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# Structural and histochemical studies on grain-filling in the caryopsis of rice (*Oryza sativa* L.)

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The endosperm and embryo that constitute the filial tissues of rice caryopsis are isolated from the maternal tissues by the absence of any symplastic continuity. Nutrients are transported to the endosperm through a single ovular vascular trace present on the ventral side of the ovary. Initially solute enters through the chalaza into the nucellar projection and then into the endosperm. At later stages transport occurs through the nucellar epidermis, centripetally towards the endosperm. The cell walls of the nucellar epidermis are provided with rib-like thickenings. A comparison of grain-filling in C<sub>3</sub> and C<sub>4</sub> cereals suggests that rice has structural features allied to C<sub>3</sub> cereals, such as wheat, but with significant differences.

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## 1. Introduction

The development and structure of rice grain are fairly well understood (Santos 1933; Juliano and Aldama 1937). Recently Jones and Rost (1989) and Bechtel and Pomeranz (1977, 1978a,b) investigated the ultrastructure of developing rice embryo and mature rice grain. The possible route of entry of assimilates into the developing rice caryopsis was studied by Hoshikawa (1984) and Oparka and Gates (1981a,b, 1982). We have previously reported on the structure of rice caryopsis in relation to yield (Ebenezer *et al* 1990, 2001), and on the histochemical localization of major storage components (Krishnan *et al* 2001). We report here our findings on the transport of assimilates and major nutrients in developing grains and the storage components in mature caryopsis of rice.

## 2. Methods

### 2.1 Plant material

An indica rice, *Oryza sativa* cv. IR50, was the central focus of study. However, several other cultivars and spe-

cies obtained from the International Rice Research Institute (IRRI), Philippines, and local sources were examined to compare and confirm the observations made on IR50. These other rices include: cv. Ponni, IR20, and ADT36 (from Tamil Nadu Agricultural University, Coimbatore), J13 (from J-Farm, Kelambakkam, Tamil Nadu) and *Oryza alta* Swallen, *O. australiensis* Domin, *O. barthii* A Chev, *O. brachyantha* A Chev & Roehr, *O. eichingeri* A Peter, *O. glaberrima* Steud, *O. grandiglumis* Proehl, *O. granulata* Nees. et Arn, *O. latifolia* Desv, *O. longiglumis* Jansen, *O. longistaminata* A Chev et Roehr, *O. minuta* J S ex C B Presl., *O. nivara* Sharma & Shastry, *O. officinalis* Wall ex Watt, *O. punctata* Kotschy, *O. ridleyi* Hook f and *O. rufipogon* Griff (from IRRI). Unless otherwise specified data and figures refer to IR50.

### 2.2 Microscopy

Free-hand sections, wax and Spurr plastic-embedded thin sections were stained with a variety of bright-field dyes and fluorochromes using standard histochemical procedures (Pearse 1972, 1980; Fulcher 1982; Harris and Oparka

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1994; Krishnan 1996). Movement of phloem-specific fluorochrome 5 (6)-carboxyfluorescein through the vascular trace and nucellar epidermis was studied by examining sections of the caryopsis at different times after placing the cut end of a pedicel in 0.01% dye solution. Specimens were examined and photographed with a Nikon Microphot-FXA provided with bright-field, dark-field, phase-contrast, Nomarski-DIC, polarized light and fluorescence modes of examinations.

### 3. Results

The term 'caryopsis' refers to the fruit of rice which at maturity consists of a thin and dry pericarp, a bulky endosperm with an outer layer of aleurone cells, and an embryo. The developing caryopsis of rice is hermetically enclosed within the space provided by two tightly clasping fertile glumes of the spikelet, the palea and lemma. A rice spikelet has other associated structures namely, rachilla, sterile lemmas, and rudimentary glumes. An abscission layer occurs immediately below the sterile lemmas. The terms 'grain' and 'paddy' should refer to this collective unit, and the term caryopsis should be restricted to the fruit of rice. One of the important but poorly understood aspects of grain-filling in rice is the relationship between the caryopsis and the sterile outer coverings, particularly the palea and lemma. In addition to offering protection against insects and fungi, the palea and lemma may contribute assimilates to the developing caryopsis, regulate water balance during grain-filling and

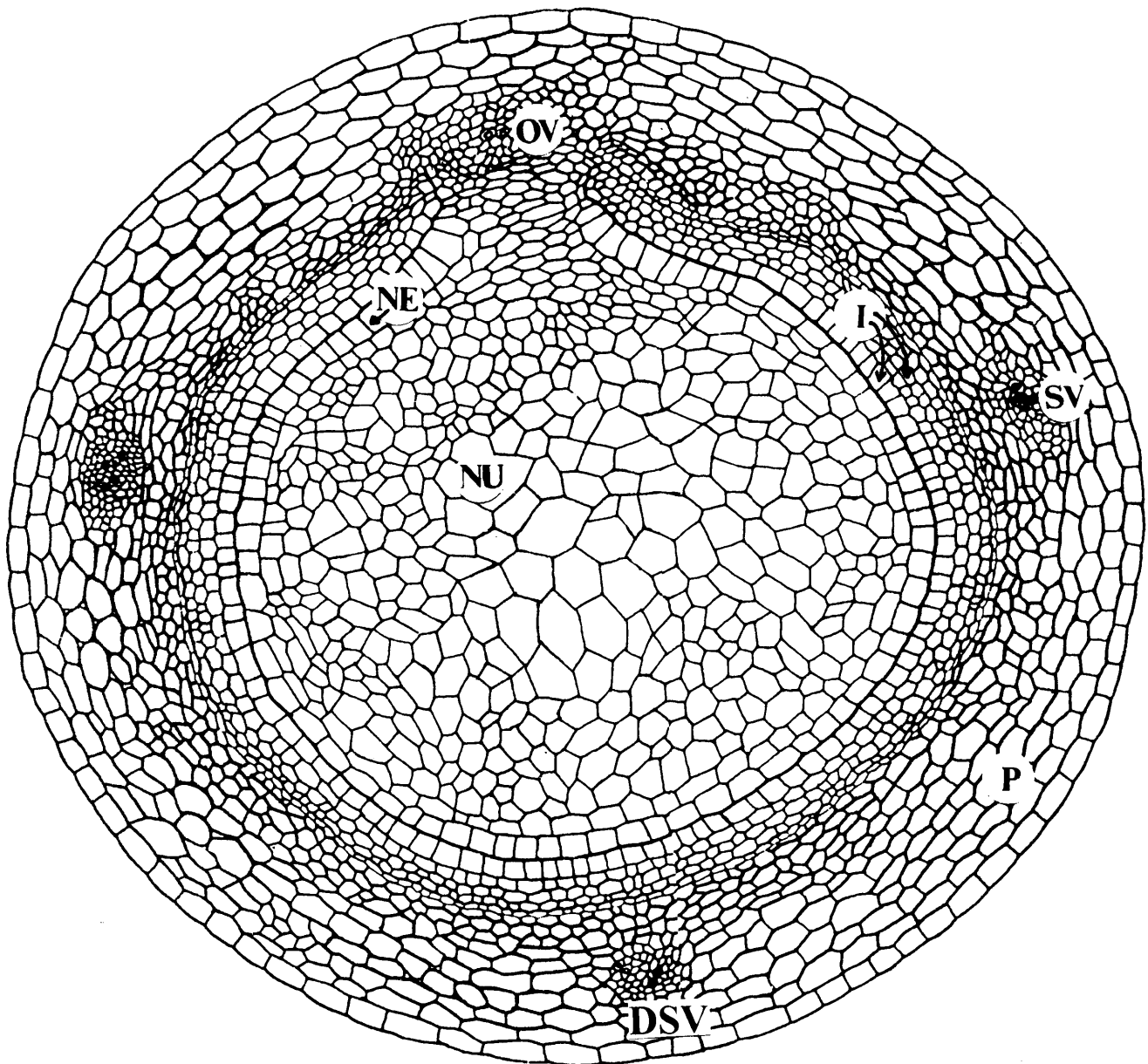
impose a limit on the size of the caryopsis (Cochrane and Duffus 1979).

#### 3.1 Development of ovary and pericarp

The changes in size and shape of the rice ovary from the time of flower opening (anthesis) to 30 days after fertilization (DAF) are summarised in figure 1. Immediately after fertilization rapid changes occur in the tissues of caryopsis: i.e. pericarp, vasculature, integuments, nucellus, endosperm and embryo. The structure of the ovary and caryopsis at three successive stages of development, as seen in transverse sections, is illustrated in figures 2–4. The ovary wall at the time of anthesis consists of about 7–10 layers of cells (figure 2). Embedded within the ovary wall are vascular tissues that supply water and nutrients to the stigma and the ovule. The outer epidermis of the ovary is covered by a thin-layer of cuticle. The inner epidermis consists of smaller cells that later develop into tube-cells. Two or three layers of subepidermal cells of the inner epidermis develop into cross-cells (figures 3, 6a). During the first six DAF the caryopsis rapidly elongates and reaches a maximum length of about 8 mm. The inner epidermal cells separate from each other, elongate longitudinally (parallel to the long axis of the caryopsis), and form an extensive network of tube-cells that enclose the developing endosperm. The functional significance of tube-cells is not known. They might offer mechanical support or may be involved in short distance transport of



**Figure 1.** Size and shape of rice caryopsis at 2–3 day intervals during development from anthesis (upper left) to maturity, 30 days after fertilization (DAF) (lower right).



**Figure 2.** Transverse section of mid-region of an ovary a few hours before anthesis showing various cell and tissue types. A dorsal stylar vascular trace is present opposite the ovular vascular trace. (DSV, Dorsal stylar vascular trace; I, integument; NU, nucellus; NE, nucellar epidermis; OV, ovular vascular trace; P, pericarp; SV, lateral stylar vascular trace.)

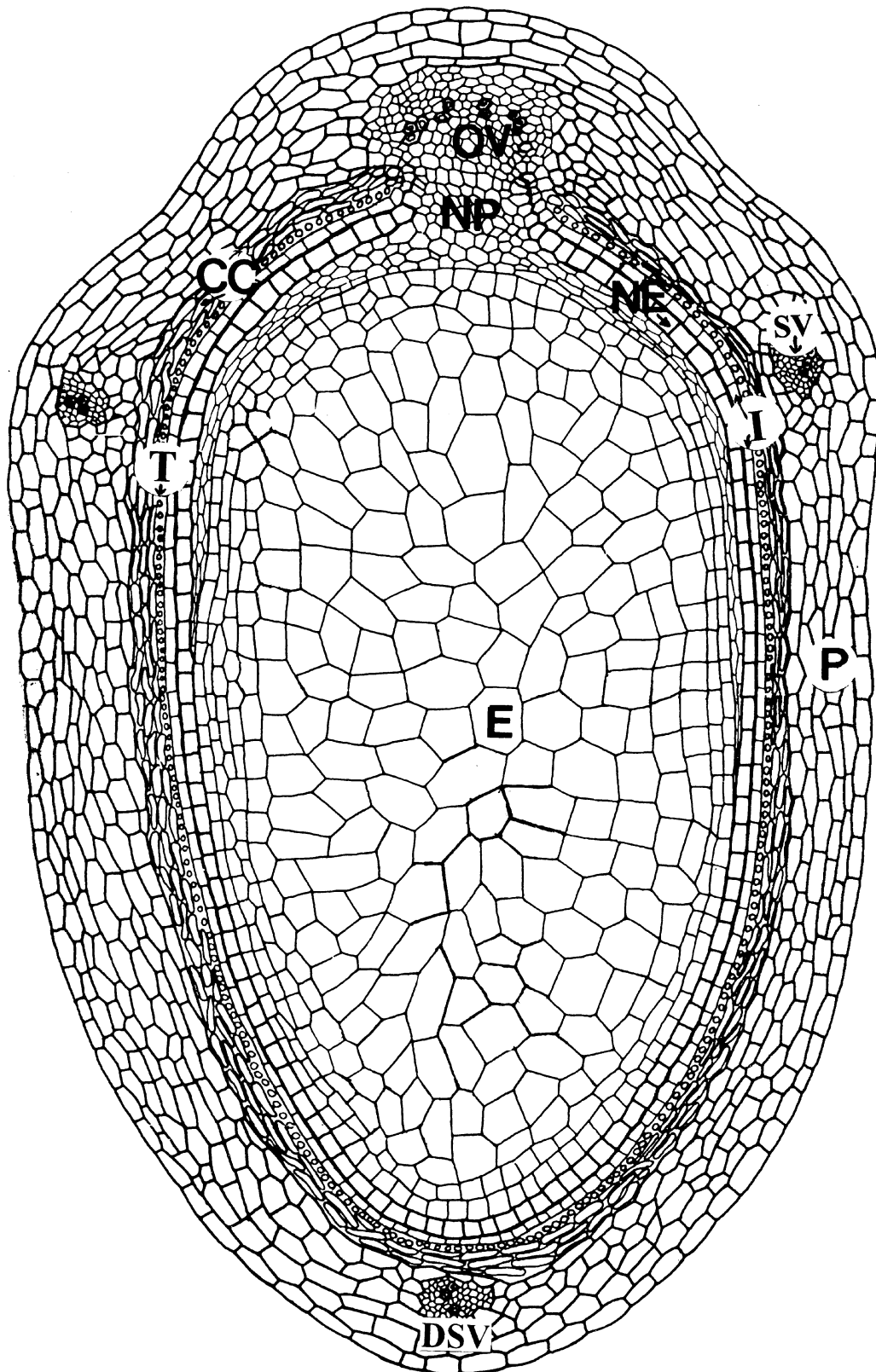
water or assimilates from one region of the caryopsis to another.

The subepidermal cells, adjacent to the tube-cell layer (inner epidermis), develop into cross-cells by elongating at right angles to the long axis of the tube-cells (figure 7d). Intercellular spaces develop between adjacent cross-cells. Each cross-cell has 2–5 chloroplasts. In transverse sections the layer appears green and excitation with blue light of a fluorescence microscope induces red fluorescence due to the presence of chlorophylls (figure 6a).

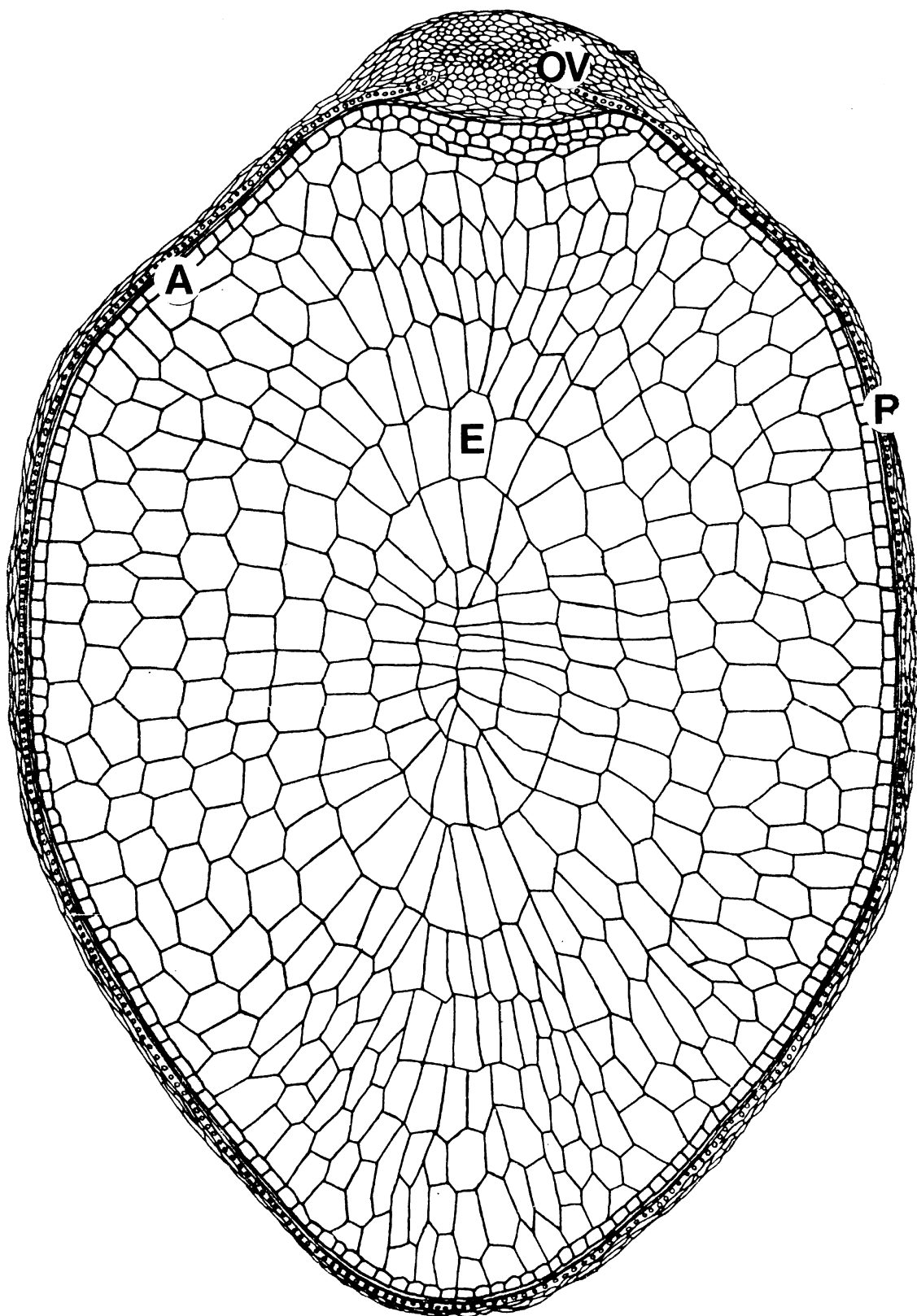
This assimilatory layer of cross-cells is in close contact with the vascular trace and surrounds the vascular trace on its outer sides. Cochrane and Duffus (1979) have suggested that the pericarp in wheat and barley may supply photosynthates to the endosperm. The cross-cells in rice may play a similar role (Ebenezer *et al* 1990).

### 3.2 Vascular traces

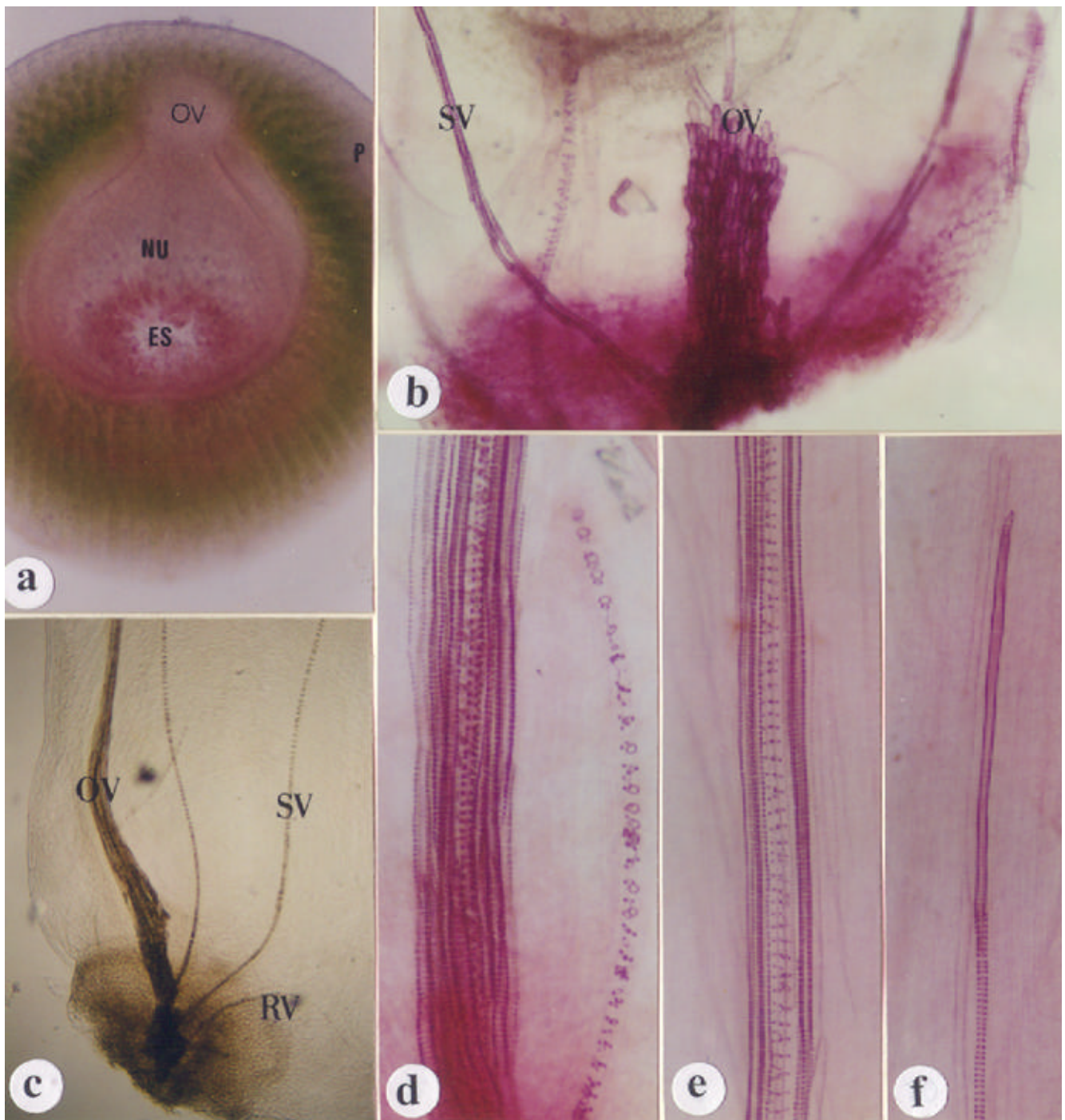
The vascular traces of the ovary are important transport components during different stages of development of the



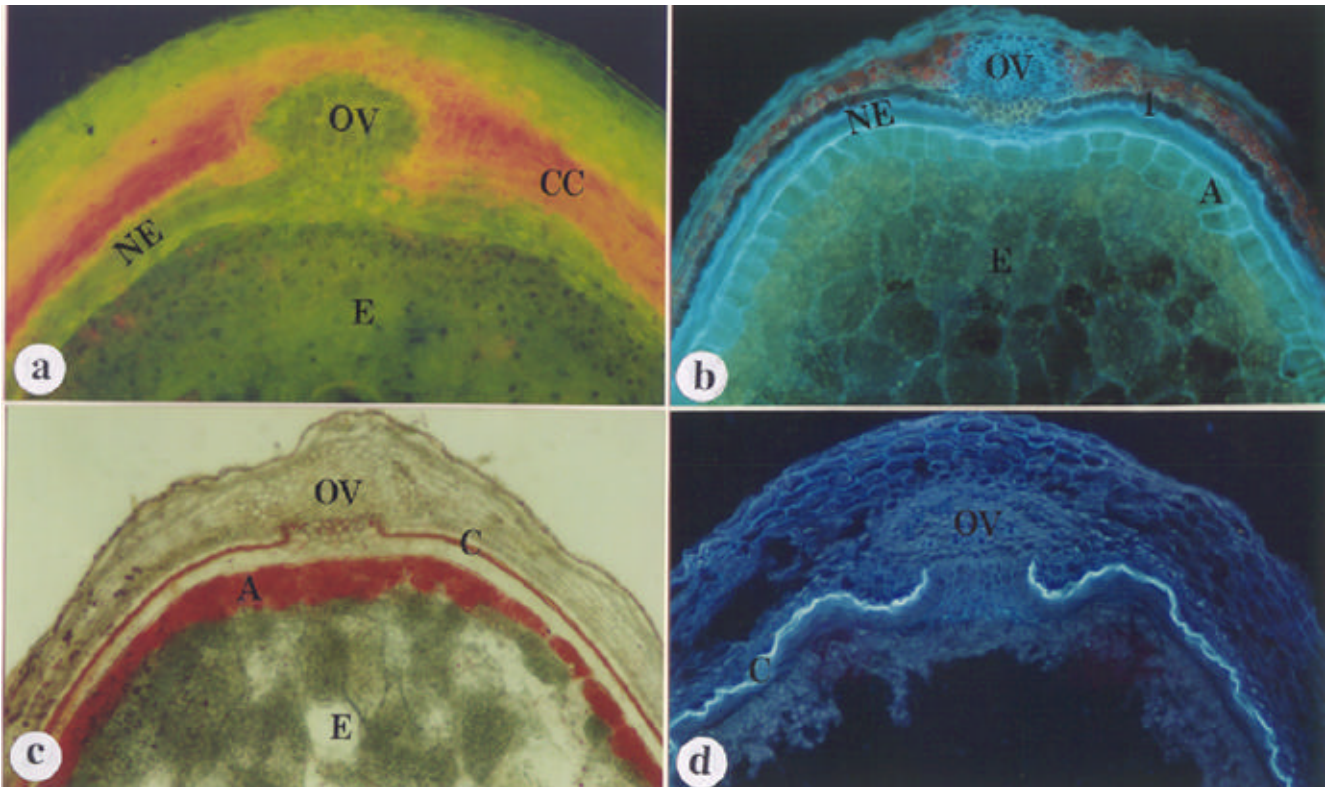
**Figure 3.** Transverse section in the mid-region of a caryopsis about 6 DAF. (CC, Cross-cells; DSV, dorsal stylar vascular trace; E, endosperm; I, integument; NE, nucellar epidermis; NP, nucellar projection; OV, ovular vascular trace; P, pericarp; SV, lateral stylar vascular trace; T, tube-cells.)



**Figure 4.** Transverse section in the mid-region of a mature rice caryopsis about 30 DAF. (A, Aleurone; E, endosperm; OV, ovular vascular trace; P, pericarp.)



**Figure 5.** (a) Early stages of enlargement of the embryo sac in a pre-anthesis ovary. Dehydrogenase activity is localized in the nucellus, particularly in the degenerating cells around the embryo sac. X125. (b-f) Cleared, whole mount of ovaries showing vascular supply. Pararosaniline hydrochloride stained. (b) Details of a short pad of vasculature located at the base of a young ovary. X250. (c) Ovary 3 DAF. The ovular vascular trace is differentiated along the length of the caryopsis. Two stylar vascular traces and the third rudimentary stylar vascular trace are also seen. X100. (d-f) Ovular vascular trace photographed at successively higher levels from the base, middle and upper portion of the caryopsis. Only a few xylem elements are seen at the apex. (f) X250. (ES, Embryo sac; NU, nucellus; OV, ovular vascular trace; RV, rudimentary stylar vascular trace; SV, stylar vascular trace.)

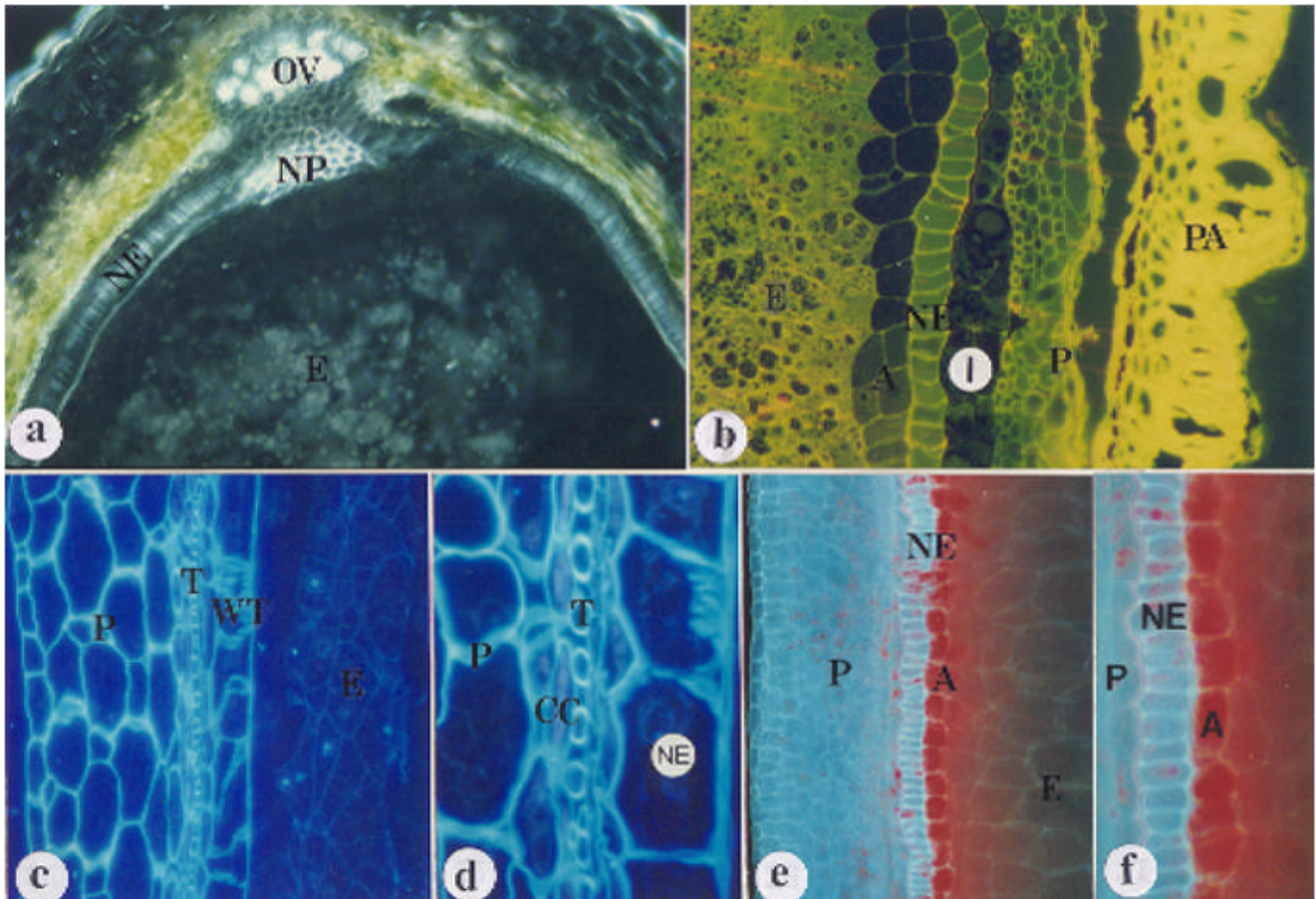


**Figure 6.** (a) Free-hand transverse section of caryopsis 6 DAF. Autofluorescence of young caryopsis under blue excitation. Chlorophylls in cross-cells fluoresce in red. X250. (b) Transverse section of caryopsis showing the tegmen derived from the inner layer of inner integument in a wild species of rice (*Oryza punctata*). Endosperm, aleurone, nucellar epidermis, integument and cross-cells are seen. X250. (c) Localization of lipids with Sudan IV in mature grain. Lipid droplets in the aleurone, and the cuticle over the nucellar epidermis are stained red. X250. (d) Fluorescence micrograph showing the cuticular layer over the nucellar epidermis. Transverse section of rice caryopsis stained with dansyl chloride. UV excitation. Excepting the OV region there is no transport pathway between the pericarp and endosperm. X250. (A, Aleurone; C, cuticular layer; CC, cross-cell; E, endosperm; I, integument; NE, nucellar epidermis; OV, ovular vascular trace; P, pericarp.)

caryopsis. Transverse sections of young ovaries usually show three vascular traces, two in lateral position and one on the ventral side. Occasionally an ovary may have a fourth vascular trace on the dorsal side (figures 2, 3, 5c). The dorsal trace, when present, and the two lateral vascular traces enter the style and stigma. These three vascular traces have no structural contact with the ovule and appear to play no physiological role in grain-filling. The presence of a dorsal stylar trace is associated with the vestigial third stigma, indicative of the putative tri-carpellary origin of the rice ovary. Nutrient supply to the ovule and the developing endosperm is carried only through the large ventral vascular trace attached to the chalaza (figures 5b–f, 8a–f). This vascular trace, wrongly described in literature as dorsal vascular trace, is really in the morphologically ventral side of the ovary. Perhaps, it should be described as an ovular or chalazal vascular trace (figure 6a–d). This vascular trace, in conjunction with chalazal and nucellar tissues, transport nutrients into the endosperm.

### 3.3 Integuments

At anthesis, the nucellus is covered by two integuments, each typically with two layers of cells. Within 2 DAF the two layers of the outer integument and the outer layer of the inner integument are absorbed. By about 3 DAF only the inner layer of the inner integument persists. In most cultivars even this layer is absorbed leaving only the cuticular remains of the integument (figures 6c, d, 8e). Thus, in IR50 rice the mature caryopsis does not have any persistent layer of cells of the integuments, and therefore, a testa or tegmen is not present. In some cultivars of rice, particularly those that are heavily pigmented, and in many wild species, the inner layer of the inner integument does persist in mature caryopsis (figure 7b). The persistent inner integument is the tegmen or the inner seed coat of the caryopsis. The cells of the tegmen accumulate a reddish-black pigmented material which appears to be tannin, as visualized through ferric chloride and nitroso-histochemical staining reactions.



**Figure 7.** (a) Transverse section of caryopsis 7 DAF showing the structure of nucellar epidermis. Between crossed polarizers cellulose thickenings on the tangential walls of nucellar epidermis are birefringent. The nucellar projection and the ovular vascular trace are also birefringent. X250. (b) Transverse plastic thin section of young caryopsis about 10 DAF. X500. (c, d) Fluorescence micrograph of transverse sections of 10-day-old caryopsis. Thin plastic sections stained with calcofluor white M2R and excited with UV. Cross-cells, tube-cells, remnants of the integuments and nucellar epidermis with wall thickenings are seen. (c) X500. (d) X1250. (e, f) Longitudinal sections of caryopsis 10 DAF stained with Sudan IV to localize lipid in aleurone cells. Nucellar epidermis and pericarp are also seen. (e) X125. (f) X250. (A, Aleurone; CC, cross-cell; E, endosperm; I, integument; NE, nucellar epidermis; NP, nucellar projection; OV, ovular vascular trace, P, pericarp; PA, palea; T, tube-cell; WT, wall thickenings.)

### 3.4 Nucellar epidermis

Like the pericarp and the integuments, the nucellus is a maternal tissue. As the embryo sac increases in size, the surrounding cells of the nucellus are progressively absorbed (figures 2, 5a). By about 5 DAF, most of the nucellar tissue is absorbed except for a prominent single layer of nucellar epidermis (figures 6a–c, 7a–f), and a small layer of tissue constituting the nucellar projection immediately below the chalazal region near the ovular vascular trace (figures 7a, 8a, d, e). Embryo development and endosperm differentiation take place within the embryo sac.

We have estimated the total number of cells in the nucellar epidermis of a single caryopsis to be about 145,000. By about 7 DAF, unique thickenings are noticed on the anticlinal walls of the nucellar epidermis (figure

7a, c). The thickenings appear as ribs of primary wall material and are cellulosic in nature as shown by histochemical tests. The thickenings may provide mechanical support to the enlarging endosperm (Ellis and Chaffey 1987). The endosperm becomes completely cellular within 10 DAF. From this stage onwards the nucellar epidermis shows signs of disintegration, and, by about 20 DAF, the nucellar epidermis loses its integrity and is obliterated.

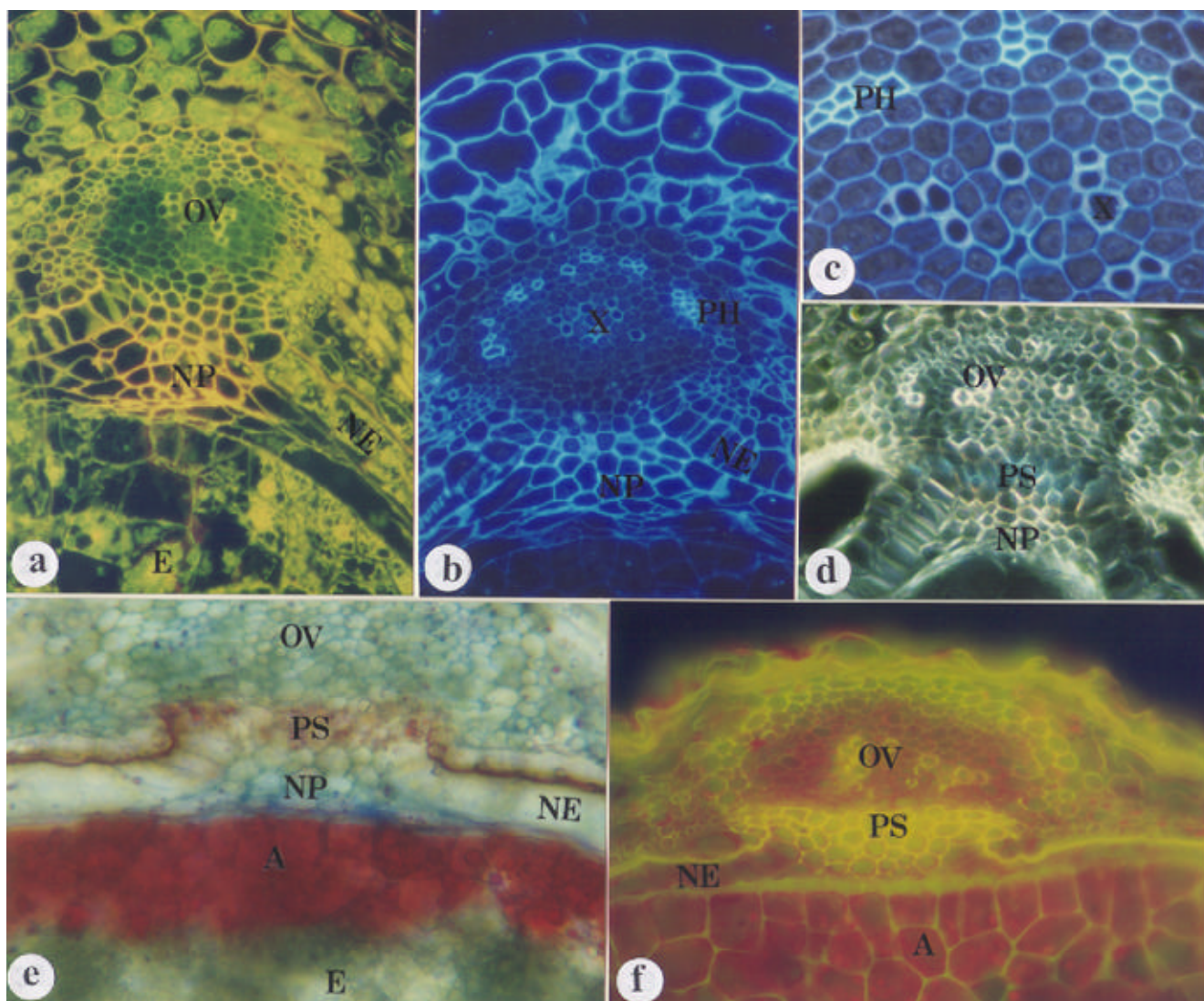
### 3.5 Chalaza and nucellar projection

Immediately below the ovular vascular trace is a zone of tissue consisting of 4 or 5 layers of cells comprising the chalaza (figure 8a, b, d, e). The chalazal region is



equivalent to the pigment strand of the wheat caryopsis (Zee and O'Brien 1970). A small zone of persistent nucellar cells, termed the nucellar projection, is attached to the chalaza. In older caryopses, the pigment strand accumulates lipoidal material (figure 8e). The cells of the nucellar projection are not naturally coloured, as in wheat. However on staining with Sudan and Nile blue A, the presence of lipids in the cells and suberin in the cell walls

can be made out (Oparka and Gates 1982). Cell walls of the pigment strand possess unusual wall properties. When viewed between crossed polarizers in the conventional position, these cells appear blue indicating the presence of additional wall encrustations (figure 8d). When the same cells are examined between crossed polarizers with a First Order Red Plate they appear to have orientation of wall material in the opposite direction as compared with the



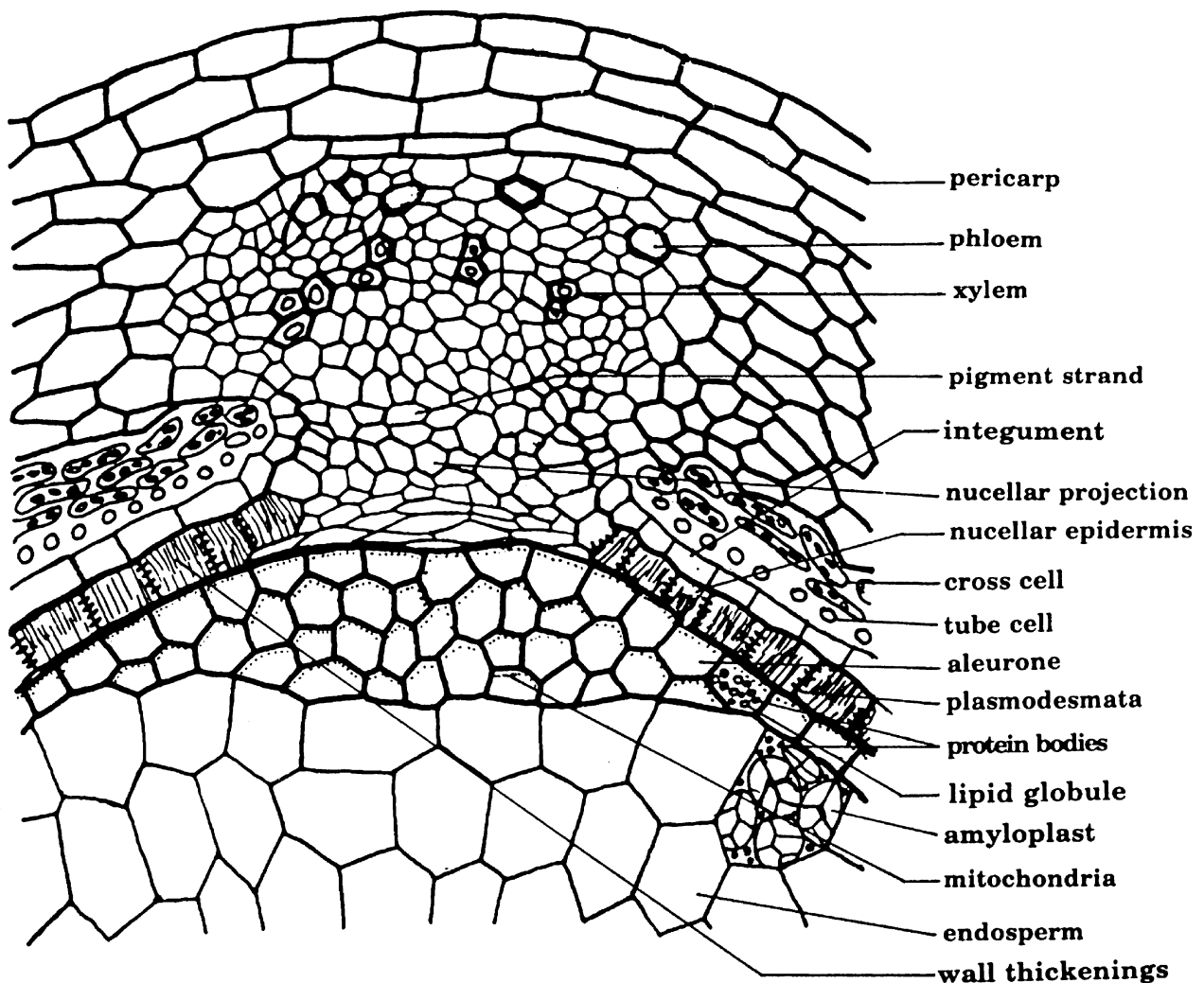
**Figure 8.** (a–f) Details of the ventral region of caryopsis showing ovular vascular trace and associated tissues. (a) Fluorescence micrograph of thin plastic transverse section stained with acridine orange. Nucellar projection is strongly fluorescing in yellow. X250. (b) Thin plastic section stained with calcofluor white M2R showing phloem, xylem, pigment strand, nucellar projection and nucellar epidermis. X500. (c) Closer view of xylem and phloem in transverse section. Thin plastic section, calcofluor white M2R stained. UV excitation. X1250. (d) Transverse section viewed between crossed polarizers without First Order Red Plate. The pigment strand cells are dichroic. X500. (e) Free-hand section stained with Sudan IV and toluidine blue O. Sudan stains the lipid in the aleurone and lipid droplets in the pigment strand. Nucellar projection stains blue. X500. (f) Fluorescence micrographs of free-hand transverse section stained with alizarin red S. Blue excitation. Aleurone appears red. The green layer between the pigment strand and the endosperm is crushed nucellus. Cuticle over the nucellar epidermis is also seen. X500. (A, Aleurone; E, endosperm; NE, nucellar epidermis; NP, nucellar projection; OV, ovular vascular trace; PH, phloem; PS, pigment strand; X, xylem.)

cell walls of the adjacent parenchyma and other cells. It is likely that the orientation of cellulose microfibrils is itself different in these cells. Alternately, deposition of suberin may alter the initial microfibrillar orientation.

### 3.6 Cuticular layers

A prominent structural feature of the developing and mature caryopsis is the presence of a cuticular layer that covers the developing endosperm. The cuticle is auto-fluorescent. Fluorochromes such as acridine orange, ali-

zarin red S, calcofluor white M2R, coriphosphine O, dansyl chloride and Nile blue A also reveal the presence of this cuticular layer (figures 6c, d, 8e). The cuticle that surrounds the endosperm and appears to be a single layer is in fact made up of at least two closely appressed layers. One layer is derived from the outer covering of the nucellar epidermis. The other is derived from the inner epidermis of the inner integument. The cuticular layer is an effective boundary that isolates the developing endosperm and its contents from the surrounding pericarp. However, the cuticular layer is interrupted by the pigment strand just below the ovular vascular trace (figure 8e).



**Figure 9.** Diagrammatic representation of a portion of the ventral side of the caryopsis seen in transverse section at rapid grain-filling stage (about 10 DAF). The pericarp, ovular vascular trace, cross-cells, tube-cells, integument, nucellar epidermis and the pigment strand constitute the maternal tissue. The endosperm, including the aleurone constitutes the filial tissue (embryo is not shown). One aleurone and one sub-aleurone endosperm cells are shown with contents. The aleurone below the nucellar projection is multilayered and the cells possess regularly arranged mitochondria. The single layer of integument and the nucellar epidermis are separated by a cuticular layer and there are no plasmodesmata between these two layers. This diagram is based on our investigations and the ultrastructural studies of Oparka and Gates (1982) and Ellis and Chaffey (1987).

### 3.7 Endosperm and aleurone layer

As the zygote develops into an embryo the primary endosperm nucleus also divides initiating the process of endosperm development. The development of endosperm belongs to the *ab initio* nuclear type; by the end of the first DAF a large number of free endosperm nuclei are located in the peripheral region of the expanding embryo sac. The endosperm turns cellular initially around the developing embryo by about the end of 2 DAF and cellularization of endosperm proceeds from this micropylar region to the chalazal end. By the third day, a peripheral layer of endosperm is established within the embryo sac. These cells divide, and through cambium-like activity produce more cells from the periphery to the centre. Cell counts based on transverse and longitudinal sections indicate that there are more than 65,000 endosperm cells on the 5th DAF. At this stage some nucellar tissue persists in the periphery of the embryo sac. During the next few days, the endosperm cells further divide and the outer layers simultaneously differentiate into aleurone and sub-aleurone cells. The endosperm cell number reaches a maximum of 75,400 by about 20 DAF. In addition, each grain has about 65,000 aleurone cells. The developing embryo as well as the endosperm store large quantities of imported nutrients. Histochemical tests are now available for the localization of stored lipids, phytin, and proteins in the aleurone cells, and the starch, protein and remnants of nuclear material in the dead endosperm cells. Only the aleurone cells of the endosperm remain living and function later during seed germination by *de novo* synthesis of enzymes involved in degradation of storage material.

### 3.8 Grain-filling

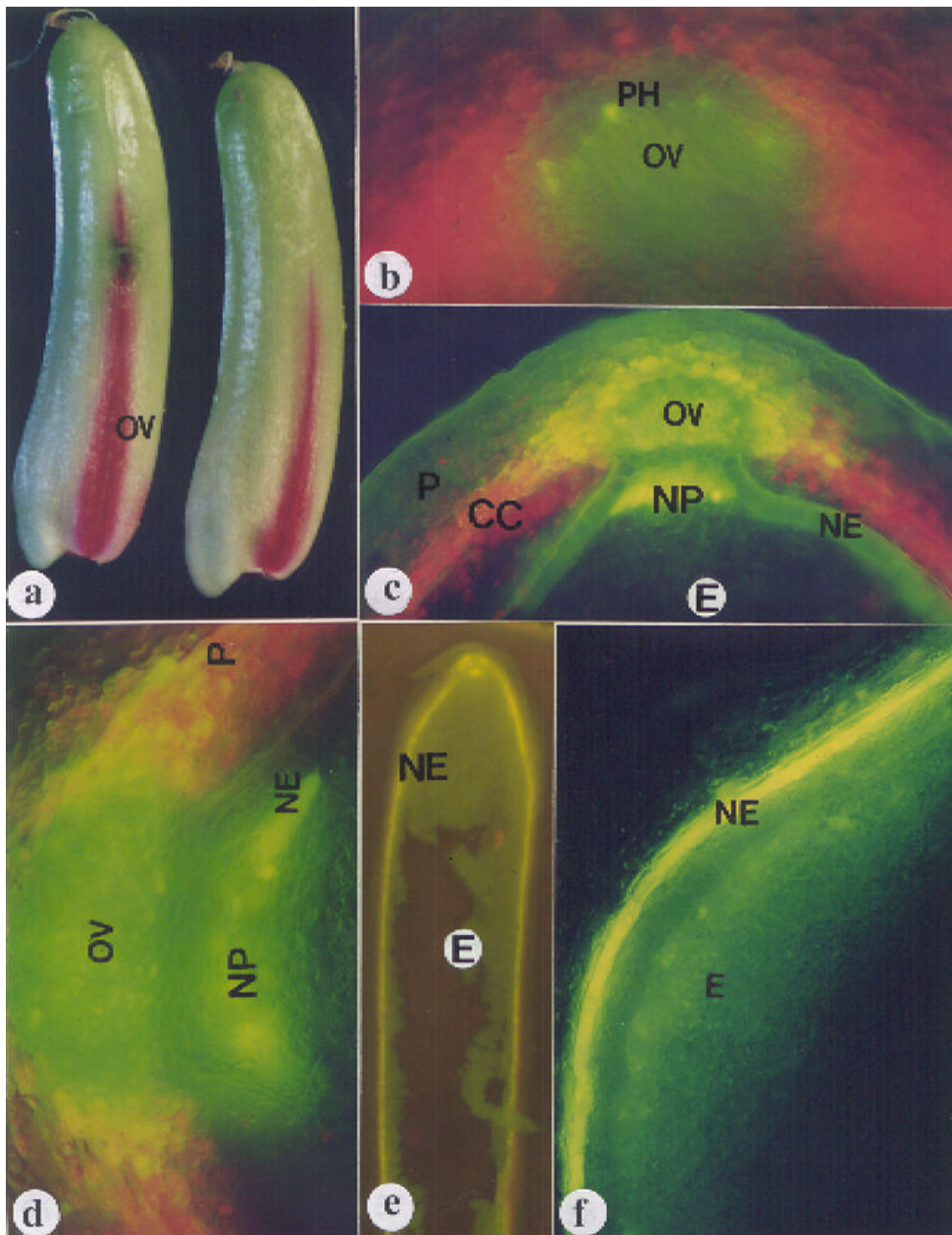
In recent years the emphasis on the study of grain-filling in cereals has been on the route of transport and post-phloem unloading and entry of solutes into the filial tissue (Ugalde and Jenner 1990a,b; Wang and Fisher 1994a,b; Wang *et al* 1994, 1995; Patrick and Offler 1995). It is now well established that in all cereals as well as in legumes the entire filial tissue (endosperm and embryo) is isolated from the maternal tissue (pericarp, placenta, vascular tissue, chalaza and nucellus) by a lack of plasmodesmatal connection between the two (Wang *et al* 1994). Symplastic continuity exists between the ovular vascular tissue and the nucellus only through a chalazal zone identified as the pigment strand in wheat (Zee and O'Brien 1970). However, solute entry beyond the nucellar projection is entirely apoplastic.

Figure 9 is a summary diagram that illustrates current knowledge of cells and tissues involved in the transport of nutrients into the rice caryopsis. This diagram is syn-

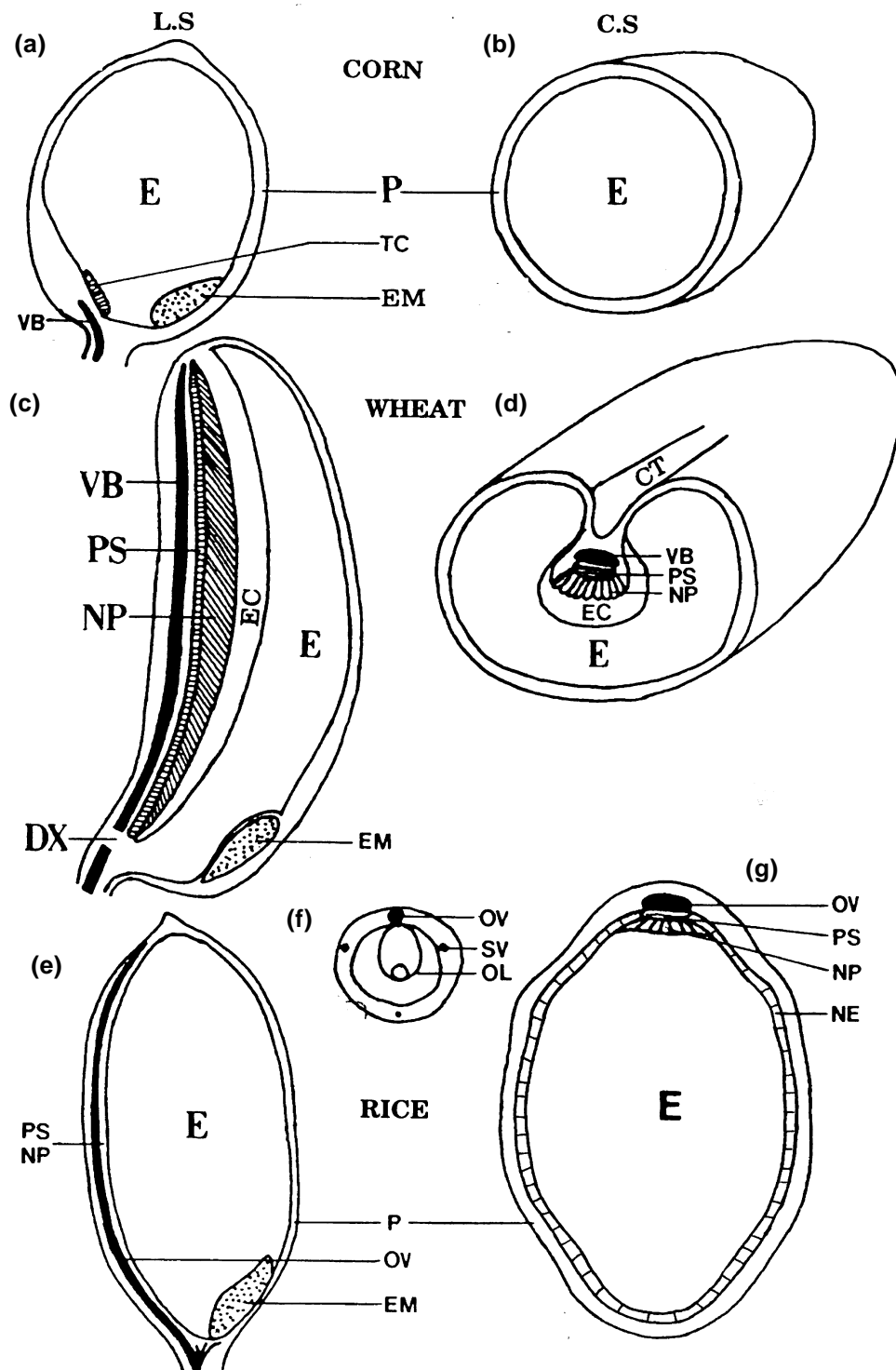
thesized from various publications and our own investigations. The ovular vascular trace is the only source of supply of nutrients to the developing caryopsis. Symplastic continuity exists between the cells of the vascular trace, chalaza, nucellar projection and nucellar epidermis. Dye-movement studies indicate that the transport of assimilates into the aleurone and endosperm may be through two overlapping pathways. During the early phase, 1–7 DAF, the nucellus below the pigment strand may be the major route of transport to the endosperm and embryo. As the nucellar tissue is used up the nucellar epidermis may become the major, and at later stages, the only route of transport (figure 10). From the nucellar epidermis that completely encircles the endosperm (except near the vascular trace) nutrients appear to be transported inwards through the apoplast. This would entail efflux from the maternal tissue (nucellar epidermis) and subsequent membrane influx by the filial tissue (aleurone cells).

## 4. Discussion

A comparison of the pattern of the transport of water, mineral nutrients and photosynthates in rice with that in wheat and corn indicates interesting similarities and differences (figure 11). Recent studies have established that the pattern of transport in tropical C<sub>4</sub> cereals such as corn and sorghum is different from those of C<sub>3</sub> temperate cereals such as wheat and barley (Wang and Fisher 1994a,b; Felker and Shannon 1980; Davis *et al* 1990). In corn, the ovular vascular trace terminates at the base of the ovule. Solute entry beyond this point occurs through apoplastic pathway along crushed placental-chalazal and nucellar cells and finally into the filial aleurone/endosperm transfer cells (Felker and Shannon 1980; Patrick and Offler 1995) (figure 11a). In sorghum the nucellar and aleurone/endosperm transfer cells are separated by a cavity known as the placental sac (Maness and McBee 1986). In wheat, a vascular trace traverses the entire length of the ovary on the side of the crease. However, a discontinuity in xylem has been noticed at the base. Solute must move from the vascular trace through a pigment strand and a nucellar projection into an endosperm cavity. From this cavity there is an influx of solute into the filial aleurone cells and further radial distribution throughout the endosperm. The situation in rice is allied to that of wheat with significant differences. There is no xylem discontinuity in rice caryopsis. Also the pigment strand and the nucellar projection in rice are not as well-differentiated as they are in wheat. In rice no endosperm cavity could be detected in our studies, although Hoshikawa (1984) described a narrow cavity below the nucellar projection. In wheat, transport occurs predominantly in a radial outward direction from the endosperm cavity, whereas in rice transport is inwards from the nucellar epidermis.



**Figure 10.** (a) Two caryopsis of *O. sativa* isolated from the spikelet after one hour of transport of Feulgen reagent through the cut end of the panicle branch. The dye has moved in the ovular vascular tissue located at the ventral side of the caryopsis. These 5-day-old caryopses are green because of chlorophyll in the cross-cells of pericarp. (b–f) Fluorescence micrographs of caryopsis showing sequential movement of carboxyfluorescein (CF) through the caryopsis. Note that the dye neither accumulates in the pigment strand nor stains it. (b) CF localized in phloem. X500. (c) CF in parenchyma cells around the trace and in the nucellar projection. X250. (d) CF in the parenchyma cells of trace and nucellar projection and entering the nucellar epidermis. X500. (e, f) CF observed in the nucellar epidermis in longitudinal and transverse sections, respectively. (e) X50. (f) X250. (CC, Cross-cells; E, endosperm; NE, nucellar epidermis; NP, nucellar projection; OV, ovular vascular trace; P, pericarp; PH, phloem.)



**Figure 11.** Diagrammatic representation in longitudinal (a, c, e) and transverse sections (b, d, g) of caryopsis during grain-filling in corn ( $C_4$  cereal), wheat and rice (both  $C_3$  cereals). (a, b), Corn. (c, d), Wheat. (e, f, g), Rice. In corn, the vascular trace terminates at the base of the ovule. Xylem discontinuity is seen in wheat. An endosperm cavity is present in wheat but is absent in corn and rice. The transection of ovary of rice in f shows the ventral vascular trace and three stilar vascular traces in the pericarp. (CT, Crease tissue; DX, discontinuity in xylem; E, endosperm; EC, endosperm cavity; EM, embryo; NE, nucellar epidermis; NP, nucellar projection; OL, ovule; OV, ovular vascular trace; P, pericarp; PS, pigment strand (chalaza); SV, stilar vascular trace; TC, transfer cells; VB, vascular trace.)

A basic knowledge of the path of assimilate transport and storage, and development of endosperm and embryo will help in improvement of yield and grain quality in rice. Broad features of transport, and the chemistry and pattern of deposition of storage material are now fairly well understood (Krishnan 1996; Krishnan *et al* 2001). Details of transport and deposit of nutrients such as sugars, amino acids and minerals across the apoplast are yet to be worked out. Significant progress has been made in recent years on the development of cereal endosperm (Olsen *et al* 1999). Immunohistochemical and molecular techniques have revealed that the endosperm of many cereals consists of five different cell types, namely the central starchy endosperm, the sub-aleurone layer, the aleurone layer, the basal endosperm transfer layer and the embryo-surrounding region (Olsen *et al* 1999). The process of cellularization is not identical in these cell types. Future studies on grain-filling in rice should explore the role of these different cell types in transport and storage of assimilates and nutrients. All cereal grains including rice, possess tube and cross-cells whose functions are not satisfactorily known. Rice is unusual among cereal grains in possessing persistent sterile structures, the palea and lemma. It is likely that the manipulation of these structures could lead to size and weight increase in rice grains (Ebenezer *et al* 2001).

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