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Isolation and Identification of Endophytic Fungi of Rauwolfia Serpentina (L.) Benth. Ex Kurz by DNA Barcoding

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Isolation and Identification of Endophytic Fungi of *Rauwolfia serpentina* (L.) Benth. Ex Kurz by DNA Barcoding

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ABSTRACT

Endophyte fungi are getting importance now a days for alternative source of valuable therapeutic compounds during their growth with host plants. Reports are available for possible association of endophytic microorganisms including bacteria, actinomycetes and fungi in different known plants having medicinal properties. *Rauwolfia serpentina* is a well-known Indian medicinal plant used in Ayurveda for treatment of snakebite, feverish illness and mental illness from ancient time and known for its antimicrobial, anti- inflammatory, antioxidants, antiproliferative, anticancerous, anti-diuretics, antifibrillar, anti-arrhythmic, anti-cholinergic, anti-dysentery, anti-diarrheal and anti-hypotensive properties. In the present study, an attempt was made to isolate endophytic fungi from leaves and stem parts of healthy young plant of *Rauwolfia serpentina* under laboratory conditions. Altogether 05 isolates namely *Aureobasidium melanogenum, Aureobasidium pullulans, Aureobasidium iranianum, Simplicillium obclavatum* and *Phoma herbarum* were obtained and identified through 18 S r RNA gene sequencing analysis. Isolated fungi were identified by sequence-based DNA Barcoding by using ITS region. Endophytic fungal DNA have been submitted to NCBI, Gene Bank and obtained Accession number for reference for further work and its phylogenetic tree was constructed by using MEGA 5.2 Software.

Keywords: *Rauwolfia serpentina*, Endophytic fungi, DNA Barcoding, ITS region, Phylogenetic tree

Abbreviations: 18 s rRNA ,18 S ribosomal ribonucleic acid; DNA, Deoxyribonucleic acid; ITS, Internal transcribed Spacer; NCBI, National Center for Biotechnology Information; MEGA, Molecular Evolutionary Genetics Analysis

1.INTRODUCTION

Rauwolfia serpentina is used as medicinal plant from ancient time for its medicinal properties. It belongs to family Apocynaceae. It is reported that powdered root of this plant has been used for treatment of snakebite, feverish and mental illness in Ayurvedic literature (Singh et al., 2016). This plant has immense therapeutic values against various diseases (Pathania et al., 2015) and have antimicrobial, antioxidant, anti-inflammatory, anticancer us, antidysentery, antiarrhythmic, anticholinergic, antidysentery, antidiarrheal, antihypotensive properties (Pal & Paul, 2020).

The various alkaloids identified in *Rauwolfia serpentina* include ajmaline, ajmalimine, ajmalicine, deserpidine, indobine, indobinine, reserpine, reserpiline, rescinnamine, rescinnamidine, serpentine, serpentinine and yohimbine etc.(Bunkar, 2017)) .Therapeutic properties are due to presence of various phytochemicals such as alkaloids, flavonoids , reserpine, serpentine etc.in the plant extracts(Gambhir et al., 2020).It is reported that 12.4 % of alkaloids and 7.35% of saponins are present in plant sample (Hussain et al.,) (Singh et al., 2016).

Various micro-organisms reside symbiotically mutualistic association with their host plants and reside in outer as well as internal soft tissues without any harmful effect to the host plant (Saikkonen et al., 1998). They may be bacteria, actinomycetes, fungi, yeasts etc. These microorganisms are known as endophytes. Endophytic fungi are one of them that protects their host from drought, unfavourable conditions and

pathogenic invaders. They produce a number of novel bioactive compounds like alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids which are beneficial for host growth and survival in unfavourable conditions. These compounds are useful in agriculture, industries and pharmaceutical industries for the production of medicine, drugs and natural biochemicals (Sahu et al., 2016).

It is reported that these endophytes may produce same bioactive compounds of their host. They can produce enzyme (Khan et al., 2016), plant growth hormones, alkaloids, non-ribosomal peptides, due to the presence of biosynthetic gene clustered in their genome (Li et al., 2016). For obtaining these secondary metabolites for antimicrobial, agricultural and pharmaceutical uses, isolations of endophytic fungi from leaves and stems of *Rauwolfia serpentina* was the objective of this study.

DNA Barcoding is a technique for characterizing species of organisms using a short DNA sequence from a standard and agreed- upon position in the genome. Species identification through barcoding is usually achieved by the retrieval of a short DNA sequence- the barcode- from a standard part of the genome from the specimen under investigation. The barcode sequence from each unknown specimen is then compared with library of reference barcode sequences derived from individuals of known identity. A specimen is identified if its sequence closely matches one in the barcode library.

The DNA barcode for fungi is Internal Transcribed Spacer regions (ITS 1,5.8 and ITS 2) gene sequence collectively called as ITS region for species identification (Senthilkumar et al., 2018).Some isolated fungal species in culture media is sticky, non-cottony in appearance, they have existed in anamorphic stage and we face difficulty in authentic identification of that specific fungal sample so we need 18 S ribosomal RNA sequencing. In this study we have isolated endophytic fungi from medicinal plant *Rauwolfia serpentina* to explore the unknown endophytic fungal diversity of this plant and identified by DNA barcoding and constructed phylogenetic tree by using MEGA 5.2 Software.

2.MATERIALS AND METHODS

2.1 Selection of Plant and Sample collection

Rauwolfia serpentina is a well-known medicinal plant of family Apocynaceae.

Healthy leaves and stems were collected as sample of *Rauwolfia serpentina* (L.) Benth. ex Kurz. from the Herbal Plant Nursery, Mana, Raipur, Department of Forest, Govt. of Chhattisgarh, India in July 2019.

2.2 Isolation of Endophytic fungi

Stems and Leaves were washed under running tap water until the surface adherents are removed. Leafy parts of fresh healthy *Rauwolfia serpentina* plants were cut into small pieces (5 mm × 5 mm) using sterile surgical blade and washed with sterile distilled water. The samples were surface sterilized by dipping into 4% sodium hypochlorite for 5 minutes and 96% ethanol for 1 minute, rinsed with sterile water and allowed to surface dry under sterile conditions. The surface sterilized samples were placed on Potato Dextrose Agar (PDA) plates amended with 50 mg/L tetracycline antibiotic to suppress the bacterial growth and incubated at 28ºC to 30 °C for 2 to 3 days. The outer bark of the stem was removed with sterile sharp blade and cut in to small pieces of inner part of the stem and inoculated on PDA media (Senthilkumar et al., 2018).

Inoculated plates were incubated at 28 °C till vegetative growth appears from inoculated tissues.

The hyphal tip of endophytic fungi growing out from the plant tissue was transferred to fresh PDA plates amended with 50 mg/L tetracycline. After incubation at 28 °C for 7 to 14 days, purity of the culture was determined by colony morphology.

2.3.3 Microscopic study of isolated fungal strains

Endophytic fungi isolated from *Rauwolfia. serpentina* were coded as S1, S2, S3, S4 and S5 Sample and studied under compound microscope after staining with Lactophenol cotton blue stain.

2.3.4 Molecular identification

Fungal Genomic DNA Extraction and 18 s ribosomal RNA sequencing

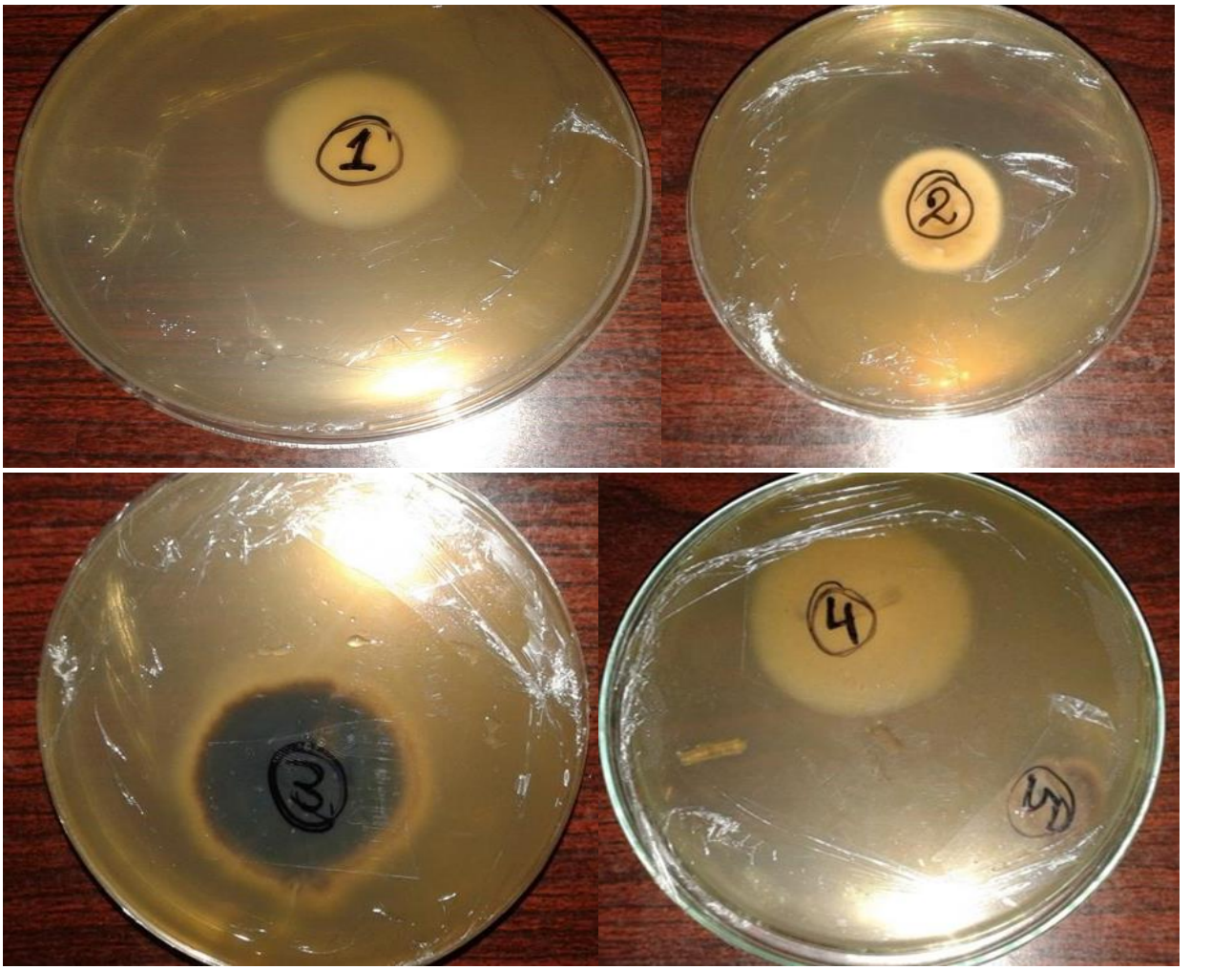
Isolated cultures were inoculated in PDB Broth at 28℃ for 7 days. After 7 days harvested cultures were centrifuged and pellets were collected. To the pellets, 50 ml. of lysis buffer (20 ml. tris HCl 400mM, 6 ml EDTA 60Mm, 0.7 ml Nacl 150 mM, 0.5 gm SDS 1%, 22.8 ml MQ) were added, mixed and incubated at room temperature for 40 min. Chloroform and isoamyl alcohol in the ratio of 24:1 to the above mixture added, centrifuged at 10000 rpm for 5 min. Supernatant were collected and added equal volume isopropyl alcohol, mixed and centrifuged at 10000 rpm for 7 min. .Supernatant were discarded and pellets were resuspended with 75 % ethanol , again centrifuged at 10000 rpm for 7 min. . 75% ethanol were discarded and pellets were oven dried and MQ water were added. Hence fungal DNA were obtained (Tripathy et al., 2017). Extracted Fungal DNA has been sent to Bio serve Biotechnologies (India) Pvt. Limited, Mallapur, Hyderabad, Telangana for molecular identification through 18 S Ribosomal RNA sequencing. The identification was confirmed solely based on the gene sequencing methodology using 18S RNA primer and primer synthesized by using Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. Investigated fungi were identified by subjecting the DNA sequence to BLASTn analysis and by using MEGA 5.2 Software, phylogenetic tree was constructed.

RESULTS AND DISCUSSION

Five endophytic fungi were isolated from the different parts (leaves and stems) of the *Rauwolfia. serpentina* collected from Herbal Plant Nursery, Mana, Raipur, Department of Forest, Govt. of Chhattisgarh, India in July 2019 as depicted in table 1. A total 32 segments (leaf 16, stem 8) of *Rauwolfia. serpentina* were processed for the isolation of endophytic fungi**.** Isolated Five endophytic fungi were identified as *Aureobasidium melanogenum, Aureobasidium iranianum Aureobasidium pullulans, Simplicillium obclavatum and Phoma herbarum.*

Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-3394.3534) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The optimal tree with the sum of branch length = 12.25508551 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree The analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 491 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011)



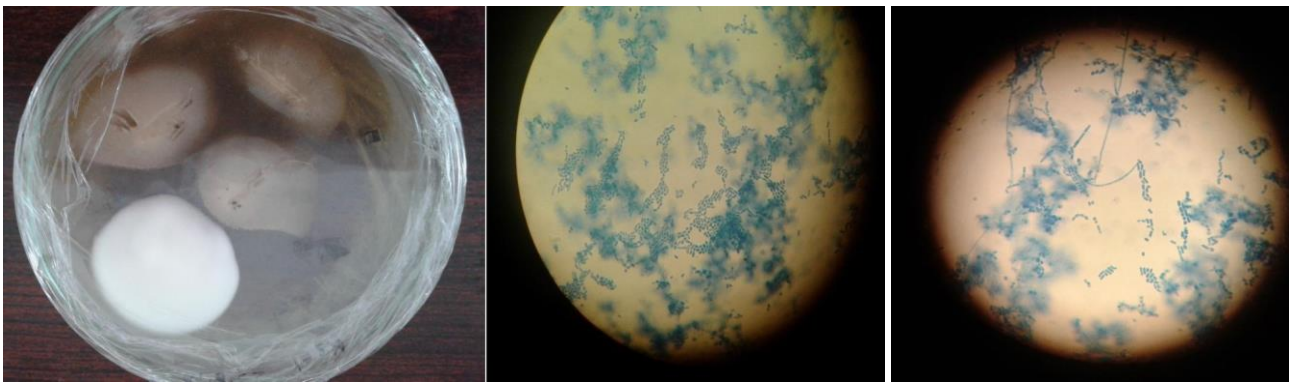


Figure 1. Fungal cultures of S1, S2,S3,S4,S5- in PDA plates and Microscopic images of Sample S2

Table 1: List of Isolated endophytic fungi

Sr.	Fungal Culture code	Identified fungi	Class
1	S1	*Aureobasidium melanogenum*	Dothideomycetes
2	S2	*Simplicillium obclavatum*	Sordariomycetes
3	S3	*Phoma herbarum*	Dothideomycetes
4	S4	*Aureobasidium pullulans*	Dothideomycetes
5	S5	*Aureobasidium iranianum*	Dothideomycetes

Sanger Sequences of five fungal samples

Sample *1* *Aureobasidium melanogenum* strain,

>MK035871.1:1-530 Aureobasidium melanogenum clone AB1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
ATACGGTGCTCAGCGCCCGACCTCCAACCCTTTGTTGTTAAAACTACCTTGTTGCTTT
GGCGGGACCGCTCGGTCTCGAG
CCGCTGGGGATTCGTCCCAGGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTAT
TAAACCGGTCGTCTGAGTTAAAA
TTTTGAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTG
GTATTCCGAGGGGCATGCCTGTT
CGAGCGTCATTACACCACTCAAGCTATGCTTGGTATTGGGTGCCGTCCTTAGTTGGG
CGCGCCTTAAAGACCTCGGCGAG
GCCTCACCGGCTTTAGGCGTAGTAGAATTTATTCGAACGTCTGTCAAAGGAGAGGAC
TTCTGCCGACTGAAACCTTTTAT
TTTTCTAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCA

Sample 2 *Simplicillium obclavatum* strain,

>MK035872.1:1-491 Simplicillium obclavatum clone AB2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

CCCTTTGTGACCTACCTTTATGTTGCTTCGGCGGTGACGCGCCGGGTTGCTCCCTCAG
GGAGCTCCCGGGACCACGCGCC

CGCCGGAGACCACAAACTCTTGATTTTGCGAAAGCAGTATTCTTCTGAGTGGCCGAA
AGGCAAAAAAACAAATGAATCAA

AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
ATAAGTAATGTGAATTGCAGAAT

TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCA
TGCCTGTTCGAGCGTCATTTCAA

CCCTCGAGCTCGTCTTCATTGACGAGATCGGTGTTGGGACCCGGCGATCGGGGACTT
TAGTTCCTCTGCCGGTCCTGAAA

TTCAGTGGCGGCCCGTTGCGGCGACCTCTGCGTAGTAACTTAACCTCGCACTGGGAC
AGCAGCGCGGCCACGCCGTAAAA

CCCCCGACTTT

Sample 3 *Phoma herbarum* strain

>MK035873.1:1-525 Phoma herbarum clone AB3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

TGAGACCGATCATCTAGAGTGCGAGCTTTGCCTGCCATCTCTTACGCCATGTCTTTTG
AGTACCTTACGTTTCCTCGGCG

GGTCCGCCCGCCGATTGGACAATTTAAACCACTTGCAGTTGCAATCAGCGTCTGAAA
AAACTTAATAGTTACAACTTTCA

ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
GTGTGAATTGCAGAATTCAGTGAA

TCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTC
GAGCGTCATTTGTACCTTCAAG

CTTTGCTTGGTGTTGGGTGTTTGTCTCGCCTTTGCGCGCAGACTCGCCTCAAAACAAT
TGGCAGCCGGCGTATTGATTTC

GGAGCGCAGTACATCTCGCGCTTTGCACTCAGAACGACGACGTCCAAAAGTACATTT
TTACACTCTTGACCTCGGATCAG

GTAGGGATACCCGCTGAACTTAAGCATATCAAAAGCCGGGAGGAA

Sample 4 *Aureobasidium pullulans* strain

>MK035874.1:1-655 Aureobasidium pullulans clone AB4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
GGGTGCTCAGCGCCCGACCTCCAACCCTTTGTTGTTAAAACTACCTTGTTGCTTTGGC
GGGACCGCTCGGTCTCGAGCCG

CTGGGGATTCGTCCCAGGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTTA
ACCGGTCGTCTGAGTTAAAATTT

TGAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACG
CAGCGAAATGCGATAAGTAATGT

GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTA
TTCCGAGGGGCATGCCTGTTCGA

GCGTCATTACACCACTCAAGCTATGCTTGGTATTGGGCGTCGTCCTTAGTTGGGCGC
GCCTTAAAGACCTCGGCGAGGCC

ACTCCGGCTTCAGGCGTAGTAGAATTTATTCGAACGTCTGTCAAAGGAGAGGAACTC
TGCCGACTGAAACCTTTATTTTT

CTAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGC
GGAGGAAAGGATCATTAAAGAGT

AAGGGTGCTCAGCGCCCGACCTCCAACCCTTTGTTGTTAAAACTACCTTGTTGCTTTG
GCGGGACCGCTCGGTCTCGAGC

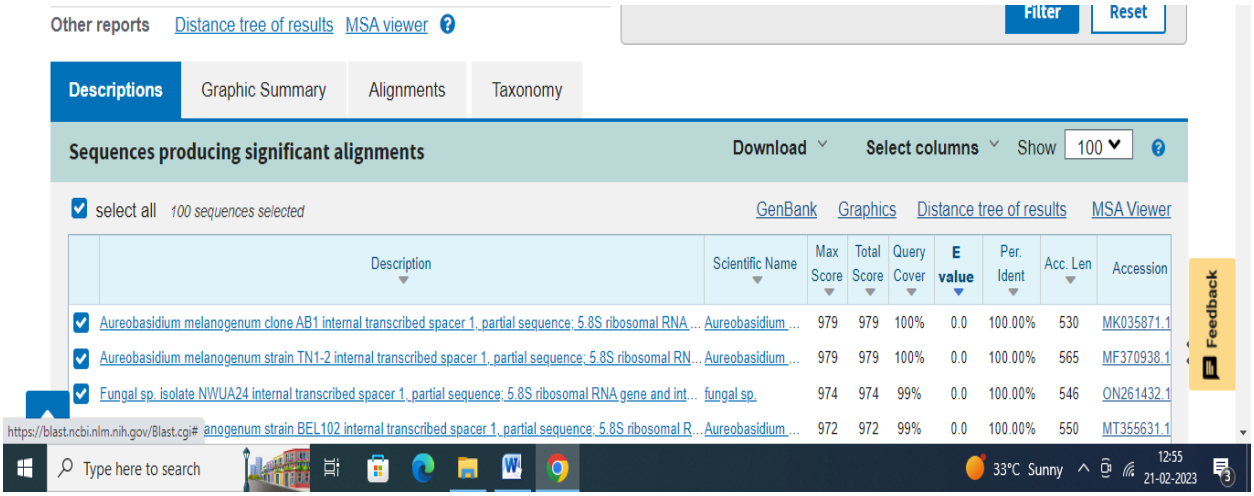
GCTGGGGATTCGTCC

Sample 5 ***Aureobasidium iranianum***

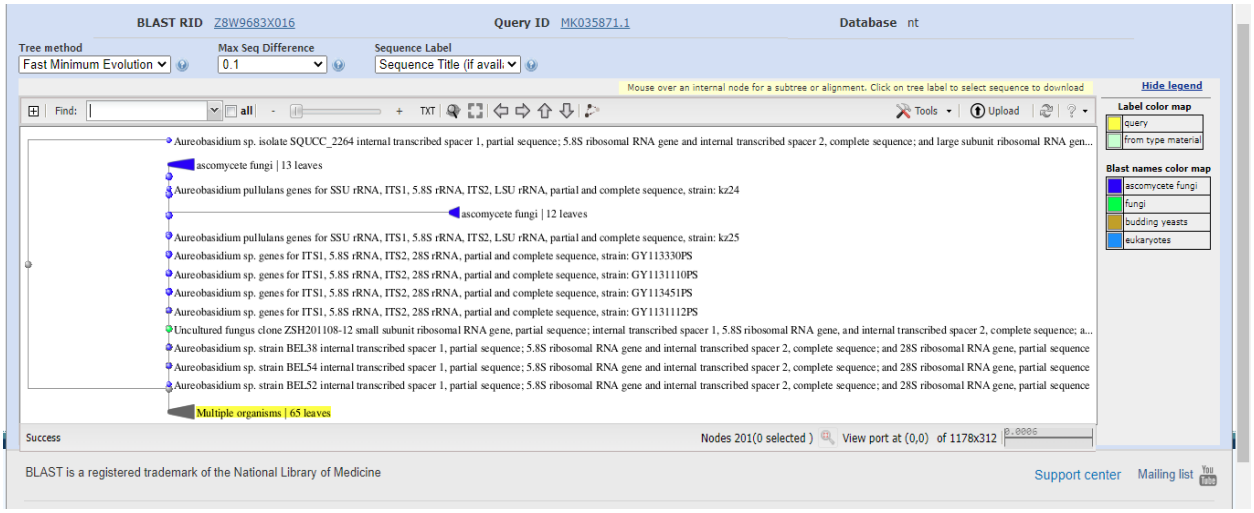
>MK035875.1:1-567 Aureobasidium iranianum clone AB5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
AAAGAGTAAGGGTGCTCAGCGCCCGACCTCCAACCCTCTGTTGTTAAAACTACCTTG
TTGCTTTGGCGGGACCGCTCGGT
CCCGAGCCGCCGGGGATCCGTCCCAGGCGAGCGCCCGCCAGAGTCCAACCAAAACT
CTTGTTAATCGTAACCGGTCGTCT
GAGTAAAAATTTTTGAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCA
TCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCCTTGGTATTCCGAGGGG
CATGCCTGTTCGAGCGTCATTACACCACTCAAGCTATGCTTGGTATTGGGTGCCGTC
CCCTTCCACGGGGGGCGCGCCTC
AAAGACCTCGGCGAGGCCTCACCGGCTTTAGGCGTAGTAGAATTTATTCGAACGTCT
GTCAAAGGAGAGGACTTCTGCCG

ACCGAAACCTTTTATTTTTTTCTAGGTTGACCTCGGATCAGGTAGGGATACCCGCTG
AACTTAAGCATATCAATAAGCGG
AGGAAAA

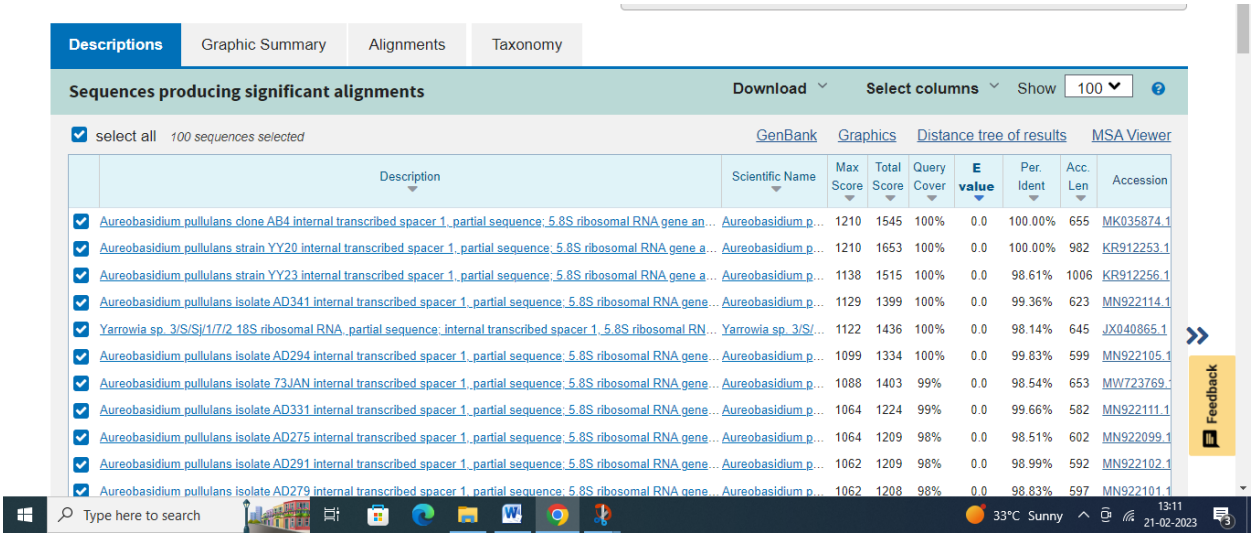
After BLASTn of *Aureobasidium melanogenum*



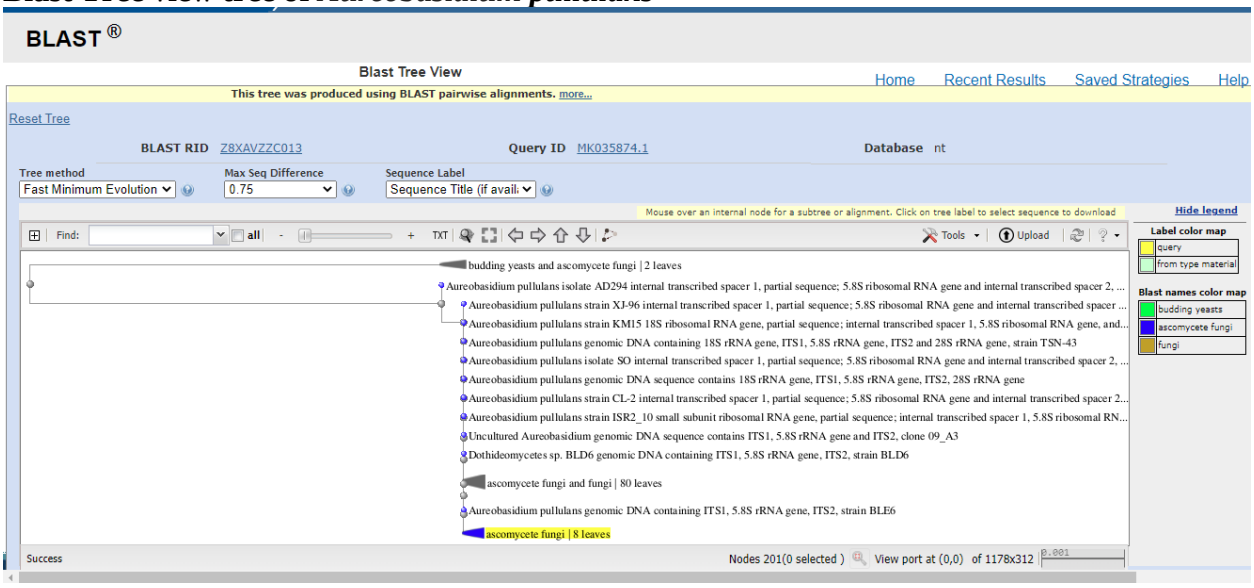
Blast Tree view tree of *Aureobasidium melanogenum*



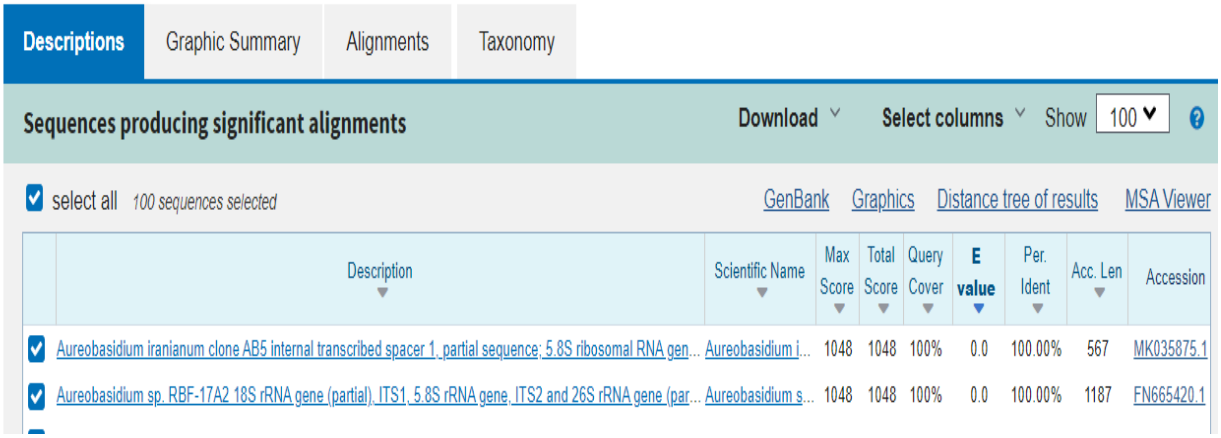
After BLASTn of *Aureobasidium pullulans*



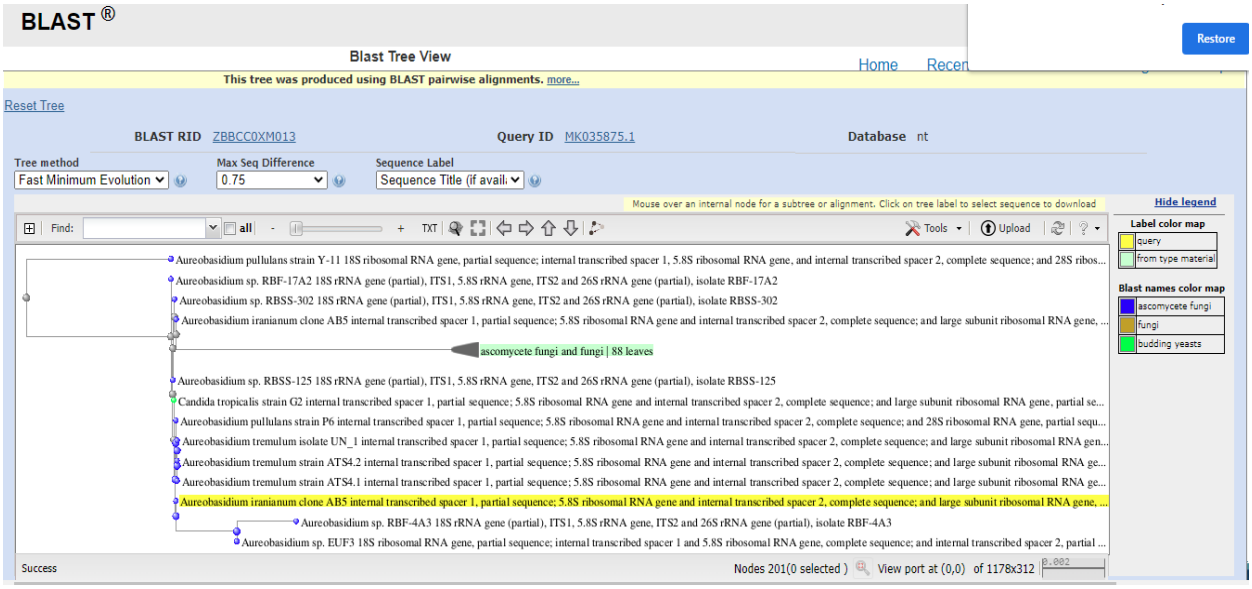
Blast Tree view tree of *Aureobasidium pullulans*



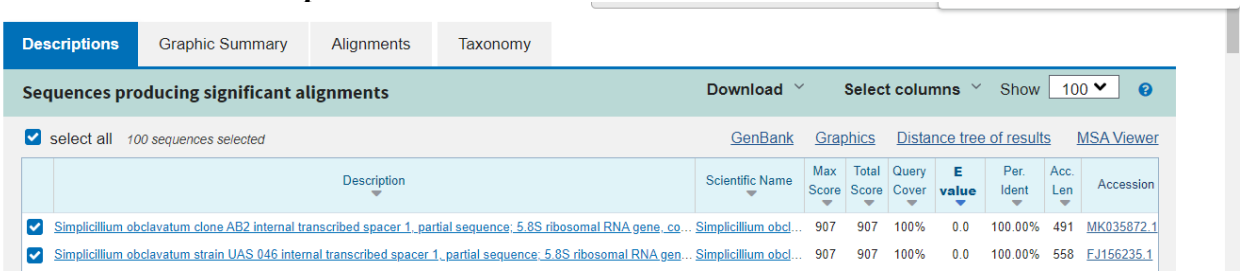
Blast Tree view tree of *Aureobasidium iranium*



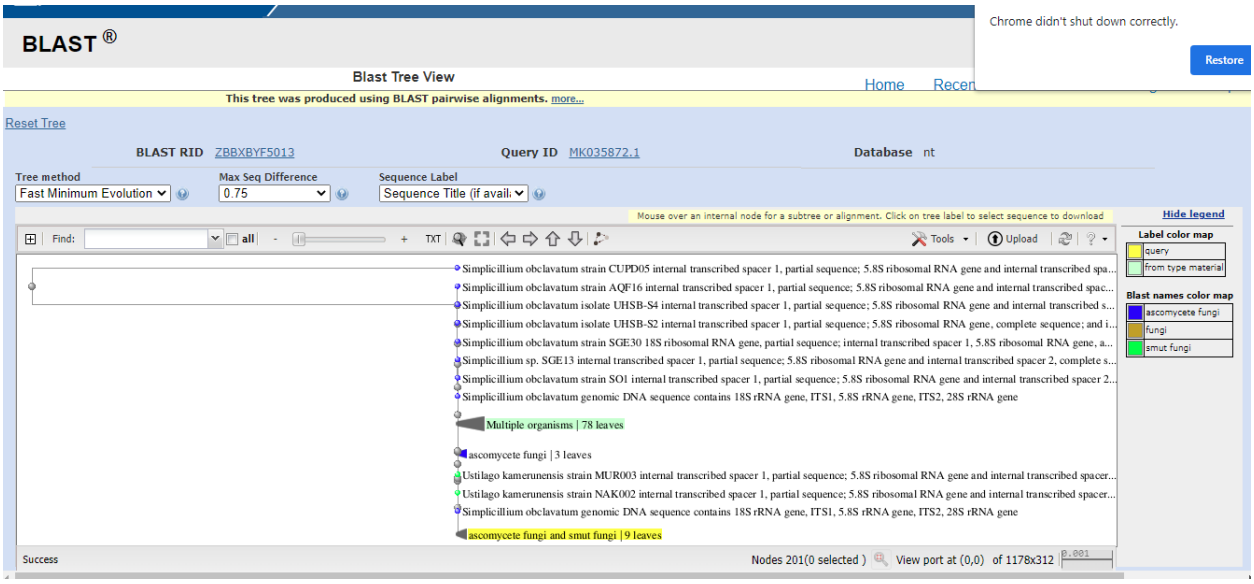
BLAST TREE VIEW



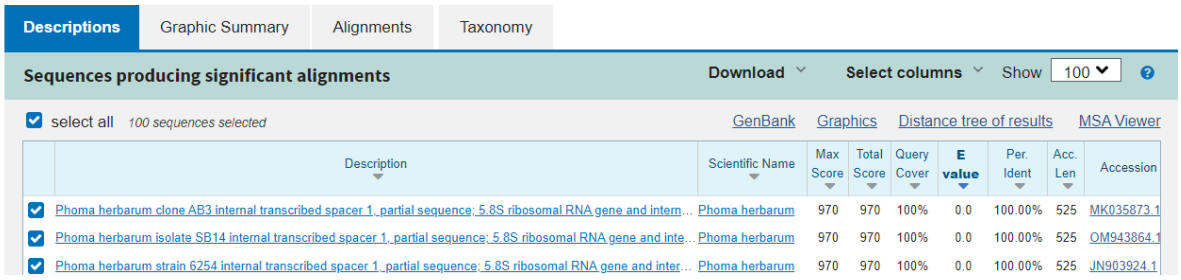
After BLASTn of *Simplicillium obclavatum*



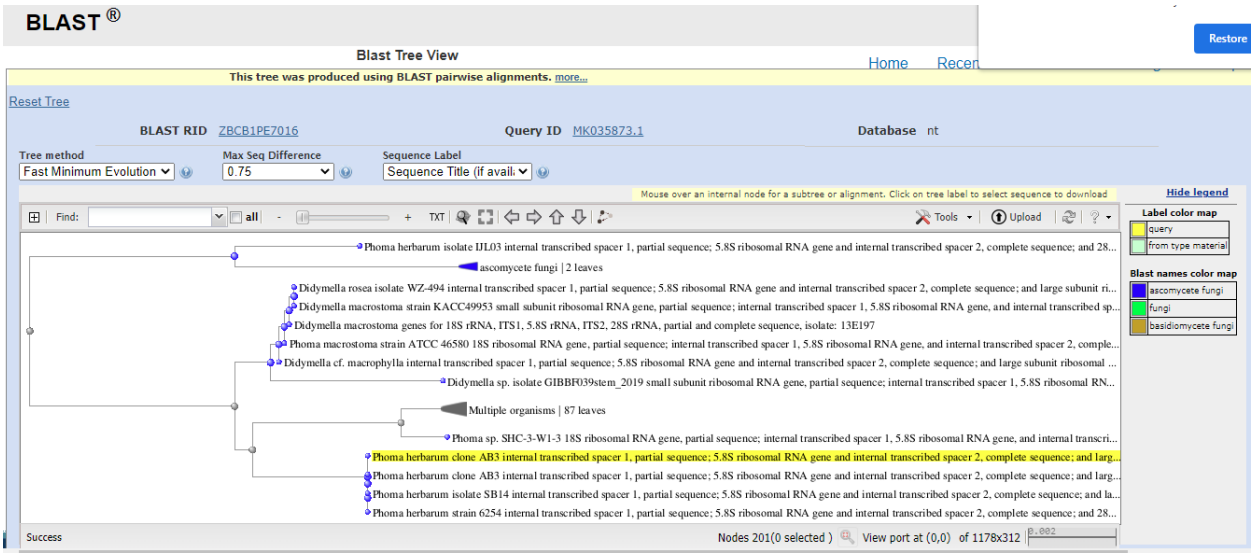
BLAST TREE VIEW



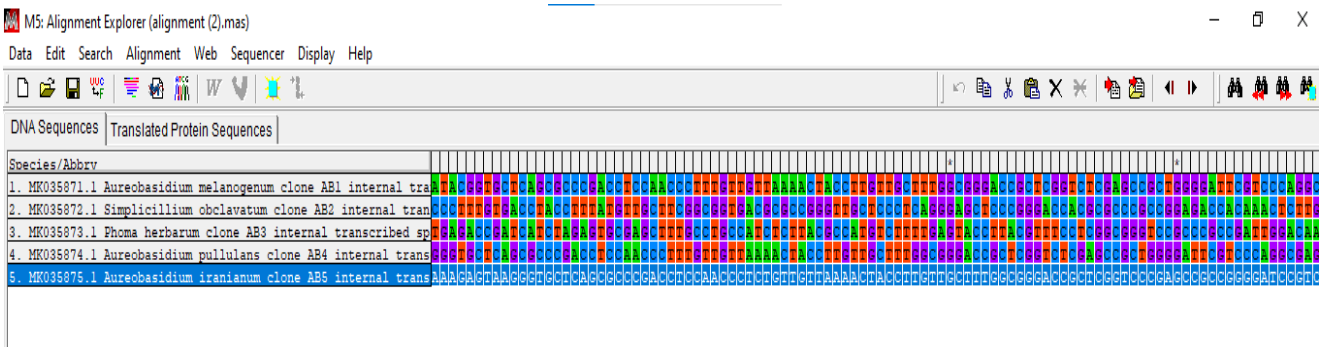
After BLASTn of *Phoma herbarum*



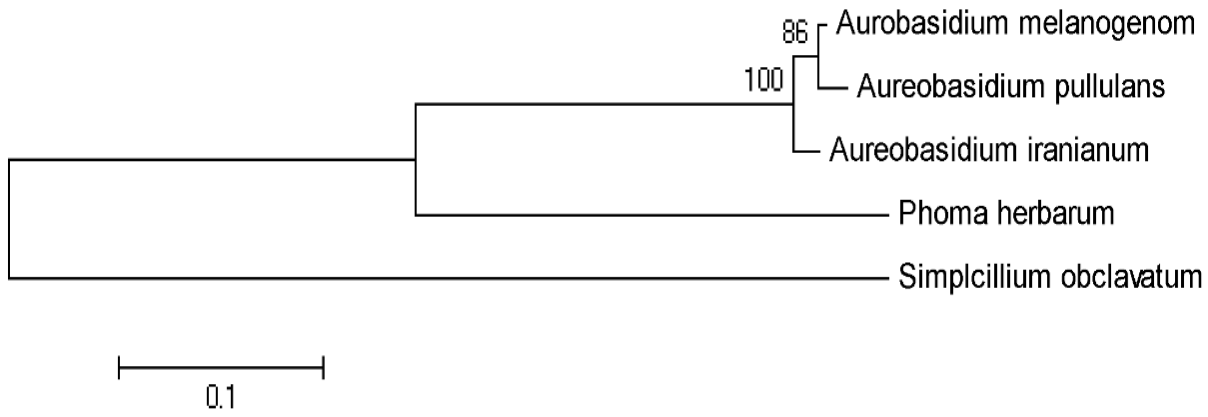
BLAST TREE VIEW of *Phoma herbarum*



Multiple Alignment of identified fungal strains



Phylogenetic Tree of identified fungal strains using MEGA 5.2



CONCLUSION

From fresh and healthy leaves of *Rauwolfia serpentina* 03 species of *Aureobasidium* were isolated of family *Aureobasidium*, 01 species of family *Simplicillium* and 01 species of family *Phoma.* These species were identified by 18 S Ribosomal RNA sequencing. In some research studies *Aureobasidium pullulans* produce secondary metabolites auxin, Exopolysaccharides pullulans which have plant growth promoting properties and bioremediation in Cadmium and Lead. New antifungal and antiviral peptides were isolated from *Simplicillium* species. These reports will help for further research to produce useful bioactive chemical substances from pure isolated fungi as well as bioremediation of heavy metals to reduce environmental pollutions.

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CONFLICT OF INTERESTS

The authors have no potential conflict of interest regarding publication of the said manuscript.

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