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PRELIMINARY SCREENING OF RESVERATROL PRODUCING ENDOPHYTES ISOLATED FROM *Vitis Vinefera* BY TECHNIQUE OF TISSUE CULTURE

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ABSTRACT

Endophytes are the important microorganisms that grow intra-and/or intercellullarly in the tissues of higher plants without causing any harm to host .They are the reservoir of bioactive substance having therapeutic potential. Resveratrol is clinically proved bioactive compound having promising health potential against various non- communicable diseases and disorders like cardiovascular diseases, cancer, neurodegenerative disease and diabetes etc. Vitis vinefera i.e. Grapevine plant is an important natural source of resveratrol. As the entophytes reside the host plant and mimics all the properties of its host, endophytes isolated from grapevine plant could be potent source of resveratrol. The objective of this study was to isolate and screen resveratrol producing endophytes from Vitis vinefera. Isolation of endophytes of V.vinefera was carried out by Plant Tissue Culture technique. Different plant parts like axillary bud, apical bud and leaf lamina segments were used. Almost seven types of fungal endophytes viz. Botrytis sp., Penicillium sp., Trichoderma sp., Fusarium sp., Aspergillus sp., Cladosporium sp. and Alternaria sp. and two bacterial endophytes like Bacillus sp. and Serratia sp were successfully isolated. It was revealed that among the different plant parts, axillary buds showed high colonization frequency for fungal as well as bacterial endophytes. The colonization frequency of Aspergillus sp. was found to be highest (11%) followed by Alternaria sp. which was (8%) whereas, Bacillus sp.and Serratia sp. showed the value of 1.6 % and 2.3 % respectively. Further, the isolated endophytes were preliminary screened to check their resveratrol production potential by ferric chloride–potassium ferricyanide chromogenic reaction .It was found that the fungal endophytes like Fusarium sp., Cladosporium sp and both the bacterial endophytes viz. Bacillus sp. Serratia sp showed negative test. The endophytes were further subjected to UV spectra scan in range of 200–400 nm. Some of the endophytes showing positive test showed maximum absorbance in range of 304nm to 308 nm. Rescreening of resveratrol -producing endophytes was carried out by Thin layer chromatography using two solvent systems viz.Toluene: Ethyl acetate: Acetic acid = 15:3:1, v/v) and Toluene: Ethyl acetate: Methanol = 25:8:1, v/v).The Rf values were found to be 0.32 and 0.31. Although, number of research has been carried out for isolation of endophytes from plants, this is the first report of isolation of endophytes by using technique of Plant Tissue Culture.

Therefore, based on the outcomes of this research, it can be concluded that Plant tissue culture technique can be efficiently used for isolation of endophytes of V.vinefera. However, additional research on resveratrol production ability of the endophytes will be needed so as to explore their commercial applications in the field of medicine, agriculture, food and cosmetic industries.

Keywords: Bioactive, Cancer, Endophytes, Resveratrol, Therapeutic, Plant Tissue Culture, Vitis vinefera

I. INTRODUCTION

Endophytes are the important components of plant micro ecological system which are systematically distributed in plant tissue, organs or cell gap of the roots, stems, leaves, seeds and fruits.[1-2].They not only participate in synthesis or transformation of the plant secondary metabolites, but act as reservoirs of novel bioactive secondary metabolites having great potential applications in the field of medicine, agriculture , food and cosmetic industries [3-7].It is reported that the endophytic fungi are symbiotically associated with the plants and can synthesize the same bioactive compounds and natural products as their host plant themselves, suggesting the possibility of intergeneric genetic exchange between the plant and the fungus; meanwhile causing no damage to the host [8].Various studies have shown that endophytic bacteria can be detected inside the endorhiza, in stems, leaves as well as inside plant reproductive organs of different host plants. [9-11].Recent advances have been made in regard to metabolite production by these micro symbionts showing their potential to produce wide range of metabolites. Resveratrol is a secondary metabolite belonging to stilbene family with great antioxidant potential. Several scientific research and clinical trials explains the high appeal of resveratrol as, it can be used against various non- communicable diseases and disorders like cardiovascular diseases, cancer, neurodegenerative disease and diabetes etc. Although there are various natural sources of resveratrol,*Vitis vinefera* i.e. grapevine plant is one of the ancient and most widely cultivated fruit crop in the world which is considered as important dietary source of resveratrol [12]. As the endophytes resides the host plant and mimics all the properties of its host, endophytes isolated from grapevine plant could be potent source of resveratrol. Therefore, in order to identify the potential isolates of endophytes capable of producing polyphenolic compound like resveratrol, the present study has been focused on isolation and preliminary screening of resveratrol producing endophytes from *Vitis vinefera* by Plant Tissue Culture technique.

II.MATERIALS AND METHODS

2.1. Collection of Plant Material

Young and healthy shoots(10-15 cm) of Black cultivars of *V.vinifera* were randomly collected from vineyards of Nashik valley, Maharashtra India during year 2017.Plant material was brought to Plant Tissue Culture Laboratory prior to few hours of experiment. Generally, younger, more rapidly growing tissues and tissues in early developmental stage are the most effective so, isolation of endophytes was carried out by using apical bud, axillary bud, and leaf lamina.

2.2. Surface Sterilization

The plant parts were rinsed with distilled water followed by soaking in solution of Bavistin (50% carbendazim) for about 20 minute and rinsing with sterile distilled water for 3-4 times. Aseptically, surface sterilization of explant was carried out by treatment in the solution of commercial bleach and few drops of Tween-20 for about 20 minutes with vigorous shaking and 3 times washing with sterile D/W. Further, it was washed with 70 % ethanol for 30 sec. followed by 3-4 times washing with sterile D/W. Finally, it was treated with 0.75 % $HgCl_2$ solution (10-15 min) followed by 3-4 times washing with sterile D/W.

2.3. Media Preparation

The MS basal medium [13] supplemented with various vitamins like Glycine, Pyradoxin, Nicotinic acid, Thiamine procured from Hi Media, Mumbai and 3% Sucrose (w/v) was used for preparation of culture media. Different growth regulators viz.6-Benzylaminopurine (Sigma Aldrich), (2,4-Dichlorophenoxy acetic acid (Sigma Aldrich),Naphthalene acetic acid (Sigma Aldrich)were added at different concentrations and pH of media was adjusted to 5.8. Prior to autoclaving, media was solidified with 0.8 % agar.

2.4. Inoculation and Maintenance of Plant Material

Sterilized plant material (1-1.5 cm) were aseptically inoculated on solid media and the cultures were maintained at 25±2ºC under 16/8 h Light/dark photoperiod cycle in the Plant Tissue Culture room. Observation of microbial colony was recorded every day.

2.5. Isolation, Purification and Morphological Characterization of Endophytes

Fungal colonies which were observed during 15 to 45 days of plant material inoculation were further transferred to Potato Dextrose Agar (PDA) and Nutrient agar (NA) media and incubated at room temperature for isolation and purification of endophytic fungi as well as bacteria respectively. Pure colonies were re-cultured on PDA and NA. All the isolated and purified endophytes were further subjected to Morphological and Cultural characterization.

2.6. Preliminary Screening of Resveratrol Producing Endophytes

Purified endophytes were further inoculated in Potato Dextrose Broth (PDB) and Nutrient Broth (NB) and allowed to incubate on orbital shaker (110 rpm), at room temperature for seven days.Then the fermentation liquid was centrifuged at 3500 rpm for 15min.The supernant was used as test sample for preliminary detection of resveratrol producing endophytes on basis of chromogenic reaction .Chromogenic agent was prepared by mixing, 0.1 % $FeCl_3$: 0.1 % K_3 [Fe (CN) $_6$] = 1:1 (v/v). 2 mL test sample was added in 2ml methanol and 2-3 drops of chromogenic agent, The blue colour with greenish tinge indicates presense of polyphenolic compound like resveratrol [14].

2.7. Ultraviolet Wavelength Scanning

Stock solution of 500 ppm was prepared by dissolving 5 mg standard resveratrol in methanol (80%) and final volume was make up to 10 ml. Then, 250 µL solution was taken from this stock solution and again volume was make up to 10ml to prepare solution of 25ppm which was further scanned by double beam UV-VIS spectrophotometer (Analytical Technologies Ltd, Model, UV-VIS 2012) in the spectral range 200–400 nm to get maximum absorption wavelength of standard resveratrol.Then, the absorbances of all the fermented liquid samples of endophytes were obtained.

2.8. Rescreening of Resveratrol Producing Endophytes by Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out on precoated silica gel plates purchased from MERK (Germany). Test samples of endophytes were loaded and air dried. Different solvent systems were tried to obtain best resolution of loaded samples. Finally, Toluene: Ethyl acetate: Acetic acid = 15:3:1, v/v) and Toluene: Ethyl acetate: Methanol = 25:8:1, v/v) were selected for detection of resveratrol. The plates were developed at room temperature in pre saturated chamber and spots were observed under UV at 254 nm. The plates were further developed by spraying the mixture of chromogenic agent prepared by mixing, 0.1 % $FeCl_3$: 0.1 % K_3 [Fe (CN) $_6$] = 1:1 (v/v). Rf value for resveratrol in the selected solvent system was calculated by using stock of pure resveratrol (25ppm) as standard [14].

III. RESULTS AND DISCUSSION

3.1. Isolation and Purification of Endophytes

Endophytes resides entirely within the plant tissues and may grow within roots, stems and/or leaves, emerging to sporulate at plant or host-tissue senescence [15-17].Therefore, altogether 500 number of different plant parts viz.axillary buds (300) apical buds (100), and leaf lamina segments (100) of Black cultivars of *V.vinifera* were processed for the isolation of endophytes by plant tissue culture technique. Only the microbial colonies observed during period of 20^{th} to 60days after initiation and sub culturing were considered for further study of endophytes .It was found that various fungal endophytes were emerged from the tissues of *V.vinefera*. It was found that no massive outgrowth of bacteria could be observed. The only indication for bacteria in the cultures was a very slight smear around the basal part of the plant part. The smear did not spread further in the medium and did not continue growing on the surface of the culture medium.

3.2. Morphological and Cultural Characterization of Endophytes

All the isolated and purified endophytes were subjected to the cultural and morphological characterization. Cultural characters such as color and nature of the growth of the colony were determined by visual observation. Morphological characteristics of the fungus like mycelia, conidiophores and conidia were microscopically studied. Among the purified isolates, almost seven types of endophytic fungi viz. *Botrytis sp., Penicillium sp., Trichoderma sp., Fusarium sp., Aspergillus sp., Cladosporium sp., and Alternaria sp.* were identified. Two bacterial endophytes like *Bacillus sp. and Serratia sp*. were identified by studying the morphology of bacterial colony, staining characteristic and biochemical tests.(Fig.1and Fig.2)

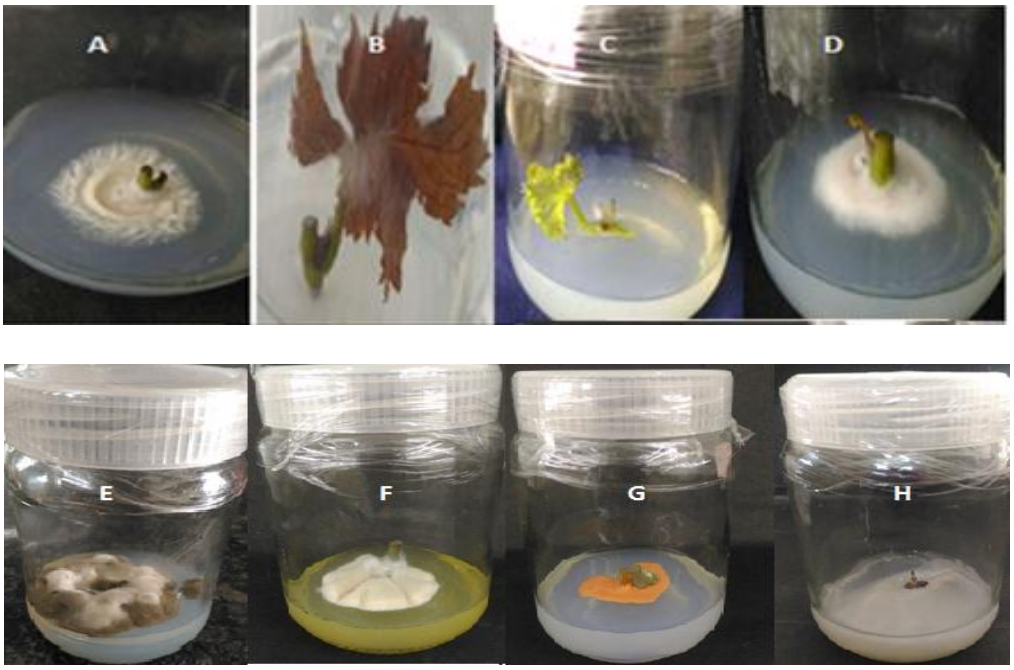


Fig.1. Diversity of endophytes isolated by Tissue Culture of *V.vinefera*

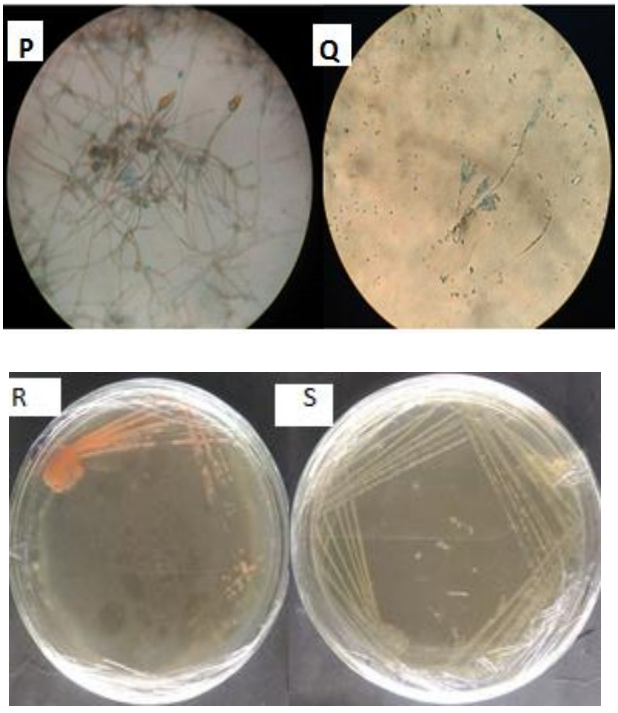


Fig.2 Microscopic Images of Purified Fungal (P and Q) and Bacterial Endophytes(R and S)

3.3. Colonization Frequency (CF %) of Endophytes

The colonization frequency (CF %) of each endophyte was calculated and determined by using the formula,

CF % = Number of segments colonized by an endophyte /Total number of segments x100 [18]. The colonization frequency of *Aspergillus sp.* was found to be highest (11%) followed by *Alternaria sp.* which was (8%) whereas, *Bacillus sp.*and *Serratia sp.*Showed the value of 1.6 % and 2.3 % respectively. It was revealed that among the different plant parts, axillary buds showed high colonization frequency for fungal as well as bacterial endophytes in comparison to apical buds and leaf lamina segments. (Fig.3)

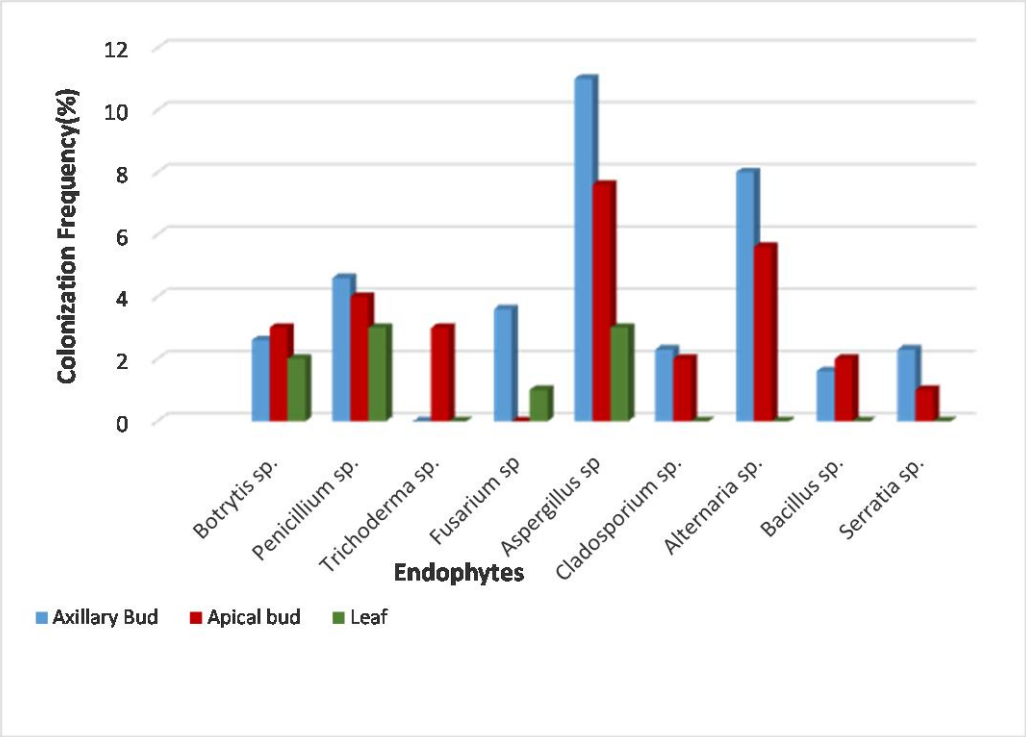
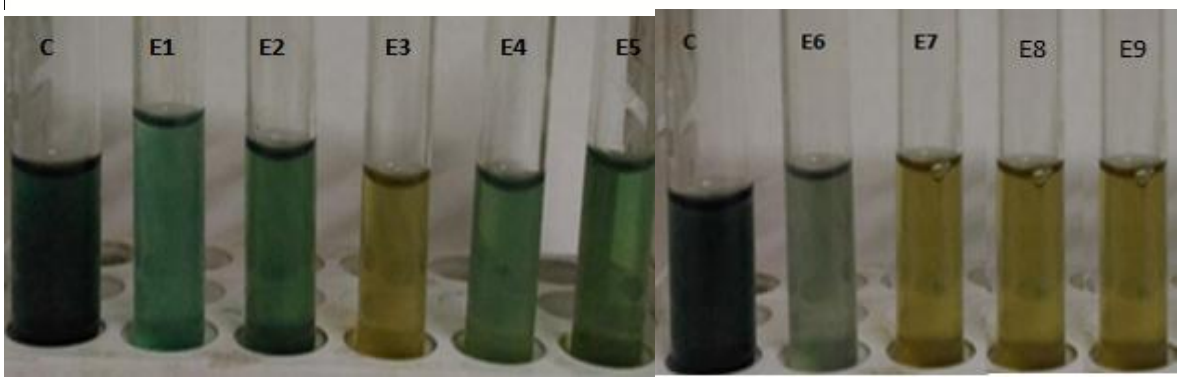


Fig.3. Colonization Frequency of Endophytes of *V.vinefera*

3.4. Preliminary Screening for Resveratrol Producing Endophytes

Preliminary screening of the test samples (Fermented liquid of endophytes) by using chromogenic reaction showed blue colouration with greenish tinge indicated presence of polyphenolic compound like resveratrol. [14]. It was found that the fungal endophytes like *Fusarium sp.*, *Cladosporium sp* and both the bacterial endophytes *viz.Bacillus sp. Serratia sp* showed no change in colour indicating absence of resveratrol. (Fig.4)



C. Positive Control, E1 - E7-*Samples of Fungal Endophytes,* E8-E9 - *Samples of Bacterial Endophytes*

Fig.4 Chromogenic Test for Polyphenolic Resveratrol Detection

3.5. Ultraviolet Wavelength Scanning

The endophytes were further subjected to UV spectra scan in range of 200–400 nm to get the maximum absorption wavelength. It was found that some of the endophytes showing positive test for presence of resveratrol showed maximum absorbance in range of 304nm to 308 nm .The maximum absorption wavelength of standard resveratrol was found to be 306 nm, indicating resveratrol production by these endophytes.

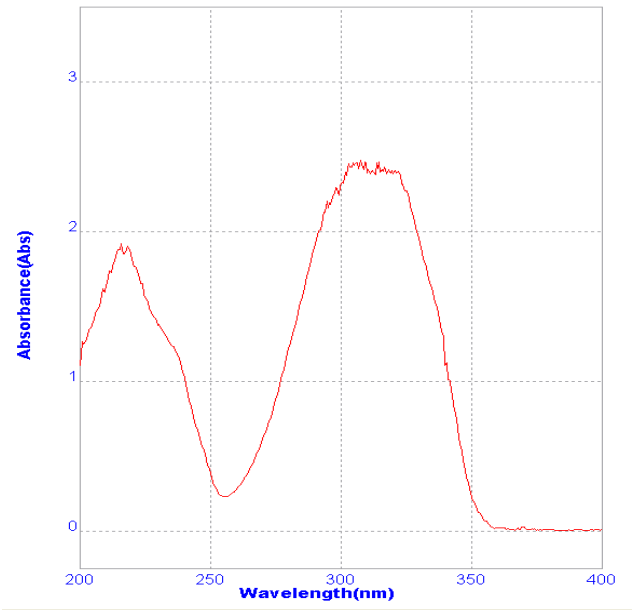


Fig.5.UV Absorption Spectrum of Resveratrol

3.6. Rescreening of Resveratrol Producing Endophytes by Thin layer Chromatography

Various solvent systems were tried to achieve a good resolution. Finally, detection of resveratrol was carried out by using two solvent systems viz.Toluene: Ethyl acetate: Acetic acid = 15:3:1, v/v) and Toluene: Ethyl acetate: Methanol = 25:8:1, v/v) respectively.The Rf values for resveratrol in the selected solvent systems were found to be 0.32 and 0.31 which were similar with Rf value of standard resveratrol.

IV. CONCLUSION

In present study, isolation of endophytes of *V.vinefera* was carried out by Plant Tissue Culture technique. Different plant parts like axillary bud, apical bud and leaf lamina segments were used. Almost seven types of endophytic fungi viz. *Botrytis sp., Penicillium sp., Trichoderma sp., Fusarium sp., Aspergillus sp., Cladosporium sp., and Alternaria sp.,* and two bacterial endophytes like *Bacillus sp., and Serratia sp.,* were successfully isolated. It was revealed that among the different plant parts, axillary buds showed high colonization frequency for fungal as well as bacterial endophytes in comparison to apical buds and leaf lamina segments.The colonization frequency of *Aspergillus sp.* was found to be highest (11%) followed by *Alternaria sp.* which was (8%) whereas, *Bacillus sp*.and *Serratia sp*. showed the value of 1.6 % and 2.3 % respectively. Further, the isolated endophytes were preliminary screened to check their resveratrol production potential. Preliminary screening of endophytes was carried out by using ferric chloride and potassium ferricyanide solution to observe greenish blue colouration indicating presence of polyphenolic resveratrol. It was found that the fungal endophytes like *Fusarium sp., Cladosporium sp.,* and both the bacterial endophytes viz. *Bacillus sp., Serratia sp.* showed no change in colour indicating absence of resveratrol.The endophytes were further subjected to UV spectra scan in range of 200–400 nm. It was found that some of the endophytes showing positive test for presence of resveratrol showed maximum absorbance in range of 304 nm to 308 nm. Rescreening of resveratrol‑producing endophytes was carried out by Thin Layer Chromatography using two solvent systems viz.Toluene: Ethyl acetate: Acetic acid = 15:3:1, v/v) and Toluene: Ethyl acetate: Methanol = 25:8:1, v/v).The Rf values for resveratrol in the selected solvent systems were found to be 0.32 and 0.31 which were similar to Rf value of standard resveratrol. Although, number of research has been carried out for isolation of endophytes from plants, this is the first report of isolation of endophytes by using plant tissue culture. Therefore, based on the outcomes of this research, it can be concluded that Plant Tissue Culture technique can be efficiently used for isolation of endophytes of *V.vinefera.* However, additional research on resveratrol production ability of the endophytes will be needed so as to explore their commercial applications in the field of medicine, agriculture, food and cosmetic industries.

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