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Arbuscular mycorrhizal association is beneficial for growth and detoxification of xenobiotics of barley under drought stress

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Abstract
Background, aim, and scope Plant growth and productivity under abiotic stresses such as water shortage or pollution are major problems which currently worry scientists in the field of food production and plant health. Since the intensification of livestock production with its associated increased demand for fodder has encouraged farmers to rely more heavily on chemical fertilizers, very often, municipal waste and wastewater sludge is considered a possible source of plant nutrients, although it might carry a significant amount of anthropogeneous pollutants. The extent to which plants react to drought, as well as how pollutants are taken up or how they act on plants, might depend on rhizosphere processes such as mycorrhizal symbioses. Therefore, it seemed timely to investigate plant defense reactions in the presence of arbuscular mycorrhizal fungi (AMF) toward a possible dangerous sewage sludge pollutant under the influence of drought.
Materials and methods This study was done with mycorrhizal plants (+AMF) and non-mycorrhizal plants (−AMF) under conditions of water shortage and certain supplements of pharmaceutics. Plant water relations, activity of plant detoxification enzymes, and phylogeny of root mycorrhization were surveyed to investigate beneficial aspects of AMF symbiosis. Plants were exposed to certain drought phases and watered with tap water containing the pharmaceutical paracetamol (acetaminophen) as a model xenobiotic with environmental relevance. Enzymes of interest for the present study were catalase (CAT) and glutathione S-transferase (GST). Assays followed published methods. GST spectrophotometric assays included model substrates 1-chloro-2,4dinitrobenzene, 1,2-dichloro-4-nitrobenzene, and 1,2-dichloro-4-nitrobenzoyl-chloride.
Results Our observations indicate H_2O_2 accumulation in roots of AMF plants and concomitant increases of catalase activities. Enzyme activities were lower in AMF plants even after exposure to both drought stress and paracetamol. AMF symbiosis seems a strategy to influence plant water tolerance and stress enzymes. Catalase activity seemed to be exhausted at the end of 56 days of drought treatment and pharmaceutics incubation, as indicated by high H_2O_2 levels at the end of the experiment. GST reactions differed between drought or paracetamol-stressed plants. A combination of both stressors resulted in lowered GST activities. Both catalase and GST activities decreased in the presence of paracetamol in the AMF roots after drought treatment. When soil water contents declined, AMF symbiosis supported leaf water content and production of biomass. During dry cycles, AMF roots achieved lower activities of cytosolic GST than that as compared to non-mycorrhizal roots. However, under drought and pharmaceutics supplements, water relations of mycorrhizal plants were more balanced than in non-mycorrhizal plants. In addition, roots showed an enhanced mycorrhization rate in those circumstances.
Conclusions We propose that the adjustment of CAT and GST activities in mycorrhizal roots under drought and xenobiotics might be caused by a decreased oxidative damage to rhizosphere because AMF plants had consis-

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tently better plant water relations and biomass production. Additional drought avoidance mechanisms indicated by the arbuscular mycorrhizal symbiosis might also contribute to the lower oxidative stress in mycorrhizal plants. Since the role of mycorrhiza in the fate of organic pollutants has not yet been understood, further ecotoxicological studies in mycorrhizosphere needs to be achieved as cooperation between plant–microbe specialists and ecotoxicologists.

Keywords Arbuscular mycorrhizal symbiosis · Drought · Enzymes · Glutathione S-transferases · Organic pollutants

1 Background, aim, and scope

Pharmaceuticals for veterinary or human use as well as personal care products increase in the environment. It was found that they were ubiquitous and pseudo-persistent due to continuous or repeated input (Halling-Sørensen et al. 1998; Daughton and Ternes 1999). Anti-inflammatory and antipyretic drugs like the acetaminophen (paracetamol) are among the most frequently used medicaments. Its appearance in the environment is considered as the result of a combination of an incomplete removal by sewage treatment and of the refractoriness with respect to abiotic and biotic (natural) transformations (Huber et al. 2009). Especially agricultural systems may receive considerable input of pharmaceuticals and their residues through the application of biosolids from waste-activated sludge of municipal wastewater treatment systems (Kinney et al. 2006), and certain medicaments have been found to accumulate in soils (Thiele-Bruhn 2003). This pathway starts with the use of liquid manure as a fertilizer on fields, in the worst case with subsequent surface runoff, leaching down to deeper soil layers and finally to groundwater, or drift during manuring with the result of an input into surface waters.

Although the concentrations at which they are normally found in the aquatic environment are in the range of micrograms to nanograms per liter (Ternes 1998; Buser et al. 1998a, b; Zuccato et al. 2000); no indications exist for the most part of them that allow ruling out of possible interactions with living organisms. In contrast, some data on antibiotics are available that indicate the capability of these molecules to exert toxic effects on algae and invertebrates (Halling-Sørensen et al. 1998) even at concentrations found in the environment.

Where toxicity testing has been conducted, the focus has been on higher organisms such as plants (Migliore et al. 2003), soil invertebrates (Baguer et al. 2000), or on the effects of antibiotics on bacterial soil function and antibiotic resistance (Cunningham 2004). In this respect, soil fungi have been virtually ignored despite being critical to ecosystem function as decomposers, mutualists, pathogens, and a food source for many other soil organisms. One argument for their exclusion from toxicological testing is that it is assumed that all fungi are "r"-type strategists, with many species ready to occupy vacated niches should the appropriate environmental conditions arise (Kapustka 1999).

However, some specialized fungi provide ecological functions that are not duplicated by any other organism within the fungal or any other kingdom. One such fungal group consists of the arbuscular mycorrhizal fungi (AMF), which form a permanent, obligate symbiotic relationship with the roots of over 80% of all plant species (Trappe 1987; Wang and Qiu 2006). Mycorrhizal fungi have been described as plant helper organisms with beneficial influence on plant nutrient acquisition and water use efficiency (Augé et al. 2001a, b; Khalvati et al. 2005). The latter might be of great importance with respect to climate change, especially water shortage, and plant productivity on poor and polluted soils.

Plants under stress are well stocked with an array of protective and repair systems that minimize the occurrence of oxidative damage. According to Smirnoff (1993), these can be divided into two categories: systems that react with active forms of oxygen and keep them at a low level, i.e., superoxide dismutases, catalase (CAT), or peroxidases, and systems that regenerate oxidized antioxidants [glutathione (GSH), glutathione reductase (GR), ascorbate and mono- and dehydroascorbate reductases]. The first group of enzymes is involved in the scavenging of O_2 radicals and H_2O_2, thereby preventing the formation of OH radicals. They are important components of the ascorbate–glutathione pathway responsible for the removal of H_2O_2 in different cellular compartments (Dalton 1995; Jiménez et al. 1997). The second group is formed by anaplerotic functions feeding the Halliwell–Asada cycle and keeping the antioxidative status of cells high. In this context, withdrawal of glutathione by other reactions, i.e., classical detoxification reactions like conjugation or chelation, have to be considered because they will also interact with the homeostasis of antioxidants (Noctor et al. 2002). In this respect, barley is a well-studied and strategically interesting crop. Its genome has been sequenced and is used in numerous studies on abiotic and biotic stresses. A better understanding of growth and physiological aspects of this crop will yield important information for other cereals and production systems.

The interaction between the organisms in the mycorrhizosphere has not well been described so far, but influences of bacteria on the defense system of plants and sensitive or hypersensitive responses are to be expected (Levine et al., 1994). Further factors like drought or xenobiotic pollution, e.g., with medicaments, will add stress to the rhizosphere. It is a question whether AMF

symbiosis can moderate adverse effects to the plant or induce defense systems. Our present study concentrates on the impact of a symbiotic soil fungus (*Glomus hoi*) on the detoxification system of barley roots (*Hordeum vulgare* L.) under conditions of restricted soil water and the presence of a pharmaceutical xenobiotic.

2 Materials and methods

2.1 Plant growth conditions

Seeds of barley (*H. vulgare* L.) were first sterilized in 0.5% NaClO solution for 15 min and then rinsed three times in H_2O dd. in Petri dishes. Seeds were then allowed to germinate in the same Petri dishes for 15 h and subsequently sown in the pots. Seeds were cultivated to grow in six pots for each treatment, with a diameter of 15 cm and a depth of 25 cm in sterilized quartz sand (0.5 mm coarse grade) in a greenhouse at 65%/70% relative humidity, day/night temperatures of 20–24°C/15–18°C, and photoperiod of 14 h at photosynthetic photon flux density of 800 μmol $m^{-2}s^{-1}$ under high-intensity incandescent light. Seven days later, the number of plants per pot was reduced to 15. Soil moisture (water potential) was measured by weighing the pots every 2 days. Water was supplied daily to maintain constant initial soil water content (24% gravimetric soil water content). After a 3-week establishment period (when plants were 21 days old), half of the plants were acclimated by six drying cycles until harvested 42, 48, or 56 days after sowing. At the end of each drying cycle, all plants received about 40 mL half-strength Hoagland solution (sterilized) to replenish to 24% soil water content. Plants in the well-watered treatments received additional tap water during the experiment. At the end of the experiment, the sand was checked for the presence of bacteria and fungi. The schedule of drying cycles and nutritional application is presented in Table 1.

Table 1 Application of water, nutrition and treatments to the experimental pots

Treatment	Watering	Hoagland solution	Drying cycles	Pharma. treatment
+AMF, WW	+	+	None	None
+AMF, Drought (D)	None	None	+	None
+AMF, D+P	None	None	+	+
−AMF, WW	+	+	None	None
−AMF, Drought (D)	None	None	+	None
−AMF, D+P	None	None	+	+

Treatments were: *+AMF, WW* mycorrhizal plant with good water conditions, *+AMF, Drought (D)* mycorrhizal plant under drought, *+AMF, D+P* mycorrhizal plant with integration of drought and paracetamol treatment, *−AMF, WW* non-mycorrhizal plant, well-watered, *−AMF, Drought (D)* non-mycorrhizal plant under drought condition, *−AMF, D+P* non-mycorrhizal plant with integration of drought and paracetamol treatment

2.2 Inoculation

For the mycorrhizal treatments, inocula of AM fungi (*G. hoi*) were provided from University of York, UK (Prof. A. Fitter) for trap culture experiments, with preformed spores (80–95 spores per 0.5 g soil), root inoculum (95% root mycorrhization), and hyphae from previous pot cultures. This inoculum was produced with *Plantago lanceolata* as host plant, cultivated in 650-mL pots in sterile sand ($ø$=0.5 μm) as growth medium. Plants were supplied with modified Hoagland solution containing reduced amounts of phosphorus (1/10). All host plants were grown in phytotrons at 65%/70% relative humidity, day/night temperatures of 20–24°C/15–18°C, and a photoperiod of 14 h at photosynthetic photon flux density of 800 μmol $m^{-2}s^{-1}$ under high-intensity incandescent light. An amount of 25 g of pre-cultivated inoculum had been identified in preliminary tests as the optimum to produce a good colonization level for the given amount of soil in each pot. The *G. hoi* inocula were placed 2–3 cm below the soil surface into the plant pots. Control treatments (−AMF) were prepared in the same manner but without inoculum (sterilized inoculums).

2.3 Evaluation of mycorrhizal colonization and symbiotic development

G. hoi root colonization was evaluated in mycorrhizal roots of each treatment after trypan blue staining (Trouvelot et al. 1986). The parameters of mycorrhizal infection were the extent of root cortex colonization (*M*%) in the whole root system. The percentage of root colonization by mycorrhizal fungi was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (*v*/*v*) according to Phillips and Hayman (1979). Mycorrhizal colonization was determined in 25 random samples of 1-cm-long root segments from each of seven plants (n=175) and percentage of root mycorrhization, and external hyphae were measured. The extent of mycorrhizal colonization was calculated according to the quantification method of Furlan and Fortin (1977).

2.4 Stress implementation

Prior to the initiation of drought, both +AMF or –AMF plants were fertilized with 60 mL of Hoagland solution with reduced phosphorus (1/10). A total of six drying cycles

were applied during plant growth by restricting irrigation for 7 days.

At the end of every drought cycle, the containers were weighed and soil water content recovered to the initial water content (24%). Drought stress was applied during the last three cycles, and the drought-stressed plants inoculated with or without mycorrhizal fungi were watered with 2 μM (300 μg L^{-1}) of paracetamol solution. This concentration was used as a representative xenobiotic pollutant concentration occurring in sewage sludge (Ternes 1998). Plant sampling was performed at the end of each of the last three drying cycles (42, 48, and 56 days after sowing). Plants were dissected and shoots and roots frozen separately.

2.5 Leaf chlorophyll content

Chlorophyll content was determined in vivo using a Minolta SPAD 502 DL meter featuring integrated data logger. With this device, it was possible to instantly measure chlorophyll content after clamping the meter over leafy tissue in less than 2 s.

2.6 Protein extraction and enzyme assays

Soluble proteins were extracted from barley root samples following the method of Schröder et al. (2002) and a modified method of Belford (2004). In short, frozen roots were ground to a fine powder with mortar and pestle and mixed with ten volumes of sodium phosphate buffer (0.1 M, pH6.5) containing 20% of glycerol, 1 mM EDTA, 1% PVP, and 14 mM DTE. The slurry was allowed to stand for 10 min on ice and repeatedly vortexed (IKA-combimag, Stauffen, FRG).

After a first centrifugation at 10,000×*g* for 10 min and a second for 1 h at 39,500×*g*, the supernatant was taken as the crude enzyme extract and the pellet discarded. The proteins present in the supernatant were subjected to ammonium sulfate precipitation. In the first step, 40% $NH_3(SO_4)_2$ was added, the extract was allowed to stand for 20 min on ice under permanent stirring, and centrifuged for 1 h at 39,500×*g*. The supernatant was saved and the pellet was discarded. The supernatant was then adjusted to 80% $NH_3(SO_4)_2$ saturation and stirred for 20 min. After the last centrifugation step, the supernatant was discarded and the pellet resuspended in 2.5 mL of sodium phosphate buffer (20 mM, pH7.0). Photometric measurements of GST enzyme activities were accomplished with the model substrates 1-chloro-2,4dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and 1,2-dichloro-4-nitrobenzoyl-chloride (p-NBoC). The assays were conducted in 0.1 M KPP buffer, pH6.4 (pH7.5 in the case of DCNB) under addition of 1 mM glutathione and 1 mM substrate concentration. Measurements were done for 5 min at the following wavelengths: CDNB, 340 nm (ε= 9.6 $mM^{-1}cm^{-1}$), DCNB, 345 nm (ε=8.5 $mM^{-1}cm^{-1}$), and p-NBoC, 310 nm (ε=1.9 $mM^{-1}cm^{-1}$) and corrected for non-enzymatic blanks. Sample amount in assays was 20% of the total volume (Habig et al. 1974). Catalase activity was determined at 240 nm (Verma and Dubey 2003). The amount of soluble protein in the samples was determined following the method of Bradford (1976).

2.7 In situ H_2O_2 detection

H_2O_2 was detected by an endogenous peroxidase-dependent in situ histochemical staining procedure using 3,3′-diaminobenzidine (DAB). Intact root samples were gently rinsed with distilled water to remove adhering sand and dried on paper tissue. The roots were placed in a solution containing 2 mg/mL DAB, vacuum-infiltrated for 5 min, and then incubated for 3 h in the dark. H_2O_2 production is visualized as a reddish brown coloration (Dongtao et al. 2002).

2.8 Relative leaf water content

The relative leaf water content (RWC) was ascertained by measuring the fresh weight, rehydrated weight on distilled water, and dry weight (DW, 80°C for 2 days) using the following formula (Turner 1986): $RWC = (FW - DW/TW - DW) \times 100$. Leaves fresh weight (FW) was determined by immediately weighing one fully expanded young leaf, which was allowed to rehydrate for 4 h by floating 1 cm from the cutting part into a covered beaker with distilled water. The rehydrated leaf was weighed (TW) to determine saturate mass, and then the leaf was dried at 70°C for 24 h to determine DW.

2.9 Statistics

Data were subjected to analysis of variance (ANOVA) with AMF plants, with drought, drought pharmaceutical treatments, and well-watered status as factors. When the main effects were significant ($P<0.05$), differences between means were tested for significance by Duncan's multiple range test (Duncan 1955).

3 Results

Exposing barley plants to drought and pharmaceutical stress led to accelerated aging and loss of leaves in all treatments. However, the beneficial effects of inoculation with *G. hoi* were significant and clearly visible in plant growth and performance.

3.1 Extent of root colonization by mycorrhizae

Roots of AMF plants were well infected by mycorrhizal fungus as shown by the presence of intraradical hyphae in roots stained with trypan blue (Fig. 4). The percentage of total root colonization was estimated as 68±6% mycorrhization rate.

3.2 Drying cycles and soil water content

The comparison of gravimetric soil water content between the plants with mycorrhizal fungi (+AMF) and plant without mycorrhizal fungi (−AMF) under conditions of drought stress is illustrated in Fig. 1. Our result showed significant differences of average soil water content ($P<0.05$, $n=6$) between +AMF and −AMF plants after the last drying cycles (42, 48, and 56 days after sowing). Soil water content decreased to 6.8% (±0.34%) and 8.5% (±0.61%) in +AMF and –AMF, respectively, in the presence of 9 ppm available phosphorus at the end of the last drying cycle (56 days after sowing). Both sets of plants were subjected to 2 μM paracetamol during the last three drying cycles (42, 48, and 56 days after sowing, respectively). A clear difference was observed between the soil water content in pots containing +AMF and −AMF plants from the end of the third drying cycle (36 days after sowing). This difference remained throughout the rest of the experiment until harvest.

3.3 Plant biomass and symbiotic development

Shoot DW was higher in the +AMF well-watered and drought pharmaceutical-stressed plants than in the corresponding −AMF plants (Table 2). In general, plants under drought and pharmaceutical stress had decreased shoot DW, but stressed +AMF plants had 49.8% more shoot DW than stressed −AMF plants. Apparently, −AMF plants were not healthy at the end of the last drying cycle (56 day after sowing) and showed numerous chloroses. Significantly different from that observation, the +AMF plants seemed morphologically healthy, with fewer and smaller chloroses. This may mainly be attributed to the beneficial contribution of AMF in host plant under stress conditions (Fig. 5).

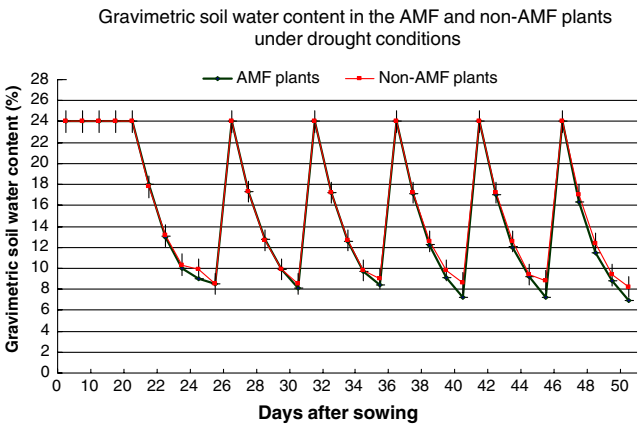

Fig. 1 Gravimetric soil water content in the plants with or without arbuscular mycorrhizal fungi (*G. hoi*) at the end of each drying cycle. *Error bars* represent standard deviation

Table 2 Effects of arbuscular mycorrhizal fungi on biomass and leaf water contents of plants with or without arbuscular mycorrhizal fungi (+AMF; −AMF) plants subjected to drought pharmaceuticals stress (D+P)

	+AMF plant WW	−AMF (control) WW	+AMF plant D+P	−AMF (control) D+P
Shoot fresh weight (g per plant)	15.56 ns	17.57 ns	6.10 a	3.73 b
Root fresh weight (g per plant)	6.60 a	4.10 b	3.10 a	1.56 b
Spike fresh weight (g per plant)	3.60 ns	3.90 ns	1.01 a	0.00
Root/shoot ratio	0.42 a	0.26 b	0.50 b	0.90 a
Relative leaf water contents (%)	98 a	100 a	78.1 a	65.9 b

Differences between means were tested by Duncans's multiple range test at $P<0.01$ level; a, b different letters in rows indicate significant differences

ns non-significant

3.4 Plant water content

Prior to the initiation of drought, both +AMF or –AMF plants had been fertilized with 60 mL of Hoagland solution with reduced phosphorus (1/10) regularly to aid them attaining comparable size (number of leaves, leaf area, shoot height). At the end of the final drought treatment in the presence of paracetamol, we found significant differences in appearance and growth (see Fig. 5), shoot, and root dry weight of plants (ANOVA, $P<0.05$; see Table 2). Also, the root/shoot ratio was affected by all treatments. Under well-watered conditions, both shoot and root dry weights in +AMF and −AMF plants were similar (see Table 2). Small growth depression was observed in the dry weight of shoots and root of +AMF as compared with the −AMF plants, which could possibly be due to the competition for photosynthates between host and fungus (Paszkowski et al. 2006; Khalvati 2005).

In plants subjected to water stress, relative leaf water contents were 78.1±2.5% and 65.9±1.8% in plants inoculated with *G. hoi* and in −AMF plants, respectively (Table 3). This demonstrates that mycorrhization had positive impacts on the leaf water relations in barley. Water use efficiency also impacted the chlorophyll contents in

Table 3 GST enzyme activities on the AMF and non-AMF roots under short-term drought conditions

GST activity of untreated controls	−AMF (WW)	+AMF (WW)	−AMF (WW)	−AMF (D)	−AMF (D)	+AMF (D)
CDNB (%)	130*		137*		203*	
DCNB (%)	−20 (ns)		−21 (ns)		−37 (ns)	
NBoC (%)	306*		−17 (ns)		53*	

Differences between means were tested by Duncan's multiple range test (Duncan and Duncan 1955)

*$P<0.01$ level)

ns non-significant

leaves of +AMF plants as compared to −AMF plants under drought pharmaceutical stress which was significantly higher than in −AMF plants (Fig. 2).

Drought will cause oxidative stress in the plants, and the accumulation of H_2O_2 during the stress condition is a known phenomenon. In an additional experiment (+AMF and −AMF barley were treated with 2 µM paracetamol for 1 week), we observed H_2O_2 accumulation in the root tissues too. After histochemical staining, the reddish brown coloration of roots indicates the presence of hydrogen peroxide (Fig. 6). The intensity of the coloration is proportional to the amount of accumulated H_2O_2. The accumulation of H_2O_2 was observed after the paracetamol exposure both in mycorrhizal and non-mycorrhizal plants; however, in mycorrhizal plants, this effect was much stronger.

3.5 Protein extraction and enzyme assays

To characterize the general behavior of barley seedling GST activities toward AMF inoculation and drought, a pilot study was performed. In this study, plants were inoculated with AMF as previously described, but only subjected to one drought treatment of 7 days. At the end of this period, they were harvested as described above.

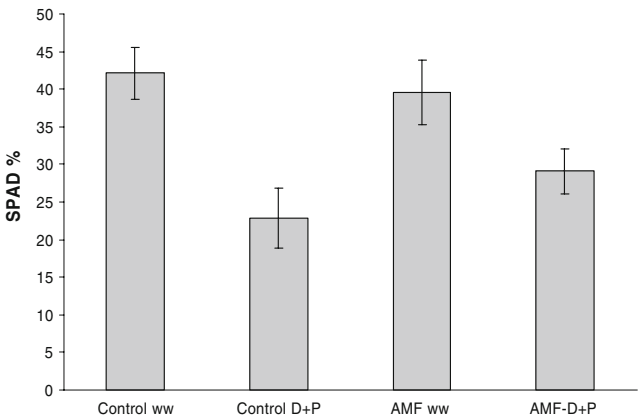

Fig. 2 Soil plant analysis development (*SPAD*) corresponding to relative chlorophyll concentration. *Error bars* represent standard deviation

The results of this pilot experiment indicate a strong effect of fungal inoculation and drought on the CDNB conjugation.

As compared to well-watered control plants, both AMF inoculation or drought treatment led to 130% higher activities, and drought-treated +AMF plants had even 200% higher CDNB–GST activity. DCNB, a closely related substrate, but substituted with two chloride groups, was conjugated at low rates and did not show significant reactions under any of the treatments. In contrast, a rarely used xenobiotic substrate, NBoC, was conjugated 306% above well-watered controls upon inoculation with the AMF. Whereas there was no significant difference in the GST activities between well-watered and drought-stressed non-mycorrhizal plants, mycorrhization as such induced NBoC GST activities in drought-stressed +AMF seedlings by 53%. Hence, the presence of *Glomus* exerts some inductive capacity on NBoC GSTs in barley (see Table 2).

Following the results of this pilot study, we investigated the effects of longer and repeated drought periods on the plants as well as the incubation with paracetamol as a typical pharmaceutical that might be present in sewage sludge and applied to fields. Because of the previously observed H_2O_2 accumulation, catalase was investigated as an additional stress enzyme. CAT mediates the degradation of H_2O_2 in stressed plants and hence is an enzyme the activity of which might provide some hints about the stress response of a given tissue. The enzymatic activities investigated also showed significant differences between +AMF and −AMF plants after being subjected to drought stress. Results obtained from each treatment applied to barley plants are shown in Fig. 3. CAT activity in roots was found to be low at around 0.15 µkat/mg protein in the well-watered −AMF control plants at all harvests. In well-watered +AMF plants, despite fungal infection, it was insignificantly higher than in controls. Contrary to this, drought caused a doubling of CAT activity in −AMF plants and a tripling in the mycorrhizal +AMF plants. When −AMF plants were treated with the pharmaceutical paracetamol and additionally subjected to drought, the previously observed increase under drought was lost, and no significant increase of CAT was found as compared to well-watered plants (−AMF WW). Here, the effect of paracetamol seems to be inhibitory on CAT activity. However, in +AMF plants 48 days after sowing, in the second drying cycle, a significant increase in CAT activity to values three times above controls was observed, but ceased after 56 days of sowing (see Fig. 3). In this case, the decreased catalase activity may well refer to the accumulation of hydrogen peroxide in the plant tissues.

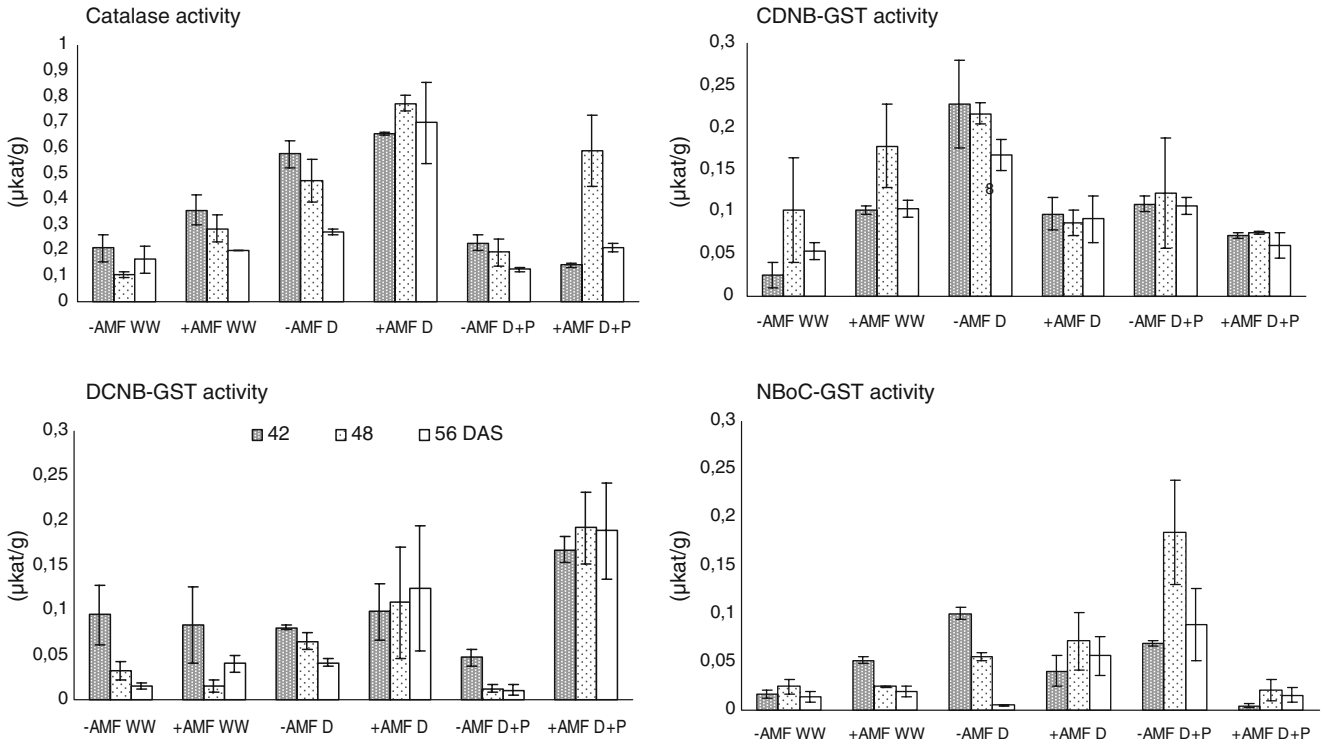

Fig. 3 GST activities in the roots of AMF plants and non-AMF plants (control) under conditions of well-watered (*WW*), drought (*D*), and a combination of drought and pharmaceutical stress (*D+P*). Plants were harvested 42 (*gray bars*), 48 (*dotted bars*), and 56 (*empty bars*) days after sowing (*DAS*), n=5. *Error bars* represent standard deviations

The course of enzyme activity for the conjugation of the model substrate CDNB is very similar to the previously described pilot study and the results obtained for CAT. Again, well-watered controls had rather low basic GST activity, and AMF inoculation led to a doubling or even tripling of GST. Drought exerted even stronger effects on the GST activity, inducing up to fivefold GST increases in −AMF plants, whereas AMF inoculation seemed to attenuate this stress reaction. Drought and paracetamol synergistically increased the CDNB conjugation twofold, and the AMF suppressed CDNB conjugation in the presence of these stressors to levels of the controls. So far, the enzymatic reactions shown are in the expected frame and demonstrate that the plants have good measures to counteract stress. However, when the less known CDNB–GST substrates DCNB and NBoC are used, the picture differs considerably.

DCNB–GST activity is present in all cytosolic extracts and all treatments at levels comparable to CDNB conjugation rates and decreases from drought cycle to drought cycle. However, DCNB conjugation showed almost contrary patterns to CDNB–GST in the extracts of both −AMF and +AMF plants. Well-watered plants (−AMF WW) had the same activities regardless of mycorrhization, and even drought did not increase the conjugation rate significantly. Only AMF inoculation in connection with drought led to an increase of DCNB conjugation, similar to the increase observed in CAT activities, and the additional stress by paracetamol induced the highest activities, with levels three to tenfold above the respective controls. Hence, DCNB activities are not responsive to drought and paracetamol alone, but clearly induced in the presence of AMF.

NBoC conjugation proceeded at lowest rates of all investigates substrates, never exceeding 0.15 µkat/mg. As depicted in Fig. 3, this GST activity reacted significantly to each treatment, except the combination of AMF, drought, and paracetamol, which was surprisingly similar to values of non-mycorrhizal plant grown under optimal watered conditions (−AMF WW). The highest induction was obtained in −AMF plants stressed by drought and paracetamol, an enzymatic answer that was not observed in the other assays (Figs. 4, 5, and 6).

4 Discussion

The presence in the environment of novel xenobiotic compounds is reported as a consequence of the massive use of chemicals in different productive fields and in everyday modern life (Paxeus 1996; Janssens et al. 1997;

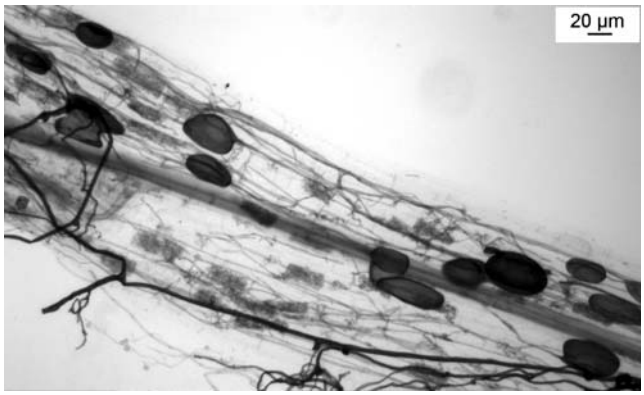

Fig. 4 Intraradical hyphae of *G. hoi* in trypan blue-stained roots of an AMF host plant 56 days after sowing

Fromme et al. 2001). Many of these substances are now considered "persistent organic pollutants," thus rising great concerns about the effects (often unknown) they could exert on living organisms (Rodgers et al. 2000). Recently, also the presence of pharmaceuticals has been documented and reported as an emerging environmental issue (Holm et al. 1995; Ternes 1998; Daughton and Ternes 1999, Kinney et al. 2006). Hundreds of tons of pharmaceuticals are annually prescribed in Europe and consequently discharged—modified or as metabolites—in sewage effluents (Hirsch et al. 1999; Buser et al. 1999; Jorgensen and Halling-Sorensen 2000). Anti-inflammatory and antipyretic drugs like the acetaminophen (paracetamol) are among the most frequently used medicaments. The appearance of drugs in the environment is considered as the result of a combination of a partial removal in sewage treatment plants and of the refractoriness with respect to abiotic and biotic (natural) transformations (Kinney et al. 2006; Huber et al. 2009).

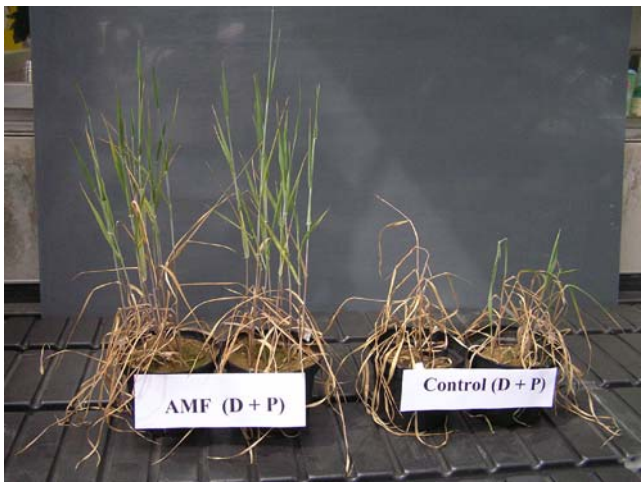

Fig. 5 Comparison of +AMF plants and −AMF plants under conditions of drought and the pharmaceutical paracetamol

The effects of these chemicals on plant health and performance have so far not been investigated in detail. Water deficit, on the other hand, is known to have profound effects on crop production. Even plants with an optimum water supply experience transient water shortage periods where water absorption cannot compensate for water loss by transpiration (Kramer and Boyer 1997). Arbuscular mycorrhizal symbiosis has been shown to increase plant tolerance to such water deficits, although the exact mechanisms involved are still a matter of debate (Augé 2001; Ruiz-Lozano 2003). This can be important for mycorrhizal plants since drought also induces oxidative stress, which, as has been pointed out, is responsible for many of the degenerative reactions caused by xenobiotics.

Glomus mycelia have the capability to increase water uptake in the rhizosphere and hence maintain plant cell hydraulic stability (see Table 2). This phenomenon may provide an appropriate way for plant roots to save individual resources and allow the mycorrhizal plant to continue producing biomass up to 49% higher than non-mycorrhizal plants under stress conditions. However, it is also well known that mycorrhizal symbiosis causes important changes in plant metabolism (Smith and Gianinazzi-Pearson 1988; Arines et al. 1993; Palma et al. 1993). However, there is little information about the response of plants in mycorrhizal symbiosis towards stress conditions. In the present case, the *H. vulgare–G. hoi* system was studied under drought and drought pharmaceutical stress conditions as an approach to understand the effects of mycorrhizal association on plant mechanisms known to cope with environmental stresses. It is well known that the antioxidant systems of plants act as important tolerance mechanisms by protecting cells against damage caused by toxic oxygen species such as superoxide radicals (O^{2-}), H_2O_2, and hydroxyl radicals (OH^-), all of which are generated under environmental as well as xenobiotic stresses (Shaaltiel and Gressel 1986; Shaaltiel et al. 1988; Asada 1994; Foyer et al. 1994).

The ASC–GSH cycle, of which the GR together with APX are important components, is one of the main antioxidant defenses in plants (Becana et al. 2000; Dalton 1995). It has been proposed that the impairment of the ASC–GSH cycle in the roots may be a common feature in the process of coping with stress (Escuredo et al. 1996). This idea agrees with recent findings by Burritt et al. (2002) who have postulated that the ability of seaweeds to prevent or reduce the production of reactive oxygen species (ROS) was due to the increased activity of the enzymes required to regenerate ascorbate and glutathione, as is the case of GR. However, we would also expect an increased CAT activity in rhizosphere of drought pharmaceutical-stressed mycorrhizal plant. We propose that other mechanisms might have contributed to the

Fig. 6 Histochemical H_2O_2 detection in the mycorrhizal (AMF) and non-mycorrhizal (control) roots exposure to 2 µm paracetamol

protection of barley plants against drought pharmaceutical-induced water use efficiency to obtain biomass. One possibility is that mycorrhizal symbiosis leads to a lower drought-induced oxidative stress in AMF plants due to primary drought avoidance mechanisms such as the higher water retention properties of a mycorrhizal soil as compared to the soil of non-mycorrhizal plants (Augé et al. 2001a, b) as well as to the ability of AMF hyphae to take up water from sources inaccessible to the non-mycorrhizal roots and transfer to the host plant (Hardie 1985; Ruiz-Lozano and Azcón 1995).

Our histochemical investigations indicate a high rate of H_2O_2 production during the paracetamol treatment to both +AMF and −AMF roots (Fig. 6). However, our observation pointed out that mycorrhizal roots endure the presence of paracetamol concentrations by emerging higher CAT activity. Similar to published data, we found increasing CAT activity in mycorrhizal plants as well as under environmental and xenobiotic stresses (Shaaltiel and Gressel 1986; Shaaltiel et al. 1988; Asada 1994; Foyer et al. 1994; Porcel and Ruiz-Lozano 2004). Our data also indicate a significantly elevated CAT activity in plants encountering the combination of two stresses.

GST activities in plants from the drought treatment were found to be higher than in well-watered plants. However, GST activity in mycorrhizal plant roots cultivated under well-watered conditions was similar or only slightly higher than non-mycorrhizal treatments (see Fig. 3). Recent reports have described mycorrhizal symbioses impact on drought and pollutant degradation (Khalvati et al. 2005; Augé 2001). The fact that the main differences in GST activity in mycorrhizal plants appeared when plants were subjected to drought and pharmaceutical stress indicates that only a small part of GST activity is induced by the reaction against AMF and possibly under the influence of excess H_2O_2. Our results obtained from assays of glutathione S-transferases activities in roots demonstrate both good adaptation to xenobiotic stress defense and the necessity for segregation of GSTs according to their natural functions. Recently, it has been demonstrated that plants are capable of conjugating paracetamol to glutathione (Huber et al. 2009), but the responsible GST isoforms have not yet been identified. The main changes in the GST activity recorded here seem to be the direct result of drought and more significantly in drought plus pharmaceutics stress, which points to a distinct group of GST isoforms with yet unknown natural functions.

5 Conclusions

In conclusion, the results obtained in this study suggest that the consistently higher water content in roots of mycorrhizal plants might have alleviated stress. Although they had increased H_2O_2 content, the mycorrhizal plants showed higher chlorophyll content and looked healthier, which indicates better tolerance towards oxidative damage. At this stage of the research, two possibilities can be envisaged to explain the low oxidative damage found in the roots of AMF plants: Either they suffered less drought stress due to a primary drought avoidance effect by the symbiosis (e.g., by direct water uptake by fungal hyphae from sources inaccessible to non-mycorrhizal plants and transfer to the host plant), which kept plants protected against the generation of ROS, or mycorrhizal infection increased the activities of a set of defense enzymes involved in the elimination of active oxygen species. Also, xenobiotic stress was reduced, and effects on GST activities, which would have been expected, were comparatively low. A specific role of certain GSTs conjugating rare substrates might not be excluded. This observation requires further attention.

This could have happened as part of the compatible reaction during mycorrhization. Mycorrhiza may be beneficial to overcome xenobiotic or environmental stress in the

rhizosphere. Further studies have to include a thorough investigation of the potential metabolism of the pharmaceutical by plants and/or the AMF partner and to identify the signals required for the induction or inhibition effect. This will help in decision-making processes whether sewage sludge or reclaimed water might be used in agricultural soils without stress to the plants.

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