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RESEARCH ARTICLE

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First Report of Zearalenone Production by *Talaromyces* Isolated from *Bidens pilosa* Using LC-q-TOF-MS and Molecular Networking

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ABSTRACT

Green leafy vegetables like *Bidens pilosa* are valuable sources of nutraceuticals worldwide. However, due to minimal human intervention, these wild-growing plants can be exposed to pollution and pathogenic microorganisms including endophytic fungi that may produce toxic secondary metabolites. Herein, metabolic profiling of methanolic extracts containing endophytic fungi isolated from *B. pilosa* was conducted using LC-q-TOF-MS and molecular networking. One of the fungi was found to produce a mycotoxin called zearalenone, which is known to be a reproductive system disruptor. The presence of zearalenone was further confirmed using an authentic standard and a series of tandem mass spectrometry approaches. ITS sequencing identified the zearalenone-producing endophytic fungus as *Talaromyces*, showing sequence similarity to various species within this genus. Findings of this study highlight the importance of exercising caution when consuming green leafy vegetables, as they may contain toxic compounds produced by endophytic fungi, despite being easily accessible and rich in valuable nutraceuticals.

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1. Introduction

Since ancient times, green leafy vegetables have been valued by humans for their nutritional benefits and medicinal properties due to their diverse chemical composition. These qualities have made green leafy vegetables like *Bidens pilosa* a focus of research interest, recently [1,2]. Despite the well-documented health-promoting properties of *Bidens pilosa*, this plant grows naturally across the globe with limited human management making it more vulnerable to environmental pollutants and microbial colonization, including endophytic microbes. Many of the endophytes (fungi or bacteria) coexist harmlessly within the host, while others may introduce potential risks, such as the production of toxic secondary metabolites particularly, mycotoxins [3]. Several endophytic fungi have been reported to produce mycotoxins in agriculturally important crops [4,5], however, studies on endophytic microbes in green leafy vegetables are still scanty in literature [6,7]. Among other mycotoxins, zearalenone, an estrogenic-like compound known to affect the reproductive system of many mammals including humans [8], has been reported in food [5]. Given the close relationship between host plants and their endophytic fungi, it is likely that chemical exchanges occur between them, thereby resulting in the contamination of green leafy vegetables with mycotoxins. To ensure food safety, research on mycotoxigenic fungi or endophytic communities in naturally growing vegetables is crucial, as these plants serve as a source of food for some households in resource limited areas across the world.

Mass spectrometry is the most widely used analytical instrumentation for detecting and characterizing secondary metabolites including mycotoxins due to its high accuracy in identifying compounds based on their unique fragmentation patterns [9]. Advancements in the field of computational metabolomics have seen the emergence of molecular networking, a tool that is currently widely used for analyzing LC-MS/MS data [10]. This approach can facilitate the identification of both known and novel mycotoxins with ease. In this study, the composition of secondary metabolites produced by endophytic fungi isolated from *B. pilosa* was analyzed

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using LC-q-TOF-MS and molecular networking techniques. This investigation aimed at identifying potentially harmful compounds that may be generated by these microorganisms. From the isolated endophytic fungi, the zearalenone producing fungi was sequenced through the ITS DNA sequencing approach.

2. Material and methods

2.1. Isolation and extraction of fungal endophytes from B. pilosa leaves

Fresh leaves from *B. pilosa* were collected at the University of Venda's experimental farm. The plant leaves were washed with tap water. To obtain endophytic microorganisms from within the leaves of *B. pilosa*, surface sterilization was done using 1% of sodium hypochlorite (*v/v*) for about 60 s, followed by a quick rinse in 70% ethanol to remove any epiphytic microbes. The surface sterilized leaves were cut into four equal pieces and incubated at room temperature on agar plates containing an antibiotic to suppress bacterial growth (composition of isolation medium: 15 g/L malt extract, 15 g/L gar and 0.2 g/L chloramphenicol in distilled water), for 14 days. The plates were checked daily for any fungal growth. Single isolates grown out from the tissues were sub-cultured into potato dextrose agar (PDA) and incubated for 14 days to obtain a homogenous culture.

2.2. Extraction of metabolites from fungal endophytes

Fungal endophytes were cultivated by inoculating selected endophyte cultures in tubes containing 20 mL of potato dextrose broth and shaken for 10 days, followed by freeze-drying for 2 days. Afterward the dried residues were mixed with 3 mL of 80% aqueous methanol and sonicated using an ultra-sonic bath for 20 min. The samples ($n = 3$) were then shaken at 40 rpm overnight using shaker to aid metabolite extraction. Using a 0.2 µm filter, the samples were filtered into 2 mL HPLC vials prior analysis on the LC-q-TOF-MS.

2.3. Liquid chromatography quadrupole-time-of-flight mass spectrometry analysis

Liquid chromatography hyphenated to quadrupole-time-of-flight mass spectrometry (LC-q-TOF-MS) model LC-MS 9030 instrument equipped with a Shim-pack Velox C_{18} column (100 mm × 2.1 mm, 2.7 um particle size) (Shimadzu, Kyoto, Japan) placed in a column oven set at 40 °C, was used for sample analysis. The data was acquired in data dependent acquisition mode and three technical replicates were done in order to ensure reproducibility. The binary solvent mixture consisting of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) was used at a constant flow rate of 0.3 mL/min. The gradient method was gradually changed from 2 to 31 min to achieve the separation of intended analytes. Briefly, the gradient conditions were as follows; 0 - 2 min: 2% B, 2 - 24 min: 60% B, 24 – 25 min: 95% B, 25 – 27 min: 95% B, 27 – 28 min:2% B, 28 - 30 min: 2% B. The analytes were monitored using a mass spectrometer detector under the following conditions: ESI negative mode; 3.5 kV interface voltage; nitrogen gas was used as nebulizer at flow rate 3 L/min, heating gas flow at 10 L/min, heat block temperature at 400 °C, CDL temperature at 250 °C and 1.70 kV detector voltage.

2.4. Molecular networking

A molecular network was created on the GNPS website (http://gnps.ucsd.edu) using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) according a published protocol [11]. Raw data from LC-q-TOF-MS analysis were converted to mzML and uploaded on WinSCP which can transfer the data online. The precursor ion mass tolerance and MS/MS fragment ion tolerance were set at 0.02 Da. The cosine score and matched peaks were set at 0.7 and 6, respectively. The maximum number of nodes that can be connected into a single molecular family was set 100 and the lowest scoring edges were removed. The output of the molecular network was visualized using Cytoscape version 3.9.0.

2.5. Multiple reaction monitoring

Liquid chromatography hyphenated to a triple quadrupole mass analyzer mass (LC-QqQ-MS) model LC-MS 8045 instrument was used to monitor and to validate the presence of zearalenone using an automated multiple reaction monitoring (MRM) approach. Using an authentic zearalenone standard, the first quadrupole (Q1) was set to allow specific precursor at m/z (317), which was further fragmented using CID at Q2 from which the resulting fragments were monitored using Q3. MRM transitions of m/z 317.0→174.8, m/z 317.0→131.0 and m/z 317.0→272.9 were used to monitor for zearalenone in all fungal analytes which tested positive according to the molecular networking.

2.6. Molecular identification

The fungi were grown at 28 °C for 7 days for genomic DNA extraction ($n = 3$). Genomic DNA was extracted

using the Quick-DNA fungal/Bacterial Miniprep Kit (Zymo, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify DNA samples with primer pairs ITS3 (5′ -GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′ - TCCTCCGCTTATTGATATGC-3′) targeting the ITS2 region that typically ranges between 450-700 bp. Each PCR reaction of 25 µl in total included 12.5 µL DreamTaq DNA polymerase mix (ThermoFisher Scientific, United States), 6.5 µL distilled water (dH2O), the final concentration 0.4 µM of each primer and 4 µL DNA template. The cycling conditions were as follow: Denaturing for 3 min at 95 °C, 30 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation for 5 s at 72 °C, then followed by a final elongation step for 10 min at 72 °C. The PCR amplicons were sequenced by Inqaba-Biotech (Pretoria, South Africa), using the aforementioned PCR primers. The phylogenetic tree based on ITS3 and ITS4 sequences were created by MEGAX software based on BLAST. The isolates were further aligned with other fungal species obtained from NCBI in order to establish homology with sequences from other fungi of different/same family using Multiple Alignment using Fast Fourier Transform (MAFFT). These sequences were aligned using MUSCLE built in MEGA [12] and visualized using gelview.

3. Results and discussion

The diversity of fungal endophytes isolated from *B. pilosa* was explored in this study and their secondary metabolites were analyzed through LC-q-TOF-MS based metabolic profiling. Molecular networking was used to establish the chemical relationships between the three isolated endophytes. As shown on Figure 1 and Figure S1 from the LC-q-TOF-MS data, compounds from the same class clustered together, forming molecular families which can enable the propagation of annotations from library matches on the GNPS platform. Upon closer analysis, one of the endophytic fungi was found to produce a compound at *m/z* 317.279 which was identified as zearalenone through molecular networking (Figure 1), therefore, highlighting the advantage of using this tool in compound identification. The identity of zearalenone was confirmed through its fragmentation pattern observed on q-TOF data. Its presence from the fungal endophytes (isolate 2) was further validated through tandem mass spectrometry (MS/MS) using an authentic standard of zearalenone (Figure 2 and Figure S2). The standard was used to validate the identification of the target compound and consistent transitions observed using the MRM method further authenticate that the compound is indeed zearalenone rather than a derivative. The product ion scan of zearalenone revealed consistent fragmentation patterns characterized by the transitions of zearalenone at *m/z* 317 (precursor ion) to its fragment ions at *m/z* 131, 175, 203 and 273 and these results are consistent with results that were reported previously [13]. Zearalenone exhibits chemical and structural similarity to the human sex hormone called 17-β-estradiol, therefore, it can bind to estrogen receptors found in particular cells thereby disrupting the hormonal balance and resulting in the development of various diseases linked to the reproductive system [8,14]. Frida et al. [15] suggested that the use of

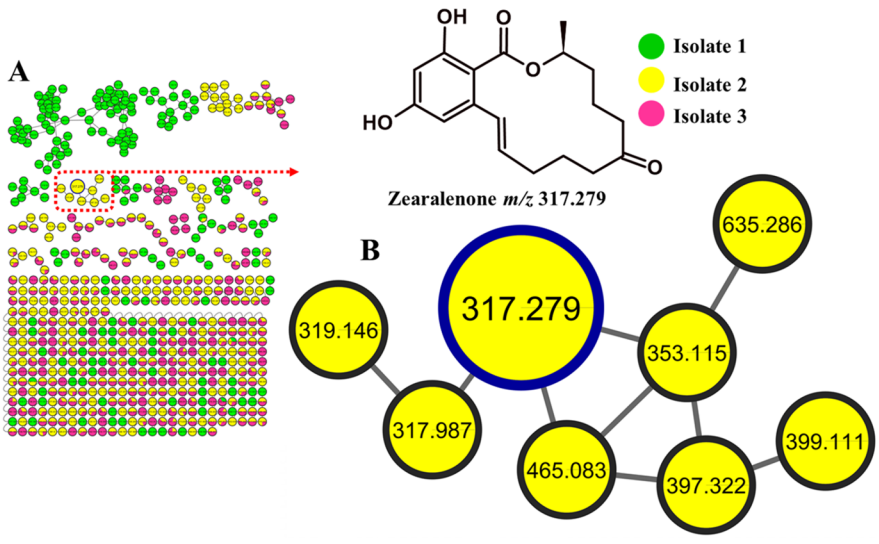

Figure 1. Classical molecular network of secondary metabolites produced by fungal endophytes isolated from *Bidens pilosa* analyzed using the LC-q-TOF-MS. Full network (A) and a molecular family (B) showing the presence of zearalenone in isolate 2 where the enlarged node represents zearalenone.

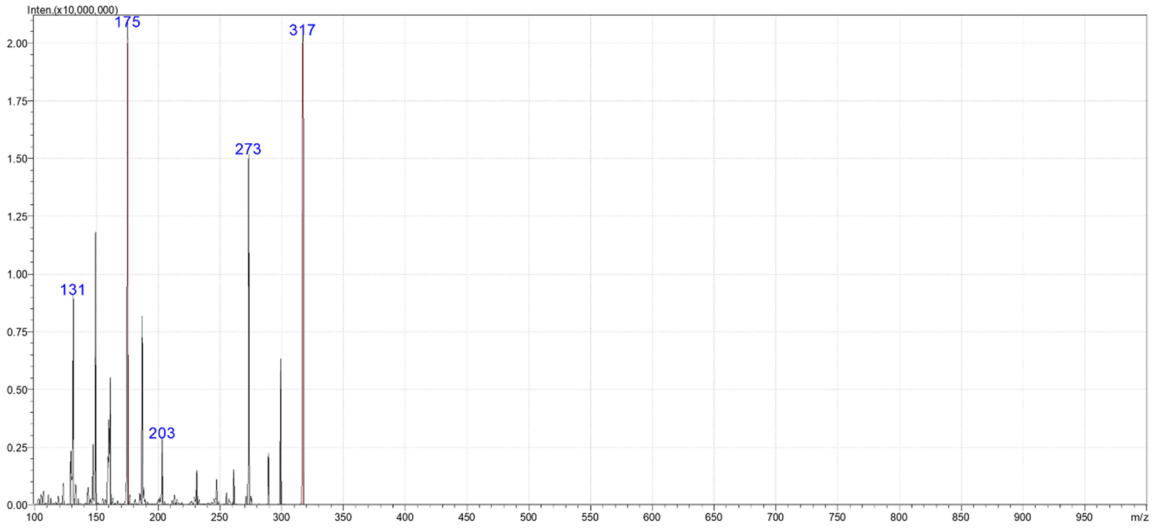

Figure 2. The product ion scan spectrum of zearalenone isolated from endophytic fungi (isolate 2) achieved through multiple reaction monitoring approach-based survey scan approach. The product ion scan of zearalenone showed a consistent fragmentation pattern characterized by the transition of zearalenone at *m/z* 317 (precursor ion) to its fragment ions at *m/z* 131, 175, 203 and 273.

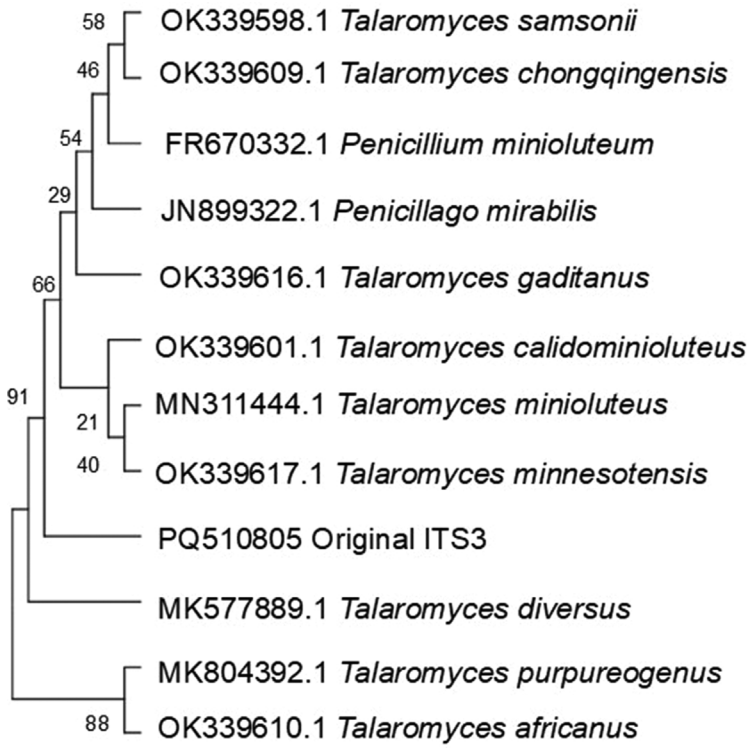

Figure 3. Phylogenetic tree constructed using the Neighbor-Joining method, showing relationships among 12 nucleotide sequences. Bootstrap values (1000 replicates) are indicated next to branches. Distances were calculated with the maximum composite likelihood method, conducted in MEGAX.

B. pilosa in the initiation of labor during childbirth by traditional practitioners could be due to the presence of bioactive compounds with estrogen-like and oxytocin-like activities. Therefore, findings of the current study suggest that the presence of zearalenone in endophytic fungi found in *B. pilosa* might be responsible for this bioactivity. There are also reports on the fast absorption of zearalenone into mammal's body and metabolized to very toxic metabolites [16]. As a result, its presence in one of the endophytes from *B. pilosa* may pose a serious food safety and health concern since this plant is used as food or medicine by various communities around the world. The health risks may also be exacerbated by long-term ingestion of these green leafy vegetables. Though, *B. pilosa* does not contain

this toxin, it is important that responsible and safe post-harvest storage practices are adhered to, in order to minimize the growth of these endophytic microorganisms which may pose serious health risks.

The current study also identified the zearalenone producing endophytic fungi (isolate 2) through the ITS DNA sequencing approach. Phylogenetic analysis of ITS DNA sequences revealed that the zearalenone-producing endophyte isolated from *B. pilosa* belongs to the *Talaromyces* genus, showing sequence similarity to multiple species, as illustrated in Figure 3. As a result, the species could not be identified with certainty. It is noteworthy that this was the first report showing endophytic fungi from this genus producing zearalenone. However, structurally similar compounds identified as talarocelic acids were reported to be produced by *Talaromyces cellulolyticus*, in a recent study conducted by Song et al. [17]. The presence of zearalenone in this isolate was confirmed through a series of experiments that showed consistent results. However, it is important to note that no trace of zearalenone was detected in *B. pilosa*, suggesting that the endophytic fungi responsible for producing zearalenone do not release it within their host. Multi-sequence alignment of the two ITS sequences obtained from positive isolates together with the sequence retrieved from NCBI is shown on Figure S3. According to this multi-sequence alignment and BLAST searches, the percentage similarity between these sequences were very high at 98%. *Talaromyces* species were initially classified as Penicillium [18]. The current study is also a first report of *Talaromyces* in *B. pilosa*.

4. Conclusion

In this study, LC-q-TOF-MS and molecular networking were employed to analyze the metabolomes of fungal endophytes isolated from *Bidens pilosa*. Among the isolated endophytes, one was found to produce zearalenone, a mycotoxin with potential to disrupt hormonal balance in both humans and animals. Identification through ITS sequencing revealed that this zearalenone-producing endophyte belongs to the *Talaromyces* genus. Moreover, this study emphasizes the combination of molecular identification and computational metabolomics in highlighting the presence of toxic secondary metabolites. While *B. pilosa* is well-regarded as a source of pharmacologically active metabolites, the production of harmful compounds such as zearalenone by associated endophytic fungi raises concerns. These findings suggest that using *B. pilosa* as a nutraceutical source needs caution, as endophytic microorganisms within the plant could pose health risks to consumers of this vegetable. Especially if the post-harvest storage is conducive for the fungi to grow and produce these undesirable toxin. Further research into the presence of such compounds across commonly consumed plants is essential for ensuring food safety and consumer health.

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Author contributions

PWM and NM carried out the experiments and wrote the first draft of the manuscript. AK and KM conducted molecular work. ANT supervised the project. BM took part in writing the manuscript. NEM ran the LC-MS and supervised the project. All the authors read the final draft of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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