Molecular & Morphological Identification of Entomopathogenic Fungus Purpureocillium lilacinum Isolated From Soil, Balakot Tehsil, Khaber Pakhtunkhwa, Pakistan

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Research Article

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Abstract

Purpureocillium lilacinum plays a crucial role as a natural biological control agent and serves as a valuable source for fungicides used in managing pests across various insect orders worldwide. The primary aim of this research was to isolate and identify *P. lilacinum* fungi from the soil of different forests and parks in the Balakot Tehsile. A total of 30 soil samples were collected, and fungal isolation was performed via the soil dilution technique. The isolated colonies were identified by the morphological characteristics of the colonies and conidia at the micro- and macro-levels by using scanning electron microscopy and also at the molecule-level standard molecular techniques, including nucleic acid extraction, ITS region ribosomal DNA amplification, and DNA sequence analysis. The fungal species isolated from the Balakot soil had 98.84–99.07% similarity with the related fungi recorded in GenBank, where they were deposited from different countries. This is the first effort to isolate *P. lilaacinum* from soil in Pakistan. In the future, it may have great economic value as a biocontrol agent in industry due to its entomopathogenic properties.

INTRODUCTION

Farmers often employ insecticides, especially chemical synthetic pesticides, to manage pests and other insects. Pesticide use has led to threats to human health, an increase in pest resistance, and ecological damage (Pathak et al., 2022). To avoid the use of chemical insecticides, environmentally friendly methods for controlling pests, such as the use of entomopathogenic fungi (EPFs), have gained widespread adoption over time (Skinner et al., 2014). Arthropods can be infected and killed by entomopathogenic fungi (EPFs), which are bioinsecticides. While they are primarily extracted from arthropod carcasses, their native environment is soil (Bihal et al., 2023). The following six fungal classes are present: Microsporidia, Oomycetes, Chytridiomycota, Entomophtoromy cota, Basidiomycota, and the most prevalent Ascomycota. The primary purpose of the EPF in the environment is to biologically control the papulation of insects (Litwin et al., 2020). *P. lilacinum* (Ascomycota: Hypocreales) (priorly *Paecilomyces lilacinus*) is a globally distributed fungus species and an EPF that is frequently found in soil and is employed as a biological nematode control agent (Baron et al., 2020), even though Triatoma infestans, a Chagas disease vector, has also been employed as a natural pathogen (Forlani et al., 2015), and its efficacy in combatting cotton aphids (*Aphis gossypii*) and red mites (Tetranychusurticae) has been confirmed (Khan & Tanaka, 2023). Moreover, it has been found to be a potential natural biological pathogen for a red fire ant and a white fly, Trialeurodes vaporariorum, and it has also been identified as a pathogen for Atta capiguara and A. laevigata, two winged female leafcutter ants (Goffre & Folgarait, 2015). Because of its entomopathogenic characteristics, it is a prevalent biocontrol agent for a variety of insect pests, including above-ground and soil-dwelling pests (Nguyen Thi et al., 2023). Entomopathogenic fungi have attracted some interest as insecticide substitutes for application in products that are stored under IPM. Entomopathogenic fungi have the potential to improve human safety and prevent the spread of epizootics (Wakil et al., 2014). Currently, *P. lilacium* has never been reported to be prevalent in Pakistani soil, and the initial stages in the formulation of biopesticides include the identification and characterization of EPFs (Dunlap et al., 2018). Fungi must be identified molecularly

since morphological features are insufficient for species-level differentiation (Minarni et al., 2021). Polymerase chain reactions have been used to amplify various DNA or RNA segments to isolate and identify EPFs. Morphological characterization was the initial method used in this regard (Sun et al., 2021). Fungal species can be identified molecularly by sequencing a PCR-amplified segment of the 18S rRNA gene using universal primers (Alsohaili & Bani-Hasan, 2018). The most widely sequenced genetic marker of fungi, the ITS region, is regularly utilized to resolve research issues related to strain identification (Salem-Bango et al., 2023).

In Pakistan, the molecular and morphological identification and isolation of *P. lilacinum* from soil have not been performed, nor has it been considered a potential biocontrol agent for insects. Thus, there is not much information present in this regard. The present research focused on determining whether Balakot soil is a potential source of *P. lilacinum* under laboratory conditions.

MATERIALS AND METHODS

Sampling site

Thirty soil samples were obtained from the following different points on Balakot Tehsil: Balakot top Hills 34°33'04"N 73°20'28"E, Balakot Natural Park 34°32'59"N 73°21'38"E and River Park 34°33'08"N 73°21'22"E. Balakot Tehsil came under Manshera district, Hazara division and Khaber Pakhtunkwa, Pakistan, as shown in Fig. 1 to 6. The samples were collected during September 2023, and the average temperature in that area is 28°C, which is suitable for purifying *P. lilacinum* (Senthilkumar et al., 2020). The soil specimens were procured from the uppermost layer, not exceeding 10 to 15 cm, by utilizing an iron spatula. Two hundred to three hundred grams of soil was cautiously placed into sterilized polyethylene bags for each collection. After that, these bags were carefully labeled according to their sampling sites and promptly stored at 4°C before being brought to the laboratory for additional processing.

Fungi isolation

The soil dilution plating method was used to extract the resulting fungus from the soil with optimization (Desoky et al., 2022). The tween solution was prepared at a concentration of 0.2% (v/v), 600 μl of solution was added to 299.4 ml of sterile water, and the solution was mixed well. Then, 9 ml of the solution was moved to each 15 milliliters Falcon tube. One gram of the soil sample was suspended in a 15 milliliters tube containing 9 milliliters of Tween 80 solution for dilution. A total of thirty dilution tubes were prepared. Then, each tube was vortexed for 30 minutes and left to settle the soil. A 450 μl of supernatant was collected and placed on a Patri plate containing PDA media. Media supplemented with the three antibiotics kanamycin (1μl/1 ml), penicillin (1μl/1 ml) and chloramphenicol (0.2 g/ml) were used to inhibit bacterial contamination. The plates were observed for one week and incubated in an incubator at 28°C (Alsohaili & Bani-Hasan, 2018). After appearing on plates, the colonies were streaked onto newer PDA plates for purification. At 4°C, the pure colonies were maintained and preserved.

Identification of isolated fungi

DNA-based molecular methods were employed alongside traditional techniques to identify the isolated fungi, involving the assessment of colony morphology and conidia characteristics at both macro and micro levels.

Morphological identification of fungal isolates

The pure culture isolate was grown in PDA for 7 to 14 days at 25°C. After that, we collected fresh mycelia using a sterilized tip and then placed them in a drop of sterilized distilled water and lactophenol blue on a clean glass slide for examination. This study focused on examining the morphology of fungal isolates, paying attention to color, shape, and size. We identified them based on measurements like hyphal and conidiophore diameter, as well as phialide and conidial shapes. To observe these details, we used a scanning electron microscope.

Examination of fungal structures by using scanning electron microscopy:

Conidia were collected from a PDA plate and suspended in deionized water with a 0.05% Tween-80 solution to create a concentration of 1 × 10^7 conidia/ml. Eight milliliters of this conidial suspension were introduced into 100 ml of SDA medium (comprising 1% peptone and 4% glucose). This medium was then incubated at 25°C and 180 revolutions per minute for a duration of 5 days. Hyphae were extracted using a vacuum suction pump and preserved with 2.5% glutaraldehyde at a temperature of 4°C for 24 hours. After rinsing with a phosphate buffer solution, the hyphae underwent dehydration with alcohol and were subsequently dried (Sun et al., 2021). The processed sample was affixed to the sample stage using a conductive adhesive and then subjected to imaging using a scanning electron microscope (ZEISS EVO MA 15, Lahore College of Women University (LCWU), Lahore).

Molecular identification of isolated fungi

Nuclic Acid Extraction:

The whole nucleic acid extraction procedure utilized the CTAB method, with optimizations made to the existing protocol (Jamil, 2020). To extract DNA, the fungal sample was manually ground in a sterile grinder within a 1.5- milliliters microtube. Then, add 450 µl of CTAB lysis buffer, 2 µl of β-mercaptoethanol, and 10 µl of 2% SDS (w/v). Following this, 30 µl of Protenase K was added, followed by vortexing for 15 to 20 seconds and then incubating at 56°C for 3 hours in a water bath. Afterward, 450 µl of lysis buffer was added to the sample, thoroughly mixed by inverting the tube, and then centrifuged at 12 thousand revolutions per minute at 25°C for 20 minutes. After adding 450 µl of a 24:1 mixture of chloroform and isoamyl alcohol to the tube, the mixture was centrifuged. The supernatant was moved to a new tube, 6 µl of RNase was added, and the mixture was incubated at 37°C for half an hour. Then, an equal amount of isomyl alcohol was added to the supernatant at the time of RNAase addition, mixed, and centrifuged. Again, a new tube was used to transfer the supernatant, and a 0.6 × volume of propanol

was added. DNA pellets were obtained through centrifugation. The DNA precipitate were washed twice with 400 µl of 70% ethanol and then centrifuged at 12 thousand revolutions per minute at 4°C for 15 min. The DNA precipitate were dried in an airtight biosafety cabinet for 25 min, dissolved in 30 µl of nuclease-free water and stored in a refrigerator at 4°C.

Polymerase Chain Reaction:

ITS1-Forward (5' TCCGTAGGTGAACCTGCGG 3') and ITS4-Reverse (5' TCCTCCGCTTATTGATATGC 3') were the universal primers used to amplify the ITS1–5.8S–ITS2 rDNA, as per the instructions of (Patidar et al., 2024). In a 25 µl reaction mixture, 12.5 µl of PCR master mix (Thermo Fisher Scientific), 5 µl of nucleic acid sample, 1.5 µl of each primer (10 pmol/µl), and 4.5 µl of sterile distilled water were added. The PCRs involved a 4-minute initial denaturation at 95°C; 25 cycles of denaturation for 50 seconds at 95°C, primer annealing for 60 seconds at 56°C, and primer extension for 60 seconds at 72°C; followed by a 9-minute final elongation at 72°C; and storage at 4°C.

Gel Electropherosis:

A negative control with no DNA template was also used in the PCR to detect any contamination. Next, 100 milliliters of 1.6% (w/v) agarose gel was made in a buffer containing 1X TAE (Tris-acetate EDTA) and 6 µl of ethidium bromide stain. The 100 bp DNA ladder (New England Biolabs), 8 µl of amplicon and negative control were loaded into the respective valves, which were electrophoretically separated at 120 V and 400 mA for 35 minutes. Then, a UV gel doc system (Bio-Rad) was used for visualization. dNTPs and unincorporated PCR primers were separated from the PCR results during purification using the QIA Fast PCR Purification Kit (Qiagen).

Sequencing and Phylogenetic Analysis:

The polymerase chain reaction product was forwarded to Lab Genetix in Lahore, Pakistan. For sequencing purposes, an ABI Prism 3100 Genetic Analyzer (Thermo Fisher Scientafic) was used for Sanger didoexy sequencing of the purified polymerase chain reaction product of the ITS gene. A BLAST (Basic Local Alignment Search Tool) search was conducted in the GenBank (NCBI) database (http://www.ncbi.nlm.nih.gov) with Entraz query can be added to limit the RefSeq to only the 177353 (i.e., BioProject) to analyze the resulting sequences with other relevant sequences, and relevant sequences were acquired. After sequencing, the BioEdit Sequence Alignment Editor (version 7.2.5) was applied to align the sequence data and obtain consensus sequences. A maximum likelihood analysis with 1000 bootstrap repeats and a phylogenetic tree of the obtained internal transcribed spacer region sequences were built utilizing Mega version 7 (Horiike, 2016).

RESULTS

Marphological identification of isolated fungus:

The identification of the isolates was based on the observation of macro- and micromorphological features. Morphological identification revealed that the isolate was Purpureocillium lilacinum because it appeared flat on media, white in the first three days, and gradually turned pink after 7 days, after which it turned from pink to purple in color with a diameter of 2.1 to 3.9 cm approximately after 7 days at 25°C, with a felty and cotton-like appearance and aerial mycelia (Figs. 6 & 7). Figures 9 &10 indicate that the lengths of the conidiophores varied widely (38.4 μm × 2.9 μm, approximate). The conidiophore presented a broom branch, and the conidia themselves were catenulate and oval to spindle-shape, and connected in a chain-like manner with a size of approximately 3.0 μm × 2.7 μm (Fig. 11) without forming chlamydo spores.

Molecular identification of isolated fungi:

The isolate was confirmed to be *Purpureocillium lilacinum* by genetic analysis of the internal transcribed spacer (ITS) region. Polymerase chain reaction was performed with ITS1 and ITS4 primers to obtain the ITS 1, 2, and 5.8S subunit coding sequences. The resulting band obtained by gel electrophoresis was approximately 490 bp in size (Fig. 12) and was subsequently sequenced and compared to database sequences for confirmation. The obtained ITS DNA sequence was submitted to the National Center for Biotechnology Information Database under GenBank accession no. PP587221.

Phylogenetic Analysis

The comparison of ITS gene sequences was utilized to establish phylogenetic relationships, leading to the construction of a dendrogram alongside other Purpureocillium species (Fig. 13). Analysis using the BLASTn program indicated 99.07% similarity between the identified *P. lilacinum* isolate (PP587221) and previously identified isolates of *P. lilacinum*, such as MW386433, MW386435, and MW386434 (Table 1). The phylogenetic tree clearly shows the close genetic affinity of our fungal isolate with other published sequences in the database. Additionally, this is the first sequence reported from Pakistan, as the most similar sequences are reported from other continents or countries, indicating fungal diversity (Table 1).

Table 1
Results of the nearest fungi taxon BLAST homology ITS1, 5.8S, and ITS2 of rDNA in NCBI.

Isolate	Spieces	GenBank Accetion	Identity %	Country	Length
XI-1	*P. lilacinum*	MW386433	99.07%	China	535 bp
XI-5	*P. lilacinum*	MW386435	99.07%	China	536 bp
XI-4	*P. lilacinum*	MW386434	99.07%	China	543 bp
TW-SP17-10B	*P. lilacinum*	MN634691	98.84%	South Africa	538 bp
SC-SP17-20B	*P. lilacinum*	MN634663	98.84%	South Africa	541 bp
RSO-AP17-32	*P. lilacinum*	MN634587	98.84%	South Africa	526 bp
CM-SP17-02B	*P. lilacinum*	MN634485	98.84%	South Africa	541 bp

DISSCUSION

The objective of this research was to provide information about the presence of the entomopathogenic fungus *Purpureocillium lilacinum.* Isolates were obtained from three different regions of Balakot.

With the objective of discovering ectomycorrhizal association strains from *C. deodara* rhizospheres, a study was carried out in 2019 at Thandiani Forest in Abbottabad, Pakistan. As a result, six new fungal strains were identified ACE4, ACE2, ACE3, ACE5, ACE1 and ACE6 of the *Talaromyces pinophilus, Aspergillus terreus*, *Aspergillus fumigatus*, *Purpureocillium lilacinum, Emmia latemar ginata*, and *T. pinophilus* strains, respectively. Both areas reported P. lilacinum since the atmospheric conditions in this region are similar to those in the current research area, including temperature and sampling technique (Jamil et al., 2020), and this is the first report on *P. lilacinum* from the nothren area of Pakistan. Othere province, such as Pujab, also reported this in the past. In the present study, the EPF *P. lilacinum* was identified and isolated from a soil sample. Previous studies have shown that a certain fungus has the potential to control various insect pests that damage vegetables (Du et al., 2021). However, until now, there was no record of this fungus being isolated from soil in Pakistan and surrounding areas. Identification and isolation of different fungal strains are primary and essential stages in developing the mycoinsecticide or biocontrol agent to control the pests (Dayanti et al., 2018). In a recent study, scientists collected over 25,000 insect samples from various districts in Punjab and found four different types of fungi that can kill insects: *L. attenuatum*, *P. lilacinum*, *M. anisopliae*, and *B. bassiana*. Interestingly, the geographical locations of the sampled areas also influenced the distribution of these sampled fungi, with P. lilacinum and L. attenuatum being relatively rare (Wakil et al., 2014). In another study conducted by researchers at 12 different localities in Punjab province, which cover almost all the province, a over all 210 soil samples were gathered from the following four various habitats: crop-filled, vegetable and fruit fields, and forest soil. Most of the entomopatohganic fungi are reported from forest habitats as compared to other habitats, and the galleria bait method was used to isolate fungi. Out of 210 soil samples, only 168 fungal isolates were retrieved and identified. Notably, eight of these isolates came from fruit soil samples, thirty-two from vegetable fields, ninety from forest soil, and thirty from crop fields. The major entomopathogenic fungi identified among these samples included *Lecanicillium attenuatum* (4.55%), *P. chlamydosporia* (9.09%), *B. brongniartii* (9.09%), *Paecilomyces lilacinus* (18.18%), *Metarhizium anisopliae* (22.73%), and *Beauveria bassiana* (36.6%). *P. lilacinus* was isolated from the forest soil, crops, and vegetable fields of Muree, which has nearly the same climatic conditions as the Balakot Tehsile region, and it was the primary study to report the diversity of EPFs and report *P. lilacinus* as a side product. They identified P. lilacinus only on the basis of morphology and did not use any moleculer anylsis for identification (Wakil et al., 2013). The same temperate continental climate as the Balakot Tehsil was found in another study conducted in Qinling Mountain, Shaanxi Province, China. However, the climate in the Qinling Mountains varies due to factors like altitude and geographical location within the range. During this investigation, they isolated Purpureocillium lilacinum from soil samples in the region. They identified this fungus by analyzing the ITS region of rDNA using universal primers specifically designed for ITS region amplification, which were also used in the present research (Lan et al., 2017).

CONCLUSION

The present research constitutes a seminal contribution by documenting the first instance of *Purpureocillium lilacinum* isolation from soil samples collected in Balakot Tehsil, Khyber Pakhtunkhwa (KPK), Pakistan. Prior to this investigation, this fungus had rarely been isolated from soil around the globe. The primary focus of this research was to identify potential areas where it could be found in Pakistan. According to the literature, this study reaffirms the multifaceted role of this fungus as a potent pathogen for nematodes, along with its efficacy in the biological control of insect populations. Extremely important is that it can be used as a commercial product due to its entomopathogenic properties, necessitating the extraction and encapsulation of secondary metabolites, which are the chemicals produced by it, and these metabolites are responsible for its antagonistic (pest-fighting) properties. Through the identification of potential habitats in Pakistan, this research lays a foundational framework ready to expedite targeted research aimed at unraveling its biocontrol potential and facilitating future industrial-scale utilization endeavors.

Declarations

Competing interests:

No conflicting interests are disclosed by the author.

Ethical Responsibilities of Authors:

The author has read, understood, and complied with the statement on "Ethical responsibilities of Authors", as found in the Instructions for Authors.

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For this research, no funding was obtained.

Author Contribution

All the research was carried out by the single author Muhammad A.S; in this paper, no other author was involved.

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during this study.

Data availability:

All the data described in this research paper are presented in tables and figures because they are real-time data.

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Figures

Figure 1

A map of Pakistan (Yellow boundries) and KPK (Red mark).

Figure 2

Amap of KPK (Blue boundry) Balkot tehsil (Black mark).

Figure 3

A map of Balkot tehsil (Black boundry) and sampling sites (Grey marks).

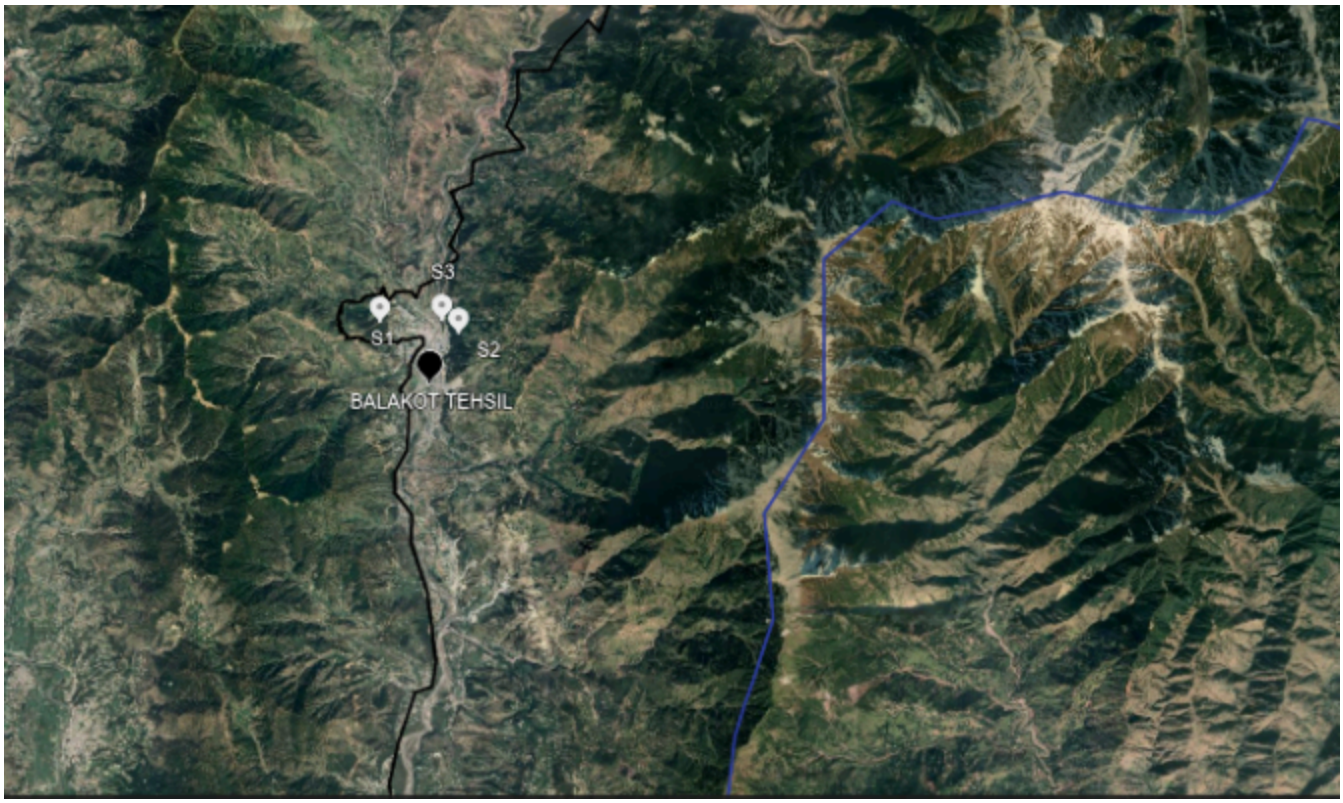

Figure 4

Samplig site of Balakot hil top (S1).

Figure 5

Sampling site of River park (S3).

Figure 6

Sampling site of Natural park (S2).

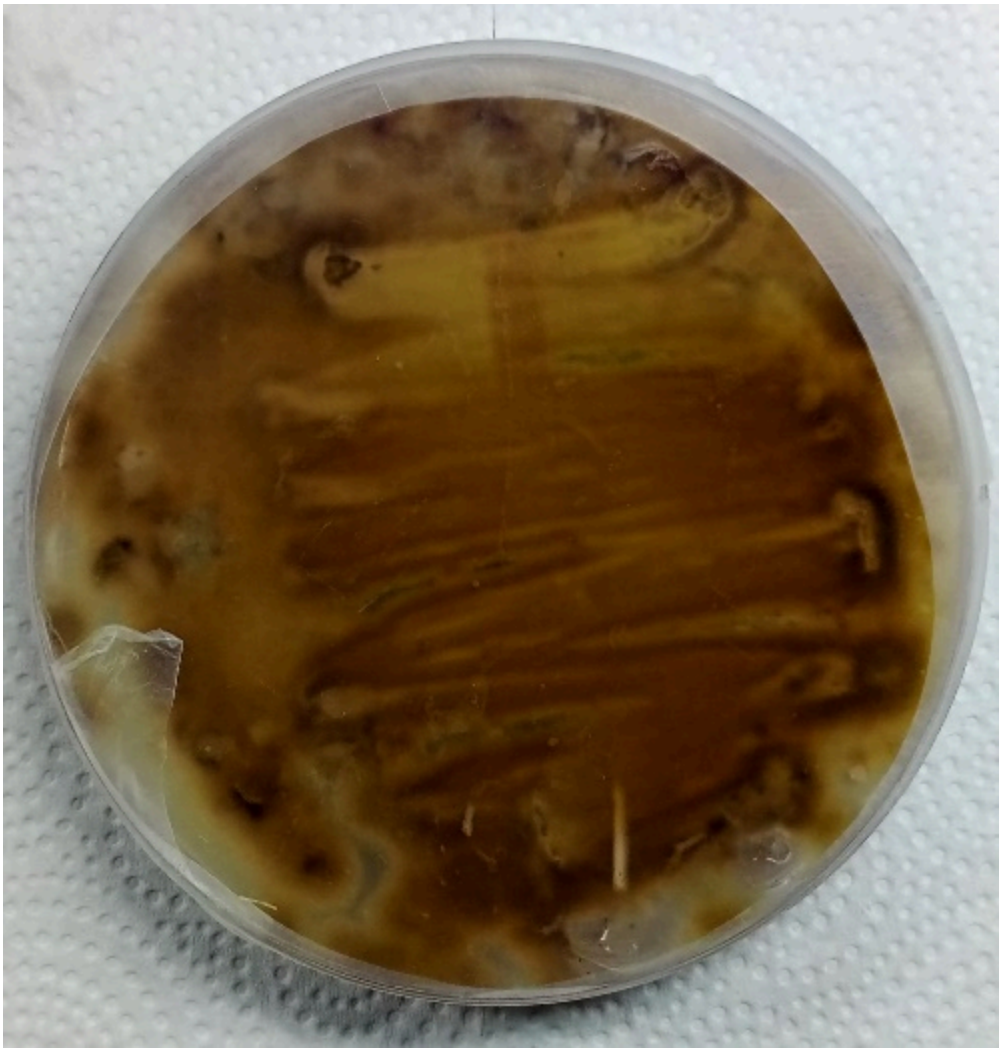

Figure 7

Front side of isolated fungus colony in between of 7 to 14 days.

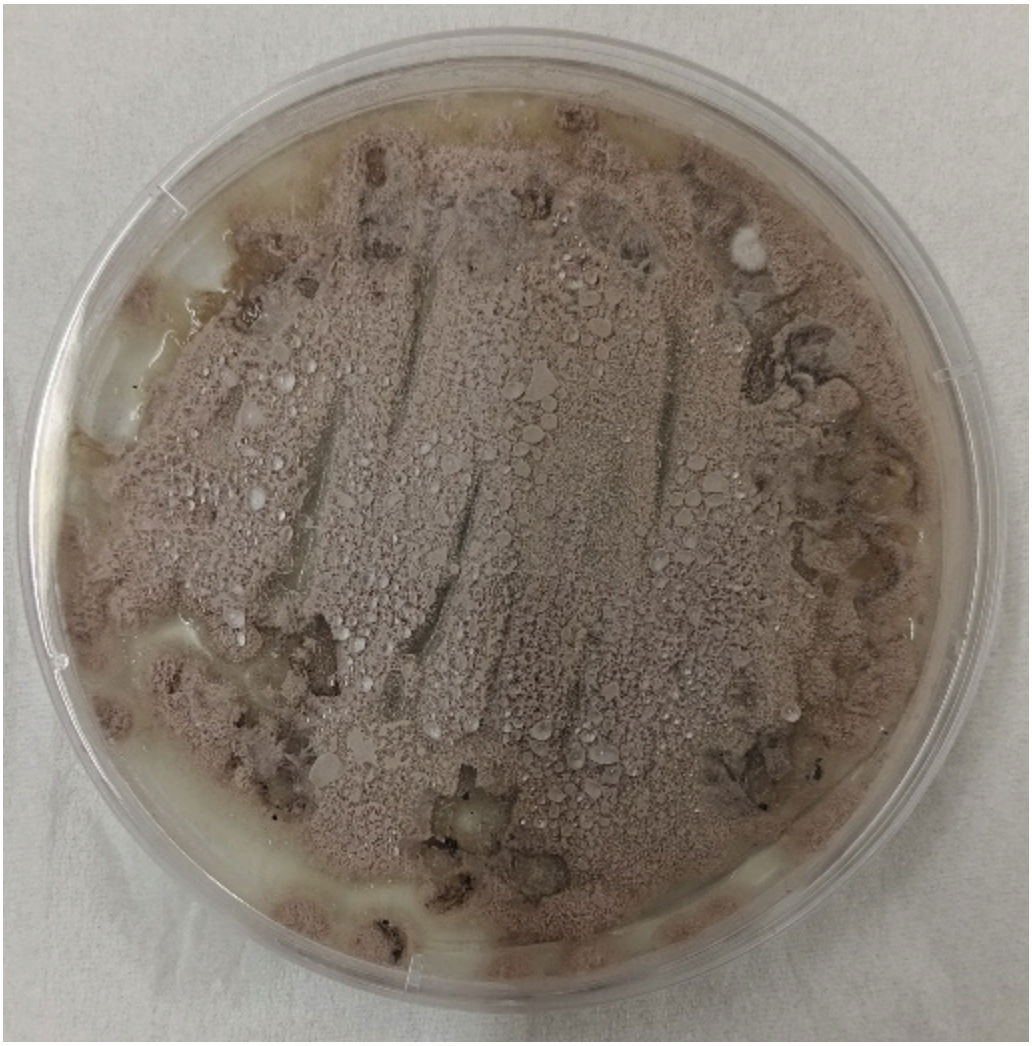

Figure 8

Back side of isolated fungs colony.

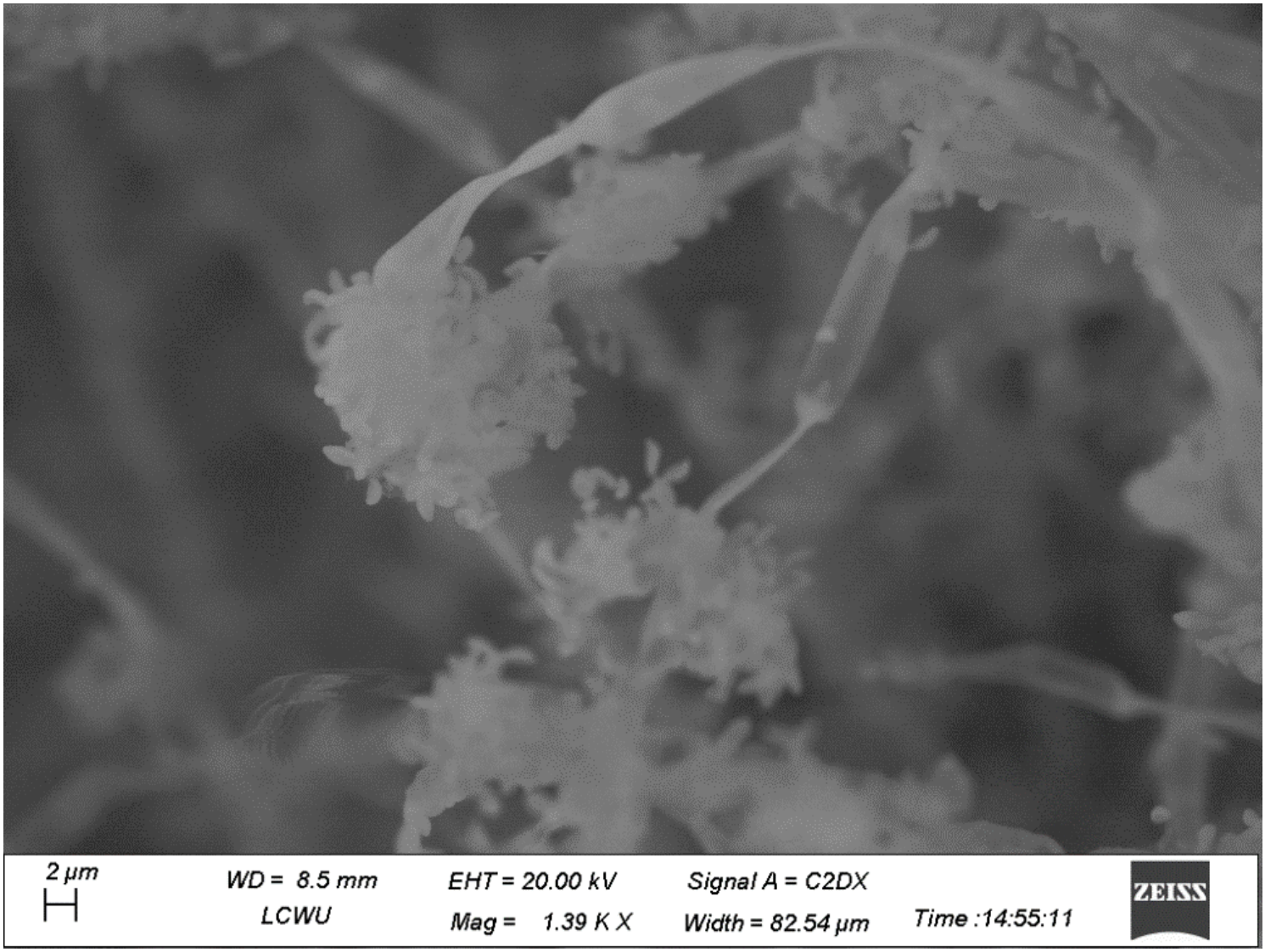

Figure 9

Scanning electron photographof conidia and conidiophores of *Purpureocillium lilacinum*.

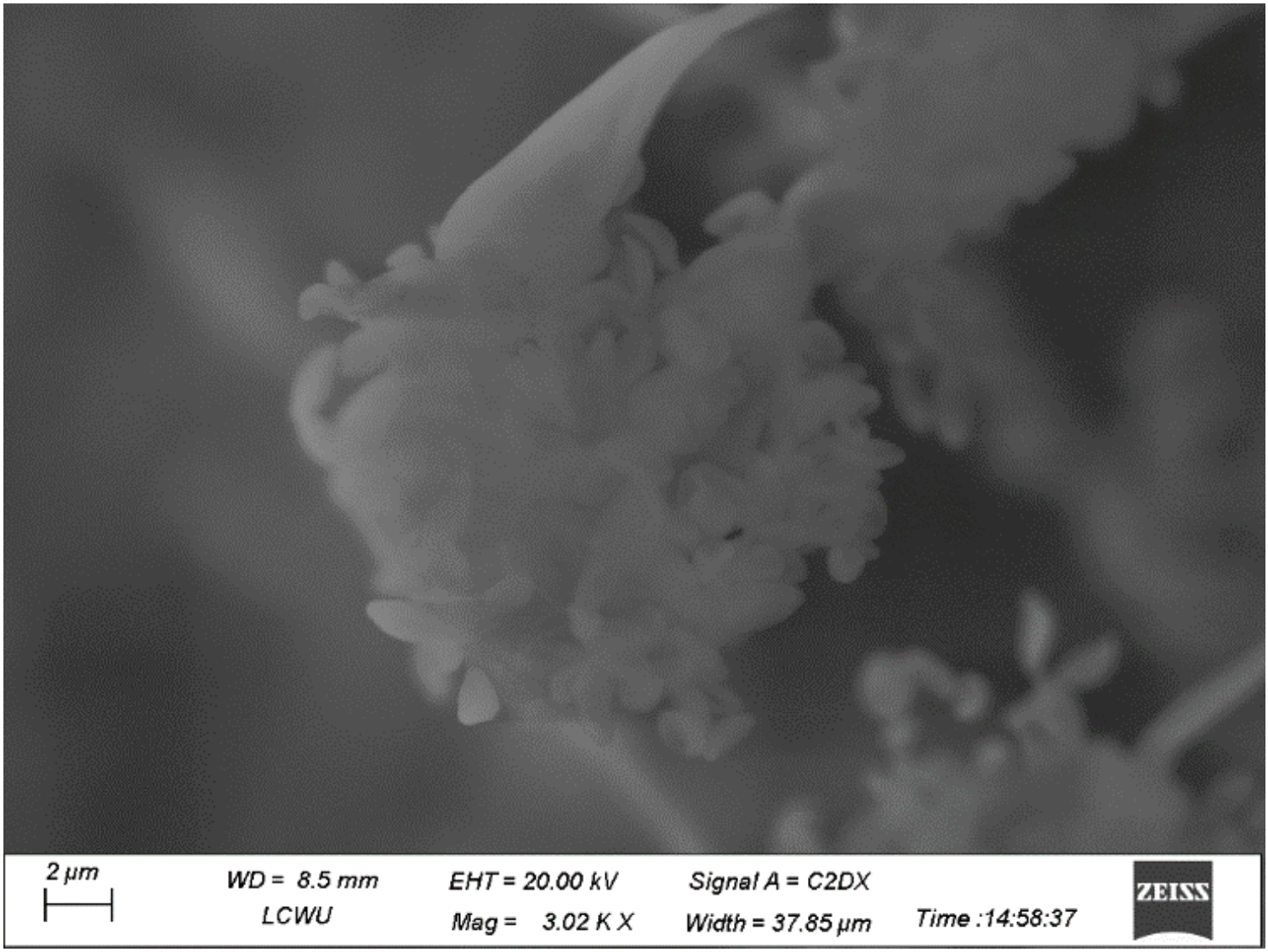

Figure 10

Scanning electron photograph of conidia and conidiophores of isolated *Purpureocillium lilacinum.*

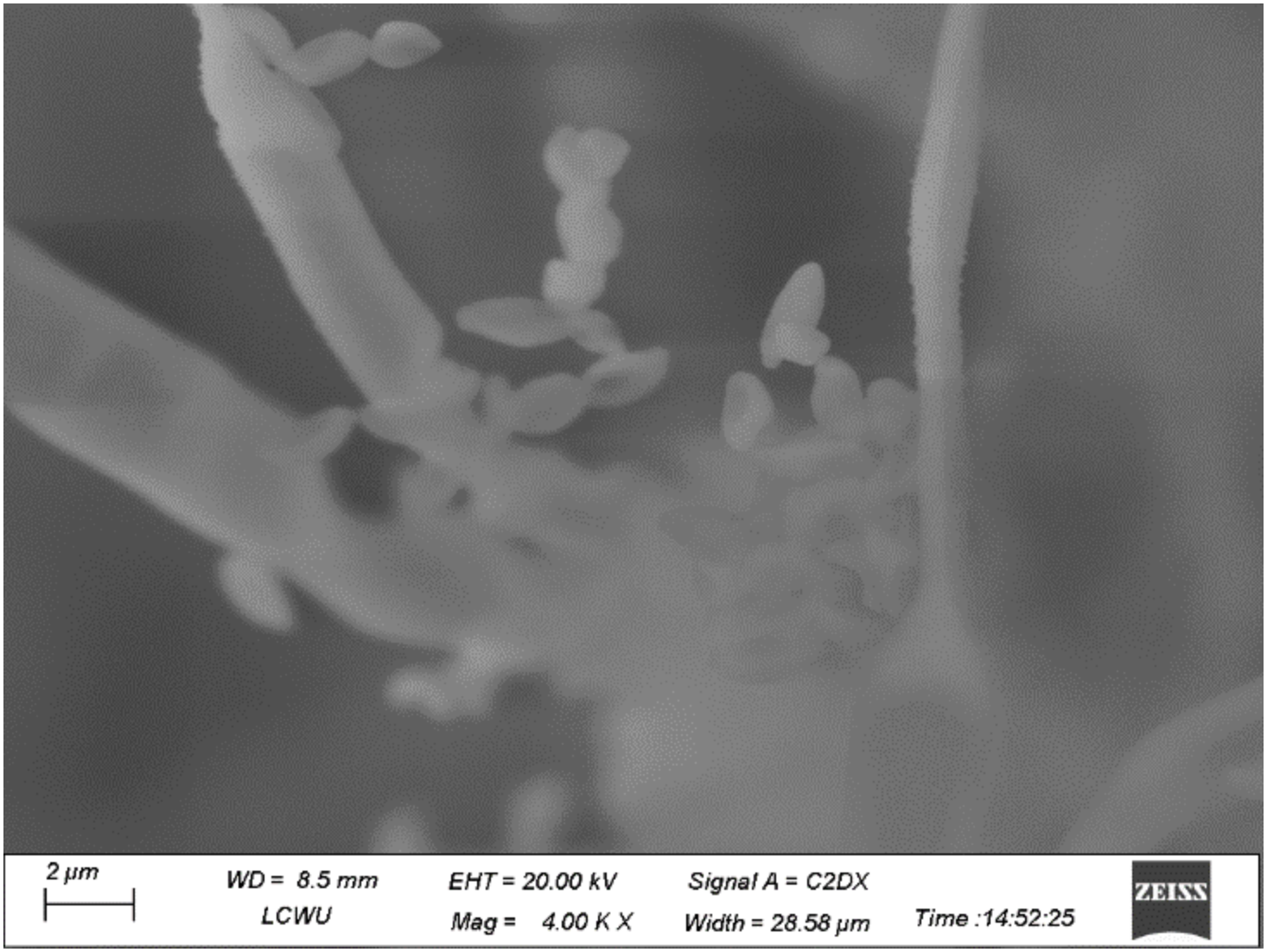

Figure 11

Scanning electron photographof more focused conidia of isolated *Purpureocillium lilacinum.*

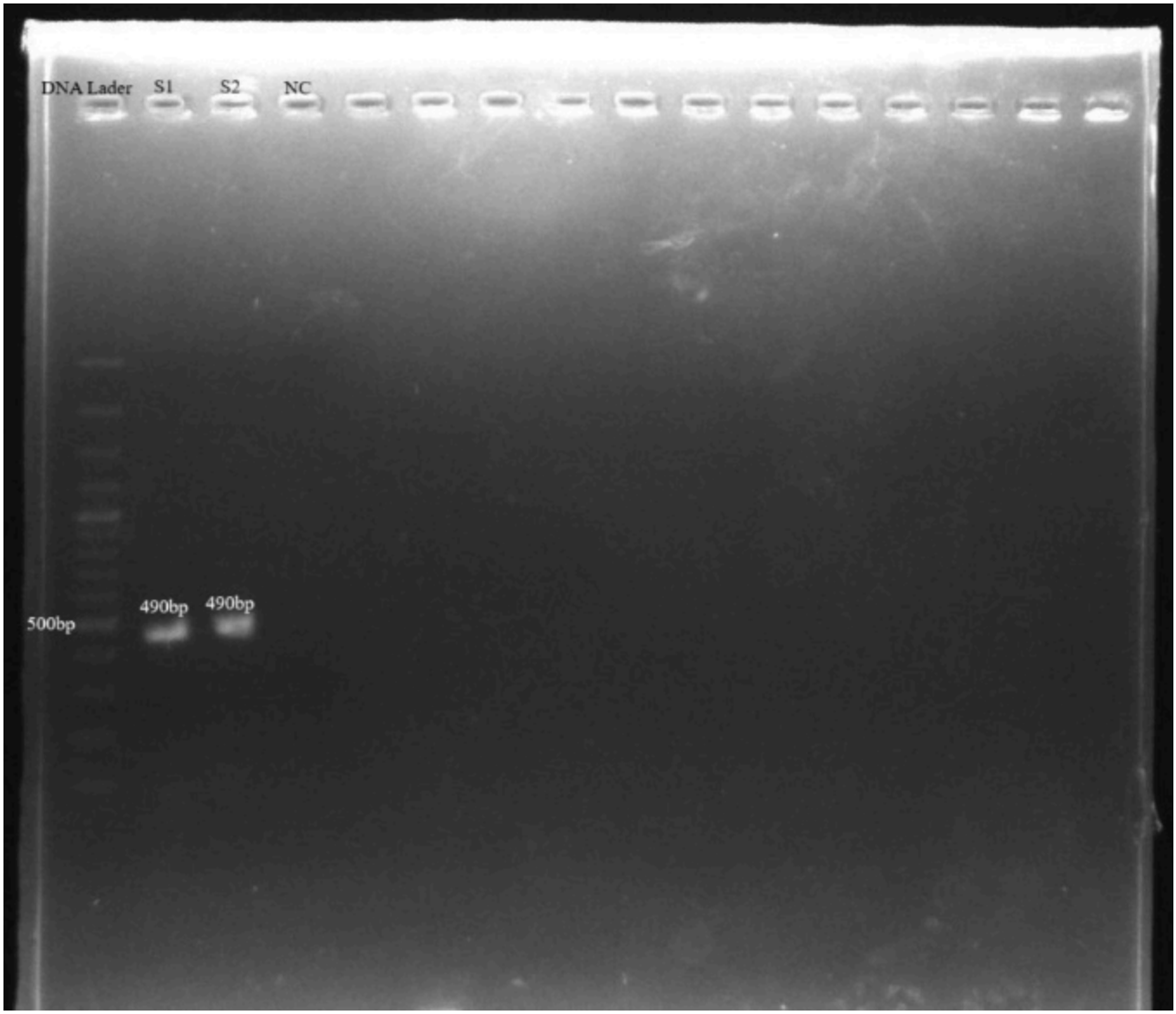

Figure 12

PCR amplification from ITS region and amplification fragment showing approximately 490bp. Valve 1: 1 DNA kb ladder; Valve 2 & 3: DNA samples, Valve 4: Negative control (distilled water).

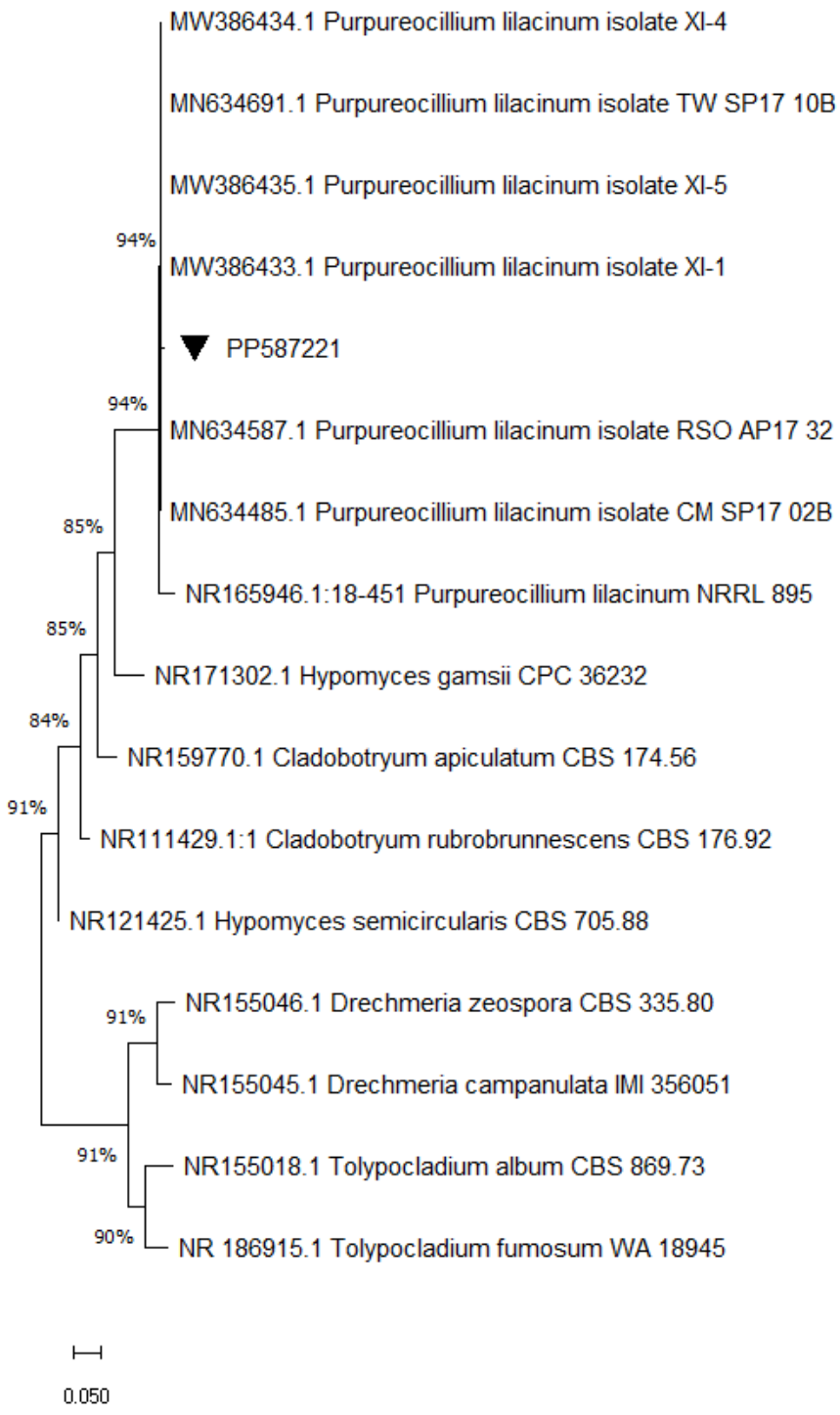

Figure 13

Maximum likelihood Phylogenatic tree of isolated *Purpureocillium lilacinum* (PP587221)based on ITS region.