Identification of *Fusarium* species as putative mycoparasites of *Plasmopara viticola* causing downy mildew in grapevines

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Abstract

Five mycoparasitic fungi were isolated from sporangiophores of *Plasmopara viticola* collected from vineyards of five grape growing regions in India. Four isolates were obtained from the *P. viticola* growth on leaf (M1, M2, M10, and M12_1) and one from growth on berry (M12_2). Morphological observations showed that all isolates belonged to the genus *Fusarium*. Phylogenetic analysis of ITS and *tef 1α* gene identified them as *F. delphinoides* (M1), *F. brachygibbosum* (M2), *F. pseudonygamai* M10, M12_1 and a *Fusarium* sp. nov. (M12_2). In the leaf disc assay all isolates showed *Fusarium* species coiling around sporangiophores of *P. viticola* and inducing lysis. They also inhibited sporangia production. To the best of our knowledge this is the first report of *Fusarium* species as putative mycoparasites of *P. viticola* in vineyards of India.

Keywords Grape · *Plasmopara viticola* · *Fusarium* · Downy mildew · Biological control

Grape downy mildew disease is caused by the oomycete *Plasmopara viticola*. The disease causes severe damage on the foliage and clusters resulting in severe crop loss. Disease control is mainly dependent on synthetic fungicides (<http://nrcgrapes.nic.in/zipfiles/Annexure%205.pdf>). *Plasmopara viticola* is a high risk pathogen for development of resistance to fungicides (Fungicide Resistance Action Committee, FRAC Pathogen Risk List 2014). In vineyards of Maharashtra, India, failure of disease control due to development of resistance in *P. viticola* to quinone outside inhibitor and carboxylic acid amide fungicides has been reported (Sawant et al. 2016, 2017). Hence biological control using a mycoparasite has become a necessity for the management of the disease (Adams 1990). In this study we report four *Fusarium* species as putative mycoparasites of *P. viticola*.

From August through December 2015, downy mildew samples were collected from vineyards in five different regions of India, Champai (Mizoram); Periyakulam (Tamil Nadu); and Sangli, Nashik, Pune regions in (Maharashtra). Each sample was placed individually in polypropylene bag and transferred to laboratory. A 25 mm disc was cut from the infected area on the leaves and placed on 0.5% water agar in 6 well plates. Each infected berry was similarly placed in a well. The plates were incubated for 5 days at 23 °C and a 12 h photoperiod in a growth chamber. After 3 days of incubation, the fungus overgrowing on *P. viticola* was isolated onto potato dextrose agar (PDA) containing 75 ppm chloramphenicol. For morphological identification, *The Fusarium Laboratory Manual* was consulted (Leslie and Summerell 2006). The isolates were grown on PDA for 7 days to observe colony and spore morphology (Olympus BX53 fitted with Jenoptik camera).

Five fungi were isolated from sporangiophores of *P. viticola*, four from infected leaves (M1, M2, M10, and M12_1) and one from infected grape berry (M12_2). The colony and spore morphology of these isolates is shown in Fig. 1. Spore morphology identified all isolates as belonging to the genus *Fusarium*.

Phylogenetic analysis was based on the sequence of the internal transcribed spacer region (ITS) and translation elongation factor 1- alpha (*tef-1α*) gene. The DNA was extracted from 5 days old fungal cultures using the Plant DNA-Mini kit (Qiagen) according to the manufacturer's instructions. The

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Fig. 1 Morphological characteristics of the mycoparasitic *Fusarium* isolates **a** colony, **b** conidia and **c** chlamydospores

ITS and *tef-1α*, genes were amplified using ITS1–ITS4, and EF1 728F–EF1 986R, forward-reverse primers respectively (Glass and Donaldson 1995; Carbone and Kohn 1999). Amplicons of each region were purified and sequenced directly in both sense and antisense directions. The sequences were submitted to NCBI GenBank and cultures have been deposited at National Centre for Microbial Resource (NCMR), Pune, India (Table 1). For phylogenetic analysis, the sequences were manually checked for any inconsistencies on Sequence Scanner and thereafter by ChromasPro software (Technelysium Pvt. Ltd., Tewantin, Queensland, Australia). An NCBI BLASTn search for ITS region of type and non-type was carried out for sequence similarity (Zhang et al.

Table 1 The phylogenetic identity of the five *Fusarium* species collected from vineyards in India

<i>Fusarium</i> spp.	Location state-district	MCC accession number	NCBI accession number		Closest similarity		Phylogenetic identity	
			ITS	<i>tef-1α</i>	Type	Non-type	Percent similarity	Percent similarity
M1	Mizoram-Champai	MCC 1343	MF073330	KY962513	<i>Fusarium delphinoides</i> CBS 116510 (EU926231)	<i>Fusarium delphinoides</i> CBS 120718 (T)	99%	<i>Fusarium delphinoides</i>
M2	Tamil Nadu-Theni	MCC 1344	MF073331	KY962514	<i>Fusarium brachygibbosum</i> NRRL 34033 (GQ505450)	<i>Fusarium beomiforme</i> NRRL 13606 (T)	99%	<i>Fusarium brachygibbosum</i>
M10	Maharashtra-Sangli	MCC 1345	MF073332	KY962515	<i>Fusarium proliferatum</i> WS4KK12 (KT581408)	<i>Fusarium circinatum</i> (NR_111885)	99%	<i>Fusarium pseudonygamai</i>
M12_1	Maharashtra-Nashik	MCC 1346	MF073333	KY962516	<i>Fusarium proliferatum</i> WS4KK12 (KT581408)	<i>Fusarium circinatum</i> CBS 405.97 (T)	99%	<i>Fusarium pseudonygamai</i>
M12_2	Maharashtra-Pune	MCC 1347	MF073334	KY962517	<i>Fusarium equiseti</i> HP7 (GQ407102)	<i>Fusarium chlamydosporum</i> var. <i>fusum</i> CBS 635.76 (AY213655)	99%	<i>Fusarium</i> sp. nov.

2000). The sequences of type and authentic cultures of other *Fusarium* species were aligned using CLUSTAL W algorithm (Tamura et al. 2011). For identification, phylogenetic trees were constructed using neighborhood joining method (NJ) in MEGA 7.0. Evolutionary distances were calculated by Kimura 2-parameter method (Kimura 1980) and presented as the units of number of base substitutions per site. Bootstrap confidence intervals were set at 50% (Saitou and Nei 1987). For phylogenetic analysis recent research papers based on the phylogeny of *Fusarium* were used (Moussa et al. 2017; Laurence et al. 2015; Al-Hatmi et al. 2016; Lombard et al. 2015; Herron et al. 2015; O'Donnell et al. 2009; Schroers et al. 2009; O'Donnell et al. 2004, 2008). The internal transcribed spacer (ITS) and *tef-1 α* genes BLASTn results for five *Fusarium* isolates is compiled in Table 1. The BLASTn results showed that isolate M1 belonged to *dimerum* species group, isolates M2, M10, M12_1 belonged to *americanum* species group and isolate M12_2 belonged to *chlamydosporum* species group. The phylogenetic position

The ability of isolated *Fusarium* species to hyperparasitize *P. viticola* was studied by leaf disc assay. Leaf discs of 25 mm diameter were cut from healthy Thompson Seedless grape cultivar and were kept on 0.5% sterile water agar in 9 cm Petri dishes with the abaxial surface up. Each disc were inoculated centrally with a 100 µl *P. viticola* inoculum containing 50,000 sporangia/ ml, incubated at 23 °C in a growth chamber with a 12 h photoperiod (Genet et al. 1997). After 24 h of incubation, the leaf discs were treated for 1 min with sodium hypochlorite (NaOCl) solution (1% w/v) for surface sterilization and washed thoroughly with sterile distilled water, blotted on sterile blotting paper and placed on fresh sterile water agar plates. On the next day conidial suspension (10^6 conidia/ml)

Fig. 3 Mycoparasitism of *Fusarium* species on *P. viticola*. **a** *Fusarium* overgrowth on downy mildew lesion on leaf, **b** *Fusarium* overgrowth on downy mildew lesion on grape berry, **c** stereo-microscopic images of *Fusarium* parasitizing *P. viticola* sporangioophores, **d** compound

microscopic observation of *Fusarium* parasitizing *P. viticola* sporangioophores, **e** SEM observation of showing coiling of *Fusarium* species around *P. viticola* sporangioophores, **f** lysis of *P. viticola* sporangioophore, **g** leaf disc bioassay showed the suppression of downy mildew growth

of each *Fusarium* isolate was applied on the discs by atomizer and further incubated for 5 days. The discs were examined under stereomicroscope (Leica, MZ 125) for hyper parasitism and the images were captured using compound microscope (Leica DMS 2500) DFC 425 digital zoom camera and Leica Application Suites (v. 4.6.0 and v. 3.7.0) (Leica Microsystems Inc., Germany). The sporangia from each lesion were harvested and counted using a hemocytometer and the percent reduction in sporangial production was calculated. Coiling of *Fusarium* species around *P. viticola* was also examined under SEM. The samples were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8 for 2 h, lesion was rinsed four times with buffer and fixed in 1% OsO₄ for 1 h. After fixation samples were rinsed four times with distilled water and dehydrated through a series of 30, 50 and 70% ethanol and followed by absolute ethanol. After dehydration, sample was mounted on specimen stub and supper coated with gold palladium and examined at 750 × under Scanning Electron Microscope (Jeol JSM 6360A).

Stereomicroscopic observations showed that *Fusarium* species formed a mycelial web over the *P. viticola* sporangio-phores (Fig. 3a–c). Compound and SEM observations showed *Fusarium* species coiling around the *P. viticola* sporangio-phores (Fig. 3d, e), and inducing lyses in sporangio-phores (Fig. 3f). All five *Fusarium* isolates inhibited sporangia production of *P. viticola* (Fig. 3g, Table 2).

Bioefficacy of *Fusarium* isolates in downy mildew control was studied in poly house on 1 year old plants of grape cultivar Thompson Seedless. Mancozeb 2 g/l (70% WP, Dhanuka Agritech Ltd., India) was used as fungicide treatment and sterile water was used as control. There were 10 replicate plants per treatment. To prepare *Fusarium* inoculum, they were grown on potato dextrose broth at 25 °C for 15 days,

the conidia were harvested by filtering through double layer of muslin cloth and adjusting the count to 1×10^6 conidia/ml. *Plasmopara viticola* inoculum consisted of 50,000 sporangia /ml. Applications were made using a hand held sprayer. Five *Fusarium* applications were made at 7 day interval, two were pre-inoculation and three were post *P. viticola* inoculation. The temperature was 23 ± 2 °C and relative humidity was $82 \pm 5\%$ during the period of experiment. Disease observations were taken 27, 33 and 39 days after first application of *Fusarium* suspension. The disease observation was recorded on 10 leaves per shoot as ratings on a 0–4 scale (Horsfall and Heuberger 1942). Percent disease index (PDI) was calculated as: {Sum of total ratings / (No. of observations × maximum of scale)} × 100. AUDPC (area under the disease progress curve) was calculated according to the equation of Campbell and Madden (1990). The data was analyzed in CRD with analysis of variance (ANOVA) using SAS (ver. 9.3; SAS Institute Inc., Cary, North Carolina, USA). The percentage data was arcsine-transformed before analysis. Means were compared using Tukey's Studentized Range Test using SAS system.

All the five isolates significantly reduced downy mildew incidence on leaves as compared to the untreated control (Table 2). The sterile water control recorded 414.57 AUDPC, while the AUDPC in the *Fusarium* treatments was significantly lower at 167.00 to 202.25 showing good control of downy mildew. Mancozeb recorded 39.81 AUDPC.

Earlier *F. proliferatum* was reported as a mycoparasite of *P. viticola* (Bakshi et al. 2001; Falk et al. 1996). This paper reports four other *Fusarium* species as putative mycoparasites of *P. viticola* with potential for use in biological control of downy mildew. Further studies on mechanism of action and bio-control of downy mildew in vineyards are required.

Table 2 Effect of *Fusarium* species on *P. viticola* sporulation and downy mildew severity

Treatment name	Leaf Disc assay	Pot assay			AUDPC
	Sporangia produced (× 10 ³)	Percent disease index (Days after application)			
		27	33	39	
Water control	56.13 ^b	3.25 (10.18) ^a	24.00 (29.31) ^a	53.13 (46.80) ^a	414.57 ^a
<i>Fusarium</i> spp.M1	15.56 ^a	2.75 (9.40) ^a	12.63 (20.66) ^b	20.75 (26.90) ^{bc}	182.50 ^b
<i>Fusarium</i> spp.M2	12.75 ^b	2.88 (9.47) ^a	13.13 (21.13) ^b	26.00 (30.57) ^b	200.00 ^b
<i>Fusarium</i> spp.M10	15.06 ^a	2.13 (8.24) ^a	10.5 (18.88) ^b	21.00 (27.20) ^{bc}	169.00 ^b
<i>Fusarium</i> spp. M12_1	13.15 ^a	1.88 (6.83) ^a	11.25 (19.46) ^b	19.00 (25.74) ^c	167.19 ^b
<i>Fusarium</i> spp. M12_2	15.28 ^a	2.25 (8.38) ^a	14.13 (22.02) ^b	24.13 (29.28) ^{bc}	202.25 ^b
Mancozeb	—	0.63 (2.83) ^b	2.875 (9.19) ^b	4.38 (11.86) ^d	39.81 ^c
CD (<i>P</i> = 0.05)	8.03	3.79	3.28	4.17	49.86

*Figures in parenthesis are Arc sin transformed values

Values followed by different letter within column are significantly different at *P* = 0.05 according to Tukey's Studentized Range (HSD) Test

The bold numbers indicate the CD value obtained by statistical analysis of the data

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