Diversity and phylogeny of resveratrol-producing culturable endophytic fungi from *Vitis* species in India

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Abstract

In the present study, resveratrol producing culturable endophytes were recovered from the Indian vineyards. Of the 145 endophytic fungi recovered, only 30.3% were positive for resveratrol production in the preliminary screening. Culture broth of only 13 isolates exhibited the actual presence of resveratrol in the range of 4.4–52.3 µg/ml by HPLC quantification. The most potent endophytic isolate producing the highest extracellular resveratrol was #19VVLPM isolated from the Pinot Noir variety. Further LC–MS analysis confirmed the similarity of fungal resveratrol with standard resveratrol. The Merlot variety exhibited the highest colonization frequency of the resveratrol-producing fungal endophytes. Using morphological methods as well as molecular phylogeny #19VVLPM was identified as *Fusarium equiseti*. It is a promising candidate for further development into a commercial strain.

Keywords *Vitis vinifera* · Resveratrol · Molecular phylogeny · Fungi · Colonization frequency

Introduction

Endophytic fungi generally colonize within the living tissues of the host plants by latent infection to obtain shelter and nutrition and therefore do not exhibit any obvious signatures of their existence. The signal molecules so produced by them to overcome inherent plant defence mechanisms purportedly play a role in aiding the host plant to combat different abiotic and biotic stresses (Busby et al. 2017; Kaul et al. 2017).

The concept of exploiting these signal molecules for crop protection or development into drug molecules has been the basis of endophytism which emerged two decades back with the discovery of *Taxomyces andreanae*, the taxol producing fungus from *Taxus brevifolia*. The production of the host metabolite by a fungus was attributed to the phenomena

of horizontal gene transfer (Schulz et al. 1993; Strobel and Daisy 2003) which can now be traced using molecular tools.

Resveratrol (RESV) (3,4,5-trihydroxy-*trans*-stilbene) was initially isolated as phytoalexin and later as a phytochemical constituent of grapevines, mulberry and Japanese Knotweed (Langcake and Pryce 1977). The medicinal properties of RESV came into the limelight with the phenomena of the “French Paradox”, which suggested that the consumption of red wine by French people prevented them from coronary heart disease despite their food habits. Alternative sources of production of resveratrol other than phytochemical extraction from *Polygonum cuspidatum* is extensively being explored to meet the global demand of this molecule.

One possible mechanisms of enhancing the production of RESV is through exploration of culturable endophytic fungi capable of producing extracellular resveratrol. The biosynthetic capabilities of the RESV-producing endophytes can be further confirmed by screening the genes of phenylpropanoid pathway, which is generally used for the production of RESV in the plants. One such gene is STS gene which converts the p-coumaroyl Co A into 4-coumaroyl CoA molecule, which along with 3 malonyl CoA molecules forms RESV (Schijlen et al. 2006; Beekwilder et al. 2006; Schwekendiek et al. 2007).

The majority of the studies from India primarily focus on the characterization and epidemiology of pathogenic

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fungi associated with *Vitis vinifera* or explains the method of crop protection. However, the present study is the very first of its kind in exploring the culturable endophytes of different varieties of *V. vinifera* growing in vineyards for their potential to produce resveratrol by the fermentation route.

Materials and methods

Plant sample collection and isolation of endophytes

The plant samples were collected from vineyards in Pune, Nashik, Bangalore and Lucknow during the month of July–August 2016/2017. Briefly, the plant samples comprised leaves and stem pieces from different varieties of *V. vinifera* viz. Merlot, Shiraz, Pinot Noir, Muscat and Wild. Three samples each of leaves and stems were collected for a variety from different selected locations, which were subsequently stored in sterile zip pouches at 4 °C till further use.

For isolation of the fungal endophytes of the plant samples, a standard procedure was adopted which comprised surface sterilization of the sample by 0.1% solution of sodium hypochlorite for 1 min followed by 70% ethanol for 45 s, then by dipping in 30% ethanol for 30 s and finally drying them aseptically. Leaf segments of 3 × 3 mm and 2 mm transverse sections of stems were subsequently prepared under aseptic conditions and placed on pre-made water agar plates (Schulz et al. 1993). The plates were then incubated at 26 ± 1 °C for 10 days with 12 h photoperiod in a cycle. Individual colonies after 10 days incubation at 26 ± 1 °C were picked from the edge of the advancing colony with a fine needle under the stereo-zoom microscope (Nikon) and transferred on to potato dextrose agar (PDA). Pure cultures of the endophytic fungi so obtained were preserved on PDA supplemented with 10% glycerol.

Culture filtrate production

Culture filtrate production was done by inoculating pre-sterilized PDB with a 5 mm mycelial plug of 1-week-old culture of the endophytic fungus and incubated at 26 ± 1 °C, 130 rpm for 10 days. After the incubation period was over, the fungal biomass was separated from the broth via filtration, followed by centrifugation at 10,000 rpm (Hitachi RXII series, Japan) for 10 min at room temperature. The supernatant so collected was passed through 0.22 µm nitrocellulose membrane (GE Health care and life Sciences, Merck, Millipore, USA) to make it cell free.

Phytochemical screening for the presence of polyphenols (resveratrol)

Three phytochemical tests, viz., chromogenic reaction, acetic anhydride test and Liebermann test were carried out to detect the presence of polyphenols (RESV).

Chromogenic reaction

In this assay, the fungal extract was subjected to ferric chloride–potassium ferricyanide colour reaction. Briefly the chromogenic agent was prepared by using 0.1% FeCl₃:0.1% K₃ [Fe (CN)₆] in the ratio of 1:1 (v/v). The reaction was started by the addition of 2 ml of sample, followed by addition of 2 ml of MeOH to which subsequently three to four drops of chromogenic agent were added. The presences of polyphenols (resveratrol) gives a characteristic blue colour. In this assay, RESV served as positive control, while uninoculated PDB was used as the negative control (Liu et al. 2016).

Acetic anhydride test

This test was used to confirm the presence of the hydroxyl group of the phenolic compound present in the culture filtrate. The reaction was initiated by adding 100 µl of acetic anhydride to 100 µl of culture filtrate, followed by the addition of 500 µl of concentrated sulphuric acid. The formation of purple or pink precipitate confirmed the presence of the –OH group of the phenolic compound in the culture filtrate in the test sample (Al-Jumaily et al. 2014).

Liebermann test

In this test, the culture filtrate was mixed with an equal amount of sulphuric acid, followed by the addition of two to three drops of 1% sodium nitrate. The appearance of a blue-violet colour in the reaction mixture confirmed the presence of phenolic compound in the culture filtrate (Al-Jumaily et al. 2014). In this test, uninoculated PDB was used as a negative control, while RESV (1 mg/ml; Sigma-Aldrich, USA) served as the positive control. The blue-violet colour of the reaction mixture indicates the presence of a free para-position in phenolic compounds.

Liquid–liquid extraction

On the basis of phytochemical screening, ethyl acetate (Merck, GR, USA) was used for recovering resveratrol from the lyophilized cell-free culture filtrates of endophytic fungi in a ratio of 3:1. The organic layers so obtained were pooled

and dehydrated over anhydrous sodium sulphate (HiMedia, Mumbai, India) and subsequently concentrated in vacuo to obtain the crude fraction. The fraction so obtained was reconstituted in methanol and was re-assayed for the presence of RESV using previously described phytochemical tests (Shi et al. 2012). Crude fractions which exhibited positive results in the preliminary test were subjected to HPLC analysis for further confirmation as well as quantification.

Quantification and confirmation of RESV

The identification and concentration of resveratrol in crude bioactive fractions was determined via HPLC (PerkinElmer-200, USA) using C18 reverse phase discovery column (Sigma-Aldrich, USA) equipped with series-200 pump, dual wavelength detector and TotalChrom Workstation software. 20 µl of the sample of pre-defined concentration was injected into the HPLC column and eluted using orthophosphoric acid (0.1%, pH 2.3 Sigma Aldrich) and acetonitrile (Sigma Aldrich), in a ratio of 45–55% as mobile phase with a flow rate of 1 ml/min. The yield of fungal RESV was determined against a standard curve of standard RESV (0.1–1 mg/ml) under similar conditions. The peak area vs. concentration of the standard as well as number of peaks and peak height was used to estimate the concentration of polyphenols (RESV) (Shi et al. 2012). The residues of fungal resveratrol from #19VVLPM, #22(P) VVLPM and standard resveratrol were analysed by LC–ESI-MS/MS (Waters, Micromass Q–TOF micro using Waters Alliance 2795 separation module) to further confirm the presence of resveratrol. The confirmation was done on the basis of molecular ion and fragmentation pattern comparison between standard and fungal resveratrol (Dincheva et al. 2011; Ramirez-Lopez and DeWitt 2014).

Taxonomic identification of potent RESV-producing endophytic isolate

Preliminary identification of endophytic fungus was done using classical morphotaxonomic tools.

Morphotaxonomic studies

For morphotaxonomic studies, the endophytic fungi were grown over different media, i.e. water agar (WA), Czapek Dox Agar (CDA), grape leaf agar (GLA), potato dextrose agar (PDA), corn meal agar (CMA), Fusarium minimal media (FMA) and synthetic nutrient deficient agar (SNA) for 15 days at 26 ± 2 °C and 98% relative humidity (with 12 h of photoperiod). Colony growth rate, appearance, along with its microscopic features like hyphae characteristics, conidia formation and other cellular bodies were critically observed under Nikon stereo zoom microscope (Nikon, Japan). All the

micrometric observations were carried out using Nikon NIS element software with at least 30 observations per structure (Ellis Martin and Ellis Pamela 1997; Barnett and Hunter 1998; Nagamani et al. 2006).

ITS-rDNA-based molecular taxonomy of selected resveratrol-producing endophytic fungus

For genomic DNA extraction, the mycelia of 3–4 days old culture (approx. 0.1–0.2 g) was scrapped off with sterile inoculation loop and crushed to very fine powder in a sterile mortar and pestle under liquid nitrogen. Further DNA extraction was done by using the Wizard Genomic DNA purification kit (Promega, Madison, WI) as per manufacturer's instructions. The internal transcribed spacer (ITS) region 1, 5.8S and 2 was amplified using ITS 1 and ITS 4 primers. The reaction mixture (25 µl) comprised 1.5U of Taq DNA polymerase in 10× Taq buffer containing 25 mM MgCl₂ (Bangalore GeNei, Bengaluru, India), 2.5 mM of dNTP, 1 µl of extracted genomic DNA and 10 mM of each primer. The conditions for the polymerase chain reaction were 96 °C for 5 min, followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s followed by final extension at 72 °C for 10 min (White et al. 1990). The ITS amplicons so obtained were examined using a 1.5% agarose gel under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software (Bio-Rad, Hercules, CA). The PCR products were purified with Wizard SV gel and PCR cleanup system (Promega, Madison, WI) as per manufacturer's instructions. The obtained PCR products were sequenced with Eurofins (Bangalore, India).

Colonization frequency of RESV-producing endophytes

Further, their diversity was calculated by finding the colonization frequency. The percentage colonization frequency (% CF) and isolation rate (IR) were calculated by the method adopted by Hata and Futai (1995) and Gond et al. (2012). Samples were incubated and growth was examined daily during 6 weeks and colonization frequency was calculated by the following formula:

$$\text{Colonization frequency (\%CF)} = \frac{\text{no. of individual fungi recorded}}{\text{total number of segments screened}} \times 100$$

Statistical analysis

All the assays were performed in triplicate and the data were represented in terms of mean $\pm$ standard deviation. The results were analysed by ANOVA followed by Tukey's

post hoc test ($p < 0.05$). Statistical analysis was performed using GraphPad Prism 7 software and $p < 0.05$ was considered significant.

Results

Isolation of endophytic fungi

A total of 145 isolates were isolated from 525 segments of leaf and stem from all the plant samples collected. Among 145 isolates, 44 isolates were recovered from Merlot variety of *V. vinifera*, 27 from wild variety, 22 from Pinot Noir, 33 from Shiraz and 19 from Muscat varieties of *V. vinifera* (Table 1).

Preliminary screening of resveratrol-producing endophytic fungi

Based on the three phytochemical tests for the detection of polyphenolic compounds, viz., Liebermann, acetic anhydride and chromogenic reaction carried out on ethyl acetate extracts of all endophytic fungi, only 44 endophytic fungal isolates exhibited the presence of polyphenolics presumably resveratrol. However,

it was expected that high RESV content would be present in isolates, viz., #20(b) VVLPM, #101VVBLK, #53VVSTBK and #18VVLPM, exhibiting the highest presence in one of the three phytochemical tests. However, the moderate presence of polyphenols (resveratrol) was exhibited by isolates #6 VVBLK, #19VVLPM and #193 VVSTPM. For further details about the preliminary screening for the presence of RESV, kindly refer to Supplementary File 1.

Quantification and confirmation of RESV

The fungal RESV gave a peak at Rt 3.36 min on reverse phase C-18 column which was similar to that of standard resveratrol by HPLC. The peak area versus concentration obtained by using standard RESV was used to quantify the fungal RESV. Out of 44 isolates, only 13 endophytic fungi produced RESV in the range of 4.4–52.3 µg/ml in liquid culture which was confirmed by HPLC analysis. Out of 13 isolates, we chose the top potent RESV producer, i.e. #19VVLPM which exhibited a concentration of 52.3 µg/ml (Table 2). Based on one-way ANOVA and Tukey's post hoc analysis ($p < 0.05$), #109VVLNM and #53VVSTBK did not give any significant difference in their mean RESV

Table 1 Colonization frequency of the endophytic fungi in different varieties of *V. vinifera*

<i>Vitis vinifera</i> variety	No. of segments of plant sample used for isolation	No. of endophytic fungal isolates recovered	Colonization frequency (%)
Merlot	115	44	38.3
Wild type	125	27	21.6
Pinot Noir	95	22	23.2
Shiraz	95	33	34.74
Muscat	95	19	20.0

Table 2 Quantification of RESV content in culture filtrate of endophytic fungi isolated from different varieties of *V. vinifera* by HPLC

S. no.	Culture code	<i>V. vinifera</i> variety	Tentative identification*	RESV production (µg/ml)*
01	#20(b) VVLPM	Pinot noir	<i>Botryosphaeria</i> sp.	15.3 ± 0.10
02	#101VVBLK	Merlot	<i>Aspergillus</i> sp.	22.4 ± 0.16
03	#53VVSTBK	Wild	<i>Aspergillus</i> sp.	23.9 ± 0.10
04	#18VVLPM	Pinot noir	<i>Botryosphaeria</i> sp.	37.3 ± 0.15
05	#6(b)VVLLK	Merlot	Unidentified	11.9 ± 0.15
06	#19VVLPM	Pinot noir	<i>Fusarium</i> sp.	52.3 ± 0.10
07	#193VVSTPM	Merlot	<i>Nigrospora</i> sp.	25.2 ± 0.10
08	#8VVGSTL	Wild	Unidentified	13.2 ± 0.06
09	#98VVSTPM	Merlot	<i>Penicillium</i> sp.	21.9 ± 0.06
10	#22(P)VVLPM	Shiraz	<i>Xylaria</i> sp.	35.4 ± 0.10
11	#4(p)VVLNM	Shiraz	<i>Fusarium</i> sp.	31.3 ± 0.11
12	#83VVLNM	Merlot	<i>Aspergillus</i> sp.	4.4 ± 0.20
13	#109VVLNM	Merlot	<i>Alternaria</i> sp.	24.1 ± 0.10

*Tentative identification is based on the morphotaxonomy of the fungus

Fig. 1 Fragmentation pattern by LC–MS analysis of **a** standard resveratrol, **b** fungal resveratrol from bioactive extract of #19VVLPM

Fig. 2 Maximum likelihood tree using Tamura and Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) #19VVLPM—*F. equiseti*

concentration among the different RESV-producing endophytic isolates (Table 2).

For further confirmation of the presence of fungal resveratrol in the bioactive ethyl acetate residue obtained from the spent broth of #19VVLPM, LC–MS/MS was carried out which revealed the presence of resveratrol apart from other constituents present such as piceatannol, stilbene, quercetin, etc., which had different retention times. The retention time of resveratrol was 1.86. Further MS analysis revealed that the fungal resveratrol from #19VVLPM and standard resveratrol exhibited the same base peak at 228.1 m/z (Fig. 1).

Taxonomic identification of potent RESV-producing endophytic isolate

The morphological as well as molecular taxonomic studies were under taken to identify the fungal isolates till species level. The morphological studies are presented in Supplementary file no. 2. The ITS sequence of #19VVLPM was submitted to GenBank with accession no. MH181157.

Subsequently, homologue ITS sequences of #19VVLPM were identified using BLAST and were used for assessing the phylogenetic association, which was analysed by preparing the maximum likelihood tree using Tamura and Nei model (Tamura et al. 2011). In this tree, #19VVLPM clustered with *Fusarium equisetum* isolate TMC as well as *F. equisetum* (Fig. 2). Based on the phylogenetic clustering of #19VVLPM and morphotaxonomic data, it was assigned the name of *Fusarium equiseti*. #19VVLPM exhibited the presence of STS gene which is responsible for the production of RESV.

Colonization frequency of RESV-producing endophytes

A total of 144 isolates belonging to 18 fungal genera were isolated from 525 segments of leaf and stem collectively. Among all the endophyte isolates recovered from *V. vinifera* in the present study, 58.2% were Hyphomycetes, 19.3% Ascomycetes, 21% Coelomycetes and 1.7% Basidiomycetes (Fig. 3). Maximum endophytic cultures were isolated from samples collected from Merlot variety, i.e. 38.3%, while the minimum were recovered from Muscat variety of *V. vinifera* (20%). The dominant fungi observed were *Aspergillus* sp., *Fusarium* sp., *Alternaria* sp., however *Botryosphaeria* sp. and *Xylaria* sp. and were reported for the first time from *V. vinifera* as endophytes.

Discussion

Endophytic fungi since the last two decades have attained remarkable recognition in the field of drug discovery and development as a sustainable source of unique and diverse bioactive compounds which can be directly or indirectly be used as drug candidates for combating major global diseases (Strobel and Daisy 2003; Zhang et al. 2006). Endophytic

Fig. 3 The relative recovery of different endophytic groups from the different locations in India

fungi generally co-exist within the plant tissues and play a crucial role in promoting host growth and adaptation to different biotic and abiotic stresses. It has been well documented that endophytic fungi co-evolve with the host plant, thereby attaining the host biosynthetic capabilities and producing phytomedicinals which are commercially used such as paclitaxel (Stierle et al. 1993), vinblastine (Zhao et al. 2011) and camptothecin (Puri et al. 2005; Kusari et al. 2009). Fungal endophytes produce a variety of compounds which belong to different classes such as alkaloids, polyphenols, terpenoids, quinones and steroids.

Exploration of endophytic fungi capable of producing resveratrol is a relatively new aspect, after its therapeutic properties came into limelight. Resveratrol has been associated with various health benefits, since it has different properties such as anti-inflammatory and anti-oxidant activity, is an anti-diabetic, can prevent cardiovascular diseases and cancer and is an anti-ageing moiety (Saxena and Srivastava 2014, Wang et al. 2013). There exist very limited data on exploration of endophytic fungi for the production of RESV. Powell et al. (1994) for the first time isolated RESV from endophytic *Acremonium* sp., and further endophytic fungi including *Alternaria* sp., *Aspergillus niger*, *Penicillium* sp. and *Botryosphaeria* sp. are amongst the recently reported producers of RESV (Wang et al. 2013; Shi et al. 2012; Liu et al. 2016).

Thus, this study was oriented towards exploring endophytic fungi associated with different *V. vinifera* varieties

in India, understanding diversity in terms of colonization frequency and finally screening them for the production of resveratrol. In this study, we reported 18 fungal genera from 525 segments, whereas González and Tello (2011) reported 51 genera from six locations in Spain, while Brum et al. (2012) reported the isolation of 550 fungal endophytes from *Vitis labrusca*. There was difference in the composition of fungal endophytes from *V. vinifera* samples collected from different geographic locations. It varies according to host species and characteristics of site, viz., vegetation, elevation, tissue type, exposure (Bills and Polishook 1994; Petrini and Fisher 1986) and age of tissue (Fisher et al. 1986; Roll-Hansen and Roll-Hansen 1979). In the preliminary screening for the presence of polyphenols, only 29.7% cultures gave a positive test. HPLC-based quantification was further helpful in identifying the resveratrol-producing fungi. Only 9.7% isolates were found to produce resveratrol which was less than that observed by Liu et al. (2016) and Shi et al. (2012) with 19.4% and 32.3%, respectively. HPLC-based quantification has been extensively used in determining the resveratrol concentration in organic extracts in other studies also (Casieri et al. 2009; Kim et al. 2008).

By using LC-MS/MS, the endophytic isolate exhibiting the highest extracellular RESV was found in isolate #19VVLMP. LC-MS/MS has been a preferred tool for quantification and confirmation of resveratrol in complex matrices such as grape juice, cranberry juice, red wine, grape concentrates and by-products of grapes (Wang et al.

2002; Careri et al. 2004). Further morphotaxonomic studies revealed that the isolate #19VVLPM and #22(P) VVLPM belonged to the genus *Fusarium*.

It has been proven that among the regions of ribosomal cistron, internal transcribed spacers (ITS) exhibit the highest probability of successful identification of the broadest range of fungi, successfully distinguishing between intraspecific and interspecific variation and therefore are used as DNA barcode marker for fungi (Schoch et al. 2012). Based on the molecular phylogeny, #19VVLPM was identified as *F. equiseti*. The RESV productions of this isolate were stable over several passages. The activity of these isolates did not decrease as reported in *Alternaria* sp. MG2 and *Botryosphaeria* sp. YG3. However, RESV production decreased severely on passaging in four cultures, viz., #83VVLNM, #6(b) VVLLK, # 8VVGSTL and # 20(b) VVLPM (Liu et al. 2016; Shi et al. 2012). This probably is attributed to the missing *STS* gene in these isolates as compared to #19VVLMP.

The endophytic mycobiota associated with *V. vinifera* in this study is dominated by hyphomycetes, which constitute about 58.8% of the total endophytes isolated. However, the dominance of Ascomycetes was reported in the study conducted on several cultivars of *V. vinifera* from the Madrid region (González and Tello 2011). The low proportion of basidiomycetes taxa found in our study is consistent with other studies (González and Tello 2011; Márquez et al. 2010). The most frequently occurring isolates belonged to the genera *Aspergillus*, *Fusarium*, *Alternaria* and *Botryosphaeria*. In previous research studies, most of these fungi have been identified in grapevines. Pancher et al. (2012) and Mostert et al. (2000) reported *Alternaria* as a dominant genus and also reported the presence of *Phoma* sp. González and Tello (2011) and Musetti et al. (2006) found that genera *Alternaria*, *Fusarium* and *Phoma* are the most frequently occurring isolates of endophytic fungi of *V. vinifera*. In another study, *Alternaria* sp. and *Fusarium* sp. were reported as dominant along with the less frequently occurring *Phoma* sp., *Diaporthe* sp. and *Phomopsis* sp. in the grapevine cultivars (Casieri et al. 2009).

Further, our focus was the exploration of potent isolate producing resveratrol under extracellular fermenting condition for possible large-scale production. In the present study, one incidental isolate #19VVLPM, *F. equiseti*, was found to exhibit the production of resveratrol under free fermenting conditions.

Conclusion

This is the first study that concurrently examined the resveratrol production of the fungal endophytes isolated from different varieties of *V. vinifera* from India. These results

indicate that *V. vinifera* harbours rich endophytic fungal community. *F. equiseti* is a promising candidate for further strain improvement to achieve resveratrol production through the fermentation route.

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Author contribution VD collected all the samples from different geographical locations between July and August 2016/2017 and then isolations were carried out under the guidance of SS. SS conceived and designed the present study, while VD performed all experiments and analysed the data. The publication was jointly written by SS and VD.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript and in the decision to publish the results.

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