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# International Journal of Phytopathology

ISSN: 2312-9344 (Online), 2313-1241 (Print)

<https://esciencepress.net/journals/phytopath>

## MOLECULAR CHARACTERIZATION OF *PENICILLIUM EXPANSUM* ISOLATED FROM GRAPES AND ITS MANAGEMENT BY LEAF EXTRACT OF *CHENOPODIUM MURALE*

Iqra H. Khan, Arshad Javaid, Syeda F. Naqvi

Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan.

### ARTICLE INFO

#### Article History

Received: January 19, 2021

Revised: March 11, 2021

Accepted: March 16, 2021

#### Keywords

*Penicillium expansum**Chenopodium murale*

Molecular identification

Blue mold

### ABSTRACT

*Penicillium expansum* Link causes an economically important postharvest blue mold disease in a number of fruits and vegetables. In the present study, this fungus was isolated from rotted grapes and identified on morphological basis. Identification of the pathogen was further confirmed on molecular basis by using four different primer pairs namely ITS,  $\beta$ -tubulin, CMD and CF under accession numbers MN752155, MN787831, MN787832 and MN787833, respectively. Leaf extract of *Chenopodium murale* was assessed for its potential to control *in vitro* growth of *P. expansum*. For this purpose, leaves were extracted in methanol and after evaporation of the solvent, the resulting extract was successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol followed by antifungal bioassays with different concentrations (1.562 to 200 mg mL<sup>-1</sup>) of each organic solvent fraction. Although all the fractions controlled the fungal pathogen to variable extents, however, *n*-butanol fraction showed the highest antifungal activity causing 45–86% reduction in the biomass of *P. expansum*. Ethyl acetate fraction was also highly antifungal and reduced fungal biomass by 44–81%. Chloroform and *n*-hexane fractions were comparatively less effective and reduced biomass of *P. expansum* by 30–72% and 11–44%, respectively. This study concludes that ethyl acetate and *n*-butanol fractions are highly antifungal in nature against *P. expansum*.

Corresponding Author: Arshad Javaid

Email: arshad.iags@pu.edu.pk

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### INTRODUCTION

Grape (*Vitis vinifera* L.) is the first fruit berry that has a long history of cultivation by humans (Morata *et al.*, 2017). It is cultivated on commercial basis in more than 75 countries with an average production of more than 23 million metric tons (FAOSTAT, 2020). It is a domesticated deciduous woody vine having numerous uses for producing table fruit, jelly, juice, wine, jam, vinegar, raisins and grape seed oil (Venkitasamy *et al.*, 2019). It is considered as a non-climacteric type of fruit having green, red or black colors that grows well in a cluster of 20 to 270 (Migicovsky *et al.*, 2017). It is enriched with essential nutrients such as minerals, vitamins, edible fibers, carbohydrates and

phytochemicals. In grapes, polyphenols are considered as the most important phytochemicals because they possess many health-promoting benefits and biological activities (Cheng *et al.*, 2020). In addition, grapes are a rich source of antioxidants such as flavones, anthocyanins, linalool, geraniol, tannins and nerol (González-Barreiro *et al.*, 2014). Due to its highly perishable nature, grape production is largely affected by a wide range of fungal pathogens (Kassemeyer, 2017). Among them, *Penicillium* is one of the most common groups of fungi that attacks on fresh table grapes during packaging and causes significant postharvest decay losses (Wang *et al.*, 2019). In recent years, *Penicillium expansum* has become the main

pathogen responsible for blue mold disease of grape in Pakistan. It represents a serious economic concern to the grape industry causing up to 60% of decay in stored fruit (Ghuffar *et al.*, 2018). It produces several toxic compounds such as chaetoglobosins, citrinin and patulin that affect the quality of processed products (Tragni *et al.*, 2021). *P. expansum* conidia typically penetrate through wounds and propagate on fruit surface even at below 0°C (He *et al.*, 2019).

Although synthetic fungicides such as fluxapyroxad, cyprodinil and thiophanate methyl are used commonly for the control of postharvest losses by *P. expansum*, however, their application on large scale has compromised their efficacy by developing resistance in pathogen populations (Samaras *et al.*, 2020). Public health concerns have stimulated the search for alternative control strategies for the effective management of *P. expansum*. In nature, plants are considered as a rich source of volatile compounds that are biologically active against fungal pathogens (Akhtar *et al.*, 2020; Javaid *et al.*, 2020; da Silva *et al.*, 2020). Many studies have focused on *C. murale* for the effective control of phytopathogens (Naqvi *et al.*, 2019; Naqvi *et al.*, 2020). It belongs to family Chenopodiaceae. It is one of the fast-growing erect annual herbaceous weed natives to Europe (Farhan *et al.*, 2019). Now, it is widespread to various parts of the world including Pakistan where it grows on different types of habitats. It grows abundantly in winter and early summer along the roadsides, cross walks, damp and waste places (Al-Batsh and Qasem, 2020). It is enriched with diterpenoids,

terpenoids, flavonoids, glycosides, sterols, sesquiterpenes, glucosinolates, alkaloids, coumarins and phenolic acids with potent antifungal activities (Belmaghraoui *et al.*, 2018). Therefore, the aim of this study was to identify *P. expansum* on molecular basis and to utilize the leaf extracts of *C. murale* for effective management of this pathogen of grapes.

## MATERIALS AND METHODS

### Isolation and characterization of *P. expansum*

Infected grapes with light brown spots were collected from Lahore, and surface sterilized in 3% sodium hypochlorite solution for 1 min followed by washing with autoclaved water. The diseased area was carried out by using a needle and placed on freshly prepared malt extract agar. The inoculated plates were kept at 30 °C for five days. The obtained colonies were re-isolated to get pure cultures and studied on the basis of macroscopic and microscopic features at different magnifications under light microscope (Vico *et al.*, 2014).

### Molecular identification using PCR

The genomic DNA of the isolated fungus *P. expansum* was isolated by CTAB method following the protocol described by Doyle and Doyle (1990). The conventional PCRs were performed by using ITS,  $\beta$ -tubulin, CMD and CF primer pairs (Table 1). The obtained PCR products were analyzed by MiSeq Illumina sequencing, USA for bidirectional sequencing and then submitted to NCBI database for BLAST alignment search.

Table 1. List of oligonucleotide primers used for the characterization of *P. expansum* at molecular level.

No.	Primer name	5' to 3' sequence	Amplicon size (bp)	Annealing temperature
1	ITS 1 Forward	TCCGTAGGTGAACCTGCGG	~ 638	60 °C
2	ITS 4 Reverse	TCCTCCGCTTATTGATATGC		
3	$\beta$ -tubulin Forward	GGTAACCAAATCGGTGCTGCTTTC	~ 471	62 °C
4	$\beta$ -tubulin Reverse	ACCCTCAGTGTAGTGACCCTTGCC		
5	CMD 5 Forward	CCGAGTACAAGGARGCCTTC	~ 576	65 °C
6	CMD 6 Reverse	CCGATRGAGGTCATRACGTGG		
7	CF 1 Forward	GCCGACTCTTTGACYGARGAR	~750	65 °C
8	CF 4 Reverse	TTTTYGCATCATRAGYTGGAC		

### Preparation of extracts

Fresh *C. murale* plant leaves were collected from Punjab University, Lahore. The collected leaves were washed under running tap water, shade dried and grinded into a fine powder. The powdered leaves (1 kg) were dipped in

methanol (5 L) for two weeks at room temperature and then passed through a filtration process by using two layers of filter paper. The obtained material was run-on a rotary evaporator to gain a thick gummy leaf extract. The crude extract was mixed in autoclaved water (200

mL) and partitioned through a separating funnel with *n*-hexane (4 × 500 mL), chloroform (200 mL), ethyl acetate (200 mL) and *n*-butanol (100 mL). The resultant solvents were run on rotary evaporator in order to get thick gummy material of each fraction (Banaras *et al.*, 2020).

#### Antifungal bioassays

A mass of 1.2 g of each fraction of leaf extract was dissolved in DMSO (1 mL) followed by the addition of malt extract (ME) broth to raise the final volume up to 6 mL (stock solution) of 200 mg mL<sup>-1</sup> concentration. The lower concentrations *viz.* 100, 50, 25, 12.5, 6.25 and 3.125 mg mL<sup>-1</sup> were prepared by serial double dilution of the stock. A control set was also prepared without plant extract and each treatment was replicated thrice. Mature culture of *P. expansum* was used to prepare spore suspension and each glass tube was inoculated with 20 µL of it and left to stand at 30 °C for 7 days. After that, fungal mats were filtered on pre-weight filter papers, dried and weighed (Khan and Javaid, 2020).

#### Statistical analysis

Data related to biomass of the *P. expansum* was analyzed by ANOVA and treatment means were separated by applying Tukey's HSD test at 5% level of probability using software Statistix 8.1.

#### RESULTS

##### Morphological identification of *P. expansum*

Morphological features showed that the mature colonies of the fungus were circular and rapidly growing attaining a diameter of 5 cm at 30 °C in 7 days. The young colonies were off white in color, which upon maturity turned into light to dark green color whereas the reverse side of the plate was pale yellow to greenish in color (Figure 1). The observed conidia were 3.25 to 3.71 × 2.56 to 3.28 µm in size, elliptical in shape, smooth surfaced having thin walls, attached with branched conidiophores. The germinated conidia form germ tubes. On the basis of morphological studies, the examined isolate was identified as *P. expansum*.

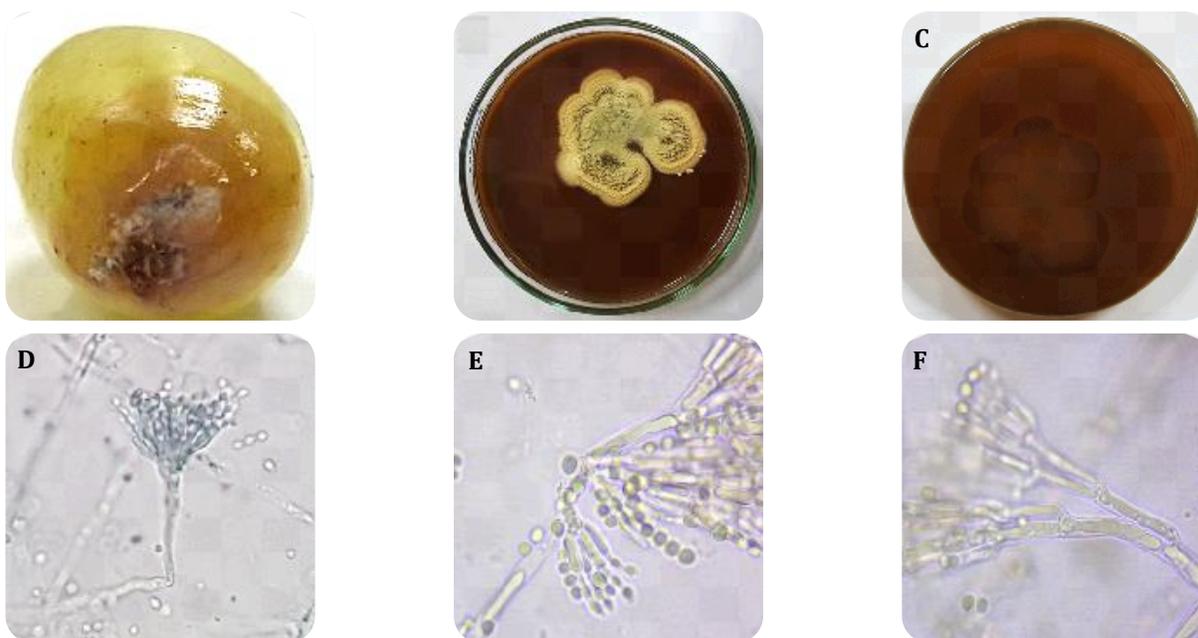


Figure 1. **(A)**- Infected grape with *Penicillium expansum* **(B)**- Mature colony of *P. expansum* on MEA, **(C)**- Colony reverse on MEA, **(D)**- Conidia at 40X, **(E & F)**- Conidia at 100X showing hyphae and conidiophores bearing conidia.

#### Molecular characterization of *P. expansum*

For more accuracy, molecular tools were used for the precise characterization of *P. expansum* as only microscopic studies are not sufficient due to huge similarities among the *Penicillium* species. Therefore, a total of four primer sets *viz.* ITS,  $\beta$ -tubulin, CMD and CF were used for the identification

of *P. expansum* genomic DNA under MN752155, MN787831, MN787832 and MN787833 accession numbers, respectively with 99–100% similarities with already deposited specimens in the GenBank. As expected, the amplified PCR products generated specific bands of the appropriate sizes as shown in Figure 2.

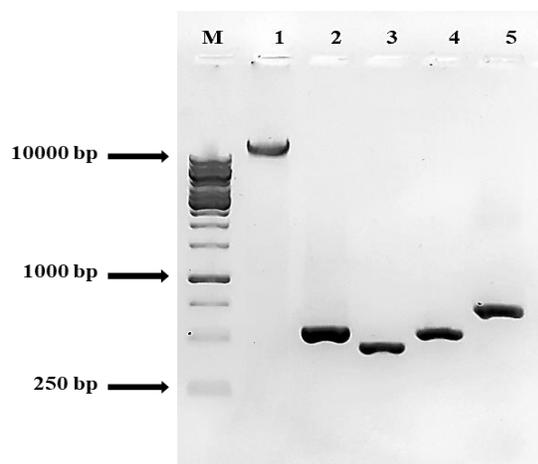


Figure 2. Agarose gel electrophoresis, (M): 1 kb DNA standard marker, (1): Genomic DNA of *Penicillium expansum*, (2): ITS1/ITS4 amplified PCR product, (3):  $\beta$ -tubulin amplified PCR product, (4): CMD5/CMD6

amplified PCR product, (5): CF1/CF4 amplified PCR product.

### Screening bioassays

Four fractions of *C. murale* leaf extract were used against *P. expansum* that showed a significant suppressive effect on the growth of the targeted pathogen. Although all the fractions variably controlled the fungal pathogen, however, *n*-butanol fraction showed the highest antifungal activity causing 45–86% reduction in *P. expansum* biomass over control. Likewise, different concentrations of ethyl acetate fraction noticeably reduced the fungal biomass by 44–81%. The other two fractions *viz.* chloroform and *n*-hexane were comparatively less effective and reduced biomass of *P. expansum* just by 30–72% and 11–44%, respectively (Table 2).

Table 2. Effect of different concentrations of *Chenopodium murale* on growth of *Penicillium expansum*.

Methanolic extract fraction	Conc. of DMSO ( $\mu\text{L mL}^{-1}$ )	Fraction conc. ( $\text{mg mL}^{-1}$ )	Fungal biomass (mg)	Decrease over control (%)
Control	1.29	0	60.2 $\pm$ 4.3 ab	-
	2.59	0	60.5 $\pm$ 2.3 a	-
	5.18	0	58.1 $\pm$ 3.4 ab	-
	10.37	0	56.4 $\pm$ 2.2 a-c	-
	20.75	0	53.6 $\pm$ 1.5 a-d	-
	41.5	0	46.2 $\pm$ 2.3 c-e	-
	83	0	30.4 $\pm$ 4.6 g-i	-
	166	0	29.2 $\pm$ 1.6 g-j	-
<i>n</i> -Hexane	1.29	1.562	53.7 $\pm$ 2.5 a-d	11
	2.59	3.125	48.8 $\pm$ 2.6 b-d	19
	5.18	6.25	46.0 $\pm$ 1.0 c-e	21
	10.37	12.5	34.1 $\pm$ 2.3 f-h	40
	20.75	25	33.4 $\pm$ 2.6 f-h	38
	41.5	50	23.5 $\pm$ 1.1 h-m	49
	83	100	21.2 $\pm$ 1.7 i-n	30
	166	200	16.4 $\pm$ 1.0 k-o	44
Chloroform	1.29	1.562	42.3 $\pm$ 2.6 d-f	30
	2.59	3.125	35.7 $\pm$ 1.4 e-g	41
	5.18	6.25	23.3 $\pm$ 2.1 h-m	60
	10.37	12.5	25.7 $\pm$ 2.0 g-l	54
	20.75	25	15.1 $\pm$ 1.0 l-o	72
	41.5	50	12.1 $\pm$ 1.6 m-o	71
	83	100	10.3 $\pm$ 1.0 no	67
	166	200	10.0 $\pm$ 1.0 no	66
Ethyl acetate	1.29	1.562	33.9 $\pm$ 1.4 f-h	44
	2.59	3.125	26.9 $\pm$ 1.1 g-k	55

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	5.18	6.25	15.9±2.2 k-o	73
	10.37	12.5	15.6±1.9 k-o	72
	20.75	25	14.5±2.1 l-o	73
	41.5	50	8.6±1.1 o	81
	83	100	10.0±1.0 no	67
	166	200	10.0±1.0 no	66
<i>n</i> -Butanol	1.29	1.562	33.1±2.2 f-h	45
	2.59	3.125	32.4±1.7 f-i	46
	5.18	6.25	25.9±1.8 g-l	55
	10.37	12.5	18.3±1.6 j-o	68
	20.75	25	7.5±1.5 o	86
	41.5	50	10.0±1.0 no	78
	83	100	10.0±1.0 no	67
	166	200	10.0±1.0 no	66
	HSD <sub>0.05</sub>		11.5	

Values with different letters in a column show significant difference ( $P \leq 0.05$ ) as determined by Tukey's HSD Test.

## DISCUSSION

In the present study, *P. expansum* was found as the causal agent of blue mold disease of grapes on both morphological and molecular bases. Blue mold decay is caused by many *Penicillium* species such as *P. expansum*, *P. chrysogenum*, *P. rugulosum*, *P. polonicum*, *P. commune*, *P. italicum*, *P. digitatum*, *P. crustosum*, *P. solitum* and *P. verrucosum* (Chen *et al.*, 2017; Papoutsis *et al.*, 2019; Duduk *et al.*, 2021). Among these, *P. expansum* is the most virulent strain that produces toxins and causes blue mold decay of peach (Jiao *et al.*, 2018), pear (Zhou *et al.*, 2018), apple (Abdelhai *et al.*, 2019), grapes (He *et al.*, 2019) and quince (Luciano-Rosario *et al.*, 2020).

As the pathogen has a wide host range, therefore, its management is very critical. For this, different fractions of *C. murale* leaf extract were used and the findings revealed that all the fractions had antifungal activities. Earlier, Naqvi *et al.* (2019) reported that different fractions of *C. murale* leaf extracts significantly reduced the growth of *Fusarium oxysporum* f. sp. *lycopersici*. Abdel-Wahhab *et al.* (2020) reported the presence of antioxidants, flavonoids and phenolic compounds with strong antimicrobial activities that might be responsible in reducing the growth of targeted fungal pathogen. *C. murale* extract also suppressed growth of *Alternaria solani* and *Penicillium digitatum* (Qasem and Abu-Blan, 1995) as well as *Macrophomina phaseolina* (Amin and Arshad, 2007). Antifungal activity of *C. murale* leaf extract could be due to presence of hexadecanoic acid, methyl ester; palmitic acid; phytol;  $\beta$ -sitosterol and methyl linolenate (Naqvi *et al.*, 2019).

The present study concludes that *P. expansum* is the causal agent of blue mold disease of grapes in Pakistan. Being a post-harvest pathogen, it produces toxins and contaminates the food products during packing and processing. For its management, leaf extract of *C. murale* was used that significantly reduced the growth of targeted pathogen. Further studies regarding methods of application and mechanism of actions of the *C. murale* leaf extract, and identification of effective antifungal compounds are necessary for effective control measures of this pathogen.

## ACKNOWLEDGMENTS

The authors wish to thank Professor Dr. Pierluigi Bonello, Molecular and Chemical Ecology of Trees, Department of Plant Pathology, Ohio State University, Columbus, USA, for providing lab facilities for molecular analysis. Authors also acknowledge the financial support by HEC Pakistan under IRSIP funding program.

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## CONFLICT OF INTEREST

All the Authors declared no conflict of interest.

## AUTHORS CONTRIBUTIONS

Iqra H. Khan did experimental work and contributed in manuscript writing. Arshad Javaid supervised the work, did statistical analysis and finalized the paper. Syeda F. Naqvi contributed in write up.

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