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Examination of the indole-diterpene biosynthesis of *Claviceps paspali* by *Agrobacterium tumefaciens* mediated gene knock out studies

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The Examination will be organized online at 10:00, 24.06.2021.

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The PhD Defense (online format) takes place at 24th of June 2021 at 15:00.
Publicity is guaranteed during the online Defense. If you are willing to participate, please indicate via email to kozaklaci85@gmail.com until 16:00, 23th of June, 2021. Due to technical reasons later sign-ups are not possible and you will not be able to join the online defense.

INTRODUCTION

Indole-diterpenes (IDTs) are small molecular weight mycotoxins, produced by a select group of ascomycetous fungi including *Aspergillus, Penicillium, Emericella, Eupenicillium, Claviceps, Epichloë, Escovopsis, Neotyphodium, Periglandula* and *Tolypocladium* species, as well as the zygomycetous fungus *Mucor irregularis*. IDTs are notorious mycotoxins that cause neurotoxic and tremorgenic symptoms in insects and mammals that consume them. This biological activity of IDTs can be attributed, at least in part, to their inhibitory effect on potassium channels in the nervous system. The mass poisoning caused by IDTs cause serious economic damage to the livestock sector year after year, but at the same time, due to their biological activity these components may form the basis for the development of new insecticides with new mechanism of action that can be used in the field of plant protection.

C. paspali is a plant parasitic Ascomycota fungus, which infects *Paspalum* species. At a specific life stage in the *C. paspali* – plant association, the fungus forms sclerotia that contain potent insect and mammalian toxins, which defend the fungus and its plant host from insect feeding and grazing by large animals. The toxins produced by *C. paspali* can be classified into two groups: IDTs and ergot alkaloids. Although ergot alkaloids are widely known and potent mycotoxins, they are not responsible for the tremorgenic symptoms caused by *C. paspali*. The reason for this is that the toxicity of the ergotamides that are produced by *C. paspali* is lower than the toxicity of ergopeptides. Ergopeptides are typically produced by *C. purpurea*, a fungus that mainly infects rye. These ergot alkaloids were responsible for the outbreak of ergotism in the middle ages. In contrast to *C. purpurea*, *C. paspali* produces large amounts of ergot alkaloids not only when it is associated with *Paspalum* spp., but also in axenic culture. This property makes this fungus important for the pharmaceutical industry.

Toxicity caused by *C. paspali* causes serious losses for agriculture, especially in the Southern Hemisphere (Australia, New-Zeeland, South Africa), but case reports from Spain, Portugal or the United States were also published. Although the sclerotium of *C. paspali* contains ergot alkaloid-type mycotoxins, the IDT compounds paspalitrem A, B and C are responsible for the poisoning. The syndrome caused by the ingestion of these toxins is called Paspalum stagger. A main symptom of the Paspalum stagger is the trembling of the head, neck and limbs. Ingestion of IDT toxins by grazing animals is usually not lethal. Animal deaths are usually caused by accidents resulting from uncoordinated movement.

Relatively little recorded information is available about toxicosis in humans caused by IDTs produced by *C. paspali*. The most notable case is the large Indian rice shortage of 1946,

when *P. scrobiculatum*, a kind of millet, was consumed more widely in certain areas of India. Several case reports from this time detailed toxic symptoms, including tremors, occurring after the consumption of millet. These symptoms were typically associated with a unique, taller and darker *P. scrobiculatum*, on which the presence of *C. paspali* sclerotia was later detected. Another important public health issue is the consumption of paspalitrem-type mycotoxins by cattle, and the possibility of the accumulation of this toxin in the milk and meat of the animals.

Until the beginning of our work, the presence and the chemical diversity of IDTs was only examined in the *C. paspali* sclerotia, but not in axenic cultures. The main IDT compounds detected in the *C. paspali* sclerotium are paspaline, paxilline, paspalinine, paspalitrem A and paspalitrem B. In addition to these main IDTs, a further seven paspaline and seven paspalitrem analogues have also been detected in the extract of sclerotia collected from the *C. paspali - P. dilatatum* association.

During IDT biosynthesis, the carbon skeleton of these molecules is synthetized by the fusion of a geranylgeranyl-diphosphate and an indole group. Almost all fungal IDT gene clusters contain a set of conserved genes which encode the biosynthesis of paspaline, the simplest cyclic IDT. A set of paspaline-like IDTs have also been detected in *C. paspali* extracts. These are likely intermediates for the biosynthesis of the paspalitrems, the main IDT mycotoxin group produced by *C. paspali*. Paspalitrems are monoprenylated paspalinine derivatives.

C. paspali is perhaps the most Janus-faced fungal species, as in addition to causing serious damage to livestock, it is widely used in the pharmaceutical industry to produce ergot alkaloid-type drugs by fermentation. Ergot alkaloid-based medications are typically semi-synthetic molecules, which are produced by the chemical modification of D-lysergic acid. Although the fermentation broth of *C. paspali* contains some free D-lysergic acid, the main products of the fermentation are the ergotamides, such as ergonovine, lysergic-carbonyl-amide, ergine and erginine. At the end of the fermentation, all ergotamide- and clavine-type ergot alkaloids are collected and converted to D-lysergic acid by alkaline hydrolysis. The resulting D-lysergic acid then serves as a precursor for the synthesis of a variety of drugs. Ergot alkaloid-type drugs are used for the treatment of migraine, Parkinson's disease and postpartum haemorrhage, amongst others. Globally, tens of tons of ergot alkaloids are produced annually using *C. paspali*, making this fungus highly important for both the pharmaceutical industry and human medicine.

Since *C. paspali* produces a number of IDTs in association with *Paspalum* grasses, it is very important to establish whether these mycotoxins are also produced during the industrial, alkaloid-producing fermentations of this fungus, and if so, how these compound could be completely eliminated from the fermentation process. Surprisingly, in spite of the importance

of this issue, the presence or absence of the IDT compounds during *C. paspali* fermentations has not been examined until our work.

AIMS

(i) Our first goal was to investigate whether *C. paspali* strain DSM833 is capable to produce IDT mycotoxins under fermentation conditions and, if so, to determine how similar the spectrum of these IDTs were to those IDTs that had been isolated from the sclerotium.

(ii) Our second aim was to optimize and adapt a genetic transformation method for *C. paspali*, and to demonstrate its applicability for the generation of stable, homokaryotic mutants. Until the publication of our work, no transformation protocol optimized for *C. paspali* was described in the literature, and despite the importance of *C. paspali*, no one has yet reported the targeted genetic modification of this fungus for any purpose. The generation of stable transformants is hampered by the fact that *C. paspali* hyphae, or even the protoplasts, contain multiple nuclei, which makes it difficult to isolate mitotically stable transformants. Therefore, in order to generate stable transformants that have undergone stable genomic integration, we decided to adapt the *Agrobacterium tumefaciens*-mediated transformation (ATMT) process to *C. paspali*, combined with a re-isolation process which eventually results in the isolation of homokaryotic transformants. By developing the technique, we not only optimized a stable transformation technology for *C. paspali*, but we were the first to develop ATMT technology for any member of the Clavicipitaceae family.

(iii) A further aim of our work was to prove the function of the hypothetical IDT gene cluster in *C. paspali* by knocking out a part of the cluster, using ATMT. For this reason, the locus containing the *idtCGBF* genes was replaced with the hygromycin phosphotransferase marker gene by ATMT, and the IDT profile of the resulting mutant was compared to that of the wild type strain.

(iv) Furthermore, we aimed to create *C. paspali* transformant strains that are devoid to IDT toxins but continue to produce ergot alkaloids undisturbed.

(v) After identifying the paspalitrem gene cluster, we decided to verify the function of the *idtP* P450 monooxygenase and the *idtF* monoprenyl transferase genes, by knocking them out and examining the IDT profile of the resulting mutants. This allowed us to gain a deeper insight into the IDT biosynthesis of *C. paspali*.

(vi) Finally, we aimed to build a model of the paspalitrem biosynthesis of *C. paspali* by analysing the metabolite profile of mutant and wild-type *C. paspali* strains and comparing these results to the available literature data.

MATERIALS AND METHODS

Fungal strain and growth conditions

Throughout this work, we used *C. paspali* DSM833, which is equivalent to the now-discontinued ATCC 13893. To produce ergot alkaloids and IDTs in laboratory conditions we used a two-stage shake flask fermentation procedure. First, we inoculated 1 mL of homogenized *C. paspali* mycelium into 60 mL inoculum medium (5% mannitol, 1% succinic acid, 0.5% soy flour, 0.2% KH_2PO_4, 0.03% $MgSO_4 \times 7H_2O$, pH 5.2), and incubated the culture for 5 days at 28°C with shaking at 4,16 Hz. A 5-mL aliquot of this pre-culture was inoculated into 60 mL of production medium (10% sorbitol, 3.5% succinic acid, 1.5% corn steep liquor, 0.05% yeast extract, 1.5% NH_4NO_3, 0.07% $MgSO_4$ x $7H_2O$, 0.0022% $FeSO_4$ x $7H_2O$, 0.001% $ZnSO_4$ x $7H_2O$, pH 5.2) in a 500 mL Erlenmeyer flask, and the resulting main culture was incubated for a further 12 days at 28°C with shaking at 4,16 Hz.

Molecular biology workflow

Genomic DNA from *C. paspali* lysates was isolated by the MagNa Pure 2.0 Nucleic Acid Isolation Robot. The mycelium was ground under liquid nitrogen in a mortar with a pestle and suspended in 400 µL sulphite buffer (0.7 M NaCl, 0.1 M Na_2SO_3, 0.1 M Tris-Cl pH 7.5, 0.05 M EDTA, 1% SDS) and digested with 3 U proteinase K and then 50 U RNase A enzymes. The lysate was centrifuged, and the supernatant was used for the isolation of high purity genomic DNA.

PCR reactions were carried out with 20 ng genomic DNA or 1 ng plasmid DNA as templates, respectively, in 50 µl reaction mixtures containing 0.2 mM of each dNTP, 1 pM of each primer, 1 µl Phusion HF DNA polymerase and 10 µl HF buffer (New England Biolabs, Ipswich, MA). Thermal cycling conditions for the PCR reactions were 180 s at 98°C for the initial denaturation, followed by 31 cycles of amplification (98°C for 10 s, 55°C for 15 s, 72°C for 30 s/kbp), and a final extension step of 60 s/kbp at 72°C.

We used Gibson assembly for the construction of the targeting plasmids. The reaction mixes contained 50 ng vector DNA with a 3-fold excess of the inserts, 10 µL Gibson Assembly Master Mix, and nuclease-free water in a total volume of 20 µL. The reaction mixtures were incubated at 50°C for 60 min prior to transformation to *E. coli* XL1-Blue chemically competent cells. Transformant colonies were selected on LB agar plates containing 25 µg/ml kanamycin.

Independent kanamycin resistant colonies were cultivated in 3-3 mL LB broth supplemented with 25 μg/ml kanamycin. Plasmids from these cultures were isolated using the EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic Inc., Toronto, Canada) and verified by diagnostic PCR and DNA sequencing.

Assembly of the gene-deletion plasmid constructs

The pAg-*idtCBGF*-KO plasmid which was used for the inactivation of the *idtCBGF* allele was constructed in two consecutive steps using Gibson assembly. In the first step, the linearized pAg-H3 vector was PCR amplified with primers pAg-F1 and pAg-R1 and fused using the Gibson Assembly Master Mix with the left targeting arm (LTA, the 1.5 kb region of the *C. paspali* DSM-833 genomic DNA located upstream of the *idtC* gene).

In the second step, the pAg plasmid already harbouring the left targeting arm was linearized by PCR and fused using the Gibson Assembly Master Mix with the right targeting arm (RTA, the 1.9-kb genomic region downstream from the *idtF* gene, amplified using primers *idtCBGF*-RA-F and *idtCBGF*-RA-R).

The *idtP* and *idtF* targeting plasmids were created in a slightly different manner. The hygromycin phosphotransferase gene (*hph*) of the pAg-H3 vector and the rest of the vector were amplified by PCR in two separate reactions. The left and right targeting arms for the *idtF* and the *idtP* genes were also amplified by PCR using *C. paspali* genomic DNA as the template and with appropriate primers. The four PCR amplicons (the *hph* gene, the rest of the pAg-H3 vector, and the appropriate left and right targeting sequences) were fused using the Gibson Assembly Master Mix, utilizing overlapping sequences between the adjacent DNA fragments at the 5′ ends of the primers. The resulting Gibson reaction products were transformed into *E. coli* XL1-Blue chemical competent cells and the transformed cells were grown on LB agar plates supplemented with 25 μg/mL kanamycin.

Plasmids from kanamycin-resistant colonies were isolated using the EZ-10 Spin Column Plasmid DNA Miniprep Kit and verified by diagnostic PCR and DNA sequencing. The correctly assembled plasmids were separately transformed into *A. tumefaciens* LBA4404 electrocompetent cells, and the transformants were selected on LB agar plates supplemented with kanamycin (25 μg/mL) and streptomycin (50 μg/mL)

A. tumefaciens-mediated transformation (ATMT) of *C. paspali* DSM833

C. paspali vegetative mycelium was collected from PDA slant agar and cultured into 50 mL of PDB broth. The culture was incubated at 28°C for 48 hours with shaking. 5 mL of this pre-culture was transferred into 50 mL fresh PDB broth and was incubated at 28°C for 48 hours with agitation. Mycelia were collected with centrifugation, washed in distilled water and suspended in induction medium (IM) containing 200 μM acetosyringone, and incubated for 8 h at 28°C with shaking at 240 rpm.

The selected *A. tumefaciens* transformant colonies were inoculated into 50 mL LB broth supplemented with kanamycin (25 μg/mL) and streptomycin (50 μg/mL) and agitated at 30°C for 48 hours. 1 mL of the pre-culture was inoculated into 50 mL fresh LB medium containing kanamycin (25 μg/mL) and streptomycin (50 μg/mL) and was cultured at 30°C for 12–16 h with shaking (until the cell density reached OD_{600}=0.2-1.0). The cells were collected by centrifugation, washed with distilled water, suspended in 50 mL IM broth containing 200 μM acetosyringone, and incubated at 30°C for 8 h with shaking. One hundred microliter aliquots of the *C. paspali* mycelia and the *A. tumefaciens* cells were mixed, spread onto IM agar plates containing 200 μM acetosyringone, and the co-culture was incubated for 2–6 days at 28°C. Co-cultivation was also attempted on IM agar plates covered with cellulose acetate ester membranes.

Selection and isolation of *C. paspali* transformants

To select transformants and inhibit the further growth of the *A. tumefaciens* cells, the IM agar plates (20 mL) were overlaid with 10 mL of top agar (PDA, 14.5 g/L) supplemented with 600 μg/mL hygromycin and 600 μg/mL cefotaxime (final concentrations calculated for the full plate: 200 μg/mL each), and the plates were incubated at 28°C for an additional 10 days. Transformation efficiency was estimated by calculating the average number of hygromycin-resistant colonies per IM plates. In the case of membrane co-cultivation, the membrane with the cells was lifted to a fresh PDA agar plate supplemented with 200 μg/mL hygromycin and 200 μg/mL cefotaxime and the incubation was continued for a further 10 days at 28°C. Transformant colonies were picked with a sterile toothpick and passed onto fresh PDA plates containing 0.5 mg/mL hygromycin. Re-isolation of the transformant colonies was repeated 4-5 times.

Analysis of the ergot alkaloid and IDT compounds produced by *C. paspali* DSM833

IDT constituents of the *C. paspali* extract were analysed with LC-MS/MS. For the sample preparation the lyophilized and homogenized mycelium was extracted with 10 mL acetonitrile/water (4:1, *v*/*v*). The supernatant was filtered and was used for direct injection for LC-MS analysis.

Ergot alkaloids were detected in the supernatants of the fermentation cultures. Five grams of the appropriate broths were transferred into 50 mL volumetric flasks, the flasks were filled up to the mark with acetonitrile:water (15:85), and the resulting solutions were filtered using a 0.22 μm membrane before injection into the HPLC system. A Waters (Milford, MA) XBridge C18 (100 × 4.6 mm; 3.5 μm) analytical HPLC column was used for the analyses, and UV detection was performed at 310 nm.

Since lysergic acid and paspalic acid co-eluted under these conditions the separation of these compounds was achieved by a different HPLC method. In this assay, the samples were diluted twofold with acetonitrile:water = 5:95 (*v*/*v*) and filtered through a 0.22 μm membrane prior to analysis. A reversed phase column (Zorbax Extend C18, 100 × 4.6 mm, 3.5 μm) was used with the UV detection was performed at 210 nm.

THE SUMMARY OF NEW SCIENTIFIC RESULTS

Optimization of ATMT for *C. paspali*

As previously no genetic transformation protocol was available optimized for *C. paspali* in the literature, our task was to develop a robust, stable transformation system which affords stable homokaryotic mutants. For this purpose, we selected the ATMT method, which has previously been successfully adapted to a number of industrially important filamentous fungi. The most critical parameter during the optimization was the *A. tumefaciens* cell density and the length of the co-cultivation period on IM agar plates. Too high *A. tumefaciens* cell density impairs the viability of *C. paspali* transformants, while too low cell density reduced the efficiency of transformation. Similarly, an overly long co-growth period negatively affected the viability of the fungus to be transformed, while a too short co-cultivation reduced the likelihood of transformation occurring. We also confirmed the necessity to supplement the IM agar with acetosyringone during the co-incubation, in order to induce T-DNA formation in *A.*

tumefaciens. The highest transformation frequency, approximately 80 hygromycin-resistant colonies per 10 mg wet *C. paspali* mycelium, was recorded at an *A. tumefaciens* cell density of $OD_{600} = 0.5$, after 4 days of co-incubation.

ATMT transformation of *C. paspali* was also performed on a cellulose acetate membrane by plating the cells on a membrane placed on induction agar and co-cultivating the fungus with *A. tumefaciens* on the membrane surface. Although this protocol was also successful, the introduction of membrane selection did not increase the efficiency of the transformation.

In order to examine the stability of the resulting transformants, we passed fifty primary hygromycin resistant colonies into fresh PDA agar containing hygromycin. All of these transformants retained hygromycin resistance and displayed vigorous growth. During subsequent re-isolations, putative *C. paspali* transformants tended to develop larger colonies within the same incubation timespans, likely due to the enrichment of the transformed nuclei at the expense of the wild type ones within the mycelia. We checked the genomic integration of the knockout cassettes by PCR amplification of the *hph* gene from the genome of the transformants. Stable gene transfer was confirmed by the presence of the *hph* gene in all the selected 12 transformants by PCR.

Indole-diterpene profile of wild type *C. paspali* DSM833 during fermentation

Until the beginning of our work, no data were available in the literature on the ability of *C. paspali* to produce IDT-type metabolites under axenic conditions. We have detected five IDT congeners in the fermentation broth: paspaline, paxilline, paspalinine, paspalitrem A and B, and a paspalitrem isomer, most likely paspalitrem C. These congeners were also the most abundant IDT components in sclerotium extracts.

Verification of the *C. paspali* paspalitrem gene cluster by inactivation of the *idtCBGF* genes

In order to prove the function of the presumed paspalitrem gene cluster of *C. paspali* DSM833, we inactivate the *idtCBGF* locus of the cluster using the ATMT method. Four out of the twelve selected and examined transformants showed the site-specific integration of the *hph* gene with the concomitant loss of the *idtCBGF* genes. Three out of the four transformants were homozygotes for the gene-knock out allele. One transformant showed the presence of both the knockout and the wild type allele, indicating that this mutant is a heterozygote. In order to

investigate the effect of the knockout of the *idtCBGF* locus on IDT biosynthesis in *C. paspali*, extracts of two selected homozygous knockout transformants were subjected to HPLC-MS/MS analysis. None of the above-mentioned IDT molecules could be detected in the extract of these strains, suggesting that the deletion of the *idtCGBF* allele completely blocked IDT biosynthesis in these mutant strains. This verified the involvement of the *idtCGBF* locus, and thus the whole biosynthetic gene cluster, in IDT biosynthesis.

Investigation of the role of the *idtP* and *idtF* genes in the paspalitrem biosynthesis of *C. paspali*

To gain insight into the roles of the *idtP* and *idtF* genes in IDT biosynthesis in *C. paspali*, we examined the IDT profiles of extracts from two representative Δ*idtP* and Δ*idtF* mutant strains, respectively, and compared them with the IDT profile of the wild-type *C. paspali*. The extract of the Δ*idtP* strain did not contain detectable amounts of paspalitrem A, paspalitrem B or paspalinine, but contained large amounts of paspalin, suggesting that the deletion of *idtP* gene in these isolates prevented the conversion of paspaline to later biosynthetic products.

Similar to the Δ*idtP* strains, prenylated IDTs were not present in the extract of Δ*idtF* isolates. In contrast, accumulation of paspalinin was observed in these extracts, suggesting that the prenylation step of paspalinin was blocked in these mutants. In addition to paspalinin, small amounts of paspalin were also detected.

Comparison of the ergot alkaloid profiles of the wild type and the Δ*idtCBGF C. paspali* strains

In order to verify whether the knockout of the *idtCBGF* genes, and the consequent complete blockade of IDT biosynthesis, affected ergot alkaloid production in *C. paspali* strain DSM-833, we examined the ergot alkaloid profiles of the wild-type and two selected Δ*idtCBGF* mutant isolates (CPIDT2 and CPIDT8) by HPLC analysis. Examination of the extracts of the fermentation broths showed that the ergot alkaloid production capacity of the wild-type and the transformant strains was not significantly different.

Wild type *C. paspali* DSM-833 produced 18.30 ± 1.05 μg/g total ergot alkaloids, including 1.25 ± 0.09 μg/g paspalic acid. The CPIDT2 and CPIDT8 knockout strains produced 19.09 ± 1.30 μg/g and 18.41 ± 0.50 μg/g total ergot alkaloids, including 1.17 ± 0.15 μg/g and 1.28 ± 0.11 μg/g paspalic acid, respectively. Lysergic acid was not detectable in the fermentation

broths of the wild type or the CPIDT2 or CPIDT8 strains. Distribution of the detected ergot alkaloids such as ergine, lysergic acid methyl carbonyl amide, ergonovine, isolysergic acid methyl carbonyl amide, erginine, and paspalic acid were similarly not affected by the disruption of IDT biosynthesis.

A model for paspalitrem biosynthesis in *C. paspali*

Functional validation of the *C. paspali* IDT gene cluster allowed us to construct a model for paspalitrem biosynthesis. The protein products of the *idtG, idtM, idtB* and *idtC* genes in the paspalitrem cluster show 53, 38, 56 and 45% identity, respectively, with the protein products of the *paxG* (geranylgeranyl diphosphate [GGPP] synthase), *paxM* (FAD-dependent monooxygenase), *paxB* (IDT cyclase) and *paxC* (prenyl transferase) genes of the paxilline gene cluster in *P. paxilli*, which catalyse the assembly of paspaline, the first stable cyclic IDT compound. Therefore, it can be hypothesized that similarly, the IdtG, IdtM, IdtB, and IdtC proteins of *C. paspali* catalyse the early steps of paspalitrem biosynthesis, resulting in the formation of paspalin.

The *idtP* and *idtQ* genes of the paspalitrem gene cluster encode two cytochrome P450 monooxygenase enzymes. The *idtP* gene product shows 41% identity to PaxP of *P. paxilli*. We propose that just as PaxP, IdtP catalyses the conversion of paspaline to 13-desoxypaxilline via the intermediate β-PC-M6 by removing the C-30 methyl group and installing the carbonyl oxygen at C-10.

The amino acid sequence of IdtQ shows 37% similarity with the amino acid sequence of PaxQ (P450 monooxygenase in paxilline biosynthesis). The role of the IdtQ is dual. First, it catalyses the C-13 oxidation of 13-desoxypaxilline, resulting paxilline. Second, it also mediates the C-7 oxidation of paxilline, which affords paspalinine. Considering that both paspalicine and paxilline were detected in the *C. paspali—P. dilatatum* association, it is reasonable to assume that IdtQ does in fact catalyse both the C-13 and the C-7 oxidations of 13-desoxypaxilline. Whether paxilline may be converted to paspalinine by IdtQ, or paxilline represents a shunt product of the pathway remains to be determined.

The next step of paspalitrem biosynthesis is the installation of the 2-methylbut-2-ene side chain to the C-21 or C-20 carbons, resulting in paspalitrem A or paspalitrem C, respectively. The most likely candidate for the prenylation reaction is the IdtF prenyl transferase, which shows 21% identity with the AtmD enzyme of *Aspergillus flavus*. Accordingly, no prenylated IDT derivatives were present in the extracts of the ΔidtF strains we generated, but an

accumulation of paspalinin was observed, clearly demonstrating that IdtF is responsible for the prenylation of paspalinin in paspalitrem biosynthesis. The final step of the paspalitrem biosynthesis is the hydroxylation of the prenyl side chain at C-32 by a still unknown oxidase to afford paspalitrem B.

DISCUSSION

C. paspali is a plant parasitic Ascomycota fungus which in the association with Paspalum spp. grasses produces large amounts of IDT mycotoxins.. Since *P. dilatatum* (the primary host plant of *C. paspali*) is a widely used forage in the Southern hemisphere, *C. paspali* causes significant losses in the agriculture every year. At the same time, *C. paspali* has been used in the pharmaceutical industry for decades to produce large amounts of water-soluble ergot alkaloids using industrial fermentation conditions.

In view of the above, it is surprising that no previous investigations were conducted to ascertain the ability of *C. paspali* to produce IDT-type mycotoxins under fermentation conditions. Furthermore, until the publication of our work, no genetic transformation method has been developed for the stable genetic modification of this fungus. Consequently, the functional verification of the ergot alkaloid or the IDT biosynthetic gene cluster in this fungus could not have been completed.

In our work, we prove the ability of *C. paspali* to produce IDT mycotoxins during axenic growth conditions.

In order to verify the function of the presumed IDT gene cluster in *C. paspali* we optimized a genetic transformation method utilizing *A. tumefaciens* gene transfer.

With the help of this method, we successfully inactivated the *idtCBGF* allele of the paspalitrem gene cluster which completely eliminates the whole spectrum of IDTs in the mutants.

In this work, we also validated the function of the *idtP* (P450 monooxygenase) and *idtF* (monoprenyl transferase) genes of the paspalitrem gene cluster, using the ATMT method. Inactivation of both genes resulted in the elimination of paspalitrem-type IDTs, but while the deletion of *idtP* led to the accumulation of paspaline, the inactivation of *idtF* resulted in the accumulation of paspalinine.

These results open new avenues for the genetic study of *C. paspali*. The optimized transformation protocol makes it possible to generate industrial strains that are able to produce ergot alkaloid metabolites on a larger scale or with a modified spectrum.

Furthermore, the targeted genetic modification of the IDT biosynthetic pathway of *C. paspali* may provide IDT variants which show reduced toxicity toward mammals, but retain toxicity towards insects, thereby providing potent but safe insecticides. This and similar applications would open new possibilities for the bioindustrial utilization of this interesting fungus.

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Candidate: László Kozák
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List of publications related to the dissertation

1. **Kozák, L.**, Szilágyi, Z., Tóth, L., Pócsi, I., Molnár, I.: Functional characterization of the idtF and idtP genes in the Claviceps paspali indole diterpene biosynthetic gene cluster.
Folia Microbiol. 65 (3), 605-613, 2020.
DOI: http://dx.doi.org/10.1007/s12223-020-00777-6
IF: 1.73 (2019)
2. **Kozák, L.**, Szilágyi, Z., Vágó, B., Kakuk, A., Tóth, L., Molnár, I., Pócsi, I.: Inactivation of the indole-diterpene biosynthetic gene cluster of Claviceps paspali by Agrobacterium-mediated gene replacement.
Appl. Microbiol. Biotechnol. 22 (7), 3255-3266, 2018.
DOI: http://dx.doi.org/10.1007/s00253-018-8807-x
IF: 3.67

List of other publications

3. **Kozák, L.**, Szilágyi, Z., Tóth, L., Pócsi, I., Molnár, I.: Tremorgenic and neurotoxic paspaline-derived indole-diterpenes: biosynthetic diversity, threats and applications.
Appl. Microbiol. Biotechnol. 103 (4), 1599-1616, 2019.
DOI: http://dx.doi.org/10.1007/s00253-018-09594-x
IF: 3.53

Total IF of journals (all publications): 8,93
Total IF of journals (publications related to the dissertation): 5,4

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

27 January, 2021