

Isolation and Identification of Endophytic microflora from *Cissus quadrangularis* Linn. of some districts of Assam

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Article

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Abstract

The present study aims at exploring the microbial diversity in one of the most important medicinal plant- *Cissus quadrangularis* Linn. Stems and leaves of the plant under study were collected from three districts of Assam. The study was conducted in two different seasons-summer and winter. 16 endophytic fungal species were isolated from the studied samples and were morphologically identified of which 5 were sterile ones. The isolation of endophytic bacteria was done on Nutrient Agar media using standard protocols. Based on the amplification of 16S rDNA amplification and comparing the results with the BLAST results and the phylogenetic tree, the organisms were identified to be- *Bacillus subtilis* and *Bacillus velenzensis*. This study will give further scope for studying the secondary metabolites from the Endophytic isolates of this plant and establish relationship for its bone healing ability.

Introduction

Endophytes are an interesting group of microorganisms that live inside the plant without harming them. Various definitions have been given by different researches to illustrate the very property of these magnificent organisms. Of all the properties one important aspect of the endophytic microflora is that their infection within the host plant is such that they do not show symptoms at least for the transient period and the microbial colonisation can be demonstrated to be internal (Stone et al., 2000). Endophytic fungi have been isolated from *Cissus quadrangularis* and the extracts of the fungal isolates have been found to exhibit bioactive potential (Nishanthi et al., 2016). In the past the diversity and the biotechnological potential of plant associated endophytic bacteria has been well documented (Quecine et al., 2014). Other than *Cissus quadrangularis* various other medicinal plants have been studied for the presence of the endophytic microflora along with their antimicrobial and other bioactive potential (Sandhu et al., 2014; Sagar et al., 2016). The medicinal properties of the plants therefore may be attributed to the endophytic microflora within the plant. Isolation of endophytic microbes from the medicinal plants may help in methods to produce bioactive substances on a large commercial scale for human welfare thereby reducing the impact of harvesting plants and environmental biodiversity (Kusari et al., 2011).

Endophytic studies on *Cissus quadrangularis* has been carried out in several parts of India but in Northeast particularly Assam it is scarce. The present study therefore aims at knowing more on the endophytic diversity of *Cissus quadrangularis* Linn. in Assam and providing scope for further research work to elucidate on the bioactive substances produced by them.

Materials and Methods

Collection of the plant sample

The plant sample (Stems and leaves) collection was done in two different seasons- Summer Season and Winter Season from three districts of Assam (Kamrup Metro, Barpeta and Biswanath Chariali, now

Sonitpur district). The samples were detached from the plants using sterilized scalpels and were kept in sterilized bags (Sandhu et al., 2014). Since the study did not involve the age of the plant parts hence the samples were collected from neither the tip of the plant nor the older parts of the plant. The Plant samples were then brought to the laboratory the next day for the study.

Ethics Statement

The plant samples were collected from the cultivated areas of the different locations under study and no specific permission was required for the same. The plant materials used in the experiment comply with relevant institutional, national and international guidelines and legislation. Plant samples were collected from the same plants for both the seasons. Only the stems and leaves of the plant samples were collected and hence no uprooting of the plant happened. This study did not involve any endangered or protected species.

Surface Sterilisation

The plant samples were at first washed properly to remove mud and dust particles. For isolating the endophytic species from the plant samples, surface sterilization is required to remove the epiphytes. The samples were first cut into small pieces and then washed in water properly to remove the dust particles. The samples were then treated with distilled water for 1 minute. The samples were then rinsed in 3% Sodium Hypochlorite solution for 30 seconds. The samples were then treated with 70% Ethanol for 60 seconds and thereafter finally rinsed in sterile distilled water (Rani *et al.*,2017). After this the samples are taken on a clean cheesecloth for soaking.

Isolation and Identification of Endophytic Fungi

Fresh PDA media was made as per the standard protocol (Varghese and Joy,2014) for isolating the endophytic fungi. The media was sterilized in an autoclave at 121⁰C for 15 minutes. The media was then inoculated with the plant samples for 6 days maintaining the temperature at 28 ± 2⁰C. Streaking fresh culture plates with the isolates was done at regular intervals till the appearance of the pure colonies. The endophytic fungal species were identified morphologically on the basis of colony morphology, shape and size of the spore (Rabha *et al.*, 2015; Nagamani *et al.*,2016). Some of the Fungal colonies have been represented in Fig. 2.

Isolation and Identification of Endophytic bacteria

Fresh Nutrient Agar Media was made as per the standard protocol (Varghese and joy,2014) for isolating the endophytic bacteria. The media was sterilised and then inoculation with the plant samples was done. The inoculated plates were maintained at a temperature of 25 ± 2⁰C. Streaking was done till the appearance of the pure colonies. For the identification of endophytic bacteria biochemical characterisation followed by Metagenomic sequencing of some of the isolates was carried out.

Biochemical test of Endophytic bacterial isolates

Gram staining, catalase test, Methyl red test, Indole test and citrate utilisation test was performed to study the biochemical nature of the isolates as per the standard protocol (Varghese and Joy, 2014) and has been reported in Table 3. Gram staining was done to differentiate between the Gram positive and Gram negative bacteria (Fig. 1).

Genomic DNA isolation

Genomic DNA of bacterial samples were isolated using a commercially available kit (Invitrogen Pure Link™ Genomic DNA Mini Kit). Isolated genomic DNA was visualised using a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Amplification of 16S rDNA

A 1500-bp sequence of bacterial 16S rDNA was amplified with primers 27F and 1492R (Frank, Reich *et al.* 2008). The 35-cycle amplification was performed in a DNA Thermal Cycler (Eppendorf Master cycler) using 6.25µl of 2X Platinum™ II Hot-Start Green PCR Master Mix (Thermoscientific), 0.25µl of 10uM concentrations of forward and reverse primers, 4µl sterile deionized water, and 2µl DNA template, for a total volume of 12.75µl. The temperature cycling program used was as follows: 1 cycle of pre-denaturation at 94°C for 5 minutes; 35 cycles at 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 1.30 minutes; and a final extension of 72°C for 10 minutes. For detection of PCR products, 4µl of the amplified DNA was run on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Sequencing

Impurified PCR product was shipped to Eurofins Genomics (Eurofins Genomics India Pvt. Ltd.) for Sanger sequencing. Purified PCR products were sequenced and analysed on an ABI 3730XL DNA sequencer.

The 16S rRNA gene sequences were compared with other 16S rRNA gene sequences available in GenBank by using the BLASTN program and aligned with similar sequences by using the MUSCLE program embedded in MEGA11. The phylogenetic tree was constructed by applying the neighbour-joining method using the MAGA11 program with 100 bootstraps.

Availability of Data and Material

All Data generated during this study has been mentioned in the paper. The datasets generated during the current study are available at 'NCBI- GenBank' with the following Accession numbers to the sequences submitted.

SUB 13361450 Seq1- OQ991266

SUB 13361450 Seq2- OQ991267

The data can be viewed in the weblink of NCBI <https://www.ncbi.nlm.nih.gov>

The supporting document related to the metagenomic data for the bacterial identification has been attached as 'Supplementary Files'.

Result and Discussion

A total of 16 endophytic fungal species were isolated including 5 sterile isolates and identified up to generic level based on their morphological features (Table 1). These endophytic species belong to Deuteromycetes, Eurotiomycetes, Sordariomycetes, Dothideomycetes and Zygomycetes of Kingdom fungi. The distribution of these fungal species varied in the different sites as well as the different seasons (Table 1). Endophytic distribution varies in different parts of the host plant and also differs with the seasonal variations (Figs. 3, 4 and 5). In all the three sites the Colonisation frequency of the endophytic fungal isolates was found to be more in the summer season as compared to the winter season except for few species. It clearly indicates that the difference may be due to the soil conditions of the area as well as the host plant. In the present study *Trichophyton rubrum* and *Absidia fusca* has been isolated from the plant samples collected from Biswanath whereas is found to be absent in the plant parts collected from Kamrup(M) and Barpeta. It may be attributed to the soil type of the area under consideration. The abundance of the Endophytic fungi is found to be more in stem as compared to the leaves in the present study (Table 2). 31.25% of the Endophytic fungal community comprises of the *Mycelia sterilia* isolated from all the plant samples of the three areas under consideration. Metagenomic sequencing can further establish the identity of these fungal species. These endophytic fungal isolates may have rich deposits of secondary metabolites that impart characteristic medicinal properties to the plant. *Alternaria* species isolated from different plants have been found to produce a variety of secondary metabolites of biological importance (Eram D *et al.*, 2018).

To establish the type of Endophytic bacterial isolate, Gram staining was done as per the standard protocol (Fig. 1). Further some biochemical tests were conducted to study the nature of the bacterial isolates. These tests included catalase test, Methyl red test, indole test and Citrate utilisation test. These tests were done as per the standard protocol. Some of the culture samples (5) based on the maximum number of colonies obtained were then processed for metagenomic sequencing. Based on the BLAST results and the phylogenetic tree, the organisms were identified to be belonging to *Bacillus* species- *Bacillus subtilis* and *Bacillus velenzensis* the former one being the dominant one. Endophytic bacteria can be a promising source of bioactive compounds. In one of the researches work it was found the extracts of the endophytic bacterial isolate from *Cissus quadrangularis* shows the presence of secondary metabolites having bioactive potential (Anwar et al., 2020). There is much scope to elucidate more on biochemical aspects of the Endophytic fungal and bacterial species that can be used to develop products for Human welfare.

Table 1
Fungal Endophytes isolated from the three districts of Assam

Sl. No.	Endophytes	Kamrup (M)		Barpeta		Biswanath	
		Summer	Winter	Summer	Winter	Summer	Winter
1	<i>Aspergillus sp.</i>	+	+	+	+	-	-
2	<i>Alternaria sp.</i>	+	+	-	-	-	-
3	<i>Aspergillus stellatus</i>	+	+	+	+	+	+
4	<i>Cochliobolus sp.</i>	-	-	+	+	+	+
5	<i>Colletotrichum</i>	+	+	+	+	+	+
6	<i>Curvularia sp.</i>	+	+	+	+	+	+
7	<i>Cladosporium sp.</i>	+	+	+	+	-	-
8	<i>Fusarium sp.</i>	+	+	+	+	-	-
9	<i>Trichophyton rubrum</i>	-	-	-	-	+	+
10	<i>Absidia fusca</i>	-	-	-	-	+	+
11	<i>Rhizopus sp.</i>	+	+	+	+	+	+
12	<i>Mycelia Sterilia I</i>	+	+	+	+	-	-
13	<i>Mycelia Sterilia II</i>	+	+	+	+	-	-
14	<i>Mycelia Sterilia III</i>	+	+	+	+	+	+
15	<i>Mycelia Sterilia IV</i>	+	+	+	+	+	+
16	<i>Mycelia Sterilia V</i>	+	+	+	+	+	+

* '+' represents present and '-' represents absence.

Table 2
Endophytic Fungi isolated from different parts of *Cissus quadrangularis* L.

Sl. No.	Name of Endophytic Fungi	Plant part	Class
1	<i>Aspergillus niger</i>	Stem	Eurotiomycetes
2	<i>Alternaria sp.</i>	Stem and Leaf	Deuteromycetes
3	<i>Aspergillus stellatus</i>	Stem	Eurotiomycetes
4	<i>Cochliobolus sp.</i>	Stem and leaf	Deuteromycetes
5	<i>Colletotrichum</i>	Stem and leaf	Sodaromycetes
6	<i>Curvularia sp.</i>	Stem	Dothideomycetes
7	<i>Cladosporium sp.</i>	Stem	Dothideomycetes
8	<i>Fusarium sp.</i>	Stem and leaf	Sodariomycetes
9	<i>Trichophyton rubrum</i>	Stem and leaf	Eurotiomycetes
10	<i>Absidia fusca</i>	Stem	Zygomycetes
11	<i>Rhizopus sp.</i>	Stem and leaf	Zygomycetes
12	<i>Mycelia Sterilia I</i>	Leaf	Class could not be established due to non-sporulation of the mycelia.
13	<i>Mycelia Sterilia II</i>	Leaf	
14	<i>Mycelia Sterilia III</i>	Stem and leaf	
15	<i>Mycelia Sterilia IV</i>	Stem and leaf	
16	<i>Mycelia Sterilia V</i>	Stem and leaf	

Table 3
Bacterial Isolates from *Cissus quadrangularis* L. and their Biochemical characterization

Sl.No.	Isolate no.	Gram test	Catalase test	Methyl red test	Indole test	Citrate Utilisation test
1	Bcpl1	+	+	+	+	-
2	Bcpl2	+	+	+	+	-
3	Bcpl3	+	-	+	+	-
4	Kmpl1	-	-	+	-	+
5	Kmpl2	-	-	+	+	+
6	Kmpl3	+	-	+	-	-
7	Brpl1	-	+	-	+	+
8	Brpl2	-	+	-	+	+
9	Brpl3	-	-	+	+	+
10	Brpl4	+	-	-	+	+

Conclusion

A single plant can harbor many endophytic species which is evident in this study. The plant samples collected from the three districts of Assam confirms this statement. A total of 16 Endophytic fungal species have been isolated and from the stems and leaves of the plant samples collected from the different locations and some variation was found in the distribution of the endophytic species in these three districts (Figs. 3, 4 and 5). Some variation was also found in the distribution of the fungal species in the stems and leaves of the plant as well. This difference in the distribution may be due to the climatic and edaphic factors of the areas under study. This research work provides further scope for elucidating more on the endophytic microflora of *Cissus quadrangularis* in other districts of Assam too.

Declarations

Conflict of Interest

The authors have no conflict of Interest.

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Author Contribution

The entire field and laboratory work in the present study was carried out by the author no. 1 Ms. Namita Deka under the supervision of Dr. Saranga Ranjan Patgiri and Dr. Bidisha Sharma. The Results were analyzed by the corresponding author while the Discussion and Conclusion part of the article was jointly prepared by the 1st and the 3rd authors.

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Figures

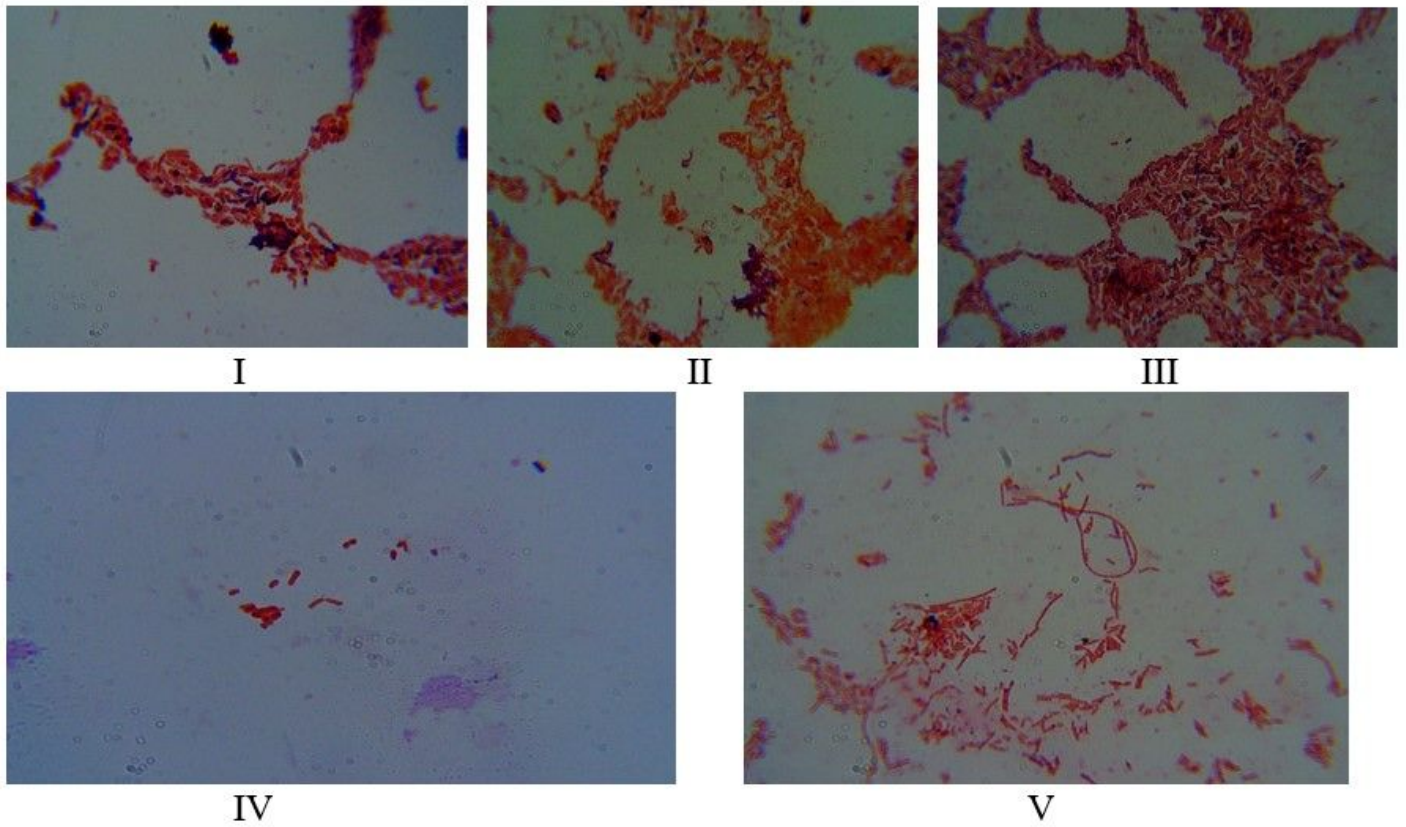


Figure 1

Microphotographs of Endophytic Bacterial isolates (I-V) of *Cissus quadrangularis* on Gram's staining.

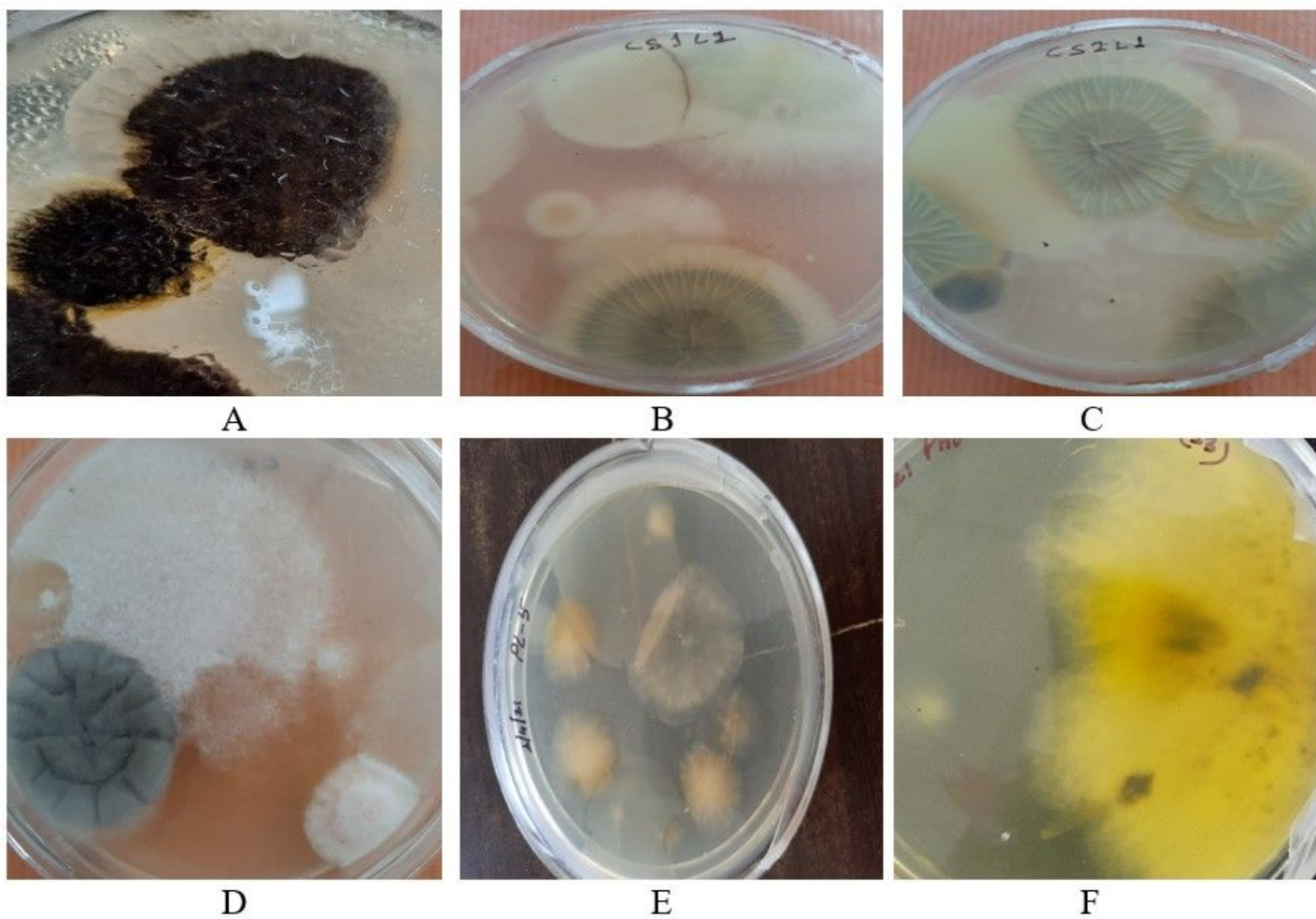


Figure 2

Photo graphs of some of the Endophytic fungal colonies (A-F) of *Cissus quadrangularis*

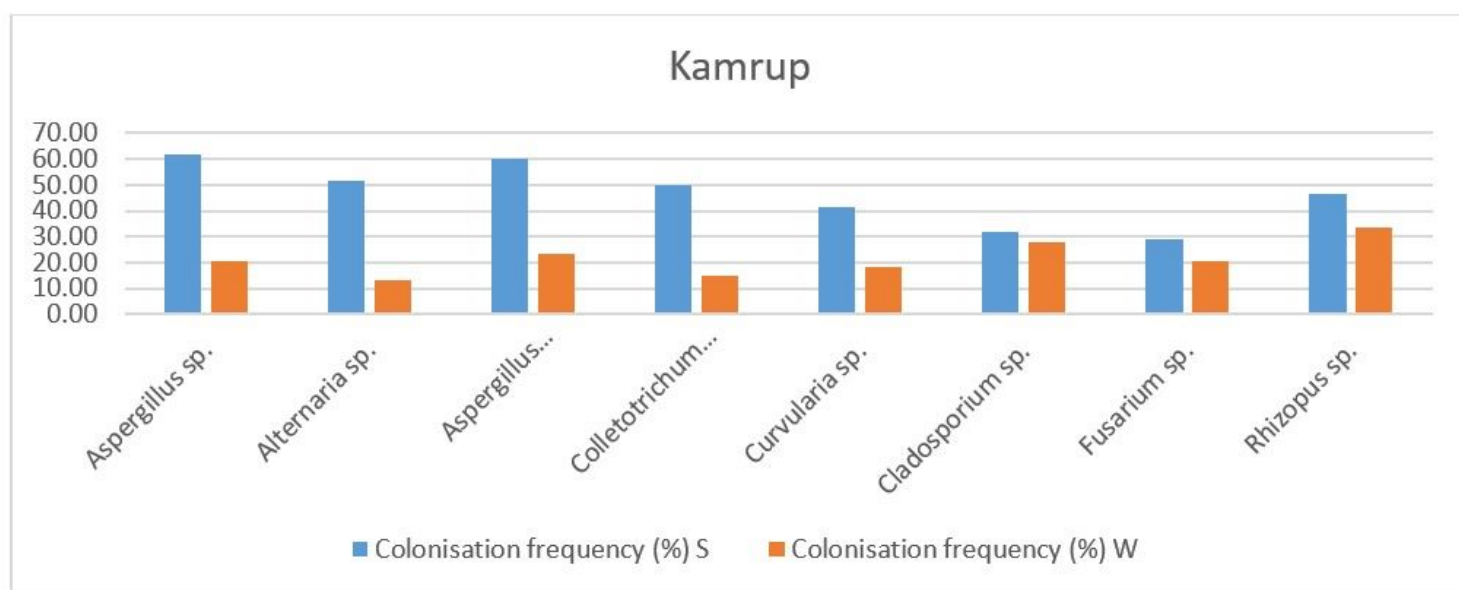


Figure 3

CFS represents colonization frequency in summer and CFW represents colonization frequency in winter.

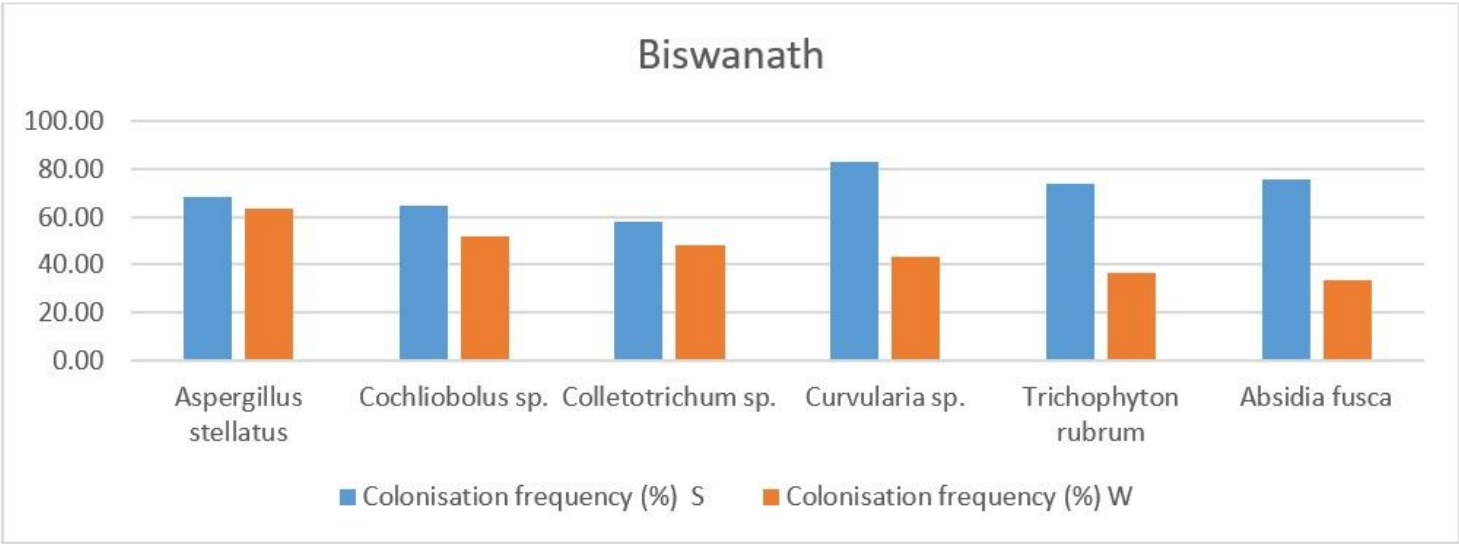


Figure 4

CFS represents colonization frequency in summer and CFW represents colonization frequency in winter.

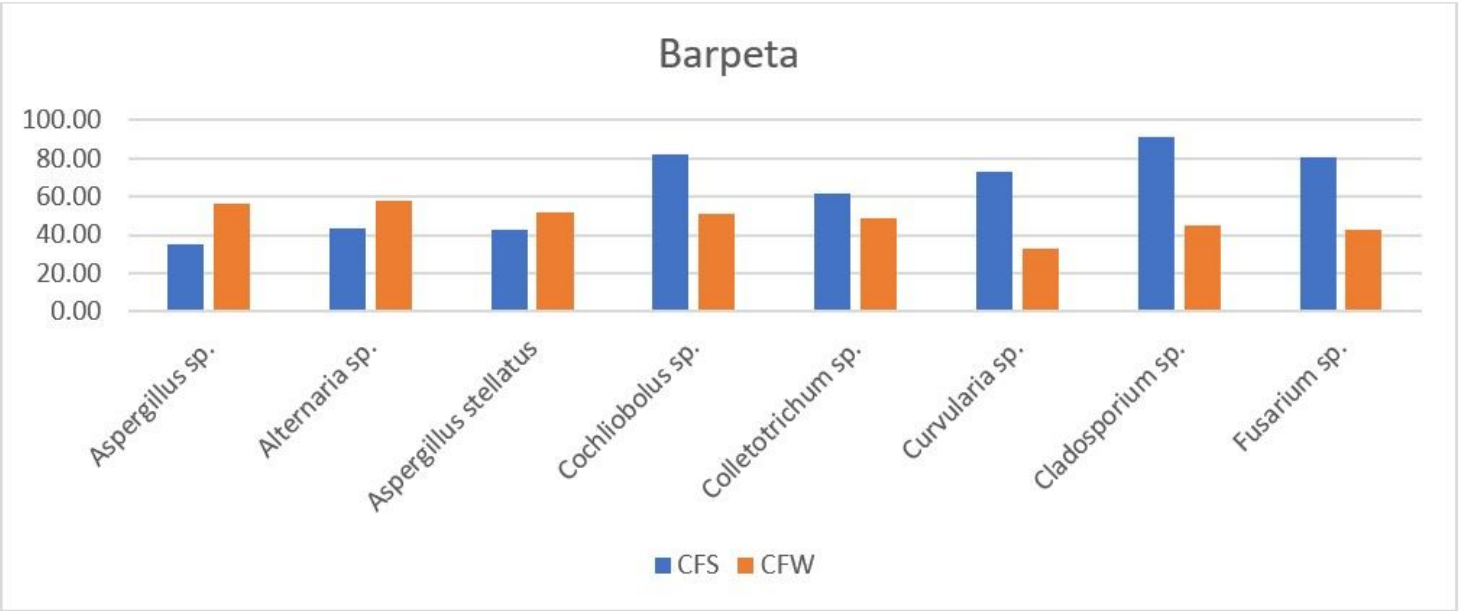


Figure 5

CFS represents colonization frequency in summer and CFW represents colonization frequency in winter.

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