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Cultural, morphological, pathogenic and molecular characterization of *Mucor fragilis* causing bunch rot of grapes in Pakistan and its bio management through plant essential oil

Salman Ghuffar*[1], Gulshan Irshad[1], Farah Naz[1], Muhammad Azam Khan[2]

[1]*Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan*[1]

[2]*Department of Horticulture, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan*[2]

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Abstract

Post-harvest disease of grape (*Vitis vinifera*) especially rot caused by *Mucor* sp. are responsible to minimize the market value of grapes. Twenty two isolates recovered from five different locations of fruit markets located in Attock district of Punjab province, Pakistsn. During morphological characterization light sulphur yellow to off white, fluffy, fast-growing colonies were observed. Width of sporangiophores ranged 6.5 to 10.5 µm; sporangia were globose to sub globose, containing yellow to orange sporangiospores. For molecular characterization, the internal transcribed spacer (ITS1, 5.8s and ITS2) regions of two pathogenic isolates were amplified using ITS1 & ITS4 primers. Sequence comparison revealed 98-100% genetic homology (Accession no.KX550076 and KY290546) with previously reported isolates of *M. fragilis*. Among, *In vitro* evaluation of five plant essential oils against *M. fragilis*. Thyme oil at 0.01% concentration showed the minimum mycelia radial growth (3.6cm) 3 days after incubation at 25±2°C as compared to control (8.0cm). The thyme (Eo) was further evaluated for the presence of antimicrobial compounds viz. terpenoids, alkaloids, phenolics and saponins employing standard protocols and found positive for the presence of all compounds. During the application of thyme oil at (0.1%) concentration on fruit bunches for the determination of decaying percentage. The result showed 9.53% decay caused by *M. fragilis* on treated bunches up to six days of storage and control was 71.34% calculated. Keeping in all view, Thyme oil possessing good inhibitory action upon *M. Fragilis* may be a potential candidate for preservation and extension of shelf-life of grapes commercially.

* **Corresponding Author:** Salman Ghuffar ✉ mominsalman2610@gmail.com

Introduction

Grapes (*Vitis vinifera* L.), are one of the most valuable fruit in Pakistan, mostly cultivated for fresh consumptions. Globally, grapes production exceeds 77.1 million tones and China is the primary producer of grapes with production of approximately 11.6 million tones followed by Italy. In Pakistan, grapes are grown over an area of 15.282 thousand ha with annual production of 66 thousand tones (FAO, 2013). Major varieties of grapes are cultivated in Pakistan including King ruby, Flame, Perlette, Wight Anab-e-Shahi and Sundar khani (Khan and Nabi, 2010).

Grapes are the big source of nutrients like vitamin A, C, calcium and iron. Due to its high vitamin and mineral contents grapes are known for their beneficial effects on cardiovascular system, digestive system, neuron degenerative system, immune system, proper functioning of kidneys, cure for asthma, helpful in curing migraine and slow down the ageing process in human body (Agarwal *et al.,* 2002). Along with the highly nutritional and medicinal values, grapes are susceptible to postharvest diseases caused by various pathogenic fungi like *Botrytis cinerea* (Javed, 2017), *Alternaria alternata* (Ghuffar *et al.,* 2017) and penicillium spp. (Ghuffar *et al.,* 2017).

Infection due to fungal pathogens during handling, transportation and storage not only responsible to lowers the shelf life but also causes significant economic losses in the commercialization phase (Gatto *et al.*, 2011). Among all biotic factors Mucor sp. is one of the most important decay pathogen of grapes causing post harvest losses at high frequency (Kassemeyer, 2009). In pakistan Mucor sp. has been already reported on citrus by (Akhtar, 2013) which are responsible to spoilage the fruit but no studies of Mucor sp. on grapes have been conducted in Pakistan which need to be full consideration for its identification and better management strategies. In recent years, consumers have become more concerned about application of chemicals in food products because synthetic preservatives release residues on foods that have negative effects on human health and environment (Zoffoli *et al.*, 2007). So there is a strong need of the time to find out some alternative control method that must be ecofriendly and safer to human health. Researchers are looking for the control of postharvest spoilage fungi using natural substances from botanical sources such as oils, extracts, among others. Plant products, especially essential oils, are one of the most promising groups of natural compounds for the development of antimicrobial agents and their use in plant protection (Reverchon, 1997). Keeping in all the view, the present study was conducted with the objective to determine the morpho-molecular identification of *Mucor* sp. in Pakistan and its bio management through different plant essential oil.

Materials and methods

Cultural and morphological Characterization

A survey was conducted from July to September, 2016 from five different locations of main fruit markets located in Attock district (33°46'07.9"N 72°21'43.0"E) of Punjab province, Pakistan. Infected grape samples were collected on the basis of symptoms (dark brown discoloration, soft and fluffy in texture on individual berries and rapidly expanded to the whole bunch shown in Fig.1 A, B) and brought to Mycology Lab at PMAS-Arid Agriculture University Rawalpindi for further processing. Infected berries were cut into small pieces and surface sterilized using 1% Clorox for two minutes.

Afterward the cut pieces were rinsed consecutively three times with sterilized distilled water and dried on sterilized filter paper for 45 sec then placed on the Petri plates. Colonies were purified by using single spore method on Czapek Dox Agar media (CDA) respectively. After 3 days the diameter of each colony was measured and identified on the basis of cultural and morphological characteristics by using taxonomic key (Ellis, 1971). In cultural characterization, mycelia growth rate, colony color, colony texture, Hyphal growth and reverse colony color were observed while in morphological characterization color, shape and size of sporangiophores, sporangia and collumella were observed by preparing slides in Lacto-phenol blue and examined under compound microscope by using 10, 40 and 100X lens respectively.

Pathogenicity Test

Pathogenicity test was conducted for the confirmation of highly virulent pathogens. For this purpose 10 µl aliquots of spore suspension (10^6 spores/ml) of fungal isolates were pipetted onto three non-wounded and four wounded asymptomatic grapes berries (seven berries per isolate). Sterile distilled water was used for a negative control. The experiment was conducted twice and berries were incubated at 25 ± 2°C in sterile moisture chambers for 3 days (Ghuffar *et al.*, 2017).

Molecular Characterization

For molecular analysis, The DNA was extracted from highly virulent isolates by using Prem Man® Ultra sample preparation Reagant (Applied Biosystem, Foster City, CA), following manufacturer instructions. The 50 µl polymerase chain reaction (PCR) mixture contained 4mM Mgc l2, 10µl 10 × Promegma buffer, 0.2mM dNTPs, 0.75µM each primer, 1.25 units of Taq polymerase (Promegma Crop., Madison, WI), and 2µl of DNA template. PCR reactions were performed in a (Model PCT-100; Mj Research Inc., Waltham MA) the rmocycler PCR conditions for amplification of isolates with ITS1 & ITS4 primers (white, 1990) included an initial denaturation step at 95°C for 2 min, followed by 30 cycles at 94°C for 1 min, 56°C for 1 min, and72°C for 1min, with a final extension at 72°C for 5min. Amplification of DNA was verified by running 6µl of the PCR product in a 1% agarose gel (Bio Rad, Herculus, CA) with 1 × Tris-borate EDTA (TBE) at 150V for 1.75h. All The PCR products were purified with Gel Band Purification Kit (GE Healthcare Bio-sciences, Pittsburgh, Pennsylvania), and sequenced by DNA sequencing and synthesis Facility at Iowa state University using DNA analyzer (Model 3730 xl; Applied Biosystems). Sequences were edited manually using Bio Edit v. 7.0.5.2 (Hall 1999). BLAST searches were performed to obtain the closest relatives sequences of the ITS sequences from the Gen Bank database BLAST program of the National Center for Biotechnology Information (NCBI) and were included in the ITS phylogenetic analysis. Sequences were aligned in the CLUSTAL-X v. 1.81 (Thompson *et al.*, 1997) and the Neighbour-Joining (NJ) analysis was performed in MEGA v. 7 (Kumar *et al.*, 2016).

Preparation of Plant Essential Oils

Leaves of thyme (*Thymus vulgare*), fennel seeds (*Foeniculum vulgare*), carum seeds (*Carum capticum*), cumin seeds (Cuminum cyminum) and garlic bulb (*Allium sativum*) dried under shade to obtain the required form. Grinder (Pascall Motorised Pestel & Mortar, Machine No. 20069) used to crush these botanical materials to fine powder. The extraction of essential oils accomplished using Soxhlet's apparatus utilizing 50g of powder in 250ml acetone. The solvent evaporated completely in a rotary evaporator at 40^0C under vacuum, following the procedure which is been reported by (Lu and He, 2010). The essential oils which obtained thus put in clean glass vials and stored in refrigerator at 4^0C.

In Vitro screening of Plant essential oils (EO) against Mucor fragilis

Fungitoxic activity of the oils tested by the poisoned food technique (Perrucci *et al.*, 1994) against Mucor sp. by making different concentrations at 0.04%, 0.06%, 0.08% and 0.1%. The concentrations of the essential prepared by dissolving the requisite amounts in 0.5 ml of 0.1% Tween 80 and then mixed with 9.5ml of Czapek dox medium. The control sets prepared similarly using equal amounts of 0.1% Tween 80 in place of the oil. The mycelia radial growth measured 72 hours after incubation, minimum mycelia radial growth will be calculated by using the following formula:

$$\text{Minimum mycelia radial growth} = \frac{dC - dT}{dC}$$

dc is mean colony diameter of control sets and dt is mean colony diameter of treatment sets. The Statistical analysis was made using the SAS (Anon., 1985). The arrangement of experiment was two factors with completely randomized design with five treatment, four concentration and three replications.

Qualitative Phytochemical analysis of Plant Essential oils

For qualitative phytochemical secreening of various metabolities such as phenolic compounds, alkaloid, Terpenoid (Evans, 1997) and Saponins (Kokate, 1999) were performed.

Application of plant essential oil on grapes against Mucor fragilis

In order to find out the efficacy of essential oils against pathogen. Mature and healthy bunches of grapes used for this experiment. The fruit bunches of control as well as of treatment sets washed in running water and surface sterilized with 0.1% sodium hypochlorite solution and then washed with distilled water. Fruit bunches treated by dipping for 3 mints at most effective concentration of essential oil by poisoned food technique and kept in perforated thermopole box (one bunch per box). The fruits inoculated by 1 ml of the standard spore suspension of decaying pathogen. For fruit inoculation spores from 3-day-old culture suspended in sterile distilled water. Each bunches inoculated by spraying with 40µl of spore suspension of *Mucor fragilis*. Decaying % was calculated by using the following formula at three days of interval.

$$\text{Decaying \%} = \frac{\text{Number of fungal infected barries in bunches}}{\text{Total number of barries in bunches examined}} \times 100$$

Three replicates were kept for treatment along with the control sets and compared with the Decay rating Scale (Table 3).

Results and discussions

Cultural and morphological Identification

Total of 22 isolates of Mucor sp. causing bunch rot of grapes were obtained from five different locations of main fruit market in Attock district of Punjab province. During cultural identification, light sulphur yellow to off white, fluffy, fast-growing colonies on the upper side and light yellow on the reverse side of the plate were observed (Table 1 & Fig. 1 C, D). Maximum width of sporangiphore was 10.5µm ± 0.15 of isolate AM22TL5 and minimum 6.5µm ± 1.25 was recorded of isolate AM1GL1 with variable length. Sporangia were globose to sub globose, numerous sporangiospores with light yellow to orange in color. Collumela was obovoid, cylindrical, ellipsoidal, subglobose exhibiting maximum length of 28.5µm ± 0.17 in isolate AM22TL5 and minimum 16.14µm ± 0.16 in isolate AM12SKL2. Least breadth 15.0µm ± 0.26 was recorded in isolate AM18PL4 and maximum 27.5µm ± 0.19 in isolate AM22TL5 (Table 2 & Fig. 1 E, F) illustrated by (Nguyen *et al*., 2016). During Pathogenicity test, six Isolates AM1GL1, AM5SUL1, AM10KL2, AM14SUL3, AM17SUL4 and AM22TL5 showed highly virulence after 3 days of inoculation (Table 2).

Table 1. Cultural characteristic of Mucor Isolates obtained from main fruit market of Attock District, Punjab, Pakistan.

S. No.	Isolates No.	Colony Color	Colony Texture	Average Colony Diameter in 3 days (cm)	Hypha Growth	Reverse colony color
1	AM1GL1	Off-White	Slightly fluffy	7.8	Slightly raised	Light yellow
2	AM2GL1	Light yellow	Fluffy	8.0	Raised	yellow
3	AM3TL1	Slightly sulphur yellow	Fluffy	8.0	raised	Light yellow
4	AM4TL1	Slightly sulphur yellow	Fluffy	8.0	Raised	Light yellow
5	AM5SUL1	Off-White	Slightly fluffy	7.6	Slightly raised	yellow
6	AM6SKL1	Slightly sulphur yellow	Slightly fluffy	7.8	Aerial	Light yellow
7	AM7KL1	Off-White	Fluffy	8.0	Slightly raised	Slightly light yellow
8	AM8PL1	Light yellow	Fluffy	8.0	Raised	Light yellow
9	AM9SUL2	Slightly sulphur yellow	Slightly fluffy	7.5	Slightly raised	Light yellow
10	AM10KL2	Light yellow	Slightly fluffy	7.3	Embedded	yellow
11	AM11GL2	Light yellow	Fluffy	8.0	Raised	Light yellow
12	AM12SKL2	Slightly sulphur yellow	Fluffy	7.8	Slightly raised	Light yellow
13	AM13TL3	Slightly sulphur yellow	Slightly fluffy	7.6	Slightly raised	yellow
14	AM14SUL3	Off-White	Fluffy	8.0	Slightly	Light yellow
15	AM15KL3	Off-White	Slightly fluffy	7.7	Slightly raised	Yellow
16	AM16GL3	Slightly sulphur yellow	Fluffy	8.0	raised	Light yellow
17	AM17SUL4	Off-White	Fluffy	8.0	raised	Light yellow
18	AM18PL4	Off-White	Fluffy	8.0	raised	Slightly Light yellow
19	AM19GL4	Slightly sulphur yellow	Slightly fluffy	7.2	Slightly raised	yellow
20	AM20TL4	Light yellow	Fluffy	7.8	Slightly raised	Light yellow
21	AM21GL5	Slightly sulphur yellow	Slightly fluffy	7.4	Slightly raised	Light yellow
22	AM22TL5	Slightly sulphur yellow	Fluffy	8.0	raised	Yellow

Table 2. Morphological Characterization of Mucor Isolates and based on mean of five reading per morphological character, ± (Standard deviation).

Sr. No.	Isolate No.	Sporangiphores Width (um)	Sporangia Shapes	Collumella Color	Shape	Width (um)	length (um)	Pathogenicity Test
1	AM1GL1	6.5±1.25	globose	light yellow	obovoid	15.39± 0.26	16.19± 0.59	+++
2	AM2GL1	6.7±1.18	-	light yellow	ellipsoidal	18.51±1.73	17.54±0.42	+
3	AM3TL1	7.6±0.19	sub globose	-	subglobose	19.56±1.27	20.31±0.26	++
4	AM4TL1	6.7±1.38	globose	orange	cylindrical	17.39±0.14	17.37±1.24	++
5	AM5SUL1	8.42±0.23	-	orange	ellipsoidal	21.54±1.26	19.47±1.63	+++
6	AM6SKL1	7.32±1.19	-	-	ellipsoidal	19.5±0.32	19.41±0.51	+
7	AM7KL1	6.5±0.29	-	-	obovoid	16.76±1.82	16.52±1.28	+
8	AM8PL1	9.34±0.19	sub globose	light yellow	cylindrical	23.5±1.25	24.27±0.54	++
9	AM9SUL2	6.54±1.21	-	-	subglobose	20.4±0.23	18.24±1.76	++
10	AM10KL2	6.56±0.32	-	-	subglobose	22.5±0.18	17.18±1.16	+++
11	AM11GL2	7.32±0.19	globose	orange	cylindrical	16.34±0	22.35±0.27	+
12	AM12SKL2	6.54±1.34	globose	-	obovoid	18.27±1.3	16.14±0.16	+
13	AM13TL3	7.8±0.51	-	-	ellipsoidal	16.0±0.15	20.84±1.46	++
14	AM14SUL3	10.32±0.19	-	orange	ellipsoidal	26.5±1.23	26.72±1.54	+++
15	AM15KL3	10.4±0.17	-	orange	obovoid	25.46±1.67	26.42±1.26	+
16	AM16GL3	7.36±1.27	-	orange	ellipsoidal	19.21±0.18	23.45±0.19	+
17	AM17SUL4	10.5±0	subglobose	light yellow	cylindrical	27.5± 1.12	27.24±1.73	+++
18	AM18PL4	6.68±0.19	-	-	obovoid	15.0±0.26	16.45±0.46	+
19	AM19GL4	6.3±1.15	-	-	subglobose	17.54±0.21	16.27±0.35	++
20	AM20TL4	7.32±0.19	globose	orange	obovoid	19.86±1.45	20.15±1.26	+
21	AM21GL5	9.34±1.21	-	-	cylindrical	24.27±0.21	25.62±1.24	++
22	AM22TL5	10.5±0.15	subglobose	-	obovoid	27.24±0.19	28.5±0.17	+++

Pathogenicity test + = low pathogenic ++ = moderate pathogenic, +++ = highly pathogenic.

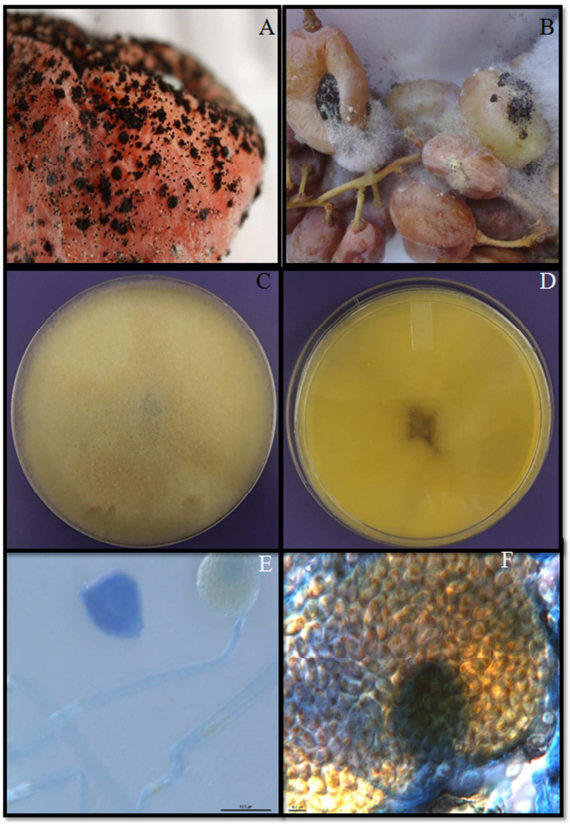

Fig. 1. (A) Dark brown discoloration on individual berry (B) Soft, fluffy, white to dark brown discoloration on the whole Bunch (C) colony color (D) Reverse colony (E) Fruiting body of *Mucor fragilis* (F) Light yellow to orange sporangiospores.

Sequencing of ITS gene and phylogenetic analysis

A total of two highly virulent isolates (Muc 01 and Muc 02) were sequenced in ITS1 and ITS4 directions. The final sequences were submitted in the public database of NCBI under the accession numbers KX550076 and KX550076 exhibiting 98-100% genetic similarity with previously reported isolates of *Mucor fragilis* available at NCBI.

Sequences were aligned in the CLUSTAL-X v. 1.81 and phylogenetic analysis was performed on ITS sequence of the isolates Muc 01 and Muc 02 from Grapes, shown in Fig. 2. Previously (Bahr *et al*., 2013) computed sequence analysis of three *Mucor fragilis* of grapes in Europe and ITS (ITS-5.8S-ITS2) region was amplified through PCR assay with the help of universal sense and antisense primers.

Table 3. Disease rating scale.

Disease Score	Bunch covered by disease
0	No symptoms
1	Up to 10%
2	11-25%
3	26-40%
4	40-60%
5	Above 60%

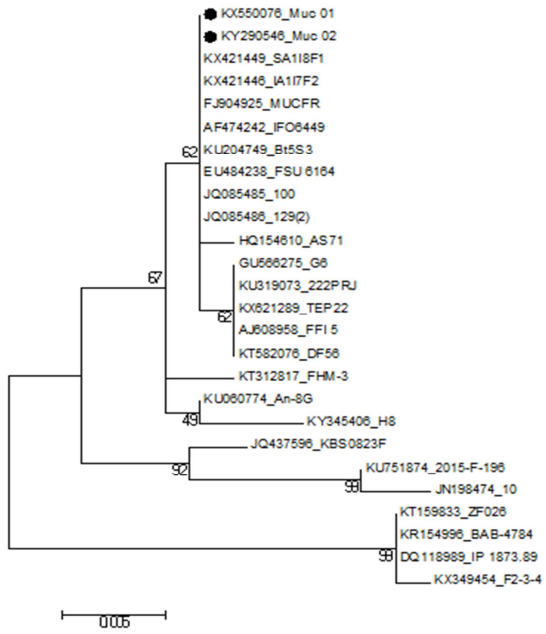

Fig. 2 Evolutionary tree of *Mucor fragilis*

In Vitro screening of Plant essential oils (EO) against Mucor fragilis

The result revealed that selected Plant essential oils showed different efficacy levels in inhibiting the radial growth of *Mucor fragilis* respectively. Statistical data indicated that Thyme oil was found most effective for controlling the growth of pathogen that showed colony diameter 4.24, 4.01, 3.89 and 3.6cm at 0.04, 0.06, 0.08 and 0.1% concentration after three days of incubation followed by Carum, Fennel, Cumin and Garlic essential oils respectively as compared to control 8cm shown in Fig. 3. According to (Abd-Alla *et al.,* 2013) result showed that during *in vitro* study, Thyme oil at 1.0 and 2.0% concentration caused a complete reduction of *P. digitatum* linear growth.

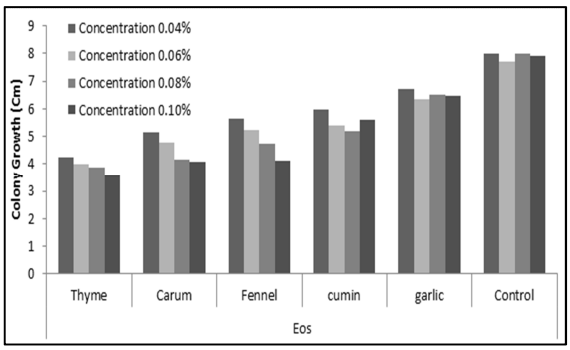

Fig. 3. *In vitro* evaluation of different Plant essential oils against *Mucor fragilis* after three day.

Qualitative Phytochemical analysis of Plant Essential oils

The result of qualitative analysis revealed that all the tested metabolites including Terpenoid, Alkaloids, Phanolic and saponins were present in Thyme essential oil (Table 4). Similar work was done by (Naz *et al.*, 2013) for the photochemical screening of *Tamarix indica* and *Tamarix passernioides* by testing twelve metabilities on different organic solvent.

Table 4. Qualitative phytochemical screening of Plant essential oils.

Sr.no	Plant Essential Oil	Phytochemical Test			
		Terpenoids	Alkaloids	Phenolic	Saponins
		Salkowski test	Wagner reagent	Ferric chloride	Foam Test
1	Thyme	+	+	+	+
2	Fennel	+	+	–	-
3	Carum	+	+	+	-
4	Cumin	-	+	-	-
5	Garlic	+	-	+	-

Key = Present (+), Absent (–)

Application of plant essential oil on grapes against Mucor fragilis

The decaying % of treatment during 3rd storage day was calculated 2.96±0.64 as compared to control (25.42±1.85) while on the 6th storage day decaying % was recorded 9.53±0.51 which covered the 1st disease score on treatment sets and control was 71.34±1.34 falls on 5th disease score. The similar methodology was reported for the Chitosan treatment against *Botrytis cinerea* and *Rhizopous stolonifer* on strawberry by (Ghouth *et al.,* 1991).

Table 5. Effect of Thyme oil on Incidence of Mucor rot after three and six days of storage.

	Storage days	Decaying % (Disease rating Scale)
Control	3	26.42±1.85 (3)
	6	71.34±1.34 (5)
Thyme oil 0.1%	3	2.96±0.64 (1)
	6	9.53±0.51 (1)

Disease rating scale (0) No symptoms (1) Up to 10 (2) 11-25 (3) 26-40 (4) 40-60 (5) above 60.

Conclusions

Morpho-molecular identification and Pathogenicity tests are reliable tools for the confirmation of *Mucor fragilis* causing bunch rot of grapes in Pakistan and Thyme oil showed maximum result for the management of this major fungal pathogen.

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