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Mycobiota of *Cnidoscolus aconitifolius* (Mill.) Phyllosphere

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was conducted to determine the mycobiota of *Cnidoscolus aconitifolius* phyllosphere using metagenomics. The phyllosphere, which is the above-ground (aerial) part of plants, is colonized by different microorganisms some of which may be pathogenic to plants and also to humans and animals.
Methodology: The mycobiota was determined by sequencing the 18S rRNA gene on Illumina MiSeq platform. The primer pair: ITS1F (5´-CTTGGTCATTTAGAGGAAGTAAT-3´) and ITS4 (5´-TCCTCCGCTTATTGACATGS-3´) were used to target the ITS regions I and II, and a portion of 28S rDNA.
Results: A total of 107 Operational Taxonomic Units (OTUs) were obtained. The mycobiota of *C. aconitifolius* had 100% Ascomycota classified into Dothideomycetes (84.15%), Eurotiomycetes (2.26%) and Sordariomycetes (12.45%). Only 1.13% of the fungi were unassigned at the class level. The core mycobiota of chaya consisted of the genera *Cladosporium* (51.70%), *Lasiodiplodia* (18.11%), *Allophoma* (6.79%), *Stagonosporosis* (2.26%) and *Aspergillus* (2.26%).
Conclusion: The economic importance of the organisms obtained were highlighted. The result from this study shows that *C. aconitifolius* phyllosphere harbors diverse fungi some of which may promote plant growth or are pathogenic to plants and/or humans.

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Keywords: Fungi; Illumina next-generation sequencing; Mycobiota; Phyllosphere.

1. INTRODUCTION

Cnidoscolus aconitifolius (Mill.) I. M. Johnston commonly called “cabbage-star” or “tree spinach” is an economic plant that is cultivated for both food and medicinal purposes [1]. *C. aconitifolius* leaves are used as vegetable to cook soup and yam portage in Nigeria and other parts of the world. The leaves have been reported to contain more nutrients than every other land-based leafy vegetable by two to three folds [2]. It is eaten in Mexico as a vegetable and also used as feed for domestic animals. It also has the ability to darken grey hair and strengthen fingernails. In Nigeria, it is used locally as a blood-booster in the rural areas especially in the Southern part of the country. Hamid et al. [3] reported that the above-ground parts of *Cnidoscolus aconitifolius* exhibit antibacterial and antifungal activities. Mordi and Akanji [4] also reported that *C. aconitifolius* have antihaemorrhagic, antihypertensive and cardiac depressant properties.

Tree spinach occurs in the wild and can also be cultivated. Propagation of *C. aconitifolius* is by seeds or by stem cuttings [1]. Cultivated varieties are propagated mostly by stem cuttings. The wild form of *C. aconitifolius* occurs in open forest, usually in open rocky localities, from 1300 metres above sea level [5,6]. *C. aconitifolius* is used as live fence-posts [1]. It is also used as mulch for vegetable gardens [7]. The edible leaves can be preserved as they have the potential to be processed and canned or frozen [7].

The phyllosphere is a microbial habitat that exists in the aerial parts of vascular plants. It is regarded as a hostile environment for the survival of microorganisms as microorganisms in this sphere need to have the ability to tolerate high ultraviolet (UV) radiations, rapid variations in humidity, temperature and heterogeneous availability of nutrients [8,9]. The phyllosphere is largely colonized through the migration of bacteria, fungi, and other microorganisms from soil, seed, air, water, or through animal-borne sources [9]. Some plant pathogens can inhabit the phyllosphere prior to an infection or in the apparent absence of an infection [9]. Filamentous fungus population in the phyllosphere ranges between 10^2 and 10^8 colony forming unit per gramme (CFU g^{-1}) leaf [10].

Understanding the modalities of the structure of microbial communities in the phyllosphere is vital for developing bio-control strategies for both plant and human pathogens [11]. Phyllosphere microbiota plays an important role in promoting plant growth through various mechanisms and also protecting plants from various diseases [12]. Microbial communities of the phyllosphere are believed to play a significant role in remediating atmospheric hydrocarbon pollutants and residual pesticides [13,14]. Phyllosphere-fungi can increase drought tolerance of plants and confer protection against plant pathogens [15,16]. Leafy vegetables significantly inhabit human pathogens in the phyllosphere which may lead to food-borne diseases [17]. It has been established that host genotype, site characteristics and seasonal changes are some of the factors that determine the structure of microbial communities in the phyllosphere [18,19]. Cordier et al. [20] investigated the variations in fungal communities of European beech, *Fagus sylvatica* L. leaf surfaces using ITS1 gene sequencing and observed a relationship between the genetic distance of beech trees and differences in fungal community structure, signifying that host genetics determines the fungal community structure on beech leaves. Plant genotype has also been identified as a determinant of fungal community structure on balsam poplar, *Populus balsamifera* L. phyllosphere using ITS pyrosequencing [21].

Earlier studies have employed the use of culture-dependent method to study and describe community structure and function. These methods are classified as low-through-put molecular techniques as they are believed to underestimate the diversity of microorganisms. All taxa present in a sample cannot be fully represented or identified using these methods. Only members of the microbial communities that can be cultured in the laboratory are represented while majority of the organisms in the habitat are left out. The advent of high-throughput molecular technologies which are also cost-effective has led to remarkable improvements in the field of phyllosphere microbiology as researchers have been able to analyse large number of samples at ease with in-depth coverage of phyllosphere microbiota present [8]. There is more information on the structure and composition of bacteria in the phyllosphere than there is for fungi [12,22]. In this study, we conducted ITS gene-based sequencing on Illumina Miseq platform to determine the mycobiota of *Cnidoscolus aconitifolius* phyllosphere. The results from this study would widen our knowledge on the fungal

community associated with *Cnidoscolus aconitifolius* phyllosphere.

2. MATERIAL AND METHODS

2.1 Sample Collection

Cnidoscolus aconitifolius leaves were obtained from Choba, Rivers State, Nigeria in April 2018. The coordinates of the sample collection location is 4.89^{o}N and 6.91^{o}E. The leaves were transferred to the lab in a zip bag prior to DNA extraction.

2.2 DNA Extraction and Illumina Next Generation Sequencing

DNA was extracted from *C. aconitifolius* leaves using Zymo Fungal/Bacterial DNA Extraction kit (Zymo Research Group, California, USA) with a slight modification as described in a previous study [23]. To analyze the total fungal community of the phyllosphere, 0.50g of leaves was used. The leaves was transferred into a sterilized mortar and homogenized with 750µl of Bashing Bead Buffer using a pestle. After homogenization, the sample was transferred to a 1.5ml Eppendorf tube. The tube containing the sample was centrifuged in a refrigerated centrifuge at 10,000 x *g* for 1 minute. 400µl of the supernatant was transferred to a Zymo-Spin III-F Filter in a collection tube and was centrifuged at 7,000 x *g* for 1 minute. 1,200µl of Genomic Lysis Buffer was added to the filtrate in the collection tube and thoroughly mixed. 800µl of the mixture was transferred to a Zymo-Spin IIC Column in a new collection tube and centrifuged at 10,000 x *g* for 1 minute. The flow through in the collection tube was discarded and the above step repeated. 200µl of DNA Pre-Wash Buffer was added to the Zymo-Spin IIC Column in a new collection tube and centrifuged at 10,000 x *g* for 1 minute. The content of the collection tube was discarded. 500µl of g-DNA Wash Buffer was added to the Zymo-Spin IIC Column in a new collection tube and centrifuged at 10,000 x *g* for 1 minute. The Zymo-Spin IIC Column was transferred to a clean 1.5ml microcentrifuge tube and 100µl of DNA Elution Buffer was added directly to the column matrix and then centrifuged at 10,000 x *g* for 30 seconds to elute the DNA.

The forward primer, ITS1F (5´-CTTGGTCATTTAGAGGAAGTAAT-3´) and reverse primer, ITS4 (5´-TCCTCCGCTTATTGACATGS-3´) were used to target the ITS region 1 between the 18S and 5.8S rDNAs, ITS region II and a portion of 28S rDNA. The samples were analyzed with 300 bp paired-end read, Illumina MiSeq, at Inqaba Biotechnology Limited, South Africa. The resulting amplicon was gel purified, end repaired and Illumina specific adapter sequence added to the 5´ end of each primer.

2.3 Processing of Sequence Reads

The reads obtained were preprocessed to check sequencing errors. Sequences that did not contain the exact match for both forward and reverse reactions were eliminated from the analysis. Sequences were trimmed with Next-generation sequencing Short Reads (*ngsShoRT*) trimmer as described by Chen et al. [24]. The ITS gene sequences were processed using QIIME v.1.9.0 (Quantitative Insights Into Microbial Ecology) pipeline as described by Caporaso et al. [25]. Sequences with less than 200 base pairs and reads with more than 2% of ambiguities were excluded from the final analysis. The UCLUST algorithm [26] was used to cluster sequences into Operational Taxonomic Units (OTUs) at a 97% identity threshold. Each OTU sequence was represented by the most abundant read. The Unified System for the DNA-based fungal species linked to the classification (UNITE) reference database [27] was used for both open reference OTU picking and taxonomic assignment for the sequences. Raw sequences of *C. aconitifolius* microbiota were deposited on NCBI (National Centre for Biotechnology Information) database under Sequence Read Archive (SRA) in GenBank as BioProject ID PRJNA592288.

3. RESULTS

3.1 *Cnidoscolus aconitifolius* Mycobiota at the Division and Class Levels

Sequences were assembled and a total of one hundred and seven (107) OTUs were successfully characterized and grouped into twenty-eight (28) genera. The fungal microbiome of *C. aconitifolius* had 100% Ascomycota classified into Dothideomycetes (84.15%), Eurotiomycetes (2.26%) and Sordariomycetes (12.45%). Only 1.13% of the sequences were unassigned at the class level. The fungal community of *C. aconitifolius* at the division and class levels is presented in Figs. 1 and 2.

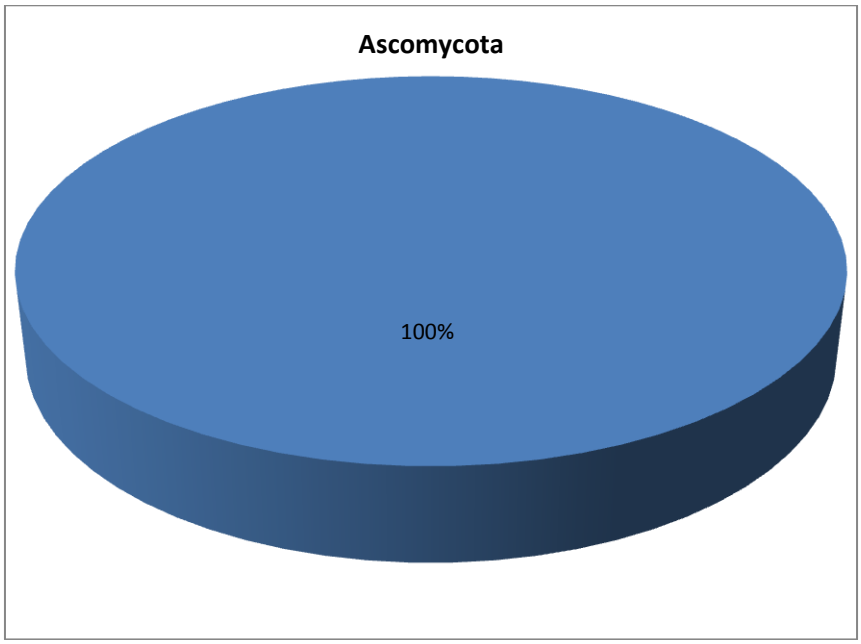

Fig. 1. Fungal division obtained from *Cnidoscolus aconitifolius* phyllosphere

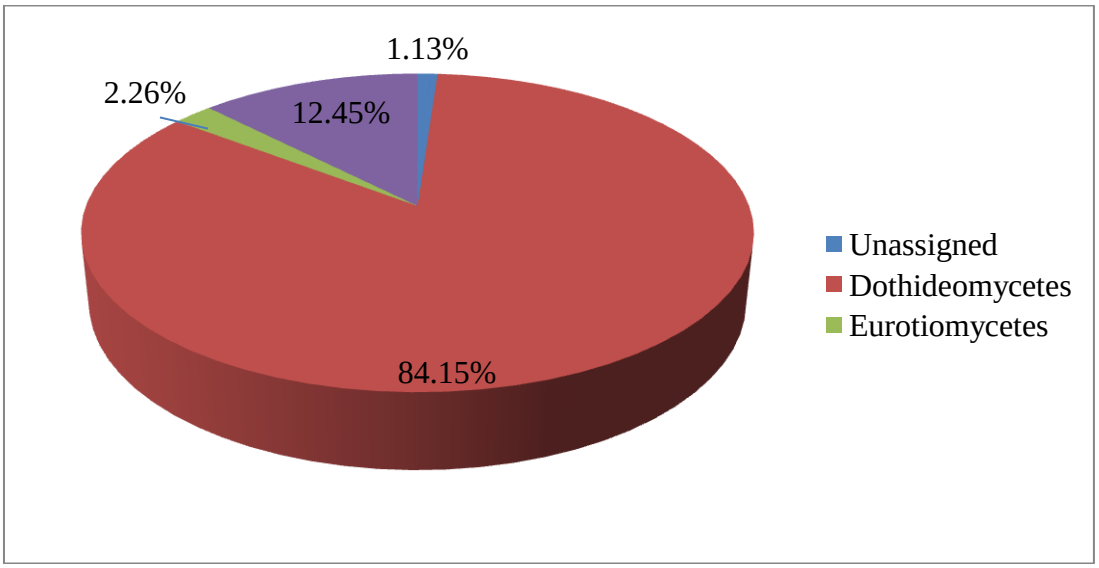

Fig. 2. Fungal classes obtained from *Cnidoscolus aconitifolius* phyllosphere

3.2 Distribution of Fungi at the Genus and Species Levels

The most represented sequences (each representing more than 1% of the total classified fungi) in *C. aconitifolius* phyllosphere belonged to the families Cladosporiaceae (52.83%), Botryosphariaceae (18.11%) and Didymellaceae (9.06%).

Out of the 107 OTUs obtained, only thirty-five (35) belonging to eight (8) taxa were successfully identified to the species level on UNITE database. The other remaining OTUs were blasted on NCBI database for species identification. This is because NCBI is a constantly updated "gene-house" database as thousands of sequences are deposited on GenBank on a daily bases. The BLAST searches revealed the match sequences of the clones against known sequences on NCBI with 86 to 100% identity. The OTU number and GenBank accession number of match sequences are listed in Table 1.

Table 1. Taxonomic affinities of OTUs with BLAST searches from NCBI Database based on their ITS sequences

OTU number	Taxonomic affinity (GeneBank Accession no.)	Percentage Similarity (%)
1	*Penicillium citrinum* (MF476066.1)	96
11	*Lasiodiplodia theobromae* (GQ469915.1)	100
27	*Aspergillius nomius* (MK841463.1)	99
39	*Phoma eupyrena* (KY765281.1)	89
42	*Lasiodiplodia theobromae* (MH251950.1)	99
50	*Penicillium citrinum* (MK852473.1)	100
57	*Allophoma minor* (MF380953.1)	100
70	*Phoma eupyrena* (KX610328.1)	99
84	*Pericona pseudobyssoides* (KU214550.1)	99
108	*Cladosporium cladosporioides* (KU182497.1)	90
112	*Acremonium charticold* (KT878345.1)	95
114	*Aspergillus flavus* (MG976497.1)	98
118	*Corynespora casiicola* (AY238605.1)	99
119	*Lasiodiplodia theobromae* (GQ469915.1)	97
127	*Cladosporium cladosporioides* (MH535968.1)	98
137	*Cladosporium tenuissimum* (MF473305.1)	89
138	*Aspergillus flavus* (MG976497.1)	100
142	*Lasiodiplodia theobromae* (GQ469915.1)	100
147	*Corynespora casiicola* (AY238605.1)	96
159	*Nigrospora sphaerica* (JQ936184.1)	96
162	*Pericoma byssoides* (KC954157.1)	99
164	*Lasiodiplodia theobromae* (MH251950)	98
185	*Allophoma minor* (MF380953.1)	97
193	*Corynespora casiicola* (AY238605.1)	97
214	*Lasiodiplodia theobromae* (MH251950.1)	100
217	*Cladosporium cladosporioides* (MH535968.1)	98
224	*Postia placenta* (KJ995944.1)	91
227	*Aspergillus violaceofuscus* (MG682503.1)	99
232	*Cladosporium xanthochromaticum* (MF473323.1)	97
235	*Lasiodiplodia theobromae* (EJ904912.1)	89
245	*Corynespora casiicola* (MH864416.1)	93
254	*Nigrospora oryzae* (MH619723.1)	87
259	*Corynespora casiicola* (AY238605.1)	89
268	*Colletotrichum gleosporioides* (MH392749)	100
272	*Corynespora casiicola* (AY238605.1)	98
274	*Schizothyrium pomi* (EF134949.1)	98
283	*Cladosporium cladosporioides* (MH535968.1)	100
287	*Aspergillus versicolor* (JQ717322.1)	99
324	*Corynespora casiicola* (MK685154.1)	99
325	*Leptosphaerulina cartarum* (KC879283.1)	99
326	*Helminthosporium asterinum* (MH178554.1)	99
327	*Cladosporium cladosporioides* (MH790419.1)	100
335	*Periconia pseudobyssoides* (KU214550.1)	99
338	*Lasiodiplodia theobromae* (GQ469915.1)	97
343	*Schizothyrium pomi* (EF134949.1)	98
345	*Lasiodiplodia theobromae* (MH251950.1)	99
349	*Cladosporium colombiae* (MH244376.1)	99
364	*Corynespora casiicola* (MK685154.1)	100
366	*Corynespora casiicola* (AY238605.1)	97
367	*Zygosporium oscheoides* (MH861194.1)	96
368	*Devriesa Lagerstroemiae* (KP197670.1)	94
369	*Cladosporium tenuissimum* (MG569541.1)	100

OTU number	Taxonomic affinity (GeneBank Accession no.)	Percentage Similarity (%)
394	*Aspergillus amstelodami* (MK267406.1)	99
395	*Pithomyces chartarum* (MH859914.1)	99
410	*Corynespora casiicola* (MK685154.1)	100
420	*Allophoma minor* (MN380953.1)	100
428	*Acremonium charticola* (KT878345.1)	94
431	*Lasiodiplodia pseudotheobromae* (FT904912.2)	89
434	*Aspergillus penicillioides* (Mh86439.1)	100
439	*Colletotrichum siamense* (MK471371.1)	100
442	*Nigrospora oryzae* (MK429852.1)	100
443	*Penicillium paxilli* (JN617709.1)	98
454	*Trichoderma harzianum* (MN046978.1)	100
457	*Helminthosporium asterinum* (AF073918.1)	96
475	*Romboustа ilealis* (LN555523.1)	99
479	*Lasiodiplodia pseudotheobromae* (FJ904834.1)	89
480	*Spegazzinia tessarthra* (JQ673429.1)	94
483	*Periconia byssoides* (MK370654)	98
506	*Simplicillium lanosoniveum* (KT878334.1)	97
510	*Periconia byssoides* (MK907734)	99
523	*Nigrospora sphaerica* (KT259476.1)	98
529	*Aspergillus gracillis* (MH858708.1)	99

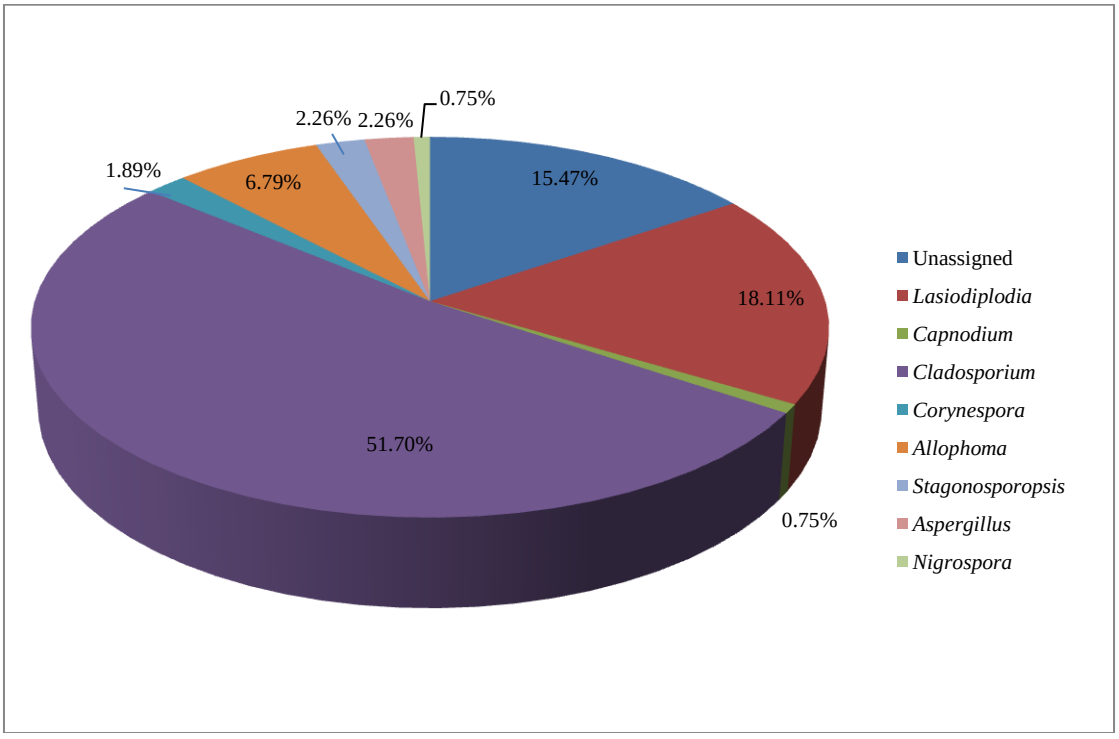

Fig. 3. Fungal genera obtained from *Cnidoscolus aconitifolius* phyllosphere

The core mycobiota of chaya consisted of the genera *Cladosporium* (51.70%), *Lasiodiplodia* (18.11%), *Allophoma* (6.79%), *Stagonosporosis* (2.26%) and *Aspergillus* (2.26%). At the genus level, the unassigned OTUs obtained 15.47% of the total OTUs. The most predominant species were: *Lasiodiplodia theobromae* (14.98%), *Corynespora casiicola* (14.98%), *Cladosporium tenuissimum* (14.98%), *Cladosporium xanthochromaticum* (14.98%), *Cladosporium cladosporioides* (14.98%), *Nigrospora oryzae* (14.98%), *Nigrospora sphaerica* (14.98%), *Stagonosporosis curcubitacearum* (14.98%), *Allophoma minor* (14.98%), *Aspergillus flavus*

(14.98%) and *Aspergillus chavelaeri* (14.98%). The fungal community of *Cnidoscolus aconitifolius* at the genus and species levels is presented in Figs. 3 and 4 respectively. A phylogenetic tree was constructed to show the relationship between the genera obtained (Fig. 5).

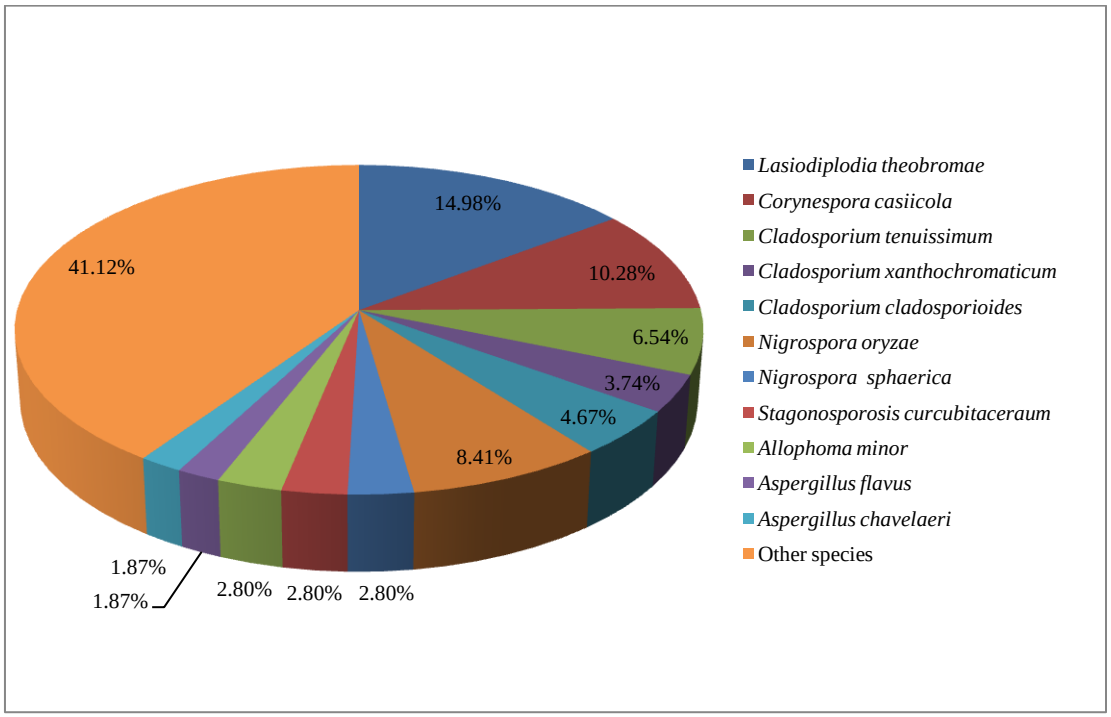

Fig. 4. Fungal species obtained from *Cnidoscolus aconitifolius* phyllosphere

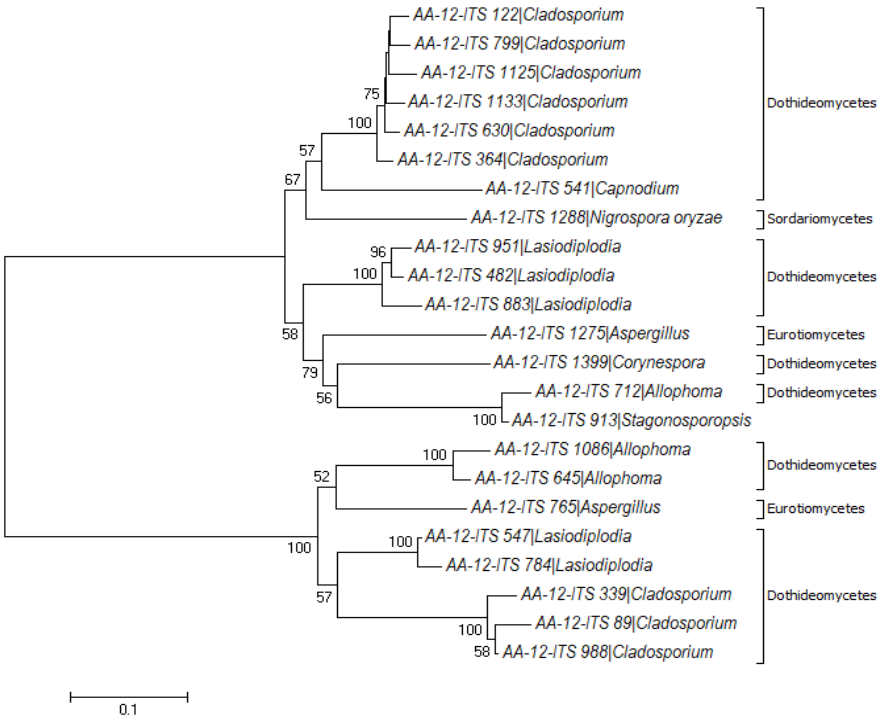

Fig. 5. Phylogenetic tree generated by maximum composite likelihood analysis based on the ITS sequences of the OTUs

4. DISCUSSION

Many authors have reported that the majority of taxa obtained from plant leaves belong to the division, Ascomycota [28,29]. Ascomycota, Basidiomycota and Chytridiomycota have also been reported as the dominant fungal divisions on plants [30,31].

Cladosporium belongs to the class Dothideomycetes, order Capnodiales and family Cladosporiaceae. Species occur in clusters of black, green or yellow spots. Different species of *Cladosporium* has been found on a variety of plants including *Phaseolus vulgaris*, *Alium porrum*, *Ananas comosus*, *Pinus ponderosa* etc; and on different parts of human samples including sputum, toe nail, lung, foot, skin, scalp, etc [32]. Ten new species belonging to the genus *Cladosporium* were reported by Sandoval-Denis et al. [28] and these species are associated with animal and human infections. *C. cladosporioides* and *C. tenuissimum* were detected in air conditioning, ventilation and heating equipments in China with *C. cladosporioides* having the highest frequency and concentration [33]. Long term exposure to *Cladosporium* is associated with allergies, asthma symptoms, and eye, ear and skin infections.

Stagonosporopsis belongs to the class Dothideomycetes, order Pleosporales and family Didymellaceae. *S. cucurbitacearum* is a fungal parasitic pathogen reported by Zhao et al. [34] to be responsible for gummy stem blight disease of pumpkin in North-east China. The disease stands as one of the major severe pumpkin diseases in North-eastern region of China and leads to huge crop losses. *S. cucurbitacearum* as a parasite inhabits its host, causing serious damage to crops but does not directly kill its host. The pathogen makes use of penetration pegs (conidial structure) at the early stage of infection, to absorb nutrients necessary for its survival [30]. Wipornpan et al. [35] also reported gummy stem blight of cantaloupe caused by *S. cucurbitacearum* in Thailand. Bhuiyan *et al*. [36] and *Vaghefi et al.* [37] reported ray blight disease of pyrethrum (*Tanacetum cinerariifolium*) caused by *S. tanaceti*. This is considered as a limiting factor to pyrethrum cultivation in Australia.

Corynespora belongs to the class Dothideomycetes, order Pleosporales and family Corynesporaceae. The genus *Corynespora* consists of mostly plant fungal pathogenic species causing diseases on various plants, world-wide. Some of the species of this genus occur as saprobes and endophytes. About 200 species of *Corynespora* have been recorded and several novel species have been reported [38-41]. *Corynespora* is synonymous with the genera, *Cercospora* and *Helminthosporium*. *Corynespora cassiicola*, a phytopathogen reduces yield of natural rubber latex in African and Asian countries. *C. cassiicola* has 375 host species. Symptoms occur as leaf and fruit decay.

The genus *Allophoma* belongs to the class Dothideomycetes, order Pleosporales and family Didymellaceae. *Allophoma* causes lesion and leaf spots on plants. *A. tropica* was first observed on lettuce in Northern Italy in 2011 [42]. *Allophoma tropica* causes leaf spot of lettuce [43]. *A. zantedeschiae* caused symptoms of small yellow lesions on lower leaves of *Papaver dubium* which eventually spread to the whole plant in Iran [44]. *A. minor* is a phytopathogenic fungus [45,46]. It was isolated as one of the pathogens responsible for lesion blight of *P. dubium* in Iran.

Aspergillus belongs to the class Eurotiomycetes, order Eurotiales and family Trichocomaceae. *Aspergillus chevalieri* was reported on peanuts in Malaysia [47]. *A. chevalieri* is a xerophilic organism which provides a favorable growth condition for other spoilage-related fungal organisms. This organism might affect the quality of plant products and lead to reduced shelf life. A study conducted by Chukwu et al. [48] indicated that *A niger*, *A. flavus* and *A. terreus* were associated with both fresh and dry tiger nuts and they can possibly endure processing treatment. The occurrence of these fungi may cause diverse effects on human health as they have the potential of producing mycotoxins [49]. *A. flavus* produces two most common aflatoxins; aflatoxins B1 and B2 [50].

Nigrospora species exist as endophytes on stems and leaves of different plant species [51] or as saprobes from leaf litter or dead larvae [52,53]. The genus consists of plant pathogenic species infecting various fruits, economic crops and ornamentals. Zhai et al. [54] reported the occurrence of *Nigrospora oryzae* on *Aloe vera* in China where it caused leaf spots. *N. oryzae* was also reported in India on *Brassica juncea* where it caused stem blight [55].

Nigrospora sphaerica has been isolated from different plants where it caused leaf spots, rots,

blight and lesions. *N. sphaerica* was reported in China as the causal agent of leaf blight of *Camellia sinensis* [56]. Alam et al. [57] reported that *N. sphaerica* caused fatal leaf spot on Kinnow mandarin (*Citrus reticulata*). *Nigrosora sphaerica* has also been reported to be associated with a disease in human. Ananya et al. [58] reported that *N. sphaerica* caused corneal ulcer in an immunocompetent woman. *N. oryzae* and *N. sphaerica* were found to cause leaf spot on date palms (*Phoenix dactylifera* L.) [59,60].

5. CONCLUSION

The use of next-generation molecular techniques has led to advances in phyllosphere microbiology. These techniques have helped researchers to know the structure of plant microbial communities, the organisms present on leaf surfaces, and what these organisms do in plants. Most of the organisms obtained in this study are plant pathogens, causing deterioration on plants, reduced quality of plant products and devastating decrease in the quantity of agricultural produces recovered after harvest. *Cnidoscolus aconitifolius* is a highly neglected and underutilized plant. Insight into the mycobiota of *C. aconitifolius* phyllosphere is the starting point for devising ways of combating the pathogenic species and increasing the yield of this plant thereby making it more available to the fast-growing population. The correct identification of microorganisms in the phyllosphere and the in-depth understanding of the interaction that exists among these organisms will help in protecting plants against pre- and post-harvest diseases.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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