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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Members of the nitronate monooxygenase gene family from *Metarhizium brunneum* are induced during the process of infection to *Plutella xylostella*

Karla Yadira Cervantes Quintero[1] · Israel Enrique Padilla Guerrero[1] · Juan Carlos Torres Guzmán[1] · Beatriz Guadalupe Villa Martínez[1] · Adelissa Valencia Félix[1] · Gloria Angélica González Hernández[1]

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Abstract

Metarhizium species are the most abundant fungi that can be isolated from soil, with a well-known biopesticide capacity. *Metarhizium* recognizes their hosts when the conidium interacts with insects, where the fungi are in contact with the hydrocarbons of the outermost lipid layer cuticle. These cuticular hydrocarbons comprise a mixture of n-alkanes, n-alkenes, and methyl-branched chains. *Metarhizium* can degrade insect hydrocarbons and use these hydrocarbons for energy production and the biosynthesis of cellular components. The metabolism of nitroalkanes involves nitronate monooxygenase activity. In this work, we isolated a family of six genes with potential nitronate monooxygenase activity from *Metarhizium brunneum*. The six genes were expressed in *Escherichia coli*, and the nitronate monooxygenase activity was verified in the recombinant proteins. Additionally, when the conidia of *M. brunneum* were grown in medium with nitroalkanes, virulence against *Plutella xylostella* increased. Furthermore, we analyzed the expression of the six *Npd* genes during the infection to this insect, which showed differential expression of the six *Npd* genes during infection.

Keywords Biopesticide · *Metarhizium* · Nitroalkanes · Nitronate monooxygenase

Introduction

Nitroalkanes are toxic compounds that can be used as solvents, fuels for rockets, and explosives (Gadda and Fitzpatrick 1999). In nature, toxic nitro-compounds, such as nitroalkanes, are synthesized by some organisms. For example, 3-nitropropionate is produced by the fungi *Penicillium atrovenetum* and *Aspergillus flavus* (Porter and Bright 1987) and some leguminous plants such as *Astragalus miser* and *Hippocrepis comosa* (Salem et al. 1995). Chomcheon et al. (2005) reported that many legumes accumulate nitro toxins such as 3-nitro-1-propionic acid and may be produced by fungal endophyte association, such as the genus *Phomosis*. These nitroalkanes could be a plant defense mechanism against pathogens. This observation suggests that many organisms face toxic compounds, such as nitroalkanes, and depend on their ability to metabolize these compounds to survive. Presently, different enzymes from plants, fungi, and bacteria that can convert nitroalkanes to less harmful products have been purified and characterized (Gadda and Francis 2010): the 2-nitropropane dioxygenase, now named nitronate monooxygenase (NMO) (EC 1.13.12.16), from *Hansenula mrakii* (Kido et al. 1976) and *Neurospora crassa* (Gorlatova et al. 1998); the 3-nitropropionate oxidase from *P. atrovenetum* (Porter and Bright 1987) and *H. comosa* (Hipkin et al. 1999); the nitroalkane oxidase (NAO) (E.C. 1.7.3.1) from *Fusarium oxysporum* (Kido et al. 1978), *Podospora anserina* (Tormos et al. 2010), *Streptomyces ansochromogenes* (Zhang et al. 2002), and *Pseudomonas aeruginosa* (Lee et al. 2013), among others. Nitroalkane oxidase is a flavoprotein that catalyzes the oxidation of neutral nitroalkanes to their corresponding aldehyde or ketone, releasing nitrite and transferring electrons to O_2 to form H_2O_2 (Fitzpatrick 2017). Nitronate monooxygenase is a flavoprotein enzyme that catalyzes the oxidative denitrification of

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✉ Gloria Angélica González Hernández
gonzang@ugto.mx

[1] Biology Department, Division of Natural and Exact Sciences, University of Guanajuato, 36000 Guanajuato, Mexico

nitroalkanes to their corresponding carbonyl compounds and nitrites (Gorlatova et al. 1998; Kido et al. 1984). NAO and NMO have differences, and their enzymatic mechanisms are distinct; additionally, NAO exclusively oxidizes neutral nitroalkanes, whereas NMO can oxidize anionic alkyl nitronates (Gadda and Francis 2010). Nitroalkane-oxidizing enzymes can convert nitroalkanes physiologically to obtain the nitrogen and carbon required for growth. In *S. ansochromogenes*, the nitroalkane oxidase NaoA is involved not only in the conversion of toxic nitroalkanes to less harmful compounds but also in growth (Li et al. 2008). In *Ralstonia solanacearum*, a putative nitropropane dioxygenase is involved in virulence (Zhang et al. 2017); in the same sense, in *Magnaporthe oryzae*, which contains five nitronate monooxygenase genes, only the *NMO2* gene is essential for mitigating nitro-oxidative cellular damage and, in rice cells, maintaining a redox balance to avoid triggering plant defenses that impact *M. oryzae* growth (Marroquin-Guzman et al. 2017).

Metarhizium species are the most abundant fungi that can be isolated from soils (Lomer et al. 2001). In addition, *Metarhizium* spp. are well known as biopesticides by their entomopathogenic lifestyle (Roberts and St Leger 2004). *Metarhizium* recognizes their hosts when the conidium of the fungus contacts the cuticle of the insect. Later, the conidium germinates on the cuticle and penetrates this barrier of defense to reach the hemocoel where it faces the defense responses of the insects (immune response and toxins) and spreads, invading the tissues of the host and causing death (Arruda et al. 2005). In the early invasion stage of the host, the fungi are in contact with the hydrocarbons of the outermost lipid layer of the cuticle. These cuticular hydrocarbons comprise a mixture of n-alkanes, n-alkenes, and methyl-branched chains in more than 100 insect species (Pedrini et al. 2007). The entomopathogenic fungi *Metarhizium* and *Beauveria* can degrade insect hydrocarbons for utilization for energy production and the biosynthesis of cellular components (Huarte-Bonnet et al. 2015).

In entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, there is a relationship between the ability to catabolize very long-chain hydrocarbons and virulence parameters (Pedrini et al. 2007). The metabolism of nitroalkanes involves the nitronate monooxygenase enzyme. To investigate whether the *Npd* genes, putatively encoding this enzymatic activity, are expressed during the entomopathogenic lifestyle of *Metarhizium brunneum*, and potentially involved in the degradation or metabolism of hydrocarbons from the host cuticle, in this work: we isolated a family of six genes with potential nitronate monooxygenase activity in *M. brunneum*. The six genes were expressed in *Escherichia coli*, and the nitronate monooxygenase activity of the recombinant proteins verified. We evaluated the ability of *M. brunneum* to grow in nitroalkanes and the virulence of the newly formed conidia. *M. brunneum* conidia produced in the presence of nitroalkanes were more virulent than the conidia grown in the absence of nitroalkanes. Additionally, we measured the expression of the six *Npd* genes during the *M. brunneum* infection to *Plutella xylostella*. The six *Npd* genes were expressed throughout the infectious cycle, with the *Npd1* gene having the highest expression.

Materials and methods

Strains and growth conditions

M. brunneum Ma10 (CNRCB MaPL10) was obtained from the entomopathogenic fungi collection from the "National Biological Control Reference Center" (WDCM 1034) and was originally isolated from *Geraeus senilis* (*Coleoptera*: *Curculionidae*) in the state of Colima, Mexico. The fungus was grown in minimal media (MM) consisting of 0.2% NH_4NO_3, 2% dextrose, 0.3% KH_2PO_4, and 2% 50× salt stock solution (25 g $MgS0_4$, 0.09 g $ZnSO_4$, 0.05 g $FeSO_4$, 0.015 g $MnSO_4$, and 0.02 g $CuSO_4$), or on Sabouraud dextrose media (SDM) to obtain the conidia or mycelium, respectively, as previously described (Morales Hernandez et al. 2010). To test the ability to grow on nitroalkanes, a drop of 5×10^4 conidia was inoculated in minimal medium (MM), supplemented with different concentrations (0.05%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%) of nitroethane, 1-nitropropane, or 2-nitropropane (catalog numbers: 130206, N22851, and 30265, respectively, from Sigma-Aldrich, Toluca, Estado de Mexico, Mexico). The samples were incubated at 28 °C for 3 days to follow growth or 14 days to measure conidiation.

To isolate the *Ncd-2* open reading frame (ORF) from *N. crassa*, strain 74-OR23-1A (FGSC#98) was employed, and the mycelium biomass was obtained by inoculating 5×10^4 conidia per milliliter in potato dextrose broth (PDB) medium and incubating at 30 °C for 48 h.

The *E. coli* DH5α strain (Invitrogen, Carlsbad, CA, USA) was used for DNA manipulations and transformations. It was maintained in Luria-Bertani medium at 37 °C (Sambrook and Russell 2001). Ampicillin (100 μg mL^{-1}) was added as required. *E. coli* BL21(DE3) pLysS (Invitrogen, Carlsbad, CA, USA) was utilized for the heterologous expression of the *Npd* cDNAs.

Nucleic acid isolation and *Npd* gene cloning

Genomic DNA was isolated from *M. brunneum* Ma10 mycelium grown for 24 h in SDM. DNA was extracted by friction following standard protocols (Sambrook and Russell 2001).

Total RNA was extracted from cells of *M. brunneum* Ma10 grown in SDM culture media or from *P. xylostella* larvae previously inoculated with conidia of *M. brunneum* Ma10

using TRIzol® Reagent (Invitrogen, Accesolab, Queretaro, Mexico) according to the manufacturer's instructions. The concentration and purity of both total RNA and DNA were determined by the ratio of the absorbencies at 260 and 280 nm. For cDNA synthesis, all samples were DNase-treated using RNase-Free DNase (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. Total RNA (1 μg) was used for cDNA synthesis, and RT-PCR was performed using the SuperScript® III One-Step RT-PCR System with the Platinum® *Taq* DNA Polymerase kit (Invitrogen, Accesolab, Queretaro, Mexico) according to the manufacturer's instructions.

To amplify the six *Npd* genes, primers for each gene were designed according to the sequence of the encoding *Npd* genes from *M. brunneum* ARSEF 3297 (KID79125, KID72326.1, KID73333.1, KID65375.1, KID78097.1, and KID61795.1) (Supplementary Table S1). The reaction mixture contained total DNA or cDNA, each primer, and PCR SuperMix High Fidelity (Invitrogen, Accesolab, Queretaro, Mexico). The amplification product was cloned into the pCR®2.1-TOPO® (Invitrogen, Accesolab, Queretaro, Mexico) or pGEM®-T Easy (Promega, Quimica Valaner, Cd. de Mexico, Mexico) vectors and sequenced. Both strands of the nucleotide sequence of the clone were generated using universal primers and specific primers based on the *Npd* genomic sequence deposited into the database (www.ncbi.nlm.nih.gov). The DNA sequence was analyzed using the Lasergene 14 program (DNASTAR Inc., Madison, WI, USA), and its identity was confirmed by comparison with published nitronate monooxygenase sequences in the database (www.ebi.ac.uk).

Neighbor-joining tree based on percentage of identity of the Npd enzymes

The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model (Jones et al. 1992). The tree with the highest log likelihood (− 8501.48) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.6233)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 1.44% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets (Kumar et al. 2016; www.megasoftware.net).

Expression of the *Npd* genes from *M. brunneum* in infected larvae (*P. xylostella*) by RT-PCR analysis

The in vivo expression analysis of the six *Npd* genes was performed according to the protocol previously described (Morales Hernandez et al. 2010). Third-instar *P. xylostella* larvae were sprayed with a conidial suspension (500 μL of 1.0×10^7 conidia mL^{-1}) at 10 lb plg^{-2}, in a Potter spray tower (Potter-Precision Laboratory Spray Tower, Burkard Scientific, Uxbridge, Middlesex, UK). The larvae were fed with a leaf disk of *Brassica oleracea var. italica* and incubated at 25 °C. Every 48 h, the leaf disk was changed. Different stages of infection were monitored over 9 days. The stages of infection were categorized as previously described (Morales Hernandez et al. 2010). At each time point during the observed infection, 10–40 larvae were collected and stored at − 70 °C until RNA extraction. Upon death, conidia from the infected insect cadavers were collected with sterile 0.1% Triton X-100 solution. From a parallel control experiment, larvae treated only with 0.1% Triton X-100 solution were collected during the experiment. RT-PCR analysis of the *Npd* genes and AJ274118 (loading control, XM_014687121, ubiquitin-conjugating enzyme) was performed to compare transcript abundance. RT-PCR was performed with specific primers (Supplementary Table S2) using the SuperScript™ III One-step RT-PCR System with the Platinum® *Taq* DNA Polymerase kit (Invitrogen, Accesolab, Queretaro, Mexico) according to the manufacturer's recommendations and as previously described (Morales Hernandez et al. 2010).

Expression of the *Npd* cDNA in *E. coli* cells

Expression of the *M. brunneum Npd* cDNAs in *E. coli* BL21(DE3) pLysS was accomplished by subcloning the *Npd* cDNA from each of the *Npd* genes into the pRSET A expression vector (Invitrogen, Accesolab, Queretaro, Mexico). The *Npd* cDNA was obtained from *M. brunneum* Ma10 amplified with the respective pair of primers from the cDNA of each one of the *Npd* genes (Supplementary Table S1) containing a *Bam*HI restriction site in the direct primers and *Eco*RI or *Hin*dIII restriction site in the reverse primers in a reaction mixture with PCR SuperMix High Fidelity (Invitrogen, Accesolab, Queretaro, Mexico). The amplification products previously purified were digested with both restriction enzymes *Bam*HI and *Eco*RI or *Bam*HI and *Hin*dIII and ligated to the *Bam*HI/*Eco*RI-digested pRSET A DNA plasmid or *Bam*HI and *Hin*dIII digested pRSET A DNA plasmid. The resulting recombinant plasmids were designed: pGG468 (*Npd1*), pGG469 (*Npd2*), pGG673 (*Npd3*), pGG674 (*Npd*4), pGG675 (*Npd5*), and pGG654 (*Npd6*), which carry the open reading frame of each of the *Npd* genes, and pGG658, which contains the *Ncd-2* from *N. crassa*. *E. coli* BL21(DE3) pLysS cells were transformed independently with

each plasmid, and colony selection occurred in LB medium supplemented with ampicillin 100 μg mL^{-1} and chloramphenicol 34 μg mL^{-1}. For recombinant protein expression and purification, bacterial cells were grown to an OD_{600} of 0.4 at 37 °C in LB liquid medium supplemented with 100 μg mL^{-1} ampicillin, 34 μg mL^{-1} chloramphenicol, and 0.1 mM IPTG for 4 h at 37 °C. Induced cells were collected by centrifugation (8000*g* for 5 min at 4 °C) and suspended in binding buffer (20 mM phosphate, 500 mM NaCl, 20 mM imidazole with 0.2 mg mL^{-1} lysozyme, and 20 μg mL^{-1} DNAse). The collected cells were lysed with a VCX 130 Vibra-Cell™ Ultrasonic Liquid Processor (CTR, Monterrey, NL, Mexico) at 130 W, 20 kHz, and amplitude of 95% over 1 min, repeating 5–6 times. Cell debris was separated by centrifugation (40,000*g* for 30 min at 4 °C), and the supernatant was passed through a 1-mL HisTrap FF column (G.E. Healthcare, Piscataway, NJ, USA) equilibrated with 50 mM phosphate buffer at pH 7.5 and 20 mM imidazole, according to the directions of the supplier. The proteins bound to the column were eluted with a gradient of 20 to 500 mM imidazole. The Npd proteins were detected at 300 mM imidazole. Fractions were stored at − 70 °C until use in nitronate monooxygenase enzymatic assays.

Enzymatic assays

Nitronate monooxygenase assays were performed according to the protocol previously described (Kido et al. 1984), with minor modifications as follows. The standard reaction mixture contained 20 mM nitroalkane (anionic form), 1.25 μL of 5 mM FMN (flavin mononucleotide), and 50 μL of the enzyme fraction, diluted to a final volume of 0.25 mL adding Britton-Robinson's buffer (pH 6.5). After incubation at 28 °C for 10 min, the reaction was stopped by the addition of 250 μL of 95% ethanol. Immediately, 50 μL aliquots of the reaction mixture were mixed with 100 μL of deionized water and used for the determination of nitrite with 50 μL of sulfanilamide and 50 μL of *N*-(l-naphthyl) ethylenediamine dihydrochloride. The reaction mixture was incubated at room temperature for 10 min in the dark. The optical density at 540 nm of the standard solutions and samples were measured in a microplate reader (Varioskan Flash™ Thermo Scientific™, Accesolab, Queretaro, Mexico). One unit of the enzyme was defined as the amount of enzyme required to produce 1 μmol of nitrite per minute. Specific activity was expressed as units per milligram of protein. Protein content was determined by the Lowry method (Lowry et al. 1951).

The Km values were calculated measuring the enzymatic activity with different concentrations of each substrate in the anionic form (1-nitropropane, 2.5, 5, 10, 20, 40, 60, 120, 140 mM; 2-nitropropane, 2.5, 5, 10, 20, 40, 60, 120 mM; nitroethane, 1.25, 2.5, 5, 10, 20, 40, 60 mM) fitting the experimental points to the Michaelis-Menten equation for one substrate.

Virulence assays

Virulence assays were performed according to the protocol previously described (Morales Hernandez et al. 2010). For each assay, ten third-instar *P. xylostella* larvae were sprayed with the LD_{90} (5×10^6 conidia) of *M. brunneum* Ma10 grown in minimal medium (MM) or MM supplemented with different concentrations (0.05%, 0.1%, 0.2%, 0.3%, 0.4%, or 0.5%) of nitroethane, 1-nitropropane, or 2-nitropropane. The larvae were fed with a leaf disk of *B. oleracea* var. *italica* and incubated at 25 °C. Every 48 h, the leaf disk was changed, and the survival was monitored every 12 h during the experiment. Three assays were performed with three replicates each. Statistical tests for survival analysis were performed using the log-rank test and the Gehan-Breslow-Wilcoxon test with the GraphPad Prism 8 software (version 8.3.0) (www.graphpad.com/scientific-software/prism).

Accession number of the *Npd* genes in the GenBank database

The accession numbers of the *Npd* genes from *M. brunneum* Ma10 are the following: *Npd1* (FR776002.1); *Npd2* (FR776003.1); *Npd3* (MN548143); *Npd4* (MN548144); *Npd5* (MN546869); and *Npd6* (MN548145).

Results

M. brunneum contains a nitronate monooxygenase gene family of six members

Analysis of the genome of *M. brunneum* ARSEF 3297 (Hu et al. 2014) revealed that it contains at least six genes with similarity to nitronate monooxygenase (nitropropane dioxygenase) genes. In this work, we isolated these six genes from the *M. brunneum* Ma10 strain. The genomic DNA and cDNA of each gene were amplified from *M. brunneum Ma*10 strain cells grown in the SDM medium using the six pairs of oligos described in Supplementary Table S1, cloned, and sequenced. The comparison between the genomic and cDNA sequences showed that the *Npd1* gene contains two introns (110 bp and 160 bp in length, respectively), and *Npd5* contains one intron (92 bp). The other genes, *Npd2*, *Npd3*, *Npd4*, and *Npd6*, do not contain introns. The Supplementary Table S3 shows the predicted amino acid number, molecular weight, and isoelectric point for each Npd protein. The DNA sequence of the *Npd1*, *Npd2*, and *Npd4* genes from the *M. brunneum Ma*10 strain is identical to the reported sequences from *M. brunneum* ARSEF 3297. The DNA

sequence of the *Npd3*, *Npd5*, and *Npd6* genes from the *M. brunneum Ma*10 strain has small differences with those from the genome sequence of *M. brunneum* ARSEF 3297 (Hu et al. 2014) (Supplementary Fig. S1).

The sequences of the predicted protein were compared in the databases of the complete genomes from different filamentous fungi, observing that all the fungi analyzed contain between 3 and 8 putative *Npd* genes in their genomes (Fig. 1). This set included some beneficial microorganisms with different lifestyles, such as *M. brunneum*, *M. anisopliae*, *Metarhizium robertsii*, *Metarhizium guizohuense*, *Metarhizium acridum*, *B. bassiana*, *Hypocrea jecorina* (anamorph *Trichoderma reesei*), *Trichoderma gamsii*, *Trichoderma harzianum*, and *Trichoderma guizohuense*. Some phytopathogenic fungi were included, such as *Colletotrichum graminicola* and *M. oryzae*, as well as the opportunistic fungus *Neosartorya fumigata* and the yeast *Williopsis saturnus*. In the analysis, an outside group was included, the protein sequence, PA1024, from the bacteria *P. aeruginosa*, an opportunistic pathogen (Ha et al. 2006).

In fungi, few nitronate monooxygenase activities have been described, in the saprophyte fungus *N. crassa* (Q01284) (Gorlatova et al. 1998) and in the phytopathogenic fungi *F. oxysporum* (Q8X1D8) (Gadda and Fitzpatrick 1999) and *M. oryzae* (MGG_02439) (Marroquin-Guzman et al. 2017). In the analyzed fungi, the putative and well-recognized nitronate monooxygenases are distributed in four main clades. Noting that the predicted proteins encoded by the six *Npd* genes of *M. brunneum* are highly conserved in the analyzed *Metarhizium* species (see Supplementary Table S4), and the two putative Npd (Npd2 and Npd3) proteins from *M. brunneum* are localized in the major clade. The second pair (Npd1 and Npd4) is localized together in a second clade. Npd1 is related to Npd4, and Npd2 is related to Npd3 with 72% identity in each pair (see Supplementary Table S5). Npd6 is related to the nitronate monooxygenase (Q01284) from

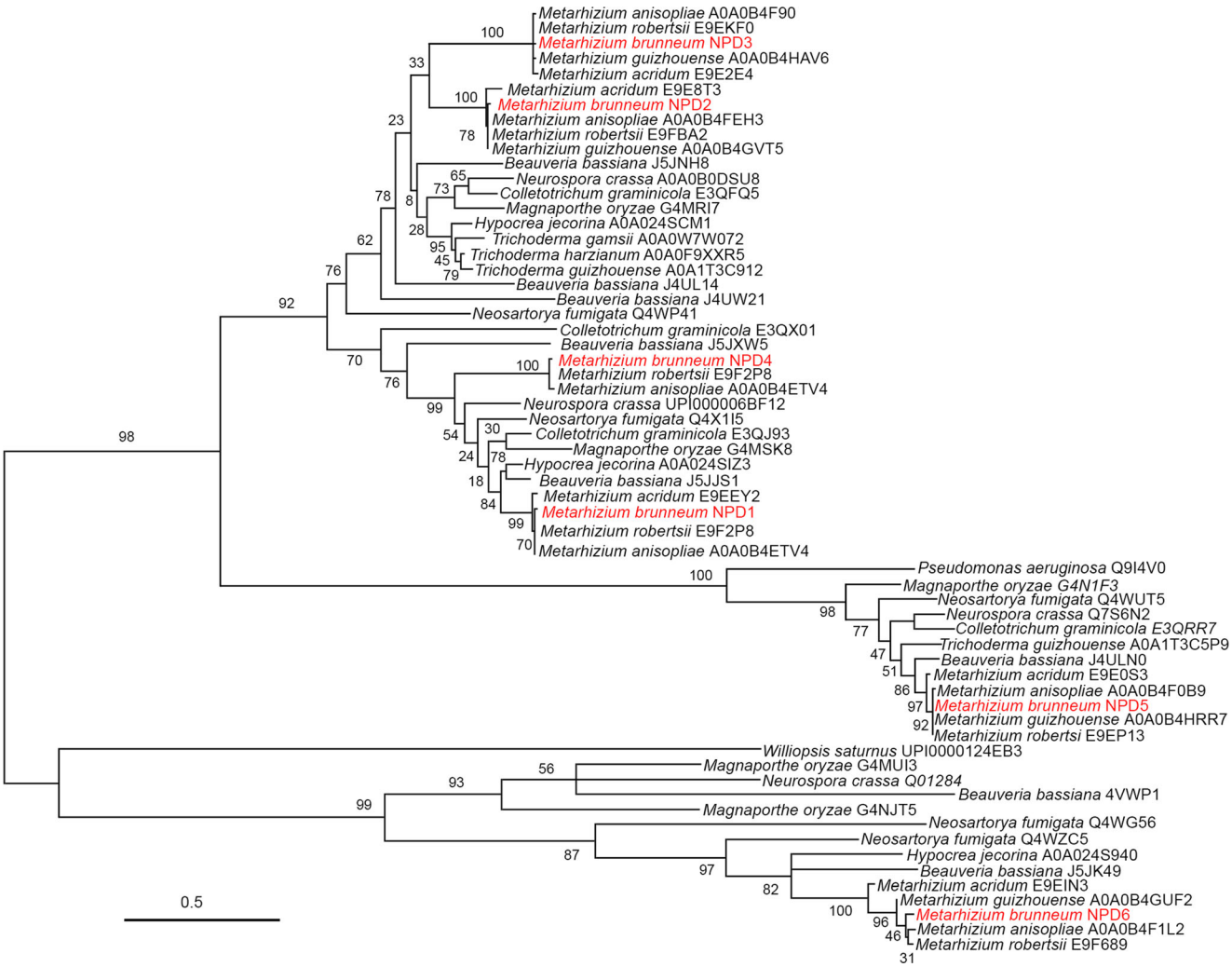

Fig. 1 Phylogeny of fungal nitronate monooxygenase. The phylogenic tree of fungal nitronate monooxygenases was inferred by using the maximum likelihood method, with 1000 bootstrap replicates, utilizing MEGA7.0 software. Names of the species are followed by their GenBank accession number

N. crassa, with an identity between the two of them of 34% (see Supplementary Table S5). Additionally, Npd2 and Npd3 are the most related to Nmo2 from *M. oryzae* (Fig. 1), a nitronate monooxygenase involved in the nitrosative stress response during blast disease (Marroquin-Guzman et al. 2017).

Comparison of the Npd protein sequences with PA1024, which was initially classified as a nitronate monooxygenase but recently identified as an NADH:quinone reductase (Ball et al. 2016), and PA4202 from *P. aeruginosa*, which have both been crystallized (Ha et al. 2006; Salvi et al. 2014), showed that the Npd proteins from *M. brunneum* contain the motifs described for NMO class II (Supplementary Fig. S2): motif I ^{14}P-I-X-Q-A-P-M-X-G-X-S-T-X-X-L-A-A^{30}; motif II ^{130}(V/I)-S-F-H-F-(G/N)-X-P^{137}; motif III ^{174}(V/I)-X-Q-G-X-E-A-G-G-H-R-G-X-F^{187}; motif IV ^{297}(P/A)-(D/E/P)-Y-P-X-X-Y-D-X-X-K-X-L^{309} (Salvi et al. 2014); and motif V ^{77}ASGGhAD(A/G)RGLhAALALGA(D/E)GhXMGTRF^{204}, described by Ha et al. (2006). Similarly, the catalytic residue His^{196} from Ncd-2 from *N. crassa* (Q01284) is conserved in the six Npd proteins from *M. brunneum*, corresponding to His^{168} of Npd1, Npd2, Npd3, and Npd4, and in Npd5 and Npd6, corresponding to His^{153} and His ^{174}, respectively. This catalytic His in all these strains is inside signature motif III, the most conserved motif of the nitronate monooxygenase enzymes.

The six *Npd* genes encode proteins with nitronate monooxygenase activity

To determine whether the proteins encoded by the *Npd* genes with similarity to nitronate monooxygenases have this activity, the ORFs of the six genes (*Npd1*, *Npd2*, *Npd3*, *Npd4*, *Npd5*, *Npd6*) and the *Ncd-2* from *N. crassa* were amplified by RT-PCR from RNA from *M. brunneum* Ma10 cells grown in MDS and *N. crassa* cells grown in PDB medium and cloned in the pRSET A expression vector and expressed in *E. coli* cells. The recombinant proteins were overexpressed and purified (Supplementary Fig. S3). The activity of the fractions eluted with 300 mM imidazole, using anionic nitroalkanes nitroethane, 1-nitropropane, and 2-nitropropane as substrates, was measured. The six Npd proteins show activity with at least one of the substrates. All these proteins showed a low Km, suggesting a high affinity for the substrates tested. The Npd1, Npd2, Npd4, and Npd5 proteins showed Km values for 2-nitropropane similar to those previously reported for Ncd-2 of *N. crassa* (Gorlatova et al. 1998) and 2Npd from *H. mrakii* (Kido et al. 1984) (Table 1). The *M. brunneum* Npd1, Npd2, and Npd4 enzymes recognize 2-nitropropane and nitroethane as substrates and have a lower affinity for 1-nitropropane. For Npd3 and Npd6, the preferred substrate is 1-nitropropane. Additionally, Npd6 recognizes nitroethane as a substrate, and Npd3 and Npd6 have a lower affinity for 2-nitropropane. Moreover, for the Npd5 enzyme, its preferred substrates are 1-nitropropane and 2-nitropropane. These results indicate that the proteins encoded by the *Npd1*, *Npd2*, *Npd3*, *Npd4*, *Npd5*, and *Npd6* genes display nitronate monooxygenase activity, showing differences in the preference for nitroalkane species, and these preferences are independent of the amino acid sequence similarity between them.

Table 1 Substrate specificity of the Npd enzymes

Enzyme	Anionic substrate	Km (mM)
*Mb*Npd1	1-Nitropropane	34.3
	2-Nitropropane	2.8
	Nitroethane	10.6
*Mb*Npd2	1-Nitropropane	13.6
	2-Nitropropane	5.6
	Nitroethane	3.4
*Mb*Npd3	1-Nitropropane	1.3
	2-Nitropropane	148.5
	Nitroethane	147.8
*Mb*Npd4	1-Nitropropane	91.4
	2-Nitropropane	4.32
	Nitroethane	45.1
*Mb*Npd5	1-Nitropropane	0.45
	2-Nitropropane	1.6
	Nitroethane	50.6
*Mb*Npd6	1-Nitropropane	12.9
	2-Nitropropane	134.9
	Nitroethane	20.1
*Nc*Ncd-2*	1-Nitropropane	8.3
	2-Nitropropane	3.1
	Nitroethane	6.0
2Np from *H. mrakii***	1-Nitropropane	3.23
	2-Nitropropane	1.6
	Nitroethane	3.13

*Data from Gorlatova et al. 1998. **Data from Kido et al. 1984

M. brunneum Ma10 is capable of growing in nitroalkanes

Once it had been determined that this fungus has nitronate monooxygenase activity, *Metarhizium* was grown in MM supplemented with nitroalkanes at different concentrations. Figure 2 a shows that *M. brunneum* can grow on nitroalkanes, with 2-nitropropane being the most toxic for the growth of the fungus. Therefore, the fungus can grow on nitroethane and 1-nitropropane at the tested concentrations. Moreover, growth in nitroethane and 2-nitropropane negatively affected the conidiation (Fig. 2b). The conidiation was almost ten times lower in nitroethane 0.3–0.5% compared with the conidiation in the absence of nitroalkane. The conidiation in 2-

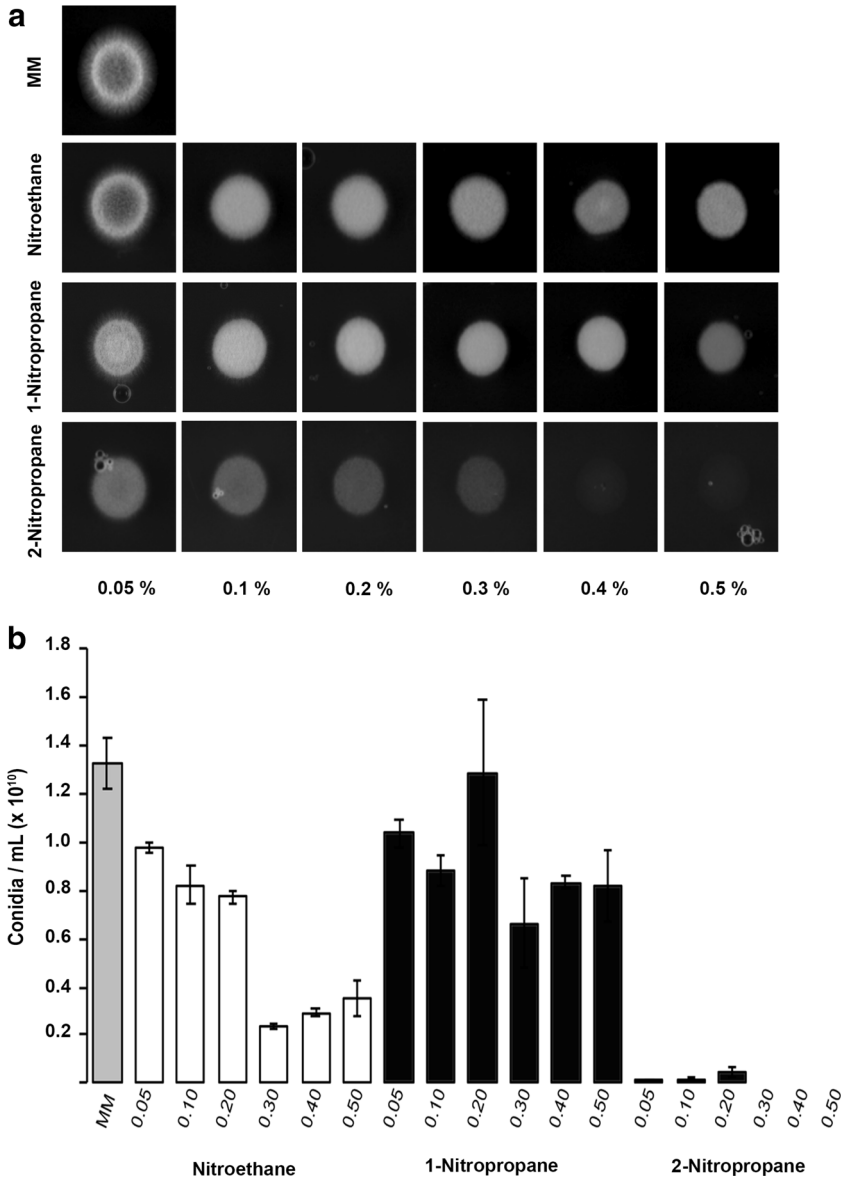

Fig. 2 Effect of the nitroalkanes nitroethane, 1-nitropropane, and 2-nitropropane on *Metarhizium* conidiation. *M. brunneum* Ma10 conidia were grown in MM supplemented with nitroethane, 1-nitropropane, or 2-nitropropane at 0.05, 01, 0.2, 0.3, 0.4, and 0.5%. **a** *M. brunneum* Ma10 colony morphology after 3 days of incubation at 28 °C. **b** *M. brunneum* Ma10 conidia number after 14 days of incubation

nitropropane was minimal. Additionally, this adverse effect on conidiation was absent when the fungus grew in 1-nitropropane.

Conidia obtained from *M. brunneum* cells grown in nitroalkanes reduce the median lethal time (LT_{50}) in *P. xylostella* larvae

Since the relationship between the fungal ability to catabolize long-chain hydrocarbons and virulence parameters has been described (Pedrini et al. 2007), we analyzed the capacity of the conidia obtained when *M. brunneum* was grown in nitroalkanes to infect *P. xylostella* larvae. In this experiment, the lethal dose 90 (LD_{90}) of conidia was applied to third-instar larvae (Morales Hernandez et al. 2010) and followed the survival of the larvae during the experiment. Figure 3 (Supplementary Fig. S4 and Supplementary Table S6) shows that in the population of larvae treated with conidia from the fungus growth on MM, it kills half of the larvae (LT_{50}) in 96 h. Moreover, the conidia obtained from MM supplemented with nitroethane (0.2, 0.3, 0.4%) or 1-nitropropane (0.05 and 0.2%) showed a statistically significant reduction of the LT_{50} (between 66 and 84 h; 31 to 12.5% reduction). Therefore, conidia obtained from *M. brunneum* cells grown in nitroalkanes increased their infective capacity.

The six *Npd* genes are expressed during *M. brunneum* infection of *P. xylostella*

Some plants, insects, and microorganisms contain nitroalkanes, potentially as defense mechanisms (Chomcheon et al. 2005; Niknam et al. 2003; Porter and

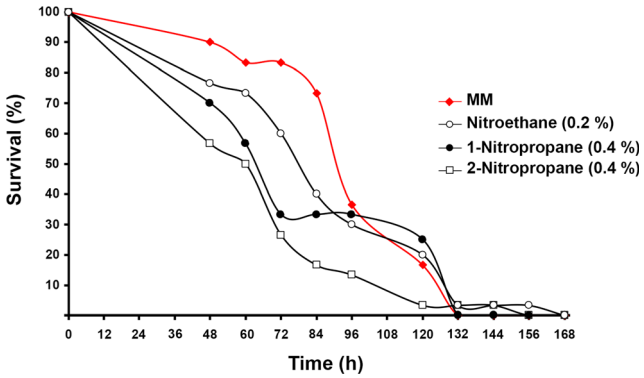

Fig. 3 Insect bioassays. Survival of *P. xylostella* larvae after the application of conidia from *M. brunneum* Ma10 grown in MM, or MM supplemented with nitroethane (0.2, 0.3, and 0.4%) or 1-nitropropane (0.05 and 0.2%)

Bright 1987; Salem et al. 1995); therefore, it is essential to follow *Npd* gene expression during *M. brunneum* infection in insects. We analyzed the relative expression of the six *Npd* genes over the course of the infection process to third-instar larvae of *P. xylostella* inoculated with conidia (Fig. 4) using the ubiquitin gene as a constitutive expression gene. This experiment showed that in early infection, the first twelve hours post-inoculation, the *Npd1* gene is the most highly expressed, followed by *Npd3* and *Npd5*. Meanwhile, the expression of the *Npd2*, *Npd4*, and *Npd6* genes is at basal levels. During the invasion process, from 24 to 72 h post-inoculation, the *Npd1* gene has the highest level of expression, followed by the genes *Npd5*, *Npd3*, and *Npd2*; meanwhile, expression of the genes *Npd4* and *Npd6* is at basal levels. Finally, during extrusion of the mycelium from the body insect and conidiation, the next 96–168 h, the *Npd* genes again increase their expression; the *Npd1* gene is the most expressed and, to a lesser extent, the *Npd3*, *Npd5*, *Npd4*, and *Npd2* genes, while the *Npd6* gene is not expressed at this stage.

The meaning of these fluctuations in the levels of gene expression of the six *Npd* genes during the life cycle of the fungus during infection to the insect is not clear. However, the expression of the *Npd1*, *Npd5*, and *Npd3* genes during this process is notorious. To understand their physiological role in the lifestyle of this fungus, we are currently constructing null mutants of each of these six *Npd* genes.

Discussion

According to Ball et al. (2016), there are over 5000 genes in the GenBank currently annotated as nitronate monooxygenases (NMOs). Nitronate monooxygenase activity was described many years ago. In the filamentous fungus *N. crassa*, it was described for the first time as nitroalkane oxidase (Little 1951), afterward as nitropropane dioxygenase (Gorlatova et al. 1998), and now as a nitronate monooxygenase (https://www.uniprot.org/uniprot/Q01284). This *Ncd-2* gene from *N. crassa* was cloned and expressed in *E. coli*, the resulting protein purified, and kinetic parameters analyzed (Francis et al. 2005). The nitroalkane oxidase (NAO) from *F. oxysporum* was expressed in *E. coli*, and the recombinant enzyme characterized. Recombinant NAO has identical kinetic parameters to the native enzyme isolated from *F. oxysporum* (Daubner et al. 2002).

In this sense, gene expression in *E. coli* and analysis of the kinetic parameters of the recombinant enzymes is an appropriate tool for the characterization of the gene products that potentially encode for this annotated nitronate monooxygenases.

In this work, the expression of six genes that encode nitronate monooxygenases in *M. brunneum* was isolated and studied. This work is the first report about the nitronate monooxygenase activity and the respective genes in the genus *Metarhizium* that display at least four lifestyles in nature: entomopathogenic, mycorrhiza, promoter of growth of plants, and saprophyte. The nitronate monooxygenase enzymes from *M. brunneum* encoded by the genes *Npd1*, *Npd2*, *Npd3*, *Npd4*, *Npd5*, and *Npd6* are active on primary and secondary short-length nitroalkanes. These enzymes display Km values similar to the nitroalkane oxidase from *F. oxysporum* (Gadda and Fitzpatrick 1999) and the nitronate monooxygenases from *H. mrakii* (Kido et al. 1984) and *N. crassa* (Gorlatova et al. 1998).

Interestingly, the analyzed fungi, saprophytes, entomopathogens, phytopathogens, and fungi with plant growth-promoting activity contain 3 or more putative nitronate monooxygenase genes. Moreover, microorganisms such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces marxianus* and, similarly, animal pathogens such as *Candida albicans*, *Sporothrix schenckii*, *Phaeoacremonium minimum*, *Trichophyton equinum*, and *Trichophyton verrucosum*, as far as we know, do not contain nitronate monooxygenase genes in their genomes (http://fungi.ensembl.org). This distribution of nitronate monooxygenase genes could be related to the environment in which each microorganism lives, considering that phytopathogens, entomopathogens, and saprophytes have a greater probability to be in contact with nitroalkanes in their niche (Chomcheon et al. 2005; Porter and Bright 1987; Salem et al. 1995).

According to the presence of nitronate monooxygenase activity in *Metarhizium* spp., the fungus was able to grow in nitroalkanes. Interestingly, we observed an adverse effect of 1-nitropropane and 2-nitropropane on conidiation, with 2-nitropropane being the most toxic, most likely because of their nitroalkane conversion into their corresponding carbonyl and nitrite increases the nitrosative stress by nitric oxide (NO) production, altering the balance of reactive oxygen species (ROS) necessary for conidia differentiation. Nitrite can be a

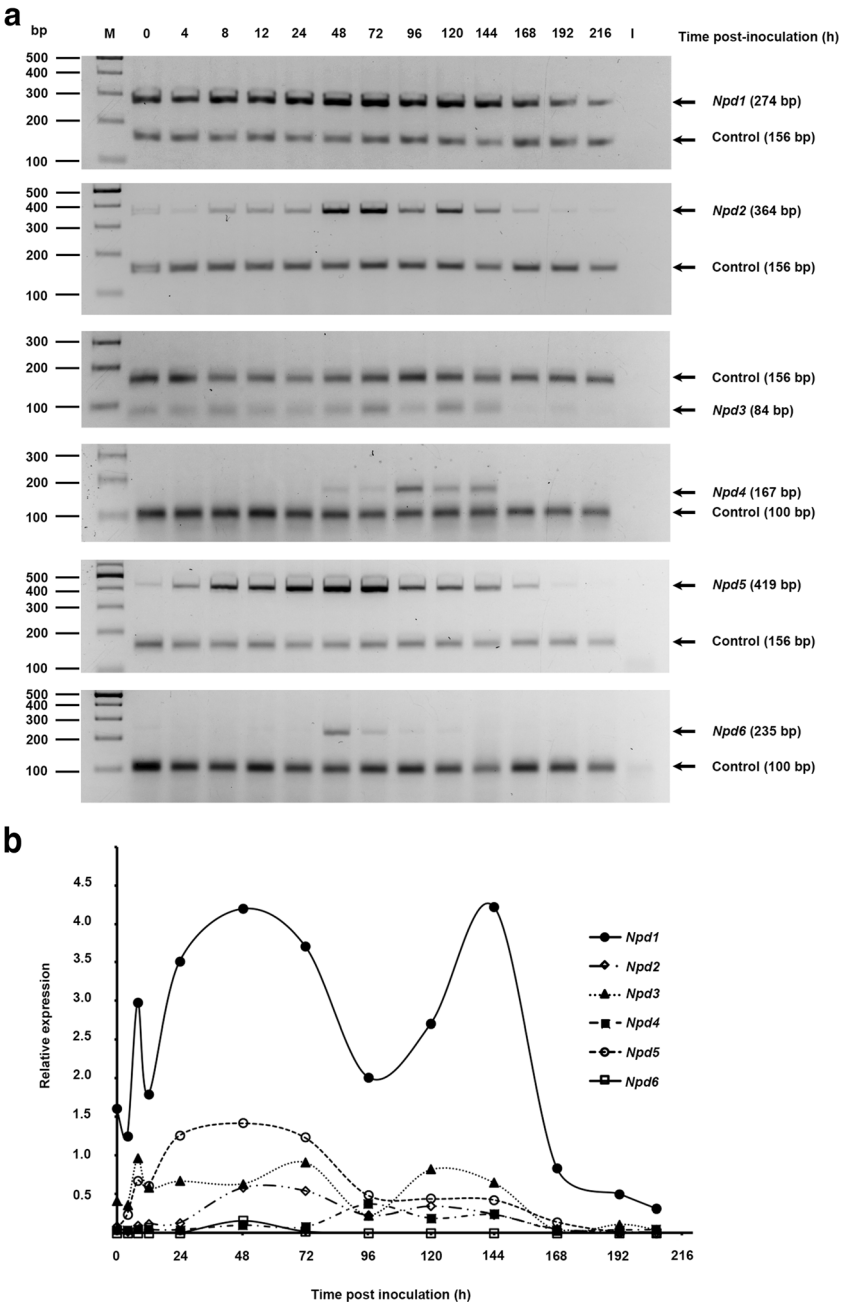

Fig. 4 Relative *Npd* gene expression during the infection process *P. xylostella*. **a** RT-PCR analysis of the six *Npd* genes during the infection of third-instar *P. xylostella* larvae with *M. brunneum* Ma10. Lane M corresponds to the DNA marker, and lanes 0 to 206 correspond to RT-PCR amplification products from the total RNA of *P. xylostella* larvae infected with *M. brunneum* Ma10 at different time points post-infection. Lane I corresponds to the RT-PCR amplification products from the total RNA from uninfected *P. xylostella* larvae. **b** Relative expression analysis measured by densitometric analysis

vital source of NO, which can modify the levels of oxidative stress, acting as a signaling molecule in some biological processes, such as responses to abiotic or biotic stresses and development in plants and reproduction in mammals (Canovas et al. 2016).

As mentioned previously, in *R. solanacearum*, a putative nitropropane dioxygenase is involved in virulence (Zhang et al. 2017); in this sense, *M. brunneum* conidia produced in the presence of nitroalkanes were more virulent than the conidia of mycelium grown in the absence of nitroalkanes. They display a shorter LT_{50} than that observed in the larvae of *P. xylostella* treated with conidia from MM without nitroalkanes. This result suggests that the conidia produced in the presence of these compounds can kill the insects faster, probably by the same mechanism suggested by Pedrini et al. (2007); the newly formed conidia contain high levels of enzymes involved in nitroalkane catabolism, which helps the fungus to increase its virulence and/or perhaps the conidia are prepared with a higher level of expression of the *Npd* genes to confront the defense mechanisms of the insect. Although the *Npd* gene expression assays on those conidia have not been performed, the genes *Npd1* and *Npd5* are the most expressed *Npd* genes in *M. brunneum* mycelium grown in minimal medium with 2-nitropropane (data not shown).

The predicted protein sequence of nitronate monooxygenases of the *Npd1*, *Npd2*, *Npd3*, *Npd4*, *Npd5*, and *Npd6* genes from *M. brunneum* contains the signature sequence motifs observed in other nitronate monooxygenases, i.e., Ncd2 (Q01284) from *N. crassa* (Gorlatova et al. 1998) and PA4204 from *P. aeruginosa* (Salvi et al. 2014). These motifs contain residues involved in binding to FMN, and importantly, signature motif III is the most conserved of all the motifs and contains the catalytic His described for the 2-nitropropane dioxygenases (Ha et al. 2006). Interestingly, the six *M. brunneum* Npd proteins contain the carboxy-terminal peroxisomal targeting signal 1 (PTS-1, (**S**/A/C)(**K**/R/H)**L**), and there are three sequence variations between the six Npd proteins: AKL, SKL, and AHL, suggesting their peroxisomal localization. The *M. oryzae* genome carries five *NMO* genes encoding putative nitronate monooxygenase enzymes: MGG_07261 (NMO1); MGG_02439 (NMO2); MGG_02593 (NMO3); MGG_09511 (NMO4); and MGG_01473 (NMO5) (Marroquin-Guzman et al. 2017), and only NMO4 and NMO5 have PTS1 targeting signals (SKL and AKL, respectively). The presence of the SKL tripeptide is not sufficient, and amino acids upstream of this sequence also contribute to the targeting, and more complete PTS1 definitions include at least ~ 10 amino acids of a protein (Notzel et al. 2016). Nevertheless, in our preliminary results by GFP fusion and localization, all the Npd proteins have peroxisomal localization in *M. brunneum* (unpublished). In fungi, peroxisomes have diverse functions: the biosynthesis of antibiotics, toxins, lysine, biotin, and secondary metabolites; the degradation of amino acids, polyamines, and H_2O_2; the oxidation of fatty acids; methanol degradation; the glyoxylate cycle; and maintenance of cellular integrity (Smith and Aitchison 2013). The presence of the six Npd proteins in the *M. brunneum* peroxisomes and the possible role in its life cycle is the next step in our research.

M. brunneum contains at least six *Npd* functional genes in its genome and is expressed differentially during infection in insect, suggesting that at least the genes *Npd1*, *Npd5*, and *Npd3* could be essential for this lifestyle. The genes *Npd1*, *Npd5*, and *Npd3* display a peak of expression during the first 12 h post-inoculation of the third-instar larvae of *P. xylostella*. This peak coincides with earlier invasion, including germination and appressorium formation. During the invasion of the insect body, the genes *Npd1*, *Npd5*, *Npd3*, and *Npd2* are expressed at different levels. Finally, during mycelial merge and conidiation over the insect body, *Npd1* and *Npd3* increase their expression levels, while *Npd5*, *Npd4*, and *Npd2* maintain a low level of expression. Only the *Npd6* gene is practically not expressed during the lifecycle of this fungus in the insect host. In this fungus, other genes, such as the *Odc1* gene (Madrigal Pulido et al. 2011), *Cat1* (Morales Hernandez et al. 2010), and *Mad1* and *Mad2* (Barelli et al. 2011), show fluctuating expression during the invasion process.

We do not know the reason why *M. brunneum* expresses these genes differentially, and if these variations are different in the *Metarhizium*-plant interaction. The deletion of each gene will be necessary to analyze their participation in the lifestyles of the fungus.

In conclusion, in this work, we demonstrated that the genes *Npd1*, *Npd2*, *Npd3*, *Npd4*, *Npd5*, and *Npd6* from *M. brunneum* encode proteins with nitronate monooxygenase activity that are differentially expressed during invasion of the host *P. xylostella*, the *Npd1* gene with the highest expression during this process. Now, we are working with the null construction of these *Npd* genes to study their participation in the different lifestyles of this fungus.

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Compliance with ethical standards

This article does not contain any studies with human participants or vertebrates, performed by any of the authors.

Conflict of interest All authors declare that they have no conflict of interest.

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