Advantages of the use of C60 fullerene/benzol solution in ultrastructure investigations: A case study of *Cycas rumphii* Miq. pollen

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Transmission electron microscopic observations on partially degraded pollen grains of *Cycas rumphii* are presented. Degradation was achieved with the use of C60 fullerene/benzol solution instead of the normally used 2-aminoethanol and potassium permanganate solution. Owing to its characteristic physical and chemical properties, advantages of this degradation agent have further been emphasized. Applying this method, a good contrast is achieved without using OsO_4 or other post-fixative agents.

FULLERENE, the ball-shaped molecules are pure, closed and hollow form of aromatic carbon. Most prevalent are C60 and C70; however, many others like C76, C78, C84 and those of higher molecular weight are also found. Since their discovery, fullerenes, have stimulated intense interest in the scientific, industrial and medical community because of their unique structure and properties. Being small particles (nano size), their application in emulsion formulation leads to uniform dispersion. The surface coating with fullerene C60 is less than 20 nm, homogeneous and smooth. These attributes help in obtaining better results in electron microscopic observations of organic entities. Keeping in view the properties of C60 fullerene/benzol, the fossil walls at molecular level were studied. In this communication, the effect of this agent on modern pollen of *Cycas rumphii* has been presented.

Living and fossil Cycadalean pollen grains have been studied using LM, SEM and TEM[1–5]. With an objective to study the resistance and biopolymer organization in ectexine, pollen grains of *Cycas rumphii* Miq. were partially degraded with the help of 2-aminoethanol and potassium permanganate (aqueous dil. 1%). A molecular system at different levels of organization was observed earlier in the extexine of these pollen grains[6]. Later, degradation of these pollen grains with C60 fullerene/benzol solution was attempted. Kedves[7], and Kedves and Frey[8] discussed the advantages of this method over the classical degradation and fragmentation with 2-aminoethanol and $KMnO_4$. Fossil *Botryococcus* colonies were degraded with C60 fullerene/benzol and quasi-crystalloid and quasi-equivalent biopolymer networks were observed[8]. The need for a more methodical experiment for systematic application has already been emphasized[8]. In the present communication, observations on the ultrastructure of ectexine of *C. rumphii* pollen after treatment with C60 fullerene/benzol solution and merkaptoethanol are discussed. This method provides an opportunity to establish the degree of resistance in ectexinous biomacromolecules that could be observed with the help of TEM without post-fixation with dilute solution of OsO_4 and lead citrate or uranylacetate. As post-fixatives, OsO_4 and lead citrate do not give good results because these get precipitated inside the material and hamper clear observations. Besides this, a gradual degradation of exine when treated with OsO_4 at higher temperature was observed[9].

After the pioneering work by John[5], several concepts have been established by different researches regarding the structure and composition of sporopollenin[3,10–14]. Comprehensive works carried out later conclude that sporopollenin is a complex of organic molecules and its structure and composition vary in different taxa[1,15–17]. To understand the processes involved in the decay and diagenetic alteration of plants as they become fossilized, it is important to study the resistant parts at the molecular level. The biomacromolecular structure and its organization in spore/pollen have been investigated by several workers[15,18,19] and its destruction in nature has been related to the determination of the pollen wall composition[20]. Differential susceptibility of the spore pollen wall to degradation has been established[15].

TEM studies revealed the presence of highly organized biomacromolecular structures in spore/pollen walls[4,18,19,21–25]. After the discovery of quasi-crystals in rapidly quenched Al–Mn alloy[2,26–28], Kedves[7,29] first published this metastable molecular system in the ectexine of *Pinus griffithii*. After partial degradation and fragmentation with magnetic stirrer, quasi-crystalloid and quasi-equivalent biopolymer structures were observed in the wall of fossil *Botryococcus braunii*[30–32]. Attempts were also made to establish the relation between two opposite biopolymer organization systems[20]. Later, biopolymer structures which may be modelled with fullerenes, were observed in partially degraded pollen walls. Attempts were made to incorporate C60 fullerene/benzol solution in the partial degradation process. Results of these experiments are summarized as follows:

(i) In partially degraded wall of *B. braunii*, highly organized biomacromolecules arranged in hexagons and pentagons were observed[8].

(ii) In *Taxus baccata*, one of the experiments revealed highly organized globular biopolymer units[32]. A regular hexagon connected with a regular pentagon was also noticed. Symmetry operations revealed that this might be a fragment of a fullerene-like biomacromolecule.

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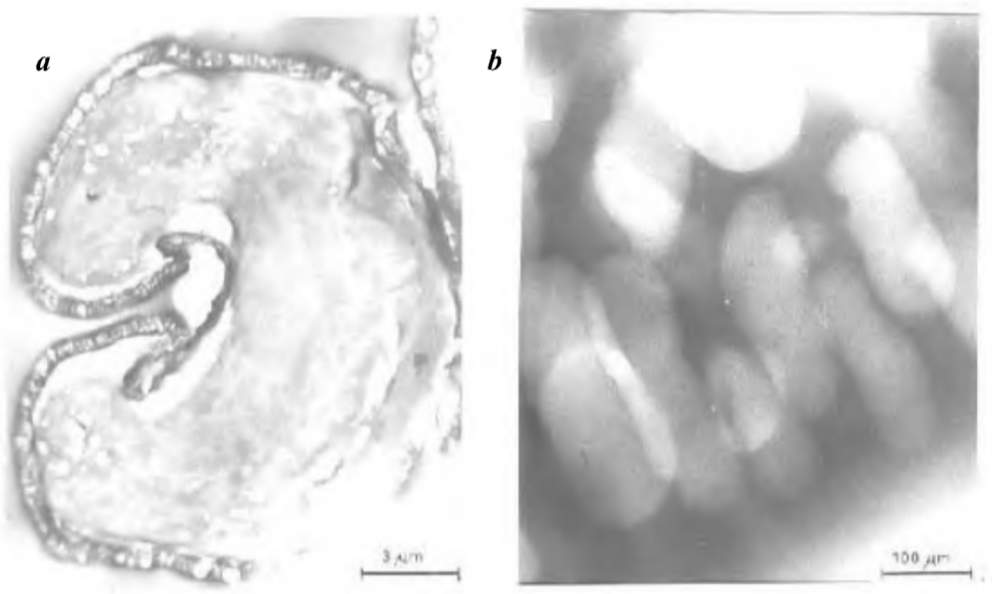

Figure 1. Pollen grain of *Cycas rumphii* Miq. ***a***, Ultrastructure in equatorial section showing degradation of intine and protoplasm. Negative no. 10107, 7500 ×. ***b***, Details of exine ultrastructure showing tectum with alveolar elements. Radial orientation of alveolar system is clearly discernible. Negative no. 13734, 250,000 ×.

(iii) In *Ginkgo biloba*, no biopolymer structures were observed but a good contrast between the ectexine and the remnant of the protoplasm was achieved[33].

(iv) In *Quercus robur*, partial degradation of the pollen wall was observed[33].

From these results it is clear that the effect of C60 fullerene/benzol is different on different biological objects, possibly indicating differences in the molecular systems and structures.

The experimental method and results of the present investigation are summarized as follows:

Dry pollen grains of *C. rumphii* (5 mg) were treated with 5 ml C60 fullerene/benzol solution for 24 h at 30°C. Material was washed with benzol, dried and kept in 5 ml merkaptoethanol for 24 h. After washing, the partially degraded pollen grains were embedded in Araldite (Durcupan, Fluka) and ultrathin sections were cut on a Porter Blum ultramicrotome. TEM pictures were taken on a Zeiss Opton EM-902 having resolution of 2–3 Å.

The ultrastructure of pollen grains through the equatorial region clearly exhibits degradation of intine and more or less degraded organelles of protoplasm (Figure 1 *a*). Well-illustrated ectexine consists of tectum, alveolar infratectal layer with radial alveolar elements and foot layer (Figure 1 *b*).

Based on these studies, it is concluded that biopolymer systems of different organization levels may be revealed in some taxa and a good contrast in the structures of ectexine could be achieved without using OsO_4 or other chemicals. The contrast in ectexinal features using fullerene/benzol solution is much better than that achieved by the traditionally applied methods for TEM investigations. It is emphasized that this method should be modified further to achieve advanced and specific standards to which, in this contribution, the authors call the attention of experimental palynologists.

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HLA-DR phenotypes and IgG, IgA and IgM antibody responses to *Mycobacterium tuberculosis* culture filtrate and 30 kDa antigens in pulmonary tuberculosis

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The role of HLA-DR genetic make-up on the IgG, IgA and IgM antibody response to *Mycobacterium tuberculosis* culture filtrate and 30 kDa antigens was studied in pulmonary tuberculosis. The study was carried out in HLA-DR typed active pulmonary tuberculosis (ATB) patients ($n = 37$), inactive (cured) pulmonary tuberculosis (ITB) patients ($n = 79$) and normal healthy subjects (NHS; $n = 46$). In ATB and ITB (cured) patients, IgG antibody (optical density at 490 nm for 1 : 3200 dilution) as measured by enzyme-linked immunosorbent assay was the predominant one than IgA and IgM antibodies. Increased IgG antibody titre to culture filtrate ($P = 0.03$) and decreased titre to 30 kDa antigen were observed with HLA-DR1-positive ATB patients than non-DR1 (ATB) patients. Moreover, HLA-DR4- and HLA-DR6-positive ATB patients showed trends toward an increased IgG antibody response to 30 kDa antigen than HLA-DR4- and HLA-DR6-negative (ATB) patients respectively. Significantly increased IgA antibody to 30 kDa antigen was observed with HLA-DR1-positive ATB patients than non-DR1 patients ($P = 0.03$). The study suggests that multiple HLA-DR molecules may regulate the IgG and IgA antibody responses to various proteins of *M. tuberculosis*. Moreover, HLA-DR phenotypes and increased IgG and IgA antibody titres may be useful to differentiate *M. tuberculosis*-infected subjects from normal subjects and cured patients with the same HLA-DR phenotypes or genetic make-up.

MYCOBACTERIAL infections are associated with the production of circulating antibodies and development of cell-mediated immune functions. Although antibody titres tend to be higher in tuberculosis patients than normal subjects, results have been disappointing for serodiagnosis of tuberculosis because of overlapping antibody response. Protein antigens of mycobacteria are characterized by cross-reactivity when analysed for antibodies obtained by experimental immunization or by those arising during natural mycobacterial infection. Species specificity is rare at the level of individual proteins, as would be expected of major proteins of functional significance[1,2]. Identification of antigens and their antigenic determinants is im-

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