Jour Pl Sci Res **36 (1-2)** 333-338 2020

Leaf Clearing: A Review

Saima Rashid Mir[1]* V.A Patil[2], B.M Shinde[1] and M.M Jana[2]

Department of Botany Prof-Ramkrishna More A.C.S College Akurdi Pune

Corresponding author e-mail : saimarashidmi@gmail.com

The clearing of leaves to reveal the leaf venation can be successfully accomplished by diverse methods. Leaf venations are an important feature for botanists and taxonomists to identify and catalogue a plant species, it has several waves of interest among morphologists and paleobotanists in studying of plant material total as such. Cleared leaves of different plant species can be stained and mounted to form ineradicable and permanent specimens applicable for research and class room study. The present paper depicts the review of literature on leaf clearing and its significance in Plant Sciences.

Keywords: Leaf clearing, Leaf venations, Plant Sciences.

INTRODUCTION

A review of literature plays an important role in depicting quantum of work done in related area of study. A brief review of literature on leaf clearing may be helpful in grasping the existing scenario, more over in understanding the significance of subject, untouched areas and hence the scope of work to be carried out.

Leaf veins are hierarchical fine lines of variant sizes and designs found on the surface of the leaf (Saba, *et al.,* 2012). This venation network is chiefly composed of lignified xylem and phloem which has various functions including transport of water and sugars, mechanical support etc (Roth-Nibelsick*, et al.,* 2001) Leaf framework including size, shape margin, leaf base, tip, veins and petiole plays important role in classification, systematic and ecology (Ellis, *et al.,* 2009) The venation patterns are important features for classification and evolution of angiosperms than the other leaf characters, because their orientation and quantitative characters are relatively stable at the species level (Fang, *et al.,* 2002; Haung, *et al.,* 2004). The plants can be identified on the basis of its external structures such as leaf, seed, fruits and flowers in accordance to the plant taxonomy theory (Goeau, *et al.,* 2013). However, in various studies leaf characteristic were found more significant in species identification (Hoshang, *et al.,* 2018). The study of leaf architecture was initially explored by paleobotanists in 1950s (Foster, A. 1936).The leaf venations are applicable to extensive areas of research, these include the evolution of leaf form and function (Boyce and Knoll, 2002) genetic and other mechanism in the ontogeny of leaf venations (Candella*, et al*., 1999) applications in systematic and evolutionary biology, veins are useful in taxonomy (Ellis, *et al*., 2009) and for studying climatic change and macro evolutionary trends (Boyce, *et al*., 2009; Brodribb and Field, 2010; Field, *et al.,*. 2011). For developmental biologists veins are the prominent features in understanding vascular patterning and tissue differentiation (Candela, *et al*., 1999; Scarpella, *et al*. 2010; Sack*, et al.* 2012). Leaf vein impressions are the most abundant plant macro fossils available to paleobotanists, thus the ability to more rigorously quantify vein geometry has the potential to aid attempts to identify fossil samples with greater phylogentic resolution (Behrenmeyer*, et al.,*1992). Levin(1929) explained that leaf venation patterns have high taxonomic value and suggested that a species has a constant number of veins that can be used for species identification Dilcher(1974) stated that a study on the nature and structure of leaf venation has significant implications for the relationship between taxonomy and phylogeny.

With suitable method of clearing the venation network of diverse leaves can be exposed and can be largely used for all the applications mentioned above. In botanical and taxonomical research the technique of

DOI:-https://doi.org/10.32381/JPSR.2020.36.1-2.42

clearing leaves has been used over centuries and a number of chemical clearing methods have been developed Bates(1931);Foster(1949; 1950); Fuchs (1963); Hearing and Nicholson (1964); Rodin and Davis (1967); Morley(1968); Gardner(1975); Berlyn, *et al,* (1976); Jackson and Snowdon (1990); Herr (1971;1993); Liang and Herr (1994); Sack, *et al.* (2006); Dunber, *et al*. (2009); Rolland-Lagan, *et al*. (2009); Scoffoni, *et al*. (2011) and Perez-Harguindeguy and Diaz (2013). There are reports in the literature regarding leaf clearing technologies using different chemical clearing agents such as sodium bicarbonate, acidified chloral hydrate, NaOH, trichloroacetic acid, trichloroethanol and phenol etc. But nearly all the chemical clearing agents used are harmful and yield fragile venation. Use of microbial culture for leaf clearing is under trial by several researchers and is eco friendly, toxicity free and the products are strong, versatile and can be used widely for a number of applications (Saima *et al*., 2019).

Various methods of leaf clearing described in the literature.

Simple method of leaf vein clearing was described by Irene(1878) who kept the leaves in a jar containing one tea spoonful of muriatic acid in one gallon of rain water and placed in warm place until the vegetable matter was softened and easily removed. The leaf clearing method developed by Coleman, *et al*. (1964) involve an initial bleaching process, acidified with the addition of acetic acid, this method was found very useful to observe fungal hyphae on oak leaves from litter and to differentiate hyphae inside the leaf from those on the surface. Lersten(1967) mentioned the use of acidified chloral hydrate as commonly used leaf clearing solution. Dilcher (1974) during the study of identification of angiosperm leaf remains, published botanical review in which Dilcher mainly focused on history of leaf preparation methods and listed many of the clearing and mounting procedures used up to the time. Mohan, *et al*. (1978) in their report mentioned that leaf clearing can be attained by soaking portions of leaves in a mixture of trichloroacetic acid and phenol in 2:1 proportion for 10 -15 minutes at 60^{0c} these can be stained with writing ink. The intensity of staining does not change even in permanent preparations and therefore specially beneficial for studying the morphology of fungi parasitic on leaves. The Leaf clearing solution consists of ethanol, chloroform, lactic acid, phenol and chloral hydrate was used for the clearing of wheat leaves for the detection of rust and powdery mildew infection in leaf tissues (Keane, *et al.,* 1988)

Clearing of leaves was attained with 5% NaOH solution followed by acetolyzation and further clearing with 5% sodium hypochlorite to study the foliar morphology of *Gunnera insignis* (Fuller and Hickey. 2005). Micheal, *et al.* (2001) reported the use of sodium bicarbonate as a clearing agent in leaf clearing. The combination of lactic acid with chloral hydrate was found effective in clearing of free hand sections of plant tissues, such sections could be mounted directly in the clearing solution for bright field microscopic observation (Alexandar, *et al.,* 2005). The method of boiling the leaves in the solution containing NaOH was reported by Murgan, *et al.* (2007) to obtain the leaf venations. Visikol clearing solution containing trichloroacetic acid and trichloroethanol was found an effective clearing agent in clearing whole mounted fresh and dried material, it renders the tissue transparent and allows the visualization of deeper layers of tissues (Thomas *et al.,* 2013). Alejandra *et al*. (2014) reported clearing by immersing the leaves in 5.0 % solution of NaOH for one to several days at 40 – 45^{O}C till the leaves were cleared. Fosters method of clearing was used by Xiao, *et al.* (2017) in studying the taxonomic and phylogenetic significance of leaf venation characteristics in Discora plants.

There are some biological methods which have been used in the past for the isolation of protoplast from the leaves. The use of microorganisms to separate plant vascular skeletons is not new. Loomis and Shull (1937) suggested the immersion of leaves in an algae tank until the mesophyll was eaten away by microorganisms, leaving an intact vascular skeleton. The recovery of rubber was done from cryptostegia to isolate and measure the full extent of the leaf veins as reported by Whittenberger, *et al.* (1945). The method utilizing an anaerobic fermentation process has been used for the isolation of protoplasts from vegetable leaves as reported by White, *et al.* (1948).

Evans, (1928; Loomis and Shull, 1937; Sharman, 1942) described methods for separating vein tissue (xylem) from other plant tissues, the method developed by Naghski and associates (1945) proved particularly adapted for Cryptostegia leaf. Whittenberger, *et al.* (1948) reported the fermentation of leaves with the pure culture of *Clostridium roseum* for the preparation of vein skeletons and epidermis from leaves of a number of species, fermentation was established in the flasks by use of a water trap and by flushing with carbon dioxide or nitrogen for 2-3 min, this anaerobic bacterium was found vigorously digesting cellulose cell walls but leaves lignified, suberized and cutinized walls essentially unaltered. Sterility and aseptic conditions were observed only in the preparation of the inoculum, for *Clostridium roseum* maintains an essentially pure culture when incubated anaerobically, even with an unsterilized leaves. The majority of leaves cleared with *Clostridium roseum* fermentation were *Liriodendron tulipifera, Ligustrum japonicum, Salix vitellina, Philodendron verrucosum, Cryptostegia grandiflora, Coleus blumei, Poa pratensis* and *Saintpaulia ionantha.*

In this way leaf clearing can be attained with both biological and chemical methods, biological methods are much safer and less destructive to tissue morphology of venation networks, but there are very few reports on leaf clearing with biological methods. The cleared leaves obtained from chemical clearing methods used for the preparations of permanent mounts cannot be used for longer periods because of the disintegrated nature of venation system, unfortunately efforts to restore the damaged mounted leaves are time consuming and expensive (Erika Gonzalez, Smithsonian Institution, personal communication, 2011). Most of the clearing methods described in the literature are based on the use of chemicals, linked with toxicity issues in which some of the chemical viz chloral hydrate, the clearing ability of chloral hydrate has been known for nearly a century and has been widely used in the examination of different plant structures (Lersten.1967) chloral hydrate is a controlled substance and is banned in several countries as longer exposure of this chemical can lead to lot of health issues (Thomas, *et al.,* 2013). The resultant product in chemical clearing methods is fragile with broken veinlet as the chemicals are harsh on specimens and caused shrinkage on tissue structure, hence deteriorated venation networks are difficult to use for any application (Saima, *et al.,* 2019). Therefore there is a need to develop non toxic and effective method of leaf clearing for plant related studies.

Discussions

Leaf clearing has enormous applications in Plant Sciences, it has been widely used for decades in plant microtechnique for preparing whole mount specimens (Alexander, *et al.,* 2005). Whole leaf preparations are much useful than thin sections in enabling the rapid examination of whole infection process including leaf surface and within leaf phases and measurement of quantitative differences between infections (Kaene, *et al.,* 1988) Clearing has been also used for the detection of various fungal diseases associated with plants and it has been found effective to assist fungal spore germination counts on several leaves (Gerrit, *et al.,* 1962). Even though leaf venations has broad scientific applications, but the study of leaf veins is often data limited (Price, *et al.*, 2011) as there are no sufficient techniques developed for long past period, results in minimum use of such techniques and hence reduction in the number of taxonomist to great extent (Lisa, W. Drew.2011). The technique of leaf vein clearing is the most effective way of exposing the inner mantle of leaves for the applications related to the Plant Sciences, thus by selecting the appropriate method of clearing that results in stronger leaf venations with unaltered morphology can be adapted for the number of applications, the same technique can be used for the preservation of endangered species which later can be utilized as fossils in study of both evolution and taxonomy.

Conclusion

The technique of clearing render the tissue transparent allowing the observation of deeper layer of cells and making it effective in research, educational and plant related applications. Different chemical methods used for clearing of leaves alter the tissue morphology. Due to toxic nature of chemicals, these methods are always linked with health issues. Hence there is a need to

develop safer and toxicity free and moreover eco-friendly leaf clearing technique. To satisfy the need of time, various researchers are trying microbial aspects to yield stronger and long lasting products. Structures obtained by leaf clearing can be effectively used for research in Plant Sciences.

Acknowledgement

We acknowledge the resources and financial support for the study was provided by the Department of Science And Technology, New Delhi, India.

References

Alejandra Vasco, Marcela Thadeo, Margaret Conover Douglas C. Daly (2014) *Applications in plant sciences.*

Alexandar Lux, Shigenori Morita, Jun Abe Kaorito (2005) *Annals of Botany.* 96:989-996.

Bates JC (1931) A method of clearing leaves. *Am Nat.* 65:28.

Behrenmeyer AK, Damuth JD, DiMichele WA, Potts R, Sues HD, Wing SL (1992) Terrestrial ecosystems through time: Evolutionary Paleoecology of terrestrial plants and animals. University of Chicago. Press, Chicago.

Berlyn GP & Miksche JP *(1976)* Botanical Microtechnique and Cytochemistry. Iowa State University Press, Ames.

Boyce CK & Knoll AH (2002) *Paleobiology.* 28:78-100.

Boyce CK, Brodribb TJ, Field TS, Zwieniecki MA (2009) *Proceedings of the Royal Society B: Biological Sciences.* 276:1771-1776.

Brodriff TJ, Field TS, Sack L (2010) *Functional plant Biology.* 37:488-498.

Coleman RE & Nickell LG (1964) A clearing technique for the examination of fungi in plant tissues. *Nature.* 942-943.

Candela H, Martine-Laborda A, Micol J.L (1999). *Developmental Biology.* 205:205-216.

Dorian Q Fuller & Leo J. Hickey (2005). The Botanical Review 71(3): 295–353.

Dilcher DL (1974) Approaches to the identification of angiosperm leaf remains. *The botanical review.* 40: 157.

Dunbar-Co S, Spork MJ, Sack L (2009) *International Journal of Plant Sciences.*172:479-489.

Ellis B, Daly DC, Hickey LJ, Mitchell J, Johnson K, Wilf P, Wing S. (2009) Mannual of leaf architecture, New York, NY, USA: New York Botanical Garden.

Evans AT (1928) *Bot. Gaz.* 85:97-103.

Fang Y, Liu J, Jiang Y (2002). *J Fujian Norm Uni (Nat Sci ed)* 18:65-9.

Foster A (1936) Leaf differentiation in angiosperms. *Bot Rev.* 2:349-72.

Foster AS (1949) Practical plant anatomy, 2nd ed. D. Van Nostrand Co.,New York, New York, USA.

Foster AS (1950) Morphology and venation of the leaf in Quiina acutangula Ducke. *American Journal of Botany.* 37: 159– 171

Field TS, Brodriff TJ, Iglesias A, Chatelet DS, Baresch A, Upchurch GR Jr, Gomez B, Mohr BAR, Coiffard C, Kvacck J (2011). *Proceedings of the National Academy of Science,* USA. 108:8363-8366.

Fuchs C (1963). *Stain Technology.* 38 141-144.

Gardner RO. (1975). *Stain Technology*. 50: 99-105.

Gerrit B & Richard Goss J (1962). *Nature.* vol no.193:1099-1100.

Goeau H, Bonnet P, Jolly A, Baki·c V, Barbe J, Yahiaoui I, Selmi S, Carre J, Barthelemy D, Boujemaa, N: et al:PI@nt net moble app.In (2013) Proceedings of the 21st ACM International Conference on multimedia.

Haung C & Haung A (2004). *Guangdong For Sci Technol.* 20:16-9.

Hearing TF & Nicholson PB (1967). *Nature London.* 201, 942-94.

Herr JM (1971) *J.Elisha Mitchell Sci. Soc.* 88:137-143.

Herr JM (1993) Clearing techniques for the study of vascular plant tissues in whole structures and thick sections. Proceedings of the Fifth Workshop/Conference of the Association for Biology Laboratory Education (ABLE), Toronto, Ontario, Canada.

Hoshang Kolivand, Bong Mei Fern, Tanzila Saba, Mohd Shafry, Mohd Rahim, Amjad Rehman (2018). *Arabian Journal for Science and Engineering.* 44:3315–3327.

Irene L. Rogers. Improvement in process of skeletonising leaves. (1878) United State Patent Office, Patent no, 211054. Application filed November 23- 1877.

Jackson BP & Snowdon DW (1990) Atlas of microscopy of medicinal plants, culinary herbs and spices. Belhaven Press, London, United Kingdom.

Keane Limongiello N & Warren MA (1988) *Australasian journal of pathology.* vol.17. 37-38.

Levin FA (1929) The taxonomic value of vein-islet areas based upon a study of the genera *Barosma, Cassia, Erythroxylon* and *Digitalis. Journal of Pharmacology and Pharmacy.* 2:17–43.

Lisa Drew W (2011) Are We Losing the Science of Taxonomy? As need grows, numbers and training are failing to keep up. BioScience, Volume 61, Issue 12: 942–946.

Lersten NR (1976) *Iowa state journal of science.* 41:481-486.

Liang D & Herr JM (1994). *Biotechnic & Histochemistry*. 69: 279 –282.

Loomis WE & Shull CA (1937) Methods in plant physiology. McGraw-Hill Book Co., Inc.New York.

Mohan Ram HY & Vijay Laxmi Nayyar (1977) *Indian Acad Sci, Vol 870B (Plant Sciences-2)* 125-127.

Morley T (1968) Accelerated clearing of plant leaves by NaOH with oxygen. *Stain Technology.* 43. 315-319.

Michael Dana N & Rosie Lerner B (2002) Preserving plant materials. Department of Horticulture Purdue University Cooperative Extension Service West Lafayette, IN.

Murugan PA, Thiyagarajan G, Ramesh K. (2007) Dry flower technology; [Online] available from http://www.indg.in/agriculture/agri-business-1/Dry-flower%20production.pdf

Naghski j, White JW, Hoover SR, Willman JJ (1945) *Jour Bact.* 49:563-574.

Perez- Harguindeguy N, Diaz S, Garnier E, Lavorel S, Poorter H, Jaureguiberry P, Bret-Harte M, Cornwell W, Craine J, Gurvich D (2013) *Australlian journal of Botany*. 61:167-234

Price Wing A & Weitz JS S (2011) Scaling and structure of dicotlydenous leaf venation network. Ecology Letters 15:87-95.

Rodin RJ & Davis RE (1967) The use of papain in clearing plant tissue for whole mount. *Stain Technology.* 42:23-28

Roth-Nibelsick A, Uhl D, Mosbrugger V, Kerp H. (2001) *A review. Annals of Botany.* 87:553-566.

Rolland-Lagan AG & Amin Pakulska M (2009) Quantifying leaf venation patterns: Two dimensional maps. *Plant journal.* 57:195-205

Saba T, Rehman A, Sulong G (2012) *J Theor. Appl Info Technol.* 17: 32-36.

Sack L & Frole K (2006). *Ecology.* 87: 483-491.

Sack L, Scoffoni C, Mckown AD, Frole K, Rawls M, Havran JC, Tran H, Tran T (2012) *Nature Communications.* 3:837.

Saima Mir, Shinde BM, Chasker MG, Jana M.M (2019) Dry Flower Technique: A Skill Development for Women Empowerment. NBRI/RS/24. Abstract book on "New Age Opportunities & Challenges for Quality, Safety & GMPS in Herbal Drug Development" held in Lucknow on February 22-23.

Scarpella E, Barkaulas M, Tsiantis M (2010) Leaf and vein development by auxin Cold Spring Harbor Perspective in Biology. 2:a001511.

Scoffoni C, Rawis M, McKown A, Cochard H, Sack L (2011) *Plant Physol.* 156:832-843.

Sharman BC (1942). *Bot. Gaz.* 103:627-629.

Thomas S. Villani, Adolfina R. Koroch, James E. Simon (2013) *Applications in plant sciences.* 1(5):1300016.

Whittenberger RT & Naghski J (1948) *American Journal of Botany.* 35:719 -7226.

Whittenberger RT, Brice BA, Copley MJ (1945) *India Rubber World.* 112:319-323.

White J. W JR, Weil L, Naghski J, Della Monica ES , Willaman JJ (1948). *Ind. Eng. Chem.* 40:293-297.

Xiao-Qin Sun, Jia–Yu Xue, Zhao Lei, Mi-Mi Li, Yan-Mei Zhang, Guang-Can Zhou, Yue-Yu Hang (2017) *Archives of Biological Sciences.* 70(00):58-58.

Received: 08-06-2020

Accepted: 12-06-2020