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Isolation and identification of fungi from Kalipati variety of Sapota fruits

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Abstract
Sapota (*Manikara zapota*) belongs to family *Sapotaceae*. This species has various properties in the traditional system of Indian medicine. In current study Kalipati variety of Sapota fruit is used. Sapota fruit is a rich source of sugar and hence it attracts number of fungal species. Two fungal species viz Aspergillus minisclerotigenes and lasiodiplodia theobromae were identified from infected fruits. In present study identification is based on microscopic and morphological characters Potato dextrose Agar, granulated medium is used for isolation of fungi and Rose bangal medium is used for pure culture. For molecular identification DNA sequencing reaction of PCR amplicon was carried out with ITS1 primer using BDT V 3.1 cycle sequencing kit on ABI 3730x1 Genetic Analyzer.

Keywords: DNA sequencing, isolation, fungi, morphological, sapota

1. Introduction
Fruits are most important constitutes of human diet, which supplies a few nutrients essential to the body, Surendranathan, 2005 [1]. Sapota belongs to family Sapotaceae and it has several properties in the traditional Indian medicinal system, Kulkarni *et al.*, 2007 [2]. Part of their nutritional values are some chemical composition that is the richest source of sugar, proteins, ascorbic acid phenolics, carotenoids and minerals like iron, copper, zinc, calcium and potassium. The main region of multiple radical scavenging potential of sapota fruits was due to its nutaceutical component, phenolics, carotenoids and ascorbic acid, Bano and Ahmed, 2017 [3].
Cultivars are identified based on fruit shape and foliage color. According to farmers, cultivars with melodious and sweeten flesh are preferred over hard flesh. Kalipati, cricket ball and Calcutta round are different varieties of Sapota fruit. Kalipati verity was chosen for this work. Kalipati- As the name shows their character, the plants have dark green leaves which look like blackish in color, spreading in branches. Fruites are ovel shaped. Seeds vary from 1 to 4 per fruit. The fruits are harvested in winter.
The fungi may be defined as non-green, nucleated thallophytes. However, mycologists have defined fungi more scientifically. The fungi include nucleated spore bearing achlorophyllous organism that generally reproduce and whose filamentous branched somatic structures are surrounded by cell walls containing chitin, Pugh, 1963 [4].
Due to some abiotic and biotic factors fungal growth is observed in the post-harvest stage. Traditional way of study includes conventional cultivation and microscopic identification, Frisvad *et al.*, 2006 [5]. Morphological identification based on colonial characters, mycelia (color, shape, size) and their morphology (conidial size, morphology conidiophore). However, identification requires good skilled mycologist, but molecular technique has been proved good for identification of fungi in an effective and easy way, Alwakeel, 2013 [6]. Stored fruits can be infected with fungi and show huge fungal diversity. Fungal infected fruits show their impact on human health and businessman with reference to post harvest losses, Jungharea *et al.*, 2015[7]. Comparative DNA sequence-based identification formats appear to be promising in terms of speed, ease, objectivity and economy for species identification. Multiple genes ranging from the universal ribosomal DNA regions ITS and the large ribosomal subunit D1-D2 to protein encoding genes such as the β-tubulin and calmodulin gene regions have been assigned to delimit species within *Aspergillus*, Balajee *et al.*, 2007 [8].

2. Material and Methods
2.1 Sample collection:
Samples were collected from SGVP (Shree Swaminarayan Gurukul Vishwavidya Pratishthanam) on 19/03/2019. Total 60 samples were collected. SGVP is a very big campus; where organic crops are grown by using organic fertilizer. Collected samples were clean at to remove soil debris and water drops were dried from fruits skin. Fruit samples were stored and incubated at room temperature for a few days. After a week of time sapota fruits were reported fully infected with fungi.

2.3 Media preparation:
PDA is commonly used media for in vitro fungal growth. Each type of fungal isolate was grown on Potato Dextrose Agar medium (PDA). For pure culture Rose Bangal media was used.

2.4 Isolation of associated fungi
Fungal species were inoculated on Potato Dextrose Agar (PDA) medium. This was done for all the samples collected from SGVP, the petri plates were incubated at room temperature. Fungal growth was observed daily. After five-six days of incubation, plates were ready with fungal colonies. For pure culture; a small portion of mycelium from each fungal colony was transferred aseptically into

fresh containing the medium used. The fungal species were purified by repeated sub-culturing.

2.5 Slide preparation:
The lacto-phenol cotton blue is used for fungal cell staining. For the preparation three components are essential: phenol; kills any living organisms, lactic acid; preserves the fungal structure and cotton blue; stains the chitin in the fungal cell wall. The samples were immersed in a drop of alcohol. Only two drops of lacto phenol or cotton blue stain is needed and then slides are observed under the microscope, Oza and Mankad, 2017 [9].

2.6 Morphological Identification:
The identification of isolation fungi up to the genus and species is possible based on macro and micro morphological characteristics, using Manual of soil fungi, Gilman, 1998[10]. A minute portion of each organism was taken by using sterile inoculating needle in aseptic condition and teased at the center of a clean glass microscopic slides containing drop of lactophenol cotton blue stain and covered with cover slips. This slide was observed in the light microscope. Genetic level identification was based on literature, identification key and standard textbooks.

2.7 Molecular Identification
Molecular identification is necessary for species level identification. Total genomic DNA was extracted according to the methodology developed in GeMBio laboratory and this was used in all tests performed, Tapia *et al.*, 2006 [11]. The gene sequence was used to carry out BLAST with the database of NCBI Genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs (Table-1). Put genome sequence in NCBI and unique Accession number (Table-2).

3. Results and Discussion
In the present investigation, the isolated fungi were examined based on cultural, microscopic and morphological characteristics. Fig: 1 shows fungal species isolate and identified in this study. Two fungal species were isolated *Lasiodiplodia theobromae* and *Aspergillus minisclerotigenes*. Bautista *et al.*, 2002 [12], was recorded *Lasiodiplodia theobromae* synonyms *Botryodiplodia theobromae* in sapota fruits.
Morphological characteristics of *Lasiodiplodia theobromae* were; dark grey colony with woolly aerial mycelium on medium and black pigmentations on back side of perti plates of medium. Morphological characters were conidial cells and septate paraphyses, hyaline immature conidia, dark bovid mature conidia with middle septum and longitudinal striation.
Morphological characteristics of *Aspergillus minisclerotigenes* were; colony surface velvety on medium, colony surface floccose, consisting of white vegetative mycelium and sparse conidial heads and dense dark sclerotia, sometime it may be supplementary conidial structures light greyish green in color, sclerotia are 150-300 mm in diameter. Conidial heads normally biseriate, but sometimes uniseriate heads occur.
16s r RNA gene sequencing analysis that fungal isolates were *Lasiodiplodia theobromae* and *Aspergillus minisclerotigenes* obtained (Table- 1 and 2).

Table 1: Description of isolate fungal species

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Sequence ID
Aspergillus minisclerotigenes isolate DTO 009-F5 small subunit ribosomal RNA gene, partial sequence	979	979	100%	0	100%	MG662408.1
Lasiodiplodia theobromae strain LASID2 18S ribosomal RNA gene, partial sequence	416	416	91%	3E-112	89.85%	KU507479.1

Table 2: Molecular based species level identification.

Sr. No.	Submission number	Accession number	Identified Organism
1	SUB794527	MT903469	*Lasiodiplodia theobromae*
2	SUB7940490	MT903466	*Aspergillus minisclerotigenes*

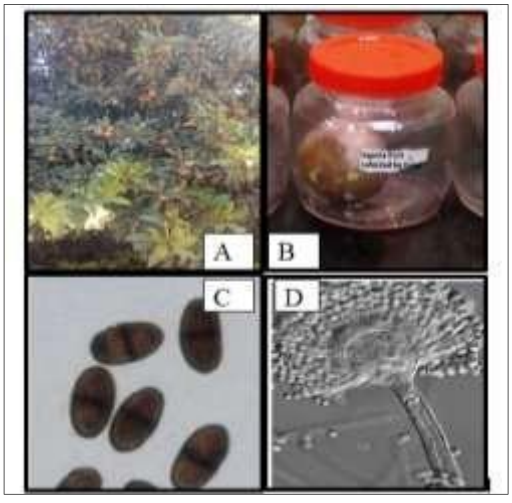

Fig 1: [A] Kalipati verity of Sapota tree, [B] infected Sapota fruit, [C] Microscopic examination of *Lasiodiplodia theobromae* and [D] *Aspergillus minisclerotigenes.*

4. Conclusion

Kalipati variety of sapota fruit showed the predominance of filamentous *Aspergillus minisclerotigenes* and *Lasiodiplodia theobromae*. In this study morphological and molocular identification of fungal species could enrich and provide valuable information to fungal diversity. This study reported that sapota is an important fruit crop and it can be considered as one of the healthy fruits because of the presence of various nutrition components. Present work gives valuable information for further research because this plant has huge potential to improve postharvest quality and marketing possibilities of sapota (chickoo) fruit life as well as nutritional quality.

5. Acknowledgement:

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6. Declaration

The authors declare no conflict of interests.

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