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Total transcriptome response for tyrosol exposure in *Aspergillus nidulans*

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A B S T R A C T

Although tyrosol is a quorum-sensing molecule of *Candida* species, it has antifungal activity at supraphysiological concentrations. Here, we studied the effect of tyrosol on the physiology and genome-wide transcription of *Aspergillus nidulans* to gain insight into the background of the antifungal activity of this compound. Tyrosol efficiently reduced germination of conidia and the growth on various carbon sources at a concentration of 35 mM. The growth inhibition was fungistatic rather than fungicide on glucose and was accompanied with downregulation of 2199 genes related to e.g. mitotic cell cycle, glycolysis, nitrate and sulphate assimilation, chitin biosynthesis, and upregulation of 2250 genes involved in e.g. lipid catabolism, amino acid degradation and lactose utilization. Tyrosol treatment also upregulated genes encoding glutathione-S-transferases (GSTs), increased specific GST activities and the glutathione (GSH) content of the cells, suggesting that *A. nidulans* can detoxify tyrosol in a GSH-dependent manner even though this process was weak. Tyrosol did not induce oxidative stress in this species, but upregulated “response to nutrient levels”, “regulation of nitrogen utilization”, “carbon catabolite activation of transcription” and “autophagy” genes. Tyrosol may have disturbed the regulation and orchestration of cellular metabolism, leading to impaired use of nutrients, which resulted in growth reduction.

1. Introduction

Tyrosol (2-(4-hydroxyphenyl) ethanol) is a natural phenolic compound that can prevent cardiovascular diseases or osteopenia and has antioxidant and anti-inflammatory activity (Giovannini et al., 2002; Puel et al., 2008). It was found in food sources such as olive oil, olive leaves, green tea and wines (Covas et al., 2003; Di Benedetto et al., 2007; Bouaziz et al., 2008). Tyrosol was also detected in the extracts of several endophytic fungi, including *Glomerella* spp., *Phyllosticta* spp. or *Phialocephala fortinii* (Guimaraes et al., 2009; Tan et al., 2015; Cui et al., 2016; Anisha and Radhakrishnan, 2017). Furthermore, tyrosol has been described as a quorum-sensing molecule in *Saccharomyces cerevisiae*, the opportunistic human pathogen *Candida albicans* and, more recently, in several other *Candida* species including *C. tropicalis*, *C. glabrata* and *C. parapsilosis* (Cremer et al., 1999; Chen et al., 2004; Chen and Fink 2006; Albuquerque and Casadevall 2012; Wongsuk et al., 2016; Rodrigues and Černáková 2020). Under physiological conditions, tyrosol influences the morphogenesis. It promotes the formation of germ tubes and mediates the yeast-to-hyphae transition in the early and intermediate periods of biofilm formation in *C. albicans*. Furthermore, tyrosol affects cell cycle, DNA replication and chromosome segregation (Chen et al., 2004; Alem et al., 2006; Wongsuk et al., 2016).

In the last decade, there has been increasing interest in the antifungal effect of quorum-sensing molecules against *Candida* species at both physiological and supraphysiological concentrations. These studies revealed that farnesol and tyrosol have potential *in vitro* and *in vivo* antifungal and anti-biofilm effect at supraphysiological concentrations in monotherapy or in combination with traditional antifungal agents in both planktonic and sessile cultures of *C. albicans* and non-*albicans* *Candida* species (Rossignol et al., 2008; Shanmughapriya et al., 2014; Cordeiro et al., 2015; Katragkou et al., 2015; Kovács et al., 2016; Arias et al., 2016; do Vale et al., 2017; Kovács et al., 2017; Monteiro et al., 2017; Mehmood et al., 2019; Kovács and Majoros 2020; Rodrigues and Černáková, 2020; Nagy et al., 2020a, 2020b; Jakab et al., 2021). In the

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case of tyrosol, the observed antifungal effect can be explained by the induced oxidative stress and the decreased expression of polyunsaturated fatty acid metabolism and ribosome biogenesis genes (Jakab et al., 2019). Although studies on the effects of quorum-sensing molecules on filamentous fungal species are also emerging (Semighini et al., 2008; Lorek et al., 2008; Wartenberg et al., 2012; de Salas et al., 2015; Onder and Oz 2021; Oiki et al., 2022), most of them focus on farnesol, and only few data are available on the consequences of tyrosol treatment on these fungi (Wongsuk and Sukphopetch 2019).

Aspergillus nidulans has been an important research organism in eukaryotic cell biology for over half a century. In addition, it was recognised as a major cause of invasive aspergillosis in patients with chronic granulomatous disease (Segal et al., 1998; Blumental et al., 2011; Henriet et al., 2013; Sabino et al., 2014). Here, we studied *A. nidulans* cultures treated with tyrosol at supraphysiological concentration to shed light on the physiological and transcriptome-based consequences of the presence of this potential antifungal agent. Understanding how filamentous fungi respond to tyrosol can be crucial in the further development of effective therapies with limited toxicity based on quorum-sensing molecules.

2. Materials and methods

2.1. Strain and culturing conditions

The *A. nidulans* FGSC A4 wild type strain and the *A. nidulans* MAD329 (*biA1, argB2, methG1, veA1, ΔphacA::argB$^+$*) and MAD817 (*biA1, argB2, methG1, veA1, p35::argB$^+$*) mutants were used in this study. MAD329 and MAD817 strains were constructed and kindly provided by Dr. M. A. Peñalva (Centro de Investigaciones Biológicas del CSIC, Madrid, Spain) (Mingot et al., 1999; Emri et al., 2003). All strains were maintained on Barratt's minimal nitrate agar plates supplemented with appropriate additives (Barratt et al., 1965) at 37 °C, and conidia, freshly harvested from 6-day-old cultures, were used in all experiments for inoculation.

For submerged cultivation, Barratt's minimal nitrate broth (100 mL aliquots in 500 mL Erlenmeyer flasks) were inoculated with 5×10^7 conidia. Cultures were incubated at 24 °C and 220 rpm for 42 h. Mycelia were harvested by filtration and re-suspended in fresh media. These cultures were further incubated at 24 °C and 220 rpm. Tyrosol (35 or 45 mM final concentration) was added after 5 h of incubation to the exponentially growing phase cultures. For data presentation, this time point (addition of tyrosol) was indicated as 0 h. In some experiments, mycelia from the 42 h cultures were transferred into fresh media containing 5 v/v % sunflower oil as sole carbon source. These cultures were treated immediately with 35 mM tyrosol, or remained untreated, and were further incubated at 24 °C and 220 rpm.

2.2. Tyrosol susceptibility assays

The growth inhibitory effect of tyrosol was assayed on Barratt's minimal nitrate agar plates (Barratt et al., 1965) supplemented with 15–45 mM tyrosol. Plates were point-inoculated with 5 μL of freshly prepared conidium suspension (1×10^5 conidia/mL physiological saline – 0.01 v/v % Tween 80 solution) in triplicates and incubated at 24 °C for 7 days or at 37 °C for 5 days (Orosz et al., 2018). In some experiments glucose was replaced with 4 g/L casein peptone, 20 g/L lactose, or 20 g/L arabinogalactan. In these cases, cultures were treated with 35 mM tyrosol and/or 0.4 g/L 2-deoxy-D-glucose (2DG) or were left untreated. Tyrosol susceptibility was characterised by the percentage of the colony diameter in tyrosol-treated cultures compared to that in the control, untreated cultures.

The growth of mycelia in the presence and absence of tyrosol in submerged cultures was characterized by the increase in dry cell mass (DCM). The DCM was measured after freeze-drying the biomass (Emri et al., 2005). In some experiments, mycelia were filtrated from the cultures at 2 h after tyrosol treatment, were washed, transferred into fresh tyrosol free Barratt's minimal nitrate broth medium and were further incubated at 24 °C and 220 rpm. The increase in the DCM was determined after18 h.

The effect of tyrosol on the germination of conidia was also tested. Freshly collected conidia were diluted with Barratt's minimal broth to 1×10^5 conidia/mL and were incubated at 37 °C for 5 h in the presence or absence of tyrosol (45 mM) in triplicates. Germinated conidia were counted under optical microscope.

2.3. Detecting glucose consumption

Glucose content of the fermentation broth was measured spectrophotometrically by the glucose oxidase – horseradish peroxidase rate assay according to Leary et al. (1992). In this reaction, 0.76 mM 4-aminoantipyrine, 11 mM phenol, 4 kU/L glucose oxidase and 1 kU/L peroxidase were dissolved in sodium-potassium phosphate buffer (0.1 M, pH 6.6). The absorbance at 500 nm was recorded immediately after addition of 10 v/v % sample (Leary et al., 1992).

2.4. Detection of tyrosol from the fermentation broth

The tyrosol content of the cell free fermentation broth was determined with micellar electrokinetic capillary chromatography (MEKC). MEKC separations were performed on a fused-silica capillary (Polymicro, Phoenix, USA) of 48.5 cm × 50 μm using 7100 CE System (Agilent, Waldbronn, Germany) coupled to UV detector. The precondition procedure was 1 M NaOH for 3 min, followed by background electrolyte (BGE) for 5 min. BGE consisted of 50 mM sodium tetraborate and 50 mM SDS (pH 9.3). Hydrodynamic sample injection (50 mbar × 2 s) was carried out at the anodic end of the capillary, and -20 kV was applied for electrophoretic separation. Detection was performed on-capillary at a wavelength of 200 nm. The Chemstation software (version B.04.02; Agilent) was used for operating the CE instrument and for processing the results.

2.5. Measuring redox imbalance and GSH and GSSG contents

The effect of tyrosol treatment on redox homeostasis was detected with 2′,7′-dichlorofluorescein (DCF-assay) as described earlier (Emri et al., 1997a). Briefly, 2′,7′-dichlorofluorescin diacetate at 10 μmol/mL final concentration was added to 20 mL aliquots of the cultures. After a 1-h long incubation at 24 °C and 220 rpm, mycelia were harvested by filtration. Cell free extract was prepared in 5 w/v % 5′-sulfosalicylic acid solution at 0 °C and cell debris was removed by centrifugation. The produced DCF was determined spectrofluorimetrically (Yin et al., 2013).

The reduced and oxidised glutathione (GSH and GSSG, respectively) contents of the hyphae were determined spectrophotometrically by the 5,5′-dithiobis(2-nitrobenzoic acid) – glutathione reductase assay (Anderson 1985) using cell free extracts prepared by 5′-sulfosalicylic acid treatment (Emri et al., 1997a).

2.6. Enzyme assays

Specific glutathione-S-transferase (GST), glutathione reductase (GR) and catalase (Roggenkamp et al., 1974; Pinto et al., 1984) activities were measured spectrophotometrically from cell-free extracts prepared by X-pressing (Emri et al., 1997a) using rate assays according to the papers indicated in parentheses. The protein concentration of cell-free extracts was measured by the Bradford method (Bradford 1976).

2.7. High-throughput RNA sequencing

In the RNAseq experiment, submerged cultures were supplemented with 35 mM tyrosol at 5 h incubation time as described above and samples were collected after 2 h exposure for RNA isolation. Total RNA

was isolated from lyophilised mycelia in three biological replicates using Tri Reagent (Chomczynski 1993). The quality of RNA was determined using the Eukaryotic Total RNA Nano kit (Agilent, Santa Clara, CA, USA) along with Agilent Bioanalyzer. Single-read 75-bp Illumina sequencing was performed using an Illumina HiScan SQ instrument (Illumina, San Diego, CA, USA) (Gila et al., 2021). Library pools were sequenced in the same lane of a sequencing flow cell and 12-15 million reads per sample were obtained (Table S1). Quality control of the reads was performed with the FastQC package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Depending on the samples, 70 % - 91 % of the reads (Table S1) were successfully aligned to the genome of *A nidulans* FGSC A4 (genome: http://www.aspergillusgenome.org/download/sequence/A_nidulans_FGSC_A4/current//A_nidulans_FGSC_A4_version_s10-m04-r14_chromosomes.fasta.gz; gff3: http://www.aspergillusgenome.org/download/gff/A_nidulans_FGSC_A4/A_nidulans_FGSC_A4_version_s10-m04-r14_features_with_chromosome_sequences.gff.gz) with the hisat2 software (version 2.1.0) (Kim et al., 2015). Differentially expressed genes (DEGs) were determined with DESeq2 (version 1.24.0) (Table S3; Love et al., 2014). RPKM (reads per kilobase million) values were calculated with the edgeR package "rpkm" function (R project; www.R-project.org/). The RNA sequencing (from library preparation to the generation of fastq.gz files) was carried out at the Genomic Medicine and Bioinformatic Core Facility, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary.

2.8. Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) assays

Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, USA) was used for RT-qPCR. Total RNA contents were isolated from lyophilised mycelia with Tri Reagent (Merck Ltd. Budapest, Hungary) (Chomczynski 1993), and the assays were carried out following the manufacturer's protocol; the primer pairs used are listed in Table S2. Oligonucleotide primers were designed with Oligo Explorer (v.1.1.) and Oligo Analyzer (v.1.0.2) software. The relative transcription levels were characterised with the ΔΔCP value. ΔΔCP is the difference between ΔCPs of the treated and untreated cultures, where ΔCP is the difference between the crossing point of the reference gene (γ-actin; AN6542) and the target gene within a sample.

2.9. Evaluation of transcriptome data

When the transcriptomes of tyrosol-treated and untreated cultures were compared, up- and downregulated genes were defined as differentially expressed genes (adjusted p-value <0.05; Deseq2), where $\log_2FC > 1$ or $\log_2FC < -1$, respectively. The FC (fold change) stands for the number calculated by the DESeq2 software using untreated cultures as reference (Table S3).

The ShiniGo platform (bioinformatics.sdstate.edu/go/) with default settings was used for the characterisation of the up- and downregulated gene sets. Only hits with a corrected p-value <0.05 were regarded as significantly enriched (Table S3). Enrichment of selected gene groups in the up- and downregulated gene sets was also studied with the "fisher.test" function of the R project (Fisher's exact test; www.R-project.org/) (Table S4).

The following gene groups were analysed:

The "Sulfur and glutathione metabolism genes" group contains genes of GSH metabolism, Cys and Met biosynthesis, the transsulfuration pathway, Met cycle and the Met salvage pathway, according to the KEGG Pathway Database (https://www.genome.jp/kegg/pathway.html) (Table S4: Dataset S1).

"Secondary metabolite cluster genes" are manually or experimentally identified secondary metabolite cluster genes according to Inglis et al. (2013). In this case, gene set enrichment analysis was carried out separately with each cluster (Table S4: Dataset S2).

"Transcription factor genes" are genes encoding known or putative transcription factors, according to the ShiniGo platform (Table S4: Dataset S3).

The "Antioxidant enzyme genes (without GSH metabolism)" group contains known or putative superoxide dismutase, catalase, peroxidase genes and elements of the glutaredoxin/thioredoxin redox system, according to Gila et al. (2022) (Table S4: Dataset S4).

"Autophagy-related genes" are genes encoded "autophagy proteins" or "authophagy-related proteins", according to Gila et al. (2022) (Table S4: Dataset S4).

"Glycolysis genes" are genes described by Flipphi et al. (2009) (Table S4: Dataset S4).

The "Lactose permease-β-galactosidase genes" group contains known and putative β-galactosidase and lactose permease genes, according to Fekete et al. (2012, 2016) and the ShiniGo platform (Table S4: Dataset S4).

"Nitrate utilization" genes include *niaD* (nitrate reductase), *niiA* (nitrite reductase), *nrtB* and *crnA* (nitrate transporter) and *fhbA* (nitrogen monoxide detoxification flavoprotein) genes (Martins et al., 2014) (Table S4: Dataset S4).

"Lipase genes" contain genes encoding known and putative "lipases", "triacylglycerol lipases" or "triglyceride lipases", collected from the FungiFun web page (https://elbe.hki-jena.de/fungifun/) (Table S4: Dataset S4).

"Heme-binding protein genes" were collected from the FungiFun web page (https://elbe.hki-jena.de/fungifun/) (Table S4: Dataset S4).

3. Results

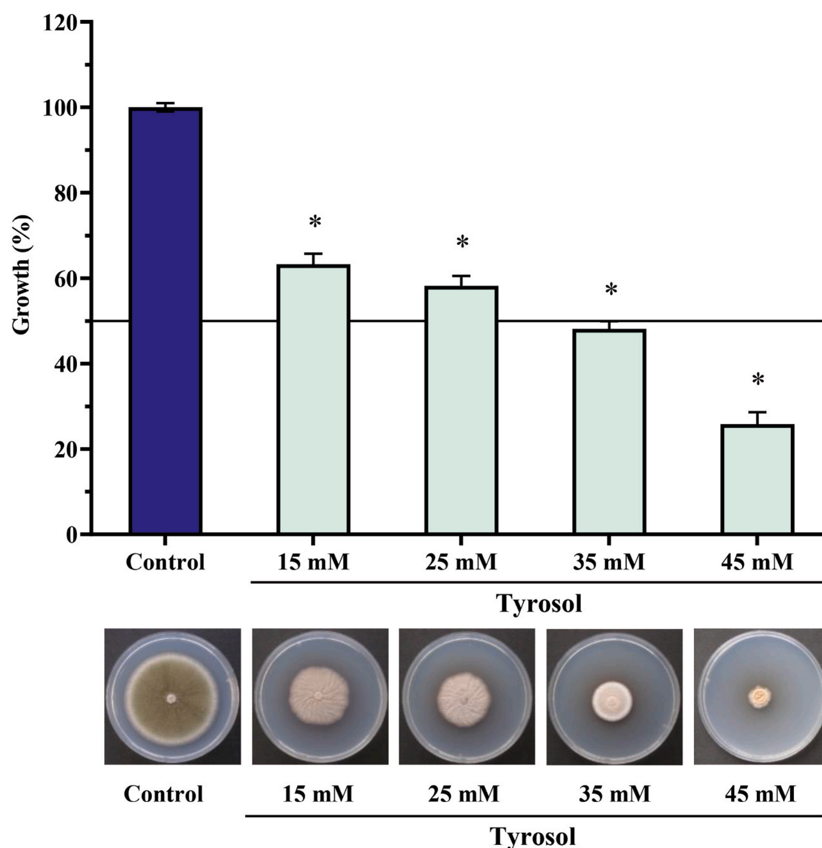
3.1. Tyrosol reduced growth, glucose consumption, the germination of conidia but did not cause oxidative stress in A. nidulans

The growth of *A. nidulans* FGSC A4 was examined on agar plates containing 15–45 mM tyrosol. Tyrosol significantly reduced the growth in a concentration-dependent manner (Fig. 1A and S1). The IC_{50} value (concentration reducing the colony diameter by 50 %) was slightly below 35 mM at 37 °C (Fig. 1A) and was between 35 mM and 45 mM at 24 °C (Fig. S1). Tyrosol, at 35 mM concentration, efficiently inhibited the growth when glucose, used as sole carbon source in the above-mentioned experiments, was replaced with casein peptone (Fig. 1B), lactose, or arabinogalactan (Fig. S2). The glucose antimetabolite 2DG completely inhibited the growth on casein peptone (Fig. 1B). Tyrosol treatment however could decrease the inhibitory effect of 2DG resulting in formation of micro-colonies (Fig. 1B). In contrast, no growth was observed on lactose or arabinogalactan when the media contained 2DG irrespectively of the presence or absence of tyrosol (Fig. S2).

The addition of tyrosol at a final concentration of 35 mM to exponentially growing submerged cultures significantly reduced both the growth (increase in the DCM) and glucose consumption after a 10-h-long incubation (Fig. 2A and B). Interestingly, when sunflower oil was used as sole carbon source instead of glucose, no growth was recorded in the presence of tyrosol (Fig. 2C). The growth of glucose containing cultures treated with tyrosol then transferred into tyrosol free fresh medium, did not differ significantly from the growth of untreated cultures (Fig. 2D), suggesting that the effect of tyrosol was rather fungistatic than fungicide.

Tyrosol significantly hindered the germination of conidia as well. Only 3.8 ± 0.2 % of conidia had germination tube after a 5 h incubation in the presence of 35 mM tyrosol. This value was 89 ± 0.2 % when untreated conidia were investigated. (The two mean values differed significantly according to the Student's t-test; $p < 0.05$, n = 3.) Thus, the reduced colony diameters observed in agar plate experiments were most likely the consequence of both the hindered germination and growth reduction of hyphae caused by tyrosol. Tyrosol treatment did not result in an increased redox imbalance according to the DCF-test and increased the GSH content of mycelia, reduced the GSSG levels (Fig. 3A).

A



B

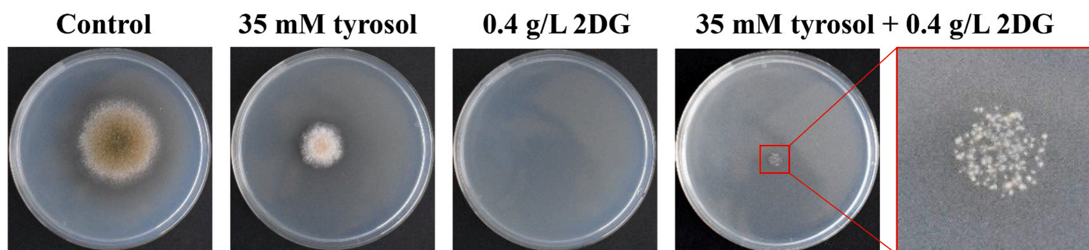


Fig. 1. Effect of tyrosol and/or 2-deoxy-D-glucose on the growth of *A. nidulans*.
Part A: Growth of *A. nidulans* FGSC A4 was examined on agar plates containing 15–45 mM tyrosol. *A. nidulans* FGSC A4 was point inoculated on Barratt's minimal agar plates and were incubated at 37 °C for 5 days. Representative photos are presented. Part B: Effect of tyrosol and 2-deoxy-D-glucose (2DG) on the growth of *A. nidulans* FGSC A4 on casein peptone as sole carbon source. *A. nidulans* FGSC A4 was point inoculated on Barratt's minimal agar plates and were incubated at 24 °C for 7 days. Representative photos are presented. The diameter of Petri-dishes was 85 mm. In the case of untreated cultures, the colony diameters were 44 ± 1 mm (n = 4), while in the case of the tyrosol treated cultures they were 20 ± 1 mm (n = 4).Mean ± SD values calculated from three biological replicates are presented. * - Significant difference between the treated and untreated cultures (Student's t-test, n = 3; $p < 0.05$).

Concurring with these observation, the specific activities of GR and catalase as antioxidant enzymes did not change; however, the specific GST activities were significantly elevated in the tyrosol-treated cultures (Fig. 3B). Degradation rate of tyrosol was weak; only 20 % of the starting value (35 mM) was decayed by the cultures after a 140 h long incubation (Fig. 3C). Deletion of the *phacA* gene encoding a cytochrome P450 dependent monooxygenase responsible for oxidation of the phenol moiety in phenylacetic acid (Mingot et al., 1999; Ferrer-Sevillano and Fernández-Cañón 2007) did not alter tyrosol susceptibility of the strain (Fig. S1).

3.2. Transcriptomic consequences of tyrosol treatment

To deeper understand the physiological consequences of tyrosol treatment for *A. nidulans,* the transcriptomes of tyrosol-treated and untreated cultures were also determined (Fig. 4, Table S1). Principal components analysis demonstrated that tyrosol treatment had a substantial effect on the transcriptome (Table S1), 2250 were upregulated ($\log_2$FC > 1) and 2199 were downregulated ($\log_2$FC < −1) in the tyrosol-exposed samples compared to the untreated controls (Fig. 4, Table S3). In the case of the 14 genes selected for RT-qPCR (Table S1), the transcriptional activities determined with RT-qPCR showed good correlation

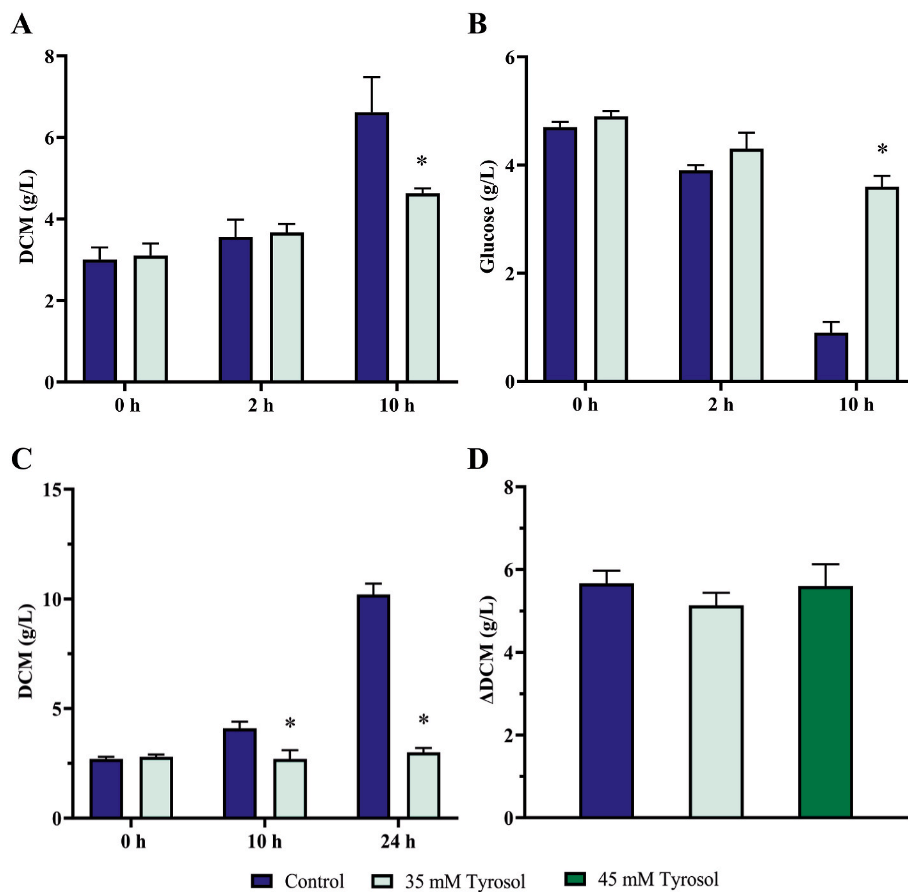


Fig. 2. Growth reduction and glucose consumption in the presence of tyrosol.
Parts A and B: Exponentially growing submerged cultures were treated with 35 mM tyrosol, and samples were taken at treatment (0 h) as well as 2 and 10 h later. Part C: Mycelia from the 42 h cultures were transferred into fresh media containing 5 v/v % sunflower oil with 35 mM tyrosol and samples were taken 0, 10 and 24 h later. Part D: Tyrosol treated and untreated cultures were transferred into tyrosol free medium and the increase in their DCM was recorded 18 h later. Mean ± SD values calculated from three biological replicates are presented. * - Significant difference between the treated and untreated cultures (Student's t-test, n = 3; $p < 0.05$).

with the RNAseq data (Pearson's correlation coefficient: 0.94) (Table S1).

The composition of up- and downregulated gene sets was characterised with gene set enrichment analyses (Figs. 5–7, Tables S3–S4). According to these data, tyrosol treatment downregulated genes related to vegetative growth, such as mitotic cell cycle, replication and translation genes (Fig. 5, Table S3). Downregulation of glycolysis, nitrate and sulphate assimilation, chitin biosynthesis and endoplasmic reticulum functions (such as phospholipid, ergosterol and glycoprotein synthesis) (Figs. 5 and 6, Tables S3–S4) was also most likely the consequence of the downregulated vegetative growth. These data concur well with the observed reduction of growth and glucose consumption in tyrosol-treated cultures (Fig. 2). Tyrosol substantially affected the metabolism of the fungus: *farA* and *farB* (transcription factor genes involved in the regulation of fatty acid use, peroxisomal functions and extracellular cutinase and lipase production; Hynes et al., 2006; Martins et al., 2014), five lipase genes as well as "Fatty acid catabolic process" and "Peroxisomal transport" genes were upregulated (Fig. 5, Tables S3–S4). Amino acid biosynthesis and catabolism genes together with "Regulation of nitrogen utilization" genes and *areA* (encoding a GATA-type transcription factor mediating nitrogen metabolite repression; Wilson and Arst 1998) were also upregulated, whereas *areB* (negative regulator of nitrogen catabolism; Wong et al., 2008) was downregulated (Fig. 5, Tables S3–S4). The *creA* gene (encoding the main carbon catabolite repressor of *A. nidulans*; Rise et al., 2016) was upregulated, and the "Carbon catabolite activation of transcription" GO term as well as the "Lactose permease-β-galactosidase" gene group were also among the upregulated gene sets (Figs. 5 and 6, Tables S3–S4). Regarding secondary metabolism, tyrosol treatment upregulated seven gene clusters (including the Aspercryptin cluster) and downregulated six another clusters (including the Emericellamide cluster) (Fig. 7, Table S4). Among the processes upregulated by tyrosol treatment, autophagy as well as glutathione metabolism (including GST genes) and metabolism of phenolic compounds (e.g., *phacA* and *phacB* genes involved in the conversion of phenylacetate) are highlighted (Figs. 5 and 6, Tables S3–S4; Mingot et al., 1999; Ferrer-Sevillano and Fernández-Cañón 2007). Bulk upregulation of genes encoding antioxidative enzymes was not observed (Fig. 6 and Table S4), whereas *napA* was upregulated and *atfA* was downregulated (Table S4). Both *napA* and *atfA* encode transcription factors important in the regulation of oxidative stress response and carbon metabolism (Mendoza-Martínez et al., 2017; Kocsis et al., 2023).

4. Discussion

A. nidulans is responsible for nearly 25 % of all fungal infections in patients with chronic granulomatous disease. The mortality rate of these infections ranges from 27 % to 32 %, and compared to *A. fumigatus*, this species has a higher virulence, invasiveness, dissemination and resistance to traditional antifungal treatments (Henriet et al., 2012, 2013).

Innovative treatment strategies interfering with quorum-sensing have become an intensely researched area in the past decade (Defoirdt, 2018; Zhao et al., 2020; Wu et al., 2020; Qin et al., 2022). Most of the published results focus on the anti-*Candida* effect of

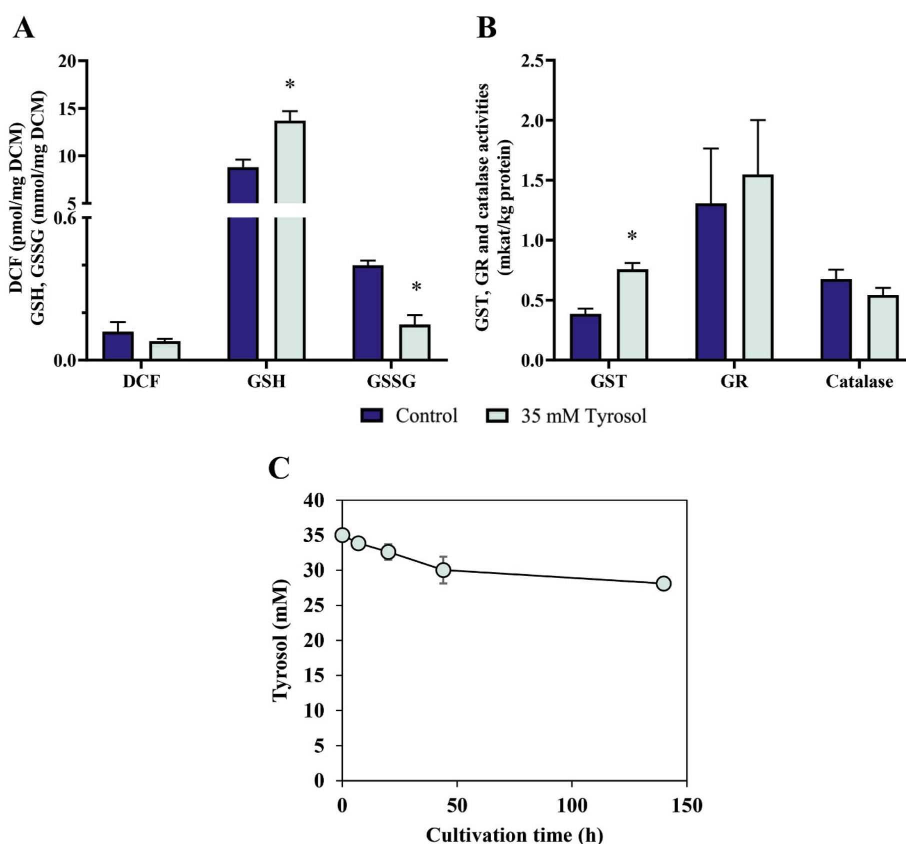


Fig. 3. Tyrosol consumption and physiological consequences of tyrosol treatment.
Part A: Effect of tyrosol treatment on redox homeostasis (DCF) and glutathione metabolism (GSH, GSSG). Part B: Changes in the specific GST, GR and catalase activities in the presence of tyrosol.Exponentially growing cultures were treated with 35-mM tyrosol, and samples were taken at 2 h after treatment. Mean ± SD values calculated from three biological replicates are presented. Part C: Changes in the extracellular tyrosol concentration. * - Significant difference between the treated and untreated cultures (Student's t-test, n = 3; $p < 0.05$).

quorum-sensing molecules, especially against *C. albicans* (Dižová and Bujdáková, 2017; Kovács and Majoros 2020; Rodrigues and Černáková 2020). The antifungal effect of yeast quorum-sensing molecules against filamentous fungi, including *Fusarium graminearum* (Semighini et al., 2008), *A. niger* (Lorek et al., 2008), *Paracoccidioides brasiliensis* (Derengowski et al., 2009), *Penicillium expansum* (Liu et al., 2010), *A. nidulans* (Wartenberg et al., 2012), *A. flavus* (Wang et al., 2014), *Scedosporium boydii, Lomentospora prolificans* (Pumeesat et al., 2017) and *A. fumigatus* (Wongsuk and Sukphopetch 2019; Oiki et al., 2022), is also well documented. These studies, however, are limited mainly to the effect of farnesol. Since tyrosol itself and in combination with farnesol, chlorhexidine gluconate, amphotericin B, azoles, and echinocandins has also good antifungal properties on *Candida* species (Chen and Fink 2006; Cordeiro et al., 2015; Arias et al., 2016; Monteiro et al., 2017; Kovács et al., 2017; do Vale et al., 2017) and even on *A. fumigatus* (Wongsuk and Sukphopetch 2019), here, we focused on the consequences of tyrosol exposure for *A. nidulans* cultures.

Tyrosol significantly inhibited the germination of conidia and the growth of *A. nidulans* cultures in the concentration range of 15–45 mM irrespectively of the applied carbon source. This result is comparable to those found with *A. fumigatus*, where 10 mM tyrosol caused a remarkable decrease in the growth of surface cultures (Wongsuk and Sukphopetch 2019). The reported minimal inhibitory concentration (MIC) values against planktonic cultures were 2.5–90 mM, >32 mM, and >45 mM for *Candida* species (Arias et al., 2016; do Vale et al., 2017; Monteiro et al., 2017), *A. fumigatus* (Wongsuk and Sukphopetch 2019) and *A. nidulans*, respectively (Fig. 1A). Focusing on sessile cells, the activity of tyrosol was limited compared to that on the planktonic counterpart of filamentous fungi. In the case of *A. fumigatus*, only 128 mM tyrosol could significantly reduce the metabolic activity of the biofilm, while for *Candida* species, the effective concentration is usually above 100 mM (Cordeiro et al., 2015; Arias et al., 2016). Regarding the tyrosol-related cytotoxic effect, only initial cell damage was demonstrated for human gingival fibroblasts (>10 mM), human gingival epithelial cells (3 mM), human salivary gland carcinoma cells (5 mM) and the colon adenocarcinoma cell line (>15 mM) (Babich and Visioli 2003; Jakab et al., 2019). Despite of its potential cytotoxic effect, Jakab et al. (2019) showed that daily tyrosol treatment (15 mM) decreased the *C. parapsilosis* burden in the kidneys of immunosuppressed mice without histological malformations (Jakab et al., 2019). Regarding other fungal quorum-sensing molecules, farnesol, phenylethanol and tryptophol (Wongsuk et al., 2016) proved to be effective against *A. fumigatus* at lower concentrations compared with tyrosol. Farnesol, phenylethanol and tryptophol could significantly reduce the growth of surface cultures at concentrations of 0.1, 10 and 1 mM, respectively, or the metabolic activity of biofilms at 8, 32, and 4 mM, respectively (Wongsuk and Sukphopetch 2019). Nevertheless, the *in vivo* efficacy of these compounds remains questionable. Farnesol has a protective effect against *C. albicans* and *C. auris* in oral and systemic mouse models (Hisajima et al., 2008; Martins et al., 2012; Nagy et al., 2020b); in addition; it caused fluconazole resistance reversion in *C. albicans* in a murine vulvovaginitis model (Bozó et al., 2016). Farnesol inhibited the release of the Th_1 cytokine interleukin-2 and gamma interferon in a mouse model (Navarathna et al., 2007), and by inducing oxidative stress, it suppressed the anti-*Candida* activity of murine macrophages (Abe et al., 2009).

A. nidulans was able to degrade tyrosol, even though the degradation rate was slow (Fig. 3C). Upregulation of GST-encoding genes (Figs. 5–6, Tables S3–S4), the increased specific GST activities (Fig. 3B) as well as

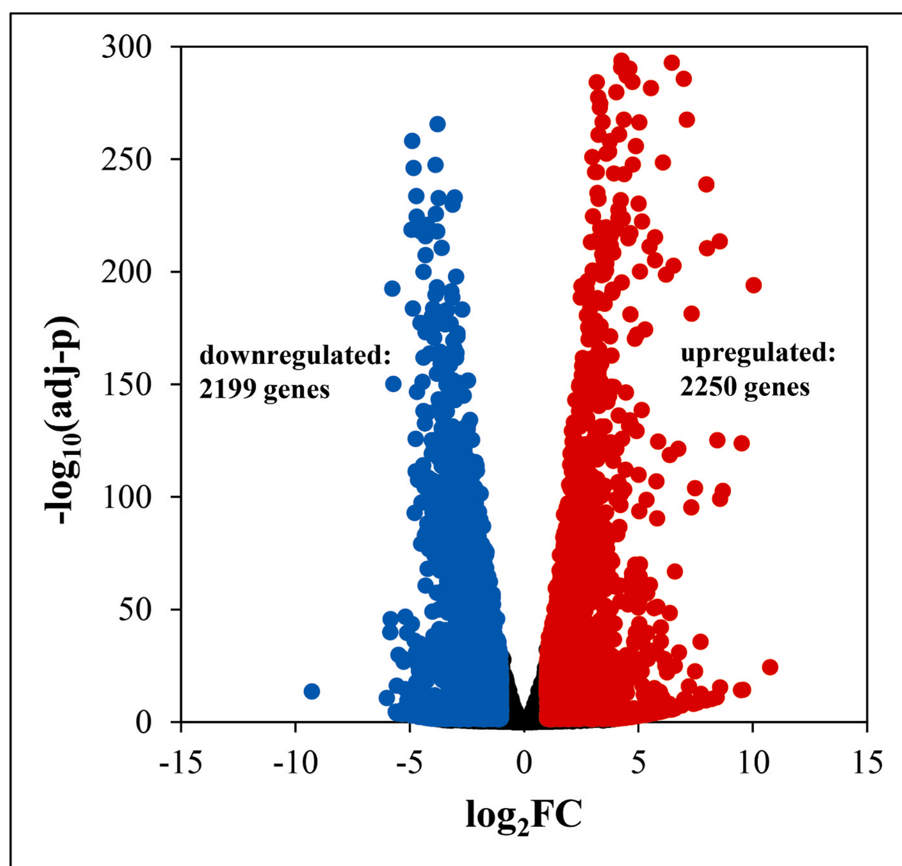
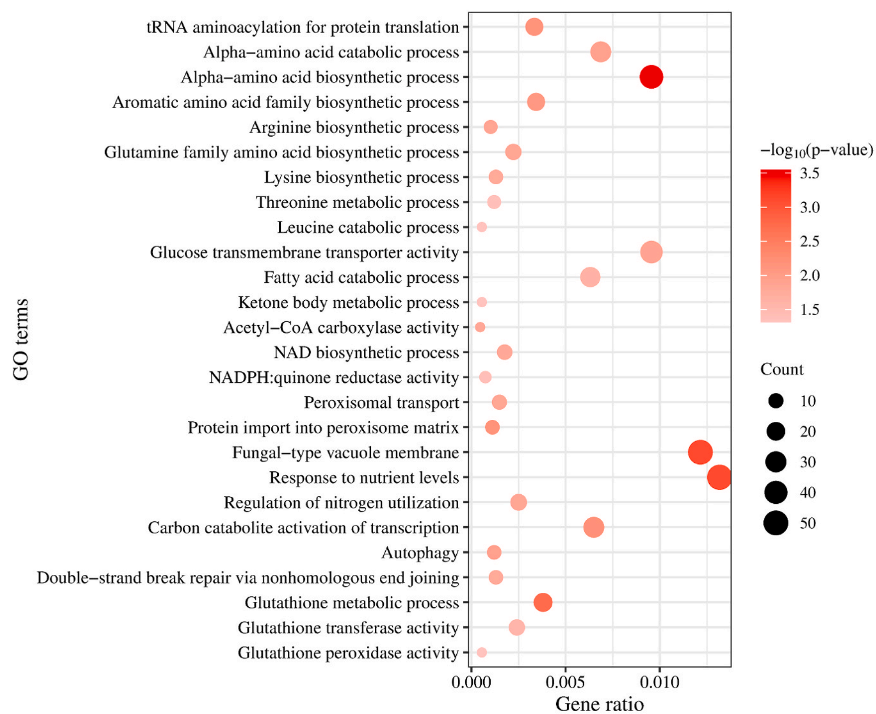


Fig. 4. Vulcano plot of the RNAseq data. Volcano plot showing statistical significance (log_{10}(adj. *p*) versus fold change (log_2FC) of RNAseq data from tyrosol treated vs control comparison. Genes where the -log10 (adj. *p*) > 300 are not shown for clarity.

the elevated GSH content of the cells (Fig. 3A) suggest that tyrosol was detoxified in a GSH-dependent mechanism. It is noteworthy that phenyl- and phenoxyacetic acids and their derivatives (e.g., 2-hydroxy-phenylacetic acid, 3-hydroxy-phenylacetic acid and 2-hydroxy-phenoxyacetic acid), which have a chemical structure similar to that of tyrosol, also induced specific GST activities, reduced the vegetative growth, increased GSH production and could even deplete the GSH pools in *Penicillium chrysogenum* at various (18–66 mM) concentrations (Emri et al. 1997a, 1997b, 2001, 2003). Nevertheless, 4-hydroxy derivatives, which harbour the hydroxyl-group in the same position as tyrosol, were unable to decrease intracellular GSH concentrations. Oxidation of the aromatic ring by monooxygenases may liberate reactive epoxide derivatives, which are subject of GST-mediated detoxification (Emri et al., 2003). Formation of such compounds from 4-hydroxy derivatives can be slow, which may explain why these compounds were unable to decrease GSH pools (Emri et al., 2001, 2003, Fig. 3A). Tyrosol can be oxidised to 4-hydroxy-phenylacetic acid in *Halomonas* spp., *Pseudomonas aeruginosa*, *Serratia marrescens*, *Pseudomonas putida*, *Arthrobacter* spp. and *Bacillus* spp. (Liebgott et al., 2008). On the other hand, phenylacetic acid can be oxidized to 2-hydroxy derivative by PhacA and, to a lesser extent, by PhacB monooxygenases allowing its degradation *via* the homogentisate pathway (Ferrer-Sevillano and Fernández-Cañón 2007). Both *phacA* and *phacB* were upregulated in our experiments (Table S3) suggesting that this pathway may contribute to the degradation of tyrosol as well. However, deletion of *phacA* did not alter tyrosol susceptibility of the strain (Fig. S1). It can be the consequence of either the low degradation rate of tyrosol (Fig. 3C) or that PhacA is not involved in the degradation of this hydroxyphenolic compound.

The mode of action of tyrosol remains to be elucidated. Our data demonstrate that tyrosol was rather fungistatic than fungicide in *A. nidulans* (Fig. 2D). It did not induce oxidative stress (Fig. 3A); however, such effect was previously observed in *C. parapsilosis* (Jakab et al., 2019). These findings demonstrate that eliciting oxidative stress can be important but not essential for the antifungal activity of tyrosol. The observed growth inhibitory effect of tyrosol on *A. nidulans* was accompanied with the downregulation of several genes related to vegetative growth (e.g., mitotic cell cycle, replication, translation, glycolysis, nitrate and sulphate assimilation and chitin, phospholipid and ergosterol biosynthesis genes) (Figs. 5 and 6, Tables S3–S4). Therefore, tyrosol may have disturbed some of these processes, leading to growth reduction.

Tyrosol occurs in plant oils primarily as fatty acid esters (Madureira et al., 2022). It is reasonable to assume that *A. nidulans* uses tyrosol liberated by secreted esterases as a signal molecule to activate the use of plant oils. It could explain why *farA* and *farB*, as well as further genes involved in triacylglycerol and fatty acid utilization and peroxisomal functions, were upregulated after tyrosol treatment (Fig. 5, Tables S3–S4). Adjusting the metabolism for plant oil use (in the absence of oils) may have disturbed glucose use and, consequently, impaired growth. However, our growth test on sunflower oil did not support this hypothesis, as tyrosol completely inhibited growth on this carbon source (Fig. 2C). Thus, the global transcriptional changes elicited by tyrosol were not beneficial and were even deleterious for the utilization of this plant oil, at least at this high concentration. Tyrosol reduced growth on casein peptone, lactose and arabinogalactan as well (Fig. 1B, S2) and prevented the glucose antimetabolite 2DG to inhibit growth completely on casein peptone (Fig. 1B). Genes involved in amino acid degradation or lactose utilization were also upregulated by tyrosol treatment on glucose (Figs. 5–6, Tables S3–S4). Moreover, upregulation of "response to nutrient levels", "regulation of nitrogen utilization" and "carbon catabolite activation of transcription" genes, including important transcription factor genes involved in the regulation of C and N metabolism,



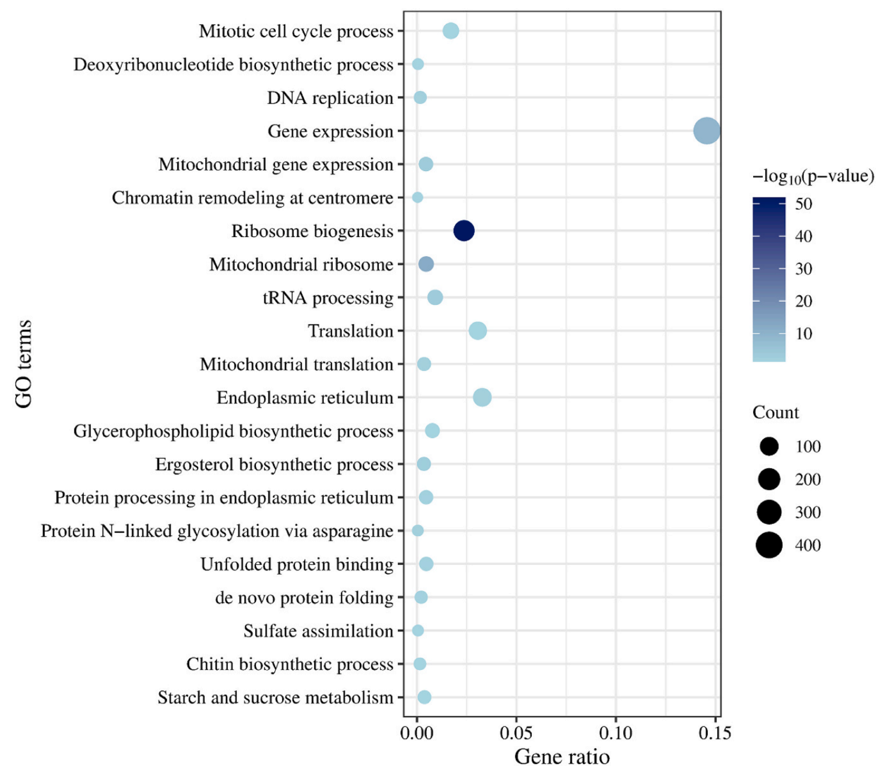


Fig. 5. Selected significantly enriched GO terms. Bubble charts represent up (A)- and down (B)-regulated genes belonging to gene groups tyrosol treated versus untreated comparison where the enrichment was significant ($p < 0.05$). The color of bubble means the significance of the corresponding GO pathway (in light color, low p values; in dark color, high p values). As well, the size of bubble means the number of different expression genes in this pathway. Only the differentially expressed genes (adj. p value of <0.05) exhibiting more than 2.0-fold increase or decrease in their transcription are shown. The full list of the significantly enriched GO terms is available in Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

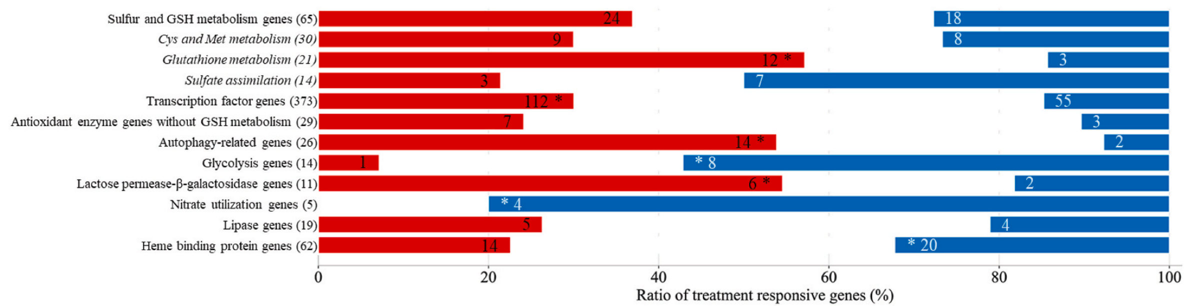


Fig. 6. Transcriptional behaviour of selected gene groups based on RNAseq data.The percentage of upregulated and downregulated genes is shown in red and blue. The full dataset is available in Table S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

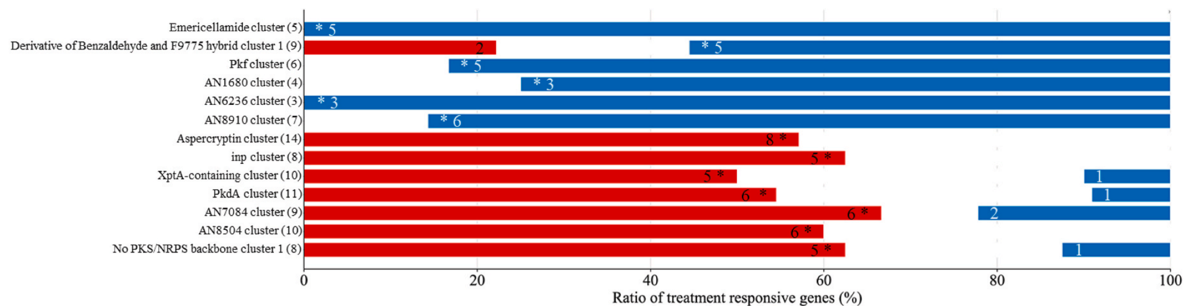


Fig. 7. Transcriptional behaviour of selected secondary metabolite clusters based on RNAseq data. Blue and red bars represent the number of genes in each category upregulated and downregulated, respectively. The full dataset is available in Table S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

such as *creA*, *areA*, *areB* and *napA* (Wilson and Arst 1998; Wong et al., 2008; Ries et al., 2016; Mendoza-Martínez et al., 2017) were also observed (Fig. 5, Tables S3–S4). These transcriptional changes suggest that tyrosol may have disturbed the regulation and orchestration of the cellular metabolism, leading to an impaired use of the nutrients presented in the medium and, hence, reduced growth. Upregulation of autophagy-related genes is common during stress responses to nutrient limitation and starvation in *A. nidulans* (Gila et al., 2022). Enrichment of these genes in the gene set upregulated by tyrosol (Figs. 5–6, Tables S3–S4) also suggests that tyrosol treatment impaired nutrient use. Nevertheless, this mode of action is consistent with the observed fungistatic nature of growth inhibition (Fig. 2D). Further studies are needed to confirm this hypothesis and to clarify whether tyrosol was harmful because it was structurally similar to a regulatory compound of *A. nidulans* or because it is a regulatory compound of this fungus and was only harmful at the supraphysiological concentration applied.

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Data availability

Supplementary material presents Supplementary Fig. S1: Effect of tyrosol on the growth of *A. nidulans* FGSC A4, MAD329 (ΔphacA gene deletion mutant), and MAD817 (reference strain for the mutant) strains. Supplementary Fig. S2: Effect of tyrosol and 2-deoxy-D-glucose on the growth of *A. nidulans* FGSC A4 on lactose and on arabinogalactan. Supplementary Table S1: Overview on RNAseq and RT-qPCR assays. Supplementary Table S2: Oligonucleotide primers used in RT-qPCR assays. Supplementary Table S3: Results of the differential gene expression and gene set enrichment analyses. Supplementary Table S4: RNAseq based transcription data of selected gene groups. Original RNAseq data obtained in this work (original fastq files, normalized count and RPKM values) have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE223010 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE223010). All other data necessary for confirming the conclusions of the article are present within the article, figures and tables.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.01.003.

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