Mycopath (2019) 17(1): 49-54 **Research Article**

Morpho-molecular identification of *Pestalotiopsis clavispora* causing post-harvest bunch rot of grapes and its management through essential oils

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Abstract

Pestalotiopsis rot caused by *Pestalotiopsis clavispora* is an important fungal post-harvest disease of grapes (*Vitis vinifera* L.) occur during transit, storage, distribution and responsible to minimize the market value of grapes. In this study, thirteen isolates of *P. clavispora* were recovered from five different locations of fruit markets located in Rawalpindi and Attock district of Punjab province, Pakistan. During morphological characterization of the pathogen, white, floccose, cottony colonies having black acervuli forming concentric rings were observed. Length and width of conidia ranged from 22.4×5.2 to 33.6×11.3µm; color of conidia was dark umber to olivacious having 4-5 septations. For molecular characterization, two representative highly pathogenic isolates (RP5PL2 and AP231GL5) were amplified using ITS1 and ITS4 primers. Sequence comparison revealed 99-100% genetic homology (Accession no. MF448223.1 and MF571908.1) with previously reported isolates of *P. clavispora*. During, *in vitro* evaluation thyme essential oil at 0.1% concentration showed significant reduction of 94% in the mycelial growth at 3rd day of incubation. During storage, application of essential oil at 0.1% significantly managed disease. It is concluded that thyme essential oil exhibited good inhibitory action upon *P. clavispora* and and it might be used for preservation and extension of shelf-life of grapes commercially.

Keywords: Grapes, Morpho-molecular identification, Pestalotiopsis rot, *Pestalotiopsis clavispora*, Thyme essential oil.

Introduction

Post-harvest diseases lead to the perishability of grapes during transportation and storage might be due to the growth of numerous fungal pathogenic microorganisms which produce some mycotoxins on fruit surface and direct effect on human health (Moss, 2002). Worldwide it is estimated that 10 to 50% of losses of perishables are due to fungal attack and among them, *Pestalotiopsis clavispora* is one of the common pathogens causing Pestalotiopsis rot of grapes. This fungal pathogen can infect the grapes during pre- and post-harvest stages under a wide range of environmental conditions. Furthermore, Infection, caused during post-harvest condition lowers the shelf life and adversely affects the market value of the fruits (Das *et al*., 2010). For the control of Pestalotiopsis rot, farmers are applying synthetic fungicides on small fruits. Besides their effectiveness against post-harvest decaying pathogens, these synthetic fungicides have some residual effect on berries' skin which may lead to the development of resistant fungi, oncogenic risk, and threats to the environment (Daferera *et al*., 2003). Therefore, worldwide many countries banned using the application of chemical fungicides on small fruits due to hazardous (Arroyo *et al*., 2007). Scientists have found some alternatives in the replacement of chemical fungicides such as Plant essential oils (EOs), which would have non-hazardous effects on the environment and easy excess to farmers (Wang *et al*., 2007). Plant essential oils (EOs) have a rich source of disease-control agents, biologically active, Wide range of natural fungicidal plant volatile compounds that have more potential to control the post-harvest diseases (Meepagala *et al*., 2002). The advantage of essential oils is their bioactivity in the vapor phase, a characteristic that makes them attractive as possible fumigants for stored product protection (Caccioni *et al*., 2002). Keeping all these points in consideration, an attempt has been made in the present piece of work to determine the morpho-molecular identification of *Pestalotiopsis* sp. in Pakistan and find out the practical applicability of essential oils of *Thymus vulgare, Foeniculum vulgare,* and *Carum capticum* in enhancing the shelf-life of grapes by protecting them from fungal rotting caused by *Pestalotiopsis clavispora*.

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 (33°46'07.9"N 72°21'43.0"E) and Rawalpindi (33°38′19.2″N, 73°01′45.0″E) cities of Punjab province, Pakistan. Infected grape samples were collected based on symptoms observations as, Initial symptoms

occurred mainly on wounded berries; following infection, the fruit skin turned reddish-brown, after which the area of discolored skin increased and whitish mycelium developed on the lesions shown in Fig. 1A and brought to fungal pathology 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 seconds then placed on the Petri plates. Colonies were purified by using single spore method on potato dextrose agar media (PDA) 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 (Guba, 1961).

Pathogenicity Test

A pathogenicity test was conducted for the confirmation of highly virulent pathogens. For this purpose, 10 µL aliquots of spore suspension (10^4 spores mL^{-1}) 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, berries were incubated at 25 ± 2 °C in sterile moisture chambers for 3 days (Ghuffar *et al*., 2018) and compared with percentage disease index (0 = berries without rot, 1 = 0–10%, 2 = 10–25%, 3 = 25–50%, 4 = 50–75% and 5 = More than 75%) for the determination of highly pathogenic isolate (Senthil *et al*., 2011).

Molecular Characterization

For molecular analysis, The DNA was extracted from highly virulent isolates by using Prem Man® Ultra sample preparation Reagent (Applied Biosystem, Foster City, CA), following manufacturer instructions. The 50 µL polymerase chain reaction (PCR) mixture contained of 4 mM Mgc l2, 10 µL of 10 × Promegma buffer, 0.2 mM dNTPs, 0.75 µM each primer, 1.25 units of Taq polymerase (Promegma Crop., Madison, WI), and 2 µL of DNA template. PCR reactions were performed in a (Model PCT-100; Mj Research Inc., Waltham MA) the thermocycler PCR conditions for amplification of isolates with ITS1 and 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, and 72 °C for 1min, with a final extension at 72 °C for 5 min. Amplification of DNA was verified by running 6 µL of the PCR product in a 1% agarose gel (Bio-Rad, Herculus, CA) with 1 × tris-borate EDTA (TBE) at 150 V for 1.75 h. 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 Neighbor-Joining (NJ) analysis was performed in MEGA v. 7 (Kumar *et al.,* 2016).

Preparation of essential oils (EOs)

Leaves of thyme (*Thymus vulgare*), fennel seeds (*Foeniculum vulgare*) and carum seeds (*Carum capticum*) were taken from herbal store located in Rawalpindi. These botanical materials were first dried under shadow, grinded well in grinder machine and subjected for extraction process through Soxhlet's apparatus followed by (Şahin *et al*., 2003). Finally, extracted EOs put in a clean glass vials and stored in refrigerator at 4°C until further tests.

***In vitro* antifungal potential of plant essential oils**

Fungitoxic activity of the oils tested by the Poisoned food technique (Perrucci *et al*., 1994) against *Pestalotiopsis* sp. by making different concentrations at 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 9.5 mL 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 formula described earlier (D'auria *et al*., 2005)

Application of plant essential oil on grapes against *Pestalotiopsis* sp.

To find out the efficacy of essential oils against pathogens, mature and healthy bunches of grapes were used for this experiment. The fruit bunches of control as well as of treatment sets were washed in running water and surface sterilized with 0.1% sodium hypochlorite solution and then washed with distilled water. Fruit bunches were treated by dipping for 3 minutes at the most effective concentration of essential oil by poisoned food technique and kept in perforated thermo-pole 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 40 µL of spore suspension of *Pestalotiopsis* sp. three days of intervals.

$$\text{Decaying } \% = \frac{No.\ of\ fungal\ infected\ berries\ in\ bunch}{Total\ number\ of\ berries\ in\ bunch\ examined} X\ 100$$

Three replicates were kept for treatment along

with the control sets and compared with the decay rating scale (0 = no symptoms, 1 = up to 10%, 2 = 11–25%, 3 = 26–40%, 4 = 40–60% and 5 = above 60 %).

Statistical analysis

The results subjected to statistical analysis using Statistix ver. 8.1. The means were separated using Tukey HSD test at $P \leq 0.05$ after ANOVA. Significant and non-significant interactions were explained based on ANOVA analysis.

Results and Discussion

Cultural and morphological identification

A total of 13 isolates of *Pestalotiopsis* sp. causing bunch rot of grapes were obtained from five different locations of main fruit market located in Rawalpindi and Attock district of Punjab province. During cultural identification, isolates were found with White, floccose, cottony with black acervuli forming concentric rings (Table 1 and Fig 1B and C). Maximum (L × W) of conidia was 33.6 µm ± 0 × 11.3±0.04 for isolate RP5PL2 and minimum 22.5 µm ± 2.3 × 5.21± 0.25 µm was recorded for isolate AP231GL5, respectively. Conidia were dark umber to olivaceous having 4 to 5 septations, while majority of isolates have 7 septations within conidia (Table 1, Fig. 1D). Similar morphological characters (conidial shape and size of *Pestalotiopsis* isolates) were also discussed by Maharachchikumbura *et al*. (2011). During pathogenicity test, isolates RP5PL2, and AP231GL5 were further used for molecular identification.

Sequencing of the ITS gene and phylogenetic analysis

A total of two highly pathogenic isolates (PSTL 01 and PSTL 06) were sequenced in ITS1 and ITS4 directions. The final sequences were submitted in the public database of NCBI under the accession numbers MF448223.1and MF571908.1 exhibiting 99–100% genetic similarity with previously reported isolates of *P. clavispora* available at NCBI. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.44076995 is shown Fig. 2. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 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 31 nucleotide sequences. All the positions containing gaps and missing data were eliminated. There were a total of 331 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The tree is rooted with *Lasiodiplodia pseudotheobromae* (MT071318.1). Previously (Deng *et al*., 2017) used the ITS region and successfully confirmed the Paesteolopsis specie as a pathogen causing post-harvest fruit rot of grapes in Korea. According to our study, this is the first report of *P. clavispora* causing post-harvest rot on grapes in Punjab, Pakistan (Ghuffar *et al*., 2018).

In vitro* screening of essential oils against *P. clavispora

Data recorded after 3rd day of incubation revealed that thyme essential oil applied at 0.06, 0.08 and 0.1% exhibited 91.2, 94 and 96.4% reduction in the fungal growth followed by fennel oil, which decreased growth by 76.1, 79.4 and 85.4%, respectively. Carum essential oil showed 58.2%, 61.1% and 64.4% reduction in the fungal growth at 0.06, 0.08 and 0.1%, respectively (Fig. 3). Similar findings regarding thyme oil treatment at 0.1 and 2.0% concentration against *Penicillium digitatum* (Abd-Alla *et al*., 2013).

Application of plant essential oil on grapes against *P. clavispora*

The decaying percentage of treatment was 3.81±2.4 during third day of storage as compared to control (26.1±1.76), while on the sixth day decaying was 11.2% ±2.26 as compared to control (78.13% ±3.45) (Table 2). Antunes and Cavaco (2010) explained some mechanism actions of plant essential oils on fruits against microbes such alteration in microbial cell permeability, strengthening of the berries' skin surface, disruption in the mycelial growth and prevent to decay of fruits.

Conclusions

Morpho-molecular identification and pathogenicity tests are reliable tools for the confirmation of *P. clavispora* causing bunch rot of grapes in Pakistan and thyme oil showed the substantial management disease caused by the fungal pathogen on the grapes.

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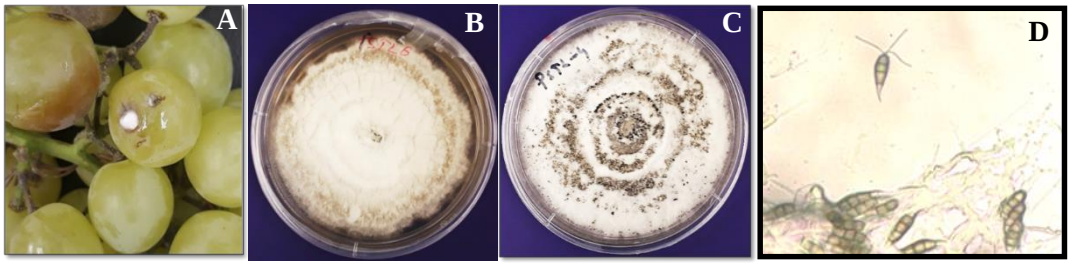

Fig. 1: (A-D) Symptomology, Cultural and morphological characteristics of *Pestalotiopsis* sp. causing bunch rot of grapes.

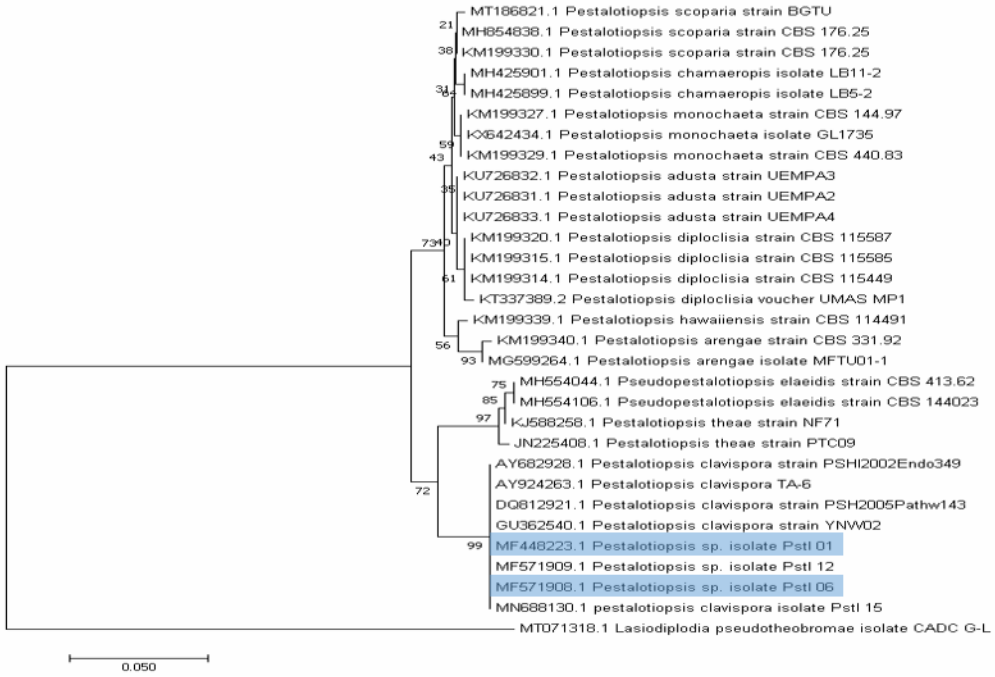

Fig. 2: Phylogenetic Tree based upon MUSCLE alignment of the ITS region of rDNA nucleotide sequences of *Pestalotiopsis clavispora* isolates causing Fruit rots of grapes.

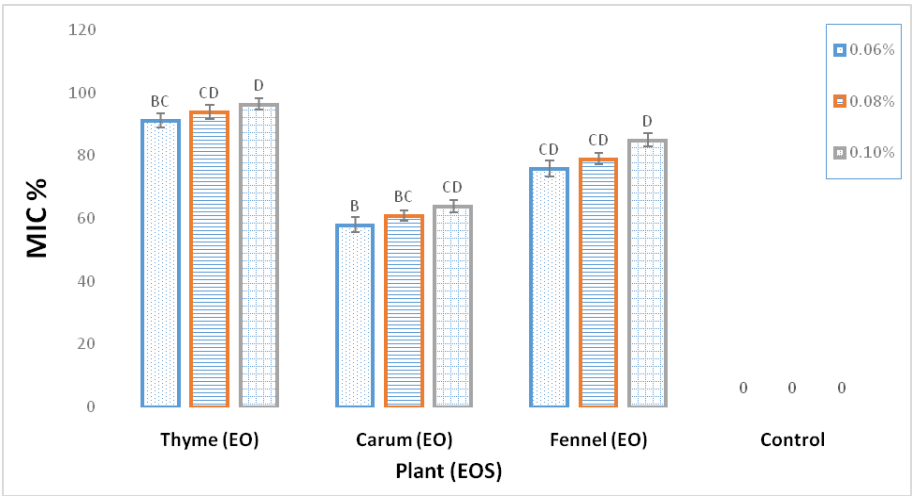

Fig. 3: *In vitro* evaluation of different plant essential oils (EOs) against *Pestalotiopsis clavispora* (Isolate: RP5PL2) after 3rd day.

Table 1: Cultural and morphological characteristics of *Pestalotiopsis* sp. obtained from the main fruit market of Rawalpindi and Attock districts, Punjab, Pakistan.

Sr.	Isolation ID	Colony		Conidia			
		Colony Color	Color	Length (µm)	Width (µm)	Septation	Pathogenicity Test
1	RP1KL1	White, floccose, cottony with black acervuli forming concentric rings	Dark umber	31.7±1.4	6.4±0.2	4	4
2	RP2TL1	White and cottony	Olivacious	29.4±0.6	7.4±0.3	5	3
3	RP3SKL1	-	Dark umber	24.1±1.5	8.4±1.1	5	1
4	RP4SUL2	-	Dark umber	29.2±0.6	9.3±0.19	4	2
5	RP5PL2	White and floccose	Olivacious	33.6±0	11.3±0.04	5	5
6	RP6GL2	Whitish and floccose	Dark umber	24.7±0.4	7.5±0.2	4	2
7	RP7KL3	White, floccose with black acervuli forming concentric rings	Olivacious	25.2±1.7	8.2±1.1	5	1
8	RP8TL3	White and cottony	Dark umber	27.3±1.4	8.3±0.2	4	3
9	A1TL4	-	Olivacious	32.3±0.46	9.6±0.27	5	3
10	A2GL4	White and floccose	Dark umber	25.3±0.6	8.7±1.21	4	4
11	A3SKL5	-	Olivacious	27.5±1.2	7.9±0.9	4	2
12	AP231GL5	White and cottony	Dark umber	22.4±2.3	5.21±0.25	5	5
13	A5TL5	White and floccose	Olivacious	26.4±1.12	8±1.7	4	3

Table 2: Effect of thyme Essential oil (0.1 %) on the Incidence of *Pestalotiopsis* rot after three and six days of storage. Whereas, **S.D** means Standard Deviation (Three number of readings), **Control*** (***P.clavispora*** as an inoculum applied), **DI*** (Decaying incidence)

Treatments	Storage days	(DI±S.D)*	Decay scoring system
Thyme essential oil 0.1% + *P. clavispora*	3	3.8^{d}±2.4	1
	6	26.1^{b}±1.76	3
*Control	3	11.2^{a}±2.26	2
	6	78.1^{c}±3.45	5

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