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# STRUCTURAL AND HISTOCHEMICAL CHARACTERS OF THE PROSOPIS TAMARUGO PHIL. SEED COAT, IN RELATION TO ITS HARDNESS

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SUMMARY

The structural and histochemical characteristics of the *Prosopis tamarugo* Phil. seed-coat have been investigated by bright-field, fluorescence and scanning electron microscopy. *P. tamarugo* seed dormancy is exclusively due to the hardness of the seed-coat. The water barrier is thought to be located in the superficial portion of the palisade cells named "cylindrical part", sited directly under the cone-like endings of the palisade cells themselves. The periclinal disposition of the "cylindrical parts" originates a line particularly evidenced by histochemical procedures of fluorescence that indicate their lipidic nature. It is believed that under natural environmental conditions a process of cracking deeper than this level permits water entry.

## 1. INTRODUCTION

Transformation of a desert ecosystem in productive agricultural land is nowadays an important and difficult task. On the other hand, it is well known that in arid lands one of the major problems is very often the high salt content. Among the species which show a great endurance to draught and salinity, the *Prosopis* spp. are very interesting from both the agronomic and economic point of view.

All parts of *Prosopis* plants are utilized; some as source of human food, others for fodder or as material for building or fuel etc. (FELKER 1979; HABIT 1981, 1985). The seeds, in particular, due to their high protein content and presence of a galactomannan gum, may have potential economic value (IRVING 1984).

Among the species of this genus, *Prosopis tamarugo* Phil. has a specific ecology and physiology and, therefore, it has been studied by a number of authors (HABIT 1985). The "tamarugal pampa', the desert ecosystem found in the first Chile region, called Tarapacà, between two mountain chains, the Andean Cordillera east and the Coastal range west, owes its name to *Prosopis tamarugo*. Tamarugo plantations introduced by man are changing this absolute desert ecosystem into an agro-ecosystem offering a concrete and considerable increase in the possible productivity in one of the most inhospitable regions of the world (RIVEROS 1981). *Prosopis tamarugo* grows, in this region, on land covered by a salt crust, varying in thickness from 10 to 60 cm. It also adapts well to soils without a salt cover, such as clay or sandy soils (HABIT 1981). Moreover the tamarugo tree may thrive

in areas where groundwater lies 10–40 or more metres deep. Some authors, such as SUDZUKI (1985) suppose that this plant is capable of absorbing atmospheric water through its foliar system. Other authors, such as ARAVENA & ACEVEDO (1985) suggest, on the contrary, that it may absorb water down deep in the soil.

As part of a broad program of FAO, Rome, for the use of stress-tolerant plants to improve productivity of arid and semi-arid regions, this research deals with the structural and histochemical characteristics of *Prosopis tamarugo* seeds, in order to clarify the cause of their seed hardness. This study has been carried out using bright-field, fluorescence and scanning electron microscopy.

## 2. MATERIAL AND METHODS

The seeds of *Prosopis tamarugo* were obtained from the FAO seed bank, Rome (Accession no. 62578) and were stored at 4°C until examined. In order to test their hardness, germination trials were performed as follows: the seeds were surface sterilized with 0,5% (W/v) sodium hypochlorite for 1 min. and then washed two or three times with distilled water. Germination tests were carried out in glass Petri dishes on four layers of filter-paper to provide to the seeds enough water without surrounding them by films of water which temporarily might inhibit germination due to paucity of oxygen. Each dish was carefully closed with parafilm as a precaution against loss of water caused by evaporation. The Petri dishes were kept in a growth chamber at alternating temperature regimes of 15°–24°C on a 24 h cycle, where the higher temperature coincided with a 12 h light period provided by Ilesa 75 W, 220 V white and red lamps, and the lower temperatures coincided with a 12 h dark period. Five replicates of 100 seeds were used and the seeds were considered germinated with the emergence of the radicle. The seeds which did not appear to be swollen after ten days of imbibition were considered hard. Various methods were employed to clarify the seed coat structure, and to localize the site and chemical nature of the barrier to water penetration in hard seeds. Whole seeds were immersed in an aqueous solution (1%) of crystal-violet for up to four months and a half (JANNERETTE 1979); every eight days some of them were hand sectioned and observed with a stereomicroscope. In order to observe them with the light microscope, the seeds were fixed for 4 h at 0–4°C with 2.5% glutaraldehyde in 0.05 M phosphate buffer pH 7.2, then dehydrated in an ethanol graded series and embedded in JB4 resin (Polyscience Inc.) (BRINN & PICKETT 1979) in BEEM capsules; or fixed for 24 h in FAA, then dehydrated in an ethanol graded series, and embedded in metyl-butyl methacrylate mixture (O'BRIEN & MCCULLY 1981) with the use of BEEM capsules. All tissue blocks were sectioned at 2–4 $\mu$m on a Reichert Om U2 ultramicrotome equipped with a glass knife.

The following histochemical reactions were carried out:

a) Safranine-fast green as a general stain; safranine aqueous solution (1%) for 15 min., fast-green alcohol 95° solution (0.5%) for 5 sec. (O'BRIEN & MCCULY 1981).
b) Toluidine Blue O (TBO) 0.05% aqueous solution for 1 min. as a general stain

(FEDER & O'BRIEN 1968).

c) Toluidine Blue O (TBO) 0.05% in acetate buffer 0.1 M at pH 4.4 and at pH 1 (by adjustment with HCl 6N) for 1 min. as metachromatic stain. (FEDER & O'BRIEN 1968).
d) Alcian Blue 8GX (AB) 1% at pH 2.5 in 3% acetic acid and at pH 0.5 in HCl 0.2 N for 20 min. (LEV & SPICER 1964), for acid polysaccharides.
e) Periodic acid-Schiff (PAS) procedure used for general polysaccharide localization (PEARSE 1985).

To study by fluorescence microscopy, the sections, embedded in methyl-butyl metacrilate mixture, were treated with periodic acid-acriflavine $SO_2$ (F-PAS) procedure for general polysaccharide localization. The F-PAS procedure is a histofluorescent test for polysaccharides employed in plant histochemistry because of its high sensitivity and selectivity in comparison to the conventional PAS (BRUNI & MODENESI 1983). By this procedure where the cellulosic/non cellulosic polysaccharide ratio in the cell wall is modified, the fluorescence turns from green to orange, depending on the specific accumulation of fluoro-chrome-polyanion band sites in a complex macromolecular structure.

Another series of seeds, unfixed, were free-hand sectioned and treated with:

a) Auramine O used for general lipids localization (HESLOP-HARRISON 1977);
b) Phosphine 3R used for general lipids localization (PEARSE 1985).

For all the cited histochemical methods control reactions were made according to the respective authors.

For SEM study, the seeds were dipped in the alcohol graded series up to absolute alcohol, then soaked in an absolute alcohol and amyl-acetate (1/1) mixture, thence in amyl-acetate. After a treatment with the Critical Point Drying Apparatus, they were mounted on stubs, coated with gold (200–220 Ä in thickness) using an Agar AIDS Sputter Coater. The specimens were then viewed with a Cambridge Stereoscan 250 MK2 at an acceleration voltage of 20 KV and photographed on Ilford FP4 film.

## 3. RESULTS

### 3.1 Histology

*Prosopis tamarugo* seeds exhibited, of course, many anatomical features of the Leguminosae seeds (CORNER 1951, 1976). In particular they showed many features described by WERKER et al. (1973) for *Prosopis farcata* (Banks & Solander) Eig. seeds. The seed coat of *P. tamarugo* may be defined by the following structures (*fig. 1A, B*):

1) a very thin cuticle (cu);
2) a layer composed of the superimposed outer portions of the outer cell walls, which are "cap" shaped (ca);
3) a palisade of columnar cells, in a single row, composed of the following shaped parts:
   a) "cones", which are the outer parts of the palisade cells (co);
   b) "cylindrical part" with the cell walls in the same direction of the cell axis (cy.p);

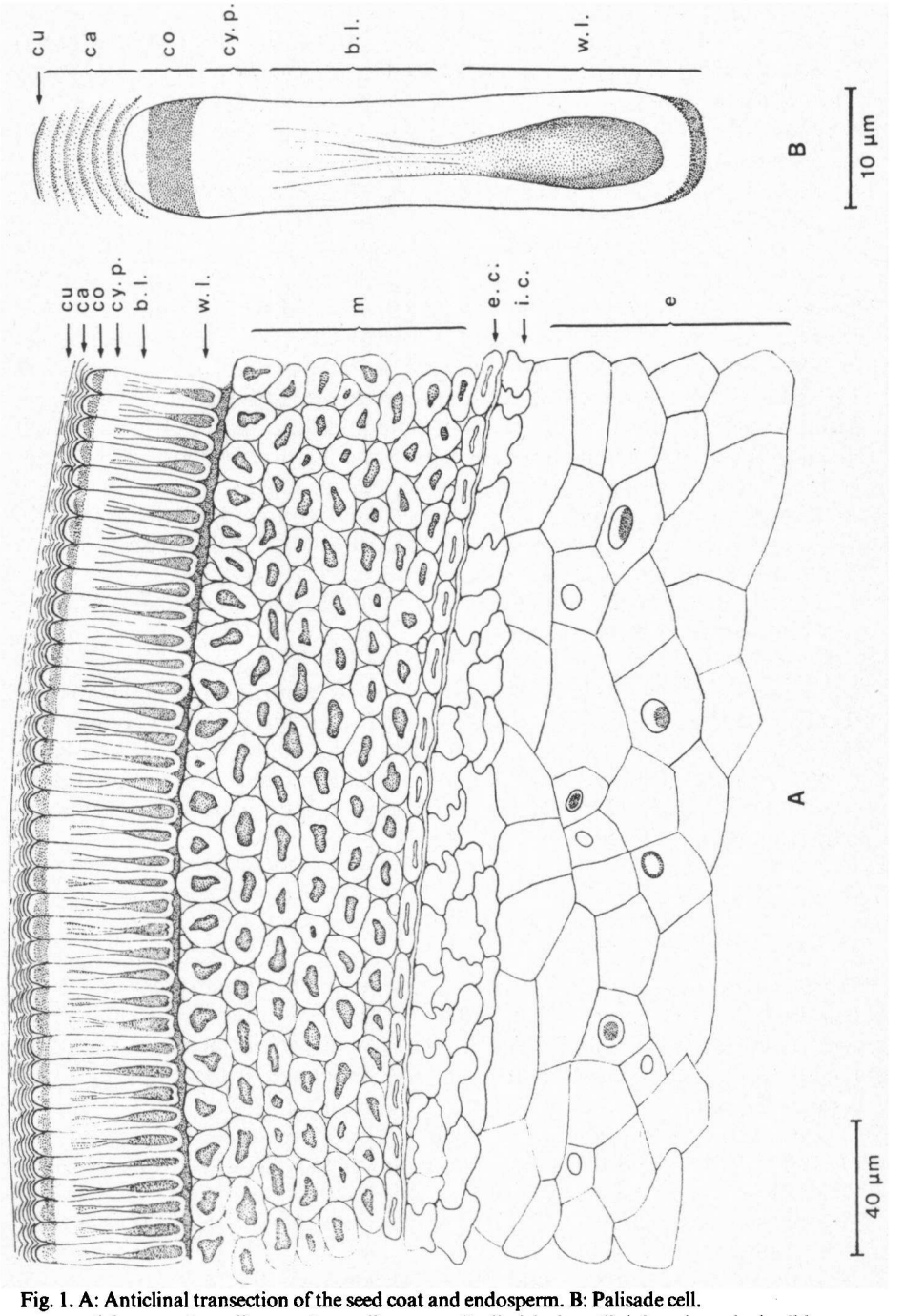


Fig. 1. A: Anticlinal transection of the seed coat and endosperm. B: Palisade cell.
cu = cuticle; ca = "caps"; co = "cones"; cy.p. = "cylindrical part"; b.l. = branched cell lumen; w.l. = wide cell lumen; m = mesophyll; e.c. = tangentially elongated cells; i.c. = irregularly rectangular cells; e = endosperm.

c) the remaining columnar part of the palisade layer, formed by an outer portion with narrow and branched cell lumen (b.l.) and an inner portion, with a wider lumen, containing the nucleus (w.l). These two parts are generally separated by the "light line" (CORNER 1951, WERKER et al. 1973). The light line, in our case, is not evident at this level;

4) mesophyll (CORNER 1951, 1976) or sclerified parenchyma cells (WERKER et al. 1973) composed of 6–12 cell rows, with thick unlignified walls (m). The first and last row of cells correspond to the hypodermal and inner epidermal hour-glass cells of CORNER (1951), in this case very irregular and not always distinguishable;
5) a row of tangentially elongated cells, which are clearly evident in the imbibed seeds, but sometimes seem more or less thorn (e.c);
6) one row or sometimes more rows, of thin walled irregularly rectangular cells, clearly visible in imbibed seeds, which, according to some authors (KHUDAIRI 1956, WERKER et al. 1973), are the remains of the inner integument. These cells could be instead, in our opinion, the remains of the nucellus (i.c.).

At the inner side of the coat lies the endosperm which is partly not cellular and rich in mucilage, partly divided in very thick-walled cells (e).

### 3.2 Water entry

Germination tests performed on a sample of 500 seeds, revealed that 60% of the seeds were readily imbibed (i.e. soft seeds), while the remaining 40% did not swell, even after ten days imbibition (i.e. hard seeds). These tests also revealed that dormancy in *P. tamarugo* seeds is not due to presence of inhibitors (chemical dormancy) nor to immature embryos (physiological dormancy), but only due to water imperviousness of the seed coat. In fact, soft seeds germinate quickly and regularly (unpublished data). Simple scarification of the unswollen seeds, and subsequent immersion in water causes immediate imbibition and successive germination.

In the test with 1% crystal violet aqueous solution, after eight days immersion in the stain, penetration of the stain in the seed coat, endosperm and embryo was evident in the soft seeds, while in the hard seeds the stain stopped on the outer part of the palisade layer; in fact only the cuticle, the layer of the superimposed "caps" and the "cones" of the palisade cells were stained. The same result was obtained maintaining the seeds for a longer period in the stain, and examining them every week over a period of 135 days (*fig. 2A-B*). SEM observations showed that the hard seeds were provided with many cracks in the cuticle and "caps" layer. This layer also could peel off from the underlying palisade cells, and impressions of the "cones" could be clearly visible on it (*fig. 3A-B-C*). In the soft seeds the whole palisade layer peeled off and showed deep cracks, revealing the mesophyll zone, that lies immediately under this layer (*fig. 3D*). *Fig. 3E* shows the internal view of a palisade fragment; on the border whole palisade cells are visible, while, in the centre of the fragment, the basis of these cells could be noticed (*fig. 3E-F*). In both hard and soft seeds SEM photographs exhibit the pleurogram (*fig. 3A, D*), which is a narrow horseshoe shaped groove in the

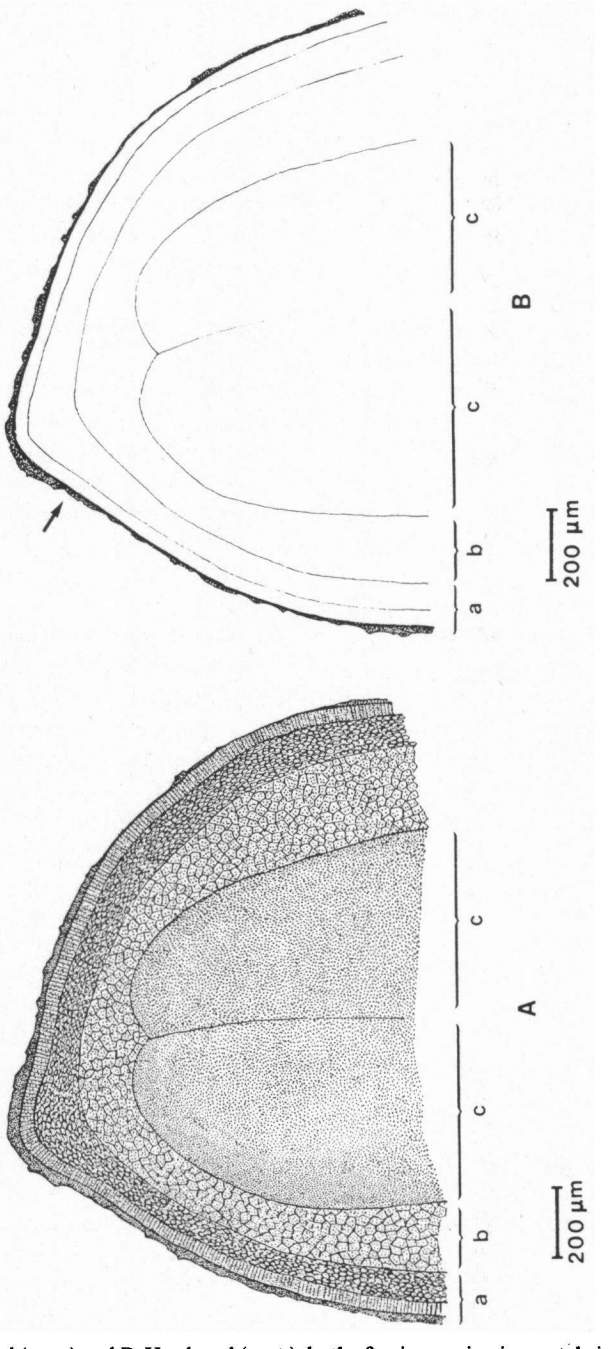


Fig. 2. A: Soft seed (part.) and B: Hard seed (part.), both after immersion in crystal violet solution. a = seed coat; b = endosperm; c = cotyledons. → hard seed permeable part, stained in violet.

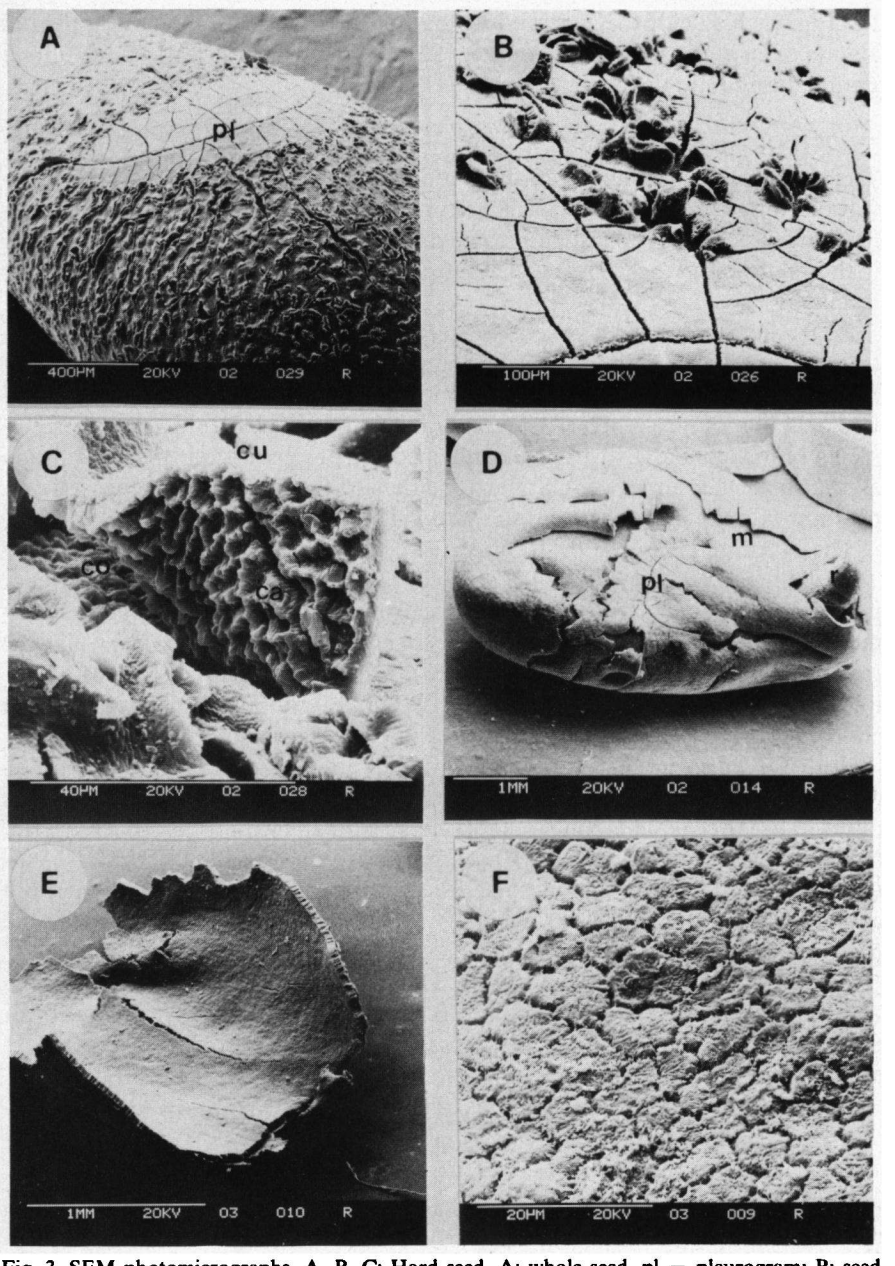


Fig. 3. SEM photomicrographs. A, B, C: Hard seed. A: whole seed, pl = pleurogram; B: seed exotesta with superficial cracks; C: particular of the exotesta shown in B, with cuticle and 'caps" detached from the underlying "cones". cu = cuticle; ca = "caps"; co = "cones".
D, E, F: Soft seed. D: whole seed, pl = pleurogram, m = mesophyll, r = radicle; E: inner view of a part of the palisade layer, detached from the mesophyll; F: particular of E showing the polygonal bases of the palisade cells.

testa on each side of the seed, following the curve of the raphe-antiraphe, but always open at the hilar end (CORNER 1951, 1976). Often the testa shows a finer granulation round the pleurogram.

### 3.3 Histochemistry

The above mentioned observations demonstrate that the barrier to water penetration in hard *P. tamarugo* seeds lies in the palisade layer. We therefore performed histochemical investigations in order to locate the position of this barrier in the palisade and to discover which substance is responsible for its hydrophobic nature.

PAS reaction showed a general affinity for the whole palisade layer, demostrating its prevalent polysaccharidic composition. The non cellular "cap" layer (ca) and the cell walls were intensely stained with red. Also the cytoplasm showed PAS reaction affinity: there was red staining at the cell basis and along the cytoplasm branches (*fig. 4A*). F-PAS confirmed the presence of polysaccharides in all the above mentioned cellular parts, demonstrating their heterogeneous composition with the polychromatic staining.

The heterogeneous composition of the cell-walls was further confirmed by TBO staining. When *P. tamarugo* seed-coat sections were stained with TBO at pH 4.4, the following parts became metachromatic (reddish-blue): the zone immediately under the "cone" tips and the inner tangential palisade cell wall. The rest of the cell wall was unstained (*fig. 4B*). At this pH several different polyanions (polyphosphates, polycarboxilic acids and polysulphates) carry a negative charge reacting metachromatically with TBO (BULLOCK et al. 1980).

With TBO at pH 1, when carboxyl groups were no longer ionized (LING LEE et al. 1977), metachromatic staining persisted only in the internal tangential walls of the palisade cells. This zone was still metachromatic with trichloroacetic acid, (which removes polyphosphates), used prior to staining with TBO pH 1( ASHFORD et al. 1975). Also the palisade cell cytoplasm showed affinity with TBO exhibiting a blue-green colour, which is a characteristic metachromasy for the phenolic substances (O'BRIEN & MCCULLY 1981).

The results of the experiments with TBO stain have been confirmed by the Alcian Blue reactions at pH 2.5 and pH 0.5 (*fig. 4C*). This basic dye shows affinity, at low pH, with faintly acid polysaccharides (with presence of –COOH groups) and with strongly acid polysaccharides (containing $-SO_4^{--}$ groups).

In the free hand transverse sections of the seed coat, autofluorescence exami-

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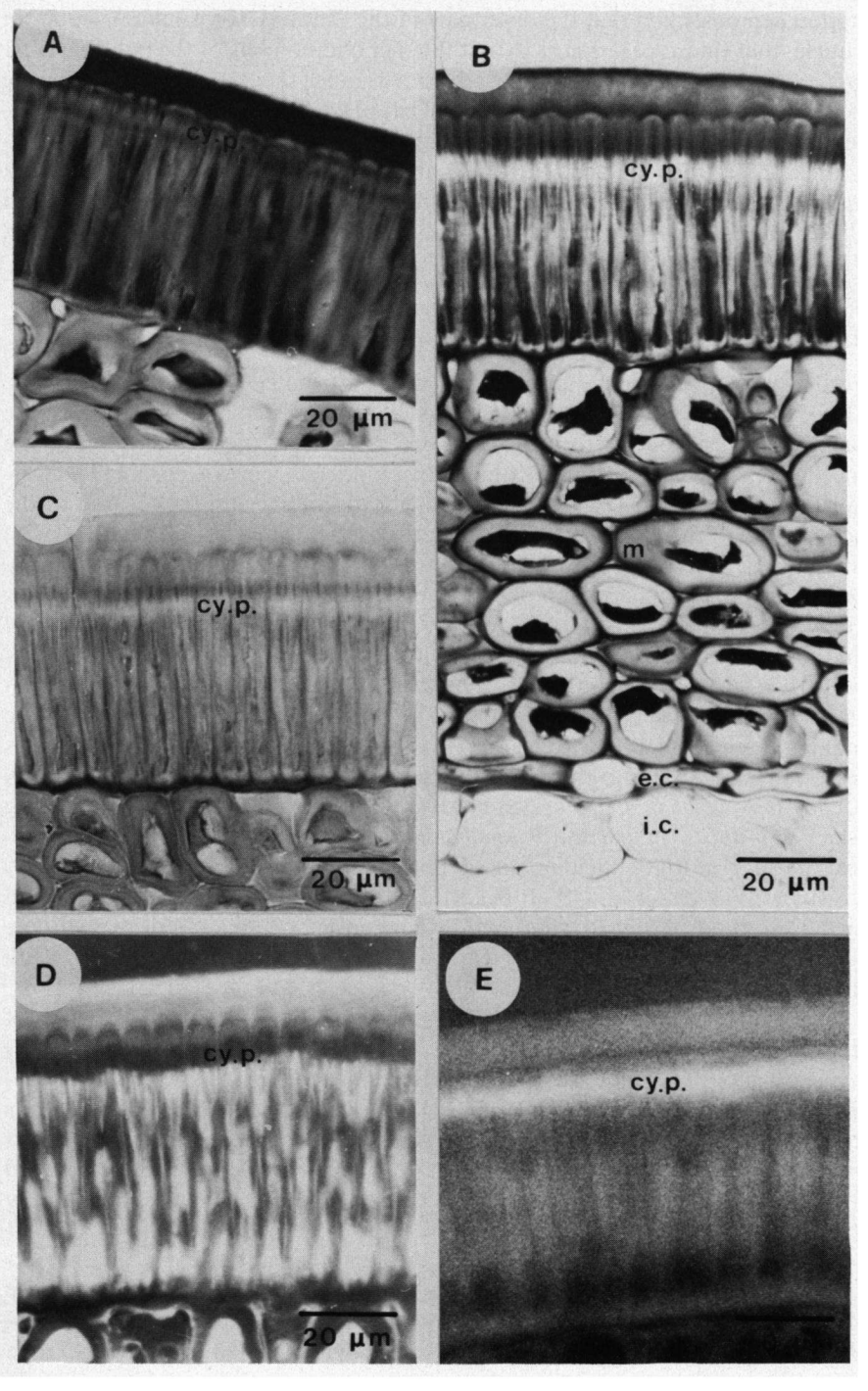
Fig. 4. Anticlinal transections of the seed coat. The "cylindrical part" of the palisade cells is shown with various staining procedures. A: Bright field micrograph, PAS; B: Bright field micrograph, TBO pH 4.4; C: Bright field micrograph, Alcian Blue pH 2.5. Notice the inner tangential cell walls strongly reacted with this dye. D: Fluorescence mircrograph, autofluorescence. The "cylindrical part" is not autofluorescent. E: Fluorescence micrograph, Phosphine 3 R. The "cylindrical part"intensely fluoresces silver-white.
cy.p. = "cylindrical part"; m = mesophyll; e.c. = tangentially elongated cells; i.c. = irregularly rectangular cells.


A
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nation demonstrated that the outer part of the "caps" layer is limited by a thin cuticle, that fluoresces orange. Also the other components of the palisade layer were autofluorescent, except a strip, located under the "cones" and under the zone which was metachromatic with TBO at pH 4.4 (*fig. 4D*). This area is situated in correspondance of the cytoplasmic branches. This strip remained unstained in sections stained with TBO at low pH. The same line was evident when free-hand sections were treated with fluorescent lipophilic stains. In fact Phosphine 3R clearly pointed out this line, which fluoresced silver-white (*fig. 4E*), while it fluoresced blue-white with Auramine O.

## 4. DISCUSSION

Seed hardness, in Leguminosae, is generally attributed to the palisade layer. However, in this single layer each part of the cell has been suggested as possible being the decisive factor for water impermeability (WERKER 1980/81). Also the mechanism by which the entrance of water is hindered, varies according to different authors. CORNER (1951) suggests that water imperviousness is due to the contraction of the walls of the palisade-cells as the seed ripens. ROLSTON (1978) mentions various waterproofing substances that involve the testa (wax, suberin, pectin, lignin, tannin and quinone derivatives). Other authors, such as MARTIN (1922) for *Melilotus alba* and CAVANAGH (1980) for *Acacia*, think that physical and sometimes chemical characteristics of the light line contribute to impermeability. RALEIGH (1930) for *Gymnocladus dioica* believes that pectic insoluble substances shrink when desiccated and may be certain chemical changes take place making them less pervious to water. HAMLY(1932) for *Melilotus alba* attributes waterproofing ability to the outer periclinal suberized walls. KHUDAIRI (1959) for *Prosopis stephaniana*, TRIVEDI et al. (1979) for some Mimosoideae, among which *Prosopis spicigera* and *P. stephaniana*, and HABIT (1981) for *Prosopis tamarugo* suggest that cuticle or wax deposits external to cuticle are impervious to water. WERKER et al. (1973) believe for *Prosopis farcata* that the first and most important barrier to water entrance is formed by the caps of the palisade cells and that the material of the caps probably becomes very compact when the seed dries.

The present structural and histochemical study clearly shows the location of the water barrier in *P. tamarugo* seeds. The tests with crystal violet solutions suggest a superficial location of this barrier, since in hard seeds the stain penetrated only cuticle (cu), "caps" layer (ca) and "cones" (co) and, even after four months and a half soaking in the stain, the latter never passed through the first outer cylindrical portion of the palisade cells (cy). SEM observations confirm this opinion demonstrating that, if the cracks on the seed surface reach only the "cones" level, the seeds do not absorb water.

The histochemical tests employed indicate that the palisade cells have a polysaccharidic heterogeneous composition; it is possible, therefore, to recognize different zones in the cell walls. Faintly acid polysaccharides or polyanion (polyanionic) pectins (HESLOP-HARRISON et al. 1984) are located in the "caps" zone

(ca) as superimposed layers; these compounds have been also found in the area immediately under the "cone" tips. On the opposite end of the palisade cells, some very acid polysaccharides (sulphates) are located in the inner tangential walls. The "cone" tips and the rest of the cell walls are mainly composed of neutral cellulosic polysaccharides. These compounds cannot constitute a hindrance to water penetration. The pectic mucilaginous layer of the "caps" and the conic tip of the palisade cells, due to their hydrophilic nature, rapidly imbibe water, as well as the other parts of the cell wall, and this also occurs in the internal portion, which is highly acidic.

Our investigation, on the other hand, points out the presence of hydrophobic substances of lipidic nature both in the thin external cuticle and in the "cylindrical part" situated under the "cones". The cuticle, however, cannot be an impediment to water penetration, being frequently cracked and, with the immediately underlying layer, often lifted, both in soft and hard seeds. We believe that the real barrier to water infiltration in the *Prosopis tamarugo* seeds consists of the hydrophobic layer, made of lipidic substances, which we name "cylindrical part" (cy). Therefore, the seeds in which the coat cracks are deeper than this level are permeable to water, while those bearing more superficial cracks stay hard.

The zone bearing these hydrophobic characteristics seems to correspond to the highly disputed "light line", a continuous line parallel to the surface of the testa. As a matter of fact, in fresh and non colored sections this layer gives the image of a bright line. It does not correspond, however, for its position, to the one described by CORNER (1951) and WERKER et al. (1973). But it is to be noticed that some variability is reported in this respect: sometimes the location of the light line in the palisade cells varies with the region of the seed coat (GASTALDO & PROFUMO 1975; SERRATO VALENTI et al. 1979), sometimes it seems also that two "light lines" are present (RALEIGH 1930).

We agree with WERKER et al. (1973) in supposing that under natural conditions a slow process of cracking of the seed coat must occur and that this natural process in the palisade layer is probably highly accelerated by temperature variations. The process of seed coat cracking, in *P. tamarugo*, involves all the testa and not only a particular region, such as the strophiole which, in *Acacia kempeana*, by heat treatment, lifts and cracks, breaking seedhardness (HANNA 1984). *P. tamarugo* lives in an ecosystem characterized by high day temperatures, greatly different day to night temperature range and intense sunlight (RIVEROS 1981). In its seeds the process of cracking could, therefore, occur in a relatively short lapse of time and thus explain the higher percentage of soft seeds in comparison to hard seeds.

The pleurogram, important from the taxonomic view point, for *Prosopis* spp. (PALACIOS et al. 1974, 1975; TROBOK 1985), does not seem to have a function in seed imbibition, although some authors (TRIVEDI et al. 1979) state that it is the center of convergence of the cracks that are responsible of water infiltration. Clarifying the exact role of this structure does not concern this work. For this purpose, in fact, it would be necessary to follow the seed development till complete maturity. This type of study, on the other hand, could also help identi-

fying the nature of the cells situated immediately exterior to the endosperm (*fig. 1A, i.c.*) and could therefore demonstrate if they are of integumental or nucellar origin.

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