest control in innovative agricultural systems.

3. Factors that influence benzoxazinone accumulation

The amount of benzoxazinoids varies highly with plant age, organ and cultivar. Investigated rye cultivars differ in the total benzoxazinoid amounts from 250 to 1800 µg g^{-1} dry tissue in young plants to about 100 µg g^{-1} or less in old plants (Reberg-Horton et al., 2005; Rice et al., 2005; Zasada et al., 2007). In rye cultivars used as mulches by Tabaglio et al. (2008), the content ranges from 177 to 545 µg g^{-1}. High differences in the concentrations among rye cultivars are also reported by Burgos et al. (1999). Water stress conditions and high temperatures increase the content of DIMBOA and DIBOA (Gianoli &Niemeyer 1997; Richardson & Bacon 1993). Nitrogen fertilization has a significant influence on the benzoxazinoid content (Gavazzi et al., 2010). In maize, we found a 3-4 fold higher benzoxazinone accumulation under sulfur deficiency conditions compared to the control plants which were cultivated under optimal nutrient supply (Fig. 1).

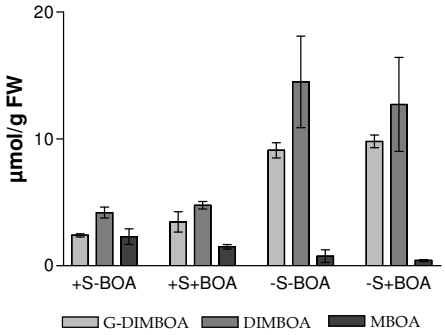

Fig. 1. Influence of sulfur on the benzoxazinoid accumulation in maize roots. Three week old plants were used for the incubation with 0.5 mM BOA (40 ml=20 µmol/g FW). Plant cultivation, BOA incubation, extraction and analyses were performed as described in Knop et al. (2007) and Sicker et al. (2001). *N* =5.

The reason for the stress induced benzoxazinoid accumulation is unclear since the biosynthesis of the compounds is developmentally regulated (Frey et al., 2009). Recently Ahmad et al. (2011) found an increased apoplastic accumulation of DIMBOA-glucoside, DIMBOA and HDMBOA-glucoside in maize leaves during defined stages of infestation with *Rhopalosiphum padi* and *Setosphaeria turtica*. Thus, the translocation of benzoxazinoids out of the cell may be an important step of a process which can lead to an increased stress tolerance and biocidal defense.

4. Effects of benzoxazolinone in maize – increase of glutathione transferase actvity and glutathione levels

Several groups investigated the mode of action of benzoxazolinones (Baerson et al., 2005; Batish et al., 2006; Sanchez-Moreiras et al., 2010, 2011; Singh et al., 2005) on *Lactuca sativa*, *Arabidopsis thaliana* or *Phaseolus aureus*. In plants BOA induces oxidative stress, membrane damage and lipid peroxidation. A prolonged exposure to high BOA concentrations (45 µmol, *Arabidopsis thaliana*) up to 8 days led to a decline in photosynthetic efficiency, induced senescence and death. At sub lethal concentrations, *A. thaliana* reacts with a strong alteration of the gene expression pattern, which comprises about 1% of the total genome. Burgos et al. (2004) found reduced densities of ribosomes, dictyosomes and mitochondria together with a lower amount of starch granules in roots of cucumber seedlings after treatment with BOA or DIBOA. These authors assume that BOA and DIMBOA induces changes in cellular ultrastructure, reduces root growth by disrupting lipid metabolism, by a decreased protein synthesis, and by a reduced transport or reduction of secretory capabilities.

Although maize roots are relatively resistant to BOA (Knop et al., 2007), their physiology is affected when exposed for 24 hours to levels considered to be non-toxic (500 µM and lower). As indicated by the marker compound malondialdehyde (MDA) lipid peroxidation is one of the earliest effects in roots of 6 to 7 days old maize seedlings. An increase is already observed after 1 min and a maximum between 5 to 40 min (Fig. 2). Subsequently, the MDA amount drops below the control value. This indicates the fast activation of mechanisms that counteract cellular damage, also an important action to avoid autotoxicity. During the next hours the level of GST activity is slightly increased (17-30% compared to +S -BOA conditions) in BOA incubated root tips of plants cultivated under optimal sulfur supply. At the same time the major detoxification product glucoside carbamate starts to accumulate (see below). Root tips from -S-plants have only about 40% of the GST activity found in +S-plants. The activity increases up to 50% during the course of incubation, but the presence of BOA has no influence (Fig. 3). Thus, -S-plants have deficits in providing GSTs that have a function in stress reactions.

The soluble plant glutathione transferases are categorized in defined classes: *Θ* (GSTF), *T* (GSTU), *Φ* (GSTT), *Z* (GSTZ), *Λ* (GSTL), dehydroascorbate reductase and tetrachlorohydroquinone dehalogenase like enzymes. Phi and tau class enzymes have a well known function in herbicide detoxification. GSTs respond to many processes that induce ROS production. Up regulating of GST gene expression can be triggered by herbicide safeners (Riechers et al., 2010). GSTs could have as well a function in the detoxification of endogenous substrates (Dixon et al., 2010). Because of the lack of *in vivo* accumulating natural glutathionylated products, Dixon et al. (2010) postulate unstable reaction products, which may decay or which are immediately transformed to other products by metabolic

channeling. GSTs may have also non-catalytic functions as transporters of unstable GS-conjugates, which can be generated spontaneously via radical formation of an acceptor molecule in presence of glutathione. In *Arabidopsis*, BOA induces the up regulation of several GST genes (Baerson et al., 2005). If these GSTs are involved in BOA detoxification pathways is unclear since glutathione conjugates have not yet been found in *Arabidopsis,* maize or other plants. However, it cannot be excluded that GSTs have a role in the transport of unstable intermediates of BOA detoxification products. This question is currently under investigation.

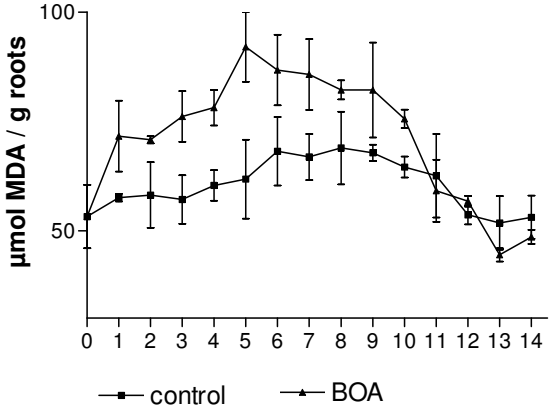

Fig. 2. Course of malondialdehyde (MDA) production in maize roots of 6-d-old seedlings. 0: control; 1-5: intervals of measurements 1 min; 6-10: intervals 15 min; 11: 90 min, 12: 3h, 13: 3h, 14: 4h after start of the incubation. Seedlings were grown and incubated as described in Schulz & Wieland (1999). Samples were prepared and MDA was determined according the method of Wong et al. (2001). *N* = 5.

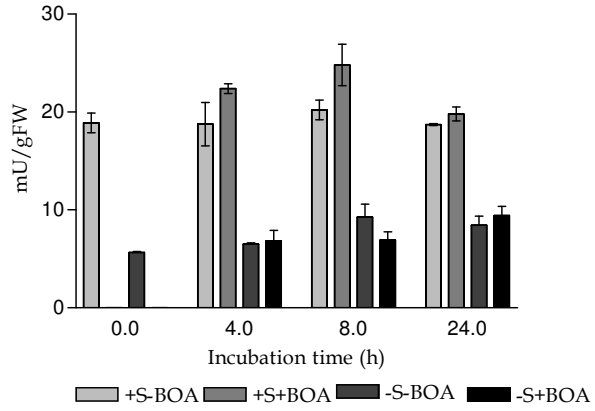

Fig. 3. GST activity in root tips of aeroponically cultured maize plants (greenhouse condition) according the method of Habig & Jacoby (1981). Sulfur deficiency was induced as described in Knop et al. (2007). *N* = 3.

The tripeptide glutathione (GSH) is the major thiol inter alia in plants and a substrate for the GSTs. The multiple functions of GSH in organisms include important roles in redox-homeostatic buffering, in cellular signaling, root development, sulfur assimilation, in defense and stress reactions and in the detoxification of xenobiotics (see review article of Noctor et al., 2011).

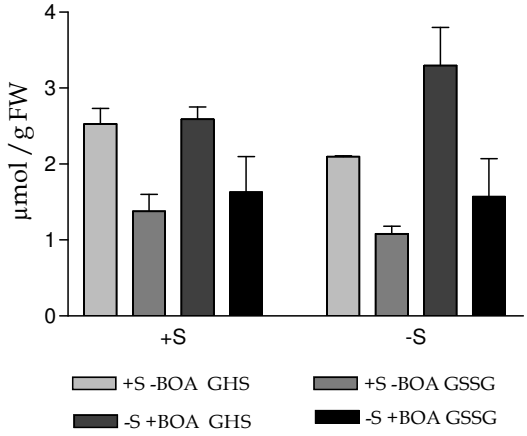

Fig. 4. Reduced and oxidized glutathione in root tips of aeroponically cultured maize plants (greenhouse). The plant material was extracted as described by Gamczarka (2005); measurement of glutathione was done according to Teare et al. (1993). *N* = 3.

The glutathione concentration measured in +S-root tips is similar to the values published by Kocsy et al. (2000). In -S-root tips, the total GHS content is decreased. However, we found slightly increased content of the total GHS in the root tips of –S-maize plants after 24 h exposure to BOA (Fig. 4). The content of GSSH in all samples indicates the induction of some oxidative stress due to the incubation conditions (see also Fig. 2), but there is a tendency of higher GSSG contents in BOA incubated plant material which indicates oxidative stress. Our results are conform to the findings that herbicides can increase the content of glutathione and the activity of associated enzymes such as glutathione reductase in herbicide tolerant plants (Kocsy et al., 2000). The response of –S-maize glutathione content to exogenously applied BOA is obviously very similar to that observed with certain herbicides. Glutathione synthetase gene induction under stress conditions is known (Hruz et al., 2008). An induction of glutathione synthesis even under -S-conditions would involve a change in the priority of the sulfur use in sulfur deficient plants and the mobilization of sulfur from sulfur containing molecules in the root tip. The exact role of glutathione in BOA detoxification is still under investigation. At present it is already unambiguous that the sulfur availability is important in the plant´s coping with BOA (see below).

5. Actin cytoskeleton, cytoarchitecture, and auxin transport

Although maize root growth is not inhibited significantly by BOA, we have scored subtle but relevant effects on the actin cytoskeleton and root apex cytoarchitecture which are

stronger exhibited in –S-plants. In cells of the meristem and transition zone, nuclei are affected in their typical central position and shifted laterally and/or axially (Fig. 5, 6). Similar effects were reported in maize root cells having affected their actin cytoskeleton due to impacts of the actin polymerization inhibitors or in mutant of maize *lilliputian* having aberrant actin cytoskeleton, irregular cell files and root anatomy (Baluška et al., 2001a, 2001b). Importantly, the actin cytoskeleton under the plasma membrane (Fig. 8), especially at the synaptic cell-cell adhesion domains is affected (Baluška et al., 2005). These domains are depleted in their abundant F-actin (Baluška et al., 1997) whereas there are over-polymerized F-actin foci assembled around nuclei of BOA-exposed root cells, shifted out of cellular centres (Fig. 6-8). Similar impacts on the cytoarchitecture were reported in maize root cells exposed to vesicular secretion inhibitor refeldin A (Baluška & Hlavacka 2005), as well as to mastoparan which is affecting phosphoinositide signalling (Baluška et al., 2001c), and to auxin transport inhibitors (Schlicht et al., 2006). Unique BOA-induced effect on the actin cytoskeleton of the transition zone cells is the prominent local assembly of F-actin patches at corners of cross-walls (plant synapses) which resemble published data of the *brk1* mutant line of *Arabidopsis* (Fig. 7C, D) in this chapter and Figure 8 in Dyachok et al., 2008). Interestingly, the BRK1 protein localizes to the cross-wall corner sites of *Arabidopsis* root apices showing aberrant actin organization both in the root cells of the *brk1* mutant and in the BOA-exposed root cells (Fig. 4). BRK1 is a component of the evolutionary conserved SCARE complex that acts as F-actin nucleator and BOA might directly target the SCARE complex. This would be a very attractive scenario and it should be tested in future. BOA is also known to inhibit activity of the PM H^+-ATPase of root cells (Friebe et al., 1997) and it is of interest to note that the actin cytoskeleton is controlling permeability of the plant plasma membrane (Hohenberger et al., 2011). Interestingly, maize mutants *lrt1* and *rum1*, and especially the *lrt1/rum1* double mutant, which are affected in the polar auxin transport, showed similar F-actin depletion at the synaptic cell-cell adhesion domains and shifted nuclei (Schlicht et al., 2006). In general, all polar auxin inhibitors resemble BOA in affecting the actin cytoskeleton especially at the transition zone of the root apex which is the most active zone with respect of F-actin rearrangements (Baluška et al., 1997, 2001a, 2001b, 2001d), the polar auxin transport (Baluška et al., 2010; Mancuso et al., 2005, 2007).

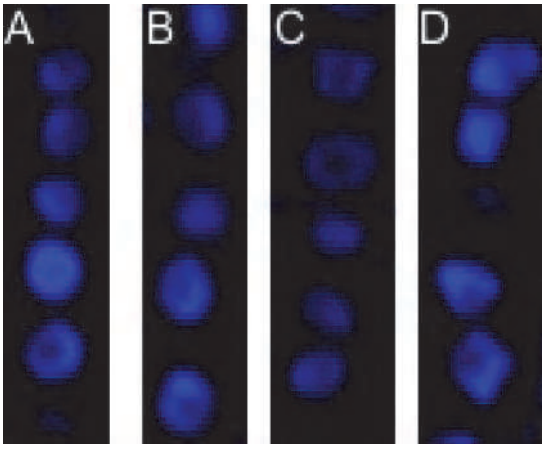

Fig. 5. Shifted nuclei (visualized with DAPI) in root apex cell files. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA.

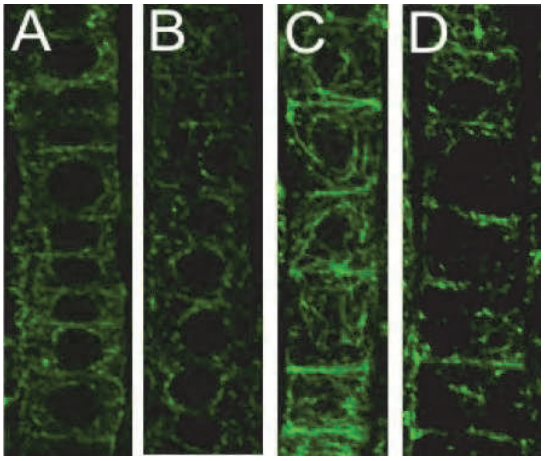

Fig. 6. Overview of the actin cytoskeleton in maize root apex. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA.

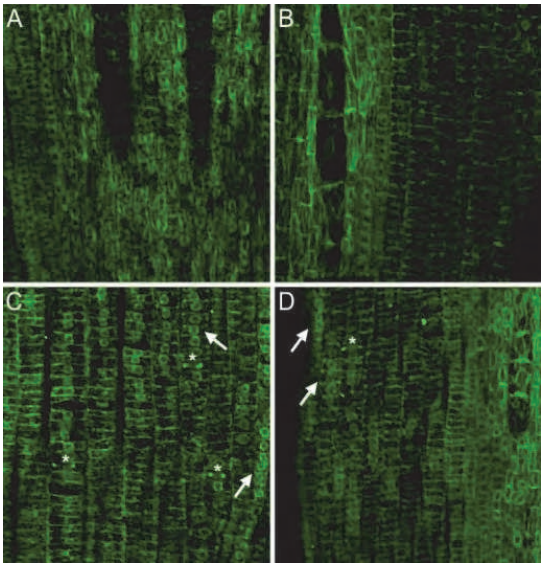

Fig. 7. Details of the actin cytoskeleton in cortical cells of the transition zone. Note the aberrant over-polymerization of F-actin in cortex cells of the BOA-exposed roots. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA

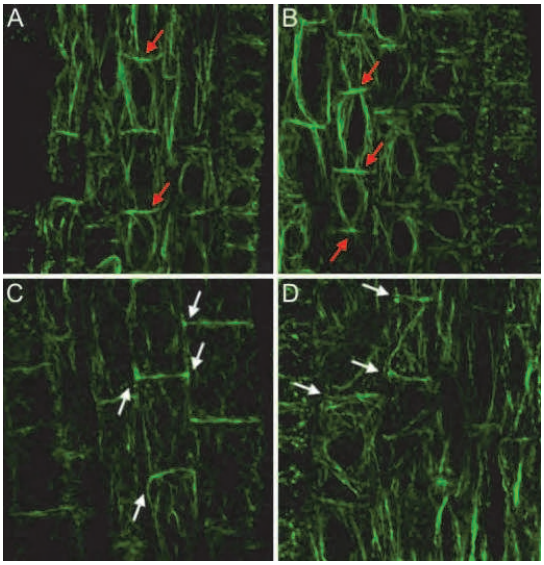

Fig. 8. Details of the actin cytoskeleton in cells of the transition zone. Red arrows indicate abundant F-actin at the cross walls (plant synapses) of pericycle and endodermis cells. White arrows indicate depleted F-actin at the cross walls (plant synapses) of pericycle/ endodermis cells of the BOA-exposed roots. Note the over-polymerization of F-actin in the cell corners. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA.

For the experiments, apical root segments (~7mm) encompassing the major growth zones were excised, fixed with 3.7% formaldehyde, and embedded in the Steedman's wax. Ribbons of 7-mm sections were dewaxed and incubated with a mouse anti-actin monoclonal antibody, clone C4 from ICN Pharmaceuticals (Costa Mesa, CA, USA) diluted 1:200 and a rabbit maize polyclonal anti-actin (gift of Chris Staiger, Purdue University, USA) diluted 1:100 in PBS buffer. After rinsing with PBS buffer and incubation with secondary antibodies, sections were mounted under a coverslip and examined in the confocal microscope Olympus Fluoview 1000.

Similarly to *Arabidopsis* roots, the actin cytoskeleton is affected by auxin transport inhibitors in similar manner as we have reported here for the maize root cells (Rahman et al., 2007; Dhonukshe et al., 2008). Importantly in this respect, BOA is known to act as anti-auxin and to block lateral root formation (Anai et al., 1996; Baerson et al., 2005; Burgos et al., 2004; Hoshi-Sakoda et al., 1994) a process well-known to be based on auxin transport in both monocots and dicots roots (Hochholdinger & Zimmermann, 2008; Peret et al., 2009). Finally, the root apex transition zone emerges as specific target of allelochemicals, particularly this unique zone of the root apex (Baluška et al., 2010). Future studies should focus on these effects of BOA on the polar auxin transport and on other processes and activities characteristic for the root apex transition zone (Baluška et al., 2010).

6. Detoxification of benzoxazolinones in higher plants

Plants react to BOA in a species- and dosage dependent manner. Generally, members of the Poaceae were found to be less sensitive to the compounds than dicotyledoneous species,

although there are exceptions. Moreover, ecotypes (for instance, *Chenopodium album* ecotypes or *Portulaca oleracea* garden forms) and varieties can differ in their accumulation and detoxification activities (Schulz unpublished). One important reason for the different sensitivity is the better developed ability of most Poaceae to reduce the toxicity benzoxazolinone(s), in comparison to dicots. Deleterious effects on the biochemistry, physiology and cell biology are therefore limited in good detoxifiers (see effects on maize described above). Interestingly, Macias et al. (2005) found an almost 100% inhibition of *Allium cepa* and *Lycopersicon esculentum* root growth whereas *Triticum aestivum* root growth was inhibited to 50 %, when low concentrations of DIMBOA-glucoside (5 µmol = 5 ml of a 1mM solution used as the highest concentration) were applied to 10 or 25 seeds in Petri dishes. It is generally known that the glucosides of benzoxazinones are much less toxic than the aglycons, but they obtained similar results with DIBOA. The growth of *Lepidium sativum* was stimulated to about 20%.

Almost all investigated higher plant species detoxify benzoxazolinone (BOA) via 6-hydroxylation and subsequent O-glucosylation (Tab. 1). *Portulaca oleracea* and a few other related species produce BOA-5-O-glucoside as a byproduct (Hofmann et al., 2006). Monocots perform, mainly or at least to a considerable portion, glucoside carbamate (Schulz et al., 2006; Schulz & Wieland, 1999; Sicker et al., 2000, 2004; Wieland et al., 1998). In contrast to BOA-6-OH and its glucoside, glucoside carbamate is not toxic up to concentrations of 1 mM and is therefore a most suitable detoxification product. First found in maize, glucoside carbamate is subsequently modified by malonylation or by addition of a second glucose molecule yielding gentiobioside carbamate (Hofmann et al., 2006). BOA-6-O-glucoside is, however, the major detoxification product when maize or other seedlings are incubated with MBOA. Glucoside methoxycarbamate occurred only in maize as a minor compound when the incubation was extended to more than 48 h. Identified stable detoxification products are illustrated in figure 9. The accumulation of BOA-6-OH is a good marker for a high sensitivity to BOA (for example *Vicia faba*). This hydroxylation product is twice as toxic as BOA and causes necrosis in the root tips within 24 h.

The BOA-detoxification process in maize roots starts with the production of BOA-6-O-glucoside. However, after 3 to 6 h glucoside carbamate accumulation is initiated. About 10 h after incubation start, this compound becomes the major detoxification product, whereas BOA-6-O-glucoside does not further accumulate although the glucosyltransferase activity responsible for glucosylation of BOA-6-OH is still abundant (Schulz et al., 2008). The increasing accumulation of gentiobioside carbamate and malonyl-glucoside carbamate 18 to 20 h after start of the incubation is a late event in the detoxification process. Avoidance of BOA uptake can be another strategy to escape the harmful effects of BOA.

In a recent study, we found a significant reduction of redroot pigweed (*Amaranthus retroflexus* L.) and common purslane (*Portulaca oleracea* L.), whereas common lambsquarters (*Chenopodium album* L.) and velvetleaf (*Abutilon theophrasti* Medicus) were moderately or not suppressed, respectively (Gavazzi et al., 2010; Tabaglio et al., 2008).

One possibility to explain the different reactions of the four weeds could be differences in the detoxification activities or accumulation characteristics that minimize the harmful effects of rye allelochemicals (BOA and related compounds). This affects a direct correlation between the benzoxazinoid content of rye mulch used in the study and weed suppression. The four warm season weeds exhibit remarkable differences in their detoxification behavior

with a high correlation to the sensitivities of the weeds previously observed in experiments with rye mulch under greenhouse conditions. These studies demonstrate for the first time that detoxification processes are important for the survival of adapted weeds in environments enriched with benzoxazinoids, such as maize, wheat or rye fields (Schulz et al., submitted). Moreover, nutrients together with stress conditions have an influence on the detoxification processes. For instance, sulfur deficiency in combination with herbicide treatment can lead to a breakdown of the BOA detoxification process in maize (Knop et al., 2007). Optimal sulfur supply seems to be an emerging factor to guarantee well functioning of detoxification pathways. This is particularly important since sulfur deficiency is increasing in many areas of the word (Scherer, 2001, 2009).

Family	**Species**	**A**	**B**	**C**	**D**	**E**	**F**
Poaceae	*Avena sativa*	xx	xx				
	Avena fatua	xxx	xx				
	Digitaria sanguinalis	xxx	x				
	Lolium perenne	xx	xxx				
	Hordeum vulgare	xx	xxx				
	Triticum aestivum	x	xxx				
	Secale cereale	x	xxx				
	Zea mays	(x)	xxx		xx	xxx	
Portulacaceae	*Portulaca oleracea* cv Gelber	xx	xx		x	x	xx
Chenopodiaceae	*Chenopodium album (ecotypeI)*	xx	x	x			
Brassicaceae	*Arabidopsis thaliana*	xxxx	x				
	Rhaphanus sativus	xxx	x				
	Diplotaxis tenuifolia	xx		x			
Amaranthaceae	*Amaranthus albus*	xxx					
	Amaranthus retroflexus	xx	xx		x	x	
Ranunculaceae	*Consolida orientalis*	xxx	x				
	Consolida regalis	xxx	x				
Apiaceae	*Coriandrum sativum*	xxx	xx				
	Daucus carota	xxx	x	x			
Asteraceae	*Galinsoga ciliate*	xxx		x			
	Helianthus annuus	xxx					
Fabaceae	*Vicia faba*	xx		xx			

Table 1. Some plant species (6-10 days old seedlings) and their major BOA detoxification products after 24 h of incubation with 0.5 mM BOA (40 ml / g FW). Maize: compounds present after 48 h are considered. Major compound: xxx; xx: minor compound: x; traces (x). **A**: BOA-6-O-glucoside; **B**: glucoside carbamate; **C**: BOA-6-OH; **D**: gentiobioside carbamate; E: malonylglucoside carbamate; F: BOA-5-O-glucoside.

There are also some hints that the ecobiochemical potential of species to detoxify benzoxazolinone drives the membership to certain plant associations (Schulz & Wieland, 1999).

A portion of the detoxification products are released again by root exudation (Sicker et al., 2002). When BOA incubated maize plants are transferred to tap water, BOA-6-O-glucoside and glucoside carbamate can be identified in the water. After several days, the compounds

cannot be detected anymore in the soluble fraction prepared from the plants. A similar result is obtained with *Galinsoga ciliata* and *Coriandrum sativum*, indicating that exudation of soluble detoxification products is a more general phenomenon. The exuded products can get in contact with endophyts and microorganisms of the rhizosphere.

7. Microbial degradation products and fate of exuded plant degradation products

Many fungi are known to be sensitive to benzoxazinones and benzoxazolinones. However, some are able to detoxify the compounds (Fig. 10). Species of *Fusarium* have been investigated for their growth in presences of benzoxazinone (Friebe et al. 1998; Glenn et al. 2001). Eleven of 29 *Fusarium* species had some tolerance to BOA, the most tolerant species was *F. verticillioides* with only one sensitive strain of the 56 ones tested (Glenn et al., 2001). The first step in the degradation of benzoxazolinone-2(*3H*)-one (BOA) is a hydrolysis yielding 2-aminophenol. This step is performed by bacteria as well, also by seed born ones (Bacon et al. 2007; Burdziak et al., 2001). 2-Aminophenol is not stable but is spontaneously dimerized to 2-amino-3*H*-phenoxazin-3-one (APO) or it can be captured by several fungi which convert the compound to N-(2-hydroxyphenyl)malonamic acid (oHPMA) and 2-acetamidophenol (AAP) (Carter et al., 1999; Friebe et al., 1998; Glenn et al., 2001, 2002, 2003). Several endophytic fungi (*Plectosporium tabacinum, Gliocladium cibotii, Chaetosphaeria sp., Fusarium sambucinum*) from *Aphelandra tetragona* are described to produce 2-amino-(*3H*)-phenoxazinone derivatives when incubated with benzoxazinones (Baumeler et al., 2000; Zikmundova et al., 2002a, 2002b). *Fusarium verticillioides*, an endophytic fungus of maize, did not convert benzoxazolinone to any known microbial degradation product when sterile grown maize seedlings were inoculated with the fungus whereas the seedlings produced their known detoxification products since gentiobioside carbamate and glucoside carbamate could be detected in the medium. APO, AAP and oHPMA can have effects on plant growth. Absorbed traces of AAP and oHPMA stimulated maize radicle growth; traces of AAP stimulated that of cress. Phenoxazinone inhibited the growth of cress radicles at concentrations higher than 500 µM, whereas maize radicles were hardly affected (Knop et al., 2007).

In another study (Schulz et al., unpublished), the growth of some representative fungi was monitored over a period of 10 days in presence of BOA and APO. Generally, BOA was always less toxic than APO. The ability to grow in presence of BOA is influenced by the availability of nutrients. Several species changed the sensitivity to BOA, when BOA had to be used as N-source.

Once released into the soil, the plant and microbial detoxification products can be degraded by fungi. All compounds are finally converted to phenoxazinone(s): The degradation work of *Botrytris cinerea* (B.cin), *Drechslera tuberose* (D.tub), *Fusarium heterosporum* (F.het), *F. verticillioides* , *F. oxysporum* (F.oxy), *F. culmorum* (F.cul) , *F. solanum (*F.sol*), Trichoderma viride* (T.vir) is presented in Fig. 11.

In the media of *Fusarium verticillioides* and *Drechslera tuberosa*, some benzoxazolinone-2(*3H*)-one (BOA) is present after the incubation with glucoside carbamate, the medium of the other fungi contained only traces of BOA. This indicates an opening of the carbamate heterocycle followed by the release of glucose. *Botryis cinerea* has only a rather limited ability to degrade

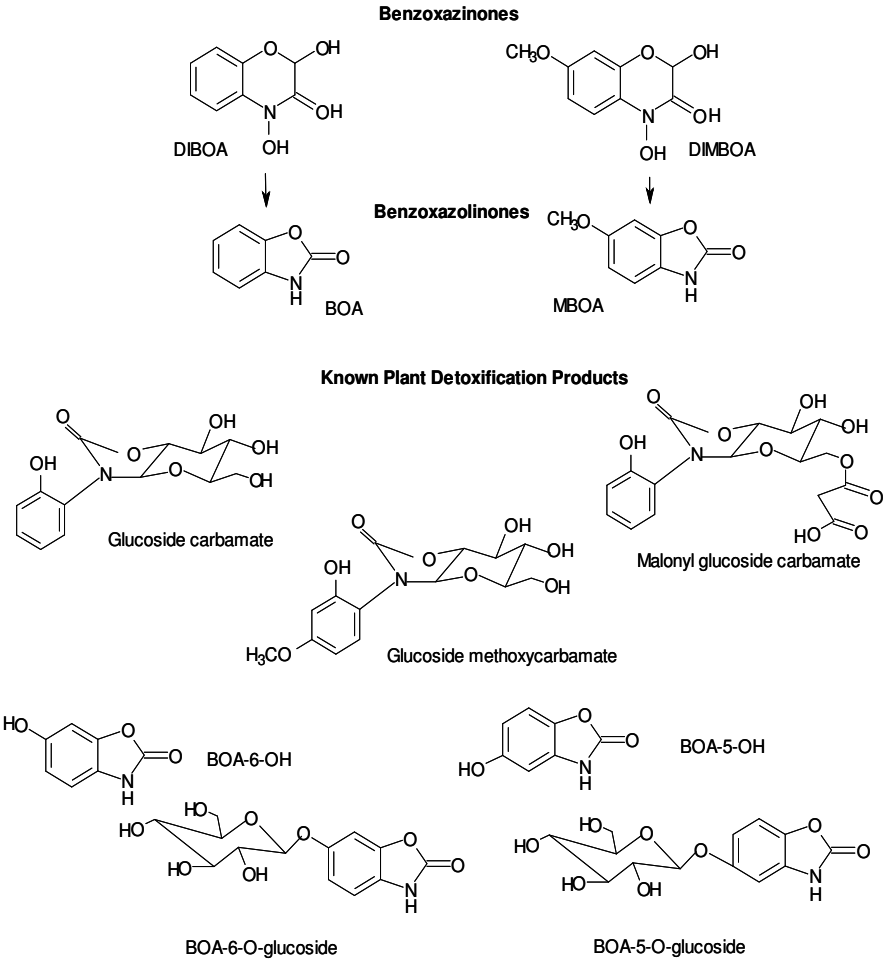

Fig. 9. Benzoxazolinone detoxification compounds produced by plants.

the compound, while *Paecilomyces farinosus* is unable to convert it. The behavior is, however, highly dependent on the different strains of a given species.

In the media of all of the species able to degrade glucoside carbamate a new, hitherto unknown intermediate occurred. The new compound was isolated for structural analysis. The ^{1}H spectrum showed signals for an *ortho*-substituted phenyl ring and well resolved signals in the sugar region with all couplings, too. The complete assignment was made by use of H,H-COSY, HMQC and HMBC. The latter technique was decided to prove that the hydrolytic ring opening of the oxazolinone precursor 1-(2-hydroxyphenylamino)-1-deoxy-ß-glucoside 1,2-carbamate (glucoside carbamate) led to a carbamic acid structure instead of a regioisomeric carbonate with 2-OH from the sugar moiety. Accordingly, H-1 of the glucose unit appears at 5.84 ppm and shows in the HMBC two cross peaks with C-3 of glucose at 74.0 ppm and the COOH group (158.3 ppm), each by coupling via three bonds.

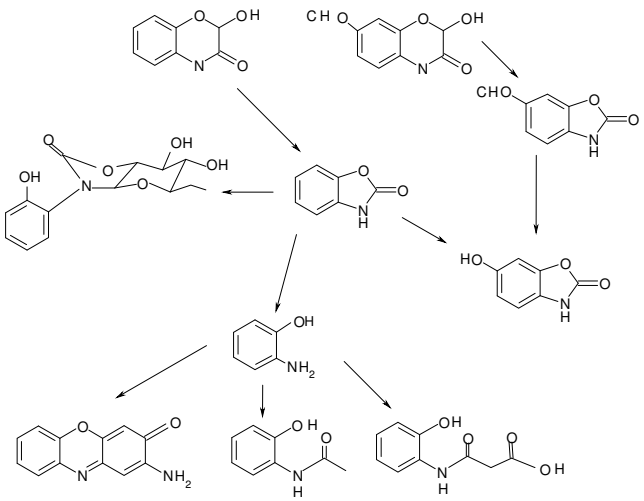

Fig. 10. Microbial degradation products derived from BOA.

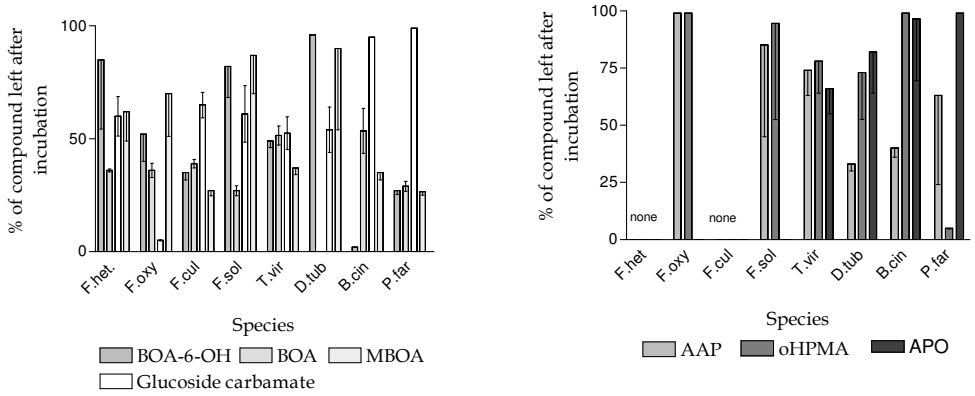

Fig. 11. Mycelial plugs from agar plates (discs 0.5-1 cm in diameter) were transferred into 250 ml flasks with 100 ml sterilized Czapek medium. When mycelia were well developed, 0.5 mg were transferred to 100 ml flasks containing sterile medium without sucrose (controls) and with addition of 10 µmol BOA, BOA-6-OH, MBOA, glucoside carbamate, AAP, oHPMA, or APO (1 µmol). Cultures were grown at 25 ^{0}C in the dark without shaking. Species which did not grow without sucrose were incubated with the different compound in presence of sucrose. BOA and 2-acetamidophenol were from Aldrich, MBOA was synthesized (Sicker 1989) as well as BOA-6-OH (Wieland et al., 1999) and oHPMA (Friebe et al., 1998). Glucoside carbamate) was prepared as described (Wieland et al., 1998; Sicker et al., 2001). The cultures were harvested after 14 days of cultivation. Mycelia were separated by filtration through 100µm nylon nets, dried between paper sheets and weighted. The medium was extracted with ethyl acetate. The organic and aqueous phases were evaporated to dryness, the residues dissolved in 70 % methanol and analyzed by HPLC. N = 5.

The following data could be obtained by MS-analysis: In the positive ion mode (with addition of formic acid for a better ionization), several peaks appear: a protonated monomer ion at 298.09216 da (exact theoretical mass 298.09213 da) besides two sodium-adducts of appropriate mono- and dimer ions at 320.07465 da (exact theoretical mass 320.07407 da) and 617.15936 da (exact theoretical mass 617.15892 da), respectively. By addition of sodium formate to the sample solution, the two last-mentioned signals increase to the most intensive peaks in the spectrum, accompanied by a further dimer peak at 639.14197 da ([2M-H,+2Na]$^+$, exact theoretical mass 639.14087 da).

In the negative ion mode, applied to the initial methanolic solution without buffer, a corresponding weak signal at 296.07845 da (exact theoretical mass 296.07758 da) appears. With ammonia as buffer this monomer signal at 296.07806 da increases and is still accompanied by a weak dimer signal. By use of a stronger base like triethylamine, the above mentioned mono- and dimers appear again, however, now accompanied by an additional ion pair of low intensity at 314.08320 da and 629.13868 da. The latter ion was already detected in the ammonia-spectra. This at first glance odd behavior can be easily understood as follows: Object of investigation is compound **1** from a well separated peak of the HPLC chromatogram. The retention time of **1** is distinctively different from that of the precursor glucoside carbamate. Hence, our findings from the mass spectra lead to the conclusion, that under the ESI conditions the carbamic acid **1** reacts almost completely back to the glucoside carbamate by dehydration. Only under strongly basic conditions signals for the intrinsic carbamic acid with the formula $C_{13}H_{17}NO_8$ appear. Thus, by means of the MS and NMR data analysis the new compound was identified as N-ß-D-glucopyranosyl-N-(2'-hydroxyphenyl) carbamic acid (**1**), (N-glucosylated carbamic acid, Fig. 12).

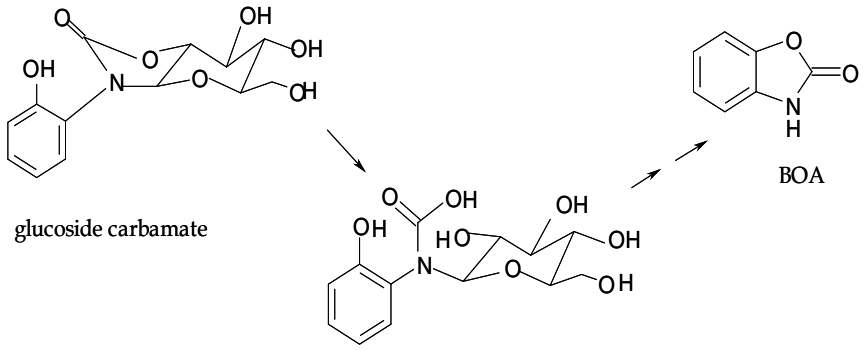

Fig. 12. Glucoside carbamate is hydrolysed to N-glucosylated carbamic acid, than deglucosylated and rearranged back to BOA. N-glucosylated carbamic acid was isolated the medium of the species mentioned in the text and purified by HPLC.

The carbamic acid feature is a rare one among natural products. Hitherto, only five representatives are known. Pallidin, a N-carboxyindole alkaloid, has been isolated from the sponge *Rhaphisia pallid* (Su et al., 1996)). Echinosulfone A, isolated from a Southern Australian marine sponge *Echinodictyum*, is a related derivative of the N-carboxyindole moiety (Ovenden et al., 1999). 1,2-Pyrrolidinedicarboxylic acid was identified as constituent of propolis balsam (Greenaway et al., 1991). N-1'-carboxybiotin has been studied in respect

of the formation of enzyme N1′-carboxybiotin complexes during biochemical transformations (Jockel et al., 2000; Legge et al., 1996). However, the feature of a glycosyl carbamic acid as found in **1** has not at all been described for natural products. The most similar compound reported is synthetic ß-D-glucopyranosyl carbamic acid (Ulsperger et al., 1958). The identification of compound 1 was possible with the help of Diana Hofmann (Universität Leipzig, Institut für Analytische Chemie) and Lothar Hennig (Universität Leipzig, Institut für Organische Chemie).

8. Degradation of 2-amino-3H-phenoxazin-3-one (APO)

Fungi differ considerably in their sensibility to APO (Fig. 13). A low sensitivity is correlated with the ability to decompose the compound. All tested strains of the *Fusarium* species, *Drechslera tuberosa* and *Trichoderma viride* are able to degrade APO. With the *Fusarium* species the compound disappeared completely. The medium of *T. viride* contains traces of several phenoxazinones, indicating that some APO is modified by substitutions. *Botrytis cinerea*, which was found to be highly sensitive to APO in the growth tests, has only a low activity to degrade the compound as well as the most sensitive species *Paecilomyces farinosus*.

Since it is rather likely that APO degradation is started by oxidation, we performed experiments to elucidate how fungi can initiate oxidation processes that result in APO destruction. When 200 nmol APO was incubated with H_2O_2 in methanolic solution at room temperature, no decrease or precipitation of the compound was observed over a period of 24 h. The same result is obtained when Czapek medium (contains 10 mg $FeSO_4$/l) is used without addition of H_2O_2 (Tab. 2). Czapek medium with H_2O_2, however, led to an almost complete destruction of APO within 24 h via several intermediates. Thus, in combination with H_2O_2 and Fe^{2+} (Tab. 2), APO is easily destroyed via Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$, in which the mechanism of radical production is still a matter of debate.

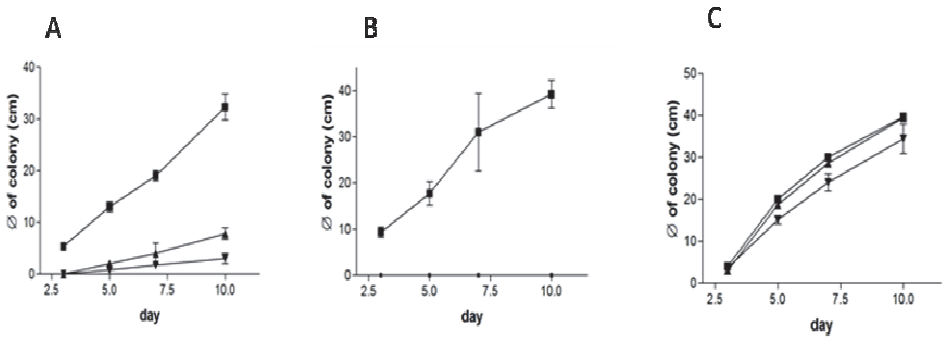

Fig. 13. Examples for the differences in the sensitivity to APO. **A**: *Botrytis cinerea* F-00646; **B**: *Paecilomyces farinosus* F-01073 and **C**: *Fusarium avenaceum* F-00475. ■: control; ▲1 µmol APO;▼2µmol APO in Czapek medium. Growing fungal mycelium was placed on the center of each Petri dish (20 cm i.d.) and incubated in the dark at 25°C. Fungal growth was measured at the 3rd ,5th ,7th and 10th day of growth. Each experiment was repeated three times. *P. farinosus* was completely inhibited by APO, *B. cinerea* strongly inhibited by both APO concentrations. *F. avenaceum* showed no inhibition.

Hyde & Wood (1997) reported on the presence of a Fe(II) oxalate complex in aerobic solution that can lead to hydroxyl radicals without any other source. Therefore we examined the excretion of oxalate by the two fungi: *Fusarium heterosporum* F-00195 as a strain able to degrade APO rather fast and *Paecilomyces farinosus* F-01073 as a strain with a low degradation activity. Oxalate determination was done with the Trinity biotech oxalate kit No. 591.The mediums of the fungi were analyzed for secreted oxalate during the first 8 h of incubation in presence of APO, the one of *F. heterosporum* was also tested for the presence of H_2O_2 (National Diagnostics hydrogen peroxide assay kit Cl-204). During the incubation period, the pH of the media was lowered from pH 7.0 to 5.5 (*F. heterosporum*) and 5.7 (*P. farinosus*). *F. heterosporum* started to secrete oxalate already 30 min after start of the incubation (Fig. 14-16). The amount strongly increased during the next hour, but drops later on. Oxalate secretion by *P. farinosus* was measurable after 4 h of incubation, but reached only about 10% of the highest *F. heterosporum* value after 8 h. The oxalate excretion profile of *F. heterosporum* corresponds to the disappearance of APO already 1 h after starting the incubation. Contrarily, *P. farinosus* started some APO degradation after 8 h. In the *F. heterosporum* medium, H_2O_2 was measurable immediately after start of the incubation and again 8 h later. Hydrogen peroxide was extremely low during the major phase of oxalate release and APO degradation. It is assumed that oxalate excretion and the release of H_2O_2 are causative for the APO degradation of all other APO insensitive *Fusarium* strains tested.

Incubation time	APO (200 nmol)	APO + H_2O_2 (35 mmol)	APO + H2O2 + Fe^{2+} (100 nmol)	APO + Czapek medium	APO + Czapek medium + H_2O_2	APO + 5 µmol oxalate + 5 µmol Fe^{2+}	Assay volume: 400 µl
Start (t 0)	1.02 nmol	1.15	0.96	1.20	1.24	1.20	Volume
End (t 24h)	1.04 nmol	1.16	0.30	1.25	0.16	0.70	analyzed by HPLC: 2 µl

Table 2. Destruction of APO in presence of H_2O_2 or oxalate and Fe^{2+} and controls. Average values of 5 independent experiments.

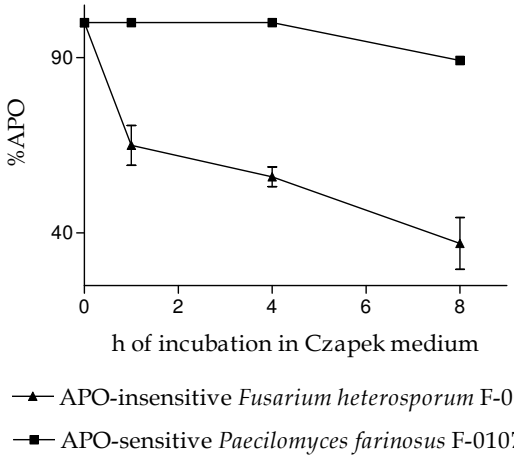

Fig. 14. Decrease of APO in the medium of *F. heterosporum* and *P. farinosus*. *N* = 3.

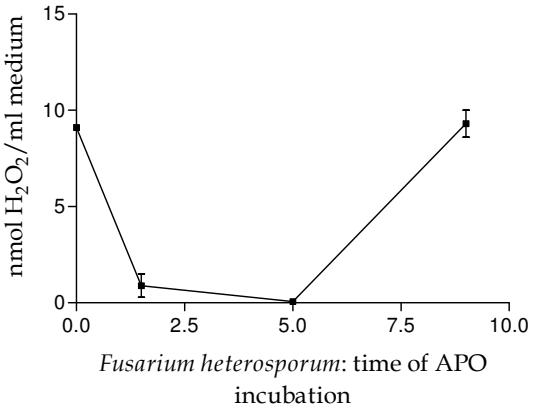

Fig. 15. During the major degradation phase, H_2O_2 is poorly present in the medium. $N = 3$.

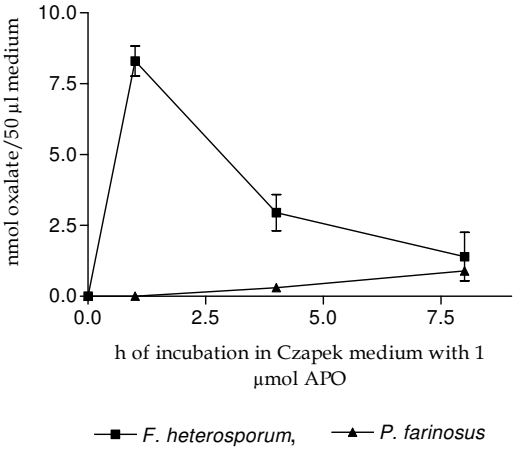

Fig. 16. High excretion of oxalate by *F. heterosporum* during the major phase of APO degradation. $N = 3$.

Many fungi are known to secrete oxalate, some in mmolar concentrations under certain conditions (Cessna et al., 2000; Dutton & Evans, 1996). According to Varela & Tien (2003) oxalate facilitates hydroxyl radical formation at low concentration. Oxalate sequestering of ferric ions are also discussed as a protection of the fungi. Moreover, certain fungi are known to release H_2O_2. According to our study, oxalate must have a function in radical formation because oxalate can replace H_2O_2 (Tab. 2). Figure 15 illustrates that the depletion of H_2O_2 during the major phase of *F. heterosporum* APO degradation is accompanied by a strong excretion of oxalate. Thus, existing H_2O_2 is used for APO degradation and excreted oxalate lead to new hydroxyl radicals.

The oxidation of BOA, BOA-6-OH, glucoside carbamate, AAP and oHPMA was also tested (100 µmol each) for degradation via Fenton reaction. None of these compounds was

destroyed in Czapek medium supplemented with H_2O_2. We assume the participation of exuded fungal enzymes that modify these compounds prior to APO production and the subsequent oxidative destruction of phenoxazinones by the Fenton reaction. It is concluded that the complete biodegradation of BOA detoxification products and phenoxazinones is a concerted action of various fungi with different metabolic properties which is probably supported by bacteria. At least the sugar moiety of the plant detoxification products can be metabolized by the microorganisms. Interestingly, Chen et al. (2010) found an increase in soil fungi after DIMBOA and MBOA application. The authors assume affects on the soil microbial community structure with a change in fungi populations in the wheat rhizosphere. Saunders and Kohn (2009) described a significant influence of benzoxazinoids on fungal endophyte communities.

Phenoxazinones are not only compounds originated from benzoxazinone degradation. They are synthesized by a variety of different organisms, for instance by members of the genus *Pycnoporus* (Sullivan & Henry 1971; Temp & Eggert 1998) or by *Streptomyces* species (Suzuki et al., 2006). Clearly, these compounds are relatively wide-spread in nature. Macias et al. (2005) reported on a life time of APO of more than 80 days in some soils. Unfortunately, neither the source of the soil nor its quality is mentioned. Also Bacon et al. (2007) take APO for a stable compound. In contrast, Krogh et al. (2006) determined $1.4x10^{-11}$ M APO as the highest concentration in sandy loam soil after incorporation of one rye seedling 300 mg^{1-} soil. The APO concentration decreased rapidly during 10 days to about 30%.

For APO degradation in nature, several soil properties such as a high diversity of microorganisms including ones that excrete chelator agents (e.g. oxalate), generation of H_2O_2, the presence of iron, and a pH lower than 6 are certainly prerequisites for starting APO degradation via Fenton reaction. Even if the life time is variable depending on the soil conditions, APO is fortunately not a stable compound but an allelochemical which can be completely degraded over time. The Fenton reaction is the key reaction in the oxidation of membrane lipids, oxidation of amino acids and biologic reactions where biological reduction agents are present. The reaction is common in chemical, biological, and environmental systems (Barbusinski, 2009; Prousek, 2007). The importance of the Fenton reaction in natural environments and in waste treatments for degradation of phenolic and other compounds has been recognized during the last two decades (Pignatello et al., 2006; Vlyssides et al., 2011).

9. Conclusion

The bio-accumulation of conventional herbicides/pesticides and their often highly toxic degradation products is a well-known problem. Another problem is their persistence in soil and ground water. Some of these compounds are not only genotoxic, carcinogenic, neurotoxic and immunotoxic but have also negative effects on the fertility of vertebrates and are toxic to bees. Moreover, plants have developed new strategies in the resistance against common herbicides (Gainesa et al., 2010; Powles &Yu, 2010). Yuan et al. (2006) summarize in their article the dramatic increase in herbicide-resistant weed biotypes which became obvious since the late 80ies of the last century. This demands innovative and environmental-friendly strategies based on sustainable resources using natural, plant–own compounds for weed control which are acceptable by consumers. Breeding crops suitable for natural product applications has to be aimed. Allelopathic concepts are more and more attractive in

agriculture. However a number of prerequisites are of importance. Among others, the selectivity and the relatively fast and complete biodegradation of the compounds is of outstanding importance to avoid environmental damage and the destruction of biodiversity. The same priority has the development of new agricultural systems. Applications of large quantities of a natural compound, perhaps booted by artificial substitutions, instead of conventional herbicides, cannot be a solution. The same problems as obtained with common herbicides will occur soon. Therefore, the reactions of plant, including detoxification strategies, of microorganisms, of plant and microbial genotypes, soil properties, fertilizer management, occurrence and degradation of the mother substances and their derivatives have to be investigated carefully prior to the use of any natural compound as a bioherbicide. This knowledge will help to design cultural systems promoting natural compounds to agricultural crops with a beneficial impact on environmental safety as well as consumers' health. Benzoxazinoids could be a group of allelochemicals that seem to fulfill several of the prerequisites. The "journey" of benzoxazolinone and its final degradation demonstrates at least some of the advantages of the use of suitable natural bioactive compounds in agriculture: highly selective efficiency and a short existence (Fig. 17). It gives also an impression of the complexity of the ecological net involved in the transformation and degradation of an allelochemical, although only a few aspects could be directed in this article.

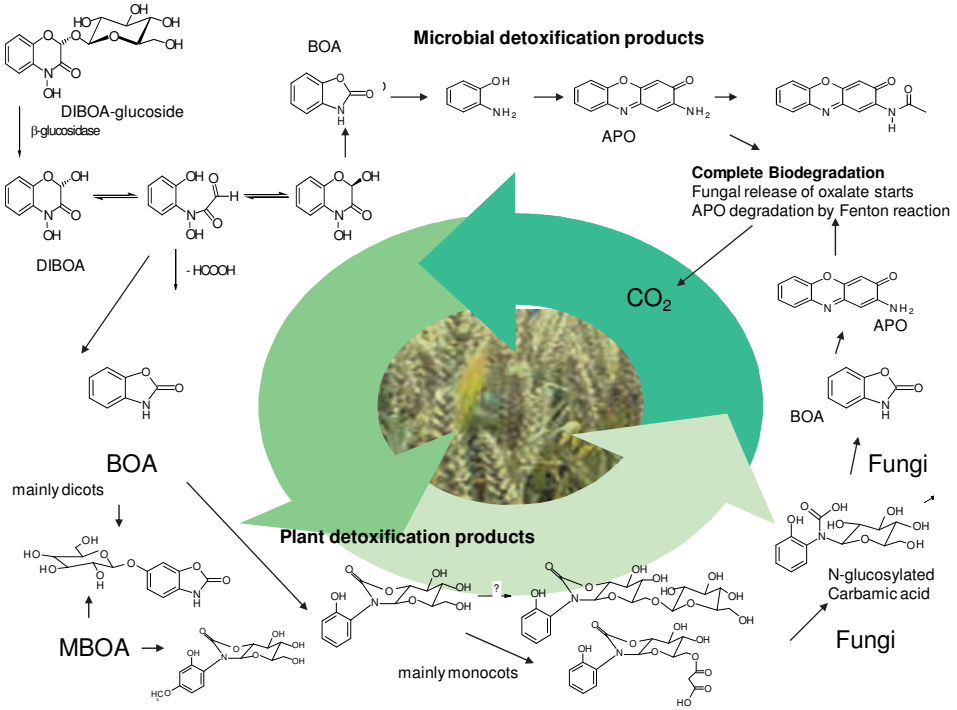

Fig. 17. Illustration of the journey. BOA is released from DIBOA degradation, can be absorbed by other plants, for example, weeds or individuals of the same species (rye, wheat, maize). Plant and microbial detoxification products can be exuded and are converted by defined microorganisms. Phenoxazinone(s) can be completely degraded via Fenton reaction.

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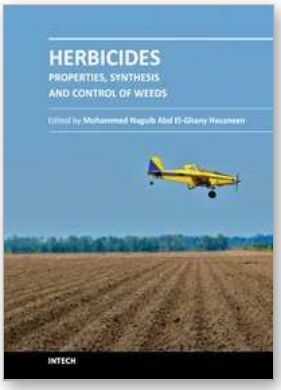

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This book is divided into two sections namely: synthesis and properties of herbicides and herbicidal control of weeds. Chapters 1 to 11 deal with the study of different synthetic pathways of certain herbicides and the physical and chemical properties of other synthesized herbicides. The other 14 chapters (12-25) discussed the different methods by which each herbicide controls specific weed population. The overall purpose of the book, is to show properties and characterization of herbicides, the physical and chemical properties of selected types of herbicides, and the influence of certain herbicides on soil physical and chemical properties on microflora. In addition, an evaluation of the degree of contamination of either soils and/or crops by herbicides is discussed alongside an investigation into the performance and photochemistry of herbicides and the fate of excess herbicides in soils and field crops.

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