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Degradation of fumonisin B1 by a bacterial strain isolated from soil

Raffaella Benedetti, Francesco Nazzi, Romano Locci & Giuseppe Firrao*
*Dipartimento di Biologia Applicata alla Difesa delle Piante, Università di Udine, via delle Scienze 208, 33100, Udine, Italy (*author for correspondence: e-mail: firrao@uniud.it)*

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Abstract

A mixed microbial culture degrading fumonisin B l was obtained from soil samples using an enrichment culture procedure. A bacterial isolate from the enrichment culture (strain NCB 1492) degraded fumonisin B1 after incubation for 3 h, as indicated by TLC and HPLC analysis. On the basis of the sequence analysis of 16S rDNA, strain NCB 1492 was related to the *Delftia*/*Comamonas* group. Thin-layer chromatographic analysis indicated the presence of metabolites in the NCB 1492 culture filtrates after degradation of fumonisin B1 supplied as sole carbon and nitrogen source in phosphate buffer. Four metabolites were identified by mass spectrometry analysis.

Introduction

Fumonisins are secondary metabolites produced by *Fusarium* spp. on naturally infected maize. Their toxic nature is of great concern for food safety, as fumonisins have been associated with human and animal diseases (Nelson et al. 1993). According to the U.S. Food and Drug Administration (2001) recommendations, to avoid possible health risks, maize should not be used for human consumption when contaminated with more than 2–4 ppm (depending on the food) fumonisins or for animal consumption when it contains more than 5–60 ppm (depending on the animal species).

Fumonisin B1 (FB1, Figure 1), the major compound of this class of mycotoxins, was characterized from cultures of *Fusarium verticillioides* (Gelderblom et al. 1988) and has since then been found to be a common contaminant of maize. FB1 is toxic to most domestic animals and epidemiological studies (IARC 1993) demonstrated that human exposure to fumonisins caused oesophageal cancer. Unfortunately, fumonisins are highly stable molecules and their detoxification by physical methods requires high pressures and temperatures, and is therefore poorly suitable for industrial processing. Trade and economical problems associated with contaminated foods and feeds, make these mycotoxins worthy targets for strategies aimed at detoxification *in planta* (Duvick 2001) or alternative strategies. Since it has been reported that fumonisins are resistant to the activity of known esterases and amine modifying enzymes (Murphy et al. 1996), for their biodegradation new enzymes and genes must be found.

In a screening program to identify fumonisin detoxifying microbes, a fungal strain was identified that performed an oxidative deamination of AP1, a derivative of FB1 obtained by chemical hydrolysis (Blackwell et al. 1999). The same research group also studied an FB1 esterase activity and further degradative steps but, to our knowledge, this was only reported in the description of a patent issued to Pioneer Hi-Bred International Inc. (Duvick et al. 1998). More recently, the isolation of two *Bacillus* spp. strain and a yeast able to partially degrade fumonisin was also reported (Camilo et al. 2000).

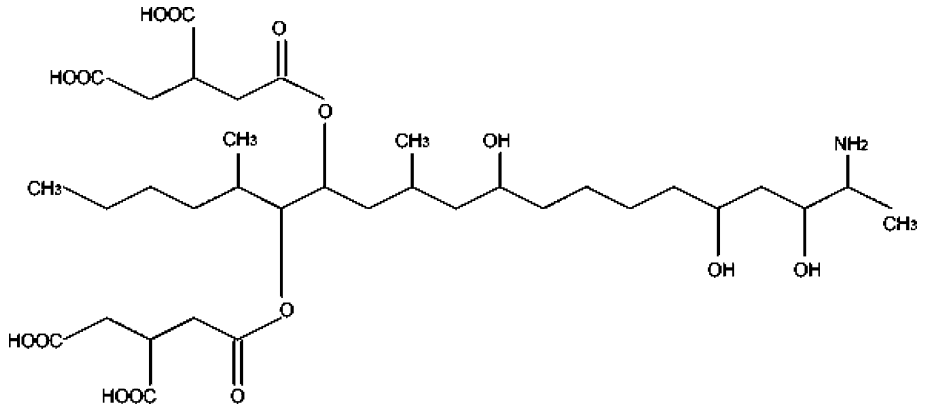

Figure 1. Structure of fumonisin B1.

We obtained a mixed microbial culture able to metabolize the fumonisin B 1 from soil samples using an enrichment culture procedure. The initial characterization of the degradation process and of the isolated bacterial strain responsible is reported here.

Materials and methods

Enrichment from environmental samples

An enrichment culture procedure similar to that of Shima et al. (1997) was used. Twenty-one soil samples were collected during spring in maize fields in Friuli Venezia Giulia, Italy. The BYE medium (Shima et al. 1997) was used for culture enrichment. It contained (per liter) 0.5 g of NH_4NO_3, 0.2 g of yeast extract (Difco), 50 mg of H_3BO_4, 40 mg of $MnSO_4 \cdot 4H_2O$, 20 mg of $(NH_4)_6Mo_7O_{24}$, 4 mg of $CuSO_4 \cdot 5H_2O$, 4 mg of $CoCl_6z \cdot 6H_2O$ and 5 mM potassium phosphate buffer (pH 7.0). Soil samples (1 g) were suspended in distilled water (10 ml), shaken and allowed to settle. After 15 min, 5 ml of each supernatant were pooled. An aliquot of the pooled supernatant (0.1 ml) was used to inoculate 1 ml of BYE medium (culture N1) supplemented with 0.5 mg/ml of FB1 (Sigma). The residual pooled supernatant was centrifuged at 10,000 rpm for 10 min, the pellet was resuspended in 1 ml phosphate buffer, and 100 μl of the suspension were added to BYE medium with 0.5 mg/ml of FB1 (culture N2). The cultures were incubated at 25 °C for 8 days with shaking. Aliquots were then transferred to fresh BYE medium supplemented with 0.5 mg/ml of FB1, and incubated for an additional 8 days. This step was repeated 5-times. The presence of FB1 in the culture media was monitored directly by TLC, as detailed below.

Isolation of FB1 degrading organisms

The cultures obtained following enrichment were treated with antibiotics to reduce the diversity of the microbial population without affecting the ability of the consortium to degrade FB1. The antibiotics used in BYE medium were: rifampicin (150 μg/μl), chloramphenicol (10 μg/μl), ampicillin (100 μg/μl). The degradation was monitored with TLC. Following the antibiotic treatment aliquots (0.1 ml) of cultures were plated onto different media: nutrient agar (Difco), nutrient agar with 10 g/l of sucrose, nutrient agar with 10 g/l of skim milk, peptone yeast extract iron agar and BYE medium agar. Plates were incubated at 25 °C for 3 days. Individual colonies were then isolated, tested for their degradation activity and stored at −80 °C after suspension in BYE with 15% (wt/vol) glycerol. Selected strains were deposited in the National Collection of Bacteria, Udine, Italy (NCB).

Analysis of FB1 degradation

The degradation of FB1 was assayed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). BYE medium as well as phosphate buffer (PBS), supplemented with 0.5 mg/ml of FB1, were

inoculated in order to obtain an optical density of 0.3 at 600 nm, then incubated in the dark for 0.5–24 h at 25 °C. TLC was carried out on silica gel 60 plates (Sigma), using a mixture of acetonitrile/water (85:15) as the mobile phase. Ten μl of cultures in BYE medium or phosphate buffer were loaded and run for 45 min, together with control samples; the TLC plate was then dried, sprayed with ninhydrin and baked at 120 °C for 15 min.

The HPLC system (Varian Analytical Instruments) was equipped with a sampling system (loop volume 20 μl), and a Prostar 363 fluorescence detector set at 335 nm excitation – 440 nm detection. A C_{18} column (5 μm particle size) was used at room temperature with methanol: water (50:50) as the mobile phase at 1 ml/min flow rate. Standard and samples were derivatized with *o*-phthaldialdehyde/2-mercaptoethanol, according to the FumoniTest kit (VICAM), before injection. Standard solutions containing 125 ng/μl, 12.5 ng/μl and 1.25 ng/μl of FB1 were included in all analyses.

A gas chromatography–mass spectrometry analysis (GC–MS) system (Saturn 2000, Varian Analytical Instruments) was used to identify volatile compounds generated by the bacterial culture in PBS buffer with 0.5 mg/ml of FB1 and a bacterial culture with PBS buffer only was used as a control. The supernatant from the cultures was reduced to dryness under nitrogen, redissolved in methanol and injected at 300 °C into a CP-SIL 8 (30 m × 0.25 mm ID) column; the column was maintained at 40 °C for 1 min and then driven at 320 °C at 10 °C/min. The carrier gas was helium (flow: 1 ml/min).

Identification of volatile compounds released by the bacterial culture was based on the comparison of the mass spectra and the retention time with that of authentic compounds analyzed under the same chromatographic conditions; if an authentic sample was not available the mass spectrum of the unknown was compared to reference spectra from libraries (Nist Chemistry WebBook 2003); in this case the chromatographic retention index of the compound tentatively identified on the ground of the mass spectrum was compared to that expected according to tentative structure (Sadtler Research Laboratories 1985). No attempts to ascertain the identity of possible isomers were made.

Taxonomic characterization of bacterial isolate

DNA was extracted from strain bacterial cultures according to Strauss (1987), and subjected to amplification by PCR with primers fD1/rP1 according to Weisburg et al. (1989). The amplified 16S rDNA was sequenced with primers fD1 and rP1. The sequence obtained was used to probe public databases using the programs WU-BLAST (Altschul et al. 1997) and SEQUENCE MATCH (available at the web site of the Ribosomal Database Project; Cole et al. 2003).

Transposon mutagenesis

To identify the gene(s) involved in the degradation of FB1 by strain NCB 1492, transposon mutagenesis was carried out as described for the related bacterium *Delftia acidovorans* (Wilson et al. 1995). *E. coli* S17–1λpyr (Vinci-Biochem) cells were transformed with the DNA of pSS240, a suicide plasmid containing the mini transposon Tn5SsgusA40 (Wilson et al. 1995), kindly supplied by Dr. J. Imperial (Universidad Politecnica, Madrid, Spain). Transformants were selected on substrate containing ampicillin. Conjugation was carried out with *E. coli* S17–1λpyr (pSS240) as donor and NCB 1492 as recipient. Numerous growth tests were carried out to identify a selective medium for the bacterium NCB 1492. Finally, a medium was found that allowed the growth of NCB 1492 but not of *E. coli* S17–1λpyr. It was based on minimal medium (Maniatis et al. 1982) supplemented with tryptophan (0.25 mM), benzyl alcohol (1.5 mM), benzoate (5 mM), *p*-hydroxybenzoate (1 mM), succinate (10 mM), glucose (0.2%). Spectinomycin (100 μg/ml) was added for the selection of transconjugants.

Results

Enrichment for degradation of FB1 and isolation of NCB 1492

With the enrichment culture procedure used, an initially small population of FB1-transforming microorganisms was increased. In both (N1 and N2) enrichment cultures the complete disappearance of FB1 in the culture filtrate was recorded by TLC after 4 repeated growth cycles (42 days), as

shown in Figure 3. Fumonisin was detected in TLC as a pink fuzzy spot with $R_f = 0.12$, while the degraded sample showed a very faint spot with $R_f = 0.05$ (Figure 2).

Three antibiotics were tested to reduce the diversity of the microbial community. Of these, only ampicillin reduced the population diversity without affecting its ability to degrade fumonisin B1 (not shown). To isolate individual bacteria from the community, the culture was plated on different media. Approximately 30 organisms could be separated on the basis of differences in colony and cell morphology. Of these 30 organisms, only one (designated NCB 1492) could completely break down FB 1 after 1 day of incubation at 25 °C (Figure 3).

Taxonomic characterization of NCB 1492

Strain NCB 1492 was a Gram negative rod. After PCR amplification of its 16S rDNA (Weisburg et al. 1991), a single fragment of 1500 bp was obtained and partially sequenced to the extent of a total of 1024 nt. According to WU-BLAST analysis, the resulting sequence was similar to the 16S rDNA sequence of bacteria belonging to the *Delftia*/*Comamonas* group, although none was more than 96% identical to that of NCB 1492. The sequence data were also submitted to the SEQUENCE MATCH program; again the 16S rDNA sequence of NCB 1492 resulted similar to members of the *Delftia*/*Comamonas* group, but with scores of 0.87 or lower. Therefore NCB1492 was tentatively assigned to the *Delftia*/*Comamonas* group, but could not be identified at the species level, representing possibly a new taxon within this clade.

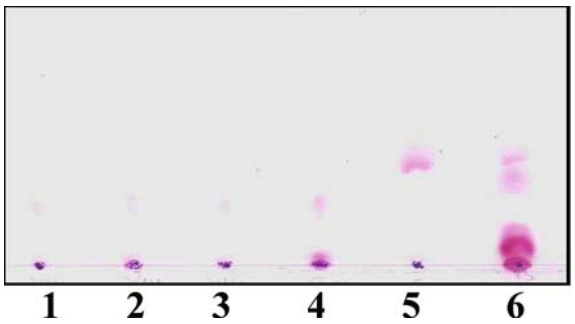

Figure 2. TLC of the supernatant of cultures N1 (lanes 1 and 3) and N2 (lanes 2 and 4) incubated for 40 (lanes 1 and 2) and 48 (lanes 3 and 4) days. FB1 (0.5 μg/ml) in water (lane 5) and BYE medium (lane 6) were used as reference.

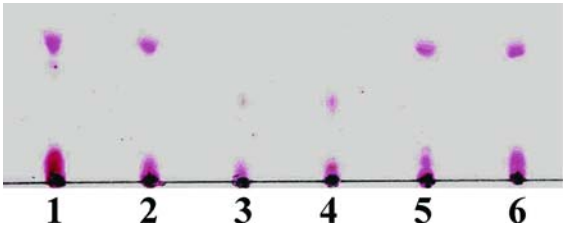

Figure 3. TLC of the supernatant of cultures of isolated bacterial strains. Lanes 1, 2, and 5: non-degrading bacterial isolates; lane 3: strain NCB 1492; lane 4: enriched culture N1; lane 6: FB1 (0.5 μg/ml) control in BYE medium.

Analysis of the degradation of FB1 by NCB 1492 in phosphate buffer

Strain NCB 1492 degrades FB1, as detected by TLC and HPLC, when grown in phosphate buffer saline (PBS) supplemented with 0.5 mg/ml of FB1 as sole carbon source. TLC analysis showed that after 24 h the FB1 spot was no longer detectable, but a new, relatively intense spot appeared with R_f larger than that of FB1 (Figure 4). The time course of the degradation of the fumonisin B1 by NCB 1492 in phosphate buffer saline (PBS) supplemented with 0.5 mg/ml of FB1 as sole carbon source was also followed by HPLC. After just 30 min of incubation at 25 °C the fluorescence peak at 6.5 min, corresponding to FB1, could no longer be detected and a new peak appeared at 2.5 min. After 1 h a fluorescence peak was detected at 4.5 min. The latter peak lowered following 2 h incubation and later no fluorescence peaks were recorded (Figure 5).

Analysis by GC–MS of the culture supernatant after 24 h of growth of NCB 1492 with 0.5 mg/ml of FB1 revealed the presence of 4 compounds, tentatively identified as heptadecanone ($C_{17}H_{34}O$), isononadecene ($C_{19}H_{38}$), octadecenal ($C_{18}H_{34}O$) and eicosane ($C_{20}H_{42}$) (Table 1, Figure 6).

Mutagenesis

From three conjugation experiments about 1000 transconjugants were obtained, and all were individually assayed for their FB1 degradation ability by TLC. No transconjugant was identified with a reduced or no ability to degrade fumonisin B1.

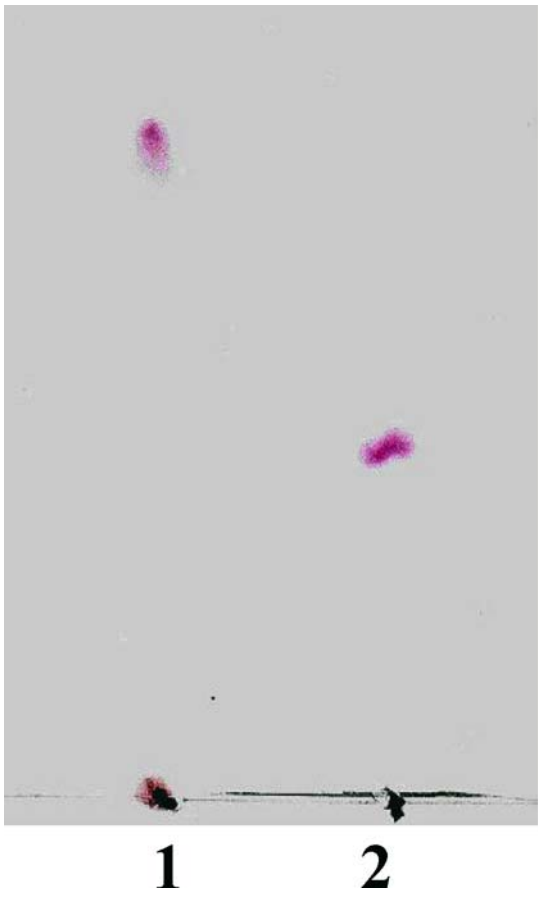

Figure 4. TLC of the supernatant of NCB 1492 in PBS buffer supplemented with fumonisin B1 (0.5 mg/ml) prior to and after incubation at 25 °C for 24 h.

Discussion

Although fumonisins are a relatively novel class of toxins, they have rapidly attracted both social and scientific attention due to their high health risk potential when consumed by humans and animals (Nelson et al. 1993). In some regions, such as the north-east Italy and other parts of the world, severe fumonisin contamination is constantly recorded on maize (Snidaro & Pavidotti 2002; Pietri et al. 2004). In those areas, prevention strategies based on risk models are not sufficient and the problem of the decontamination of commodities needs to be faced. Unfortunately, the drastic physical and/or chemical means required may be detrimental for the treated commodities. Consequently microbial degradation is advantageous, as it could reduce the toxicity without affecting food and feed quality.

For some mycotoxins, such as ochratoxin A (Abrunhosa et al. 2002), degrading enzymes are quite frequent in nature. Conversely, other mycotoxins, such as deoxynivalenol and the fumonisins are difficult substrates for biodegradation. Recently Shima and co-workers (1997), isolated from soil some bacteria, taxonomically related to the *Agrobacterium*/*Rhizobium* clade, that detoxify *in vitro* deoxynivalenol into 3-ketodeoxynivalenol.

In this work, we evaluated the potential of microbial decontaminators present in soil where maize has been grown in monoculture for several years. In fact, we were able to isolate a bacterial strain which efficiently degraded FB1. NCB 1492 breaks down native FB1 even if supplied as the sole carbon source. The process appears to be extracellular in the initial phase, as HPLC analysis revealed the transformation of fumonisin in the culture supernatant into other compounds during the first 2 h of incubation. Later on, no compounds that could be derivatized with *o*-phthaldialdehyde/2-mercaptoethanol were detected in the culture supernatant, suggesting that the modified compound is either deaminated or taken up by the bacteria. In the presence of only phosphate buffer the subsequent enzymatic processes lead to the accumulation of final products, four of which have been tentatively identified as heptadecanone, isononadecene (b), octadecenal and eicosane; none of the above listed compounds was reported to have toxic activity (Nagao 1983; Yamaguchi et al. 2000; Han & Chen 2002). On the basis of the results, we speculate that NCB 1492 possesses fast deamination (and possibly esterase) activities; conversely, degradation of the aliphatic chain (remaining after release of tricarbossilic acids) in phosfate buffer appears to be slow, require coreactants or the metabolic contribution of other organisms for its completion.

Elimination of the amino group may result in the production of a compound that reacts with ninhydrin but not with *o*-phthaldialdehyde/2-mercaptoethanol, or could not be detected under the conditions used in this work for HPLC analysis. The enzymatic degradation of fumonisin was found to be more extensive when carried out in a complete medium rather than in phosphate buffer; however, even in phosphate buffer the initial steps are carried out very rapidly. Thus, our inability to identify by transposon mutagenesis mutants impaired in FB1 degradation may be explained by

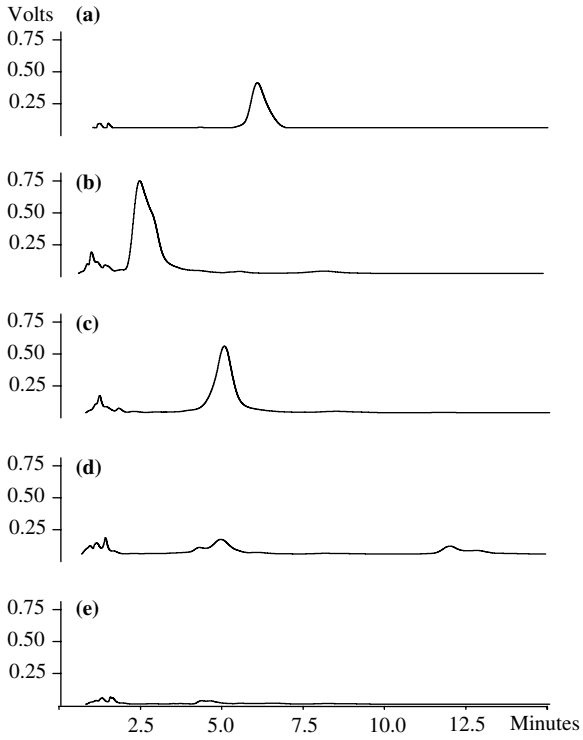

Figure 5. HPLC of supernatant of a culture of NCB 1492 in PBS buffer supplemented with fumonisin B1 (0.125 mg/ml) incubated at 25 °C for 0 min (a), 30 min (b), 60 min (c), 120 min (d) and 180 min (e).

the hypothesis that the enzymes involved in the initial steps of this process are also involved in the primary metabolism of the bacterium.

The close phylogenetic relationship between this bacterium, *Delftia acidovorans* and other organisms belonging to the Comamonadaceae, wide spread in soil and water, confirms the ability of this group of bacteria to degrade unusual molecules, such as those of herbicides (Benndorf & Babel 2002), the industrial polluting agent 1,3 dichloropropene (Katsivela et al. 1999), aniline and derivatives (Boon et al. 2001) and aryl sulfonates (Junker & Cook 1997). In those examples, often one or more of the genes involved in biodegradation are carried by plasmids. This does not appear to be the case of NCB 1492, as no plasmids could be detected in total DNA preparations.

Table 1. Volatile compounds tentatively identified by GC–MS in the bacterial culture in PBS buffer with 0.5 mg/ml of FB1

Compound[a]	Major fragments (m/z)	Retention index	Identity	Formula	Identification[b]
A	41,43,57,71,141	1878	Heptadecanone	$C_{17}H_{34}O$	spec + ret ind
B	41,43,55,69,97	1919	isononadecene	$C_{19}H_{38}$	spec + ret ind
C	41,43,67,81,83	1971	octadecenal	$C_{18}H_{34}O$	spec + ret ind
D	41,43,57,71,85	2000	eicosane	$C_{20}H_{42}$	auth std

[a]Compound: letters are as in Figure 6.
[b]Identification: "auth std" means that the spectrum and retention time of the unknown were compared to those of an authentic standard injected under the same chromatographic conditions; "spec + ret ind" means that the identification is based on the comparison of the spectrum of the unknown to those from reference libraries and the retention index to that expected on the ground of the tentative structure.

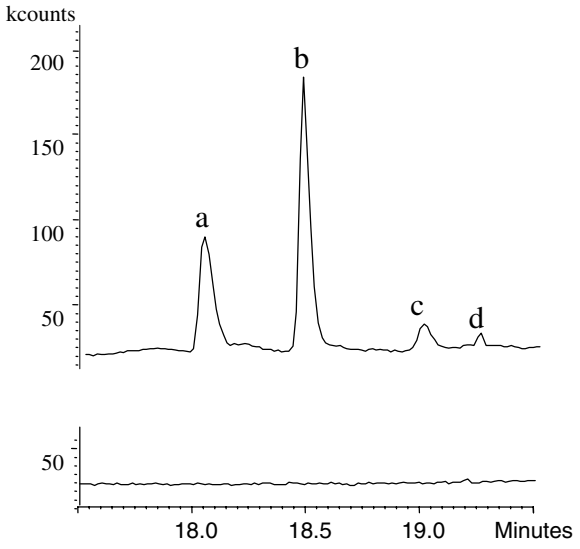

Figure 6. GC–MS of the supernatant of a 24 h old culture of NCB 1492 in PBS buffer with (upper) and without (lower) the supplement of fumonisin B1 (0.125 mg/ml). The *Y* axis represents the total ion current. The four peaks were tentatively identified as heptadecanone (a), isononadecene (b), octadecenal (c) and eicosane (d).

Acknowledgements

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References

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

Abrunhosa L, Serra R & Venancio A (2002) Biodegradation of ochratoxin A by fungi isolated from grapes. J. Agric. Food. Chem. 50: 7493–7496

Benndorf D & Babel W (2002) Assimilatory detoxification of herbicides by *Delftia acidovorans* MC1: induction of two chlorocatechol 1,2-dioxygenases as a response to chemostress. Microbiology 148: 2883–2888

Blackwell BA, Gilliam JT, Savard ME, DavidMiller J & Duvick JP (1999) Oxidative deamination of hydrolysed fumonisin B(1) (AP(1)) by cultures of *Exophiala spinifera*. Nat. Toxins 7: 31–38

Boon N, Goris J, De Vos P, Verstraete W & Top EM (2001) Genetic diversity among 3-chloroaniline and aniline-degrading strains of the *Comamonadaceae*. Appl. Environ. Microbiol. 67: 1107–1115

Camilo SB, Ono CJ, Ueno Y & Hirooka EY (2000) Anti-*Fusarium moniliforme* activity and fumonisin biodegradation by corn and silage microflora. Braz. Arch. Biol. Tecnol. 43: 159–164

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM & Tiedje JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 31: 442–443

Duvick J (2001) Prospects for reducing fumonisin contamination of maize trough genetic modification. Environ. Health Perspect. 109(Suppl. 2), 337–342

Duvick J, Rood T & Wang X (1998) Fumonisin detoxification enzymes. U.S. Patent # 5,716,820

Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG & Horak RM (1988) Fumonisin – Novel mycotoxin with cancer-promoting activity produced by *Fusarium moniliforme*. Appl. Environ. Microbiol. 54: 1806–1811

Han B & Chen Z (2002) Behavioral and electrophysiological responses of natural enemies to synomones from tea shoots and kairomones from tea aphids, *Toxoptera aurantii*. J. Chem. Ecol. 28: 2203–2219

IARC (1983) Evaluation of the carcinogenic risks to humans: some food additives and naturally occurring substances. 31International Agency for Research on Cancer, Lyons France 279–291

Junker F & Cook AM (1997) Conjugative plasmids and the degradation of arylsulfonates in *Comamonas testosteroni*. Appl. Environ. Microbiol. 63: 2403–2410

Katsivela E, Bonse D, Kruger A, Strompl C, Livingston A & Wittich RM (1999) An extractive membrane biofilm reactor for degradation of 1,3-dichloropropene in industrial waste water. Appl. Microbiol. Biotechnol. 52: 853–862

Kuske CR, Banton KL, Adorada DL, Stark PC, Hill KK & Jackson PJ (1999) Population of *Fusarium* spp. in soils from the arid regions and effect of water potential on their growth. J. Food Prot. 62: 1326–1332

Maniatis T, Fritsch EF & Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor., N.J.

Murphy PA, Hendrich S, Hopmans EC, Hauck CC, Lu ZB, Buseman G & Munkvold G. (1996) Effect of processing on fumonisin content of maize. In: Jackson LS, Devries JW and Bullerman LB (Eds) Fumonisins in Food, (pp 323–334). Plenum Press, New York

Nagao M (1983) Involvement of electron transfer system on microsomal fatty acid chainz elongation. Hokkaido Igaku Zasshi 58: 390–399

Nelson PE, Desjardin AE & Plattner RD (1993) Fumonisins, mycotoxins produced by *Fusarium* species: Biology, chemistry, and significance. Annu. Rev. Phytopathol. 31: 233–252

Nist Chemistry WebBook (2003) NIST Standard Reference Database Number 69 – March, 2003 Release. http://webbook.nist.gov/chemistry/

Pietri A, Bertuzzi T, Pallaroni L & Piva G (2004) Occurrence of mycotoxins and ergosterol in maize harvested over 5 years in Northern Italy. Food Additives & Contaminants 21: 479–487

Sadtler Research Laboratories (1985) The Sadtler Standard Gas Chromatography Retention Index Library. Vol 1–4, Sadtler Research Laboratories, Inc., Philadelphia, Pennsylvania

Shima J, Takase S, Takahashi Y, Iwai Y, Fujimoto H, Yamazaki M & Ochi K (1997) Novel detoxification of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. Appl. Environ. Microbiol. 63: 3825–3830

Snidaro P & Pavidotti M (2002) Rinnovare la tecnica di coltivazione del mais per migliorare qualità della granella. Notiziario ERSA 14(1–2), 15–17

Strauss WM (1987) Preparation of genomic DNA. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Siedman JG, Struhl JA and Smith K (Eds) Current Protocols in Molecular Biology, (pp 2.4.1–2.4.5). Greene Publishing and Wiley-Interscience, New York

U. S. Food and Drug Administration (2001) Guidance for Industry on Fumonisin Levels in Human Foods and Animal Feeds. Dockets Management Branch (HFA-305). U.S. Food and Drug Administration, Rockville, MD

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 173: 697–703

Wilson KJ, Sessitsch A, Corbo JC, Giller KE, Akkermans AD & Jefferson RA (1995) Beta-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. Microbiology 141: 1691–705

Yamaguchi T, Miyamoto K, Yagi S, Sato AM & Takeuchi M (2000) Detection of plasmalogen from plasma low density lipoprotein and high density lipoprotein in carp, *Cyprinus carpio*, and rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 127: 339–346