Chapter – 5

Methods and Techniques in Collection, Preservation and Identification of Lichens

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1. Introduction

The word 'lichen' has a Greek origin, which was referred to the superficial growth of fungus like organism on the bark of olive trees. Theophrastus, the Father of Botany coined the term 'lichen' during 300 BC and introduced this group of plants to the scientific world.

Lichen is a combination of two organisms, an alga and a fungus, living together in symbiotic association. Sometimes instead of an alga a cyanobacterium (blue-green alga) may be present in the lichen thallus. The algal component in the lichen is generally called as 'photobiont' as it contains photosynthetic pigment. The fungal part is called as 'mycobiont' (myco = fungus). The photobiont and the mycobiont loose their original identity in symbiotic association and the resulting organism is the lichen which behaves as a single organism, both morphologically and physiologically. Hence, the lichen is called as a composite organism. The lichen thallus is made 90% of mycobiont which provides shape, structure and colour to the lichen. In a lichen thallus whatever visible from outside is fungal part, which holds algae inside its body (Fig. 1) and therefore, the lichens are placed within the Kingdom - Fungi. The fungi present in lichens are called as 'lichenized fungi'. Among the 20,000 lichen species present in the world, 98.9% of them belongs to Ascomycetes group while Bacidiomycetes and Deuteriomycetes groups are represented by only 0.1% and 1%, respectively.

Fig. 1. Vertical section of a lichen thallus

2. Nature of Symbiosis

Schwendener (1867), a Swiss Botanist demonstrated for the first time the dual nature (presence of both fungus and alga) of lichen thallus. There after a great debate started discussing the nature of relation between the fungus and the algae. It was proposed that the relationship is 'parasitism', where fungus is a parasite on alga. It is observed that the haustorium of fungal hypae enter in to algal cells for drawing nutrients. However, this theory is discarded, because in parasitism the host would ultimately die and finally the association will come to an end, whereas in case of lichen the algal fungal association is permanent. Sometimes the relation is called 'controlled or balanced parasitism', because the fungus does not kill the alga, but at the same time it will not allow alga to flourish. It can also be called as 'helotism' or master and slave relationship, where fungus is the master and the alga is a slave. Some lichenologists called the relation an 'endosaprophytism'. The fungi are usually saprophytes and thrive on dead organic matter. Inside some lichens dead algal cells were observed and hence it was thought that fungus may be feeding on them. However, most of the lichenologists believe it is 'mutualism'. The alga by having photosynthetic pigments prepares food and supplies it to fungus; the fungus in turn provides shelter to the alga and supplies water and nutrients. Due to the difference of opinion regarding the nature of relationship between fungus and alga, it is suggested to call the relation as 'symbiosis', which simply means 'living together'.

3. Lichen Growth and Growth Forms

Lichens are very slow growing organisms. They grow just few millimeter or centimeter in a year. Lichens can grow in diverse climatic conditions and on various substrates. The lichens growing on bark or tree trunk are called corticolous, on twigs as ramicolous,

on dead wood - legnicolous, on rocks and boulders - saxicolous, on moss - muscicolous, on soil - terricolous and on evergreen leaves – foliicolous (Fig. 2A-G). The lichens can grow under water rocks, but not purely in water or ice. The lichens are widely distributed in almost all the phytogeographical regions of the world. Adequate moisture, light, altitude, unpolluted air and undisturbed substratum favours luxuriant growth of lichens. By their appearance the lichens can be grouped into three main growth forms.

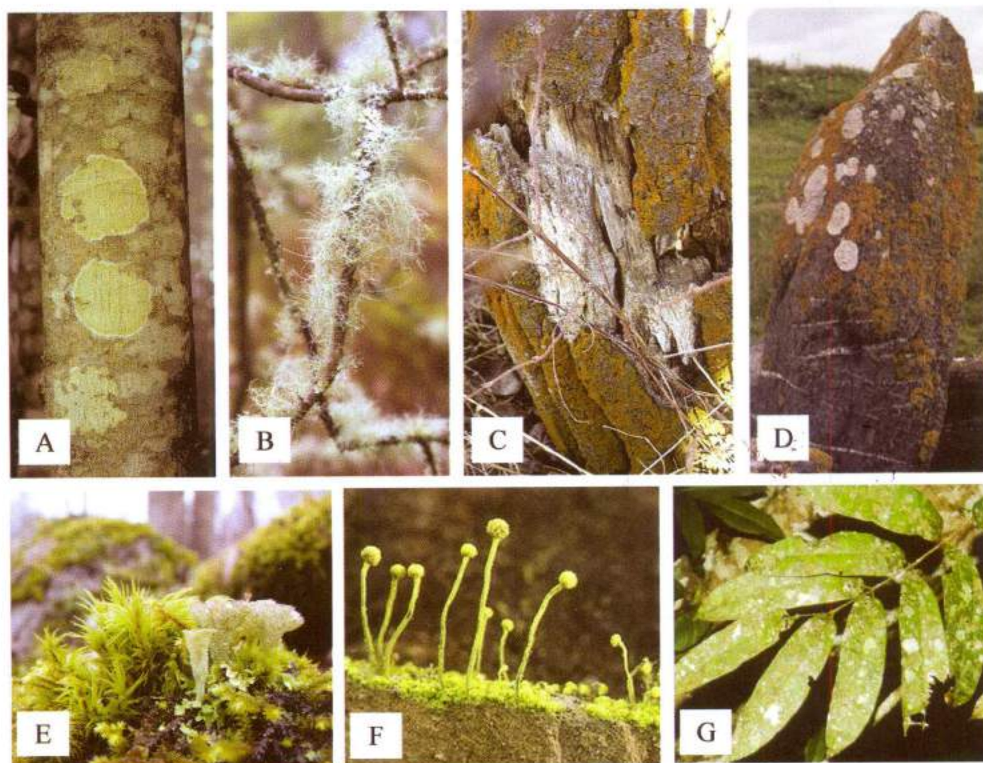

Fig. 2. (A) Lichen growing on tree bark (corticolous), (B) On twig (ramicolous), (C) On dead wood (legnicolous), (D) On rock (saxicolous), (E) On moss (muscicolous), (F) On soil (terricolous), and (G) On leaf (foliicolous).

3.1. Crustose lichens

The lichen thallus is closely attached to the substratum without leaving any free margin. The thallus usually lacks lower cortex and rhizine (root like structure). Such lichens have to be collected along with their substratum for the study (Fig. 3A).

3.2. Foliose lichens

Foliose lichens are also known as leafy lichens. The thallus in this case is loosely attached at least at the margin. Such lichens can be collected by scraping them from the substratum (Fig. 3B).

3.3. Fruticose lichens

Here the lichen thallus is attached to the substratum at one point and remaining major portion is either growing erect or hanging. The lichen usually appears as small shrub or bush (Fig. 3C).

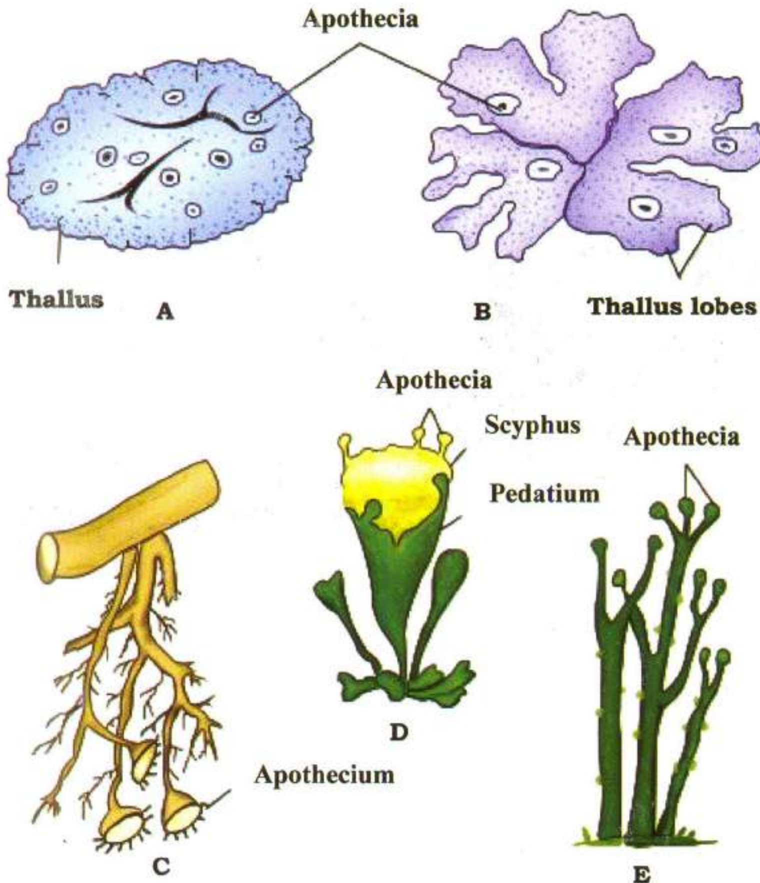

Fig. 3 (A) Crustose lichen, (B) Foliose lichen, (C) Fruticose lichens (D-E) Dimorphic lichen

There are few intermediate categories of growth forms such as:

(a) **Leprose lichens.** Here the lichen thallus is powdery or granular, does not form perfect smooth thallus.

(b) **Placodioid lichens.** In this case the lichen thallus is closely attached to the substratum at centre and lobate or free at the margin, but lacking rhizine.

(c) **Squamulose lichens.** Here the lichen thallus is in the forms of minute lobes, having dorsiventral differentiation.

(d) **Dimorphic lichens.** In this case single lichen thallus has the characters of both foliose/ squamulose and fruiticose lichens. Here the leaf like structures are called 'phyllocladia' and erect, stem like structures are called as 'podetia' (Fig. 3D-E).

All the above mentioned growth forms of lichens can be arranged according to their imaginary evolution as; Leprose (pioneer) → Crustose → Placodioid → Squamulose → Foliose → Dimorphic → Fruticose (latest). The leprose, crustose, some placodioid and squamulose lichens are generally called as 'microlichens', because of their smaller size and mostly require microscope for their identification. The foliose, dimorphic and fruticose lichens on the other hand are called as 'macrolichens'. The macrolichens have comparatively larger thallus and a hand lens or dissection or stereozoom microscope or is sufficient for identification.

The crustose, foliose, squamulose, placodioid and sometimes dimorphic forms of lichens usually grow in a circular and centrifugal manner. The rough and uneven surface of the substratum may change the shapes of the thallus. The leprose lichens forms irregular patches of thallus on the substraum. The fruticose lichens of smaller size usually grow erect while larger ones hang from the substratum with their growing point located at the tips.

Differentiating lichens from other groups of plants

The non-lichenized fungi, algae, moss, liverworts (bryophytes) are the plants, which grow on rocks, bark and soil, and may confuse with lichens at least for the beginners in the field. However, lichens can be easily differentiated from these plants. The lichens are never greener as algae, liverworts and mosses. Foliose lichens in the moist places or in wet condition may look greener, but have thick, leathery thallus while liverworts have non-leathery and slimy thallus. The dimorphic forms of lichens such as *Cladonia* may confuse with the leafy liverworts and mosses. However, leafy liverworts and mosses have dense small leaf like structures throughout the central axis of the plant, while in case of dimorphic lichens the squamules of semicircular shape are usually present at the base of the central axis or sparce throughout. Algal mat are usually found in water-flooded habitat. The beginners may confuse the dried algal mat on rocks and bark for lichens. By spraying some water on these mat one can make out whether it is algal mat or lichen.

The non-lichenized fungi are the most confusing ones with crustose lichens in the field. Such fungus usually forms patches with loosely woven hyphae, which will be evident under lens. The lichens on the other hand form smooth, perfect thallus. The fungus are usually whitish in colour and lichens are usually grayish, off white, yellowish, yellowish- green or sometimes bright yellow or yellow orange in colour. The lichens by having algal cells inside exhibit greenish tinge in colouration. The lichen thallus usually bears cup like structures called apothecia, or bulged, globular structures called perithecia or finger like projections called isidia or granular, powder like structure called soredia. Some crustose lichens belonging to family Graphidaceae bear worm like structures, which are nothing but modified apothecia. While collecting lichens it is necessary to look for such structures with the help of hand lens. When a lichen thallus does not have any such structures, it becomes difficult to differentiate it from fungus.

In any case it is observed that a beginner usually collects fungus and other plants in place of lichens. Usually fungus of various colour (mostly appearing like mushroom) are confused for lichens and collected by the beginners. Such specimens can be identified by taking a thin section of the thallus and studying them under a microscope. If the section contains both fungal tissue and algal cells, then the specimen is lichen, otherwise it is something else. In India basidiolichens (looking like mushroom) are rare or absent.

4. Methods

4.1. Collection and preservation of lichens

The micro or macrolichens are visible to the naked eye in the field. However, a hand lens, preferably of 10×, is necessary to examine the structure of the thallus and confirm while collecting the lichens. A sharp, flat edged chisel (1 to 2 inch) and a hammer (1 to 2 kg weight) are the tools required for collecting lichens. Carpenter use such flat chisels and it can have either wooden or plastic handle. Sometimes sharp, hard knife can also be used. The pointed, long chisel and heavy hammer are recommended for collecting lichens growing on rocks. Polythene packets (small (6 × 12 inch) and bigger sizes), rubber bands, labeling stickers, Global Positioning System (GPS), a field notebook, pen, pencil, plant press, old news papers or blotters, nylon ropes, collection bags, herbarium packets are the other necessary items needed during lichen collection trip.

The lichens are usually collected along with their substratum irrespectively of their growth form. Only the lichens that are very loosely attached to substratum are scraped out and collected. In case of saxicolous lichens smaller pieces of the rocks are collected. Collection of lichen samples in 'sufficient' amount (at least 2 thallus) is necessary, as the material will be used for detailed microscopic as well as chemical studies. Care should be taken to collect intact thallus, at least the margins should be clearly visible in the specimens.

In case of corticolous lichens one should try to collect superficial bark to avoid damage to the trees. The collected lichen samples are transferred to the polythene packets, labeled and closed with the help of rubber bands. Several such packets are then transferred to larger polythene or collection bags. One can also keep the collected material in newspaper or blotter packets. The lichen specimens should not be kept in polythene packets for longer duration as they spoil due to fungal attack and colour of the thallus will also change. After returning from field all the specimens should be transferred to newspaper or blotter packets. The lichen specimens on wet barks should be kept in plant press and tied tightly. Otherwise the bark gets curled up as it dries, makes uncomfortable to preserve in herbarium packets and will give a shabby look. No poisoning methods are available for lichen preservation. Lichen samples are thoroughly dried and preserved in the herbarium packets.

The lichen herbarium packets should be of thick, white or brown hand made paper. The hand made paper sheet of dimension 13.5 × 11.5 inches is folded lengthwise twice and then side ways to produce the packets of dimension 7 × 5 inches with upper flap of 3.5 inches to stick the label (Fig. 4). The herbarium label should contain the information on name and family of the lichen (which can be written after the identification), details of locality, altitude, longitude, latitude, date of collection, a reference number, collectors name and notes on its substratum and any other interesting observations. After the identification name of the person who identified (determined) the specimen along with date can also be mentioned.

The dried lichen specimen should be pasted on to a thick, hard paperboard of dimension 6.5 x 4.5 inches (little less than the total packet size) and then placed inside the herbarium packet. The board also should have the same reference number as on the label. A herbarium packet should have specimen belonging to single species and mixtures should be avoided. Once the packets are ready they can be stacked in rectangular boxes (like shoe box) and such boxes can be kept inside the almerahs or wooden cupboards.

The method for collecting lichens depends on the objective of the study. For a simple floristic study the method mentioned above is sufficient. However, for air pollution, ecological or phytosociological study the data gathering method may be different. Hence, the objective and the methodology should be clear before starting the field work. Only representative few samples should be collected and bulk collection should be avoided.

3.5 inch
5 inch
5 inch
Fold here towards front
11.5 inch
Step 1

Fold here towards front
Step 2

Fold here
Fold here
7 inch
Step 3

Label
Step 4

LICHEN HERBARIUM
NAME OF THE INSTITUTE (ACRONYM IF HERBARIUM IS RECOGNISED)

Name ....
Family ....
Locality & altitude ....
Date .... No. ....
Collector .... Det. ....
Notes ....

Lichen herbarium label

Fig. 4. Method of preparing lichen herbarium packet

4.2. Identification of lichens

The collected lichen specimens are initially segregated according to their growth forms. Within the growth forms the specimens can be further grouped according to the type of fruiting bodies (apothecia, perithecia, sterile) (Fig. 5).

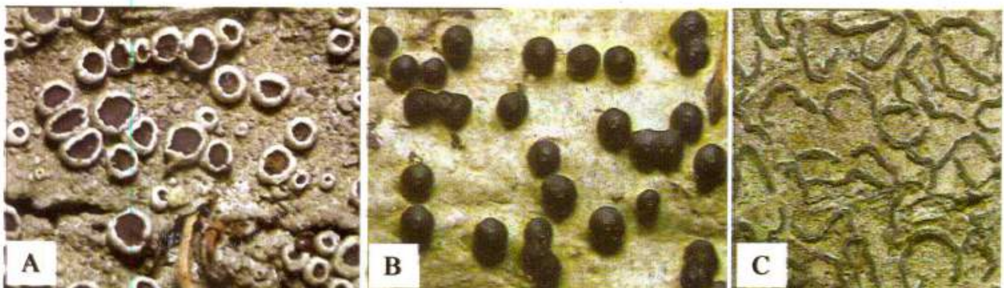

Fig. 5 (A) Apothecia, (B) Perithecia, (C) Streched apothecia, lirellae

The lichens are identified by studying their morphology, anatomy and chemistry. The micro and macrolichen keys of Awasthi (1991, 2007) are the important literature referred for identification of Indian lichens. The beginner should have a glossary of technical terms while identifying the lichens. Illustrated glossary given in 'Lichen flora of Great Britain and Ireland' (Purvis *et al.* 1992) would be very useful. A botany student or one with mycological background can better follow the terminology and identification keys.

The morphological and anatomical characters to be observed in a lichen specimen differ from genus to genus or group to group. However, some common characters to be noted are given in Fig. 6.

4.2.1. Morphology

The morphological characters of a lichens specimen are studied under dissection or stereo microscope. Type of thallus or growth form (leprose, crustose, foliose, squamulose, dimorphic, fruticose), its shape (irregular, circular) and size should be recorded.

(a) Upper surface

The colour of the thallus, texture (smooth, rough, warty), presence of finger like projections (isidia), granular structures (soredia), fine powder (pruina), black dots (pycnidia) and whitish decorticated areas (pseudocyphellae) have to be noted. The branching pattern, length and breadth of marginal lobes, presence of hair like structures (cilia) in case of foliose lichens has to be noted. In case of fruticose lichens length of the thallus, branching pattern, flatness or cylindricalness of the thallus has to be noted down (Fig. 7).

The morphology of fruiting bodies have to be studied separately. In case of apothecia, shape [rounded or stretched (Fig. 5)], size, attachment (stalked or not), colour and texture of the margin and disc, presence or absence of powder (pruina) on the disc, shape of the disc (convex or concave) are essential characters. In case of perithecia, its colour, shape, size and the position of its opening (ostiole, apical or lateral), single or grouped has to be noted.

Some lichens thallus emits florescence (yellowish, bluish) when observed under UV light due to the presence of lichen substance called lichexanthone. Such lichens are examined by keeping them in a closed UV lamp chamber and exposing UV light with wavelengths 254 and 365 nm.

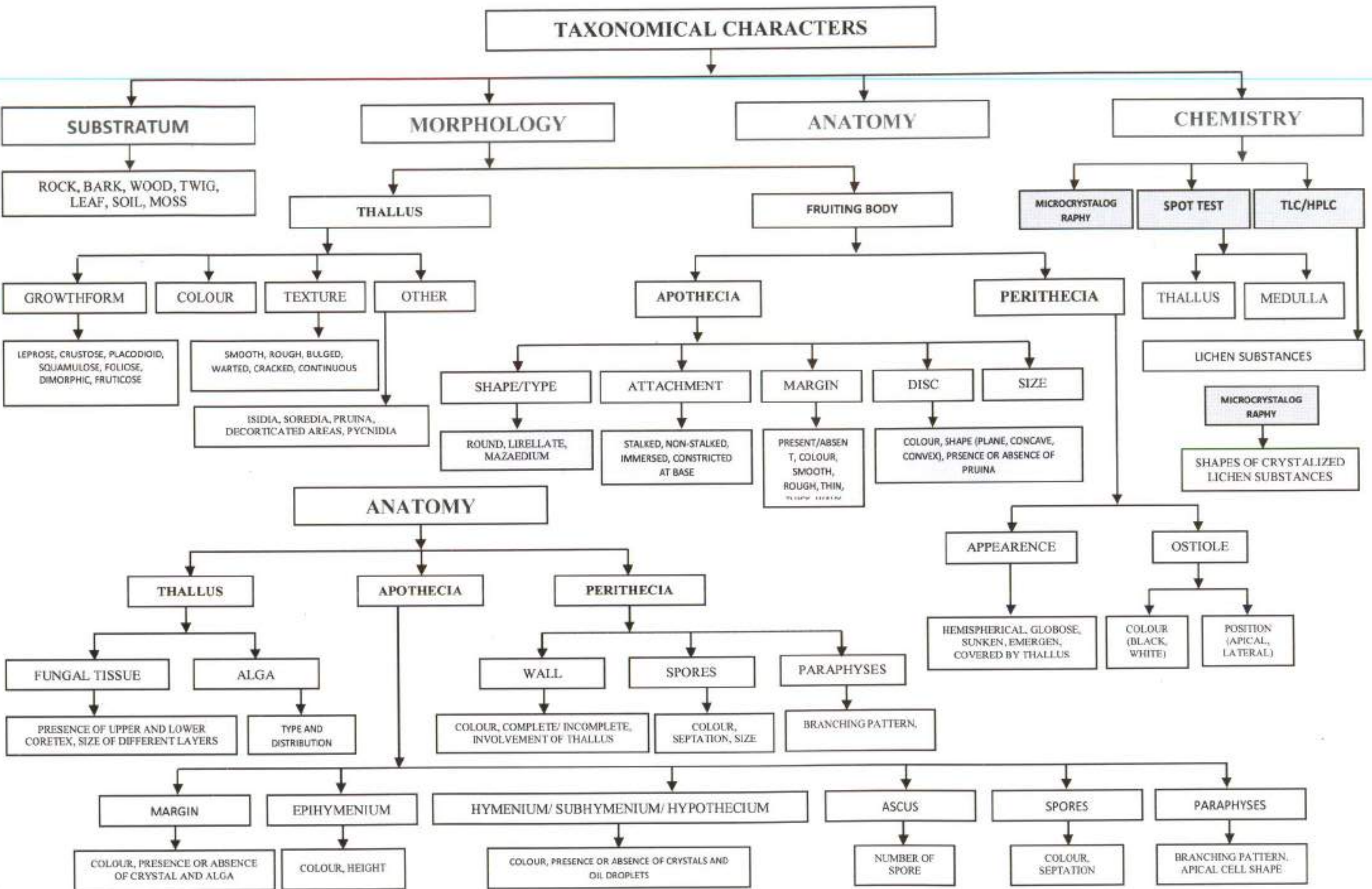

Fig. 6. The important characters to be observed for identification of lichens

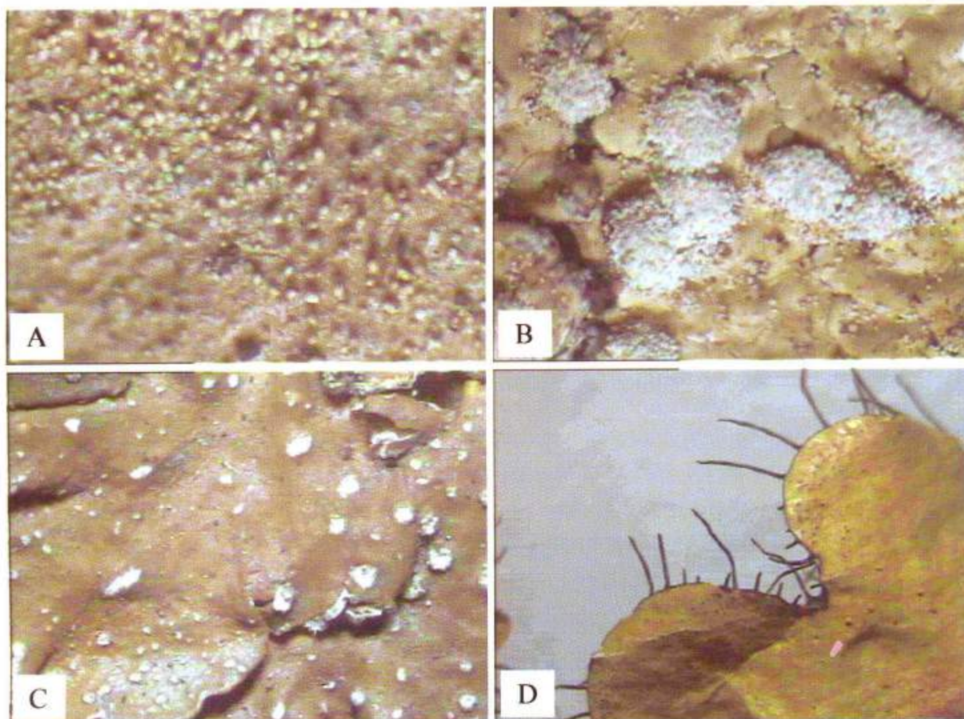

Fig. 7. (A) Isidia, (B) Soredia, (C) Pseudocyphellae, (D) Marginal cilia

(b) Lower surface

The lower surface of only foliose lichens can be seen as it is absent in crustose lichens while dimorpohic and fruticose lichens do not show dorsiventral differentiation. The colour of lower surface, presence of any pores (cyphaellae, pseudocyphellae), presence or absence of rihizines (root like structures), their colour, distribution, branching, abundance are to be noted.

4.2.2. Anatomy

The anatomy of lichen thallus and fruiting bodies is examined under compound microscope with minimum magnification of 40X. The anatomy of the thallus is occasionally studied to see the thickness of various layers (upper cortex, algal layer, medulla, lower cortex), type of algae and their distribution (stratified – heteromerous or uniform – homeomerous) and arrangement of fungal hyphae (vertical or horizontal) within the thallus. The section of thallus can be cut with snapper or razor blade by keeping the fragment of thallus in potato or papaya pith. Microtome sections are very helpful but time consuming. Just to check the type of algae present in the thallus one need not cut a section. By the colour of the thallus one can make out the type of alga (at least group) present within. Lichen with blackish, bluish, slate grey thallus usually has blue green alga, while grayish, yellowish, brownish,

greenish thallus has green alga. However, it is better to confirm the type of alga present by the following easy procedure. The algal layer of the lichen thallus is exposed by scraping the upper cortex with blade and alagal part (which appears dark green, blue green, black) is picked up with blade or needle, transferred to the slide and examined under microscope.

The anatomical character of fruiting bodies (ascocarp) is very important identification aids especially in case of crustose lichens. The type of spore (simple, septate), colour (hyaline, brown), their shape, size, number of spores in a spore-sac (ascus), colour of ascocarp wall (exciple), presence or absence of crystals and algal cells in the wall, colour and height of different layers (hymenium, epi and subhymenium, hypothecium) within in the ascocarps are to be noted (Fig. 8). The branching pattern and arrangement of paraphyses, shape and colour of apical cell are important character to be noted.

The thin, hand section of ascocarp is taken with the help of blade while it is still attached to the thallus or substratum and by viewing through dissection or stereo microscope. The ascocarp is made wet with a drop of water before cutting the section. Few sections are enough to observe the character.

The sections of thallus and ascocarps are mounted with plain water to observe basic characters. The lactophenol cotton blue and other stains can be used to colour different tissue as per requirement. Semi-permanent slides can be prepared by adding a drop of glycerol water mixture (1:1) to the section slide and sealing the cover slip with quick fix.

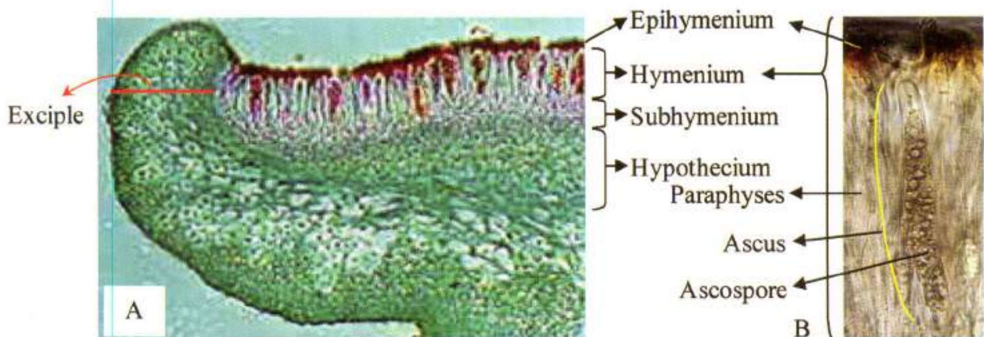

Fig 8. (A) Vertical section of apothecia showing anatomical structures, (B) Part of hymenium showing ascus, ascospores & paraphyses

4.2.3. Chemistry

Lichens produce more than 1000 secondary metabolites that are popularly known as lichen substances. Out of the 1000 lichens substances around 950 are unique to the lichens and are not available in any other groups of plants, only small portion of about 50 – 60 occur in other fungi or higher plants (Elix and Ernst-Russel 1993). For example,

the anthraquinone parietin, the orange pigment that is common in most 'Teloschistales' occur in non – lichenized fungal genera *Achaetomium, Alternaria, Aspergillus, Dermocybe, Penicillium* as well as in the vascular plants *Rheum, Rumex* and *Ventilago.* The lecanoric acid also occurs in fungus *Pyricularia,* while the sterol, brassicasterol of higher plant is also available in the lichens. Most of these lichen substances act as an important character for identification of lichens (chemotaxonomy). The lichen substances are identified by performing colour spot test, microcrystalography, thin layer chromatography (TLC) or by high performance liquid chromatography (HPLC).

4.2.3.1. Colour spot test

Three chemical reagents commonly used for colour spot test are aqueous potassium hydroxide (K), bleaching powder or aqueous solution of calcium hypochlorite (C) and aqueous solution of paraphyenldiamine (Pd). K-test is performed either on upper surface of thallus (cortex) or on the medulla by exposing it with blade, or on both. A drop of K solution is placed on the cortex or medulla and colour reaction is noted. Usually C and Pd test are performed on the medulla and colour changes are recorded. KC-test is performed by applying K solution first and then immediately C soloution over earlier K solution drop. The colour of the cortex or medulla changes due to presence of particular lichen substances in lichen thallus (Fig. 9). Sometimes Iodine (I) test is also performed on the cortex to check the presence or absence of polysaccharides. William Nylander in 1860s introduced I, K, C and KC test. Later Asahina *et al.* introduced Pd test in 1930s (Culberson 1969). The composition of the reagents is given below and the possible mode of action is given in the box 1.

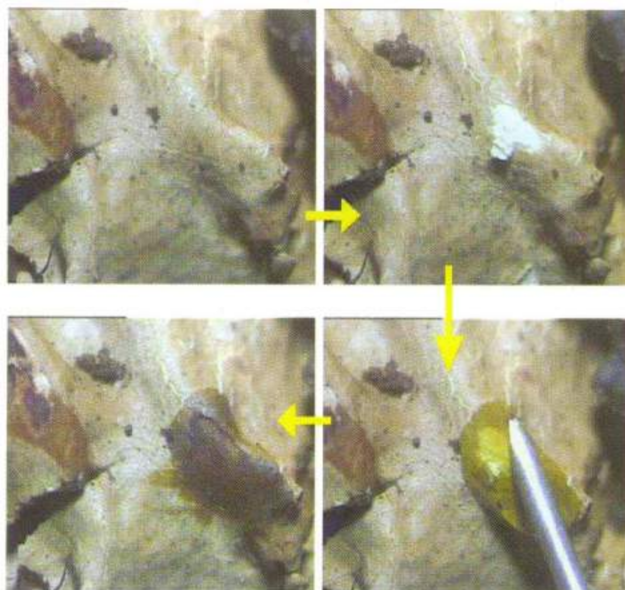

Fig. 9. Performing colour test on lichen thallus

K = 10% aqueous KOH solution
a. Turns yellow then red with most *o*-hydroxy aromatic aldehydes.
b. Turns bright red to deep purple with anthraquinone pigments.

C = saturated aqueous $Ca(OCl)_2$ or common bleach (NaOCl) solution
a. Turns red with *m*-dihydroxy phenols, except for those substituted between the hydroxy groups with a –CHO or $-CO_2H$.
b. Turns green with dihydroxy dibenzofurans.

KC = 10% aqueous KOH solution followed by saturated aqueous $Ca(OCl)_2$ or common bleach (NaOCl) solution
a. Turns yellow with usnic acid.
b. Turns blue with dihydroxy dibenzofurans.
c. Turns red with C- depsides and depsidones which undergo rapid hydrolysis to yield a *m*-dihydroxy phenolic moiety.

PD = 5% alcoholic *p*-phenylenediamine solution
a. Turns yellow, orange or red with aromatic aldehydes.

Box 1. The colour spot test reagents and their possible reactions

(a) Reagent 10% K

Potassium hydroxide pellets 10 g dissolved in 100 ml of distilled water. The reagent should be prepared fresh, it absorbs carbon dioxide from the air and gradually becomes ineffective, it should be replaced when it becomes cloudy. Confirmation test can be performed on the medulla of *Parmelinella wallichiana* or any other lichen which gives K+ red colour.

(b) Reagent C

One part of calcium hypochlorite should be added to double the volume of distilled water (1:2 ratio) and shaken well. The reagent may be allowed settle down and supernatant solution can be used for the spot test. The reagent should be prepared fresh and it should be discarded when it stops emitting chlorine smell. The confirmation test can be performed on the medulla of *Puctelia borreri* or any other lichen that gives C+ pink colour.

(c) Reagent Pd (Steiner's solution)

The *para*-phenylenediamine 1 g, sodium sulphite 10 g, detergent liquid 0.5 ml are dissolved in 100 ml of distilled water. The reagent should be prepared fresh; oxidation of the reagent gives false result and should be discarded. Confirmation test can be performed on the medulla of *Parmelinella wallichiana* or on the lichen which yield Pd+ orange colour. *Para*-phenylenediamine is a suspected carcinogen which should be handled with great care.

4.2.3.2. Microcrystalography

The method for crystalization of lichen substances was introduced by Asahina and Shibata (1954). The experiment is usually carried out on the glass slide. Small pieces of lichen are placed at the centre of the thallus and chemical substances present in it are extracted on to the slide by dropping acetone over it. The acetone drops should be smaller and each drop should be added after the evaporation of the earlier drop. It gives concentric white rings of crystals around the lichen pieces. Then the material should be removed gently and a drop of crystallizing reagent is added over the concentric rings. After placing a cover-slip on the crystallizing agent the slide should be gently warmed over the spirit lamp and then allowed to cool down and to form crystals (Fig. 10). Then the slide is observed under compound microscope and the lichen substances are identified based on the shape of the crystals. The compositions of various crystallizing reagents are given in the box 2.

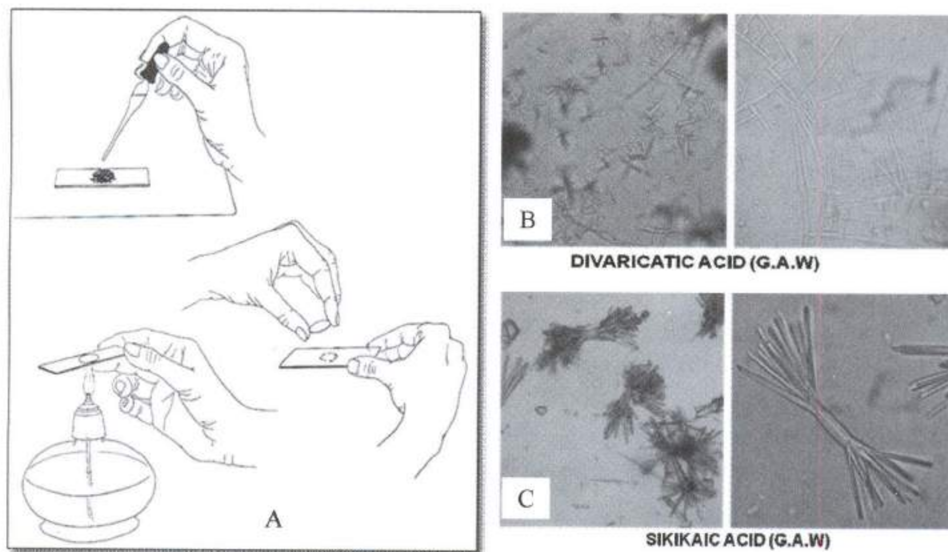

Fig. 10. (A) Procedure involved in microcrystallography, (B) Crystals of Divaricatic acid in GAW, (C) Crystals of Sikikaic acid in GAW.

G.E.: Glycerine-acetic acid, 1:3.
G.A.W.: Glycerine-alcohol-water, 1:1:1.
G.A.*o*-T.: Glycerine-alcohol-*o*-toluidine, 2:2:1.
G.A.An.: Glycerine-alcohol-aniline, 2:2:1.
G.A.Q.: Glycerine-alcohol-quinoline, 2:2:1.

Box 2. Various crystallizing reagent used in microcrystallography

Microcrystalography is believed to be more accurate than spot tests. However, may be difficult to identify mixture of substances with this method, and also minor substances may be undetectable. This method is superseded by more accurate Thin Layer Chromatography (TLC) method.

4.2.3.3. Thin layer chromatography

Many lichen substances are undetectable in colour spot test or the colour spot test may not give proper result. In such cases TLC have to be performed. In TLC various lichen substances present in a lichen thallus get separated as spots on TLC plates. With the help of lichen TLC manuals, these spots can be identified. The TLC method was standardized by Culberson in 1950s and it is now a widely used technique (Culberson 1969). It is a simple, relatively inexpensive, speedy method and helps accurate recognition of secondary metabolites. The steps involved in performing TLC are given below.

i. Extraction of lichen substances

Fragments of the thallus are placed in small test tubes and few drops of Acetone are added to it. The lichen substances in the thallus get extracted in to the acetone. The test tubes should be numbered serially including the control sample.

ii. Preparation of TLC plate

Silica gel pre-coated thin aluminium plates are used for the TLC and 20 × 20 cm Merck 60F is highly recommended. A line is drawn at 2 cm from base of the plate (loading line) and another at 15 cm (finishing line). On the 2 cm line several spots are marked at equal distances. The number of spots should be corresponding to the number of test tubes of samples which are needed to be checked for chemistry. The minimum distance between two spots should be 0.8 cm. Farer the spots on the loading line lesser would be the overlapping of developed spots. The width of the plate can be cut in to various sizes as per requirement and number of samples.

iii. Loading

The acetone extract in the test tube is spotted on the TLC plate with the help of capillary tube. The number on the test tube with the extract should be corresponding to the number on the TLC plate. The capillary tube should be thin and separate tube should be used for each extract to avoid contamination. The spot on the TLC plate should be concentrated enough and it can be achieved by repeated loading of the extract on the same spot.

iv. Preparation of solvent system and TLC tank

Different organic solvent can be used to separate the compounds present in the lichens (Box 3). Solvent should be freshly prepared and older solvent give false result. Rectangle specimen jar can be used as TLC tank and it should be covered with glass lid. The tank is made air tight by applying grease or Vaseline at the rim of the jar where the glass lid

touches. In side the TLC tank towards back side filter paper sheet can be placed. The wet filter paper provides uniform vapourous atmosphere inside the tank and help separation better of lichen compounds. The quantity of the solvent should be sufficient enough but should be just below 2 cm loading line of TLC plate. If the bottom of the TLC tank is not flat a broader glass slide can be placed at the base to provide flat 'flat form' for placing TLC plate.

Solvent A = Toluene–dioxane–acetic acid (180:45:5) is reputed to owe its distinctive characteristics to the ability of dioxane to associate with phenolic hydroxy groups.

Solvent B = Hexane–methyl *tert.*-butyl ether–formic acid (140:72:18) gives good separation of compounds that differ only slightly due to the length of side chains or the number of *C*-methyl substituents.

Solvent C = Toluene–acetic acid (170:30) is an excellent general solvent for a wide variety of different compounds.

Solvent E = Cyclohexane–ethyl acetate (75:25) is recommended for less acidic compounds, that have high R_f values in solvents A, B, and C (e.g. many pigments, esters, triterpenes: (Elix *et al.* 1988).

Solvent G = Toluene–ethyl acetate–formic acid (139:83:8) is particularly useful in separating compounds with relatively low R_f values in solvents A, B and C (e.g. β-orcinol depsidones, secalonic acids).

Box 3. Different solvent systems and their composition

v. Running

The spotted TLC plate is placed inside the TLC tank. The solvent rises up on the TLC plate passing through the loaded spots. The heavier lichen substance within the spot settles down near to the base of the plate while the lighter substances are carried away as the solvent rises upwards; hence the lichens substances get separated. The solvent is allowed to touch the finishing line drawn at 15 cm and then removed out of the TLC tank. The process takes about 40 – 50 minutes.

vi. Colouring the spots and charring

The lichen substances separated on the TLC plate are usually invisible or paler in colour. They are made more visible by spraying colouring reagent and heating. 10% sulphuric acid solution is sprayed over the TLC plate. The spray particles of solution should be very fine and it should just wet the plate, overflow of the solution should be avoided. Good quality glass spraying gun should be used for this purpose. After spraying

the TLC plate is kept in the hot air oven for few minutes. The oven should be pre-heated 110° C before keeping the plate. The TLC plate can be taken out of the oven once the spots are developed properly after 3 – 5 minutes.

vii. Identification of the spots

The spots appeared on the TLC plate are identified as per their colour and the distance they travelled from the loading point. The distance travelled by a lichen substance (spot) is either referred as Rf value or Rf class and are calculated as follows,

Rf value = (distance travelled by substance (spot) ÷ distance travelled by solvent) × 100

Rf class = Divide TLC plates into approx. 7 equal parts from the loading line to the last spot (atranorin), each division is a Rf class. If *Parmelinella wallichiana* is used as control, it gives 2 spots, salazinic acid and atranorin. When the TLC plate is divided into 7 parts from loading line to atranorin, the salazinic acid spot appears at Rf class 2. Hence, Rf classes of salazinic acid and atranorin can be used for referring other spots. The lichen sample containg norstictic (Rf class 4) acid and atranorin can also be used as control. TLC manuals are available for identification of the lichen substances (Fig. 11).

Observation of TLC plate under shortwave UV lamp is sometimes necessary before and after heating. The spots appear differently while some spots emit fluorescence and such spots are marked with pencil. These are added characters for identification of lichen substances.

viii. Identification of fatty acids

After removing the TLC plate from the tank it is sprayed with distilled water. The fatty acids appear as oily, grey spots as the water dries up. They are circled with pencil as dotted lines. The suphuric acid solution is sprayed only after this step and then the plate is heated. The fatty acid spot does not give any colour after heating. The fatty acids are also identified based on their Rf class or Rf value.

4.2.3.4. High performance liquid chromatography

This technique provides a powerful complement to the established TLC methods. The bonded reverse phase columns are used here and all the aromatic lichen products are suitable for analysis with this method. Samples are dissolved in methanol and injected in to the appropriate portion column, through which an appropriate solvent or sequence of solvents is passed under high pressure. The substances separated and detected using UV detector. The retention time (Rt or time of passage) and peak intensity are recorded by a chart recorder. HPLC is also used to measure either absolute or relative concentrations of lichen compounds, because the peak intensity (area under curve) is

proportional to the concentration. Most workers use HPLC to detect lichen compounds combine this technique with TLC and/or mass spectrometry to verify the identification of the peaks.

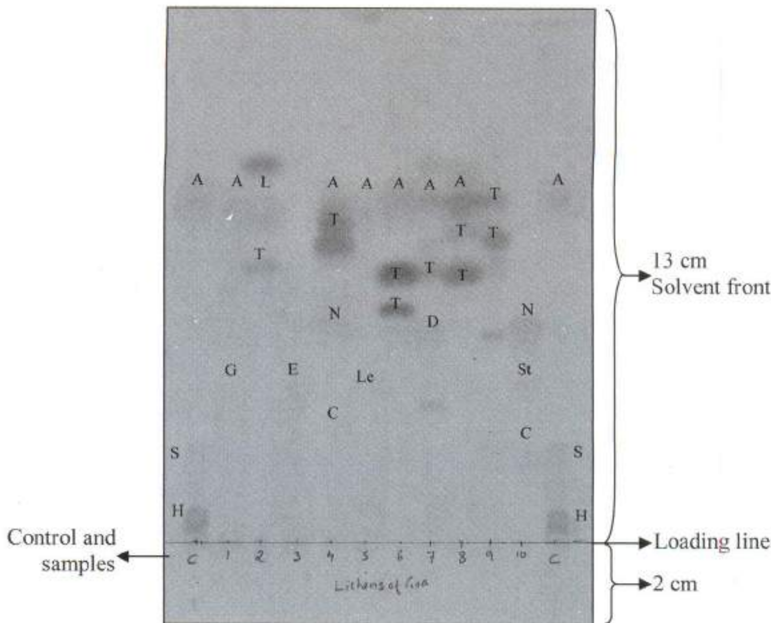

Fig. 11 A. Thin Layer Chromatogram of some lichens. C. Control (*Parmelinella wallichiana*), 1. *Parmotrema sancti-angelii*, 2. *Pyxine cocoes*, 3. *Roccella montagnei*, 4. *Pyxine cylindrica*, 5. *Parmotrema tinctorum*, 6. *Heterodermia diademata*, 7. *Dirinaria aegialita*, 8. *Lecanora cenisia*, 9. *Buellia disciformis*, and 10. *Graphis capillacea.* A – atranorin, S – salazinic acid, H – hypostictic acid, G – gyrophoric acid, L – Lichenoxanthone, T – triterpen, E – erythrine, N – Norstictic, C – constictic acid, Le – Lecanoric acid, D – divericastic acid, St – stictic acid.

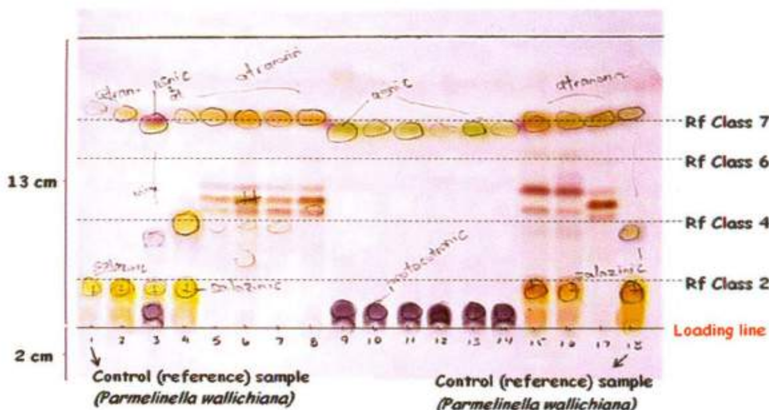

Fig. 11 B. Another example for developed TLC plate

There are several other methods used for identification of lichen compounds, but later discontinued because of their inefficiency. These techniques involve Paper chromatography, High Performance Thin-layer Chromatography (HPTLC), Gas Chromatography and Lichen Mass Spectormetry (GCLMS).

The identification of lichens involves combination techniques. Many macrolichens specimens can be identified with their external morphology and colour spot test, and rarely anatomy of thallus or fruiting bodies. Whereas in case of microlichens, identification mostly involves observation of anatomical details of fruiting bodies along with colour spot test and TLC. While observing the anatomical details of fruiting bodies sometimes the sections are also treated with reagents K, P, and rarely with C, and colour reaction is noted. As discussed earlier, characters to be observed and techniques to be applied for identification of lichens differ from group to group or genus to genus. As chemistry plays a very important role in identification of lichens, it also helps in segregating complex group of lichens. Hence, taxonomic significance of lichen chemistry is discussed here with a few exmples.

4.2.3.5. Taxonomic significance of lichen chemistry

The chemical constituents of lichen can be categorized in to two major classes: 1. Primary metabolites, and 2. Secondary metabolites. Primary metabolites are intracellular products, which are directly involved in the metabolic activities of the lichens such as growth, development and reproduction. They include proteins, amino acids, polyols, carotenoids, polysaccharides and vitamins, which are bound to the cell walls and protoplasts. They are often water soluble and can be extracted with boiling water. The primary metabolites are either of fungal or algal origin or both. They are also non-specific and present in free living alga, fungus, higher plants and other organisms. The secondary metabolites in lichens are of fungal origin. They are produced by utilizing the primary metabolites through three major pathways: 1. Acetyl-polymalonyl pathway, 2. Mevalonic acid pathway, and 3. Shikimic acid pathway. These secondary metabolites are not involved in the direct metabolism of lichen. They are the byproduct of primary metabolism and biosynthetic pathways. They act as storage substances, few have an important ecological role, but role of several secondary metabolites is poorly known. The secondary metabolites are deposited on the surface of the hyphae rather than within the cells, hence they are called as extra-cellular compounds. Popularly they are known as lichen acids or lichen substances. Secondary metabolites are insoluble in water, but can be extracted using organic solvents.

5. Biochemical Systematics

The first chemical test conducted on lichen thalli for taxonomic purpose was carried out by Nylander in 1860 (Hale 1983). He detected the presence of various colourless lichen substances by spotting chemical reagents on lichen thallus to produce

characteristics colour change (spot test). In recent times spots test is much standardized and one of the important steps in identification of lichens. Nylander separated C+ red *Cetrelia olivetorum* from the identical *C. cetrarioides* (C-); however he was condemned at the time by most of the lichenologists (Fig. 12A,B). Today species discrimination on the basis of chemistry has become inseparable procedure in lichen taxonomy. Lichenologists have employed "chemical characteristics" in the taxonomy of lichen-forming fungi for nearly 120 years! They have employed a wide variety of terms/classifications method to these chemical population variations including; SPECIES, SUBSPECIES, VARIETIES, FORMS, "Chemovars", "Chemical Strain No.__", "Chemotypes" and "Chemical races". However, before jumping into any conclusion Lichenologists must be satisfied with answers to following questions; Do differences in chemical composition mean two individuals (which may look the same) belong to different species? Are there ecological, morphological, geographical correlations with the observed chemical differences? What taxonomic weight (if any) should be applied to these differences?

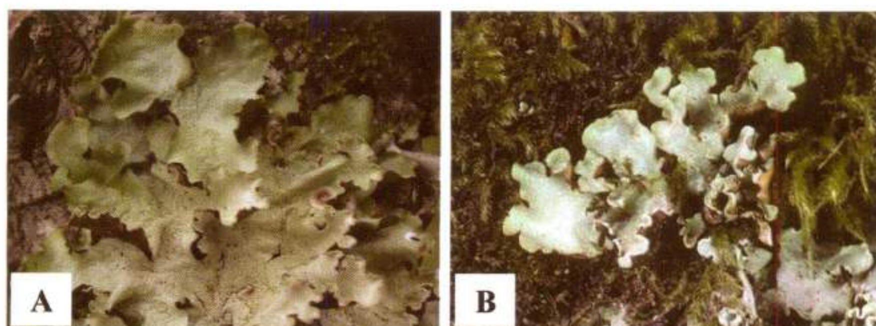

Fig. 12. (A) *Cetrelia olivetorum* (C+ red) and (B) *C. cetrarioides* (C⁻), separated by W. Nylander in 1860 based on the colour test.

The secondary metabolites are stored either in the cortex or in medulla of lichen. The cortical substances usually have an ecological role and most of them act as light screens (e.g., usnic acid, atranorin, chloroatranorin, anthraquinones, pulvinic acid derivatives, xanthones). They may absorb sunlight to warm up quickly in colder regions so as to initiate active metabolism of the thallus. They act as a filter and regulate the amount of sunlight reaching to sensitive algal layer. Given their apparent physiological importance it seems likely that their formation would have evolutionary significance. Some of the cortical substances are correlated with higher taxonomic ranks, example – at generic level presence of vulpinic acid is the characteristic feature of genus *Lethraria* (Fig. 13). Morphologically similar looking genera *Physcia* and *Phaeophyscia* can be identified on the basis of presence or absence of atranorin, which is present in former species and absent in later species. Similarly, *Flavopunctelia* (usnic acid present) and

Punctelia (usnic acid absent) (Fig. 14 A,B) are separated. Superficially, the usnic acid containing lichen would have yellowish tinge and atranorin yield yellow colour for K spot test. At family level presence of anthraquinones and particularly parietin is characteristics of Teloschistaceae.

Fig. 13 *Lethraria columbiana*

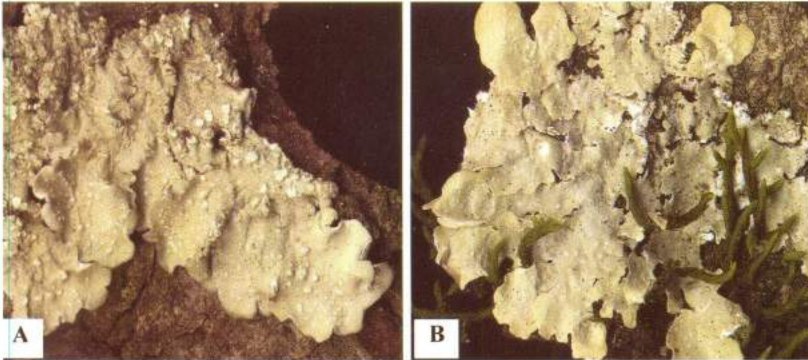

Fig. 14. Example for role of cortical chemistry in biochemical systematics. (A) *Flavopunctelia* (usnic acid present), (B) *Punctelia* (usnic acid absent).

The medullary chemistry of lichen is usually complicated. The secondary metabolites present in the medulla of lichen are used primarily as discriminators at the species level but also occasionally at generic or suborder level. For example, *Cetralia* (with orcinol derivatives) and *Platismatia* (with fatty acids or beta-orcinol derivatives) are separated on the basis of their chemistry). The discovery of chemical differences often led to an appreciation of the importance of previously overlooked morphological features as in *Punctelia subrudecta* with lecanoric acid and a pale tan lower surface, and *P. borreri* with the related tridepside gyrophoric acid and a black lower surface (Fig.15 A,B). Fortunately, most morphologically defined species have a constant chemistry, irrespective of their geographical origin, substrate or ecology, and this justifies the use of chemistry in lichen taxonomy. Within a complex of morphologically similar species, three common patterns of chemical variation are observed; replacement of compounds, chemosyndromic variation, and accessory type compounds.

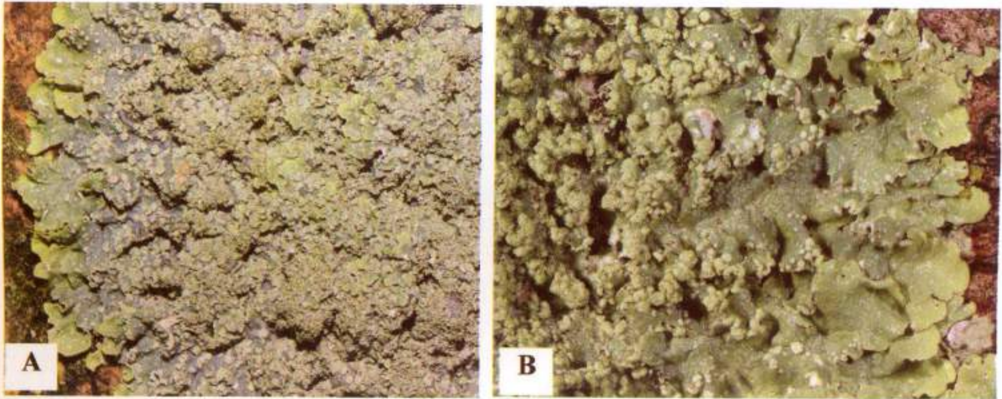

Fig. 15. Example for role of medullary chemistry in biochemical systematics. (A) *Punctelia subrudecta* (Lecanoric acid present, (B) *Punctelia borreri* (Gyrophoric acid present).

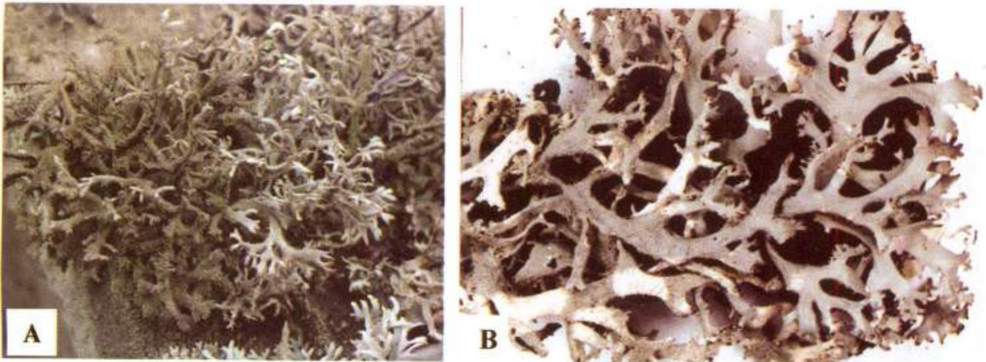

Fig. 16 (A) *Pseudoevernia furfuracea,* (B) *P. conssocians*

In case of replacement, simple replacement of one compound with another takes place and the replaced compound would be from same family of chemicals or from same biosynthetic sequence (biosequential). Morphologically these lichen populations are sometimes indistinguishable, but they have well defined, constant variations in chemical composition. This can be explained with the help *Pseudoevernia furfuracea* (Fig. 16A), which has three chemical races; an olivetoric acid race from northern Europe; a physodic acid containing race from southern Europe and north Africa; and a lecanoric acid containing race from North America. Biogenetically the first two races appear closely related, the metabolites can be considered biosequential, because one can be derived from the other by a single biosynthetic step. But the third race is not related as the lecanoric acid is biosynthetically remote from the other two compounds. It is now generally accepted that, when there is a biogenetic demarcation allied with a biogeographical separation, such taxa should be recognized as species and the North American taxon is distinguished as *P. conssocians* (Fig. 16 B). Further studies on first

two races of *P. furfuracea* revealed that though they possess distinctive, but overlapping chemistries do not show significant habitat ecology or morphological differences, hence considered as single species. It is suggested that chemical variation among lichen species should be genetic rather than being environmentally determined. The occurrence of intermediates in an area indicates that such races are either in the process of speciation or hybridization is occurring between the races.

The chemosyndrome refers to a group of biosynthetically related metabolites and in this pattern of chemical variation the major metabolite (or metabolites) in any one taxon is invariably accompanied by minor quantities of several bio-sequentially related substances. Further, the major constituents of one species become minor in related taxa and vice versa. Hence a true chemical intermediate cannot simply be defined as containing both of two replacement compounds, but would have to contain both chemical constellations in comparable concentration. Several examples are available among lichens for the occurrence of chemosyndrome. In *Cladonia chlorophea* group about 14 chemotypes are available, while in *Usnea longissima* (Fig. 17A) seven chemical strains are identified. Sometimes it is used for discrimination of species, which is logically wrong. Example, chemosyndromic variation leading to misidentification of species can be seen in case of *Relicina samoensis* complex (Box 4).

Species (distribution)	Echinocarpic	Conechinocarpic	Hirtifructic	Gyrophoric	Fatty acids	Distribution
R. samoensis	major	minor	—	—	—	Pan-Pacific
R. amphithrix	major	minor	—	—	—	Australia/Indonesia
R. terricrocodila	major	minor	trace	—	—	Australia
R. fijiensis	—	—	major	—	—	Fiji
R. niuginiensis	—	—	major	trace	minor	Papua New Guinea
R. relicinula	—	—	—	—	major	Indonesia

Box 4. Chemosyndromic variation in *Reclicina samoensis* complex

Fig. 17. (A) *Usnea longissima,* (B) *Ramalina siliquosa*

Ecology plays an important role in differentiation of chemical races. Different chemical races were found to be ecologically sorted into distinct habitats in their range of sympatry. For example, different species within the *Ramalina siliquosa* (Fig. 17B) complex are recognized due to the difference in chemistry and habitat preferences. *R. siliquosa* complex occur on maritime rocks on the coast of Europe (microhabitat preferences); the one which occupies lowest zone is identified as *R. cuspidata* and it has stictic acid; *R. crassa* is most sheltered species that grows in land-facing habitats and contains hypoprotocetraric acid; while *R. stenoclada* occupies the region in between and contain norstictic acid. Here all the three species do not have distinct morphological difference and the lichen substances are biogenetically related. They could have been chemical races rather than species. Culberson (1986) of the opinion that the ecological and biological characteristics of the major chemotype should be better considered as sibling species rather than as components of traditional morphological species. The population of sibling species would have reproductive isolation along with ecological but may not have morphological differentiation.

Accessory metabolites occur sporadically in a species, in addition to constant constituents but usually have no correlation with any morphological or distributional variations. Hence have less or no taxonomic significance. Such compounds commonly occur as accessory compounds in more than one species and often vary in quantity from deficiency to abundance. On the contrary in North Carolina the edaphic preferences of *Cladonia* along with difference in chemistry separates them into two distinct species. *C. polycarpoides* prefers "clay-type" soils and contains norstictic acid, while *C. polycarpia* prefers "sandy" soils and contain an additional substance, atranorin along with norstictic acid (Fig. 18 A,B). Here the chemical characteristics are correlated with ecological, but not morphological. Hence, it could have been a variety rather than a different species.

Fig. 18 (A) *Cladonia polycarpia* (atranorin and norstictic acid) grows on "sandy" soils; (B) *C. polycarpoides* (norstictic acid) occur on "clay-type" soils

Cell wall polysaccharides

These are primary metabolites and are polymorphic storage products of lichens require different techniques for their detection. Some of the well known polysaccharides in lichen are lichenan, isolichenan, and galactomannan. Some polysaccharides are taxonomically significant at the highest levels of classification. For example presence chitin, chitosan, or cellulose in the cell wall is a feature that helps define the classes of fungi. This indicates the conservative features of the taxa in evolution and helps tracing the phylogeny. In lichen taxonomy utility of cell wall polysaccharides can be seen in family Parmeliaceae where four types of lichenan can be identified in four distinct groups; isolichenan, *Xanthoparmelia*-type lichenan, *Cetraria*-type lichenan, and an intermediate-type lichenan. Chemically the polysaccharides differ in stereochemistry of the glycosidic bonds. These lichenan differ mostly in their staining properties with iodine and hence can be easily identified (Box 5). The morphologically similar lichen genus *Hypogymnia* (Fig. 19A) contains *Cetraria*-type lichenan, while *Menegazzia* (Fig. 19B) contains isolichenan. The polysaccharides are also used as one of the primary discriminators to differentiate yellow parmelioid genera, *Psiloparmelia* and *Flavoparmelia* (containing isolichenan) from *Arctoparmelia* (*Cetraria*-type lichenan) and *Xanthoparmelia* (*Xanthoparmelia*-type lichenan).

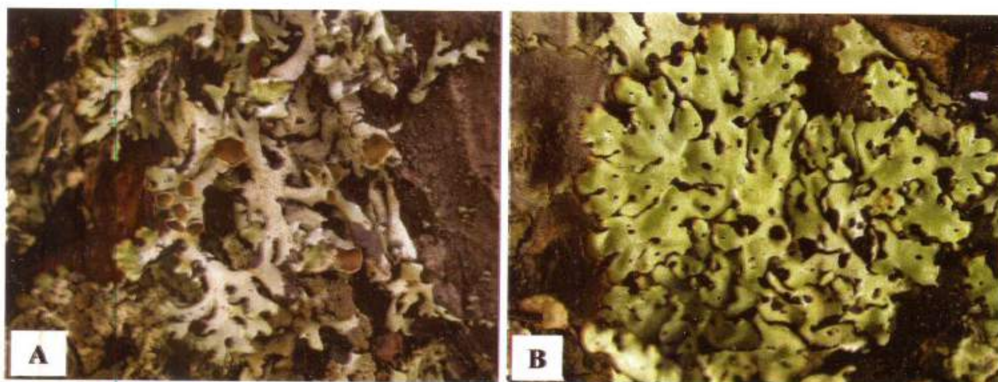

Fig. 19. (A) *Hypogymnia* with *Cetraria*-type lichenan, (B) *Menegazzia* with Isolichenan

To resolve taxonomic dilemma due to varying chemical, morphological, ecological and geographical characteristics four patterns can be illustrated.

Pattern 1. Replacement of one substance by one or more biogenetically distinct substances.

A. Correlated with morphological and (or strong) ecological differences = SPECIES

B. Correlated with major geographical differences = SPECIES

C. Not correlated with morphological or geographical differences = NONE

Polysaccharide	20–0.15% IKI	0.15% LPIKI	1.5% IKI	CaIKI	ZnIKI	SIKI	Meltzers
Isolichenan	blue	pale blue	bluish	bluish	bluish	—	bluish
Cetraria-type lichenan	—	—	red	deep red	—	red ppt.	orange
Xanthoparmelia-type lichenan	intense blue	—	red	deep red	purple	red ppt.	deep red
Intermediate-type lichenan	pale blue	—	red	deep red	—	red ppt.	red

IKI, iodine, potassium iodide solution; LP, lactophenol; S, 10% sulfuric acid; Ca, calcium chloride; Zn, zinc chloride; Meltzers Reagent, chloral hydrate + iodine potassium iodide.

Summarized from Common (1991).

Box 5. Four types of lichenan present in Parmeliaceae and their reaction with reagents.

Pattern 2. Replacement of one substance by one or more biogenetically closely related substances

A. Correlated with morphological and distributional differences = SPECIES

B. Correlated with local geographical differences or tendencies = VARIETY

C. Correlated with ecological or microhabitat differences = VARIETY

D. Not correlated with morphological, ecological or geographical differences = NONE

- Can be called as chemotype, chemical strain or race

Pattern 3. Presence of one or more unreplaced (additional) substances

A. Correlated with major geographical differences = SPECIES

B. Correlated with differences in ecological amplitude = VARIETY

C. Correlated with local distributional differences or tendencies = VARIETY

D. Not correlated with morphological, ecological or geographical differences = NONE

Pattern 4. Variations in concentration of particular substances = NONE

A. Correlated with light intensity

B. Correlated with heavy metal contents of substrate

C. Correlated with any other ecological factor

In biochemical systematics or chemotaxonomy of lichens considerable controversy still remains. American, Australian and Japanese workers generally recognize chemical variation at the species level while European lichenologists prefer to "lump" but are more likely to recognize subtle morphological traits. However, most morphologically defined species have a constant chemistry, irrespective of their geographic origin, substrate or ecology. This clearly justifies the use of chemistry in lichen taxonomy. So

far only about 6000 lichens are screened for metabolites, approx. 30% of known species are studied, remaining 70% are yet to be explored. The structures of several metabolites are unknown, while biosynthetic pathway has to be revisited with molecular approaches. Apart from taxonomy the commercially important metabolites should be identified and the genes responsible for their production should be recognized for mass production and commercialization and hence "Lichen products" should be popularized.

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