Research Article

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Studies of *Penicillium* species associated with blue mold disease of grapes and management through plant essential oils as non-hazardous botanical fungicides

Abstract: Blue mold caused by *Penicillium* species is a major fungal disease threatening the viticulture industry in Pakistan, responsible for deteriorating the quality of grapes during handling, transportation, and distribution. Identification-based approaches of *Penicillium* species provide a better strategy on accurate diagnosis and effective management. In this study, 13 isolates were recovered from symptomatic grape bunches at five main fruit markets of Rawalpindi district, Punjab province. Based on morphological data and multi-loci phylogenetic analysis, the isolates were identified as two distinct species viz. *Penicillium expansum* (eight isolates) and *Penicillium crustosum* (five isolates). Meanwhile, the pathogenicity test of *Penicillium* isolates presented by the inoculation of grape bunches showed various degrees of severity. For improving the fruit quality and eliminating the needs of synthetic fungicides, botanicals such as thyme (*Thymus vulgaris* L.), fennel (*Foeniculum vulgare* L.), garlic (*Allium sativum* L.), ginger (*Zingiber officinale* L.), and carum (*Carum capticum* L.) essential oils (EOs) at concentrations of 0.6, 0.9, 1.2, and 1.5 mg/mL were evaluated. *In vitro* studies indicated that thyme EO showed a highly significant reduction of fungal growth. Furthermore, the experiments related to reducing the decay development and average weight loss percentage of grapes revealed similar findings. Based on the results of this study, it is recommended that thyme EO can be used as an eco-friendly botanical fungicide against *Penicillium* spp. causing blue mold disease.

Keywords: blue mold disease, essential oils, green botanical fungicide, *Thymus vulgaris*, *Vitis vinifera* L.

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1 Introduction

Grapes (*Vitis vinifera* L.) are a widely cultivated fruit in Pakistan for fresh consumption, covering over an area of about 15,000 ha with an annual production of 57,000 tons [1]. Due to the nutritive and economic values, it was considered as the "queen of fruit" [2]. Despite being known for the most nutritious and medicinal values, grapes are one of the perishable fruits that have a short shelf life of about 2–3 days at ambient temperature. The susceptibility to *Penicillium* genus is the main cause towards reduced shelf life, deteriorating the berries and increasing market losses up to 50%. This pathogen not only is responsible for weight loss, colour changes, and softening of berries but also produces mycotoxins that may be harmful to human [3]. Penicillium is the most important genus of saprophytic fungi, with over 400 described species distributed worldwide [4]. Symptomology and morphological characterization are traditional approaches for species-level identification of *Penicillium* species; therefore, modern molecular techniques such as PCR amplification and sequence analysis have been successfully proved an effective tool for the diagnosis of this fungi with associated species [5]. The internal transcribed spacer (ITS) rDNA sequence is the commonly used universal sequenced marker for the identification of fungi up to the species level. Unfortunately, the ITS sequence diagnosis is not enough for *Penicillium* species distinguishing among all closely related species [6]. Because of the limitations associated with the ITS, several additional gene regions, including β-tubulin and calmodulin, have been used to distinguish closely related *Penicillium* species [7,8]. Previous literature indicated that the management of postharvest decaying pathogens is accomplished by several synthetic fungicides (chemical methods), which badly affect the physical structure of grape berries and pose serious health hazards in humans [9]. These synthetic fungicides on fruits cause two major problems: first, contamination of fruits with fungicidal residues, and second, they induce resistance in the pathogen populations. Food safety is one of the major concerns related to fresh fruits and

vegetables. According to the World Health Organization (WHO) and United Nations Environment Program, it is estimated that millions of individuals associated with agriculture in developing countries suffered severe pesticide toxicity and approx. 18,000 die every year [10]. Researchers have successfully found some alternatives for the control of postharvest spoilage fungi in the form of botanicals such as plant essential oils (EOs) [11]. Plant EOs also known as secondary metabolites are natural, volatile, and aromatic liquid compounds that are non-toxic to humans, highly effective against postharvest pathogens, and can be further exploited to replace hazardous environment deteriorating artificial fungicides [12,13].

Keeping in view the aforementioned facts, the overarching goal of our work is to find a rapid, convenient, and accurate way to identify *Penicillium* species involved in blue mold and develop a novel strategy to control this pathogen infecting grapes employing plant EOs, which are environmentally sustainable, economical, and easy to access.

2 Materials and methods

2.1 Cultural, morphological characterization, and mycotoxin detection

A survey was conducted in July 2016 in five different main fruit markets of Rawalpindi (33°38′19.2″N, 73°01′45.0″E) district. Infected samples of grapes cv. Perlette were collected based on symptoms as initially brown followed by lesion expansion and later on bluish mycelium spread on the surface of the whole bunch shown in Figure 1. For isolation, small portions of about 3–5 mm^2 of symptomatic fruit samples were excised at the lesion margin. The segments were surface disinfected with 2% sodium hypochlorite for 2 min, rinsed twice in distilled water, dried out on sterilized filter paper for 2 min to remove excess moisture, and placed on Petri dishes (4–5 segments per dish) containing potato dextrose agar (PDA) medium. The Petri dishes were incubated at 28 ± 2°C for 3 days and checked regularly for fungal colonization. After 3 days, the diameter of each colony was measured and identified based on cultural and morphological characteristics by using fungal taxonomic keys [14]. To obtain a preliminary analysis of mycotoxins (patulin and citrinin), the isolates were cultured in potato dextrose broth at 25°C for 7 days and extracted using an organic solvent extraction method [15]. A total of 20 mL of potato dextrose broth from growing cultures was extracted three times with 25 mL of ethyl acetate by shaking vigorously for 1 min each time. Then, the organic phases were combined. Five drops of glacial acetic acid were added to the combined organic phase solution, and the solution was evaporated to dryness in a 40°C water bath under a gentle stream of nitrogen gas. The dried residue was immediately dissolved in 1 mL of acetic acid buffer solution. Acetate buffer was prepared by adding 0.45 mL of glacial acetic acid and 0.245 g of sodium acetate trihydrate to 40 mL of double-distilled water (ddH$_2$O). The pH was adjusted to 4.0 with glacial acetic acid. The volume was then adjusted to 50 mL with ddH2O. The toxins in the extracted sample were subjected to thin layer chromatography [16].

2.2 Pathogenicity test

A pathogenicity test was conducted for the confirmation of highly virulent isolates. For this purpose, asymptomatic

Figure 1: Symptomology of blue mold disease associated with *Penicillium* spp.

grape bunches were surface sterilized with running tap water. Injuries were made with the help of sterile needle to fruits up to 5 mm diameter and placed in a disc of fungal isolates, whereas control bunches were inoculated with distilled water. Bunches were placed in a sterile thermo-pole box (one bunch per box) and stored at 25°C for 3 days. Three replicates were used for each treatment [17]. The disease is scored with modification following [18] 0–5 disease rating scale, where 0 = berries without rot, 1 = 0–10%, 2 = 10–25%, 3 = 25–50%, 4 = 50–75%, and 5 = more than 75%.

2.3 Molecular characterization

DNA was extracted and purified by using Prepman DNA extraction kit (Thermo Fisher) and GeneJet purification kit, respectively. Each source culture was derived from a single conidium on PDA medium. Mycelium of each isolate was used for DNA extraction. Amplification of the extracted DNA was performed by using PCR. For molecular identification of *Penicillium* species, primers specific for ITS, benA, and CaM loci were selected for PCR amplification (Table 1) Polymerase Chain Reaction (PCR) was carried out by using the programmable thermocycler Bio-Rad T100 as follows: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 72°C for 1 min, and a final extension at 72°C for 5 min. PCR product was analysed on 1% agarose gel electrophoresis by staining with ethidium bromide as a staining agent. After positive band detection on a gel, all isolates were subjected to purification amplified DNA by using the GeneJet purification kit (KT-00701) by following the standard protocol of the manufacturer. After purification, the PCR products were sent to Macrogen Korea Inc. for sequencing in both directions through Sanger Sequencer. The sequences generated in this study were aligned and submitted to GenBank and accession numbers were obtained. Datasets of the ITS, ben A, and CaM genes were used for phylogenetic analysis. After confirmation of isolates, sequences were aligned by the MUSCLE (Multiple Sequence Comparison by Log-Expectation) method [19], and the neighbour-joining method was performed in MEGA v. 7 for phylogenetic analysis [20].

2.4 Preparation of plant EOs

Matured leaves of thyme, seeds of fennel and carum, clove of garlic, and rhizome of ginger were obtained from Rawalpindi herbal store. These botanical materials were ground well in a grinder machine and subjected to the extraction process through Soxhlet apparatus by following ref. [21]. Finally, the extracted plant EOs are put in clean glass vials and stored in a refrigerator at 4°C until further tests.

2.5 *In vitro* screening of plant EOs

2.5.1 *In vitro* contact assay method

To find out the effect of different plant EOs on mycelial growth of *Penicillium expansum* and *Penicillium crustosum*, poisoned food technique was used. For this purpose, 50 mL of prepared PDA medium was kept in 100 mL conical flasks, sterilized for 20 min, and kept under the sterilized hood to cool up to 60°C, and then plant EOs were added to each flask and shook gently to prepare PDA medium with concentrations of 0.6, 0.9, 1.2, and 1.5 mg/mL. PDA containing known concentrations of plant EOs was poured into 9 cm Petri plates. Then, 5 mm plugs of 7-day-old culture of *Penicillium* spp. were kept in the centre of each Petri plate, whereas in control sets PDA free of any EOs was used. After that Petri dishes were incubated at ±25°C for 3 days, and finally, mycelial growth inhibition percentage (*I*%) was recorded by using the following formula [22]:

$$I\% = (d_c - d_t)/d_c \times 100, \tag{1}$$

Table 1: Primers used for *Penicillium* species identification

Gene	Primers	Sequences (5'→3')	Length (bp)
ITS	ITS 1F	CTTGGTCATTTAGAGGAAGTAA	~600
	ITS 4R	TCCTCCGCTTATTGATATGC	
β-Tubulin (benA)	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	~550
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
Calmodulin (CaM)	CF1	GCCGACTCTTTGACYGARGAR	~750
	CF4	TTTYTGCATCATRAGYTGGAC	

Table 2: Antifungal compounds, tests, and colour indications

Anti-fungal compound	Tests	Colour indications
Terpenoids	Salkowski test	Dark blue
Alkaloids	Wagner reagent	Dark brownish to black
Phenols	Ferric chloride	Yellowish to green
Saponins	Saponin test	Foam-like appearance

where d_c is the diameter of control and d_t is the diameter of treatment. Analysis was replicated three times.

2.6 Qualitative phytochemical analysis of plant EOs

To find out the antifungal compounds such as phenolics, alkaloids, terpenoids [23], and saponins [24] in plant EOs various phytochemical tests were performed (Table 2).

2.7 Comparison of the fungi toxicity of the selected plant EO with synthetic fungicides

The efficacy of the oils was compared with some fungicides, viz. benzimidazole, diphenylamine, phenylmercuric acetate, and zinc dimethyl dithiocarbamate, by the usual poisoned food technique.

2.8 Fungitoxic properties of the selected plant EO

The effect of increased inoculum density of the test fungus on fungitoxicity of the oil was studied by following ref. [25] at their respective mycelial inhibition concentration (MIC) values. The effect of storage and temperature on the fungitoxicity of the EO was evaluated according to ref. [26]. The range of fungitoxicity of the oil was determined at their respective toxic and hypertoxic concentrations by the poisoned food technique.

2.9 Physicochemical properties of the selected plant EO

The oils were standardized through physicochemical properties, viz. specific gravity, refractive index, acid number, saponification value, ester value, and phenolic content, which were estimated by following ref. [27].

2.10 Application of plant EO in grape bunches

After *in vitro* screening and phytochemical analysis, the selected plant EO is used to find out the efficacy against major both *Penicillium* spp. causing blue mold of grapes. For this purpose, bunches of grapes cv. Perlette are used, which are surface sterilized with distilled water, dipped for 2 min in the prepared plant EO, placed on the perforated thermo-pole box (one bunch/box), and finally the inoculum is sprayed with 40 μL of spore suspension.

2.10.1 Decaying incidence percentage (DIP) determination

Decaying percentage was calculated by using the following formula at 3 days of interval:

$$DIP\% = A/B \times 100, \tag{2}$$

where A is the fungal infected berries in a bunch and B is the total berries in bunch examined. Three replicates were kept for treatment along with the control sets and scored with the decay rating scale, where $0 = $ no symptoms, $1 = $ up to 10%, $2 = 11–25\%$, $3 = 26–40\%$, $4 = 40–60\%$, and $5 = $ above 60%.

2.10.2 Measurement of average weight (AW) loss

The AW loss measured after 3 and 6 days of storage and calculated by using the following formula followed in ref. [28]. The layout of the experiment included three replications of each treatment along with the control set:

$$AWL\% = (A − B)/A \times 100, \tag{3}$$

where A is the original fruit weight after storage and B is the final fruit weight.

2.11 Statistical analysis

The results were subjected to statistical analysis using Statistix ver. 8.1. The mean values were separated using Tukey's HSD test at $P \leq 0.05$ after analysis of variance (ANOVA). Significant and non-significant interactions were explained based on ANOVA.

3 Results

3.1 Cultural and morphological identification, mycotoxin detection, and pathogenicity analysis

Thirteen isolates of *Penicillium* spp. causing blue mold of grapes were recovered from five different locations of main fruit markets in Rawalpindi district. Based on their morphology and diversity with respect to location from which the isolates were obtained, the isolate number, source, and morphological characteristics viz. colony colour, shape of conidiophore, length, width of conidia, and conidial shape are listed in Table 3. Using traditional means, the 13 isolates were identified tentatively as belonging to two different *Penicillium* species: *Penicillium expansum* (eight isolates: Pen 01, Pen 02, Pen 03, Pen 06, Pen 08, Pen 11, Pen 17, and Pen 18) and *Penicillium crustosum* (five isolates; Pen c 02, Pen c 03, Pen c 04, Pen c 05, and Pen c 05) shown in Figure 2.

In preliminary mycotoxin analysis, *P. expansum* isolates (Pen 01, Pen 03, Pen 08, and Pen 18) produced both patulin and citrinin, whereas Pen 02 and Pen 17 produced only patulin. In the case of isolate Pen 11, low level of mycotoxins was detected. For *P. crustosum*, isolates Pen c 04 and Pen c 05 produced both mycotoxins, whereas Pen c 03 and Pen c 06 did not produce detectable mycotoxin (Table 3).

Pathogenicity test revealed that five isolates of both *Penicillium* spp. viz. Pen 01, Pen 03, Pen 08, Pen c 04, and Pen c 05 were highly virulent, causing more than 75% rotting on bunches and fall fifth in the disease rating scale (Table 3).

3.2 Sequence analysis of *Penicillium* spp. and phylogenetic analysis

Three sets of primers were chosen to amplify three marker genes (ITS, BenA, and CaM) from the 13 *Penicillium* isolates. Using these primers, amplified sequences were submitted to GenBank, and their accession numbers are listed in Table 4. The initial species identifications based on traditional criteria were largely confirmed by sequence analysis.

Phylogenetic trees were made with each individual gene: ITS (Figure 3), benA (Figure 4), and CaM (Figure 5). In building the phylogenetic tree, MUSCLE alignment was made between 13 sequenced isolates of *Penicillium* spp. amplified with three marker genes along with previously identified reference sequences of *Penicillium* spp.

available in the databases. For phylogenetic analysis of ITS, β-tubulin, and calmoduline regions of *Penicillium* spp. The reference sequences that were used are *P. atrofulvum*, *P. anatolicum*, *P. bilaiae*, *P. adametzioides*, *P. sclerotiorum*, *P. adametzii*, *P. aurantioviolaceum*, *P. carneum*, *P. brunneoconidiatum*, *P. araracuarense*, *P. brasilianum*, *P. brefaldianum*, *P. caperatum*, *P. alfredii*, *P. brocae*, *P. atrovenetum*, *P. bovifimosum*, *P. atramentosum*, *P. glandicola*, *P. rabsamsonii*, *P. astrolabium*, *P. bialowiezens*, *P. canescens*, *P. abidjanum*, *P. allii-sativi*, *P. chrysogenum*, *P. cellarum*, *P. paneum*, *P. commune*, and *P. brevicompactum*, respectively. Furthermore, *Phytophthora parasitica* was used as outgroup among all amplified regions during phylogenetic tree construction shown in Figures 3–5.

3.3 *In vitro* evaluation of plant EOs against *Penicillium* spp.

3.3.1 *In vitro* contact assay method

The effects of five plant EOs at four different concentrations viz. 0.6, 0.9, 1.2, and 1.5 mg/mL were tested on mycelial growth of two *Penicillium* spp. (*Penicillium expansum*; isolate ID: Pen 3 and *Penicillium crustosum*; isolate ID: Pen c 04) under *in vitro* conditions.

3.3.1.1 Efficacy of plant EOs against *Penicillium expansum*

In vitro application of plant EOs revealed that from the five selected plant EOs, thyme EO showed maximum effectiveness to inhibit the growth of highly pathogenic isolate (Pen 03) of *P. expansum* (81.3%, 84.8%, 89.7%, and 93.6%) at all concentrations, i.e., 0.6, 0.9, 1.2, and 1.5 mg/mL after 3 days of incubation (Figure 6) followed by fennel EO with reduced growth inhibition (67.2%, 74.0%, 76.2%, and 84.5%) in comparison to control in which none of the growth inhibition was observed. However, garlic EO was found least at all concentrations in the range of 30–60% during the third day of observation.

3.3.1.2 Efficacy of plant EOs against *Penicillium crustosum*

Similar results were also achieved regarding thyme EO efficacy against *Penicillium crustosum* (Pen c 04),

Table 3: *Penicillium* spp. isolate ID with morphological characteristics (under colony) heading: CC = colony colour and microscopic characterization (under conidiophore), S = shape (Conidia) heading, S = shape, L = length, B = breadth, and mycotoxin production of **13** *Penicillium* isolates; ± standard deviation calculated by number of readings = 30

S. no.	Isolation ID	Location (source)	Colony CC	Conidiophore S	Conidia S	L (µm)	B (µm)	Mycotoxin detection[a] Patulin	Citrinin	Pathogenicity[b] test
1	Pen 1	Rawalpindi	White to bluish green sporulation lacking white margins	Mono-verticillate	Ellipsoidal	3.16 ± 0.14	1.3 ± 0.7	+	+	5
2	Pen 2	Gujar Khan	White to bluish green sporulation lacking white margins	Mono-verticillate	Spherical	3.17 ± 0.2	2.6 ± 0.6	+	−	3
3	Pen 3	Rawat	White to bluish green sporulation lacking white margins	Mono-verticillate	Ellipsoidal	3.34 ± 1.15	2.4 ± 0.25	+	+	5
4	Pen c 02	Rawalpindi	White to greyish-green sporulation with white margins	Tetra-verticillate	Ellipsoidal	3.3 ± 0.45	2.3 ± 1.2	±	±	4
5	Pen c 03	Islamabad	White to greyish-green sporulation with white margins	Tetra-verticillate	Spherical	3.02 ± 0.04	3.5 ± 0.9	−	−	1
6	Pen 6	Texilla	White to bluish green sporulation lacking white margins	Mono-verticillate	Subglobose	3.17 ± 0.2	1.6 ± 0.2	−	+	3
7	Pen 8	Gujar Khan	White to bluish green sporulation lacking white margins	Mono-verticillate	Ellipsoidal	4.18 ± 1.8	2.6 ± 0.89	+	+	5
8	Pen 11	Islamabad	White to bluish green sporulation lacking white margins	Mono-verticillate	Spherical	3.2 ± 1.8	1.6 ± 0.3	±	±	4
9	Pen c 04	Rawalpindi	White to greyish-green sporulation with white margins	Tetra-verticillate	Ellipsoidal	3.6 ± 0.13	2.6 ± 1.2	+	+	5
10	Pen 17	Texilla	White to bluish green sporulation lacking white margins	Mono-verticillate	Subglobose	3.2 ± 1.3	1.4 ± 0.5	+	−	3
11	Pen 18	Rawat	White to bluish green sporulation lacking white margins	Mono-verticillate	Ellipsoidal	3.34 ± 1.15	1.96 ± 1	+	+	5
12	Pen c 05	Islamabad	White to greyish-green sporulation with white margins	Tetra-verticillate	Spherical	3.24 ± 0.1	1.8 ± 0.5	+	+	5
13	Pen c 06	Rawalpindi	White to greyish-green sporulation with white margins	Tetra-verticillate	Ellipsoidal	3.26 ± 1.1	2.6 ± 1.2	−	−	2

[a]The mycotoxins patulin and citrinin were detected by organic solvent extraction followed by thin layer chromatography (TLC) separation and visualization under UV light at 365 nm wave length; ±: low level faint band on TLC plate and it is unclear whether the mycotoxin is produced or not; +: mycotoxin present; −: mycotoxin not detected. [b]Pathogenicity test = (0) no bunch rot, (1) 0–10%, (2) 10–25%, (3) 25–50%, (4) 50–75%, and (5) more than 75%.
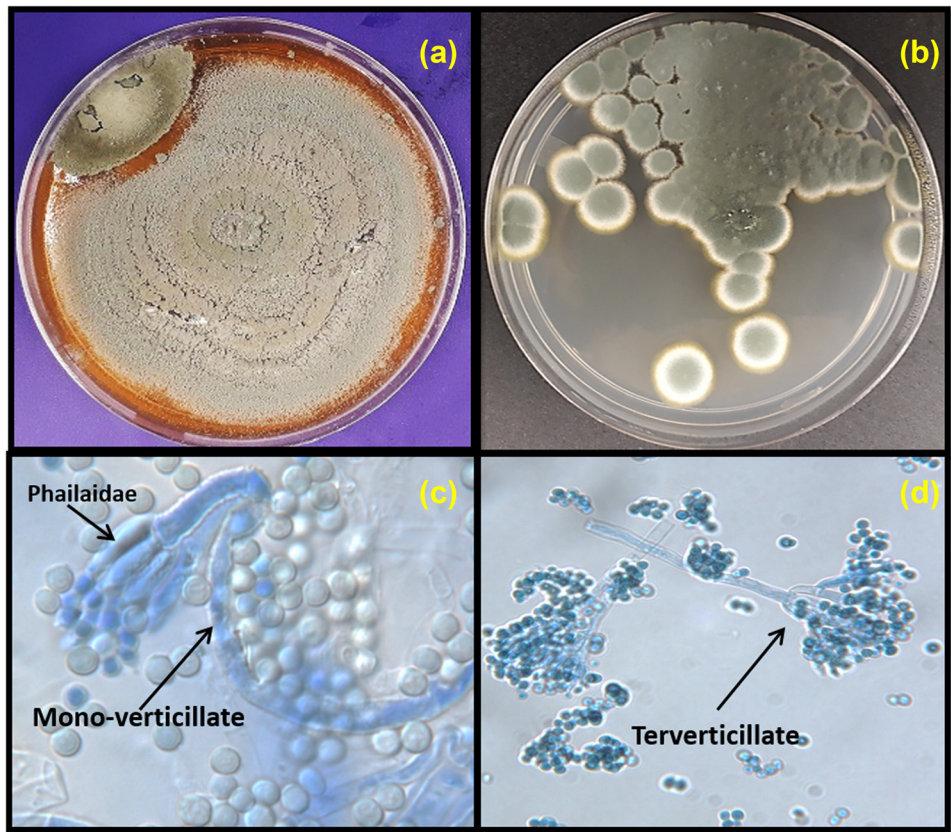
Figure 2: (a–d) Morphological characteristics of *Penicillium* spp. (a and c) Bluish-green colony colour without white margins along with mono-verticillate conidiophore identified as *Penicillium* expansum. (b and d) Greyish green colony colour with white margins and tetra-verticillate conidiophore identified as *Penicillium* crustosum.

Table 4: Accession numbers of amplified nucleotide sequences from *Penicillium* spp. isolates

Penicillium spp.	Isolation ID	ITS	ben A	CaM
Penicillium expansum	Pen 01	MT294660.1	MT387276.1	MT387286.1
Penicillium expansum	Pen 02	MT294661.1	MT387277.1	MT387287.1
Penicillium expansum	Pen 03	MT294662.1	MT387278.1	MT387288.1
Penicillium expansum	Pen 06	MT294663.1	MT387279.1	MT387289.1
Penicillium expansum	Pen 08	MT294664.1	MT387280.1	MT387290.1
Penicillium expansum	Pen 11	MT294665.1	MT387281.1	MT387291.1
Penicillium expansum	Pen 17	MT294666.1	MT387284.1	MT387294.1
Penicillium expansum	Pen 18	MT294667.1	MT387285.1	MT387295.1
Penicillium crustosum	Pen c 02	MT298909.1	MT387257.1	MT387267.1
Penicillium crustosum	Pen c 03	MT298910.1	MT387258.1	MT387268.1
Penicillium crustosum	Pen c 04	MT298911.1	MT387259.1	MT387269.1
Penicillium crustosum	Pen c 05	MT298912.1	MT387260.1	MT387270.1
Penicillium crustosum	Pen c 06	MT298913.1	MT387261.1	MT387271.1

showing maximum inhibition of 82.1%, 87.5%, 92.4%, and 95.3% at four concentrations, i.e., 0.6, 0.9, 1.2, and 1.5 mg/mL followed by fennel EO (70.9%, 75.8%, 83.6%, 90.3%), whereas garlic EO showed less inhibition effectiveness 45.2%, 50.3%, 53.2%, and 55.1% while none of the growth inhibition appeared under control conditions (Figure 6).
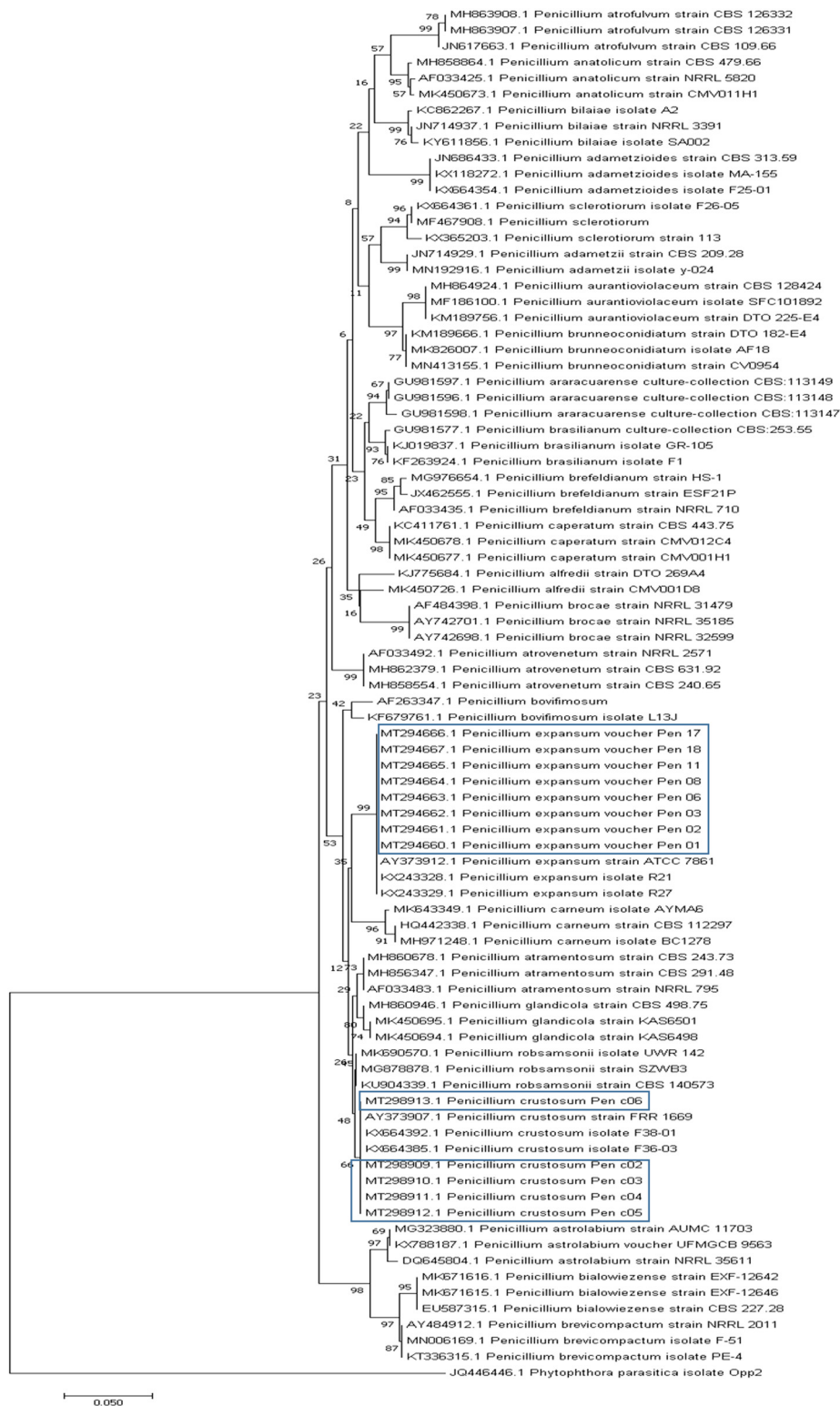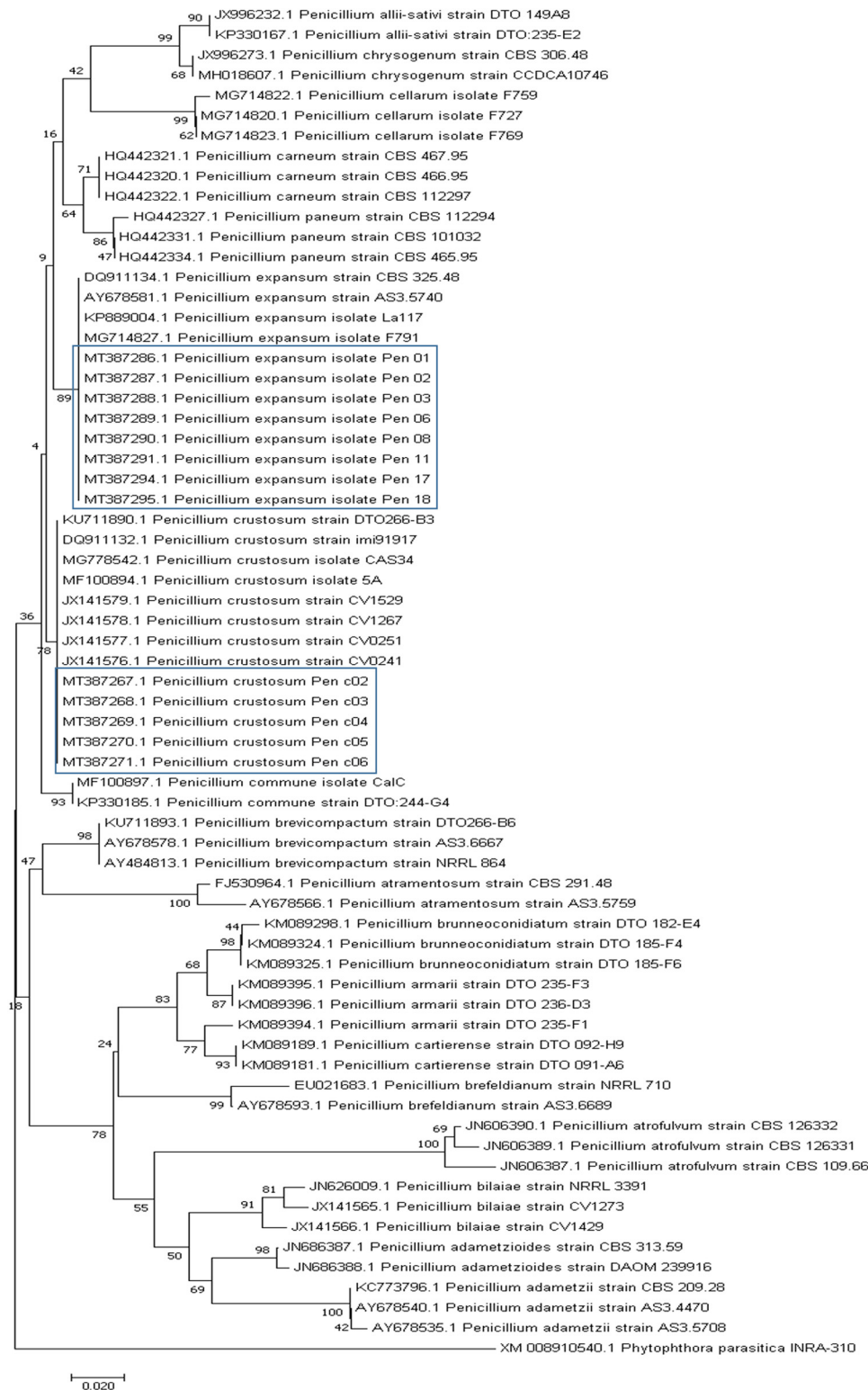
Figure 3: Phylogenetic tree based upon MUSCLE alignment of the ITS region of rDNA nucleotide sequences of *Penicillium* isolates causing blue mold disease of grapes. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.995574 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. 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 evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 86 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 275 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The tree is rooted with *Phytophthora parasitica* isolate (JQ446446.1).
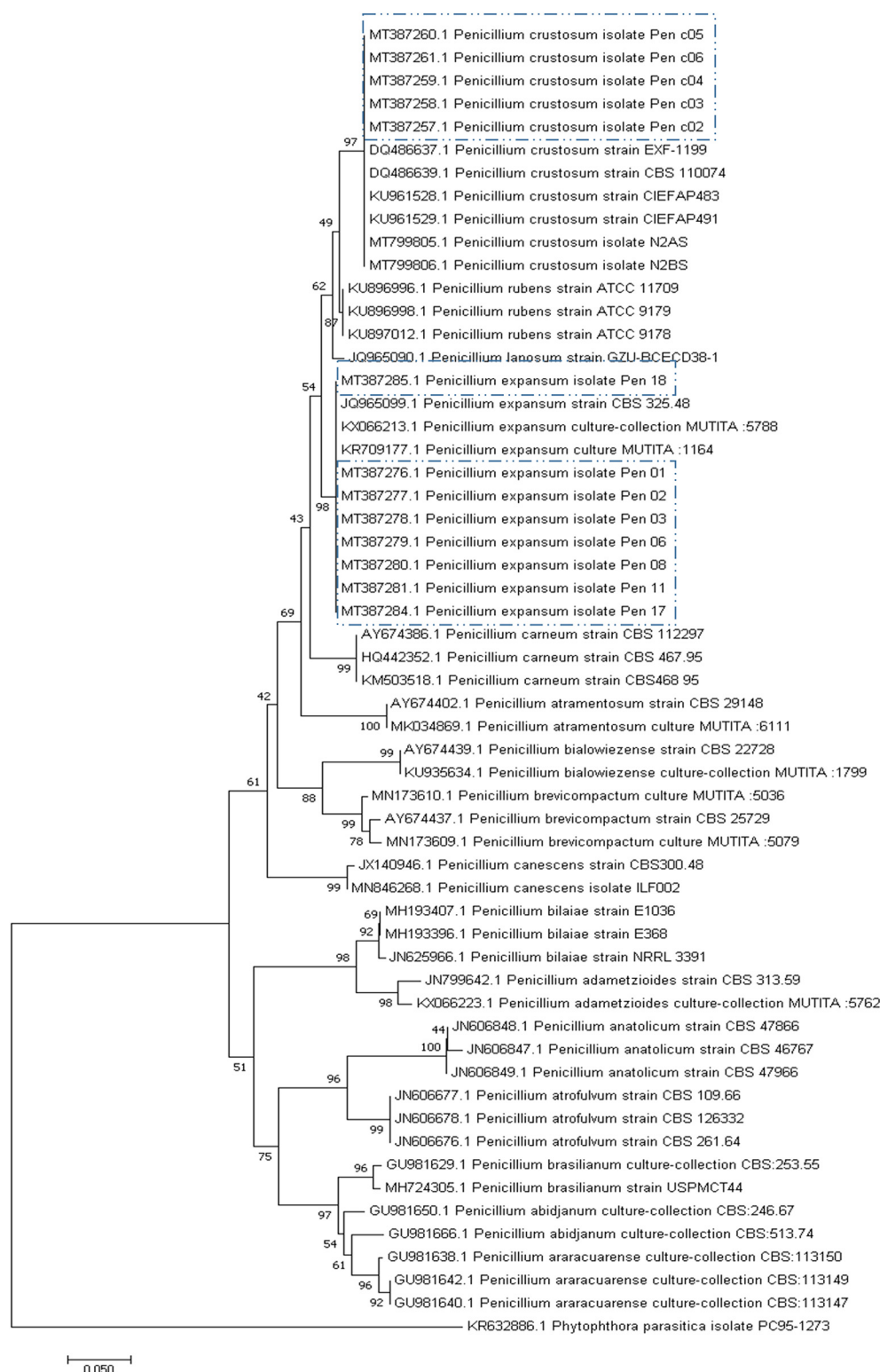
Figure 4: Phylogenetic tree based upon MUSCLE alignment of the β-tubulin region nucleotide sequences of *Penicillium* isolates causing blue mold of grapes. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 1.55153106 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. 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 evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 57 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 181 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The tree is rooted with *Phytophthora parasitica* isolate (KR632886.1).

Figure 5: Phylogenetic tree based upon MUSCLE alignment of the calmodulin region nucleotide sequences of *Penicillium* isolates causing fruit rots of grapes. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.99983118 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. 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 evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 67 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 181 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The tree is rooted with *Phytophthora parasitica* isolate (XM_008910540.1).
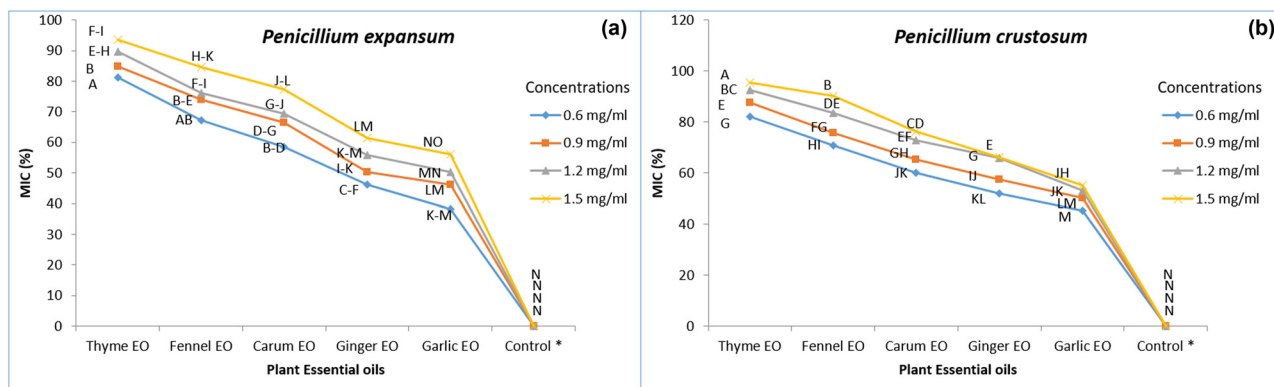
Figure 6: Effect of plant EOs at four concentrations (0.6, 0.9, 1.2, and 1.5 mg/mL) on growth inhibition of (a) *Penicillium expansum* and (b) *Penicillium crustosum*. Mean values in the columns separated by Tukey's HSD test at $P \leq 0.05$ followed by the same letters are not significantly different from each other; Control* (without application of plant EO).

3.4 Qualitative phytochemical analysis of plant EOs

Results of qualitative phytochemical analysis showed that based on colour indication, thyme EO has all tested metabolites such as terpenes (dark blue), alkaloids (dark brownish to black), phenols (yellowish to green), and saponins (foam-like appearance) (Figure 7). Fennel and carum EOs have three anti-microbial compounds viz. alkaloids, terpenes, and phenolics. Ginger EO has two phenols and saponins, whereas garlic EO has one (phenolic) antimicrobial compound (Table 5).

3.5 Comparative efficacy of the thyme EO with some prevalent synthetic fungicides

MIC of synthetic fungicides viz. benzimidazole, zinc dimethyldithiocarbamate, and phenyl mercuric acetate was found to be 7, 4, and 3 mg/mL against *P. expansum*, whereas 6.2, 4, and 2.8 mg/mL against *P. crustosum* which were higher than the most effective thyme oil at 1.5 mg/mL tested in this study (Table 6). Thus, this oil is more efficacious than the synthetic fungicides.

3.6 Fungitoxic properties of the thyme EO

According to the results obtained, it was observed that thyme EO inhibited the mycelial growth of both *Penicillium* spp. The treatment sets containing 32 discs of the test fungi indicating the efficacy of the thyme oil to tolerate high

inoculum density of test fungus are shown in Table 7. This is the potential of this oil to be exploited as botanical fumigant. It was found that thyme oil remained active for 20 months. The oils remained fungitoxic in nature at different temperatures between 10°C and 45°C, showing the thermostable nature of their fungitoxicity.

3.7 Physicochemical properties of thyme EO

The yield of thyme oil was 0.6%. Furthermore, this EO was yellowish green in colour and pleasant in odour. The oil was found to be soluble in all the tested organic solvents. The specific gravity of thyme oil was found to be 0.92, and the phenolic content was also found in this oil during observations (Table 8).
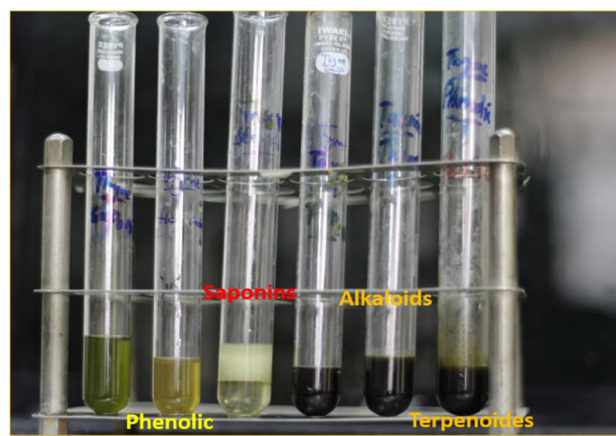
Figure 7: Qualitative phytochemical analysis of plant EO containing anti-fungal compounds.

Table 5: Detailed phytochemical analysis of plant EOs

S. no.	Plant EOs	Phytochemical test			
		Alkaloids Wagner reagent	Terpenoids Salkowski test	Phenolics Ferric chloride	Saponins Foam test
1	Thyme EO	+	+	+	+
2	Carum	+	+	+	−
3	Ginger	−	−	+	+
4	Garlic	−	−	+	−
5	Fennel	+	+	+	−

+: present; −: absent.

Table 6: Comparative efficacy of the thyme EO with some prevalent synthetic fungicides, ± standard deviation calculated by number of readings = 6

	Penicillium expansum	*Penicillium crustosum*
	Minimum inhibitory concentrations (mg/mL)	
Fungicides		
Benzimidazole	7 ± 1.6	6.2 ± 1.32
Zinc dimethyldithiocarbamate	4 ± 0.6	4 ± 1.1
Phenylmercuric acetate	3 ± 1.2	2.8 ± 0.2
Plant EO		
Thyme EO	1.5 ± 0.8	1.5 ± 0.5

3.8 Application of thyme oil on grapes against *Penicillium* spp.

3.8.1 Decay incidence percentage

Thyme EO coating was effective against *Penicillium* spp. at 1.5 mg/mL concentration. Results revealed that DIP of (thyme EO + *Penicillium expansum* inoculum)-treated bunches was 9.64% after the third day of storage at room temperature as compared to the control set (28.63%) in

which only inoculum was applied. However, on the sixth day DIP was recorded to be 18.24% and ranked the second disease score in treatment (T1) (thyme EO + *Penicillium expansum* inoculum), whereas in the case of control (T0) DIP was 92.35% and ranked the fifth disease rating level (0–5 scale). Similar results found in the case of *Penicillium crustosum* revealed that DIP with application of 1.5 mg/mL of thyme EO on Perlette cultivar was calculated to be 8.1% after the third day in comparison with control which showed 26.23%. A 20.46% decay was recorded on Perlette bunches against *Penicillium crustosum* after the sixth day of storage as compared to 90.5% for control (Table 9).

3.8.2 AW loss assessment

AW of 525 g Perlette bunches was calculated in an electrical balance before the application of thyme EO at 1.5 mg/mL concentration against *Penicillium* spp. (*P. expansum* isolate and *P. crustosum*). After the third day of application of treatment set T1 (thyme EO 1.5 mg/mL conc. + *Penicillium* spp. inoculum) on grape bunches, the minimum AW loss percentage was recorded as 12.8% and 14.7% against *P. expansum* and *P. crustosum* as compared to control (T0), and weight loss% increased up to 42.3% and 45.2% where only inoculums were applied. However,

Table 7: Effect of increased inoculum density on fungitoxicity of the thyme oil

No. of fungal discs	*Penicillium expansum* Approx. number of spores	Growth of *P. expansum*		*Penicillium crustosum* Approx. number of spores	Growth of *P. crustosum*	
		Treatment	Control		Treatment	control
2	$1{,}564 \times 10^3$	−	+	$1{,}590 \times 10^3$	−	+
4	$3{,}128 \times 10^3$	−	+	$3{,}180 \times 10^3$	−	+
8	$6{,}256 \times 10^3$	−	+	$6{,}360 \times 10^3$	−	+
16	$12{,}502 \times 10^3$	−	+	$12{,}720 \times 10^3$	−	+
32	$25{,}004 \times 10^3$	−	+	$25{,}440 \times 10^3$	−	+

(−) indicates no growth of test fungus, (+) indicates growth of test fungus.

Table 8: Physiochemical properties of thyme EO

Parameters	Thyme EOs
Colour	Yellowish green
Odour	Pleasant
Specific gravity	0.92
Optical rotation	(−) 16°C
Refractive index	1.49 at 24°C
Solubility	
Acetone	Soluble (1:1 conc.)
Absolute alcohol	Soluble (1:1 conc.)
90% alcohol	Soluble (1:1 conc.)
Ethyl acetate	Soluble (1:1 conc.)
Benzene	Soluble (1:1 conc.)
Chloroform	Soluble (1:1 conc.)
Hexane	Soluble (1:1 conc.)
Methanol	Soluble (1:1 conc.)
Phenolic content	Present

Table 9: Effect of thyme oil on DIP of *Penicillium* spp. after 3 and 6 days of storage

Treatments		Storage days	DI* (%)	Decay rating scale
Penicillium expansum				
T0	Control*	3	28.63[b]	3
		6	92.35[d]	5
T1	Thyme oil 1.5 mg/mL + Penicillium expansum	3	9.64[a]	1
		6	18.24[c]	2
Penicillium crustosum				
T0	Control*	3	26.23[b]	3
		6	90.5[d]	5
T1	Thyme oil 1.5 mg/mL + Penicillium crustosum	3	8.1[a]	1
		6	20.46[c]	2

Decay rating scale: (0) no symptoms, (1) up to 10, (2) 11–25, (3) 26–40, (4) 40–60, and (5) above 60.
Control* (only *Penicillium* spp. as an inoculum applied) – DI* (decaying incidence) The mean values of decaying percentage of "Perlette variety" given in the columns are analysed by Tukey's HSD test at $P \leq 0.05$. The values are an average of three replicates. The data for statistical analysis included (two treatments, two-storage days, one concentration, and three replications).

after the sixth day of storage AWL% of 26.6% and 29.2% was recorded in thyme oil treatment with inoculum (*P. expansum* and *P. crustosum*), whereas in control, this percentage increased to 52.4% and 54.2%, respectively (Figure 8).

4 Discussion

In this study, morphological characteristics and multi-locus phylogenetic analysis were used to identify 13 isolates on symptomatic fruit bunches located at five different locations of Rawalpindi district, Punjab province, Pakistan. The genus causing blue mold was classified into two distinct species viz. *Penicillium expansum* (eight strains, 61.4%) and *Penicillium crustosum* (five strains, 31.2%). Study revealed that the colony appearance of *Penicillium expansum* showed significant difference from *Penicillium crustosum*, which had dense white to bluish green sporulation lacking white margins, whereas colony colour of *P. crustosum* appeared white to greyish-green sporulation with white margins on PDA medium. Next, we examined other morphological characteristics such as conidiophore and conidia. The shapes of conidia were ellipsoidal, subglobose, and spherical but varied in size. In addition, the shape of conidiophore varied in both species, mono-verticillate type of conidiophore was observed in *P. expansum* isolates while conidiophore having tetra-verticillate shape was found in *P. crustosum*. These are consistent with the results of Hema and Prakasam (2013) who observed that *P. expansum* and *P. crustosum* appeared in different colony colours, i.e. bluish to green varying

from species to species [29]. A similar study was also conducted by Pitt (2009) regarding morphological characteristics of *Penicillium* spp. having mono-verticillate conidiophore in *P. expansum* while tetra-verticillate conidiophore observed in *P. crustosum* [30]. Taken together, our results suggested that the morphological characteristics of *P. expansum* were different from *P. crustosum* to some extent, but still not accurate enough to classify the species within a species complex. Therefore, morphological characteristics and phylogenetic analysis were carried out to identify *Penicillium* species in this study. Phylogenetic analysis divided 13 isolates into *P. expansum* and *P. crustosum*, respectively. Capote et al. (2012) revealed that DNA analysis has become the most critical and precise method to characterize the fungal species and sketching the phylogenetic relationship between them [31]. The ITS region of DNA has been widely applied in phylogenetic studies of fungi due to highly conserved and can be easily investigated using PCR amplification [32]. However, the ITS sequence information alone cannot be used to place a fungus at the species level. In this study, we use the ITS with combination of β-tubulin and calmodulin an effective marker for species identification by following the research work which identified the other fungal genera such as *Alternaria* [33], *Aspergillus* [34], and *Botrytis* [35] by using the same marker genes. In future work with *Penicillium*
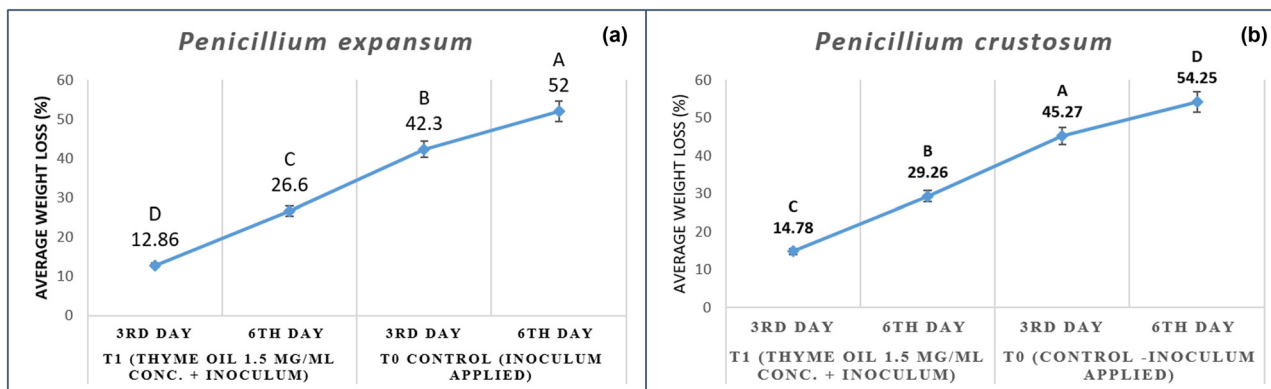
Figure 8: (a and b) Average weight loss (AWL) assessment of thyme oil against *Penicillium* spp.

species identification and genome sequencing, we are developing other more effective markers, with an emphasis on distinguishing the blue molds that cause postharvest decay. In our pathogenicity tests, both *Penicillium* species are pathogenic to grape bunches, suggesting that *Penicillium* spp. can be a huge potential threat to the viticulture industry in Pakistan during storage conditions. Therefore, this study is valuable for guiding the prevention and management of blue mold of grapes. Identification of *Penicillium* species and their pathogenicity on grapes help us to elucidate the relationship between *Penicillium* population and grape bunches in Pakistan. This research provided us a useful information for the control of blue mold and promote a sustainable and healthy development of Pakistan's viticulture industry.

For the management of postharvest pathogens, previous studies illustrated that frequent use of chemical fungicides is the primary means of controlling the postharvest pathogens but have some limitations due to fungicidal resistance among fungal population and high development cost of these synthetic chemicals. Therefore, researchers have successfully introduced green revolution in the form of botanicals as the replacement of synthetic fungicides because of non-toxic residual effect, eco-friendly green synthesis, and public acceptance [36]. In this study, we examined the antimicrobial activities of thyme, fennel, carum, ginger, and garlic EOs against highly pathogenic isolates of *Penicillium* spp. viz. *P. expansum* and *P. crustosum* causing blue mold disease and found that thyme EO had considerable effect on the growth rate of tested fungus both under *in vitro* conditions and application on grape bunches. The results of this study confirmed a strong inhibitory potential of thyme EO against *Penicillium* spp. *Thymus vulgaris* is an effective fungitoxic agent previously studied by several scientists against fungal pathogens [37]. The mechanism of thyme EOs involved inhibition of hyphal

growth, interruption in nutrient uptake, disruption of mitochondrial structure, and eventually disorganization of fungal pathogens [38]. Similarly, Pina et al. (2004) found that thymol is the major active component in thyme EO and has a fungicidal activity, resulting mainly in cell lysis and extensive damage to the cell wall and the cell membrane of postharvest fungal pathogens [39]. In the past, researchers Teixeira et al. (2013) have studied various chemical compounds present in the aerial part of *Thymus vulgaris* such as thymol 46.6%, linalool 3.8%, cymene 38.9%, camphene 1%, α-pinene 3.3%, and β-pinene 1.2%, which showed fungal toxicity against postharvest fungal pathogens [40]. Cakir et al. (2005) reported that phenols, terpenes, and alkaloids are biologically active compounds in thyme oil having anti-fungal properties to control the postharvest decay of fruits [41]. The present study investigated that on comparing the MIC of thyme oil with some synthetic fungicides, the oil was found more active with less concentration and thermostable in nature which is in agreement with the findings of Tripathi and Dubey (2004) concluded that antifungal potency of *O. sanctum* (200 ppm), *P. persica* (100 ppm), and *Z. officinale* (100 ppm) was found to be greater in comparison to some prevalent synthetic fungicides viz. benomyl (3,000 ppm), Ceresan (1,000 ppm), and Ziram (5,000 ppm), respectively [42]. Antunes and Cavaco (2010) explained some mechanism actions of thyme EO during the application on fruits against microbes such as those responsible to alter microbial cell permeability, strengthen the berries' skin surface, disrupt the mycelial growth, and prevent the decay of fruits, respectively [43]. Previously other researchers such as Lopez et al. (2010) also checked the effect of thyme (*Thymus vulgaris*) oil on four cultivars of apples against *Penicillium digitatum* for the determination of DIP and found that thyme oil at 0.1% concentration showed maximum efficacy as compared to fennel and lavender EOs [44]. Our result is in agreement

with the results of Shamloo et al. (2013) who stated that application of thyme EO on sweet cherries during the storage period acts as a physical barrier against moisture loss from fruit skin, prevent weight loss and fruit shrinkage, decrease respiration rate of fruits, and delay senescence [45].

5 Conclusions

This research provided a comprehensive factual picture of blue mold disease of grapes from Pakistan through proper morphomolecular identification of associated *Penicillium* spp. In conclusion, thyme oil with strong fungitoxicity, thermostable nature, long shelf life, fungicidal nature against the test fungus, lower MIC in comparison to synthetic fungicides and the efficacy to withstand high inoculum density has all the desired characteristics of an ideal fungicide. Meanwhile, could also be recommended as a botanical fungitoxicant. These natural compounds are highly degradable with no bioaccumulation in plant, can be effective against postharvest pathogens, and can be further exploited to replace hazardous environment deteriorating artificial fungicides. The results of this study can be served as a guide for the selection of EOs and their concentrations for further *in vivo* trials aimed at fungicide development.

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