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CONTEMPLATION OF APPLICATIONS OF SCANNING ELECTRON MICROSCOPY IN TAXONOMY WITH SPECIAL REFERENCE TO PHERMATOLOGY

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ABSTRACT

The present review deals with the basics of Scanning Electron microscopy (SEM) and analyses the applications in taxonomic studies with phermatology. The popularity of SEM over TEM (Transmission electron microscopy) and applications of TEM in scientific elucidations including plant taxonomy have also been discussed. SEM studies strengthen morphological description of surface characteristics of leaves, pollen, floral parts, fruits and seeds revealing both conservative and variable data thus helping in studying various aspects of biodiversity. It is greatly helpful in discrimination of taxa at the levels of family, genus, species and infra-specific categories. SEM also provides tools to view microevolution by revealing sculpture of spermoderm of seeds. Moreover it provides pharmacognosy with tools for correct identification of crude drugs and also proves meaningful in identification of seeds of the Plant Genetic Resources (PGR) at varietal levels thus ensuring quality control and prevention of adulteration. SEM is also helpful in ecological studies through assessment of seed-bank potential of waste- and fertile- lands, monitoring of habitat status, i.e. xeric, mesic and hydric states, revealing trophic relationship and co-evolution and monitoring the environmental state.

Key words: Taxonomy, Scanning Electron microscopy, phermatology, pharmacognosy, Plant Genetic Resources (PGR), Ecology

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Taxonomy and electron microscopy:

Conventionally, taxonomy is defined as that branch of biological science which is concerned with identification, nomenclature, classification and determination of interrelationship of living organisms. Technically it is defined as the study and description of variations in living organisms, investigation of causes and consequences of these variations and manipulation of the data thus obtained in forming a system of classification. Being a classical discipline, taxonomy has been providing services to all other disciplines of science-both pure and applied based on living organisms. Although the term 'plant taxonomy' in principle relates itself to all types of plants its usage concerns 'angiosperms', the highest evolved group of plants having seeds enclosed in fruits. Systematic botany and plant systematics, the alternative terms for plant taxonomy convey the same message.

Identification of a species is the most critical task of a taxonomist which necessitates application of his instinct, knowledge and skill. Often identification is defined as the art and science of recognizing a living entity and distinguishing it from others. Traditional taxonomy or alpha taxonomy has been using the most comprehensive morphological tools for readily identifying a species. Modern taxonomy or omega taxonomy has been further to morphology making use of other data sources like anatomy, phytochemistry, embryology, palynology, serology, genetics, molecular biology, ecology etc. to confirm identification especially of infraspecific taxa and know more about the species and its dynamic attitude.

Since morphology as a tool fails to resolve with unaided eyes such finer distinctions which delimit very close species i.e. the closely related ones with very little exomorphic differences. In view of this Blair and Turner (1972) introduced *micromorphology* as one of the tools to establish identification of plant and plant parts, solve taxonomic problems and to contribute to plant classification as one of the integrative approaches (Cronquist, 1988 and Takhtajan, 1997). Electron microscopy has started contributing valuable information to systematics in general and has in many instances solved problems arising from incomplete or partial taxonomic data collected from different sources and by different techniques. Specific applications of thin-sectioning (TEM), Freeze-etching (FE) and scanning electron (SEM) microscopy have been revealing ultra-structural features of different plant groups and contributing to alpha, beta and omega taxonomy of prokaryotes, algae, fungi, lichens, bryophytes, pteridophytes and spermatophyte (Cole and Behnke, 1975).

SEM: A comprehension

Scanning electron microscope, familiar in its abbreviated form as SEM, is a microscope that resolves objects (samples) using electrons instead of light to form an image. It images a sample by scanning it with a high-energy beam of electrons in a raster scan pattern which interact with atoms at or near the surface of the sample. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition, and other properties such as electrical conductivity.

In the most common or standard detection mode, secondary electron imaging or SEI, the SEM can produce very high-resolution images of a sample surface, revealing details less than 1 nm in size. A wide range of magnifications is possible, from about 10 times (about equivalent to that of a powerful hand-lens) to more than 500,000 times, about 250 times the magnification limit of the best light microscopes. In a typical SEM, an electron beam is thermionically emitted from an electron gun fitted with a tungsten filament cathode. Tungsten is normally used in thermionic electron guns because it has the highest melting point and lowest vapour pressure of all metals, thereby allowing it to be heated for electron emission, and because of its low cost. One must take care to see that sample is free from particles, stable when put under vacuum and able to emit adequate secondary electrons. The surface should develop least surface charges for stability of the sample.

Advantages of SEM over traditional photomicroscopes are worthwhile. Due to the very narrow electron beam, SEM images have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample. Moreover the SEM has a large depth of field, which allows more of a specimen to be in focus at one time. The SEM also has much higher resolution, so closely spaced specimens can be magnified at much higher levels. Since electromagnets are used in place of lenses, the researcher has much more control in the degree of magnification. All these advantages, as well as the actual strikingly clear images, make the scanning electron microscope one of the most useful instruments in taxonomic research today.

TEM vis a vis SEM:

TEM (Transmission electron microscopy) is a microscopic technique which involves transmission of a beam of electrons through an ultra-thin specimen or its section and interaction with the specimen as it passes through. The interaction of the electrons transmitted through the specimen results in an image which is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film or by a sensor such as a CCD camera.

An TEM has greater resolving power than a light microscope and can reveal the structure of smaller objects because electrons have wavelengths about 100,000 times shorter than visible light photons which can magnify objects by as much as 10,000,000 times whereas ordinary, non-confocal light microscopes are limited up to at best about 200 nm resolution and up to 2000x magnifications.

The first TEM was built by Max Knoll and Ernst Ruska in 1931 and the first commercial TEMs were released in 1939. TEMs are capable of imaging at a significantly higher resolution than light microscopes, due to the small de Broglie wavelength of electrons, thus enabling the instrument's user to examine fine detail of objects even as small as a single column of atoms, which is thousands times smaller than the smallest resolvable object in a light microscope. Contemporarily used electron microscopes still remain based upon Ruska's prototype and are capable of two million-power magnification, as a very useful scientific instrument for biological studies both phenomenal and organizational. TEM which forms a major analysis method in a range of scientific fields, in both physical and biological sciences especially in revealing internal nanostructure, cellular ultrastructure, sieve-tube plastids etc.TEM is extensively used in in cancer research, virology, materials science as well as pollution, nanotechnology, and semiconductor research.

TEM has been proved to be of use in plant taxonomy after realization of the taxonomic importance of sieve element plastids by Dr. Dietmar Behnke, Professor of Heidelberg University Germany. Sieve element plastids (SE plastids), derived from proplastids as other plastids, constitute a type of plastid occurring exclusively in sieve elements and characteristically contain starch and/or proteins. They are classified based on their components into S-type (with starch only) and P-type with proteins ±starch. This categorization is worthwhile in linking them characteristically with taxa for being highly family- . order- and often subclass-specific and useful in angiosperm classification and taxonomic resolutions (Behnke, 1965, 1976, 1977; Behnke and Barthlott, 1983). S-Type of SE-plastids is characteristic of Hamamelidae, Rosidae & Orders Plumbaginales, Polygonales. P-types based on protein configuration are further subdivided into sub-types as P-I found in the sub-class Magnoliidae; PII in Monocots and some members of Aristollochiaceae; PIII in the subslass Caryophyllidae [PIIIa-globular-crystalloid- in the suborder Caryophyllineae, PIIIb –hexagonal- in the suborder Chenopodineae, PIIIc- withoud crystalloid- in the suborder Phytolaccineae], PIV in the order Fabales; PV in the families Ericaceae, Rhizophoraceae and PVI in the family Buxaceae. No other ultra-structure of plants till date has been found to be useful in taxonomy. Since the methodology of TEM is very critical and ultra thin sections of plant specimens are needed which reveal hardly any taxonomic marker other than SE-plastids, SEM has become more popular in taxonomy not only for its simplicity but also the efficacy to reveal both conservative (taxonomically suitable) and variable (eco-, physio-logically useful) surface characteristics of leaves, pollen, floral parts, fruits and seeds. Moreover comprehensive descriptive terms have also been made available to us by Barthlott (1981, 1984).

SEM protocol:

Today, SEM is utilized extensively in biology, forensic and medical sciences. This instrument is getting easier to use with the progress of electronics and introduction of new techniques. Any researcher can now take micrographs after short-time training in its operational procedure. The protocol for studying spermoderm characteristics is very simple and economic especially when institutional instrumentation facilities like USIC, BU (University Instrumentation Centre, Burdwan University) are available. The seeds collected fresh from plants or harvested fruits or from cold storage can be used. Only the dry, clean, mature seeds are to be directly mounted on adhesive tapes, placed on stubs and subsequently palladium gold coated. Now these stubs can be used for studying the surface pattern of the seeds using different magnifications of a Scanning Electron Microscope (e.g. SEM Hitachi, Models- S-4100, S530 etc. Japan) with a processor for photography. The photographic images can be retrieved in compact discs wherefrom prints of the same can be obtained.

Methods thus appear to be very simple and can be accomplished through the following steps:

- Fixation: The sample is fixed by a wide range of chemicals depending upon its nature.
- Dehydration or drying with Air, chemicals, liquid nitrogen for very low temperature drying and liquid carbon dioxide for Critical Point drying (CPD).
- Mounting of the dried specimens on stubs with the help of a double sided sticky tape conducted by silver paint.
- Coating with gold-palladium alloy by Ion Sputter (Bio-rad SC-500).
- High vacuum generation and focusing of electron beams in the specimen chamber of the microscope.
- Detection of secondary electrons and photographic capturing of image maintaining optimum resolution, depth of field and clarity.

Taxonomy and SEM:

Scanning electron microscopy (hereinafter abbreviated to SEM) can reveal both conservative and variable surface characteristics, the former being important taxonomically and the latter in assessing environmental impact on the species in temporal and spatial scales. However SEM appears to be very familiar for its efficiency to integrate itself with both alpha and omega taxonomy. By revealing the extent of unevenness and sculpturing i.e. relief of the surfaces of such plant organs as leaves, spores, pollen, petals, sepals, fruits, seeds etc. SEM gives information about their micromorphological characteristics. By revealing the morphological details of the structure of sex organs (gynoecia and androecia) at different stages of development and reproduction SEM contributes to the understanding of reproductive biology of the species.

SEM studies of biological structures is one of the best possible ways to reveal conservative surface characters with three dimensional qualities which provide clues to the understanding of systematics and phylogeny. Although sections of biological samples are also clearly viewed through SEM, its role in viewing surface sculpture is more valued.

The taxonomic approach based on SEM has been working very extensively and successfully on pollen i.e. microspores of angiosperms. That the scanning electron microscopic studies on epidermal and cuticular characters of leaf and seed surfaces can be very successfully used in delineating species have also been well established (Hardin, 1979; Wilkinson, 1979 Barthlott, 1981, 84; Soladoye, 1982; Wurdac, 1986; Paul and Nayar, 1987; Das *et al*., 1995; Sahai *et al.*, 1997; Kameswari and Muniyama, 2001; Biswas and Mukherjee, 2011). The present work reveals the possibilities of application of SEM in resolving surface characters of seeds for taxonomic studies.

Sem in phermatological studies:

The term 'Phermatology' was coined by Doweld (1997) to describe the study of seeds. As realized, seeds by virtue of their conservative characters, although often of small size, are ideal materials for taxonomic investigation. It is acknowledged that morphologic features of different seed structures provide a wide research field; these characters play an important role on the identification of taxa (Vaughan 1968; Corner, 1976) and have traditionally been used to solve systematic and phylogenetic problems. However for addressing the problems to discriminate seeds of different taxa remarkably sharing exomorphic features certain improved technology capable of resolving micromorphology of spermoderm or more precisely and comprehensively the free surface of the seed coat is necessary. It is the SEM which is the best technique to reveal the surface architectural pattern and relief of the spermoderm of seeds and convey information useful in systematics, ecology, genetics and phylogeny i.e. evolutionary perspectives of plants.

Seed length and width, seed shape, cell shape, and cell ornamentations are some qualitative and quantitative characters of importance. Spermoderm characters i.e. seed coat sculpturing being conservative have proved their worth to serve as excellent taxonomic markers (Skvortsov and Rosanovitch, 1974; Brisson and Peterson, 1976 and Seavey *et al.,* 1977). One advantage of seed coat micromorphology is the availability of elaborate meaningful terminologies worked out by Barthlott (1981, 1984) and Stearn (1992) for describing epidermal cell structure, seed coat surface patterns and the sculptural variations that are commonly found.

Ever since Davis and Heywood (1963) proposed scanning electron microscopy as a potential taxonomic tool, further to the classical text of Corner on dicotyledonous plants (1976) SEM- studies were completed on seed surface patterns of various taxa with considerable success Matthews and Levins, 1986; Matthews *et al*., 1994; Brahmi and Bhat; 2004; Hassan *et al.,* 2005; Adams *et al.,* 2005; Verma *et al*., 2014). The following studies and many others cover different levels of taxonomic hierarchy from the specific (Arias and Terrazas, 2004), generic (Abu Sbaih and Jury, 1994a & 1994b) to the intergeneric (Buss *et al.,* 2001; Yong-Ming *et al.,* 2011), and to the subtribal (Chase and Pippen, 1988), subfamilial (Varadarajan and Gilmartin, 1988), and familial (Musselman and Mann, 1976) levels. Seeds (and fruits) have also been surveyed within particular geographic regions, e.g. in Central and Eastern Europe by Bojansky and Fargasova (2007). Jensen (1998) also provided a very useful bibliography on seed morphology and taxonomic implications. Arias and Terrazas (2004) used morphometric analyses on seed coat features in Orchidaceae. The usefulness of micromorphological studies on seeds of *Orobanche* in relation to differentiating taxa was demonstrated with a key by Plaza *et al.* (2004) to distinguish species or groups of species. Characters of the epidermal seed coat cells of *Orobanche* species proved to be very helpful in this respect. Ornamentation of the periclinal walls could be used to discriminate four morphological types. Other features related to the anticlinal walls of the cells, such as thickness, presence/absence of a narrow trough, or relative depth, all contributed to the characterization of a large number of species.

While studying by scanning electron microscopy procedure the microstructure of 15 species seed coat in *Genisteae* (*Fabaceae*) of two regions Estrelles *et al.*(2006) could show the clarity in differences between the studied areas; so that the area of observation is relevant in order to find comparable patterns among different taxa. That the seed micromorphology with a few exceptions is of significant taxonomic value in distinguishing taxa at the species level was shown by Mostafavi *et al*., (2013) in form of variation in 20 species of *Minuartia* L. (Caryophyllaceae) representing 2 different subgenera (i.e. *Minuartia* subgen. *Spergella* (Fenzl) McNeill and Minuartia subgen. *Minuartia*).

Summing up:

An elucidation of relevance of Scanning Electron Microscopy to linking seed surface pattern with taxonomic resolutions is certain to highlight the following utilitarian perspectives.

i. Strengthens morphological description as a conservative data source and helps in biodiversity studies for having high efficiency in discrimination of taxa at the levels of family, genus, species and infra specific taxa.

ii. Provides tools to view microevolution (sculpture of exine of pollen and spermoderm of seeds).

iii. Provides tools for correct identification of the seeds of PGR (Plant Genetic Resources) so as to ensure quality control and prevention of adulteration.

iv. Provides important data source to

pharmacognostic characterization of crude drug yielding plants. The spermoderm sculpture and leaf surface epicuticular pattern can prove worth in preparation of the 'standards' useful in prevention of adulteration and controlling quality of drugs

v. Helpful in ecological studies

(a) Assessment of seed-bank potential of any land especially of wastelands by scanning the mineral matrix of top-soil/spoil and identifying the seeds in them.

(b) Monitoring of water-status of habitats through identification of xeric, mesic and hydric states.

(c) In studying trophic relationship with insects and their co-evolution.

(d) In identification of ecoclines (different biotypes resulting from habitat heterogeneity) along environmental gradients.

While winding up the present discourse it can be said that in spite of realization of the potential of the SEM-features of plant parts to accomplish traditional as well as modern plant taxonomy, the work so far done, especially on seed surface ornamentation pattern, are meager and inadequate. There is, thus, a tremendous scope to undertake research programmes on conservative surface and have deeper insight into the taxonomic identity of plants and address problems concerning their inter- and intra-relationship and phylogeny with the comprehensive but powerful tool of SEM.

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