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MOLECULAR CHARACTERIZATION OF ENDOPHYTIC FUNGUS *BOTRYOTINIA FUCKELIANA* FROM *SARACA INDICA*

V. Pushpa Rani*[1], J. Delphine Jaya Mary[2], M. Parimalam[3], G. Meena Kumari[4], and K. Deepak Kumar[5]

*[1-5]PG & Research Department of Advanced Zoology and Biotechnology, Loyola College, Nungambakkam, Chennai-600 034. Tamil Nadu, India.

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***Corresponding Author**
V. Pushpa Rani
PG & Research Department of Advanced Zoology and Biotechnology, Loyola College, Nungambakkam, Chennai-600 034. Tamil Nadu, India.

ABSTRACT

The present study was focused on molecular characterization of endophytic fungus from ethnomedically important plant Ashoka tree ***(Saraca indica)*** in view to amplify their DNA region. Endophytic fungus which affects the Ashoka tree leaves ***(Saraca indica***) has been identified from this study by various techniques, which was already used by other fungus identification and molecular characterization method. Genomic DNA was isolated through Qualitative and Quantitative analysis. **18S rRNA** region was amplified through PCR standardization. Based on the differences in RNA genes the fungus has been identified and classified through Ribotyping method. The name of the endophytic fungus was identified as ***Botryotinia fuckeliana*** from the percentage of similar endophytic fungus appeared in histogram. This study will help to discover the causes and preventive mechanism of ***(Botryotinia fuckeliana)*** endophytic fungus in ***Saraca indica***.

KEYWORDS: *Botryotinia fuckeliana, Saraca indica,* DNA, Molecular Characterization, 18S rRNA.

INTRODUCTION

Botryotinia fuckeliana (de Bary) Whetz. (teleomorph of ***Botrytis cinerea*** Pers.) Incites grey mould disease on at least 250 different host plants ***(Jarvis, 1980).*** The pathogen is well known for causing heavy yield losses on many **economically important crops**, including grapevine and horticultural and ornamental crops, especially in temperate areas worldwide ***(Jarvis, 1980).*** The control of grey mould is not simple because fungicide sprays are often

required at times close to harvest and hence there is a risk of leaving high levels of residues on fruits and vegetables. Furthermore, ***B. fuckeliana*** is well known for its broad variability and adaptation; it is recognised as a **"high-risk"** pathogen for its ability to acquire resistance to fungicides, causing low effectiveness of sprays in the field **(Brent, 1995; Brent and Hollomon, 1998).** Although several groups of fungicides with different modes of action are available against the pathogen, the widespread occurrence of acquired resistance has indeed been experienced with most of them. ***Guido. et al., (2007).***

Botryotinia fuckeliana (de Bary) Whetz., teleomorph of ***Botryis cinerea*** Pers., is the causal agent of grey mould. The fungus is a **cosmopolitan, necrotrophic** and **polyphagous pathogen** causing **heavy yield losses** on **numerous crops**, such as grapevine, horticultural and ornamental crops, glasshouse crops, etc., in all worldwide temperate areas. Moreover, it induces one of the most important postharvest diseases on fruit and vegetables. The main fetures of ***B. fuckeliana*** and grey moulds have been reviewed exhaustively on several occasions ***(i.e. Verhoeff et al., 1992; Elad et al., 2004; Williamson et al., 2007). Angelini et al.,(2012).***

Asoka is the most ancient tree of India, generally known as a **"ashok briksh",** botanist known as a ***Saraca asoca*** (Roxb.), De. Wild or ***Saraca indica*** belonging family ***Caesalpinaceae.*** **Asoka or ashoka** is a Sanskrit words which means **"without sorrow"** or which that gives no grief. Ashoka is one of the most **legendary** and **sacred trees** of India. ***(Pradhan et.al., 2009). Saraca indica*** is highly regarded as a **universal panacea** in the **ayurvedic medicine**. It is one of the **universal plant having medicinal activities.** ***Saraca indica*** has been greatly used as **traditional medicine** for women related problems, such as **leucorrhoea, menorrhagia, dysfunctional uterine bleeding, bleeding haemorrhoids etc (ayurvedic pharmacopoeia of India. 2001).** The **antimicrobial activity** of the stem and bark of ***Saraca indica*** have been evaluated against standard strain of **Staphylococcus aureus, Escherichia coli, Salmonella typhimurium** ***(Shilpakala Sainath et al, 2009).*** The leaves of ***Saraca indica*** also evaluated for **anthelmintic activity** ***(Manjunath et al, 2006; Nayak et al, 2011),*** analgesic and antipyretic activities ***(Pradhan et al, 2010),*** **CNS depressant activity** ***(Yadav et al, 2008).Pal et al.,(2014).***

Mathew. N et al.,(2009) evaluation the **larvicidal potential** of the extracts of commonly available medicinal plants ***Saraca indica/asoca (F: Caesalpiniaceae).***

Fig: 1 Ashoka Tree *(Saraca indica)*

The Ashoka tree is one of the **ethno medically important plant**. Therefore the **molecular characterization** of **endophytic fungus** from **ethno medically important plant** Ashoka tree leaf ***(Saraca indica)*** in view to amplify their DNA region is the first step which enables us for further more detail studies.

MATERIALS AND METHODS

Sample Collection

The plant biomass (leaf) samples were randomly and aseptically collected from Ashoka tree in Chennai. The sample were aseptically collected and placed in sterilized containers and transported to the laboratory for processing.

Extraction and inoculation of endophytic fungus from Ashoka leaf

The leaves were thoroughly washed in running tap water followed by sterile distilled water, and fine slurry was prepared by taking 200 gm of leaf with 100 ml of sterile water. Leaf extract is taken and fungus is allowed to grow in Petri plate. After that the extract is inoculated with the fungus, after 3-5days of incubation culture develops. These fungus is further develops in a nutrient broth for full growth of the fungus. It took around 3 days. Nutrient broth is kept in a shaker at 37^{o}C.The fungus was developed and isolated by grinding with liquid nitrogen. ***Verma and Kharwar (2006).***

DNA Isolation CTAB method

CTAB extraction buffer of 10ml were added and mixed to wet the entire powder. It was placed in 65^{0}C water baths for 30 min. Cooled and equal volume of $CHCl_3$: IAA (24:1) added. Then it was mixed, centrifuged for 10 min at full speed. Aqueous supernatant were transferred to a new tube. Equal volume of isopropanol was added. The spooled DNA was rinsed with 70 per cent ethanol. Air dried and it was added to 1-5 ml TE containing 20ug/ml

RNase suspended samples were placed at 65^0C in water bath or allowed pellets to resuspend overnight at 4^0C. ***Sarvananda et al., (2016).***

Qualitative Analysis- Agarose gel electrophoresis

Agarose gel with 1X TAE buffer was prepared and stained with 2µlof ethidium bromide. Samples were loaded with loading dye (2µl of loading dye is used). Electrophoresis of DNA fragments at 50volts were done. DNA fragments are visualized under the UV trans-illuminator. ***Sarvananda et al., (2016).***

DNA Quantitative Analysis -Spectrophotometric Method

DNA sample was quantified by spectrophotometric quantification method. 5µl of DNA sample was added with 2995µl of TE Buffer and with the help of glass cuvette readings were taken in spectrophotometer. ***Sarvananda et al., (2016).***

PCR analysis

To perform several parallel reactions, a master mix containing buffer was prepared, dNTPs, primers and Taq DNA polymerase in a single tube which and aliquoted into individual tubes and template DNA solutions are then added and finally the enzyme was added. The amplification of 32 cycles using the following reaction was carried out. ***Fredricks et al., (2005).***

Ribotyping

DNA is extracted from a colony and then restricted into discrete-sized fragments. Then DNA transferred to a membrane and probed with a region of the rRNA operon to reveal the pattern of rRNA genes. The pattern is recorded, digitized and stored in a database. Databases for identification of fungus are used in this method. ***Syec et al., (2010), Pei et al.,(2009).***

Blast (Basic Local Alignment Search Tool)

Each colour represented a particular percentage of similarity. Below the Histogram, the lists of best hits are given along with E-score, links and Gi number. We can identify the maximum similarity sequences based on the score by viewing the actual alignment. ***Johnson et al., (2008).***

RESULTS AND DISCUSSION

The Molecular Characterization of Endophytic fungus ***Botryotinia fuckeliana*** from ***Saraca indica*** has been studied through various methods and the results are given below:

Extraction and inoculation of endophytic fungus from Ashoka leaf

From the fine slurry of Ashoka leaves the endophytic fungus ***Botryotinia fuckeliana*** was developed and inoculated. Shown in **Fig:2.**

Fig: 2 Inoculated fungus (*Botryotinia fuckeliana)*

Genomic DNA isolation(Cetyl trimethyl ammonium bromide) Method

The nucleic acid was isolated this is a basic necessity for genome characterization, gene mapping procedures and for the identification and isolation of genes for genetic engineering. Fig :3 shows that DNA bands are clearly seen in lane: 5 and lane 6.

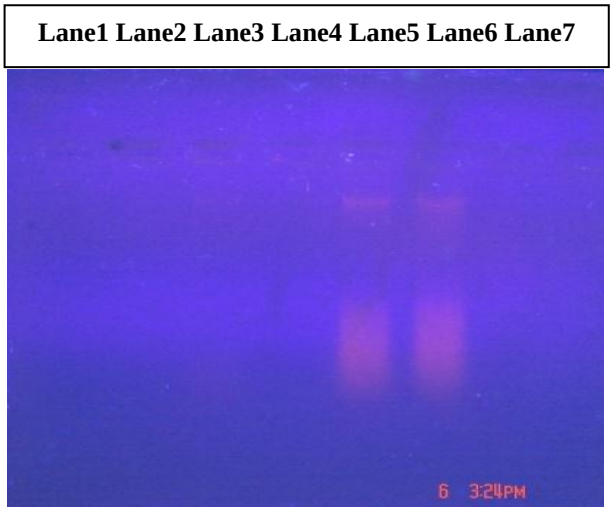

Fig: 3 Genomic DNA isolation CTAB Method

[Lane 1: genomic DNA1, Lane 2: genomic DNA2, Lane 3: genomic DNA3 Lane 4: genomic DNA4, Lane 5: CTAB DNA1, Lane 6: CTAB DNA 2, Lane 7: Ladder].

Qualitative Analysis - Agarose gel electrophoresis

Through this effective method DNA fragments are separated, identified and purified.

DNA Quantification-spectrophotometric Method

To quantify DNA, DNA quantification was done before performing DNA profiling PCR.

PCR Reaction

CTAB DNA 2 exhibits a clear band of 18S rRNA region was amplified through PCR standardization.

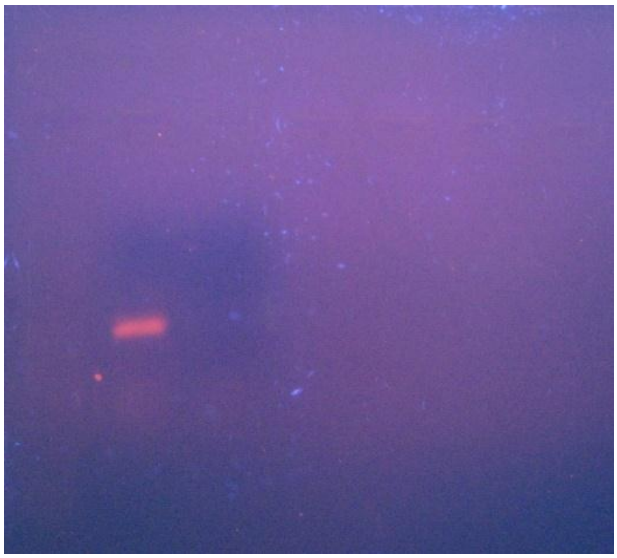

Fig:4 [CTAB DNA 2 Showing a clear band of 18S rRNA region after the PCR standardization.]

Ribotyping

Based on the differences in RNA genes the fungus has been identified and classified through Ribotyping method.

BLAST Analysis

The amplified product was sequenced and nucleotides of DNA. **(Fig: 5)** Sequences are done through Blast analysis. The statistical searching program having parameters like length of the query length of the database, gap penalty, effective HSP scores. Thus above results given score with query sequence producing significant alignment with the database of protein. **Score: 872E-value: 0.0.** The graphical representation for sequence alignment is shown in **(Fig: 6).** The name of the endophytic fungus was identified as ***Botryotinia fuckeliana*** from the percentage of similarity of endophytic fungus appeared in **histogram.**

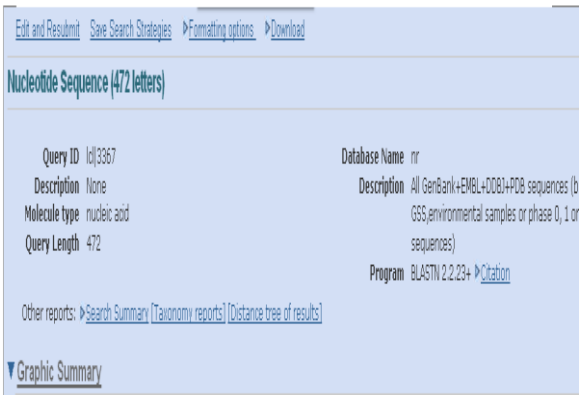

Fig: 5 Blast: Nucleotide Sequence

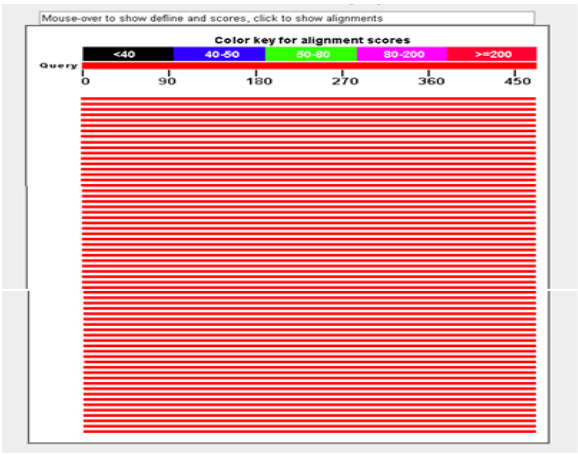

Fig: 6 Graphical Representation for sequence alignment

Endophytic fungus *Botryotinia fuckeliana* (Fig:2) which affects the Ashoka tree leaves **(*Saraca indica*) (Fig: 1)** has been identified from this study by various techniques, which was already used by other fungus identification and molecular characterization method. ***Verma and Kharwar (2006).***

Therefore the molecular characterization of endophytic fungus from ethno medically important plant Ashoka tree leaf ***(Saraca indica)*** in view to amplify their DNA region is the first step which enables us for further more detail studies.

CONCLUSION

Therefore this study reveals that endophytic fungus ***Botryotinia fuckeliana*** has been identified from ethanomedically important plant Ashoka tree ***Saraca indica.*** This will be help to discover the causes and preventive mechanism of ***(Botryotinia fuckeliana)*** endophytic fungus.

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