Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Spl Issue [1] January 2023 : 182-186.

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Soil indigenous microbial flora for the preparation of leaf venation of leaves

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Abstract
Leaf venation is an important feature for botanists and taxonomists to identify and catalogue a plant species because venation orientation and quantitative characters are relatively stable at the species level. Present methods of leaf clearing are mostly chemical-based, which are harsh on specimens and cause damage or shrinkage to tissue structure, aside from causing toxic effects on the biota of the soil. The current study attempted to establish a non-toxic and cost-effective biological leaf clearing method for the preparation of leaf venation of leaves using indigenous microbial flora of soil extract. Cellulosic digestion due to the enzymatic activity of indigenous microbial flora leading to leaf vein skeleton formation was reported in Ficusreligiosa, Ficusnuda, Ficusbenjamina, Ficusheterophylla, Artocarpuslakucha, and Artocarpusheterophyllus with different exposure time periods.
***Keywords**: Leaf clearing, Leaf venation, Plant studies, Soil indigenous microbial flora.*

Received 29.10.2022 Revised 23.11.2022 Accepted 19.12.2022

INTRODUCTION

Leaf architecture is the hierarchical arrangement of different sizes and patterns of veins found on the surface. The arrangement of leaf veins creates a distinct and intricate pattern known as leaf venation. Leaf architecture, viz., the shape, size, margin, leaf base, tip, veins, and petioles, is correlated with plant evolution and has systematic significance in plant identification and classification[1].Leaf venation is a key to understanding vascular patterning and tissue differentiation, and the leaf architecture impressions are essentially very relevant for plant macrofossils available to paleobotanists and aid in identifying fossil samples with greater phylogenetic resolution. Leaf venation pattern plays a very important role in the identification of incomplete plants, e.g., sterile specimens, archaeological remains, and fragmentary fossils of non-reproductive organs [2]. Leaf vein clearing has been widely used for decades in plant microtechnique for preparing whole mount specimens [3]. Whole-leaf preparations are much more useful than thin sections in enabling the rapid examination of the whole infection process, including the leaf surface and within-leaf phases, and the measurement of quantitative differences between infections [4]. It has also been used for the detection of various fungal diseases associated with plants and has been found effective for assisting fungal spore germination counts on several leaves [5]. Even though leaf venation is broad, there are numerous scientific applications for studying leaf veins. However, data is frequently scarce [6]. Because there are insufficient techniques developed for the long past period, such techniques are used less frequently, resulting in a significant reduction in the number of taxonomists [7]. The technique of leaf vein clearing is the most effective way of preserving leaves. Thus, by selecting the appropriate method of clearing that results in intact leaf venations with unaltered morphology can be used in the preservation of endangered species, which can later be used both as fossils in the study of evolution and in the identification of plant material [8]. Therefore, for all the studies mentioned above, it is important to have a way to observe even the smallest details of veins. To this end, numerous leaf vein clearing techniques have been developed. Leaf vein clearing methods are designed to degrade the interveinal tissue of leaves without damaging the venation architecture. The interveinal tissues of leaves are traditionally cleared using a variety of chemical techniques, such as chloral hydrate, sodium bicarbonate, trichloroethanol, trichloracetic acid, and sodium hydroxide [9–11]. Unfortunately, the majority of these chemical treatments are harsh on the specimens and result in structural damage or shrinkage. Cleared leaves obtained through a chemical-based method are fragile with broken veinlets as the chemical is harsh on specimens and causes tissue structure shrinkage [12]. Additionally, the chemicals pollute the environment and are caustic. Hence, there

is a need to develop a technique that entirely removes the interveinal tissue without damaging the venation pattern and is also non-toxic and environmentally acceptable. The majority of reports on leaf vein clearing so far found in the literature include the use of chemical methods. On the other hand, there are hardly any reports in which microbial activity was used. The use of microorganisms as a medium for making transparent leaves is economically and environmentally friendly [13]. Soil is a natural nutrient medium that is widely available. The soil is composed of organic and inorganic matter, which favours the nourishment of a variety of microorganisms. The indigenous microbial flora of soil is primarily composed of avariety of bacteria, viz., cellulolytic Clostridia and Bacillus [14]. Fungi, viz., Aspergillus, Fusarium, Rhizopus, Penicillium, etc.[15]. Actinomycetes, viz., cellulolytic streptomyces, Nocardia, etc. [16]. Due to their extensive repertoire of enzymes, microbes have the capacity to degrade a wide range of organic natural materials, converting complex polymers into monomers [17]. Taking into consideration the enzymatic potential of soil microbial flora, in the present study, an attempt was made by using a soil extract containing indigenous microbial flora for leaf clearing.

MATERIALS AND METHODS

(i) Selection of plants:

The present study entitled "Soil indigenous microbial flora for the preparation of leaf venation of leaves",was carried out at the Research Laboratory, Department of Botany, Prof. Ramkrishna More Arts, Commerce, and Science College, Akurdi, Pune, during 2020–2022. Six plant species from the family Moraceaewere chosen for the investigations on the preparation of leaf venation of leaves. Of these, two are from the Genus Artocarpus, including *Artocarpuslacucha* and *Artocarpusheterophyllus*, and four belonging to the Genus Ficus, including *Ficusreligiosa, Ficusnuda, Ficusbenjamina*, and *Ficusheterophylla.*

Selection of leaves from plants under study:

All six healthy plants fully grown leaves were chosen, gathered, and brought to the laboratory in polythene bags. These leaves had a firm texture and were free of insect or environmental damage. These leaves were then thoroughly washed to remove any dust, if any, and were used in the study.

(ii) Preparation of soil extract:

In the study, a soil extract containing microbial flora was utilised in order to exploit the
Indigenous microbial flora for leaf clearing. It was made as follows:
(iii) A 50 g sample of fertile soil was collected in polythene bags from an area rich in decomposed leaf matter (and thus rich in leaf decomposing microorganisms). The soil sample thus collected was crushed with a mortar and pestle and then used. A total of 40 g of soil sample was added to 400 mL of sterile, distilled water and homogenized. This sample was filtered and centrifuged at 2000 rpm for 30 minutes to settle any soil particles. The supernatant was collected and used.
(iv) **Sterilization of supernatant**
To kill indigenous microbial flora from the supernatant, 200 ml of the supernatant, as obtained by the above-mentioned method, was sterilised in a flask at 121 oC for 15 minutes in an autoclave. The sterilised solution thus obtained was then used at a 10% concentration for the treatment of leaves.
To study the effect of indigenous microbial flora on leaf clearing, experiments were conducted in three sets, as detailed below.
Set I Flask containing 180 mL of sterile distilled water + 20 mL of soil extract (supernatant without sterilization) and leaves in submerged conditions.
Set II Flask containing 180 mL sterile distilled water + 20 mL of sterile supernatant of soil extract and leaves in submerged conditions.
Set III Flask containing 180 mL sterile distilled water leaves in submerged conditions.
A total of eighteen sets, three for leaves of each of six selected plants as detailed, were prepared and used separately, and kept for clearing with intermittent observation. After an optimal period, the almost-cleared leaves were taken out of the solution and kept in a vessel of clean water. The tissue between veins was carefully removed by brushing from the middle rib to the leaf edge, so as to obtain intact leaf venation as much as possible withoutdamage, and results were recorded.

RESULTS AND DISCUSSION

The results of three sets for each of six selected plants were obtained and compared; it was found that clearing of leaves occurred in Set I but not in Sets II and III. The clearing of leaves in Set I was attributed to the enzymatic activity of indigenous microbial flora in soil extract. No clearing effect was observed with treatment as per Set II, as there was no living microbial flora due to the use of sterilised supernatants of soil extract and no enzymatic activity at all. No clearing of leaves was found with sterile distilled water in Set III due to the lack of microbial flora in it. These reports clearly indicated the role of

indigenous microbial flora, which showed the digestion of interveinal tissue and hence the development of leaf venation.

The duration required for the degradation of interveinal tissue and the formation of leaf venation varies with different plant materials. *Ficusreligiosa* (Fig. 1) and *Ficusnuda* (Fig. 2) were cleared in seven days. *Ficusbenjamina* (Fig. 3) was cleared in eight days. *Ficusheterophylla* (Fig. 4) was cleared in ten days. *Artocarpuslacucha* (Fig. 5) was cleared in five days, and *Artocarpusheterophyllus*(Fig. 6) required nine days to clear.

The indigenous microbial flora of soil extract cleared the leaves (family Moraceae); the microorganisms were found to vigorously disintegrate the chlorophyll and other green contents of the cell, leaving behind an intact network of veins. The use of microorganisms to separate plant vascular skeletons is not new. The corn stems were retted after a few days in a warm place [18]. The immersion of leaves in an algae tank was suggested until the mesophyll was eaten away by microorganisms, leaving an intact vascular skeleton [19]. The method utilising an anaerobic fermentation process has been used for the isolation of protoplasts from vegetable leaves [20]. The recovery of rubber was done from *Cryptostegia* to isolate and measure the full extent of the leaf veins [21]. Our results are similar to the results obtained by these workers.

In investigating the effect of time on the clearing of various leaves with consortium, it was observed that the leaves showed some time variation in the formation of a complete network of veins, in which most of the leaves were cleared in a week's time but thick leaves took more time. Similar results of time variation in leaf vein clearing were obtained by Saima [13]. Whittenberger [21] reported that the time required for the completion of fermentation varies with the type of leaf, although 2-3 days are sufficient for leaf skeletonization. The preparation of vein skeletons from the leaves of a number of species of Clostridium roseum was reported. However, thick xeromorphic leaves require a longer period than loosely compacted mesomorphic ones. Alejandra [22] observed that the clearing of leaves can take from one day to several weeks, depending upon various leaf qualities like size, thickness, and chemistry.

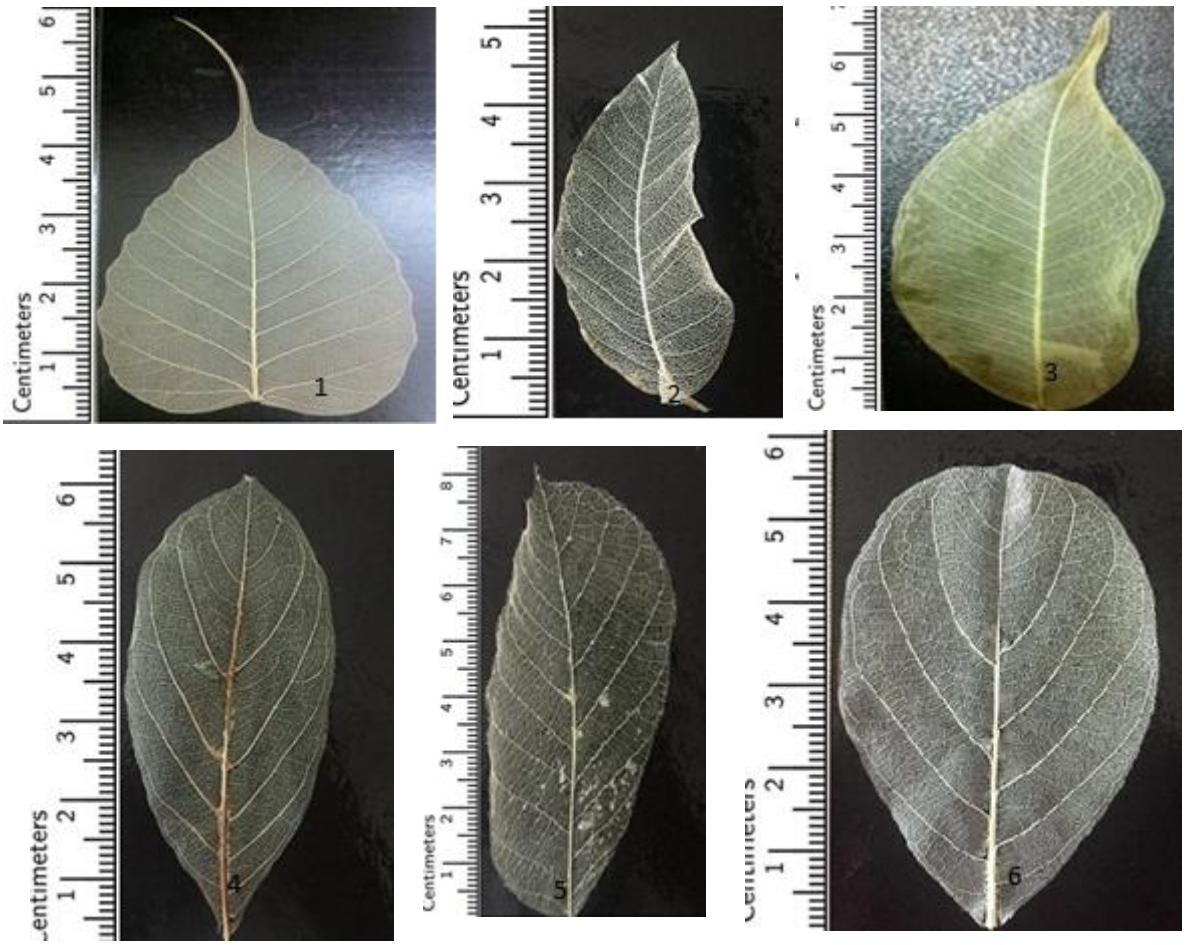

Fig.1. *Ficusreligiosa*. Fig.2. *Ficusnuda*. Fig.3. *Ficusbenjamina*. Fig.4. *Ficusheterophylla* Fig.5.*Artocarpuslacucha*. Fig.6.*Artocarpusheterophyllus*.

CONCLUSION

The techniques of leaf clearing render the tissue transparent, enabling the observation of deeper layers of cells and making it effective for applications in research, teaching, and taxonomical studies. Microbial flora in the soil clears the leaves without harming the venation architecture. Thus, soil extract is effective in leaf clearing as it contains a variety of microorganisms with lignocellulose digestion capacity. This method of leaf clearing is safer and less toxic.

Acknowledgement

The authors thankfullyacknowledge the financial support for the study was provided by the Department of Science and Technology, New Delhi, India. The generous support for carrying out the study in the laboratory of Department of Botany, Prof Ramkrishna More Arts Commerce and Science College Akurdi, Pune, Maharashtra is also acknowledged.

Conflict of Interest Disclosure

Conflict of interest declared none.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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CITATION OF THIS ARTICLE

R.M. Saima and B.M. Shinde: Soil indigenous microbial flora for the preparation of leaf venation of leaves, Bull. Env.Pharmacol. Life Sci., Spl Issue [1]: 2023:182-186.