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# Biotransformation of *Trichoderma* spp. and Their Tolerance to Aromatic Amines, a Major Class of Pollutants

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*Trichoderma* spp. are cosmopolitan soil fungi that are highly resistant to many toxic compounds. Here, we show that *Trichoderma virens* and *T. reesei* are tolerant to aromatic amines (AA), a major class of pollutants including the highly toxic pesticide residue 3,4-dichloroaniline (3,4-DCA). In a previous study, we provided proof-of-concept remediation experiments in which another soil fungus, *Podospora anserina*, detoxifies 3,4-DCA through its arylamine *N*-acetyltransferase (NAT), a xenobiotic-metabolizing enzyme that enables acetyl coenzyme A-dependent detoxification of AA. To assess whether the *N*-acetylation pathway enables AA tolerance in *Trichoderma* spp., we cloned and characterized NATs from *T. virens* and *T. reesei*. We characterized recombinant enzymes by determining their catalytic efficiencies toward several toxic AA. Through a complementary approach, we also demonstrate that both *Trichoderma* species efficiently metabolize 3,4-DCA. Finally, we provide evidence that NAT-independent transformation is solely (in *T. virens*) or mainly (in *T. reesei*) responsible for the observed removal of 3,4-DCA. We conclude that *T. virens* and, to a lesser extent, *T. reesei* likely utilize another, unidentified, metabolic pathway for the detoxification of AA aside from acetylation. This is the first molecular and functional characterization of AA biotransformation in *Trichoderma* spp. Given the potential of *Trichoderma* for cleanup of contaminated soils, these results reveal new possibilities in the fungal remediation of AA-contaminated soil.

growing proportion of xenobiotics of anthropogenic origin are contaminants of natural environments, particularly soils. Estimates indicate that, in a typical soil, up to 80% of the biomass, with the exception of plant roots, is of fungal origin (1, 2). Fungi outcompete bacteria in many degradation processes or synergize their activity (3). They efficiently secrete into the surrounding medium a large array of enzymes involved in cellulose/lignin degradation that have been shown to have xenobiotic-metabolizing enzyme (XME) potential (1, 4, 5). They also produce and encounter many secondary metabolites (6), including harmful polyketides, cyclic peptides, and alkaloids, for which they must have detoxifying enzymes with xenobiotic-metabolizing potential (7). The identification of mechanisms by which soil fungi can tolerate harmful chemicals is important to understanding their adaptation to stressful environments. In addition, deciphering molecular mechanisms underlying this tolerance may lead to novel pharmaceutical and biotechnological applications, including soil bioremediation (8). Indeed, Harms and colleagues recently noted that the potential use of fungi in bioremediation has not received the attention it deserves (9).

The fungal genus *Trichoderma* (Ascomycetes, Hypocreales) includes cosmopolitan, free-living, or mycoparasitic fungi that are very common in soil and root ecosystems (10). They are frequently found on decaying wood, compost, or other organic matter (11). *Trichoderma reesei* (teleomorph *Hypocrea jecorina*), a saprotroph, is widely used in industry as a source of cellulases and hemicellulases for the hydrolysis of plant cell wall polysaccharides (12), and *T. virens* (teleomorph *Hypocrea virens*) is a mycoparasite used in biological control because of its efficient colonization of plant roots (13). *Trichoderma* spp. produce a wide diversity of secondary metabolites and possess high intrinsic resistance to

toxic compounds (14). Although these fungi have been extensively studied, including through “omics” approaches (15), very little is known regarding their XME (16). Because of the possible interactions of *Trichoderma* spp. with numerous xenobiotics of anthropogenic origin (pesticide derivatives, industrial chemicals), there is a need for the accurate identification of XME in these fungi.

In a pilot study, we performed proof-of-concept remediation experiments in which *Podospora anserina*, through its arylamine *N*-acetyltransferase 2 (NAT2) enzyme, detoxified aromatic amines (AA), a major class of occupational or environmental pollutants (17), including the highly toxic pesticide residue 3,4-dichloroaniline (3,4-DCA) (18). NATs are intracellular XMEs that catalyze the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the nitrogen or oxygen atom of arylamine-based xenobiotics and their hydroxylated metabolites (19). However, the role of NATs in fungal degradation mechanisms remains largely unknown. Here, we have characterized two arylamine NATs in *T. virens* and one in *T. reesei*. Although NAT enzymes

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contribute to the AA tolerance of *T. reesei*, we show that, unlike *P. anserina*, *T. reesei* and *T. vires* may utilize another, unidentified, metabolic pathway for detoxication of AA aside from acetylation. Given the potential of *Trichoderma* spp. for the remediation of contaminated soil (14), our results offer new perspectives in fungal bioremediation.

## MATERIALS AND METHODS

**Materials.** All of the aromatic compounds used in this study (sulfamethoxazole [SMX], sulfamethazine [SMZ], sulfadiazine [SDZ], sulfapyridine [SP], hydralazine [HDZ], 5-aminosalicylate [5-AS], atrazindesethyl [ADE], atrazindesisopropyl [ADP], 2,6-diethylaniline [2,6-DEA], 2-ethyl-6-methylaniline [2,6-EMA], 3,4-DCA, 3,5-DCA, 3-chloro-4-methylaniline [3,4-CMA], 4-isopropylaniline [4-IPA], 4-bromoaniline [4-BA], benzidine [BZ], 2-aminofluorene [2-AF], and 4-iodoaniline [4-IA]), acetyl-CoA, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Acetyl-4-IPA and acetyl-3,4-DCA were purchased from Interchim (Montluçon, France). Acetyl-BZ was synthesized by ITODYS UMR CNRS 7086.

**Strains and culture conditions.** Strains of *T. vires* (CBS 430.54) and *T. reesei* (CBS 383.78) were used for all experiments. These strains were grown in potato dextrose agar (PDA) medium or in M2 synthetic medium (0.25 g · liter<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.3 g · liter<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 0.25 g · liter<sup>-1</sup> MgSO<sub>4</sub>, 0.5 g · liter<sup>-1</sup> urea, 5 g · liter<sup>-1</sup> yeast extract) with trace elements (2.5 mg · liter<sup>-1</sup> C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>, 2.5 mg · liter<sup>-1</sup> ZnSO<sub>4</sub>, 0.5 mg · liter<sup>-1</sup> CuSO<sub>4</sub>, 125 µg · liter<sup>-1</sup> MnSO<sub>4</sub>, 25 µg · liter<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>, 25 µg · liter<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, and 25 µg · liter<sup>-1</sup> H<sub>4</sub>FeNO<sub>8</sub>S<sub>2</sub>-12H<sub>2</sub>O), and vitamins (0.05 mg · liter<sup>-1</sup> thiamine and 0.25 µg · liter<sup>-1</sup> biotin). Strains were incubated at 27°C in the dark and without shaking.

**Molecular cloning and protein expression and purification.** The NAT open reading frames of *T. vires* and *T. reesei* were cloned after PCR amplification of the exonic sequences from genomic DNA and reassembly of these separately amplified exons by an overlap extension PCR strategy with the Phusion High Fidelity DNA polymerase according to the manufacturer's recommendations (New England BioLabs, Evry, France). The amplification of *T. vires* NAT1 exons 1 and 2 was done with primers 5'-AGCCATATGACTGCATACTCGCAAGACC-3' (sense) and 5'-TTGCTGCAGTGAGTCATGCCACCGTATCGTGGG-3' (reverse) and primers 5'-ACGATACGGTGGCAGTACTGACTGCAGCAATATCGTCA-3' (sense) and 5'-CCGCAAGCTTTCATGATACTGCACTCTTGGCAA-3' (reverse), respectively. *T. reesei* NAT1 exons 1 and 2 were amplified with primers 5'-CAGCCATATGGAGGCGCCCTACACC-3' (sense) and 5'-TTGGCGCAGTGAGTCATGCCTCCGTATCGAAGACCA-3' (reverse) and primers 5'-TCGATACGGAGGCTGACTCACTGCCCAACATC A-3' (sense) and 5'-CCGCAAGCTTCTATGCAACTGCACCTTTCGCG-3' (reverse), respectively. Restriction sites are underlined, and regions of complementarity used for overlap extension are in bold. Amplified products were inserted into the NdeI/HindIII sites of expression vector pET28a (Invitrogen, Saint Aubin, France). Inserts were verified by DNA sequencing, and cDNA analysis confirmed the exon-intron boundaries. *Escherichia coli* C41(DE3)/pGro7 cells transformed with (HYPVI)NAT1 or (HYPJE)NAT1 carrying plasmid pET28a were used to produce and purify 6×His-tagged recombinant protein. The production and purification of recombinant NAT enzymes have been described elsewhere (19). The purified (HYPVI)NAT1 and (HYPJE)NAT1 enzymes were dialyzed against phosphate-buffered saline. Protein concentrations were determined by the Bradford assay (Bio-Rad, Marnes-la-Coquette, France), and purity was assessed by SDS-PAGE.

**Gene transfer and expression in *P. anserina*.** Because *Trichoderma* and *Podospira* are related genera, the *T. reesei* NAT coding sequence was expressed with its own promoter. The (HYPJE)NAT1 sequence was amplified with *Pfu* DNA polymerase (Promega, Charbonnières, France), the appropriate genomic DNA, and primers 5'-TGATGCTGCTGAACCAAG C-3' and 5'-GGGCACACTGGATTGACTCT-3'. The PCR product obtained was cloned into *Swa*I-digested plasmid pCIB4. This plasmid per-

mits integration in single copy into an expressed region of the *P. anserina* genome (20). Recombinant plasmid was selected and cut with *Asc*I. The resulting product was transformed into a Δ*Pa*NAT1/2 Δ*mus52::GenR* mutant strain. In this strain, the *mus52* subunit of the KU complex involved in nonhomologous recombination is inactivated and DNA integration proceeds almost exclusively by homologous recombination. Nourseothricin-resistant transformants were obtained and crossed with the Δ*Pa*NAT1/2 mutant strain. In the progeny, we obtained strains devoid of the *P. anserina* NAT genes and lacking the Δ*mus52::GenR* mutation but containing a single copy of the *T. reesei* NAT gene. No mutation was found in the coding sequence and the surrounding regions.

**Enzyme assays and determination of kinetic parameters.** NAT activity was measured in a DTNB assay as described previously (4). Briefly, purified recombinant enzymes and arylamine substrates (500 µM final concentration) were mixed in the wells of a 96-well enzyme-linked immunosorbent assay plate and preincubated (37°C, 2 min) in buffer (25 mM Tris-HCl, pH 7.5). Acetyl-CoA was added to start the reaction (400 µM final concentration), and the plate was incubated at 37°C. The total reaction volume was 100 µl. The reaction was quenched with 25 µl of DTNB (5 mM) in guanidine hydrochloride buffer (6.4 M guanidine-HCl and 0.1 M Tris-HCl, pH 7.3). A plate reader was used to measure absorbance at 405 nm. All assays were done in triplicate under conditions giving a linear initial rate. Controls were carried out in the absence of enzyme or acetyl-CoA. The amount of CoA in the reaction mixture was determined by comparison with a standard curve obtained with DTNB. Kinetic analyses were performed by varying the AA substrate concentrations. Kinetic parameters (*V*<sub>max</sub> and *K*<sub>m</sub>) were determined by nonlinear regression analysis, with the Kaleidagraph program (Synergy Software). The *k*<sub>cat</sub>/*K*<sub>m</sub> ratios were used to compare the catalytic efficiencies of recombinant enzymes on different aromatic substrates.

**Assays of tolerance to AA.** The AA tolerance of fungi was assessed in M2 agar medium. Increasing AA (3,4-DCA, BZ, and 4-IPA) concentrations were added to the medium. Sensitivity was screened by assessing radial growth for 3 days at 27°C. Data are presented as means ± standard deviations (SDs) of three independent experiments. One-way analysis of variance was performed, followed by Dunnett's method with SigmaStat 3.0 (Systat Software Inc.).

**In vivo acetylation assays and detection of acetylated AA by HPLC.** Fungi were grown for 3 days at 27°C on a PDA dish covered with a sterile cellophane sheet. The mycelium was harvested by scraping the cellophane sheet. The fungal dry mass (0.2 g) was then inoculated into potato dextrose liquid medium supplemented with 250 µM aromatic compound (3,4-DCA). The rate of acetylation of AA by fungi was measured by high-performance liquid chromatography (HPLC) at different time points of incubation (27°C). Control experiments were performed with 250 µM AA in PD liquid medium without fungi and incubation at 27°C. For the remediation of 3,4-DCA (20 mg · kg<sup>-1</sup>) contamination of soil (Terre végétale, NFU-44-551; Truffaut, Paris, France), sterilized soil samples (3 g) were inoculated with *T. reesei* or *T. vires* (0.6 g of mycelium) and incubated for 3 days at 25°C. At different time points, aliquots (0.5 g) of soil were mixed with acetone (1 h, 300 rpm). The soil was then sonicated in a water bath for 30 min to extract the aromatic compounds. Samples were centrifuged, and then the supernatants were harvested and dried in a vacuum (Concentrator plus; Eppendorf, Le Pecq, France). For analysis, dried samples were placed in methanol (200 µl). AA and acetyl-AA were analyzed by reversed-phase HPLC (Shimadzu HPLC system interfaced with the LabSolution software). Samples were injected onto a Kromasil Eternity C<sub>18</sub> column (length, 100 nm; internal diameter, 4.6 mm; particle size, 2.5 µm) at 40°C. The mobile phase used for separation consisted of two eluants; solvent A was 20 mM sodium perchlorate buffer (pH 3.0), and solvent B was acetonitrile. Compounds were separated by the following discontinuous gradient at a flow rate of 1.5 ml · min<sup>-1</sup>. The initial concentration of 5% in solvent B increased to 20% over 2.5 min and remained stable for 2.5 min; this was followed by a decrease to 5% over the next minute, and the initial conditions were then maintained for 5 min.

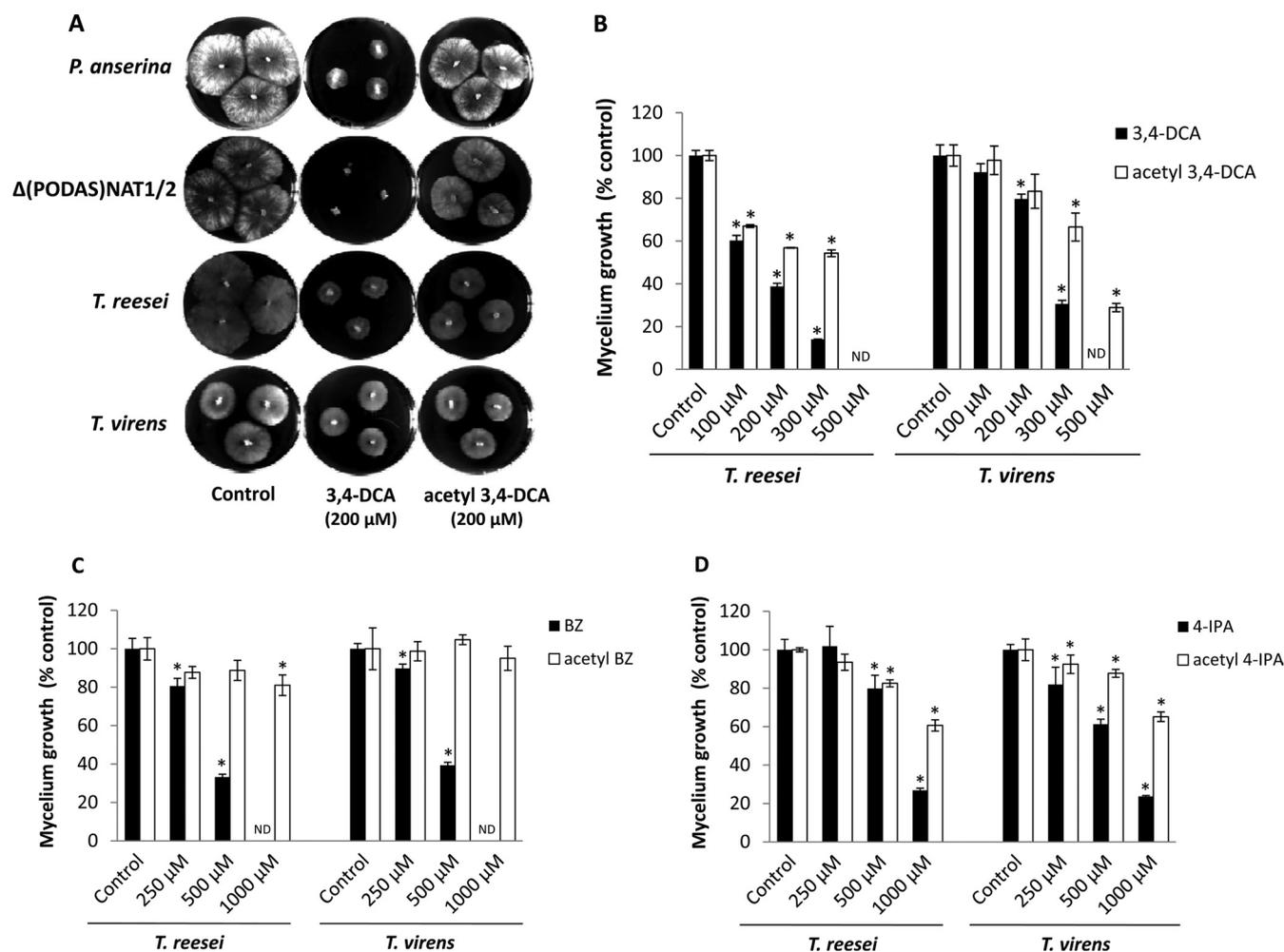


FIG 1 AA tolerance of *T. virens* and *T. reesei*. (A) Eight-centimeter petri dishes containing the *P. anserina*  $\Delta(PODAS)NAT1/2$  mutant, *T. reesei*, or *T. virens* grown on M2 agar medium with 3,4-DCA or acetyl-3,4-DCA. Photographs were taken after 3 days of growth at 27°C. (B to D) Rates of growth of *T. virens* and *T. reesei* on M2 agar medium in the presence of 3,4-DCA or acetyl-3,4-DCA, 4-IPA or acetyl-4-IPA, and BZ or acetyl-BZ, at the indicated final concentrations. Data are presented as means  $\pm$  SDs of three independent experiments. \*,  $P < 0.05$  compared with the control. ND, not detectable.

The products were monitored spectrophotometrically at 254 and 280 nm and quantified by integration of the peak absorbance area by employing a calibration curve established with various known concentrations of AA and acetyl-AA. Data are presented as means  $\pm$  SDs of three or five independent experiments. One-way analysis of variance was performed, followed by Tukey's method with SigmaStat 3.0 (Systat Software Inc.).

**Extract preparation and enzyme assays.** Fungi were grown for 3 days at 27°C in M2 liquid medium. The fungal mass was harvested by filtration. Dry biomass was subjected to high-speed shaking with metal beads (Tissue Lyser II; Qiagen, Hilden, Germany) and 1 volume of lysis buffer (20 mM Tris-HCl [pH 7.5] 100 mM NaCl, 1 mM EDTA, 0.05% Triton X-100) supplemented with 1 mM dithiothreitol and protease inhibitors. After centrifugation (10 min, 10,000 rpm, 4°C), supernatants were removed and used for enzyme assays. The rate of acetylation of AA by fungal extracts was measured by HPLC as previously described.

## RESULTS AND DISCUSSION

**Assessment of AA tolerance of *Trichoderma* spp.** The AA and acetyl-AA tolerance of *Trichoderma* spp. was screened by assessing radial growth as described previously (17). The strain that was most resistant to 3,4-DCA at a concentration of 200 μM (corresponding to 2 g · kg dry agar medium<sup>-1</sup>) was *T. virens*; the resis-

tance of *T. reesei* was similar to that of *P. anserina*. As previously shown (17), the growth of a *P. anserina*  $\Delta(PODAS)NAT1/2$  mutant devoid of both NAT genes was dramatically impaired in the presence of 3,4-DCA. In contrast, fungal growth was less impaired in the presence of 200 μM acetyl-3,4-DCA (Fig. 1A). The AA tolerance of *T. virens* and *T. reesei* was further analyzed with three AA (4-IPA, BZ, and 3,4-DCA) at different concentrations. Growth inhibition was dependent on the AA concentrations used (Fig. 1B, C, and D). Fungal growth was completely inhibited in the presence of 500 μM 3,4-DCA (Fig. 1B). In the presence of BZ at the same concentration (500 μM), the growth of *T. reesei* and *T. virens* decreased by 67 and 60%, respectively (Fig. 1C), while 4-IPA (500 μM) inhibited their growth by 20 and 39%, respectively (Fig. 1D). Overall, both *Trichoderma* species appeared to be more sensitive to 3,4-DCA than to BZ and 4-IPA. Next, fungal tolerance to acetyl-AA was assessed. In the presence of acetyl-4-IPA (1 mM), acetyl-3,4-DCA (300 μM), or acetyl-BZ (500 μM), *T. reesei* and *T. virens* growth was globally increased between 40% (acetyl-4-IPA and acetyl-3,4-DCA) and 60% (acetyl-BZ) over the radial-growth values observed in the presence of AA (Fig. 1B, C,

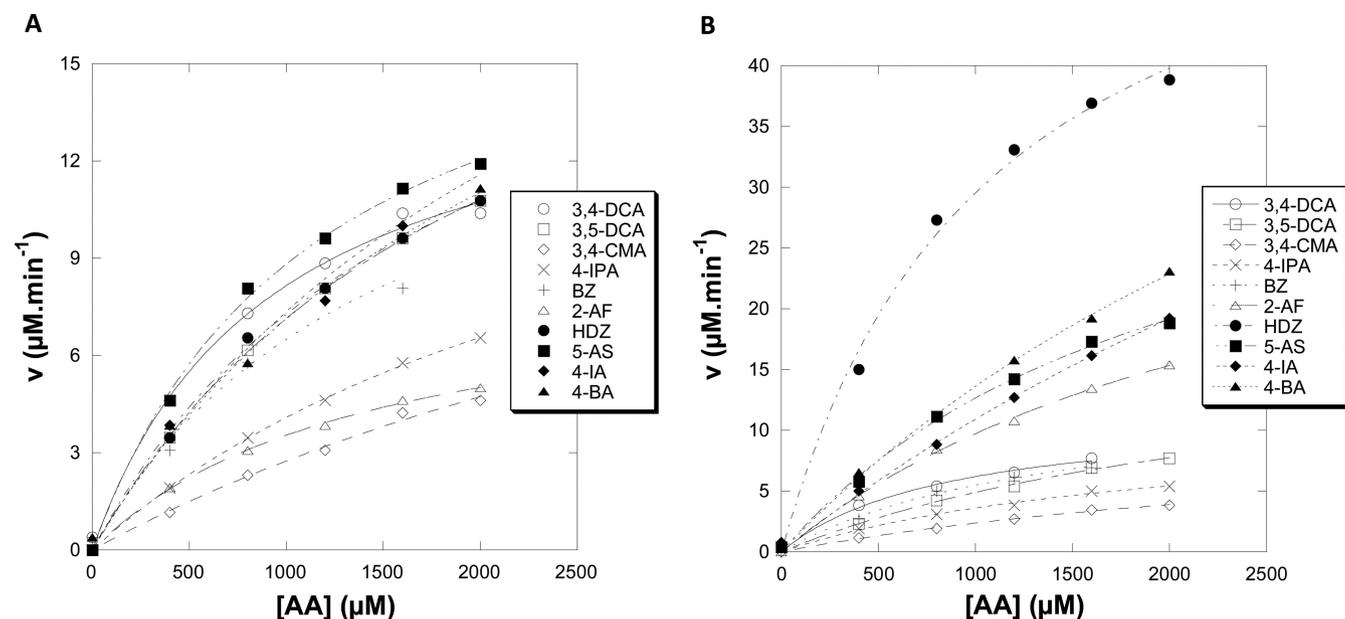


FIG 2 Functional characterization of (HYPVI)NAT1 and (HYPJE)NAT1. The Michaelis-Menten kinetics of (HYPVI)NAT1 (A) and (HYPJE)NAT1 (B) with typical NAT substrates are shown.

and D). Thus, acetylation appeared to markedly increase the AA tolerance of *Trichoderma* spp. This observation was consistent with previous reports. Indeed, using a *Vibrio fischeri* bioluminescence assay, Tixier and colleagues showed that aromatic compounds derived from phenylurea herbicides [3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron), chlorotoluron, and 3-(4-isopropylphenyl)-1,1-dimethylurea (isoproturon)] were more toxic than their corresponding acetyl metabolites (18). Moreover, Martins and colleagues demonstrated that *N*-acetylation of AA is a key mechanism underlying the AA tolerance of *P. anserina* (17). Therefore, we investigated whether the *N*-acetylation pathway has the same protective function in *Trichoderma* spp.

**Trichoderma NAT gene identification.** In a previous analysis of the complete genome sequences of a wide diversity of fungi, two NAT genes were identified in *T. virens* [(HYPVI)NAT1 and (HYPVI)NAT2; GenBank accession numbers [BN001414.1](#) and [BN001413.1](#), respectively] and only one was identified in *T. reesei* [(HYPJE)NAT1; GenBank accession number [BN001412.1](#)] (21). Phylogenetic analysis showed that the two *T. virens* NAT genes were orthologous to the *P. anserina* PaNAT1 [renamed here (PODAS)NAT2 in accordance with arylamine NAT gene nomenclature] and PaNAT2 [now (PODAS)NAT1] genes, while that of *T. reesei* was orthologous to (PODAS)NAT1 (21). A multiple-sequence alignment (see Fig. S1 in the supplemental material) showed that the percentages of identity between *Trichoderma* NAT isoforms and the corresponding *P. anserina* NAT range from 39 to 42%. The sequence identity between *T. virens* NAT isoforms is only 23% but reaches 32% with *P. anserina*. As is the case with *P. anserina*, *T. virens* NAT1 is shorter than the NAT2 isoform, which is a particularly large isoform for an NAT (305 and 341 amino acids, respectively). The two *Trichoderma* NAT1 isoforms that have been extensively studied here have more than 80% sequence identity. Structurally, NATs from *Trichoderma* spp. contain the well-described NAT-specific functional motifs and harbor the catalytic triad found in all arylamine NATs (19).

**NAT enzyme purification.** His-tagged recombinant enzymes (HYPVI)NAT1 and (HYPJE)NAT1 were expressed in *E. coli*, and highly purified proteins were obtained and assessed by SDS-PAGE (see Fig. S2A in the supplemental material). A molecular mass of 36 kDa was consistent with the predicted value for (HYPVI)NAT1 and (HYPJE)NAT1. Immunoblotting (see Fig. S2B) with an anti-His tag antibody confirmed these results. After purification, the yields of recombinant enzymes (HYPVI)NAT1 and (HYPJE)NAT1 from 1 liter of cell culture were 4.7 and 1.8 mg, respectively. (HYPVI)NAT2 from *T. virens* was also cloned, produced, and purified (data not shown).

**Characterization of (HYPJE)NAT1 and (HYPVI)NAT1 activities.** To test whether recombinant *Trichoderma* NATs are enzymatically active *in vitro*, we analyzed their catalytic activities and substrate profiles with a series of AA substrates (see Table S1 in the supplemental material) that were chosen for their potential toxicity. These substrates included drugs (SMX, SMZ, SDZ, SP, HDZ, and 5-AS), pesticide residues (ADE, ADP, 2,6-DEA, 2,6-EMA, 3,4-DCA, 3,5-DCA, 3,4-CMA, 4-IPA, 4-BA), and industrial chemicals (BZ, 2-AF, and 4-IA). By-products of aniline-derived herbicides (such as 3,4-DCA) are among the most toxic chemicals found in soil (22, 23). Since (HYPVI)NAT2 did not show significant activity in response to these substrates, they were not used in subsequent experiments. (HYPVI)NAT1 and (HYPJE)NAT1 were found to acetylate the same 10 substrates (HDZ, 5-AS, 3,4-DCA, 3,5-DCA, 3,4-CMA, 4-IPA, BZ, 2-AF, 4-IA, and 4-BA) among the 18 substrates tested (see Table S1). Apparent Michaelis-Menten parameters  $V_{max}$  and  $K_m$  were determined for these 10 substrates (Fig. 2), and catalytic efficiencies ( $k_{cat}/K_m$ ) were calculated (Table 1). (HYPVI)NAT1 efficiency was higher for 7 of the 10 substrates tested (1.5- to 7.5-fold), and similar to that of (HYPJE)NAT1 for 3,4-CMA, 4-IPA, and BZ. In the presence of 5-AS, the catalytic efficiency of (HYPVI)NAT1 ( $14,504 \text{ M}^{-1} \cdot \text{s}^{-1}$ ) was 7.5 times as high as that of (HYPJE)NAT1. The catalytic efficiencies of (HYPVI)NAT1 and (HYPJE)NAT1 with 2-AF, a pro-

TABLE 1 (HYPJE)NAT1 and (HYPVI)NAT1 catalytic efficiencies on aromatic NAT substrates

Class/compound	Short name	$k_{cat}/K_m$ ( $M^{-1} \cdot s^{-1}$ ) <sup>a</sup>	
		(HYPVI)NAT1	(HYPJE)NAT1
Pesticide residues			
3,4-Dichloroaniline	3,4-DCA	340	124
3,5-Dichloroaniline	3,5-DCA	194	77
3-Chloro-4-methylaniline	3,4-CMA	682	694
4-Isopropylaniline	4-IPA	1,352	1,330
4-Bromoaniline	4-BA	164	84
Other arylamines			
Benzidine	BZ	5,455	5,024
Hydralazine	HDZ	5,336	2,615
5-Aminosalicylate	5-AS	14,504	1,919
2-Aminofluorene	2-AF	6,203	1,406
4-Iodoaniline	4-IA	295	196

<sup>a</sup> Errors of triplicate values were at a maximum of  $\pm 5\%$ .

totypic NAT aromatic substrate, were 50 and 200 times less, respectively, than that of the most efficient NAT studied, *P. anserina* NAT1 [(PODAS)NAT1,  $k_{cat}/K_m = 290,000 M^{-1} \cdot s^{-1}$ ] (17). For the highly toxic pesticide derivative 3,4-DCA, the catalytic efficiencies of (HYPVI)NAT1 and (HYPJE)NAT1 were 510 and 1,400 times less, respectively, than that of (PODAS)NAT1 ( $k_{cat}/K_m = 170,400 M^{-1} \cdot s^{-1}$ ). Conversely, the catalytic efficiencies of (HYPVI)NAT1 and (HYPJE)NAT1 with 3,4-DCA were 12 and 4 times as high, respectively (data not shown), as that of the plant symbiotic bacterium *Mesorhizobium loti* (17, 24, 25).

As a means of demonstrating the existence of functional NAT enzymes in *T. virens* and *T. reesei*, the *ex vivo* *N*-acetylation capacity of total fungal extracts was assessed with 2-AF. Acetyl-CoA-dependent acetylation of 2-AF was found to occur in *T. virens* and *T. reesei* extracts. The activity of *T. virens* extract was 1.5 times as high as that of *T. reesei*. When the rate of 2-AF acetylation was measured (in triplicate) in the presence of mycelium extracts, acetyl-CoA (1 mM), and 2-AF (1 mM), the NAT activity of *T.*

*virens* was  $98.7 \pm 7.4 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  and that of *T. reesei* was  $61.8 \pm 2.5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  (means  $\pm$  SDs of three independent experiments). Moreover, the activities of *T. virens* and *T. reesei* extracts were, respectively, 25 and 40 times lower than those of *P. anserina* (data not shown). Overall, our data demonstrate that extracts from *Trichoderma* species contain functional NAT enzymes. To characterize the NAT pathway in more detail, we investigated the capacity of fungi to acetylate AA *in vivo*.

***In vivo N*-acetylation by *T. virens* and *T. reesei* and 3,4-DCA tolerance.** In the environment, organic pollutants can be degraded by different biological (26), chemical (27), or photochemical (28) processes. The *N*-acetylation of aniline-derived herbicides has been shown to detoxify them (18, 29) and has been reported to occur in soil bacteria such as *Pseudomonas* spp. (30–32) and *M. loti* (24). For instance, the herbicide metobromuron is metabolized to 4-BA, which is detoxified by certain soil microbes to acetyl-4-BA (26, 29). This acetylation pathway has been characterized in the filamentous fungus *P. anserina* (17). Tixier and coworkers (18) reported the acetylation of 3,4-DCA by four other fungi (*Aspergillus niger*, *Beauveria bassiana*, *Cunninghamella echinulata*, and *Mortierella isabellina*); however, the corresponding metabolic pathways of these fungi have not been characterized. BLAST analysis of its genomic sequence shows that *A. niger* has five putative NAT genes (21) and *B. bassiana* appears to have two, but the genomic sequences of the other two species are not yet available. Few studies have reported the metabolism of AA by fungi. Basidiomycete laccases or lignin peroxidases can attack the free amino group of alkylated and chlorinated anilines, leading to the formation of toxic dimers (33), as well as oligomers of AA (34). Only *Phanerochaete chrysosporium* is known to completely mineralize 3,4-DCA after the formation of conjugate intermediates (35).

To investigate the capacity of *Trichoderma* spp. to acetylate AA *in vivo*, fungal cultures were performed in the presence of 250  $\mu\text{M}$  3,4-DCA, a concentration allowing significant fungal growth (Fig. 1B). We assessed the stability of AA and acetyl-AA in potato dextrose culture medium. The half-life of 3,4-DCA was 2.7 days,

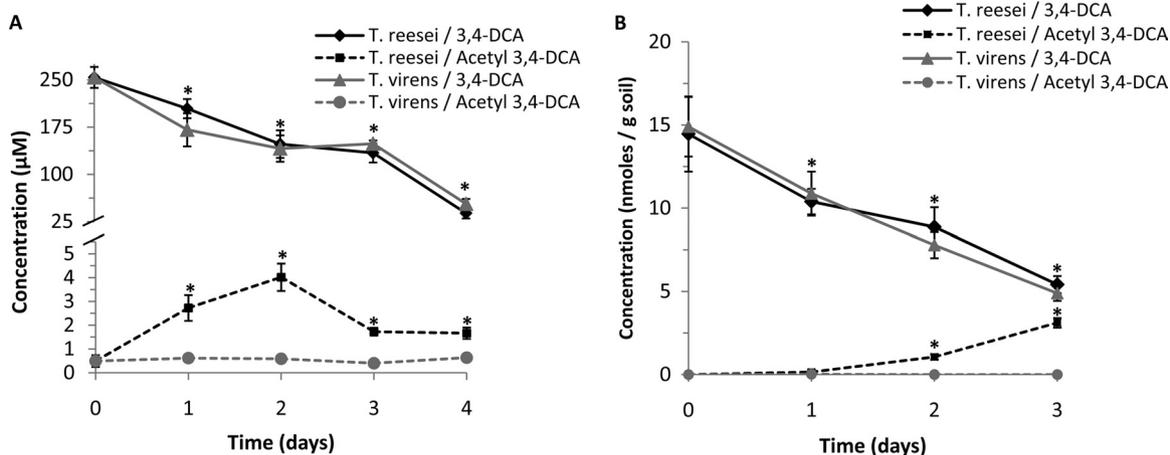


FIG 3 *In vivo* acetylation of 3,4-DCA by *T. virens* and *T. reesei*. (A) *T. reesei* or *T. virens* were grown in PDA liquid medium in the presence of 250  $\mu\text{M}$  3,4-DCA. At different time points, 3,4-DCA and acetyl-3,4-DCA were detected in the growth medium and quantified by HPLC. Data are presented as means  $\pm$  SDs of three independent experiments.  $P < 0.05$  compared with the control. (B) *T. reesei* or *T. virens* was used to inoculate soil contaminated with 3,4-DCA ( $20 \text{ mg} \cdot \text{kg}^{-1}$ ). At various points, 3,4-DCA and acetyl-3,4-DCA were extracted from soil samples and analyzed by HPLC. Data are presented as means  $\pm$  SDs of five independent experiments. \*,  $P < 0.05$  compared with the control.

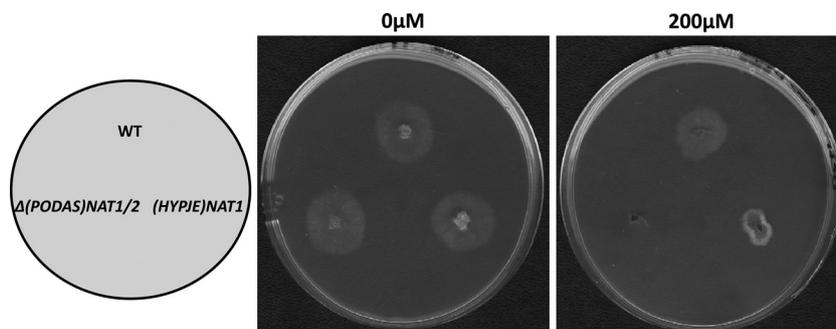


FIG 4 Resistance of transgenic *P. anserina* to 3,4-DCA was observed after 6 days of growth on M2 agar supplemented with the indicated concentrations.

but acetyl-3,4-DCA was stable for up to 4 days (data not shown). AA concentrations were thus normalized to account for the abiotic degradation of AA. After 4 days of incubation with *T. reesei*, 85% of the 3,4-DCA disappeared from the culture medium and 1.6% of the substrate present was acetylated within 2 days (Fig. 3A). Under the same conditions, 79% of the 3,4-DCA disappeared from the medium with *T. virens* without detectable formation of acetyl-3,4-DCA (Fig. 3A). Interestingly, in the *T. reesei* culture, the acetyl-3,4-DCA concentration decreased between 2 and 4 days of incubation (Fig. 3A). Tixier and collaborators observed this phenomenon in association with an increase in the 3,4-DCA concentration (18). Here, in the presence of *T. reesei*, acetyl-3,4-DCA appeared to be biotransformed rather than deacetylated into 3,4-DCA. To confirm these results, remediation of soil contaminated with 3,4-DCA ( $20 \text{ mg} \cdot \text{kg}^{-1}$ ) was performed after inoculation of a fungal biomass (Fig. 3B). As shown previously, AA concentrations were normalized to account for the abiotic degradation of compounds. 3,4-DCA concentrations decreased 62 and 67% after 3 days of incubation with *T. reesei* and *T. virens*, respectively (Fig. 3B). Acetyl-3,4-DCA was observed in the soil with *T. reesei*. Indeed, 22% of the substrate present was acetylated within 3 days (Fig. 3B). Overall, *Trichoderma* spp. appeared to efficiently remediate 3,4-DCA in soil. However, our data suggest that NAT-independent transformation is solely (in *T. virens*) or mainly (in *T. reesei*) responsible for the observed removal of 3,4-DCA.

To assess whether *T. reesei* NAT activity contributes to AA tolerance, the gene encoding (HYPJE)NAT1 was transferred into the *P. anserina*  $\Delta(\text{PODAS})\text{NAT1/2}$  mutant strain lacking both endogenous NAT genes (17). This strain is highly sensitive to 3,4-DCA. When the rate of 2-AF acetylation by fungal extracts was measured (in triplicate) in the presence of mycelium extracts, acetyl-CoA (1 mM), and 2-AF (1 mM), extracts of the transgenic strain showed NAT activity significantly higher than that of the  $\Delta(\text{PODAS})\text{NAT1/2}$  mutant strain ( $37 \pm 0.5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ). In addition, this activity ( $512 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ;  $P < 0.01$ ) was close to the value obtained with wild-type (WT) *P. anserina* ( $808 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ;  $P < 0.01$ ). Introduction of the recombinant (HYPJE)NAT1 gene into *P. anserina* triggered increased resistance to AA similar to that of WT *P. anserina*, showing that the *T. reesei* NAT gene is fully functional in promoting 3,4-DCA acetylation and tolerance in *P. anserina* (Fig. 4).

*Trichoderma* spp. have been shown to tolerate high concentrations of toxicants, likely because of efficient detoxification pathways (9). *In vivo*, the toxic compound 3,4-DCA is metabolized in

a few days in the presence of both *Trichoderma* species (Fig. 3). The *N*-acetylation pathway appears to be part of the mechanisms that enable *T. reesei* to metabolize aromatic compounds. Interestingly, the introduction of the (HYPJE)NAT1 gene into the *P. anserina*  $\Delta(\text{PODAS})\text{NAT1/2}$  mutant strain still led to tolerance of 3,4-DCA, confirming that the *N*-acetylation pathway is a mechanism, if not the only one, used by *T. reesei* to tolerate aromatic compounds. On the other hand, acetyl-3,4-DCA is not detected in *T. virens*. The intrinsic acetylation level in *T. virens* may be underestimated since acetylated compounds may be readily metabolized and hence not accumulate. However, *T. virens* and, to a lesser extent, *T. reesei* likely utilize another, unidentified, metabolic pathway for detoxification of AA aside from acetylation.

*T. virens* and *T. reesei* show high 3,4-DCA tolerance (Fig. 1). Different levels of contamination with AA pollutants have been observed, depending on their origin (agriculture or industrial). In agricultural soil, 3,4-DCA release from the recommended use of plant protection agents [e.g., 3-(3,4-dichlorophenyl)-1,1-dimethylurea, *N'*-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea (Linuron), or *N*-(3,4-dichlorophenyl)propanamide (Propanil), applied at around 2 to 6 kg of the active ingredient  $\cdot \text{ha}^{-1}$ ] could lead to variable 3,4-DCA concentrations ranging from  $0.02$  to  $2 \text{ mg} \cdot \text{kg} \cdot \text{kg dry soil} \cdot \text{liter}^{-1}$ . Nevertheless, considering the nonextractable bound residues in soil, the predicted environmental concentrations may range from  $0.85$  to  $5 \text{ mg} \cdot \text{kg} \cdot \text{kg dry soil} \cdot \text{liter}^{-1}$  (36). Our experiments showed that, even at much higher 3,4-DCA concentrations (corresponding, in our experiments, to  $2 \text{ g} \cdot \text{kg dry agar medium}^{-1}$ ), *Trichoderma* spp. mediate efficient detoxification of this compound (Fig. 1A and B). In addition, these fungal species have several characteristics that make them potentially usable for bioremediation. Indeed, *T. virens* is a successful colonizer because it is able to remove competition by mycoparasitism (37) and *T. reesei* is widely used in industry and its production on a large scale is well mastered (12, 38).

Rather than the use of single fungal species for future remediation strategies, assessment of the potential efficiency and complementarity of well-characterized species, including combinations of rhizosphere-competent *Trichoderma* strains (14), may be more relevant. In a preliminary proof-of-concept study, we investigated the capacity of *T. virens*, *T. reesei*, and *P. anserina* to grow together. After coloration of coculture plates by the trypan blue exclusion method (39), neither growth inhibition (antibiosis) nor important hyphal interference (40) was evident (see Fig. S3 in the supplemental material). These results reveal novel possibilities for the

use of fungal association in the development of new bioremediation strategies.

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## REFERENCES

- Durrieu G. 1993. *Écologie des champignons*. Masson, Paris, France.
- Gilbertson RL. 1980. Wood-rotting fungi of North America. *Mycologia* 72:1–49.
- Boer W, Folman LB, Summerbell RC, Boddy L. 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol. Rev.* 29:795–811.
- Brooke EW, Davies SG, Mulvaney AW, Pompeo F, Sim E, Vickers RJ. 2003. An approach to identifying novel substrates of bacterial arylamine *N*-acetyltransferases. *Bioorg. Med. Chem.* 11:1227–1234.
- Tortella GR, Diez MC, Duran N. 2005. Fungal diversity and use in decomposition of environmental pollutants. *Crit. Rev. Microbiol.* 31:197–212.
- Misiek M, Hoffmeister D. 2007. Fungal genetics, genomics, and secondary metabolites in pharmaceutical sciences. *Planta Med.* 73:103–115.
- Glenn AE, Bacon CW. 2009. FDB2 encodes a member of the arylamine *N*-acetyltransferase family and is necessary for biotransformation of benzoxazolinones by *Fusarium verticillioides*. *J. Appl. Microbiol.* 107:657–671.
- Mougin C, Boukcim H, Jolival C. 2009. Soil bioremediation strategies based on the use of fungal enzymes, p 123–149. *In* Singh A, Kuhad RC, Ward OP (ed), *Soil biology: advances in applied bioremediation*. Springer, Berlin, Germany.
- Harms H, Schlosser D, Wick LY. 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. *Nat. Rev. Microbiol.* 9:177–192.
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. 2004. *Trichoderma* species—opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2:43–56.
- Błaszczak L, Popiel D, Chelkowski J, Koczyk G, Samuels GJ, Sobierski K, Siwulski M. 2011. Species diversity of *Trichoderma* in Poland. *J. Appl. Genet.* 52:233–243.
- Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, Schoch CL, Yao J, Barabote R, Barabote R, Nelson MA, Detter C, Bruce D, Kuske KR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M, Brettin TS. 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat. Biotechnol.* 26:553–560.
- Velazquez-Robledo R, Contreras-Cornejo HA, Macias-Rodriguez L, Hernandez-Morales A, Aguirre J, Casas-Flores S, Lopez-Bucio J, Herrera-Estrella A. 2011. Role of the 4-phosphopantetheinyl transferase of *Trichoderma virens* in secondary metabolism and induction of plant defense responses. *Mol. Plant Microbe Interact.* 24:1459–1471.
- Harman GE, Lorito M, Lynch JM. 2004. Uses of *Trichoderma* spp. to alleviate or remediate soil and water pollution. *Adv. Appl. Microbiol.* 56:313–330.
- Lorito M, Woo SL, Harman GE, Monte E. 2010. Translational research on *Trichoderma*: from 'omics to the field. *Annu. Rev. Phytopathol.* 48:395–417.
- Del Carratore R, Carratore RD, Gervasi PG, Contini MP, Beffy P, Maserti BE, Giovannetti G, Brondolo A, Longo V. 2011. Expression and characterization of two new alkane-inducible cytochrome P450s from *Trichoderma harzianum*. *Biotechnol. Lett.* 33:1201–1206.
- Martins M, Rodrigues-Lima F, Dairou J, Lamouri A, Malagnac F, Silar P, Dupret JM. 2009. An acetyltransferase conferring tolerance to toxic aromatic amine chemicals: molecular and functional studies. *J. Biol. Chem.* 284:18726–18733.
- Tixier C, Sancelme M, Ait-Aissa S, Widehem P, Bonnemoy F, Cuer A, Truffaut N, Veschambre H. 2002. Biotransformation of phenylurea herbicides by a soil bacterial strain, *Arthrobacter* sp. N2: structure, ecotoxicity and fate of diuron metabolite with soil fungi. *Chemosphere* 46:519–526.
- Dupret JM, Rodrigues-Lima F. 2005. Structure and regulation of the drug-metabolizing enzymes arylamine *N*-acetyltransferases. *Curr. Med. Chem.* 12:311–318.
- Déquad-Chablat M, Nguyen T, Contamine V, Hermann-Le Denmat S, Malagnac F. 2012. Efficient tools to target DNA to *Podospora anserina*. *Fungal Genet. Rep.* 59:21–25.
- Martins M, Dairou J, Rodrigues-Lima F, Dupret JM, Silar P. 2010. Insights into the phylogeny or arylamine *N*-acetyltransferases in fungi. *J. Mol. Evol.* 71:141–152.
- Dearfield KL, McCarroll NE, Protzel A, Stack HF, Jackson MA, Waters MD. 1999. A survey of EPA/OPP and open literature on selected pesticide chemicals. II. Mutagenicity and carcinogenicity of selected chloroacetanilides and related compounds. *Mutat. Res.* 443:183–221.
- Harvey PJ, Campanella BF, Castro PM, Harms H, Lichtfouse E, Schäffner AR, Smrcek S, Werck-Reichhart D. 2002. Phytoremediation of polyaromatic hydrocarbons, anilines and phenols. *Environ. Sci. Pollut Res. Int.* 9:29–47.
- Rodrigues-Lima F, Dairou J, Diaz CL, Rubio MC, Sim E, Spink HP, Dupret JM. 2006. Cloning, functional expression and characterization of *Mesorhizobium loti* arylamine *N*-acetyltransferases: rhizobial symbiosis supplies leguminous plants with the xenobiotic *N*-acetylation pathway. *Mol. Microbiol.* 60:505–512.
- Silar P, Dairou J, Coccagn A, Busi F, Rodrigues-Lima F, Dupret JM. 2011. Fungi as a promising tool for bioremediation of soils contaminated with aromatic amines, a major class of pollutants. *Nat. Rev. Microbiol.* 9:477.
- Van Eerd LL, Hoagland RE, Zablotowicz RM, Hall JC. 2003. Pesticide metabolism in plants and microorganisms. *Weed Sci.* 51:472–495.
- Sarmah AK, Sabadie J. 2002. Hydrolysis of sulfonylurea herbicides in soils and aqueous solutions: a review. *J. Agric. Food Chem.* 50:6253–6265.
- Katagi T. 2004. Photodegradation of pesticides on plant and soil surfaces. *Rev. Environ. Contam. Toxicol.* 182:1–189.
- Tweedy BG, Loepky C, Ross JA. 1970. Metobromuron: acetylation of the aniline moiety as a detoxification mechanism. *Science* 168:482–483.
- Travkin VM, Solyanikova IP, Rietjens IM, Vervoort J, van Berkel WJ, Golovleva LA. 2003. Degradation of 3,4-dichloro- and 3,4-difluoroaniline by *Pseudomonas fluorescens* 26-K. *J. Environ. Sci. Health B* 38:121–132.
- Vol'nova AI, Surovtseva EG, Vasil'eva GK. 1980. Acetylation of 3,4-dichloroaniline by representatives of the genus *Pseudomonas*. *Mikrobiologiya* 49:167–170.
- Westwood IM, Holton SJ, Rodrigues-Lima F, Dupret JM, Bhakta S, Noble ME, Sim E. 2005. Expression, purification, characterization and structure of *Pseudomonas aeruginosa* arylamine *N*-acetyltransferase. *Biochem. J.* 385:605–612.
- Hoff T, Liu SY, Bollag JM. 1985. Transformation of halogen-, alkyl-, and alkoxy-substituted anilines by a laccase of *Trametes versicolor*. *Appl. Environ. Microbiol.* 49:1040–1045.
- Kremer S, Sterner O. 1996. Metabolism of 3,4-dichloroaniline by the basidiomycete *Filobolus* species TA9054. *J. Agric. Food Chem.* 44:1155–1159.
- Arjmand M, Sandermann H. 1985. Mineralization of chloroaniline/lignin conjugates and of free chloroanilines by the white rot fungus *Phanerochaete chrysosporium*. *J. Agric. Food Chem.* 33:1055–1060.
- IHCP. 2006. 3,4-Dichloroaniline (3,4-DCA) CAS no: 95-76-1 EINECS no: 202-448-4, summary risk assessment report. Institute of Health and Consumer Protection (IHCP), European Commission, Ispra (Varese), Italy.
- Schuster A, Schmoll M. 2010. Biology and biotechnology of *Trichoderma*. *Appl. Microbiol. Biotechnol.* 87:787–799.
- Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas-Flores S, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atana-

- sova L, Cervantes-Badillo MG, Challacombe J, Chertkov O, McCluskey K, Couplier F, Deshpande N, von Döhren H, Ebbole DJ, Esquivel-Naranjo EU, Fekete E, Flipphi M, Glaser F, Gómez-Rodríguez EY, Gruber S, Han C, Henrissat B, Hermosa R, Hernández-Oñate M, Karaffa L, Kosti I, Le Crom S, Lindquist E, Lucas S, Lübeck M, Lübeck PS, Margeot A, Metz B, Misra M, Nevalainen H, Omann M, Packer N, Perrone G, Uresti-Rivera EE, Salamov A, Schmoll M, Seiboth B, Shapiro H, Sukno S, Tamayo-Ramos JA, Tisch D, Wiest A, Wilkinson HH, Zhang M, Coutinho PM, Kenerley CM, Monte E, Baker SE, Grigoriev IV. 2011. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome Biol.* 12:R40.
39. Silar P. 2005. Peroxide accumulation and cell death in filamentous fungi induced by contact with a contestant. *Mycol. Res.* 109:137–149.
40. Silar P. 2012. Hyphal Interference: self versus non-self fungal recognition and hyphal death, p 155–170. *In* Witzany G (ed), *Biocommunication of fungi*. Springer, New York, NY.