



Kynurenine pathway as alternative biosynthetic pathway for fusaric acid in *Fusarium oxysporum* f.sp. *cubense*

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Abstract

Banana fusarium wilt caused by *Fusarium. oxysporum* f.sp. *cubense* race 4, has already become one of the main diseases in banana production. Previous research suggests that Foc can secrete two phytotoxins, respectively, fusaric acid and beauvericin, to contribute to its pathogenicity when it infects bananas. A gene cluster *FUB* consisting of 12 genes has been identified for fusaric acid biosynthesis in several *Fusarium* spp, including *Fusarium. oxysporum* f.sp. *cubense* race 4, *F. oxysporum* f.sp. *lycopersici* etc. In the present study, using reverse genetic research methods, we report the evidence for the kynurenine pathway as alternative biosynthetic pathways of fusaric acid in *F. oxysporum* f.sp. *cubense* 4. In the meanwhile, our experiments suggests that the beauvericin might not directly contribute to the pathogenicity to banana of *F. oxysporum* f.sp. *cubense* 4.

Keywords Banana fusarium wilt · Fusaric acid · *FUB* gene cluster · Kynurenine pathway · Beauverin

Introduction

Banana Fusarium wilt, a soil-borne disease caused mainly by *F.oxysporum* f.sp. *cubense* 4 (Foc4), has already become one of the main diseases in banana production (Ploetz 2006, 2015). Followed by rhizome vascular colonization, Foc4 can penetrate into banana roots from the intercellular space of the epidermis and wounds, and then spread upwards to the banana corms and pseudostems, causing diseases such as corm browning, leaf yellowing, and plant death (Warman and Aitken 2018).

Previous studies have shown that when Foc4 infects bananas, it can secrete two phytotoxins, fusaric acid (IUPAC Name: 5-butylpyridine-2-carboxylic acid or 5-butylpicolinic acid) and beauvericin (IUPAC Name: 3,9,15-tribenzyl-4,10,16-trimethyl-6,12,18-tri(propan-2-yl)-1,7,13-trioxa-4,10,16-triazacyclooctadecane-2,5,8,11,14,17-hexone), to contribute to its pathogenicity (Pegg and Langdon 1987; Li et al. 2013; Dong et al. 2014). However, one research has suggested that the amount of beauvericin secreted by Foc4 during the infection process is very low (Li et al. 2013), whether is one of phytotoxins to its pathogenicity remains uncertainty.

Fusaric acid, formed by adding a butyl group to the 5-position C of 2-picolinic acid (IUPAC name: pyridine-2-carboxylic acid or 2-picolinic acid), is a non-host-specific toxin produced by plant pathogenic fungi-Fusarium in the process of their infecting hosts (Bacon et al. 1996; Niehaus et al. 2014a). This may induce ROS bursts, thus resulting in undue lipid peroxidation harmful for cellular membrane system (Singh and Upadhyay 2014), which allows the direct diffusion of water from those damaged cells, eventually giving rise to leaf wilting and yellowing (Dong et al. 2014). The butyl side chain of fusaric acid can be modified to form various homologous compounds, such as 9-hydroxy fusaric acid (IUPAC name: 5-(3-hydroxybutyl)pyridine-2-carboxylic acid), 8-hydroxy fusaric acid(IUPAC name: 5-(2-hydroxybutyl) pyridine-2-carboxylic acid), 9,10-dehydro fusaric acid (IUPAC name:5-but-3-enylpyridine-2-carboxylate) (Crutcher et al. 2017),

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10-hydroxy-11-chloro fusaric acid (IUPAC name: 5-(4-chloro-3-hydroxybutyl)pyridine-2-carboxylic acid) and fusaricates A (IUPAC name: 2,3-dihydroxypropyl 5-butylpicolinate) (Liu et al. 2016). The parent nucleus of fusaric acid is 2-picolinic acid which also possesses some phytotoxicity, yet is significantly lesser phytotoxicity than fusaric acid. Addition of alkyl groups to the 5-position C of 2-picolinic acid results in a gradual increased phytotoxicity when the length of the alkyl group increases. Thus, phytotoxicity is increased in the order of 2-picolinic acid < 5-ethylpicolinic acid < 5-propylpicolinic acid < 5-butylpicolinic acid (i.e., fusaric acid) < 5-pentylpicolinic acid. Nonetheless, compared with 5-pentylpicolinic acid, 5-heptylpicolinic acid has lower toxicity (Stipanovic et al. 2010).

Previous research has suggested that fusaric acid is a secondary metabolite derived from polyketones (Brown et al. 2012). It is suggested by the universally accepted rule for fungi that, genes that participate in secondary metabolite biosynthesis are generally located near each other in the chromosome for the sake of forming the gene cluster (Keller and Bennett 2005; Shwab and Keller 2008). Typically, the *FUB* gene cluster, which includes one gene that encodes polyketide synthase (*FUB1*) together with 11 additional genes (*FUB2-FUB12*), is recognized to be involved in the biosynthesis of fusaric acid.

Based on sequence similarity analysis, this cluster gene is conserved in *F. fujikuroi*s, *F. verticillioides*, *F. oxysporum* f.sp. *lycopersici*, *F. oxysporum* f.sp. *cubense* (Liu et al. 2019). According to the gene functions and annotations in this gene cluster, a putative biosynthetic pathway, the *FUB* gene cluster pathway, has been proposed (Studt et al. 2016).

Foc4 is likely to use *FUB* gene cluster for the synthesis of fusaric acid, just as that in *F. oxysporum* f.sp. *lycopersici*, because they are the species with identical evolution (Ding et al. 2018). However, in the Foc4 infection process to bananas, the conditions required for high-yielding fusaric acid through the *FUB* gene cluster pathway, such as high nitrogen and intermittent light (Wiemann et al. 2013; Niehaus et al. 2014b), are not available that there was no enough nitrogen and light in plant root vascular tissues. According to the transcriptomes of Foc4, the expression of *FUB1* gene was down-regulated by 9.13 times in natural conditions than on a medium containing sufficient nitrogen (Guo et al. 2014).

Previous studies on biosynthesis of fusaric acid were conducted under laboratory culture conditions, such as the optimal medium for toxin-producing, alternating darkness and light, and sufficient nitrogen supply, which show great difference from natural conditions. As everyone knows, natural nutritional conditions can provide more precursors and inducers for the biosynthesis of secondary metabolites, thereby activating more biosynthetic pathways to produce more types of secondary metabolites. Therefore, in previous studies, the species of phytotoxin produced by *Fusarium* may be lost due to the culture medium.

Additionally, the study has also shown that in Foc4 and *F. oxysporum* f.sp. *lycopersici*, even if the *FUB* pathway was completely interrupted, it remained the pathogenicity to its host (Brown et al. 2015; Ding et al. 2018). These studies suggest that there might be other mycotoxins, or alternative biosynthetic pathways of fusaric acid in Foc4 or *F. oxysporum* f.sp. *lycopersici*.

In most organisms, 2-picolinic acid is synthesized using tryptophan as the precursor via the kynurenine pathway (Fig. 1). Since fusaric acid is a structural analog of 2-picolinic acid, and it can also be formed by introducing butyl side chains in certain steps of the 2-picolinic acid biosynthesis pathway, we hypothesize that kynurenine (IUPAC name: 2-amino-4-(2-aminophenyl)-4-oxobutanoic acid) pathway may be an alternative synthetic pathway for fusaric acid.

To test this hypothesis, in the present study, all five *ACMSD* (aminocarboxymuconate-semialdehyde decarboxylase, EC 4.1.1.45) genes at the kynurenine pathway in Foc4 that catalyze the decarboxylation reaction on 2-amino-3-carboxymuconate semialdehyde, two *HAD* genes (3-hydroxyanthranilate 3,4-dioxygenase, EC 1.13.11.6) at the same pathway that catalyze 3-hydroxyanthranilic acid (IUPAC name: 2-amino-3-hydroxybenzoic acid) to intermediate 2-amino-3-carboxymuconate semialdehyde, were respectively knocked out or interfered. Thereafter, this study examined the fusaric acid and 2-picolinic acid products generated in mutants, together with the pathogenicity and toxicity of mutants to banana seedlings or leaves. To confirm the role of beauvericin in the pathogenesis of Foc4, the beauvericin synthetase (*BbBEAS*) gene was deleted and the phenotypes related to pathogenicity were also investigated.

Materials and methods

Fungal strains, media and growth conditions

The wild-type strain of Foc4 (Abbreviations: Foc4-WT) used in the present study was B5 which was from Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences. The potato dextrose broth (PDB) or potato dextrose agar (PDA) medium were used to culture wild-type and mutant Foc4. To investigate fungal biomass and spores, 1 mL of fresh spores (10^7 spores per mL) from the wild-type and mutant Foc4 were inoculated in 100 mL of PDB and cultured on a rotary shaker for 8 d, at 28 °C and 180 rpm. Subsequently, the mycelia were harvested and fungal dry weight was determined. Using a hemocytometer, the spores were counted (Li et al. 2014). Three replicates were done for each sample. The metabolite products of wild-type and mutant Foc4 used for HPLC analysis were from PDB shaking culture solution for 8 days at 180 rpm at 28 °C (100 mL of PDB in 500 mL erlenmeyer

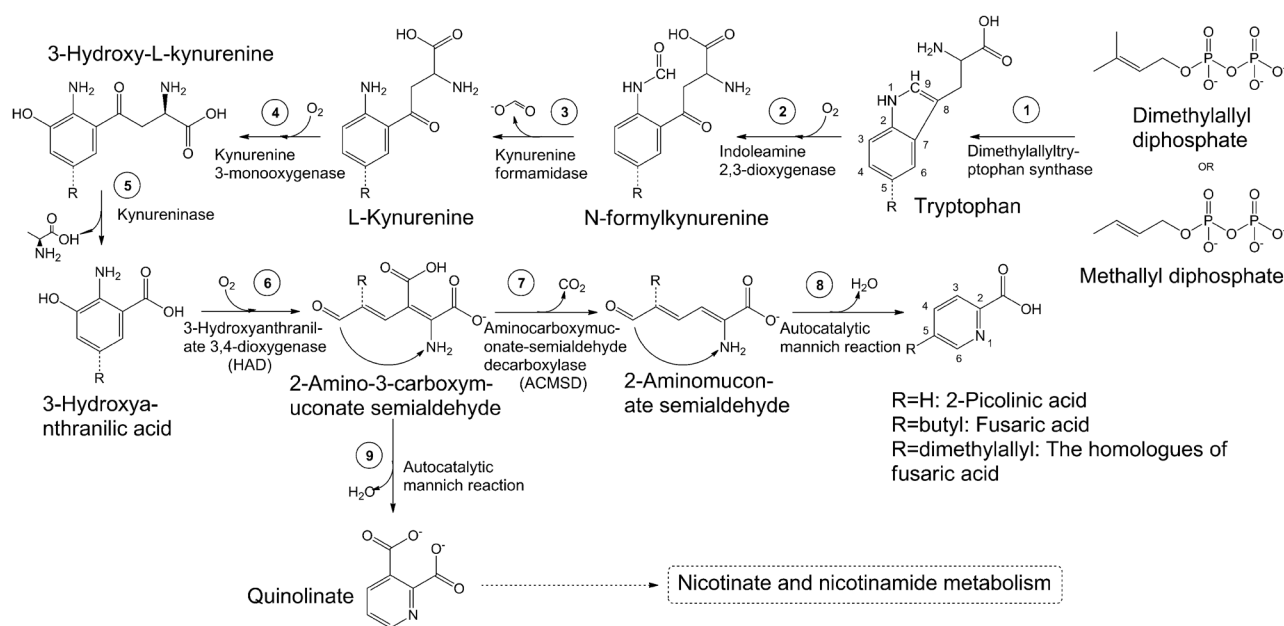


Fig. 1 The kynurenine pathway for 2-picolinic acid biosynthesis of *F. oxysporum* from KEGG database, redrawn with chembiodraw software

flask incubated with two 3-mm² mycelia plugs from a culture grown on PDA). Three replicates were done for each sample. Before conducting HPLC analysis, the three replicates were merged.

Bioinformatics analysis

The mRNA and DNA sequences of *FUB1* and *FUB7*, and the beauvericin synthetase mRNA sequence in *Foc4* were determined through homology alignment using the alignment search tool (BLAST) (Schäffer et al. 2006). The *FUB* gene cluster sequences (GenBank accession numbers: FOXG_15235 ~ FOXG_15248) of *F. oxysporum* f.sp. *lycopersici* were used as query sequence (Brown et al. 2015). The GenBank accession numbers of mRNA and DNA sequences of *FUB1* are XM_031217894.1 and FOIG_16450. In addition, those of *FUB7* are XM_031217909.1 and FOIG_16458 respectively. The beauvericin synthetase gene mRNA sequence (GenBank accession numbers: XM_031217181.1) in *Foc4* was determined by employing the same method, in which the released beauvericin synthetase amino acid sequence (GenBank accession number: ACI30655) was used as query sequence.

The amino acid sequences of aminocarboxymuconate semialdehyde decarboxylase (ACMSD) and 3-hydroxyanthranilate 3,4-dioxygenase (HAD) were retrieved through searching the protein sequences within *Foc4* (NCBI taxonomy id: 61,366) from NCBI. Then, these amino acid sequences were used as query sequences to search their mRNA and DNA sequences using the alignment search tool (BLAST). Five amino acid

sequences are annotated as aminocarboxymuconate semialdehyde decarboxylases (ACMSD) in *Foc4*. Their GenBank accession numbers at NCBI are EXL91397.1, EMT63964.1, EMT74385.1, EMT67436.1, and EMT72782.1. Besides, their mRNA GenBank accession numbers are XM_031216795.1, XM_031204437.1, XM_031213692.1, XM_031212087.1 and XM_031199773.1. Their corresponding GenBank accession numbers of DNA sequences are FOIG_15435, FOIG_05693, FOIG_12755, FOIG_11516, FOIG_02463, respectively. Two amino acid sequences are annotated as 3-hydroxyanthranilate 3,4-dioxygenase (HAD) in *Foc4*. Their GenBank accession numbers are EMT65309.1 and EXL91398. Their corresponding mRNA GenBank accession numbers are XM_031200655.1 and XM_031216796.1. In addition, corresponding GenBank accession numbers of DNA sequences are FOIG_03029 and FOIG_15436, respectively.

DNA preparation and DNA fragment assembly

The *Foc4*-wild and its mutant DNAs used as template for PCR analysis were obtained from their harvested mycelia using the Flexigene DNA Kit (Qiagen, Germany) in accordance with its operation manual.

Enzymatic assembly of overlapping DNA fragments for constructing vectors was carried out in gibbon DNA assembly method with Gibson Assembly Master Mix (2×) Kit (NEB, America) (Gibson 2011). All primers used for polymerase chain reaction (PCR) were obtained from BGI-Shenzhen, China.

Targeted gene deletion or interference and complementation of the mutants

To construct knockout vectors for *FUB1*, *FUB7* and *ACMSD* genes, the respective upstream homologous arm of these genes was amplified with the primer pairs up-f-in//up-r and the downstream homologous arms were amplified with the primer pairs down-f//down-r-in (Table S1). Both up-r and down-f primers contained overlap sequences from the resistance cassette. This study designed a pair of primers up-f-//down-r (Table S1), which were respectively located in upstream of the up-f-in primer site of the upstream sequence of each gene and in downstream of the down-r-in primer site of the downstream sequence of the same gene, so as to verify the genes knocked out.

Hygromycin was used as resistance marker for all *ACMSD* genes knockout experiments, and G418 for *FUB1* and *FUB7* genes. The hygromycin resistance cassette HygR, consisting of the hygromycin B phosphotransferase gene *hph* (Gritz and Davies 1983) and TrpC promoter, was amplified using the primer pair Hph-f//Hph-r from the pCT74 plasmid (Lorang et al. 2001). The G418 resistance cassette (Table S2) synthesized chemically by BGI-Shenzhen, was amplified with the primer pair R-f-G418//R-r-G418 (Table S1). The gibbon DNA assembly method was employed to connect the upstream fragment, the resistance cassette fragment and the downstream fragment of the target genes. The assembly product was used as a template, and up-f-in//down-r-in as primers to amplify linear knockout vectors for the targeted genes by high-fidelity Taq enzyme PrimeSTAR®HS (Takara Bio, Japan) (Fig. S1).

To construct complementary vector for the *ACMSD* gene, the hygromycin resistance cassette of the pCT74 plasmid was replaced with the G418 resistance cassette. In addition, the *GFP* gene expression cassette was replaced with the DNA sequence from ~1000 bp before the initiation codon to ~500 bp after the termination codon of the *ACMSD* gene (Fig. S2).

To construct RNA interference vectors for the *HAD* genes, the *GFP* gene ORF of pCT74 plasmid was replaced by a sequence consisting sequentially of a forward promoter (RP27 promoter), a reverse terminator (Terminator), a forward terminator (Terminator), and a reverse promoter (ToxA promoter). A 100~250 bp long sequence located within 250 bp behind the target mRNA start codon was chosen, which was less than 17% similar to no target mRNAs in *Foc4* total mRNA, as the interference sequence for each target *HAD* gene. Then, to obtain the *HAD* gene RNA interference vectors, the interference sequence was inserted between the forward promoter and the reverse promoter of the modified pCT74 plasmid (Fig. S3).

All vectors were transformed separately into fungi in accordance with the preparation and transformation protocol of fungal protoplast (Leng and Zhong 2012). The transformants were selected on the PDA medium containing

antibiotics ($200 \mu\text{g}\cdot\text{mL}^{-1}$ hygromycin B or G418). The transformants were identified by PCR.

RNA extraction and expression analysis

Mycelia cultured for 96 h in PDB were filtered through the filter paper and ground within the liquid nitrogen. RNA was extracted using the Rneasy@Plant Mini Kit (Qiagen, Germany). Residual DNA was removed by DNA-free@DNase Treatment and Removal kit (Ambion, America). The RNA was reversed to cDNA with M-MLV transcriptase (Invitrogen, America) and oligo(dT) primers (Ambion, America).

The real-time qPCR procedure was performed using a Mx3000P QPCR System (Agilent, America) in a 25 μL reaction system, which consisted of cDNA, $1\times$ Fast Sybr@Green Master Mix (ABI, America), and primers of the two *HAD* genes, or the reference gene *GAPDH*. Data were analyzed from three biological replicates. The expression of each gene was normalized using the formula $2^{-(\Delta\Delta\text{CT})}$ according to the manufacturer's instructions. Primers for QPCR to the two *HAD* genes are listed in Supplemental Table S1.

Quantification of fusaric acid, 2-picolinic acid and beauvericin

To exclude mycelium and spores, the PDB culture solution of *Foc4*-wild and the *Foc4*-mutants was filtrated with Waterman filters. 100 mL filtrated solution was dried by vacuum freeze-drying and then redissolved with 10 mL ultrapure water, followed by filtrating through 0.45- μm filters. Waters E2695 Alliance HPLC system with Comatex C18-CB (4.6 \times 250 mm) was employed to analyse fusaric acid, 2-picolinic acid and beauvericin. Since these compounds are highly polar, sodium dodecyl sulfate was added to the acid mobile phase as ion-pairing reagents to delay their retention time. The mobile phase was contained by 10% acetonitrile and 90% water with 0.05% SDS and 1% H_3PO_4 . The detective wavelength was 272 nm for fusaric acid, 2-picolinic acid, whereas 254 nm for beauvericin. The flow rate of the mobile phase was $1 \text{ mL}\cdot\text{min}^{-1}$. Peak areas of fusaric acid after HPLC-DAD analysis and integration referenced to the dry weight and normalized to the wild-type level (Fig. S4).

Toxicity test to banana leaves

Semi-quantitative tests were performed using banana leaves. The old leaves from the adult banana plant were got rid of petioles and then teared into long strips ~15 mm in width and ~200 mm in length,

followed by inserting in a 15 mL tube containing 5 mL of the filtrated culture solutions. These treatments were placed on the windowsill and exposed to natural light, and observed the leaves yellowing every day. The experiment had three replicates.

Quantitative tests were performed by relative electrical conductivity of leaf strip so as to evaluate leaf membrane injury. When leaves showed symptoms in semi-quantitative testing, 1 g leaf samples were collected from each treatment, and washed with deionized water to remove the adsorbed electrolytes. The washed leaf segments (1 g) were immersed in 40 mL deionized water, and shaken gently. The 100 μ L extraction of the leaves was diluted to 2 mL with deionized water, and the conductivity of the diluted leaf extraction was measured using an electrical conductivity meter (DDS-11A, Leici Instrument Co., China). A second conductivity measurement was made by employing the same method after the same leaf tissues were boiled in a 100 °C for 30 min and then cooled to room temperature. Relative leaf membrane injury was calculated as a ratio between the first and second conductivity measurements.

Virulence assay to banana seedlings

Thereafter, virulence tests of WT, mutants, and the complemented strains were performed in the tissue culture-derived banana plantlets with 6–7 leaves. Banana root inoculation experiments were conducted with a concentration of 10^5 microconidia per mL of the indicated strains and disease symptoms were observed at 30 days after inoculation as described by Li et al. (Li et al. 2011). For each plantlet, based on the extent of vascular browning in the corms, disease severity was recorded and graded as 1–7, withering and yellowing of the leaves.

Grade 1 indicated no vascular browning; grade 2 indicated the vasculars of corms were not brown, while the junctions of the roots and corms were; grade 3 indicated the browned vasculars were up to 5%; grade 4 indicated the browned vasculars were up 6% to 20%; grade 5 indicated the browned vasculars were up 21% to 50%; grade 6 indicated the browned vasculars exceeded 50% and grade 7 indicated the vasculars were completely browned; Disease incidence was measured as the percentage of symptomatic plantlets (grades 2–7) over the total number of inoculated plantlets. Five plantlets were used for each treatment. The disease index was given by.

$$\text{Disease index} = \left[\sum (N_{\text{each}} \times G_{\text{each}}) / (N_{\text{tot}} \times G_{\text{highest}}) \right] \times 100\% \quad (1)$$

in which N_{each} represented the number of the diseased plants at each grade; G_{each} represented the disease severity

grade; N_{tot} represented the total plants investigated; G_{highest} stood for the highest disease severity grade.

Results

Functional analysis of FUB1 and FUB7 genes involved in biosynthesis of fusaric acid in Foc4

The *FUB1* and *FUB7* were chosen to study for their functions in Foc4. In the Foc4 wild (Foc4-WT) background, the *FUB1* and *FUB7* genes were deleted. The knockout mutants of *FUB1*, *FUB7* genes were named Foc4- Δ FUB1, Foc4- Δ FUB7, respectively.

The HPLC analysis of the mutant cultures demonstrated that the fusaric acid production of the Foc4- Δ FUB1 and Foc4- Δ FUB7 mutants was reduced by 80% and 35% compared with the Foc4-WT (Fig. 2a), respectively. This result confirmed that in Foc4, the *FUB* gene cluster also shows the responsibility for fusaric acid synthesis.

No measurable changes in colony morphology, spore or mycelium growth were observed in the mutants of Foc4- Δ FUB1, Foc4- Δ FUB7 and Foc4-WT on PDA agar medium (Fig. 2b). However, the mutants growing in the PDB liquid medium for 8 days had a decreased biomass and spore production, to be specific, the biomasses of Foc4- Δ FUB1 and Foc4- Δ FUB7 decreased by 20% and 11%, respectively, whereas their spore production decreased by 83% and 74%, respectively (Fig. 2c).

As the host of Foc4 is *Brazil banana*, we used its leaf strip to test the toxicity of Foc4- Δ FUB1, Foc4- Δ FUB7. After soaking for 5 days with the filtrated culture solution of each strain, the yellow degree of the leaf strip treated with Foc4-WT was over 80% and the relative leaf membrane injury was 24.55%. However, fresh water was 0% and 5.21% respectively. The yellow degree of the leaf strip treated with Foc4- Δ FUB1 and Foc4- Δ FUB7 was 30% and 50% respectively. The relative leaf membrane injury was 11.29% and 15.24% (Fig. 3a, b). It indicated that the decreased yellow degree and the relative membrane injury of leaf strip in Foc4- Δ FUB1, Foc4- Δ FUB7 had less toxicity to banana leaves. The results of the mutant's toxicity test on leaf strip were roughly consistent with the decrease in fusaric acid content in their culture.

According to the virulence tests on banana seedlings, both mutants of Foc4- Δ FUB1 and Foc4- Δ FUB7 did not completely lose their virulence. The disease incidence and disease index of Foc4- Δ FUB7 were 100% and grade 5, separately, which were close to those in wild type; and those of Foc4- Δ FUB1 were 60% and grade 4, respectively (Fig. 3c, d). These results indicate that there exists some correlation between the yield of fusaric acid and virulence of Foc4.

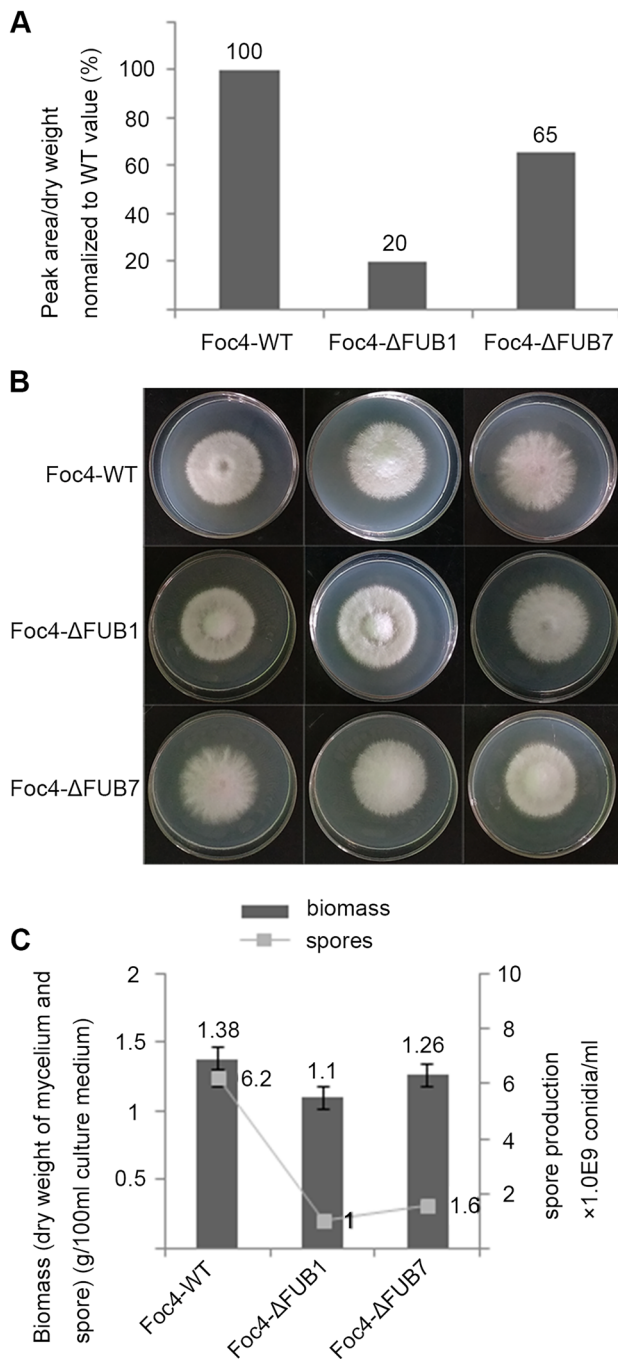


Fig. 2 Some biological phenotypes of Foc4-ΔFUB1, Foc4-ΔFUB7 and Foc4-WT. **a** Fusaric acid products of Foc4-ΔFUB1, Foc4-ΔFUB7 and Foc4-WT. The strains were cultivated for 8 days in the PDB liquid medium; and the obtained culture filtrates after being 10 times concentrated were analyzed. Peak areas were related to the dry weight and normalized to the wild-type level; **b** The growth of Foc4-ΔFUB1, Foc4-ΔFUB7 and Foc4-WT on PDA agar medium; **c** The affection of the deletion of *Fub1* and *Fub7* genes on the biomass and spore production of Foc4 growing in PDB liquid culture medium. The average number of the biomasses and spores were from five independent experiments each with five replicates

Functional analysis of ACMSD and HAD genes including in kynurenine pathway in Foc4

Under the Foc4-WT background, the 5 DNA sequences of *ACMSD* were deleted, respectively, and 5 *ACMSD* gene knockout mutants were produced, which were named Foc4-ΔEMT63964, Foc4-ΔEMT67436, Foc4-ΔEMT74385, Foc4-ΔEMT72782 and Foc4-ΔEXL91397, respectively.

Based on the mRNA sequences, two RNA interference vectors were constructed for the *HAD* genes (EMT65309.1, EXL91398.1) and then were transformed into Foc4 protoplast via PEG-mediated, producing 2 *HAD* RNAi mutants named Foc4-RNAi-65309 and Foc4-RNAi-91398, respectively. According to RT-qPCR analysis of *HAD* genes, in the two RNAi mutants, the transcript levels of the *EMT65309.1* and *EXL91398.1* genes in Foc4-RNAi-65309 were reduced by 65% and 4%, while those in Foc4-RNAi-91398 decreased by 5% and 63%, respectively, compared with those in Foc4-WT (Fig. 4a).

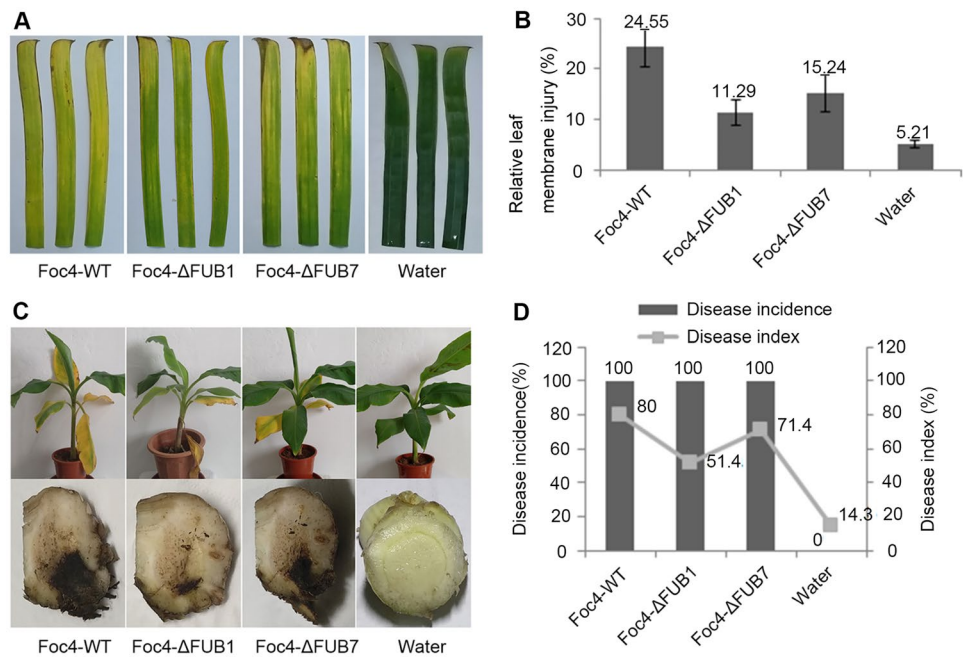
Based on HPLC analysis of the mutant culture solution indicated, the productions of fusaric acid were unchanged in mutants of Foc4-ΔEMT63964, Foc4-ΔEMT67436, Foc4-ΔEMT72782, and Foc4-ΔEXL91397 in comparison with WT, while their productions of 2-picolinic acid were a little reduced. Nevertheless, the productions of fusaric acid and 2-picolinic acid were significantly reduced in the mutant of Foc4-ΔEMT74385, and RNA interferences of Foc4-RNAi-65309 and Foc4-RNAi-91398 in comparison with Foc4-WT (Fig. 4b). Subsequently, the complementary Foc4-ΔEMT74385-cp strain for the mutant Foc4-ΔEMT74385 was constructed. Meanwhile, the results showed that the productions of fusaric acid and 2-picolinic acid in the Foc4-ΔEMT74385-cp strain were restored to those of Foc4-WT (Fig. 4b), proving that the EMT74385.1 is involved in the biosynthesis of fusaric acid and 2-picolinic acid.

On PDA agar medium, the mutants of Foc4-ΔEMT74385, Foc4-RNAi-65309, Foc4-RNAi-91398, Foc4-ΔEMT74385-cp and Foc4-WT had the similarity in the growth rate and colony characteristics (data not shown). However, when the mutants of Foc4-ΔEMT74385, Foc4-RNAi-65309 and Foc4-RNAi-91398 were growing in the PDB liquid medium, both biomass and spore production were reduced, which were similar to Foc4-ΔFUB1 and Foc4-ΔFUB7 (Fig. S5).

The yellow degree of the leaf strips treated with both Foc4-WT and Foc4-ΔEMT74385-cp were up to 80%. Those of Foc4-ΔEMT74385, Foc4-RNAi-65309 and Foc4-RNAi-91398 were between 40 to 60%. Besides, the fresh water was 0%.

The relative leaf membrane injury proportions of Foc4-WT and Foc4-ΔEMT74385-cp were up to 31%, while those of Foc4-ΔEMT74385, Foc4-RNAi-65309 and Foc4-RNAi-91398 were between 19 and 22%, and that of fresh water was 6.7% (Fig. 4c, d).

Fig. 3 Virulence assay of Foc4-WT, mutants Foc4- Δ FUB1 and Foc4- Δ FUB7. **a** Semi-quantitative toxicity tests of strain filtrated culture solution onto banana leaves; **b** Effects of strain filtrated culture solution on relative injury in the banana leaves. Values were from three replicates; **c** Disease symptoms on banana tissue plantlets and its corms were observed after 30 days of inoculation with the indicated strains, Foc4-WT was used as a positive control treatment and sterile water as a negative control (water); **d** Disease incidence and disease index indicated disease severity. Values were from five replicates



Disruption of beauvericin biosynthesis did not reduce the toxicity of Foc4

In the Foc4- Δ EMT74385 background, the beauvericin synthetase (*BbBEAS*) gene was deleted. The obtained *BbBEAS* gene knockout mutant was named Foc4- Δ EMT74385: Δ BbBEAS. The beauvericin production of the Foc4- Δ EMT74385: Δ BbBEAS after being cultured in PDB liquid medium was not detected (Fig. S6).

Moreover, it was shown in leaf toxicity analysis that, the yellowing degree (Fig. 5a) and the relative leaf membrane injury (Fig. 5b) of the leaf strips treated with Foc4- Δ EMT74385: Δ BbBEAS were not significantly different from those of Foc4- Δ EMT74385. The virulence tests on banana seedlings showed that Foc4- Δ EMT74385: Δ BbBEAS caused the disease incidence of 100% and the disease index of grade 5, which is higher than that by Foc4- Δ EMT74385 in which the disease incidence and disease index was 80% and grade 4 respectively (Fig. 5c, d). According to the result, in Foc4, the knockout of the beauvericin synthase gene could interrupt beauvericin biosynthesis. However, it neither weakened the toxicity of Foc4 to banana leaves, nor weakened the its pathogenicity to banana seedlings.

Deletion of both EMT74385.1 and FUB1 genes resulted in greatly reduced pathogenicity of Foc4

In the Foc4- Δ FUB1 background, the *EMT74385.1* gene involved in kynurenine pathway was further deleted. The obtained double gene knockout mutant was named

Foc4- Δ EMT74385: Δ FUB1. HPLC analysis of the mutant culture solution indicated that the content of fusaric acid in Foc4- Δ EMT74385: Δ FUB1 was lower than that in the single-knockout mutant Foc4- Δ EMT74385, and almost could not be detected (Fig. 6). This suggests that the kynurenine pathway is not only responsible for the biosynthesis of 2-picolinic acid, but it may also contribute to the biosynthesis of fusaric acid.

The leaf toxicity of the double knockout mutant was significantly lower than that in the single knockout mutants of Foc4- Δ EMT74385 and Foc4- Δ FUB1, and the wild type (Fig. 7a, b), showing consistence with the decreased content of fusaric acid. Additionally, the double knockout mutant showed a little leaf toxicity, which was almost the same as the sterile water (Fig. 7a, b), and the Foc4- Δ EMT74385: Δ FUB1 did not cause any fusarium wilt symptoms in banana plantlets and its corm (Fig. 7c, d).

These results indicate that the *EMT74385.1* involved in kynurenine pathway in Foc4 played an important role on the biosynthesis of fusaric acid and fusarium wilt symptoms in Banana.

Discussion

The fusarium acid biosynthesis cluster genes (*FUBs*) have been reported in four species of *Fusarium*, respectively, *F. verticillioides*, *F. kujikuroi* and *F. oxysporum* f.sp. *lycopersici* and Foc4, which are consisted of twelve genes, including two transcription factors, nine enzyme genes and a transporter gene (Brown et al. 2015; Ding et al. 2018).

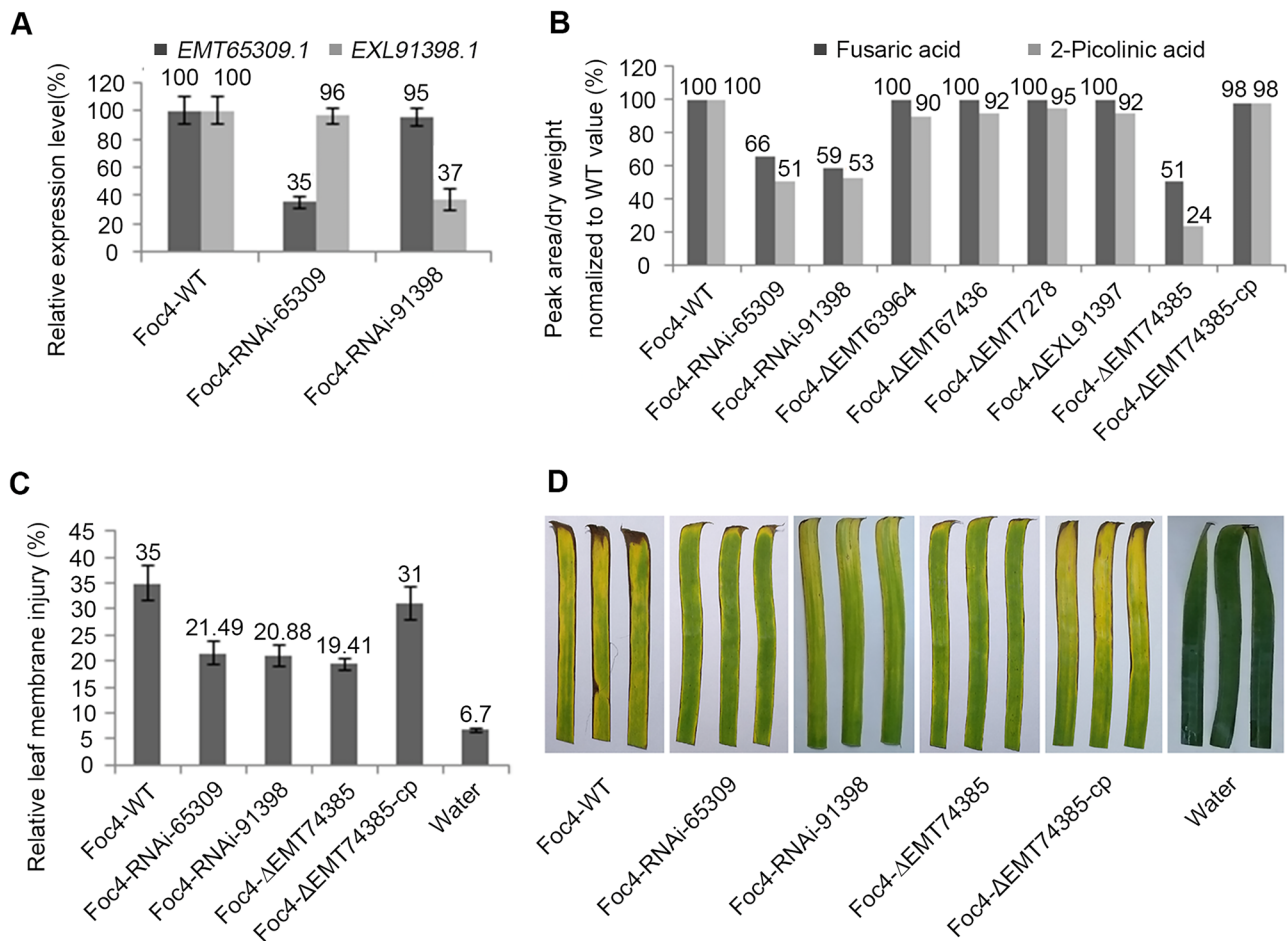


Fig. 4 Some biological phenotypes and virulence assay of the mutants which some genes in kynurenine pathway were knocked out or interfered. **a** The transcript levels of the two HAD genes, EMT65309 and EXL91398 in Foc4-WT and its RNAi mutants Foc4-RNAi-65309, Foc4-RNAi-91398; Data represent an average of three replicates; **b** The relative products of fusaric acid and 2-picolinic acid in Foc4-ΔEMT63964, Foc4-ΔEMT67436, Foc4-ΔEMT72782, Foc4-ΔEXL91397, Foc4-ΔEMT74385, Foc4-RNAi-65309, Foc4-

RNAi-91398, Foc4-ΔEMT74385-cp to Foc4-WT. The strains were cultivated for 8 days in the PDB liquid medium; and the obtained culture filtrates after being 10 times concentrated were analyzed. Peak areas were related to the dry weight and normalized to the wild-type level; **c** Effects of the strain filtrated culture solution on relative injury in the banana leaves. Values were from three replicates; **d** Semi-quantitative toxicity tests of the strain filtrated culture solution to banana leaves

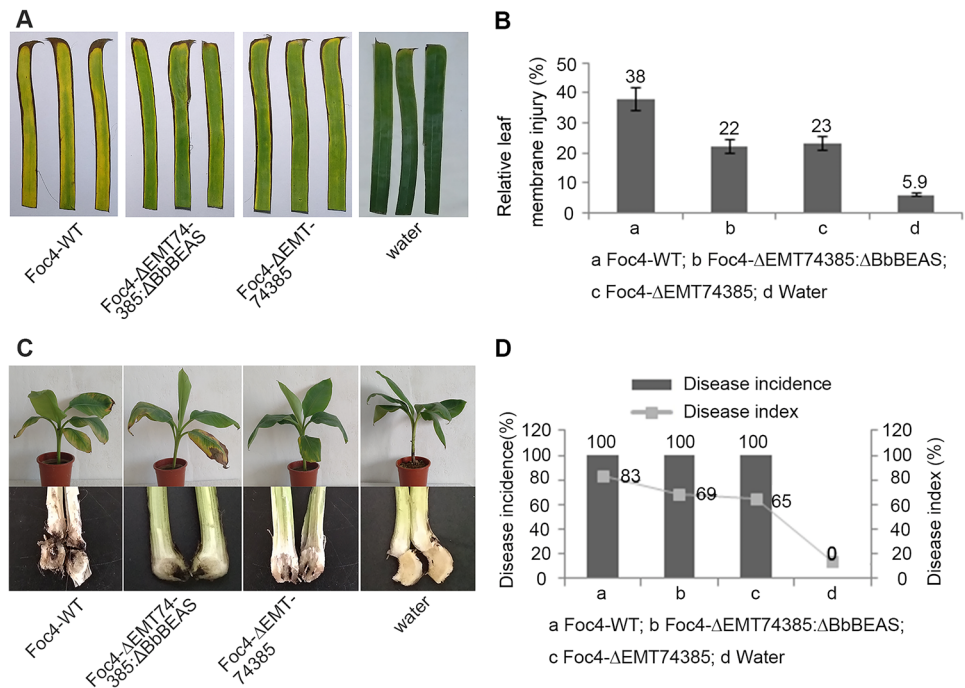
Based on the study of *FUB* genes function through molecular genetics, chemical analysis, and bioinformatics, a fusaric acid biosynthetic pathway that is mainly composed of the *FUB* genes was proposed (Studt et al. 2016).

However, the pathogenicity tests of the *FUB1* mutant in *F. oxysporum* f.sp. *lycopersici* and *F. verticillioides* showed that the mutant had a significant decrease in fusaric acid production and toxicity to leaves while they still remained pathogenic to their host (Brown et al. 2015). Zhaojian knocked out the *FUB4* gene in *Foc4*, and the production of fusaric acid was not detected in *FUB4* gene knockout mutant. However, the mutant has not been completely lost its pathogenicity, and it still remained disease incidence of ~40% and disease index of ~30% relative to

the wild-type strain (Ding et al. 2018). It is possible that *Foc4* uses more than one biosynthetic pathways to synthesize fusaric acid during infecting its host. In the present study, the results showed that *FUB1* and *FUB7* mutants in *Foc4* caused the decreased yield of fusaric acid, among which, *FUB1* mutant had more significant reducing effect than *FUB7* mutant, but it did not completely lose fusaric acid production.

That the *FUB1* mutant still produced a little fusaric acid and had pathogenicity to bananas might result from an alternative biosynthetic pathway of fusaric acid in *Foc4*. The chemical structure of fusaric acid is similar to that of 2-picolinic acid, in which the hydrogen on the 5-position carbon of 2-picolinic acid is substituted by a butyl. We speculate that the synthetic

Fig. 5 Virulence assay of Foc4-WT, Foc4- Δ EMT74385 and Foc4- Δ EMT74385: Δ BbBEAS. **a** Semi-quantitative toxicity tests of strain filtrated culture to banana leaves; **b** Effects of strain filtrated culture on relative membrane injury in the banana leaves. Values were from three replicates; **c** Disease symptoms on banana tissue plantlets and its corms were observed after 30 days of inoculation with indicated strains, Foc4-WT was used as a positive control treatment and sterile water as a negative control (water); **d** Disease incidence and disease index indicated disease severity. Values were from five replicates



pathway of 2-picolinic acid might be an alternative biosynthetic pathway for fusaric acid.

Alternative biosynthetic pathways of some secondary metabolites have been reported, such as pyrimidine in *Salmonella typhimurium* (Petersen and Downs 1996), aldosterone in rats (Müller 1980), bile acid in animals (Duane and Javitt 2002), polyamines in *Vibrio cholerae* (Gardiner et al. 2010), Brassinolide in plants (Yong-Hwa et al. 1997) and menadione in microorganisms (Hiratsuka et al. 2008).

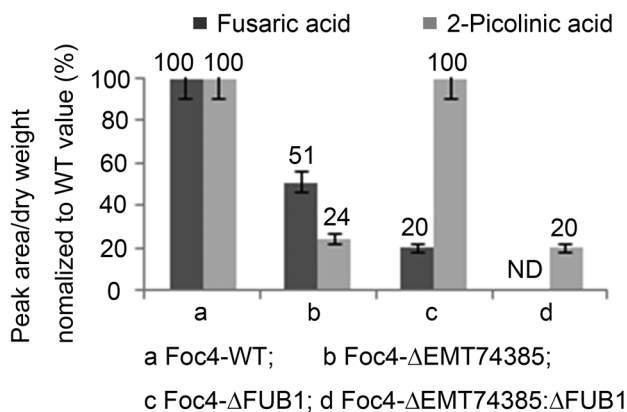
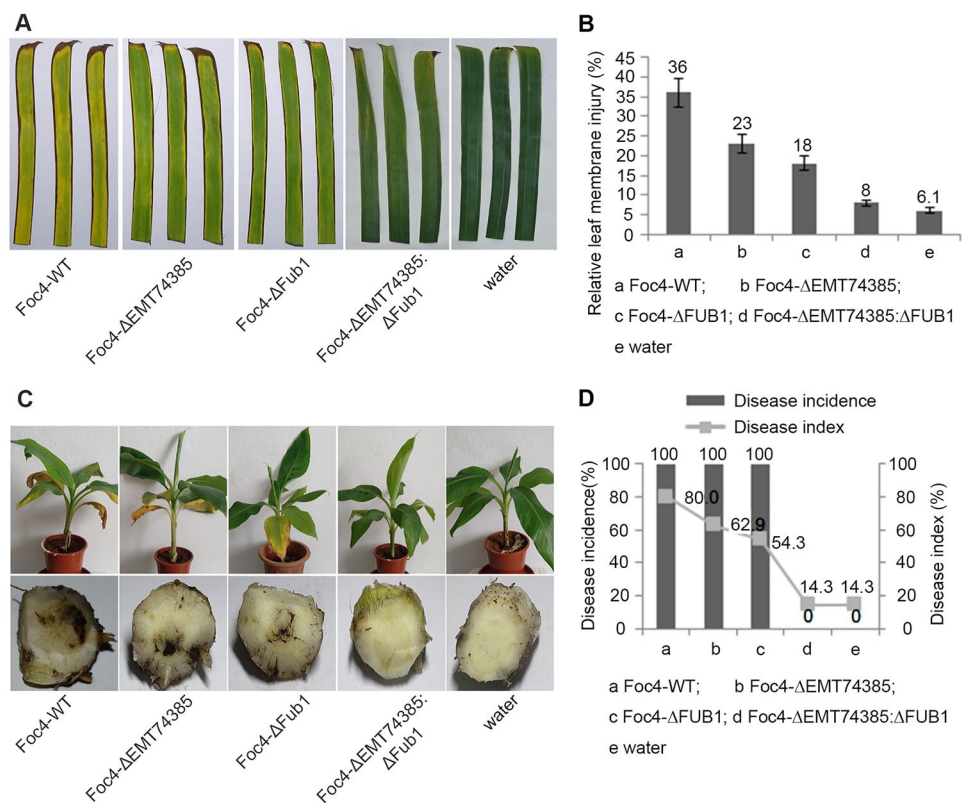


Fig. 6 Fusaric acid products of Foc4- Δ EMT74385: Δ FUB1, Foc4- Δ EMT74385, and Foc4- Δ FUB1 relative to Foc4-WT. The strains were cultivated for 8 days in the PDB liquid medium; and the obtained culture filtrates after being 10 times concentrated were analyzed. Peak areas were related to the dry weight and normalized to the wild-type level

The toxicity of 2-picolinic acid could induce HR-like reactions in rice suspension cells (Zhang et al. 2004), which is considered to be a toxin produced by rice blast pathogen *magnaporthe grisea*. However, its toxic concentration on plant leaves is much higher than that of fusaric acid (Stipanovic et al. 2010). In our toxicity tests on banana leaves, its toxic concentration is 100 times that of fusaric acid (data not shown). When we respectively knocked out one of the five *ACMSD* genes in the kynurenine pathway that might be responsible for 2-picolinic acid synthesis, the production of 2-picolinic acid was significantly reduced. Meanwhile, the production of fusaric acid was also reduced in the Foc4- Δ EMT74385. This suggests that the *ACMSD* enzyme encoded by this gene could not only accept 2-amino-3-carboxymuconate semialdehyde as a substrate to catalyze the production of 2-picolinic acid, but also accommodate 2-amino-3-carboxy-5-butylmuconate semialdehyde as a substrate to catalyze the production of fusaric acid. There are five *ACMSD* genes in Foc4, and the knock-out of only one *ACMSD* gene contributes to significant phenotypic changes. This may be due to the low expression of the other four *ACMSD* genes, or their substrate specificity is not biased towards the synthesis of fusaric acid. In comparison with the Foc4-WT and Foc4- Δ EMT74385-cp, the yellow degree and relative leaf membrane injury of leaf strips treated with the mutants were significantly reduced, indicating that the mutants's toxicity to leaves was reduced. The results of the mutant's toxicity tests on leaf strips were roughly in consistence with the decrease in the content of fusaric acid in their culture.

Fig. 7 Virulence assay of Foc4-WT, Foc4- Δ EMT74385, Foc4- Δ FUB1 and Foc4- Δ EMT74385: Δ FUB1. **a** Semi-quantitative toxicity tests of strain filtrated culture to banana leaves; **b** Effects of strain filtrated culture on relative membrane injury in the banana leaves. Values were from three replicates; **c** Disease symptoms on banana tissue plantlets and its corms were observed after 30 days of inoculation with indicated strains, Foc4-WT was used as a positive control treatment and sterile water as a negative control (water); **d** Disease incidence and disease index indicated disease severity. Values were from five replicates



A reverse genetic study for single gene is not sufficient to infer the synthetic pathway of a metabolite, as this gene may function in another pathway. Therefore, we interfered with the expressions of *HAD* genes that their coding enzymes are responsible for the previous step reaction of the ACMSD-catalyzed reaction on the kynurenine pathway. Additionally, RNA interferences of the two *HAD* genes also resulted in the same phenotype as *EMT74385* gene knock-out mutant, which had a reduced yield of 2-picolinic acid and fusaric acid, and reduced leaf toxicity. The results from *HAD* interference and *ACMSD* mutant suggest that the kynurenine pathway responsible for the biosynthesis of 2-picolinic acid might also provide an alternative biosynthetic pathway for fusaric acid.

Attaching butyl to the corresponding position of the intermediate in the kynurenine pathway can finally form fusaric acid. One candidate scheme refers to that dimethylallyl or methylallyl is transferred to the C₅ position on the indole ring of tryptophan (IUPAC name: (2S)-2-amino-3-(1H-indol-3-yl) propanoic acid) by DMATS (dimethylallyltryptophan synthase, EC 2.5.1.34 or EC 2.5.1.B31) (Fig. 1).

It is generally believed that DMATS shows high flexibility for aromatic substrates (Yu and Li 2012; Li 2009), yet has highly substrate specificity for dimethylallyl. However, several studies have indicated that it can accept methylallyl diphosphate as a donor for transfer. For example, both FgaPT2, a 4-DMATS (4-dimethylallyltryptophan synthase,

EC 2.5.1.34) from *Aspergillus fumigatus* and 5-DMATS (5-dimethylallyltryptophan synthase, EC 2.5.1.B31) from *Aspergillus clavulatus* can transfer methylallyl groups to the C₄ or C₅ position on the indole ring of tryptophan (Liebhold, Xie and Li 2012).

There are four tryptophan dimethylallyl transferases in Foc4 and some of them may have similar substrate specificity to FgaPT2 and 5-DMATS, which transfer dimethylallyl or methylallyl to the C₅ position on the indole ring of tryptophan, thus producing 5-(2-trans-butenyl)-L-tryptophan.

The double bonds on butenyl of 5-(2-trans-butenyl)-L-tryptophan might be reduced, resulting in the formation of fusaric acid. Even if this double bond is not reduced, the end product 5-(2-trans-butenyl)-2-picolinic acid would be still almost as toxic as fusaric acid (Stipanovic et al. 2010). However, the speculative reaction requires experimental evidences that would be our following research.

Beauvericin is considered to be another toxin produced by Foc4. Higher concentration beauvericin (20 μ M) can cause obvious symptoms in banana seedlings (Li et al. 2013). However, the content of beauvericin in the diseased banana plant is extremely low, and which is mainly distributed in the fruit and then in the pseudostem. The least amount is in the leaves (Li et al. 2013), and thus it is not the cause of leaf yellowing. In order to avoid of masking the toxicity of low yield of beauvericin by the high yield of fusaric acid in wild-type strains, and to bring the role

of beauvericin in the pathogenic process to the surface, we deliberately knocked out the beauvericin synthase gene in the Foc4- Δ EMT74385 background in which the production of fusaric acid was reduced. However, the leaf toxic and pathogenicity to its host still remain. In addition, our experiments have also shown that the interruption of beauvericin synthesis did not affect the pathogenicity of Foc4. The double mutants deleted with the key genes in the *FUB* and the kynurenine pathways of fusaric acid synthesis have almost completely lost their pathogenicity, even though it can still produce beauvericin. Therefore, beauvericin might not be a toxin for fusarium wilt symptoms in Banana.

Our research shows that only fusaric acid can be considered as a Foc4 toxin. Moreover, it has alternative synthetic pathways. In the early stage of infection that the Foc4 mycelium just touches the root surface of the host plant, and then penetrates into the vascular tissue along and between the epidermal cells of root, the Foc4 mycelium does not expose to the high nitrogen conditions required for *FUB* genes high expression (Pitel and Vining 1970). At this stage, the fusaric acid synthesized mainly by the kynurenine pathway contributed to the invasion of Foc4. When Foc4 mycelium invaded the vascular tissue of the banana, the host cells cytosol was released due to the damage of the cell membrane by the fusaric acid. The nitrogen in the cell cytosol stimulates the expression of the *FUB* gene cluster of Foc4 to synthesize more fusaric acid so as to help progression of disease.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1007/s13313-021-00788-y>.

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