

Regulatory Effects of Nitric Oxide on Reproduction and Melanin Biosynthesis in Onion Pathogenic Fungus *Stemphylium Eturmiunum*

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Abstract

The formation of propagules (conidia and ascospores) is the critical stage for the transmission of the pathogenic fungus *Stemphylium eturmiunum*. However, how the development of these propagules is regulated remains to be fully understood. Here, we show that nitric oxide (NO) is necessary for the formation of conidia and pseudothecia in *S. eturmiunum*. Application of NO scavenger carboxy-CPTIO (cPTIO) or soluble guanylate cyclase (sGC) inhibitor NS2028 abolishes the formation of conidia and pseudothecia. In the culture of *S. eturmiunum*, supplement of NO-releasing compound sodium nitroprusside (SNP) results in an increased formation of conidia at 0.2 mmol/L, and pseudothecia at 2 mmol/L. SNP supplement also triggered increased biosynthesis of melanin, which can be inhibited partially upon the addition of either arbutin or tricyclazole, the specific inhibitors for 3,4-dihydroxyphenylalanine (DOPA) and dihydroxynaphthalene (DHN) synthetic pathway, respectively. Intriguingly, enhanced melanin biosynthesis triggered an increased formation of propagules; while its inhibition impaired their formation. The SNP-induced increment in the formation of propagules can be also compromised upon supplement of cPTIO or NS-2028. RT-PCR analysis showed that SNP at 0.2 mmol/L promoted transcription of the genes encoding the conidiation co-regulators *brlA*, *abaA*, and *wetA*, and inhibited at 2 mmol/L. In contrast, application of SNP at 2 mmol/L increased transcription of the genes encoding *mat1*, and *mat2*, the genes related to sexual reproduction, and the transcription of two DHN melanin synthetic genes *pks1* and *pks2*, and the key gene *tyr* for DOPA melanin biosynthesis. However, the increased transcription of these genes is down-regulated or blocked upon supplement of cPTIO or NS-2028. Thus, NO regulates asexual and sexual development, as well as melanin synthesis in *S. eturmiunum* possibly through NO-sGC-GMP signaling pathway.

Introduction

The foliar fungal pathogen *Stemphylium eturmiunum* is a homothallic filamentous ascomycete causing severe leaf blight of onions (Fernandez and Rivera-vargas 2008). In addition, *S. eturmiunum* is also a postharvest spoiler of fresh tomatoes (Andersen and Frisvad 2004; Trinetta et al. 2013). The infection at the lesions of fresh tomatoes by the fungus results in the production of toxic metabolites infectopyrone and macrosporin (Andersen and Frisvad 2004). In natural habitats, *S. eturmiunum* develops asexual conidiophores for conidiation, and sexual fruiting bodies pseudothecia for producing ascospore (Simmons 2001). The conidia that easily reach the host alveola are the major infective propagules (Muñiz-Paredes et al. 2017). Notably, the conidial cell wall, directly in contact with host cells, consists of melanins in addition to the presence of α -(1,3)-glucan and proteinaceous rodlets. The fungal melanins, synthesized via DOPA or DHN pathway (Chang et al. 2019) both possess the potential to scavenge free radicals (Pacelli et al. 2020). In filamentous fungi, the presence of melanins in conidial cell wall confers the resistance to the attack of free radicals from host organisms, which, in turn, increases their pathogenicity for effective infection and transmission (Chamilos and Carvalho 2020; Amin et al. 2014; Cunha et al. 2010). The sexual fruiting body of fungi was constructed by tightly interwoven melanin-contained hyphae (Engl et al. 2007). Similar to conidia, the unique structure of pseudothecia also

confers fungi the capacity to survive in harsh environment (Lambou et al. 2008; Zhao et al. 2017). Thus, the involvements of melanin in the construction of conidia and pseudothecia endow the resistance of fungi against environmental insults and the effective infection and transmission (Pal et al. 2014).

Asexual sporulation is the most common mode for reproduction and transmission in filamentous fungi (Park et al. 2012) and is timed and genetically programmed (Adams et al. 1998; Lee et al. 2010). In *Aspergillus*, the conidial sporulation is determined by a central regulatory pathway, where the transcriptional factors *brlA*, *abaA*, *wetA* coordinate conidiation-specific gene expression and determines the order of gene activation during the formation of conidiophores and maturation of conidia (Boylan et al., 1987; Mirabito et al. 1989).

Similar to asexual sporulation, the formation of pseudothecia is also regulated by multiple genes. Previously, we showed that the development of pseudothecia in *S. eturmiunum* is regulated not only by the genes encoding MAT1, MAT2, and the protein in G-protein signaling pathway but also by *magI*, the gene responsible for the synthesis of arachidonic acid (Zhao et al. 2019). Treatment of *S. eturmiunum* with 5-azacytidine (5-AC) resulted in the complete silence of *magI* followed by disruption in the development of pseudothecia and melanin synthesis. Interestingly, the impaired development of pseudothecia can be restored by external supplement of arachidonic acid (Zhao et al. 2019), which implicates the significance of arachidonic acid, the common source for the genesis of oxylipins (Aukema et al. 2016), in sexual development of *S. eturmiunum*.

The successful survival of fungi in nature depends on the efficient communication with their surroundings (Hassan et al. 2019). In filamentous fungi, the communication with habitats is achieved by complex signaling systems that determine the fungal proliferation, development, and in some cases virulence (Kozubowski et al. 2009). The well-known signaling pathways in fungi include the protein kinase A/cyclic AMP (cAMP), protein kinase G/cyclic GMP (cGMP), protein kinase C (PKC)/mitogen-activated protein kinase (MAPK), lipid signaling cascades, and the calcium-calcineurin signaling pathway (Kozubowski et al. 2009). PKG/cGMP pathway, which seems to exist in filamentous fungi, is activated by free radical molecule NO (Zhao et al. 2020). The NO, highly diffusible within the cell or through cell membrane (Lancaster 1997), can work as a transient, local, intra- or intercellular signaling molecule in miscellaneous biological systems including fungi (Culotta and Koshland 1992). In fungi, NO signaling is involved in conidiation, formation of sexual fruiting bodies, and regulation of secondary metabolism (Zhao et al. 2020). Established evidences have shown that NO can function as the activator of sGC able to bind selectively with the hemoprotein in sGC even in the presence of oxygen (Boon and Marletta 2005). The binding of NO with Heme iron triggers the change sGC conformation and subsequently activates its catalytic domain, where GTP can be catalyzed to cyclic GMP (cGMP) (Friebe and Koesling 2003). The produced cGMP then combines with PKG, which constitutes central downstream mediator of NO-cGMP-PKG signaling pathway, and orchestrates pathway-specific cellular response through the phosphorylation of phosphorylation-dependent transcriptional factors (Contestabile 2008).

Studies in the past decades have shown that NO-mediated signaling networks are involved in conidiation (Wang and Higgins 2005; Gong et al. 2007), the formation of parasitic structure of appressoria (Prats et al. 2008), asexual structures of sporangiophores (Maier et al. 2001), and sexual structures of cleistothecia in *Aspergillus* species (Baidya et al. 2011). In our previous attempts to unravel the regulatory mechanisms for sexual development of *S. eturmiunum*, we found that treatment of the fungus with 5-AC resulted in the formation albinism phenotype and the silence of the genes responsible for melanins synthesis in addition to *magI*. Application of SNP partially restored biosynthesis of melanins in 5-AC treated *S. eturmiunum*. Intriguingly, SNP supplement in non-treated *S. eturmiunum* increased the number of conidia and pseudothecia. However, how NO signaling affects asexual and sexual development remains to be established. In this study, we used SNP as the external NO source to probe the regulatory effects of NO on conidiation, sexual fruiting body formation, and production of melanin. Our data showed that NO-induced increment in conidiation, formation of pseudothecia, and melanin accumulation is possibly mediated by NO-cGMP signaling pathway, where NO works as a sGC activator triggering the signal transduction cascade.

Materials And Methods

Strains and growth conditions

Wild type and 5-AC-treated (the albinism mutant) *S. eturmiunum* strains were obtained from the Key Laboratory for Biology of Vegetable Diseases and Insect Pests of Shandong Province, Department of Plant Pathology, Shandong Agricultural University, Tai'an, China, and stored at -80 °C in 15% glycerol and routinely maintained and grown in CM medium at 26 °C (Zhao et al. 2019). *Aspergillus nidulans* LO8030 was kindly donated by Dr. Caly Wang from the University of Southern California.

Effects of exogenous NO on development of *S. eturmiunum*

The effects of exogenous NO on the development of *S. eturmiunum* were we assayed by supplementing sodium nitroprusside dihydrate (SNP, Sigma, St. Louis, USA) into the culture *S. eturmiunum* at concentrations of 0, 0.2, 2, 4, 8 mmol/L, respectively. For determining the possible pathways of NO signaling, the SNP-supplemented or SNP-non-supplemented cultures were also simultaneously supplemented either with NO scavenger cPTIO (Beyotime, Shanghai, China) at a concentration of 1 mmol/L or sGC specific inhibitor NS-2028 (Beyotime, Shanghai, China) at a concentration of 18 µmol/L, and cultured at the same conditions. All cultures were incubated for 28 d in the continuous dark at 26 °C to avoid the possible light effect of fungal development and SNP instability followed by observation for asexual and sexual development, as well as melanin accumulation. All the treatments were analyzed at least triplicate.

Gene transcription analysis

For RNA extraction, the mycelia were grounded with pestle and mortar in liquid nitrogen followed by extraction using Trizol (Takara, Ishiyama, Japan) as previously described (Lee et al. 1997). cDNA was

generated using the Prime Script™ RT reagent kit (Takara, Ishiyama, Japan). Gene transcription analysis was conducted by quantitative real-time PCR (RT-PCR). The forward (F) and reverse (R) oligonucleotide primer sequences were listed in Table 1. RT-PCR was carried out using the Fast SYBR Green Master Mix (Thermo Fisher Scientific, USA). Gene expression levels were normalized using the endogenous control gene *actin*. To characterize the changes of mRNA levels of the genes influenced by NO, we employed Ct value of the initial amount of the candidate mRNA in a sample, as a quantitative measurement of gene expression strength under specific experimental conditions and simultaneously performed RT-PCR of the genes in the samples treated and untreated by SNP followed by double Δ Ct analysis. The relative mRNA level was indicated by the exponentiation of $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001). Every experiment was independently performed three times.

Table 1 Primers used for all experiments

Primers	Nucleotide sequences (5– 3)	Purpose
<i>actin</i> -F	5'-CATCCAGCTCAAGGACATCAT-3'	qRT-PCR for <i>actin</i>
<i>qactin</i> -R	5'-GTCGGATCAAGGTTTCATCTTCT-3'	
<i>qmat1</i> -F	5'-ATACTGGCTACAGGGCAAATC-3'	qRT-PCR for <i>mat1</i>
<i>qmat1</i> -R	5'-GGACGAAGTTGGGAACAAGA-3'	
<i>qmat2</i> -F	5'-CGCATTTGGCACAACCTATC-3'	qRT-PCR for <i>mat2</i>
<i>qmat2</i> -R	5'-GGCTATACTTGTAGTCGGGATG-3'	
<i>qbrlA</i> -F	5'-GTCCAGACATTCACCCCCAG-3'	qRT-PCR for <i>brlA</i>
<i>qbrlA</i> -R	5'-CTTGTTGACATTGGGCTGGC-3'	
<i>qabaA</i> -F	5'-TCAAGATCCTGACGGAAGCG-3'	qRT-PCR for <i>abaA</i>
<i>qabaA</i> -R	5'-AACGCAATGTTCAAGCGGTC-3'	
<i>qwetA</i> -F	5'-CACCTCGACCTCACACTTCC-3'	qRT-PCR for <i>wetA</i>
<i>qwetA</i> -R	5'-GGATGGCGATGAGACTGGAG-3'	
<i>qtyr</i> -F	5'-ATGGCATCGCTACTACATC-3'	qRT-PCR for <i>tyr</i>
<i>qtyr</i> -R	5'-GATAGGGCAGGTGAGTTCT-3'	
<i>qpks1</i> -F	5'-TGGGATCTTGTGGAAGCATAC -3'	qRT-PCR for <i>pks1</i>
<i>qpks1</i> -R	5'-GTGCATAGACCGACAACCTCTT-3'	
<i>qpks2</i> -F	5'-CTTGCGGTTGGAGCATTG -3'	qRT-PCR for <i>pks2</i>
<i>qpks2</i> -R	5'-TTGTTCGTGGGTCGGTAATC-3'	
<i>tpks1</i> -F	5'-tccttctcctgatcgctagcATGAACGTCCTCATTTTCGGAG-3'	For <i>pks1</i> transformation
<i>tpks1</i> -R	5'-acagaataactctcgctagcTTATAGCTTAAGACCTTGCTGGATC-3'	
<i>tpks2</i> -F	5'-tccttctcctgatcgctagcCTCTACCGACCCTCTCACCC-3'	For <i>pks2</i> transformation
<i>tpks2</i> -R	5'-acagaataactctcgctagcTCCAACGTCAGCAACCCATC-3'	
<i>testpks1</i> -F	5'-GCGAGACCGCATTCTAACT-3'	Test <i>pks1</i> transformants
<i>testpks1</i> -R	5'-TGTCAAGCTCCTCACGGAAC-3'	
<i>testpks2</i> -F	5'-AGGATACTGTGGGGGTCTCC-3'	Test <i>pks2</i> transformants
<i>testpks2</i> -R	5'-TCAATCGGGTGGTCAAGAGC-3'	

Assay of NO levels and NO producing capacity activity

For assaying NO levels and NO producing capacity, the mycelia were withdrawn and rinsed twice with pure water and disrupted with mortar and pestle in liquid nitrogen. The well-powdered mycelia were then used for the assay of NO production and NO producing capacity. NO levels were determined by total NO assay kit (A012-1-2, Nanjing Jiancheng Bioengineering Institute, China), and its levels were indicated as micromolar per milligram protein ($\mu\text{mol}/\text{mg prot}$). NO producing capacity in homogenates was estimated using a total NOS assay kit (A014-2-1, Nanjing Jiancheng Bioengineering Institute, China). The protein content was determined by Coomassie Brilliant Blue using BSA (High-Tech Innovation, Inc., Shanghai, China) as standard. NO producing capacity was demonstrated as units (U). One unit of NO producing capacity stands for the number of micromoles of NO produced by 1 mg protein in 1 min (Zhao et al. 2015). Each experiment was independently performed at least three times.

Pathways for melanin biosynthesis

The pathway for melanin synthesis was determined by the application of specific pathway inhibitors. Dihydroxyphenylalanine (DOPA) pathway was assayed by supplementing the specific inhibitor arbutin (Alfa Aesar, USA) at a final concentration of 30 mg/L (Gong et al. 2013), and the dihydroxynaphthalene (DHN) pathway was assessed by the addition of the pathway specific inhibitor tricyclazole (Adamas, China) at a final concentration of 30 mg/L (Pal et al. 2014) into the cultures, respectively. The CM media were inoculated by placing a mycelium plug cut from the margins of an actively growing colony, and incubated at 26 °C for 7d followed by observation of growth and pigmentation. Every experiment was independently performed three times.

Tyrosinase activity

Tyrosinase activity was assayed as described (Lejczak et al. 1987; Likhitwitayawuid and Sritularak, 2001) with slight modification. Briefly, 20 mmol/L phosphate buffer (pH 6.8), 2 mmol/L L-DOPA (Sigma, St. Louis, USA), and protein extracts were mixed and reacted at 30 °C for 30 min. Then the formation of dopachrome was measured at 475 nm. Tyrosinase activity was demonstrated as units (U). One unit of tyrosinase activity stands for the number of micromoles of dopachrome produced by 1 mg protein in 1 min. The test was performed in triplicate.

Melanin extraction

Melanin extraction from the fungal biomass was performed as described previously (Pal et al. 2014) with a slight modification. In brief, the mycelia were crushed with 2 mol/L NaOH (pH 10.5) and placed for 48 h. Next, the mixture was centrifuged at 3500 g for 15 min and the resultant supernatant was mixed with 6 mL of a mixture solvent consisting of chloroform, ethyl acetate and ethanol (2 mL each), and vortexed

for 5 min at room temperature to remove lipids. After removal of the organic layer, the aqueous layer was acidified by 2 mol/L HCl to pH 2.5 followed by incubation overnight at room temperature and then centrifuged at 3500 g for 15 min to remove carbohydrates and proteins. The resultant precipitate was dried at room temperature, and dissolved in 2 mol/L NaOH followed by centrifugation at 3500 g for 15 min. The supernatant was then acidified with 6 mol/L HCl. And centrifuged at 3500 g for 15 min. The resultant precipitate was washed with distilled water and dried at room temperature, and used until further analysis.

Heterologous expression of the genes involved in biosynthesis of DHN melanin

A. nidulans LO8030 (Chiang et al. 2013) was used as heterologous host to express the genes involved in DHN melanin biosynthesis. For constructing the heterologous transformants, one 1.5 Kb up- and downstream of the two *pks* genes were fused with *pyrG* by fusion PCR using the primers listed in Table 1. The fused DNA fragments were then transformed to *wA* locus in *A. nidulans* LO8030 following the protocol described previously (Brock et al. 2008). All constructions were confirmed by diagnostic PCR.

Protection of conidial germination under UV irradiation

The conidia were irradiated by UV (30 W) at a distance of 65 cm for 15 min. After exposure to UV, the conidia were suspended in 20% Tween 80 and reconstituted to a final concentration of 500 cfu/mL using a hemocytometer. A total of 200 μ L was pipetted to plate spreading on GMM agar media. For germination assay, the conidia-spread plates were incubated at 37 °C for 24 h. The germination rate was calculated by the number of germinated conidia divided by the number of conidia in 200 μ L conidia suspension.

Statistical analysis

All determinations were carried out in triplicate and the results are expressed as mean \pm standard deviation (SD). Excel was used to compare the difference between means by *t*-tests. Values of $p < 0.05$ were considered significant.

Results

Phenotype changes of *S. eturmiunum* induced by different levels of NO

The morphogenesis in filamentous fungi often correlates with cellular levels of NO (Chiuchetta and Castro-Prado 2005; Vieira et al. 2009). To probe how NO regulates the development of filamentous fungi, we conducted the culture of onion pathogenic *S. eturmiunum* by supplementing SNP. Under laboratory growth conditions, the fungal colony was densely dotted with brown-colored conidia, and was more densely dotted upon exposure to 0.2 mmol/L SNP. However, the brown-colored conidia were reduced and gradually disappeared upon exposure to SNP at 2, 4, and 8 mmol/L, respectively (Fig. 1A).

Next, we chose 0.2 mmol/L and 2 mmol/L SNP as the external NO source to observe NO-induced changes of phenotype. To confirm the regulatory roles of NO in the changes of phenotype, we also supplemented NO specific scavenger cPTIO (1 mmol/L). The addition of cPTIO resulted in the retarded

mycelial growth and the presence of more albinism mycelia on the surface of colonies especially in control and the cultures supplemented with 0.2 mmol/L SNP (Fig. 1B). To test the possible mechanisms of NO-induced phenotype changes of *S. eturmiunum*, we further used sGC specific inhibitor NS-2080 (18 μ mol/L) to block the formation of cGMP. Intriguingly, addition of NS-2080 resulted in the presence of more albinism mycelia and retarded mycelial growth in control cultures and the cultures containing 0.2 mmol/L SNP (Fig. 1B).

Involvements of NO in the formation of conidia and pseudothecia

As a signaling molecule, NO is involved in conidiation (Wang and Higgins 2005; Gong et al. 2007), and the formation of sexual cleistothecia in *Aspergillus* species (Baidya et al. 2011). To test whether NO affects the formation of the propagules in *S. eturmiunum*, we also assayed its conidiation and the formation of pseudothecia in the cultures supplemented SNP, cPTIO, and NS-2080, respectively. In the control cultures without SNP supplement, conidiation was observed on day 28 post inoculation. Morphologically, the produced conidia intertwine tightly with mycelia, and can hardly be isolated from cultures for counting. To this end, we portrayed conidiation qualitatively by visually comparing the density of the conidia present in the same square of culture plates in different treatments. In the control cultures, the promoted conidiation was observed upon exposure to 0.2 mmol/L SNP, and was largely reduced upon exposure to 2 mmol/L. The presence of cPTIO compromised conidiation in the SNP-supplemented cultures and totally abolished in control cultures. Intriguingly, application of NS-2080 resulted in the impairment of conidiation even in the presence of SNP (Fig. 2A). SNP supplement also increased the formation of pseudothecia especially in the cultures exposed to 2 mmol/L SNP. Similarly, application of cPTIO and NS-2080 inhibited the formation of pseudothecia (Fig. 2B). To further identify the effects of NO on the formation of pseudothecia, we also prepared the cultures exposed to 4 mmol/L and 8 mmol/L SNP to observe the correlation of NO levels with the number of pseudothecia. As shown in Fig. 3A, the maximum number of pseudothecia was observed upon exposure to 2 mmol/L SNP and largely reduced in the cultures exposed to 4 and 8 mmol/L (Fig. 3A). Similar to conidiation, application of cPTIO and NS-2080 all reduced the number of pseudothecia especially in control and the cultures exposed to 0.2 mmol/L SNP (Fig. 3B).

External NO application changes the expression of genes involved in conidiation and the formation of pseudothecia

In *Aspergillus* molds, asexual development is orchestrated by BrlA, AbaA, and WetA transcriptional cascade (Lind et al. 2018). To testify the relevance between conidiation and expression of the three transcription factors in *S. eturmiunum* upon external NO application, we conducted the RT-PCR assay of the genes encoding BrlA (GenBank accession number: MT925646), AbaA (GenBank accession number: MT925645), and WetA (GenBank accession number: MT925644). SNP application at 0.2 mmol/L triggered the maximum increase in transcription levels of *brlA*, *abaA*, and *wetA*. Interestingly, application of cPTIO and NS-2080 all reduced expression levels down to 0.62 folds for *brlA* (Fig. 4A), 0.64 folds for

abaA (Fig. 4B), and 0.59 folds for *wetA* (Fig. 4C), respectively. Similar to conidiation, exposure to 2 mmol/L SNP also caused the maximum increase in transcription levels of *mat1* (up to 4.8 folds) and *mat2* (up to 3.8 folds), the two genes involved in sexual reproduction, and reduced the expression levels of *mat1* and *mat2* upon application of cPTIO and NS-2080, respectively (Fig. 4D, F).

NO-stimulated biosynthesis of melanins correlates with increased conidiation and formation of pseudothecia

Biosynthesis of melanins in fungi plays a crucial role in defending fungal cells against environmental stresses. Accumulation of melanin in fungal propagules contributes to virulence and capacity to survive in harsh surroundings (Valiante et al. 2016). Interestingly, exposure to 2 mmol/L SNP stimulated production of melanins. Application of cPTIO and NS2080 compromised melanin biosynthesis especially in control and the cultures exposed to 0.2 mmol/L SNP (Fig. 5). To determine the possible types of melanins produced in *S. eturmiunum*, we further supplemented arbutin and tricyclazole, the two specific inhibitors for DOPA and DHN biosynthetic pathways, respectively. Upon addition of arbutin or tricyclazole, the dark-brown colonies were bleached to albinism mycelia (Fig. 6A). In addition, supplement of the two inhibitors for melanin biosynthesis also impaired conidiation (Fig. 6B) and the formation of pseudothecia (Fig. 6C, D).

NO production correlates with the biosynthesis of melanin in *S. eturmiunum*

NO is involved in many physiological processes of filamentous fungi and the production of NO has been demonstrated in many fungal species although the search for the genes coding for NOS-like proteins (NOSL) in fungal genomes remains unfruitful (Li et al. 2010). To test the capacity to produce NO by *S. eturmiunum*, we used the term “NO-producing capacity” to stand for NOS-like activity. In addition, we also assayed NO levels under the supplement of SNP, arbutin, and tricyclazole, respectively. Similar to other NO-producing filamentous fungi, NO-producing capacity was also observed in *S. eturmiunum*, and was reduced following supplement of SNP. Reduction in NO-producing capacity was also seen in the cultures supplemented with arbutin or tricyclazole. Intriguingly, simultaneous supplement of SNP and arbutin, or SNP and tricyclazole further reduced NO producing capacity (Fig. 7A). Accordingly, NO levels were also reduced evidently in the cultures supplemented with arbutin or tricyclazole (Fig. 7B). Notably, following the reduction in NO levels, melanin accumulation was decreased remarkably even in the presence of SNP (Fig. 7C). In order to further correlate NO production and melanin biosynthesis, we analyzed NO-producing capacity in 5-AC-treated *S. eturmiunum*. Obviously, 5-AC treatment resulted in the impairment in NO production, the activity of tyrosinase, as well as melanin biosynthesis, which coincides with the cultures supplemented with arbutin and tricyclazole (Fig. 8A, B). Genome analysis resulted in the annotation of two polyketide synthase genes possibly involved in DHN-melanin synthesis (PKS1 and PKS2, GenBank accession numbers: MT925643 and MT925643) (Pal et al. 2014; Akamatsu et al. 2010), and one tyrosinase gene *tyr* (GenBank accession numbers: MT925641) for DOPA-melanin biosynthesis (Choi et al. 2012; Kumar et al. 2015). Intriguingly, SNP treatment resulted in 9.4 folds increased transcription for *pks1*, 5.4 folds increased transcription for *pks2*, and 7.4 folds increased transcription for *tyr*. In contrast,

5-AC treatment down regulated I to 0.19 folds in transcription for *pks1*, 0.065 folds for *pks2*, and 0.094 folds for *tyr* (Fig. 8C). To further identify the function of *pks1* and *pks2*, we also constructed the heterologous transformants of *pks1* and *pks2* using *wA* locus in *Aspergillus nidulans* LO8030. Heterologous expression of *pks1* and *pks2* in *wA* locus resulted in the presence of brown pigment in the conidia of either *pks1* mutant or *pks2* mutant (Fig. 9A). Moreover, the two transformants showed similar germination rate with conidia of *A. nidulans* after UV irradiation (Fig. 9B). Collectively, melanin biosynthesis is also dependent on cellular levels of NO in *S. eturmiunum*.

Discussion

The formation of conidia and pseudothecia of the onion pathogenic *S. eturmiunum* is the critical stage for its transmission. However, how the development of the two propagules is regulated remains to be fully understood. In the study, we showed that NO is necessary for conidiation and the formation of pseudothecia in *S. eturmiunum*. Application of NO scavenger cPTIO abolishes the formation of conidia and pseudothecia, as well as melanin production. In control cultures supplement of SNP results in an increased formation of conidia at 0.2 mmol/L, and pseudothecia at 2 mmol/L. SNP supplement also triggered increased biosynthesis of melanin, which can be inhibited upon addition of either arbutin or tricyclazole, the specific inhibitors for DOPA and DHN melanin synthetic pathway, respectively. Moreover, the enhanced biosynthesis of melanin coincides with the increased formation of the two propagules that are impaired following the inhibition of melanin biosynthesis (Fig. 10).

The small free radical NO is a short-lived but highly reactive diatomic gas (Brüne 2010). Because it is highly diffusible within the cell and through cell membranes (Lancaster 1997), NO functions as a transient, local, intracellular or intercellular signaling molecule in diverse biological systems (Culotta & Koshland 1992). One of the important mechanisms that NO regulates physiological processes of living system is the binding of NO with transition metals of metalloproteins such as sGC, a hemoprotein that has evolved to bind selectively with NO (Boon & Marletta 2005). The activation of sGC by NO results in the production of the secondary messenger cyclic GMP (cGMP) (Friebe & Koesling 2003) that binds to protein kinase G (PKG), and forms the central downstream mediator of NO-cGMP signaling pathway, and mediates pathway-specific cellular responses via the phosphorylation of phosphorylation-dependent transcriptional factors, such as the cAMP-response-element-binding protein (Contestabile 2008), and activates its downstream targets (Tomankova et al. 2017).

In filamentous fungi, endogenous production of NO correlates with mycelial growth and conidia formation (Marcos et al. 2020). In *A. nidulans*, increased production of NO was detected in the transition from vegetative growth to conidiation (Marcos et al. 2016). Similar to *A. nidulans*, our results showed that the presence of NO-producing capacity followed by intracellular production of NO correlates with the regular conidiation, which is compromised following the addition of NO scavenger cPTIO. This indicates that maintaining a certain level of cellular NO underpins the vegetative growth and asexual development in *S. eturmiunum*. Interestingly, addition of NO-releasing compound SNP resulted in an evident reduction in NO-producing capacity (Fig. 7A), which suggests that NO-producing capacity can be self-regulated in

response to alteration of the cellular NO levels. In addition, SNP supplement at concentrations lower than 0.2 mmol/L also promotes conidiation, which implicates that external application of NO at lower concentrations favors the transcription of *brlA*, *abaA*, and *wetA*, the three transcriptional factors involved in conidiation (Chen et al. 2020).

Cellular NO levels also affect sexual development in filamentous fungi (reviewed by Zhao et al. 2020). In *Aspergillus nidulans*, the increase in cellular NO levels by disrupting the genes encoding flavohemoglobin or supplementing NO-releasing compound promotes the formation of cleistothecia (Baidya et al., 2011). Similar to *A. nidulans*, supplement of SNP at 2 mmol/L increased transcription of *mat1* and *mat2* followed by increased formation of pseudothecia, which indicates that the higher NO levels also tend to induce sexual development in *S. eturmiunum*.

Melanins production in fungi is believed to protect hyphae and propagules from environmental stresses (Bell and Wheeler 1986; Butler and Day 1998), and to serve as a virulence factor (Langelder et al. 2003; Nsanchuk and Casadevall, 2003). Disruption of melanin biosynthesis compromised fungal ability for survival and longevity (Engh et al. 2007). In *Pestalotiopsis microspore*, the production of polyketide-derived DNH melanin is required for the formation of integrated conidia and viability in addition to morphogenesis, germination and viability (Yu et al. 2015). Moreover, production of DNH melanin is also linked to the development of sexual fruiting body, and is controlled by specific regulatory genes involved in sexual differentiation in *Sordaria macrospora* (Engh et al. 2007). In our study, external addition of NO also promotes the transcription of the two genes encoding two polyketide synthases, and the gene coding for tyrosinase followed by increased melanin accumulation and formation of conidia and pseudothecia. Notably, inhibiting biosynthesis of DOPA and DNH melanin all lead to the impairment in the formation of conidia and pseudothecia. This indicates that biosynthesis of both DOPA and DNH melanin is required for asexual and sexual development in *S. eturmiunum*.

The melanins present in fungal cells are involved in their protection from UV radiation desiccation, salinity and oxidation (Pacelli et al. 2020). Melanins in phytopathogenic or human pathogenic fungi are also involved in the protection against ROS attack from host defense (Papon et al. 2020). In filamentous fungi, most ascomycetes produce DHN-melanin (Gonçalves et al. 2012). While in the ubiquitous human-pathogenic fungus *Aspergillus fumigatus*, the pathogen that causes fatal lung infection in immunocompromised individuals, produces both DOPA and DNH melanins (Langfelder et al. 2003). The presence of DNH melanin in conidia protects from phagocytic uptake and ROS-induced intracellular killing by frugivorous amoeba *Protostelium aurantium* and disrupts its autonomous defense (Ferling et al. 2020). In *Sporothrix schenckii*, production of DOPA melanin in the presence of tyrosine in fungal cell confers more resistance to nitrogen-derived oxidants and UV irradiation (Almeida-Paes et al. 2012). In our study, heterologous expression of *pks1* and *pks2* all resulted in the production of pigmented conidia with resistance to UV irradiation. In this context, the production of DOPA and DNH melanins found in phytopathogenic *S. eturmiunum* and their tight connections with the formation of conidia and pseudothecia implicate that this fungus possesses more resistance to the attacks from environmental stressors than those producing DNH melanins alone.

The primary sensor of NO soluble guanylate cyclase (sGC) orchestrates NO-cGMP-PKG signaling pathway, and has been implicated in many essential physiological processes and disease conditions in mammals (Kang et al. 2019). Several studies have reported that sporulation in filamentous fungi is promoted by application of external NO but inhibited by sGC inhibitor L-NAME or ODQ. However, sporulation can be restored by exogenous cGMP (Zhao et al. 2020). In our study, NO promoted formation of conidia and pseudothecia, and melanin production can also be compromised by sGC specific inhibitor NS-2080 even in the presence of SNP. This implicates the existence of NO-cGMP-PKG signaling pathway in *S. eturmiunum* that mediates the increased transcription of the genes encoding the downstream regulators for conidiation, formation of pseudothecia and melanin biosynthesis.

It should be noted that, in our study, in contrast to the increased formation of conidia and pseudothecia by lower concentrations of SNP (≤ 2 mmol/L), external supplement of SNP at higher concentrations (≥ 4 mmol/L) inhibited or compromised the formation of the two propagules and melanin biosynthesis. This indicates that higher concentrations of NO can covalently binds with thiols in active cysteine residues (Amal et al. 2019; Heinrich et al. 2013), leading to S-nitrosylation of active proteins and subsequent negative feedback of NO signaling (Zhao et al. 2016).

This study revealed the NO-mediated mechanisms in regulating asexual and sexual development of onion blight pathogen *S. eturmiunum*. Further studies should be directed to the identification of downstream transcription factors of NO-cGMP-PKG, which is undertaking in our lab. However, our data reveal, for the first time, that the cellular levels of NO determines the fate of asexual and sexual development of *S. eturmiunum*. Moreover, the specific requirement for NO by *S. eturmiunum* in the development of conidia and pseudothecia implicates a possible strategy to curb the transmission of this onion pathogen by applying higher concentration of NO-releasing compound to impede its asexual and sexual development.

Conclusion

Our results confirmed that NO is necessary for conidiation and the formation of pseudothecia in *S. eturmiunum*. Conidiation is promoted upon exposure to 0.2 mmol/L SNP, while pseudothecia formation is enhanced upon exposure to 2 mmol/L SNP. Application of NO scavenger cPTIO and sGC specific inhibitor NS2080 abolishes their formation of conidia and pseudothecia, as well as melanin production. In addition, melanin accumulation in the fungus is achieved either by DOPA and DHN melanin synthetic pathway, which correlates with its asexual and sexual development, and confers the capacity for *S. eturmiunum* to transmit and infect effectively even in harsh environment.

Abbreviations

5-AC: 5-azacytidine; cAMP: cyclic AMP; cGMP: cyclic GMP; cPTIO: carboxy-CPTIO; DHN: dihydroxynaphthalene; DOPA: L-dihydroxyphenylalanine; MAPK: mitogen-activated protein kinase; NO:

nitric oxide; PKC: protein kinase C; PKS: polyketide synthase; sGC: soluble guanylate cyclase; TYR: tyrosinase

Declarations

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Adherence to national and international regulations

Not applicable.

Authors' contributions

YZ, XZ and WZ designed the experiments, analyzed the data, and WZ and YZ wrote the manuscript. YZ, WY and MS performed the experiments. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Competing interests

The authors declare that they have no competing interests.

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Figures

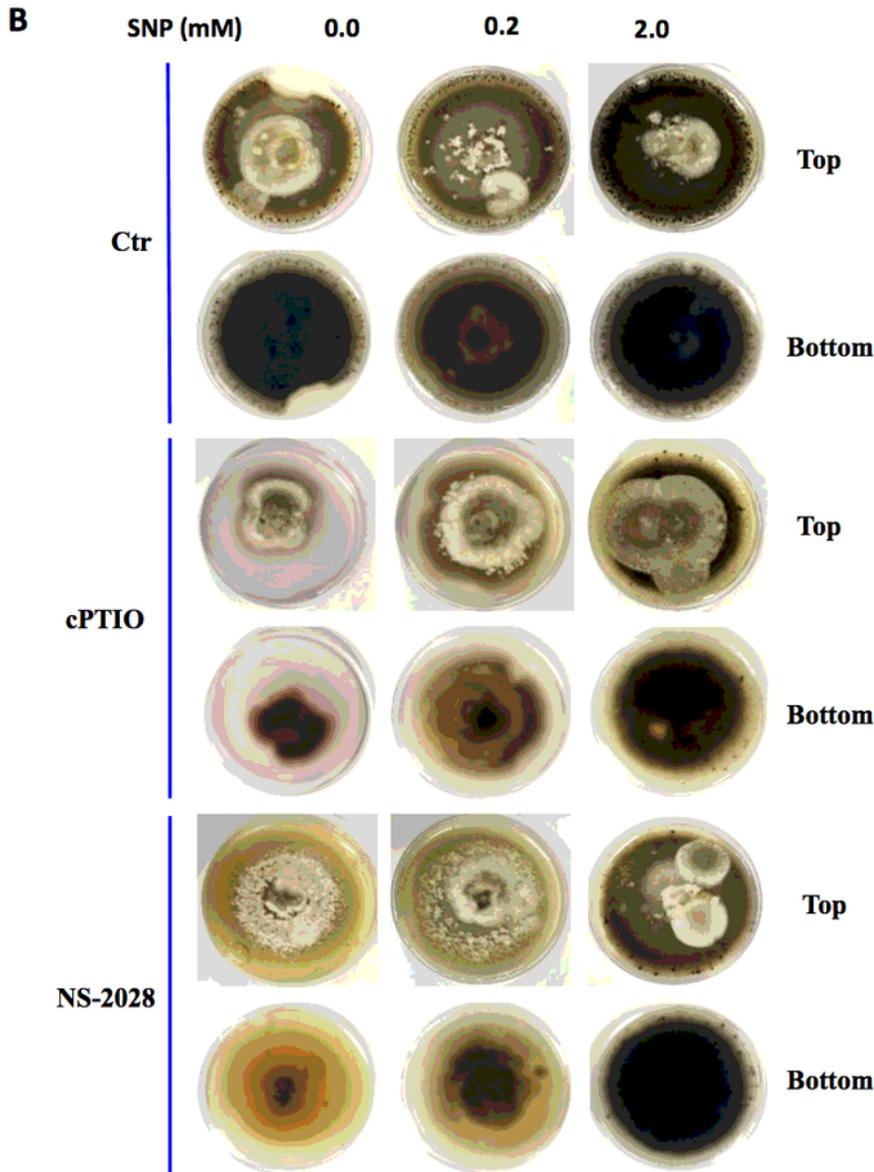
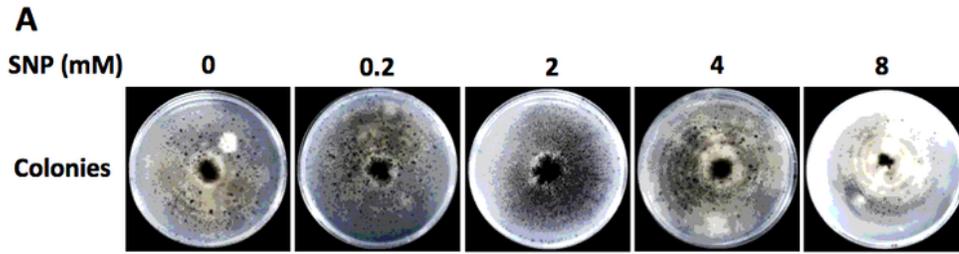


Figure 1

Effects of nitric oxide on growth and development of *S. eturmiunum*. (A) Changes in phenotype induced by SNP at concentrations of 0, 0.2, 2, 4 or 8 mmol/L, respectively. (B) Changes in phenotype in the absence or presence of SNP (0.2 mmol/L and 2 mmol/L), cPTIO (1 mmol/L) or NS-2028 (18 μ mol/L). SNP, sodium nitroprusside; cPTIO, arboxy-PTIO; NS-2028, inhibitor of sGC.

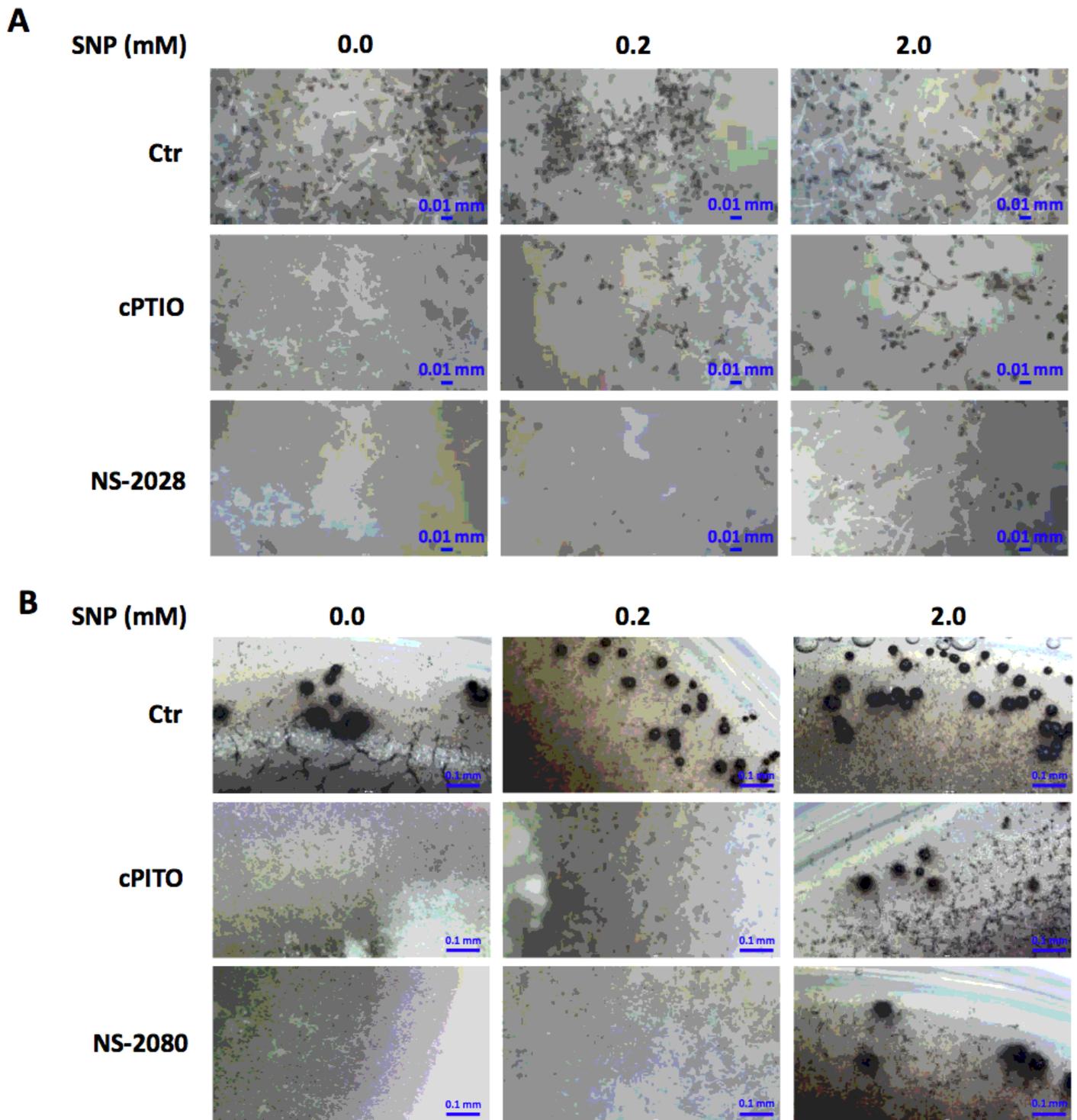


Figure 2

Propagules formation of *S. eturmiunum* in presence of SNP and/or NO scavenger and sGC inhibitor. (A) Conidia formation in the absence or presence of SNP at concentrations of 0.2 and 2 mmol/L, or the presence of cPTIO at 1 mmol/L or NS-2028 at 18 μ mol/L. The blue bar = 0.01 mm. (B) Pseudothecia formation in the absence or presence of SNP at concentrations of 0.2 and 2 mmol/L, or the presence of

cPTIO at 1 mmol/L or NS-2028 at 18 μ mol/L. The blue bar = 0.1 mm. SNP, sodium nitroprusside; cPTIO, carboxy-PTIO; NS-2028, sGC inhibitor.

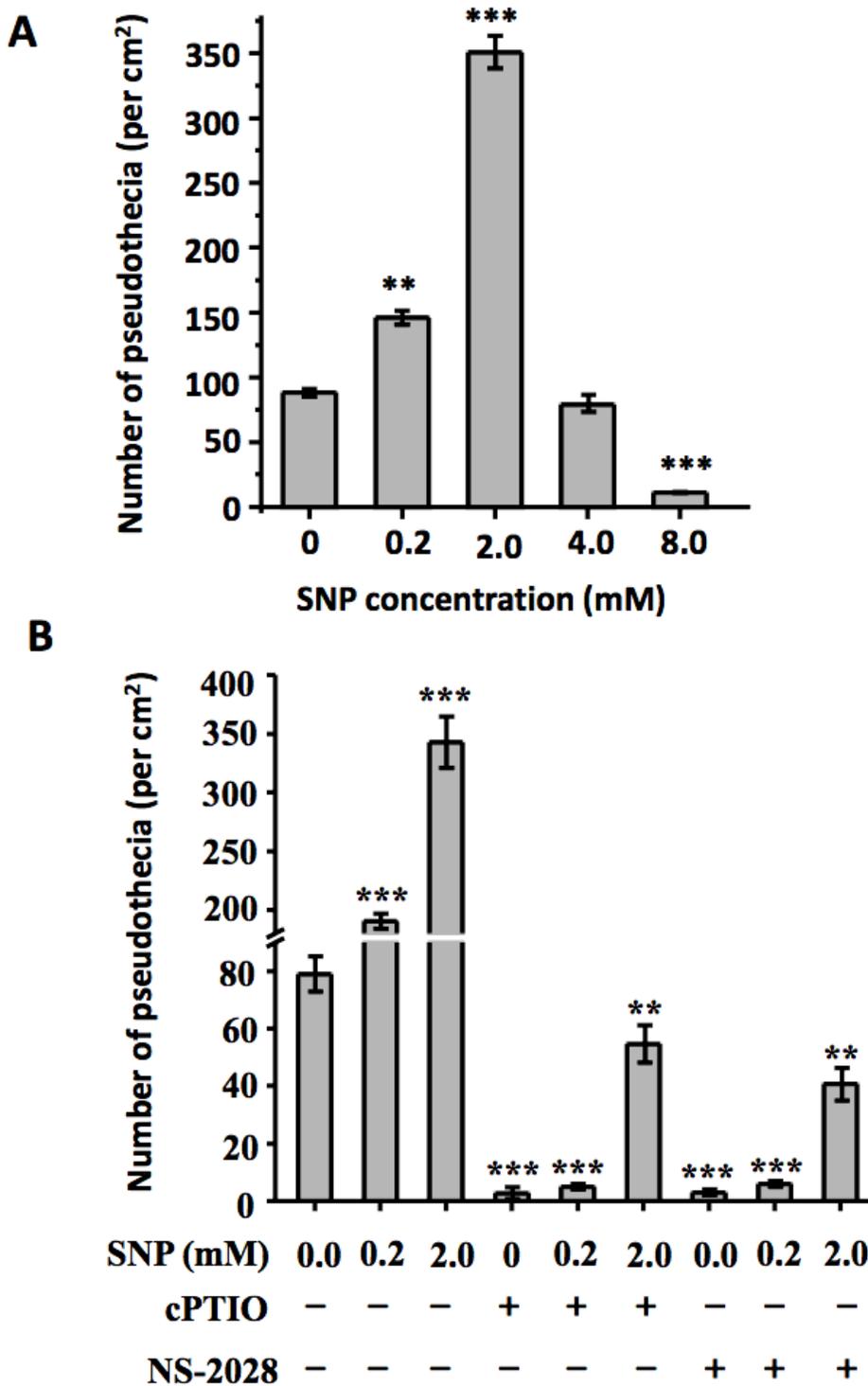


Figure 3

Effects of nitric oxide on pseudothecia formation in *S. eturmiunum*. (A) Numbers of pseudothecia induced by SNP at the concentrations of 0, 0.2, 2, 4 or 8 mmol/L, respectively. (B) Numbers of pseudothecia in the cultures in the absence or presence of SNP at concentrations of 0.2 and 2 mmol/L, or

the presence of cPTIO at 1 mmol/L or NS-2028 at 18 μ mol/L. SNP, sodium nitroprusside; cPTIO, carboxy-PTIO; NS-2028, inhibitor of sGC. Values are means \pm SD (n=3), error bars represent SD. ** P<0.01, *** P<0.001 by t test.

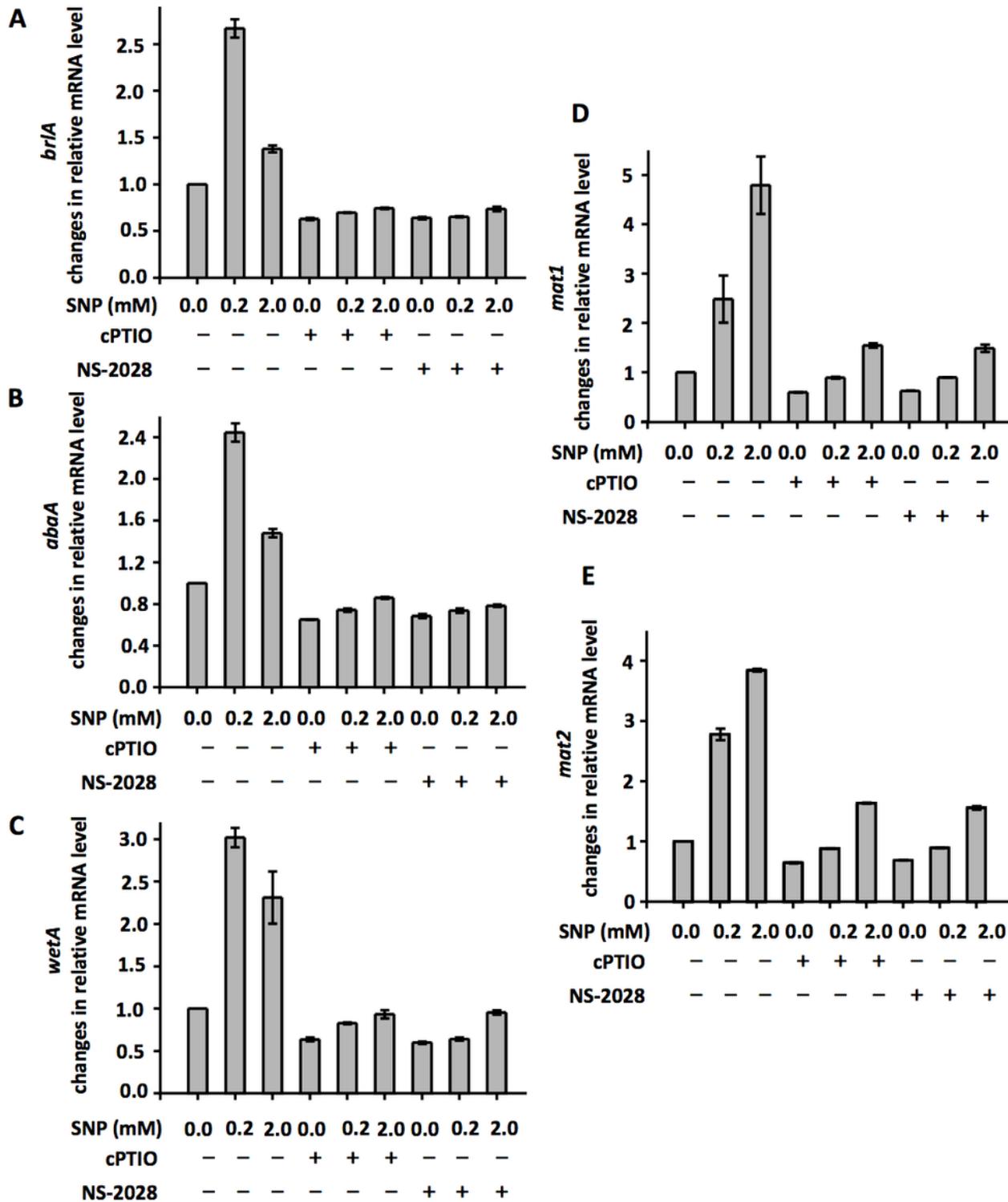


Figure 4

Changes in expression of genes involved in asexual and sexual development in *S. eturmiunum*. (A) Relative expression levels of *brlA*. (B) Relative expression levels of *abaA*. (C) Relative expression levels of

wetA. (D) Relative expression levels of mat1. (E) Relative expression levels of mat2. Actin was used as internal reference. Data are the average of at least three independent experiments, and err bars are the standard error of the mean.

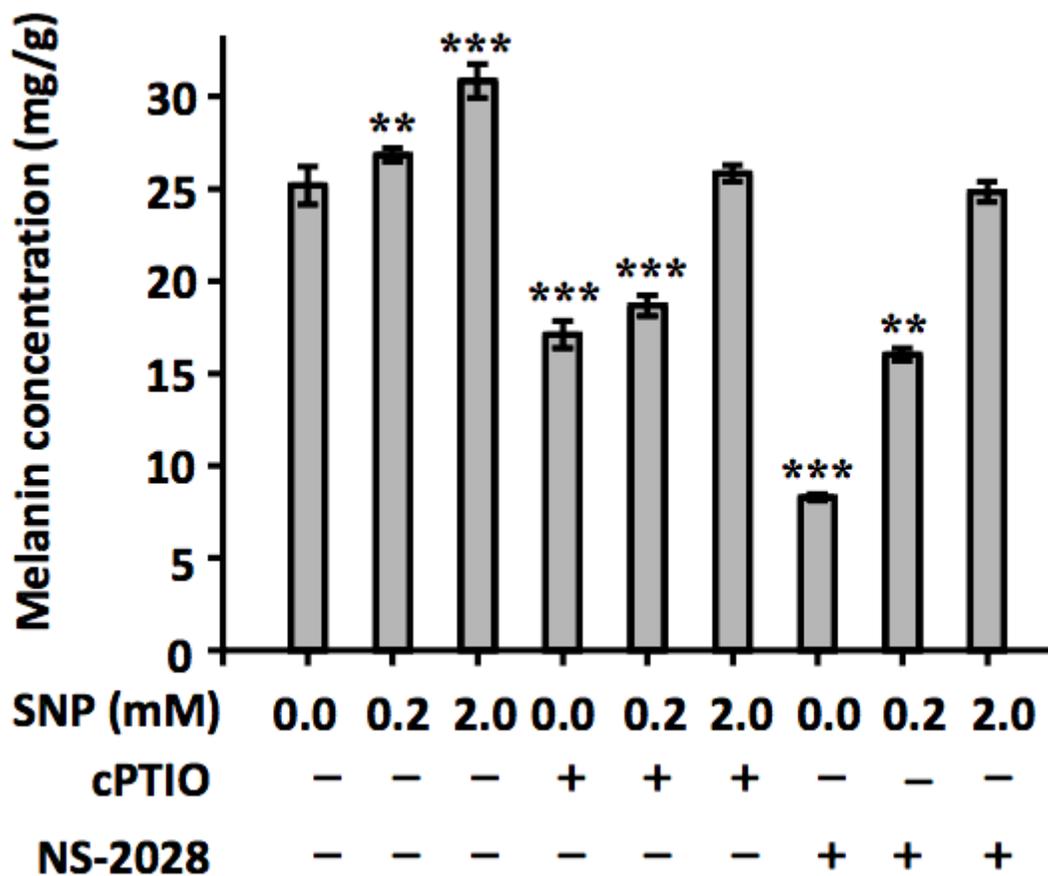


Figure 5

Accumulation of melanin in absence or presence of SNP, or simultaneously with cPTIO or NS-2028. SNP at 0.2 or 2 mmol/L, cPTIO at 1 mmol/L, NS-2028 at 18 μ mol/L. SNP, sodium nitroprusside; cPTIO, carboxy-PTIO; NS-2028, inhibitor of sGC. Values are means \pm SD (n=3), error bars represent SD. ** P<0.01, *** P<0.001 by t test.

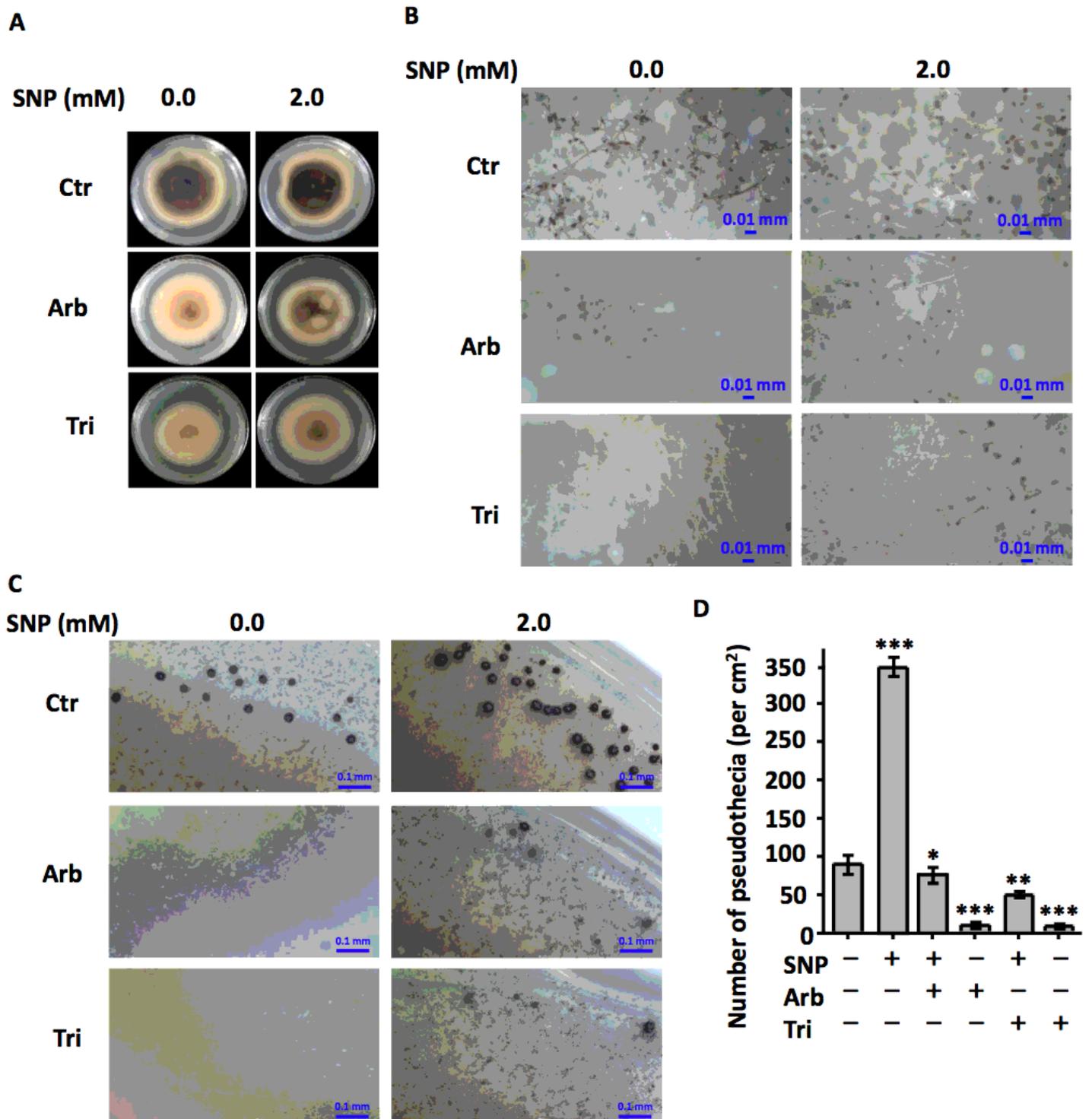


Figure 6

Correlation between melanin biosynthesis and propagules formation in presence of SNP and/or arbutin and tricyclazole. (A) Changes in phenotype. (B) Conidia formation. The blue bar = 0.01 mm. (C) Pseudothecia formation. The blue bar = 0.1 mm. (D) Numbers of pseudothecia. SNP at 2 mmol/L, arbutin at 30 mg/L, tricyclazole at 30 mg/L. SNP, sodium nitroprusside; Arb, arbutin; Tri, tricyclazole. Values are means \pm SD (n=3), error bars represent SD. * P<0.05, ** P<0.01, *** P<0.001 by t test.

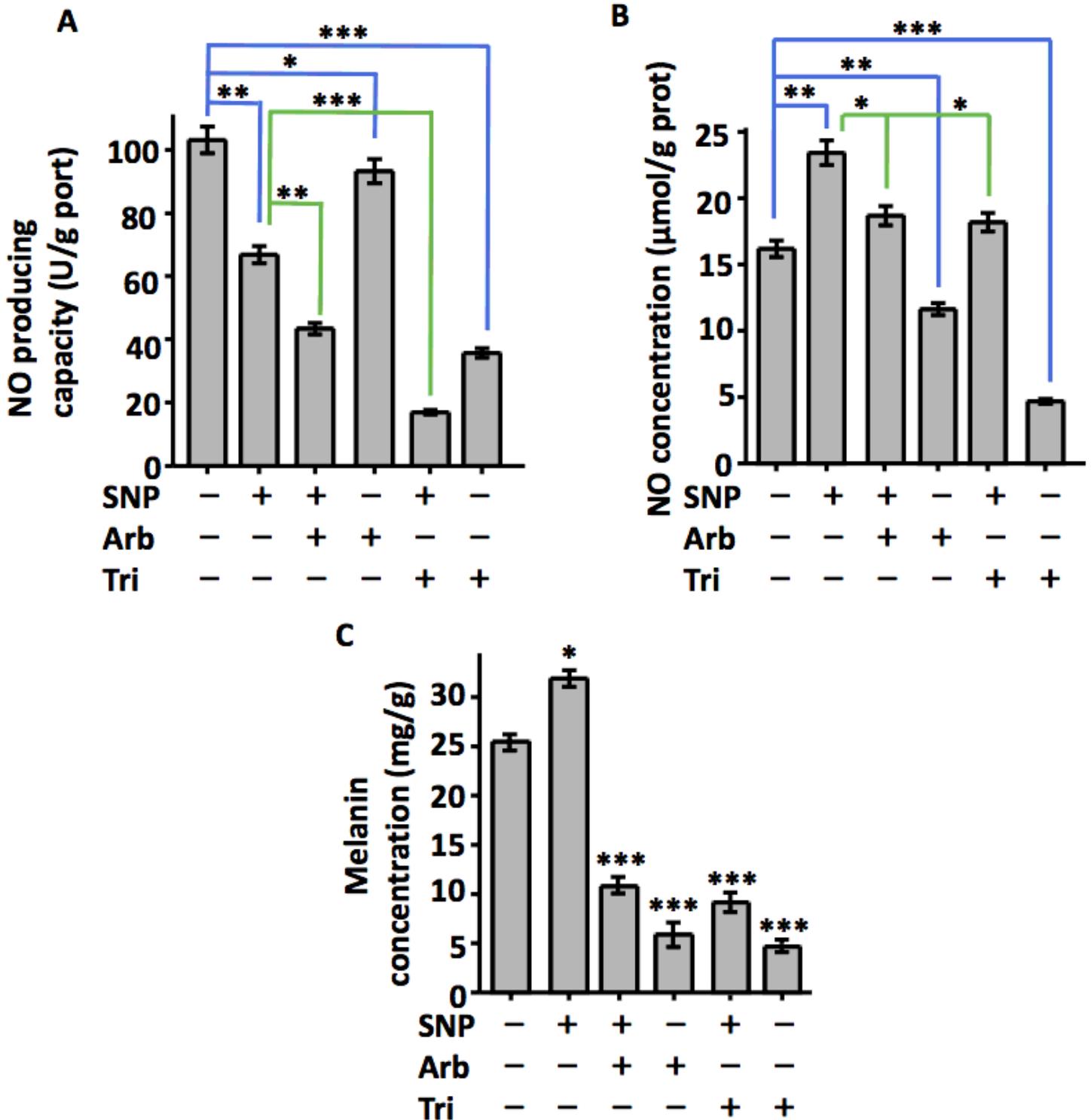


Figure 7

Effect of SNP and/or melanin synthesis inhibitors on NO and melanin production in *S. eturmiunum*. (A) NO producing capacity in the absence or presence of SNP (2 mmol/L), arbutin (30 mg/L) or tricyclazole (30 mg/L). (B) Concentration of NO in the absence or presence of SNP (2 mmol/L), arbutin (30 mg/L) or tricyclazole (30 mg/L). (C) Accumulation of melanin in the absence or presence of SNP (2 mmol/L),

arbutin (30 mg/L) or tricyclazole (30 mg/L). SNP, sodium nitroprusside; Arb, arbutin; Tri, tricyclazole. Values are means \pm SD (n=3), error bars represent SD. * P<0.05, ** P<0.01, *** P<0.001 by t test.

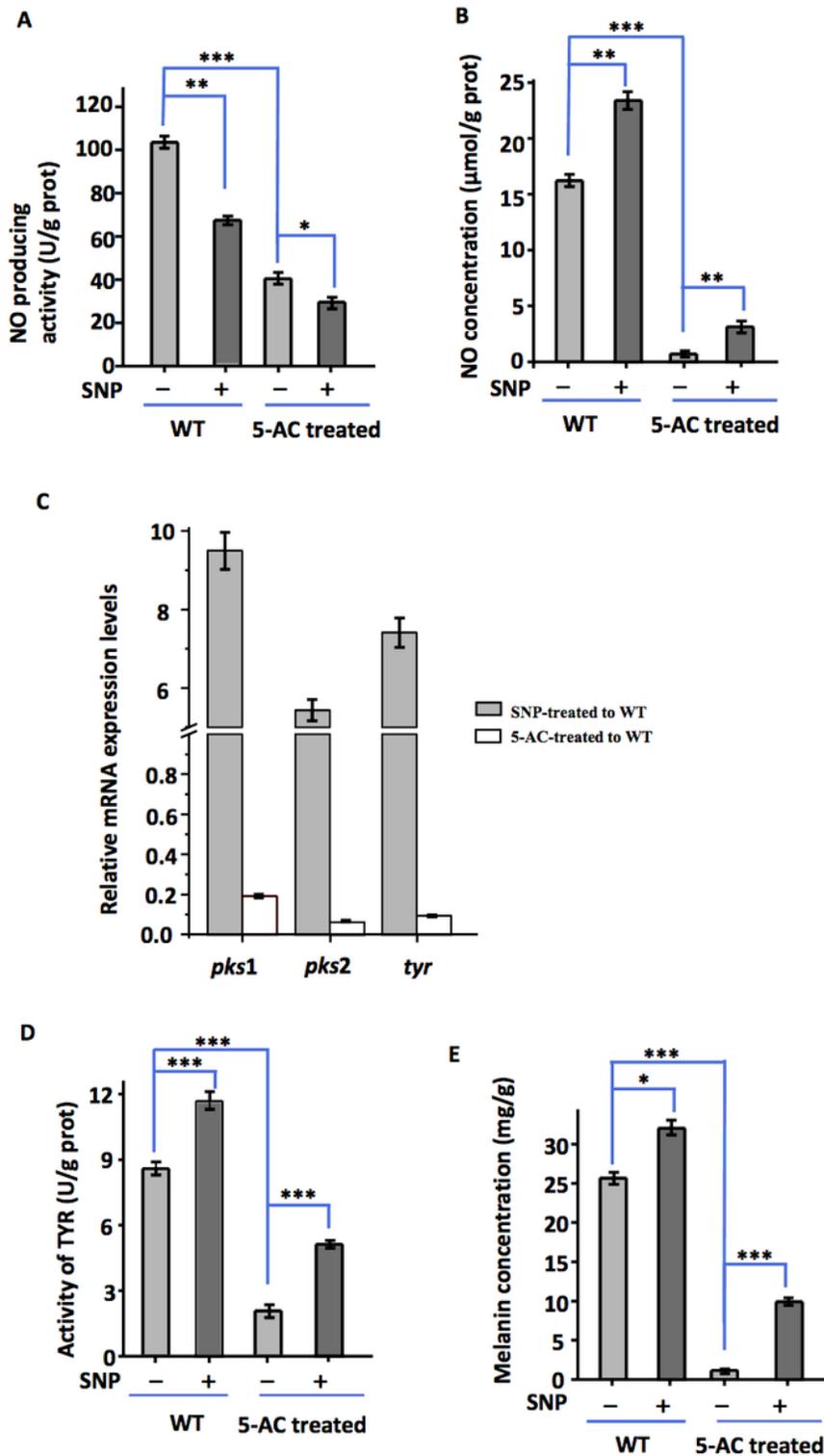


Figure 8

Effect of SNP on NO and melanin formation in wild-type and 5-AC treated *S. eturmiunum*. (A) Effect of SNP (2 mmol/L) on NO producing capacity. (B) NO concentration in wild type and 5-AC treated *S. eturmiunum* induced by SNP (2 mmol/L). (C) Relative mRNA expression levels of *pks* and *tyr* related to

the melanin synthase in the wild type and 5-AC treated *S. eturmiunum* (D) Tyrosinase activity in wild type and 5-AC treated *S. eturmiunum*. (E) SNP (2 mmol/L) induced changes in melanin accumulation. SNP, sodium nitroprusside. Actin was used as internal reference. Values are means \pm SD (n=3), error bars represent SD. * P<0.05, ** P<0.01, *** P<0.001 by t test.

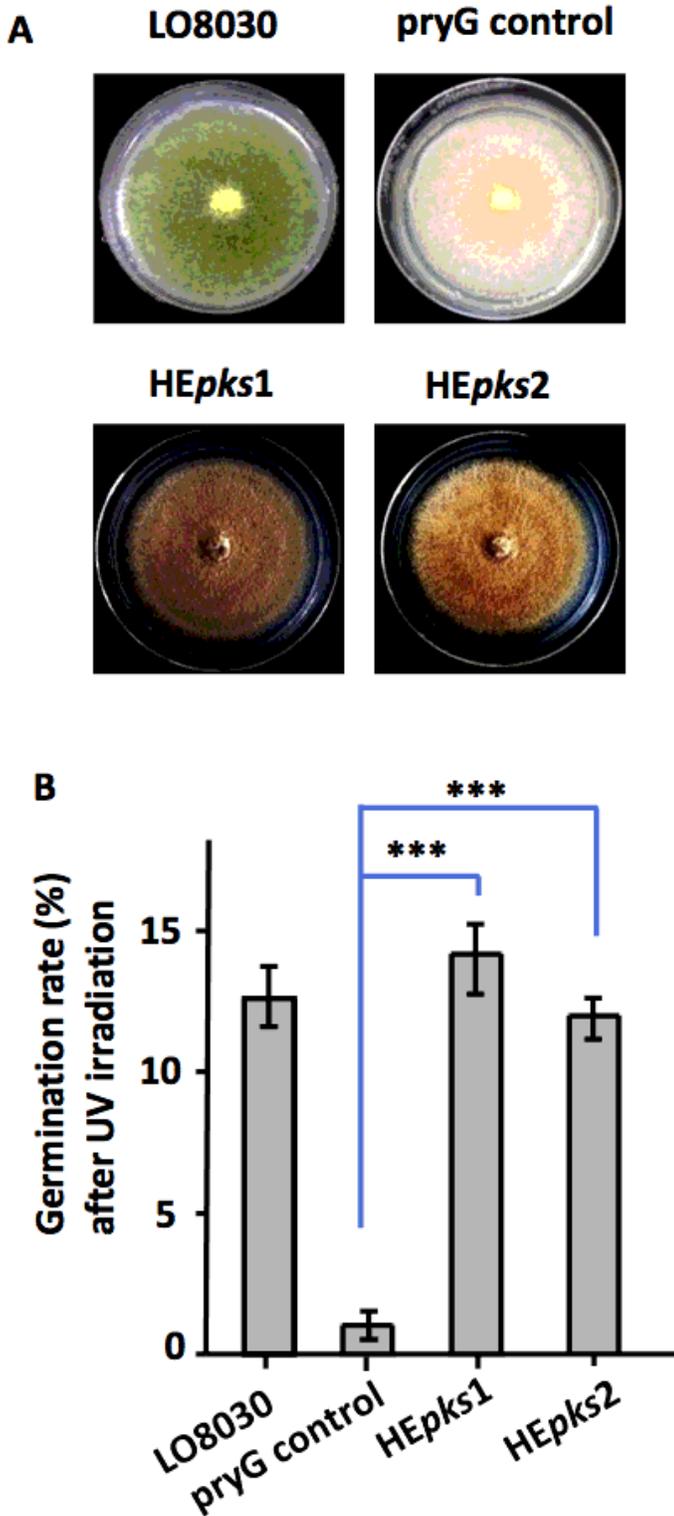


Figure 9

Heterologous expression of pks and resistance of the conidia in the transformants against UV irradiation. (A) The genes encoding PKS1 and PKS2 are responsible for producing the dark-brown pigments. (B) Conidia germination upon exposure to UV irradiation. The conidia were irradiated by UV (30 W) at a distance of 65 cm for 15 min. LO8030: *Aspergillus nidulans* LO8030; pyrG control: pyrG related wA in *A. nidulans* LO8030; HEpks1: heterologous expression of pks1 in host strain *A. nidulans* LO8030; HEpks2: heterologous expression of pks2 in host strain *A. nidulans* LO8030. Results are means \pm SD (n=3), error bars represent SD. *** P<0.001 by t test.

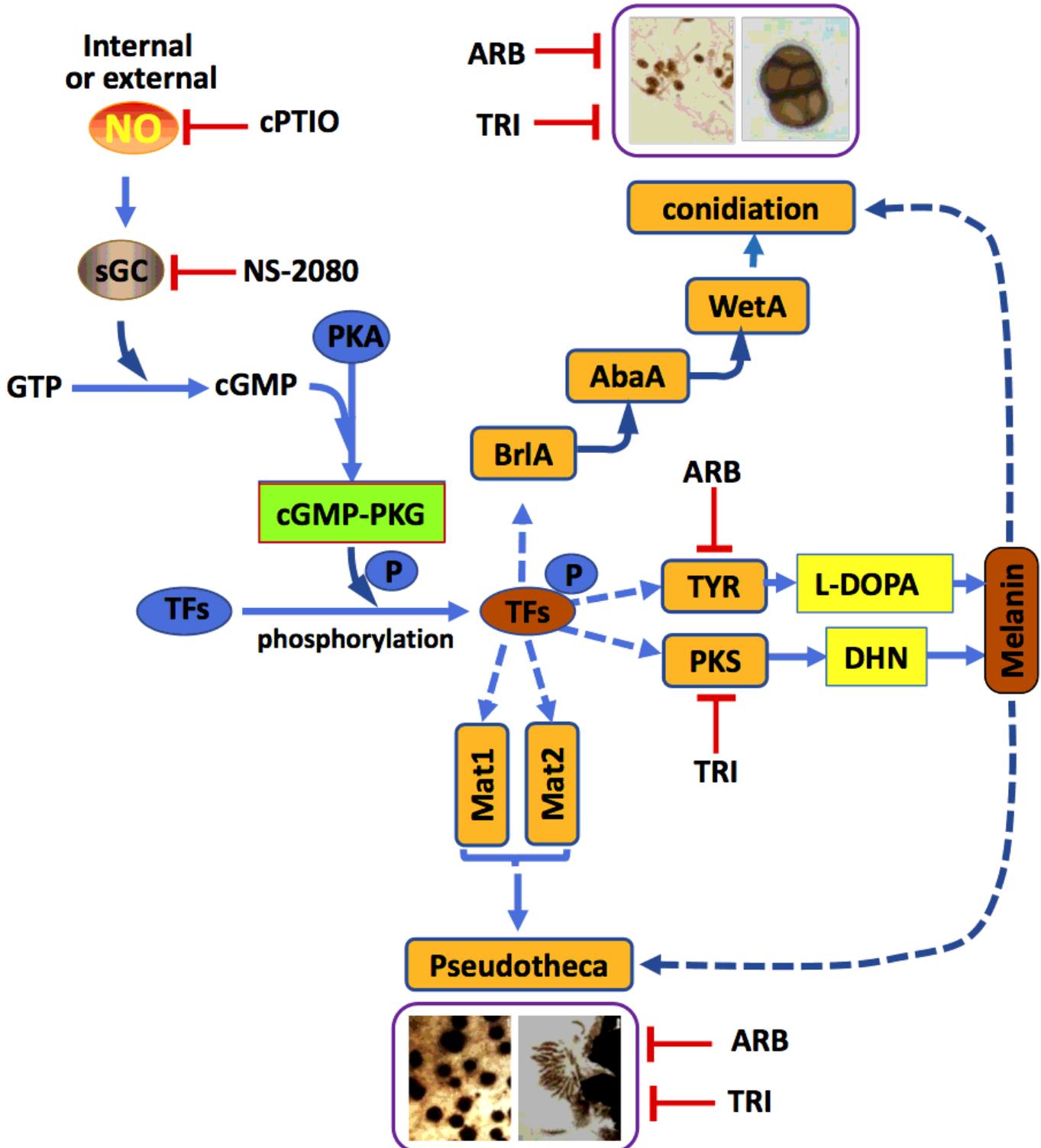


Figure 10

The proposed mechanism mediated by NO in propagules formation and melanins production in *S. eturmiunum*. DHN, dihydroxynaphthalene pathway; DOPA, 3,4-dihydroxyphenylalanine pathway; NO, nitric oxide; PKS, polyketide synthase; SNP, sodium nitroprusside; TYR, tyrosinase; cPTIO, carboxy-PTIO; NS-2028, inhibitor of sGC; Arb, arbutin; Tri, tricyclazole.