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Original article

Isolation and molecular characterization of causal agent of blue mold on *Allium cepa* L. and its control by *Pennisetum flaccidum* Griseb

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ABSTRACT

Blue mold pathogen, isolated from infected *Allium cepa* L., was identified as a *Penicillium* species through morphological and molecular characterisation. Internal Transcribed spacer (ITS) region of ribosomal DNA (rDNA) was utilised for DNA sequencing. Basic Local Alignment Search Tool (BLAST) analysis has found the maximum similarity index of the fungus to be 82.39% with the Uncultured *Penicillium* clone (Accession: MF535522). So, the isolated *Penicillium* specie is the first reported specie of the genus that infects onion. A phylogenetic tree was constructed to establish a relationship of the isolated fungus with the most relevant species reported on GenBank. Extracts of *Pennisetum flaccidum* Griseb. were evaluated against the isolated fungus as a potential biocontrol agent. Among the five tested methanol concentrations (0.5%, 1%, 1.5%, 2% and 2.5%) of each plant part (root, inflorescence and foliage), 0.5% root extract showed maximum growth retardation, i.e. 89%. For bioassay-guided fractionation, the root extract was partitioned in *n*-hexane, chloroform, *n*-butanol and ethyl acetate. Ethyl acetate (1%) was proved to be the most potent one. Phytochemical screening has confirmed the occurrence of terpenoids, tannins, saponins and alkaloids. The applied molecular approach has deduced that the *Penicillium* specie collected from Pakistan might be novel. This study can be concluded that *P. flaccidum* contains potent phytochemicals which might be used as antifungal agent against *Penicillium* species.

1. Introduction

Blue mold rot is a severe food concern deterioration worldwide. Different *Penicllium* species are responsible for the rot. Its anemochory mode of spore dispersal provides a high risk of disease spread during storage and transportation at temperatures range 21–25 °C (Hussain et al., 2010). In Pakistan, 24 different species of *Penicillium* have been reported infecting various food crops (Khokhar and Bajwa, 2014).

Allium cepa L., commonly known as onion, belongs to the family Liliaceae, easily attacked by *Penicillium*. It holds prime importance in Asian cuisine and is of immense importance worldwide due to its therapeutic and nutritional value (Sohail et al., 2011). Globally, it is the second most cultivated crop to suffice the absolute demand (FAOSTAT, 2001). China and India are the primary producers of the plant (Kumar et al., 2016). However, Pakistan ranks 8th as a universal producer with a production rate of 1.94 million tons (FAOSTAT, 2011). Annually, 30–40% of onion gets wasted due to fungal attacks (Khokhar and Bajwa, 2014). Correct identification of the pathogen is the fundamental step for its effective control (Kumar et al., 2016).

Various classical (morphological and cultural analysis) and modern approaches (physiological, biochemical and DNA studies) have been described for systematics of fungi (Samson and Pitt, 2019). In the last decade of the 20th century, DNA sequencing proved itself as the most authentic technique for specie identification (Ratnasingham and Hebert, 2007; Masters et al., 2019). The regions used for DNA-sequencing can be Internal transcribed spacer regions (ITS), nuclear large subunit ribosomal DNA, partial elongation factor-1 sequences (EF-1), protein-coding regions and partial-tubulin A sequences (Ben-A). The barcode region for fungi is 400–800 base pair (bp) (Seifert et al., 2007; Raja et al., 2017). ITS region is the most considerable one due to its uniqueness for each specie. Moreover, the region can easily be amplified from

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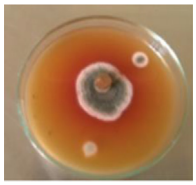
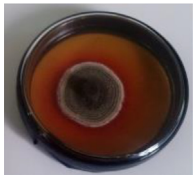
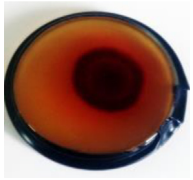
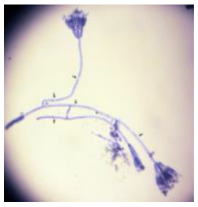
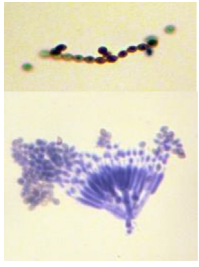
Table 1
Morphological characterisation of the isolated blue mold pathogen maintained on 2% MEA.

Characters	Description	Picture
Macroscopic characterization:		
Colony Size	Diameter: 2.89 ± 0.03 cm of 7 days old culture	
Colony growth pattern	Circular colonies; Growth in concentric circles	
Colony texture	Flat and velvety	
Colony colour (top)	Colourless and mucilaginous texture formed around the inoculum initially, which turned to white, olive green and finally sea-green in colour. Yellow and tinge pink masses were also observed occasionally upon the colony. Blood red colouration originates from the bottom of the colony extend towards the top	
Colony colour (bottom)	Blood red to reddish-brown colouration, which saturates with the ageing	
Microscopic characterization:		
Hyphal characters	Branched hyphae; erect, septate and verticillate conidiophore	
Conidiophore diameter	3.5 μm	
Metulae dimensions	15 × 5 μm	
Phialide dimensions	12 × 6 μm	
Conidial characters	Ellipsoidal and spherical; Average diameter: 2.0 × 2.5 μm	

degraded DNA sources. ITS1F and ITS4 are the appropriate primers for Ascomycota (White et al., 1990; Izzo et al., 2005; Fernandez and Kennedy, 2016).

Fungal pathogens destroy one-third of food crops each year (Fisher et al., 2012). Various chemical, biological and physical methods are practised to control yield losses (Gan et al., 2006). Chemical treatment is the most adopted option due to its ease of application and long-lasting results. Some of the important fungicides used against *Penicillium* include Imazalil (Enilconazole sulfate), Fludioxonil [4-(2, 2-Difluoro-1, 3-benzodioxol-4-yl)-1H-

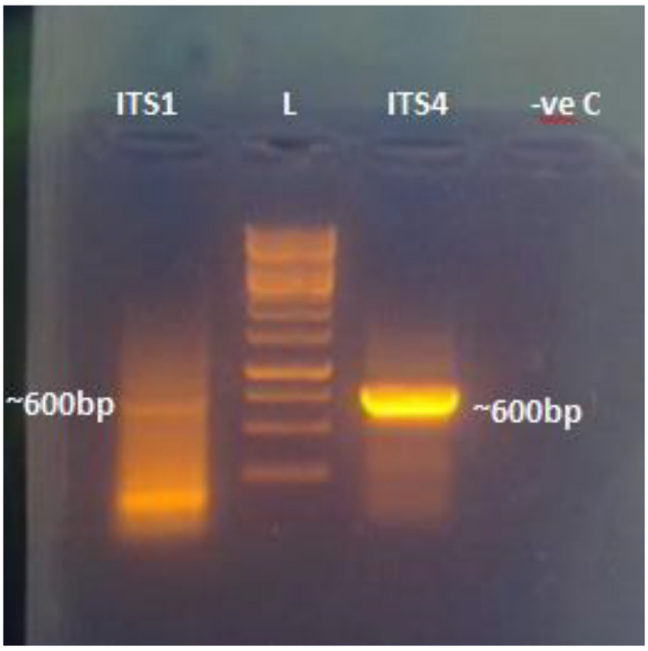


Fig. 1. The amplification results of ITS region through polymerase chain reaction. 'L' represents the standard DNA ladder of 1 Kb, ITS1 and ITS1 region amplified showing ~600 bp bands that were sliced out for DNA sequencing.

pyrrole-3-carbonitrile], Captan (N-trichloromethylthio-4-cyclohex ene-1, 2-dicarboximide) etc. (Ivic et al., 2013). On the other hand, fungicides serve as a rich source of environmental pollution. These fungicides execute unwanted metabolic change in plants and due to inefficient degradation cause health problems in humans (Axelstad et al., 2011). Therefore, fungicides safer for non-target specie should be formulated (Komarek et al., 2010). Based on medicinal value, plant extracts are studied as biocontrols against various phytopathogenic fungi (Anuagasi et al., 2017; Bashir et al., 2019; Khan et al., 2018; Anwaar et al., 2021).

Several species of Family Poaceae (Tribe: Panicoideae) are reported for antimicrobial potential, and fungi suppressing proteins have also been isolated out of them (Ratha et al., 2012; Singh et al., 2015). Analysing the relevant importance of the family and tribe, *Pennisetum flaccidum*. has been tested as a biocontrol against the isolated *Penicillium* specie. The plant is native to various sub-tropical and tropical areas of the world. So, the present study, aimed to isolate and identify (morphological and molecular) the pathogen of blue mold disease and its control by using extracts of *P. flaccidum*.

2. Materials and methods

2.1. Collection and isolation of blue mold pathogen

The blue mold rotten onion was picked up from a vegetable market situated on Multan Road, Lahore, Pakistan (Latitude: 31.32 °N; Longitude: 74.33 °E; 688 feet altitude above sea-level). A pure colony of the pathogen was obtained on 2% MEA (malt extract agar) medium.

2.2. Morphological characterisation of the test pathogen

Morphological features of the colony were noted and matched with the former studies and the key (Domsch et al., 1993; Pitt and Hocking, 2009). The colony's texture, color (top and bottom), diameter, and growth pattern were used as macroscopic diagnostic parameters. For microscopic diagnosis, slides of the 7 days old fungi were examined under the compound microscope (Model. MX 4000) and the pictures were taken. The Motic Image Plus 2.0 software was used to note various fungal structures from the pictures.

2.3. Molecular characterisation of blue mold pathogen

ITS region of the fungi was amplified by PCR (Mullis, 1990). The primers combination used was: forward ITS1 fungal accession number KP794160, i.e. (5′ TCCGTAGGTGAACCTGCGG 3′) and reverse ITS4 fungal accession number JQ034359, i.e. (5′ TCCTCCGCTTATTGATATGC 3′). The respective ITS bands (~600 bp) were segregated through agarose gel electrophoresis and were eluted by a gel purification kit (FavorPrep™, Favorgen, Biotech Corp). Macrogen, Inc (Korea) did the sequencing of the samples. The BLAST analysis was performed to surge for the identical sequence from the GenBank. The phylogenetic tree of the closely related ITS sequences was constructed through MEGA 6.0 by the neighbour-joining method.

2.4. Pathogenicity test

A pathogenicity test was performed following the method (De Lange et al., 1998). Healthy onions were peeled off and surface sterilised with 70% ethanol. One hole per onion was punched (5 mm wide and 10 mm deep). The onion holes were sealed by the mycelial plugs. The inoculated onions were then placed inside the sterilised polythene cups and were sealed with aluminium foil. The setup was then allowed to incubate at 25 °C for 7 days. The pictures were clicked and subjected to Motic Image Plus 2.0 software to note the dimensions of the lesions. The scale was considered for the grading of lesions (Nova et al., 2011).

2.5. Biocontrol of the blue mold pathogen with P. flaccidum extracts

2.5.1. Preparation of methanol extracts of the plant

Entire plants of *P. flaccidum* were collected from University of the Punjab, Lahore, Pakistan. All parts of the plant (roots, inflorescence and foliage) were separated; thoroughly cleaned and sun-dried at 35 ±°C and relative humidity was 20–25 RH. After drying the plant material was ground to make a fine powder. Each powdered part (100 g) was extracted in methanol (500 mL) for 7 days with occasional stirring. The extracts were filtered using double-layered muslin cloth and were then evaporated at room temperature. The resulting crude extracts were stored in glass vials at 4 °C to be used for further assays.

Table 2
The DNA sequence of the isolated Blue mold pathogen (TZITS- *Penicillium* sp.).

TGTTCTGGGGGTGAGGTCTGTGGTTCATGTGCCGCCATGTCTTTGTACCTTGTTGTTTCGGTGCGCCCCTCTTGCTTTCGCCTGGCCGATACATCCGCCGGGCCAGAGGTTTAACG
AAACACTATTGAATTATGTCTGAAGATTGCCAAATTATGAATTACTCAATAAAACTTTCTTTCCGGATCTCTCTCTTCTCGCCGGCATCAATAAAGAACGCAAATGAATTCAGTAA
CTAATGTTAATTATTTAATTGAATCAATCATCCAGTCATTGAACATTGATTCCTCCGCCTGTCCTTACGGGGGGCCTGCCTGACCGAGCTTTCTTGCTGCCCTCCCCGGCGGTGG
GATTGAGGGATTCTCTCCCCCCAGCTACGGGTCCGATTGGAAAGGATTGGACTGAGTCCGGCTGTCTAGCGCATGGGGCTTCTCCACCCATTATGTTTATCCAGCTCGTT
CAATGGTACAACCAATCATCCTTTTTTCAGGTTGACCTCGGATCACGACGGGATACCCGCTTAACTTAAGCATATCGATAGAATGAGGAATGAACTT

2.5.2. Antifungal activity of the methanol extracts against the fungus

In vitro antifungal activity of the extracts was checked using the protocol of (Jabeen et al., 2014). The five concentrations *viz.* 0.5%, 1.0%, 1.5%, 2.0% and 2.5% were formulated by mixing the methanolic plant extracts in 2% ME liquid media. The suitable volumes were calculated using the formula: $M_1V_1 = M_2V_2$. The commercial fungicide "metalaxyl + mancozeb 72% WP" (manufactured by Zhejiang Heben pesticide and chemicals company limited) was mixed with 2% ME media in the same series to maintain a positive control. However, the negative control was maintained as plain 2% ME media. So Each treatment was supplemented with Chloramphenicol (5 mg mL^{-1}) to refrain from any bacterial growth. Each treatment [Positive control (metalaxyl + mancozeb 72% WP), negative control (plain ME media), 0.5%, 1.0%, 1.5%, 2.0% and 2.5%)] was maintained in triplicates in completely randomized design (CRD).

The 5 mm mycelial plugs were added to each sample, and the set-up was left undisturbed at 30 °C. After a week, the mycelia from each treatment were harvested on separate filter papers and their growth inhibition percentage was calculated after noting the dry weight of the mycelia. The formula applied for growth inhibition percentage is:

$$GI(\%) = \frac{GT - GC}{GC} \times 100$$

Wherease, GI = Growth inhibition; G_T = Growth in treatment; G_c =- Growth in control

2.5.3. Bioassay-guided fractionation

The root extract was noted to be the most potent among all parts of the plant, hence, considered for bioassay-guided fractionation (Jabeen et al., 2014). Root powder (50 g) was extracted in methanol (100 mL) and was evaporated at room temperature. In a separating funnel, the crude extract was fractioned in organic solvents (*n*-hexane, chloroform, *n*-butanol and ethyl acetate) in raising the order of polarities. The fractions were evaporated at room temperature. Stock solution (20% w/v) for each extract was prepared in distilled water. Two concentrations viz. 0.5% and 1.0% were prepared for each extract using formula: $M_1V_1 = M_2V_2$. Posi-

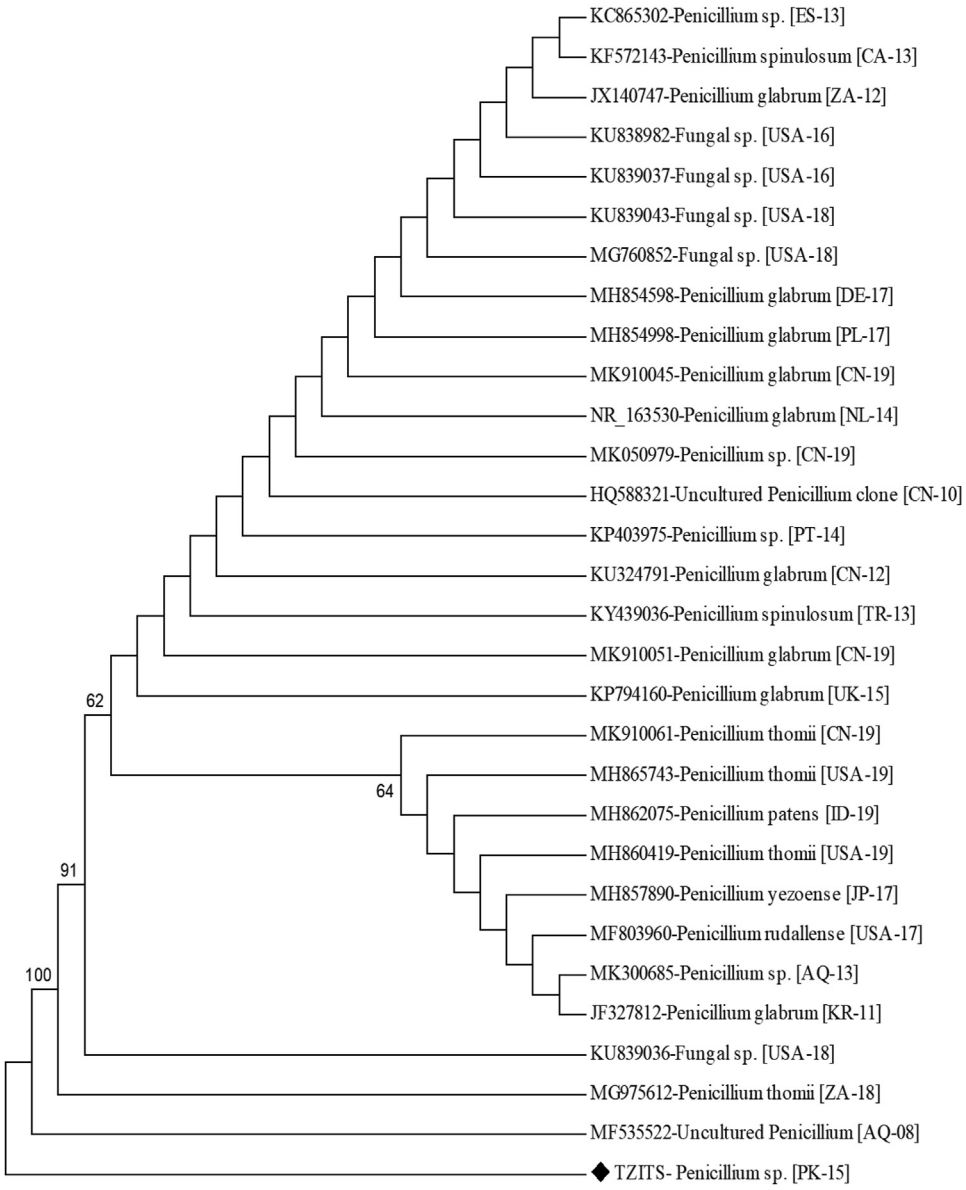


Fig. 2. Phylogenetic tree depicts relationship of the isolated *Penicillium* sp. (TZITS-Penicillium sp.) with 29 closely related species. Length of the horizontal branches represents genetic differences among the sequences, while the vertical branches are arbitrary. The sequences of closely related species were retrieved from GenBank.

Table 3
The data of the pathogenicity test.

Sample name	Lesion diameter	Grade	Inference	Picture
A	7.6 ± 0.4 mm	2	Mildly Pathogenic	
B	8.6 ± 0.5 mm	2	Mildly Pathogenic	
C	10.1 mm	2	Mildly Pathogenic	

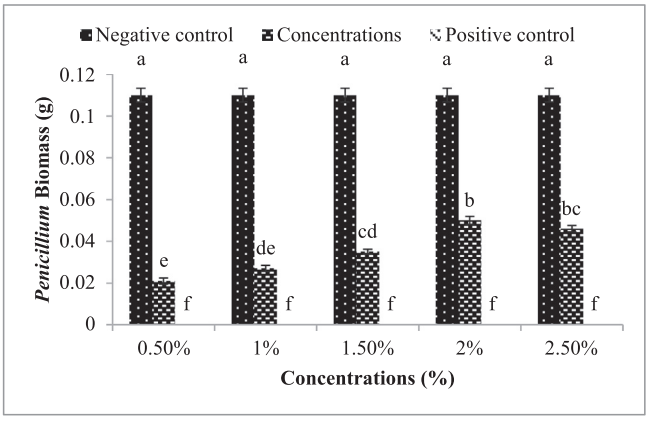


Fig. 3a. Root methanol extracts in various concentrations (0.5–2.5% on X-axis) suppressing the *Penicillium* biomass (Y-axis). The positive and negative controls were maintained as standards against each concentration. The standard error for each replicate is denoted by vertical bars, and the significance of the results is represented by alphabetical letters on the top of each bar.

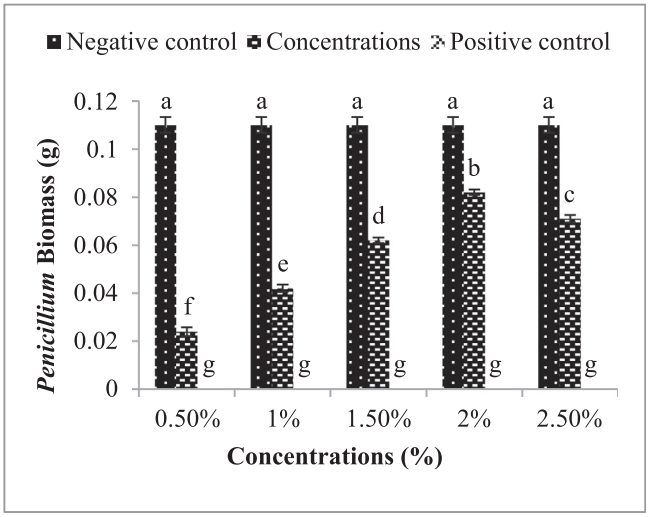


Fig. 3b. Foliage methanol extracts in various concentrations (0.5–2.5% on X-axis) suppressing the *Penicillium* biomass (Y-axis). The positive and negative controls were maintained as standards against each concentration. The standard error for each replicate is denoted by vertical bars, and the significance of the results is represented by alphabetical letters on the top of each bar.

tive control was prepared with the commercial fungicide (metalaxyl + mancozeb 72% WP) in the same concentrations. Whereas negative control with no treatment was prepared in distilled water. All of the treatments and controls were supplemented with Chloramphenicol (5 mg mL^{-1}). The inoculations were done by placing 5 mm mycelial plugs in the centre of each plate and were left undisturbed for a week. Colony diameters were measured from each plant, and growth inhibition (%) was calculated using the formula:

$$GI(\%) = \frac{GT - GC}{GC} \times 100$$

Wherease, GI = Growth inhibition; G_T = Growth in treatment; G_c =- Growth in control

2.6. Phytochemical profiling

Phytochemical profiling of *P. flaccidum* root powder was performed to screen possible phytochemicals that might be responsible for suppressing the fungal growth (Edeoga et al., 2005; Parekh and Chanda, 2007).

2.7. Statistical analysis of antifungal activity

The antifungal activities were performed using a randomised complete block design. The data were analysed statistically applying a one-way analysis of variance (ANOVA) and Duncan's multiple range test (5% significance) described by Steel et al. (1997).

3. Results and discussion

3.1. Morphological characterisation of the blue mold pathogen

The incidence of blue mold infection on onion in Lahore is comparable with the previous data collected from the same region

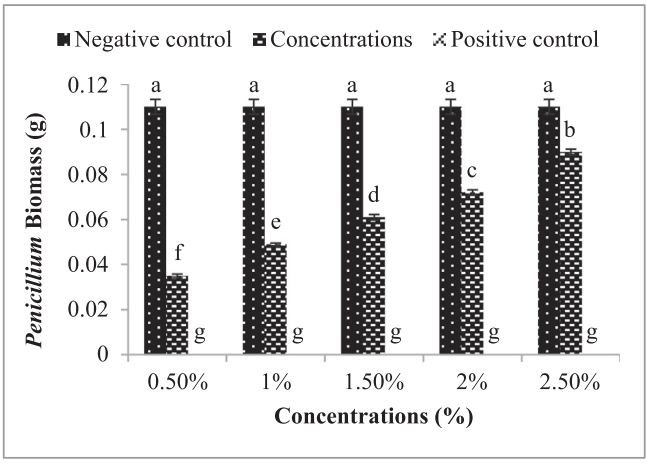


Fig. 3c. Inflorescence methanol extracts in various concentrations ((0.5–2.5% on X-axis) suppressing the *Penicillium* biomass (Y-axis). The positive and negative controls were maintained as standards against each concentration. The standard error for each replicate is denoted by vertical bars, and the significance of the results is represented by alphabetical letters on the top of each bar.

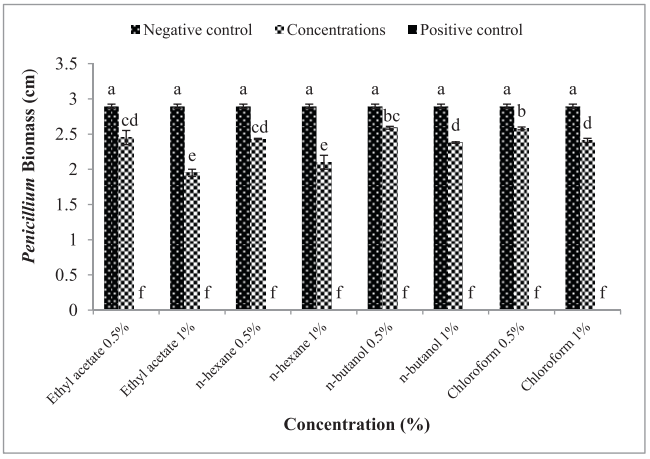


Fig. 4. Isolated fractions in two concentrations (0.5% & 1% on X-axis) inhibiting *Penicillium* biomass (Y-axis). The positive and negative controls were maintained as standards against each concentration. The standard error for each replicate is denoted by vertical bars, and significance of the results is represented by alphabetical letters on the top of each bar.

(Khokhar and Bajwa, 2014). Since the mold is responsible for notable yield losses worldwide. Diagnosis is the crucial step of treatment (Kumar et al., 2016). The present study used a combination of the techniques for a complete diagnosis of the fungus responsible for blue mold rot in the onion. Through macroscopic and microscopic analysis, the isolated pathogen was identified to be a *Penicillium* spp. (Table 1). Since the morphological studies are limited to identify specie in Fungal Systematics. Therefore, the study has paired up the classical approach with the modern molecular method, i.e. DNA sequencing.

3.2. Molecular characterisation of the blue mold pathogen

ITS region of rDNA comprising 600 bp (Fig. 1) was sequenced. The sequence of the isolated fungus is shown in (Table 2). BLAST analysis of the DNA sequence was done to find out the identical sequence on GenBank. The analysis has found an Uncultured *Penicillium* clone (Accession: MF535522) showing 82.39% similarity

Table 4
The data of phytochemical screening of root extracts of *P. flaccidum*.

Phytochemicals	Test name	Result	Inference
Alkaloids	Dragendroff's test	Brown precipitation	+++
Coumarins	Fluorescence response test	No yellow fluorescence observed	–
Flavonoids	Ammonium test	No yellow colouration observed	–
Glycosides	Keller-killani test	No ring formation	–
Phlobatannins	1% HCl test	No precipitation	–
Saponins	Frothing test	White emulsion formation	++
Tannins	Braemer's test	Cyan colouration	+
Terpenoids	Salkowski test	Red-green ring formation	++

'+' represents slight amount, '++' represents moderate amount, '+++' represents high amount, and '–' represents absence of the respective phytochemical in the sample.

index with the sequence results of the *Penicillium* spp. The cut-off value was used jointly with phylogenetic analysis to delimit and identify unidentified fungus sequences to species level. Since it seems likely that the Uncultured *Penicillium* clone does not belong to the same species because its cut off value is 82.39% that is below the proposed cut off value for specie delimitation (97%). A phylogenetic tree has been constructed to establish the isolated *Penicillium* spp. with closely related species (Fig. 2). The sequences of 29 closely related *Penicillium* spp. have been retrieved from the GenBank.

The analysis proved that the isolated *Penicillium* specie from Lahore is the first reported specie of the genus. The finding can be justified from the previous work in which Khokhar and Bajwa (2014) reported 14 new *Penicillium* species in Pakistan on various hosts. The novel species of *Penicillium* have also been reported from various regions around the world Please write down the name of regions of the world (Langlois et al., 2014; Visagie et al., 2016; George et al., 2018).

3.3. Pathogenicity test

The pathogenicity test was found to be positive. According to the scale for rating of pathogenicity (Nova et al., 2011), all the lesions formed were categorised as grade 2, i.e. mildly pathogenic (Table 3).

3.4. In vitro antifungal activity of methanol extracts of P. flaccidum

An effective biocontrol of the isolated *Penicillium* spp. has been proposed in the study. For this purpose, *in vitro* evaluation of the antifungal activity of *P. flaccidum* extracts against the fungus was done. The positive control (metalaxyl + mancozeb 72% WP) showed maximum inhibition in test fungal growth as this totally inhibited the biomass of Penicillium. The methanol root extract (0.5%) was also found most effective in suppressing the *Penicillium* species growth, i.e. 89%. While the rest of the concentrations also generated promising results by controlling fungal growth in the range of 58–89% compared to negative control (plain MEA medium) (Fig. 3a). Foliage extract (0.5%) suppressed fungal growth up to 78.2% (Fig. 3b). The same concentration of inflorescence was highly potent with the inhibition rate of 68% compared to negative control (Fig. 3c). Overviewing the trend, it can be concluded that the lower concentrations of the plant have shown the best results as a bio-fungicide.

Further, root extract is the most efficient in suppressing *Penicillium* growth. The trends of fractions in suppressing fungal growth are illustrated (Fig. 4). The 1% ethyl acetate fraction was the most potent among other fractions and has controlled the fungal growth up to 32% compared to negative control. However, in case of posi-

tive control no germination of the test fungus was observed. different species of the family Poaceae, *Panicum maximum* and *Eleusine coracana* have also been reported to show antimicrobial activities (Kanife et al., 2012; Singh et al., 2015).

3.5. Phytochemical profiling

P. flaccidum was tested for the presence of phytochemicals. Saponins, terpenoids, alkaloids and tannins are present in the plant's roots (Table 4). Other members of the family have also been reported to possess similar phytochemicals (Okaraonye and Ikewuchi, 2009). However, saponins, tannins and alkaloids are famous for antimicrobial activity (Evans et al., 2002). Recently Naeem et al., (2020) suggested that phytochemicals present in *Ocimum sanctum* and *Nicotiana tabacum* might be responsible for antifungal potential against tested Aspergillus species. Khan et al., (2018) reported that *Euphorbia hirta* contains flavonoids, tannins, alkaloids,

phlobatannins and coumarins which can reduce the germination of pathogenic fungus *Colletotrichum gleosporioides.*

4. Conclusion

The present study concluded that the DNA sequence of the blue mold pathogen used in the study does not match any sequence present on the GenBank. Therefore, it might be the newly reported specie of *Penicillium* collected from Lahore, Pakistan. This study also proposes that *P. flaccidum* (root) can be utilized as biocontrol agent against *Penicillium* specie.

CRediT Author statement

Tehzeeb Zubairi: Data curation, Investigation, methodology. **Khajista Jabeen:** Data curation, Investigation, methodology. **Sana Khalid:** Data curation, Investigation, methodology. **Sumera Iqbal:** Data curation, Investigation, methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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