

# Pre-harvest rindstain of ‘Encore’ mandarin: initial histological signs of epicarp disturbance and extent of the disorder

M.I. Maia <sup>a,\*</sup>, M.C. Medeira <sup>a</sup>, M.J. Pinto <sup>b</sup>, A.M. Duarte <sup>b</sup>

Departamento de Fisiologia Vegetal, Estação Agronómica Nacional, Quinta do Marquês, e  
2780-505 Oeiras, Portugal

<sup>b</sup> Universidade do Algarve, UCTA, Gambelas, 8000-117 Faro, Portugal

## Abstract

Pre-harvest rindstain in ‘Encore’ mandarin starts by the injury of a few epidermal cells. The connection between the damaged epidermis and the collapsed layers of flavedo, extending in parallel with rind surface was only visible on cross-sections containing the narrow zone of injured epidermal cells. The individual cell alteration including loss of membrane integrity and the accumulation of large amounts of osmiophilic materials in the cytoplasm are the result of a degrading process. Minute disruptions in the cuticle may provide shelters for resting forms of amoeboid or plasmodial-like organisms living on the rind. Our results suggest that these organisms could be involved in the disorder expression.

**Keywords:** Citrus; Histology; Plasmodial organisms; Pre-harvest; Peel-pitting; Rind blemish; Rind injury; Ultrastructure

## 1. Introduction

Rindstain in ‘Encore’ mandarin (*Citrus nobilis* Lour. × *Citrus deliciosa* Ten.) is a rind disorder that arises as chlorotic spots. This disorder decreases the commercial value although the fruit palatability is not impaired (Vitor *et al.*, 1999). Externally this disorder resembles other rindstains caused by climate, mechanical injury, chemical treatments or post-harvest chilling (Freeman, 1976; Agustí and Almela, 1989; Arpaia *et al.*, 1991; Duarte and Guardiola, 1995).

In previous works, the first stages of pre-harvest rindstain or ‘peel-pitting’ were described (Medeira *et al.*, 1999; Maia and Medeira, 1999). It was verified that the first visual symptoms of the disorder mainly appeared in the portions of the rind most exposed to solar radiation, corresponding to a parenchymal flattening and collapse of cell layers, increasing in parallel with the epidermis.

The first signs of cellular damage in parenchymatous cells of the flavedo seemed to be associated with abnormal internal organisation of the plastids, great vesiculation of the cytoplasm followed by degradation of cytoplasmic membranes.

Vitor *et al.* (1999) verified that the injured tissues had an increase of senescence rate in localised epicarp cells associated with the degradation of cell membranes, in a metabolism that seemed to be modulated by the oxidative modifications that occur in cell compartments. The same authors indicated that high light intensities may induce an oxidative stress in the ‘Encore’ epicarp which is characterised by an increase of peroxidation and degradation of cytoplasmic membranes (Vitor *et al.*, 2000).

The observations of ‘Encore’ early spotted zones revealed the presence of resting or germinated forms of several organisms associated with the epidermis (Medeira *et al.*, 2000). The association of these organisms with the first signs of epidermal cell damage led us to search for the contribution of these organisms in pre-harvest rindstain, as well as for the evolution and extent of the disorder along with time.

Special attention is given to the initial aspects of the lesion and it is our goal to enhance knowledge of the causes of this disorder, based on a detailed structural rind study of the spotted areas.

## 2. Materials and methods

### 2.1. Incidence of rindstain

The observations have been carried out in a commercial orchard of 12-year-old trees of ‘Encore’ hybrid mandarin (*Citrus nobilis* Lour. × *Citrus deliciosa* Ten.) grafted onto Troyer citrange, located in the south of Portugal (Algarve). The orchard was watered as needed, using a sprinkler irrigation system. In five trees, 60 fruits distributed all around the canopy were monitored every second week, recording the presence and the intensity of rindstains. The observations were extended from the first appearance of the rind spots until colour-break.

### 2.2. Histological observations

The observations were carried out on very small spotted areas, corresponding to the beginning of the disorder. About 100 rind damaged areas encircled by healthy tissue were cut in fragments of about 1 mm<sup>3</sup> which were processed for light, transmission and scanning electron microscopy (SEM), in order to cover all the spotted area.

### 2.3. Light microscopy (LM) and transmission electron microscopy (TEM)

Fragments of tissue were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4 °C. The specimens were washed three times for 30 min in the same

buffer, post-fixed in 1% osmium tetroxide in the same buffer, stained in 1% buffered uranyl acetate, dehydrated in an ethanol gradient to absolute ethanol and embedded in LR White resin. Sections of 1–2  $\mu$ m and 60–80 nm were cut in an LKB-ultramicrotome IV using glass knives. Thick sections were observed by LM, stained in 0.05% toluidine blue in 1% sodium carbonate.

The sections for TEM were obtained from the areas where LM observations revealed the injury of epidermal cells. These thin sections were stained in an aqueous saturated solution of uranyl acetate for 30 min and post-stained in a 3% aqueous solution of lead citrate at room temperature for 15 min. The observations were made with a Philips 300 Transmission Electron Microscope at 80 kV.

## 2.4. SEM

About 100 fragments of flavedo were fixed, post-fixed and dehydrated by the same procedure used in the preparation of the material for TEM. Specimens were critical point dried and mounted on the metal stubs using a neutral varnish. Each sample was coated with a thick layer of gold using the ‘sputter-coating’ technique and scanned with an ISI Scanning Electron Microscope, model DS-130, at 10 kV accelerating voltage.

## 3. Results

The rindstain in “Encore” mandarin started as small chlorotic spots mainly in the areas of the rind most exposed to solar radiation (Fig. 1). The disorder started by early September, after summer high temperatures and when the relative humidity increased.

The proportion of affected fruits increased with time, during the observation period. The intensity of the disorder, as measured by the number of rindstains per fruit, showed the same pattern as the proportion of affected fruits (Fig. 2).



Fig. 1. Chlorotic spots of a mandarin fruit.

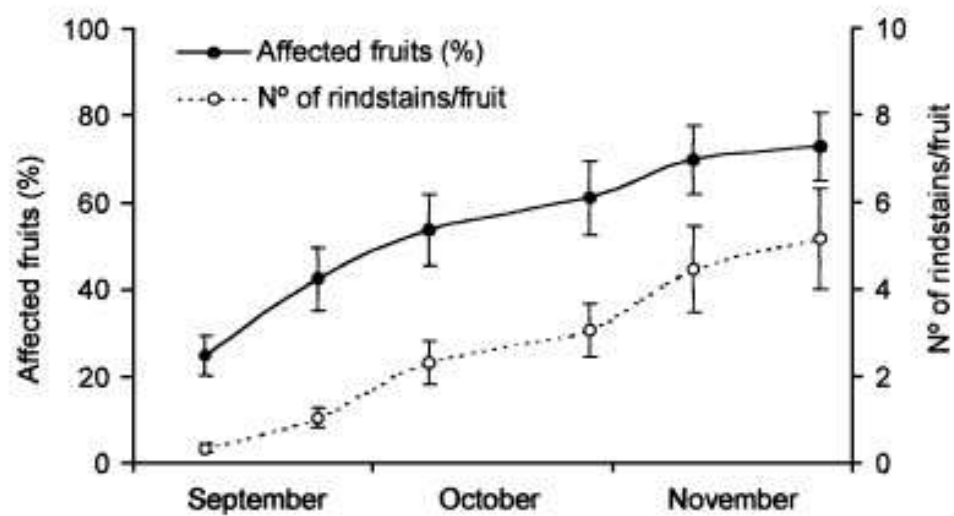


Fig. 2. The time course of on-tree rindstain development in 'Encore' mandarin fruits. Vertical bars represent  $\pm$  standard errors of the means (S.E.M.).

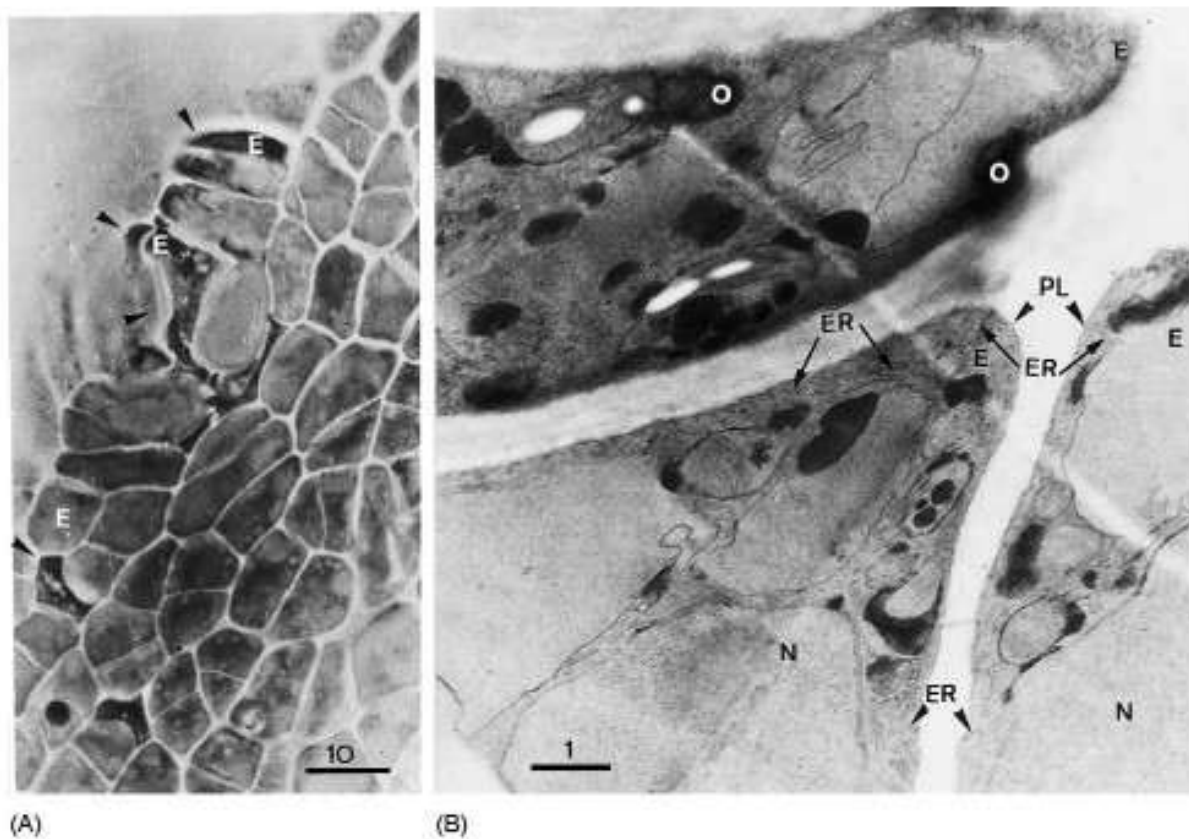


Fig. 3. The start of lesions in epidermal cells. (A) Alterations of some epidermal (E) and sub-epidermal cells (arrows) in very small areas (using LM). (B) The injured cell accumulated osmiophilic materials (O) undergoing degradation of cell membranes. Contiguous cells still preserved their integrity. The plasmalemma (PL) is well preserved. Abundant endoplasmic reticulum (ER) can be seen at cell periphery. Nucleus (N) (using TEM).

At the beginning of lesion development, epidermal and sub-epidermal cell disorganisation was seen in very small areas (arrows) (Fig. 3A).

The damaged cells accumulated osmiophilic materials undergoing degradation of cytoplasmic membranes, although contiguous cells still preserved their integrity. The plasmalemma was well preserved and abundant endoplasmic reticulum was observed at cell periphery (Fig. 3B).

The observation of sequential sections revealed that there was continuity of lesion between small damaged areas in the epidermis and injured and flattened layers of flavedo extending in parallel with rind surface (Fig. 4).

The aspects of healthy flavedo at the same developmental stage of the fruit can be observed on Fig. 5A–C. The thick cuticle, epidermal and sub-epidermal cells were preserved. Plasmalemma, tonoplast, mitochondria and cell membranes maintained their integrity.

Associated with the beginning of the injury process, thin external threads that seemed to cross the cuticle were observed by LM. They were seen inside the cuticle and cell wall of the epidermal cells that started to be disorganised (\*) (Fig. 6A and B).

In the same samples observed by TEM, we could see resting forms with different sizes and shapes, with shrivelled and thin walls on the epidermal cells. Highly vesiculated protoplasmatic masses were seen being released from these bodies (Fig. 7A and B). The



Fig. 4. Cross-section of the flavedo in the central region of the spotted zone (using LM). It is evident the continuity of cell injury between the small damaged area of the epidermis (arrows) and the lesioned and flattened parenchymatous layers of the flavedo (five-point stars).

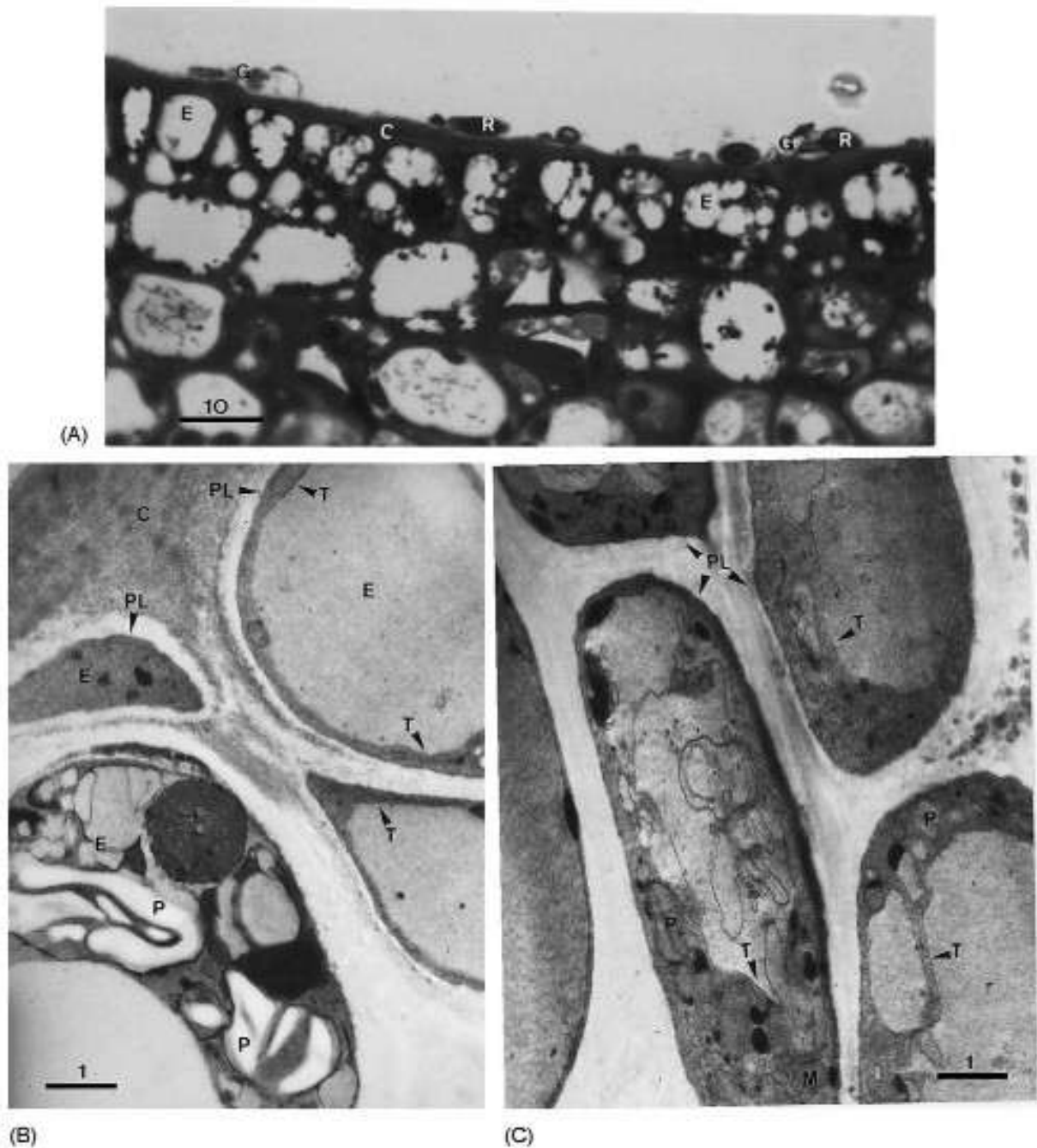


Fig. 5. Cross-sections of healthy flavedo at the same developmental stage of Fig. 3. (A) Epidermal (E) and sub-epidermal cells are apparently healthy. Resting (R) and germinated bodies (G) are on the cuticle (C) (using LM). (B, C) Thin sections of epidermal and sub-epidermal cells (using TEM). Plasmalemma (PL), tonoplast (T), plastids (P), mitochondria (M) and cell membranes maintain their integrity.

protoplasmatic masses were multinucleated, plasmodial like (Fig. 7C) and seemed to penetrate the cuticle and cell wall (Fig. 7D, arrows).

The apparently healthy external surface of the rind observed by SEM showed the stomata and characteristic epicuticular waxes with minute disruptions (arrows) (Fig. 8A). The external surface of lesioned rinds was covered by numerous aggregates of 1–4 m bodies (Fig. 8B).

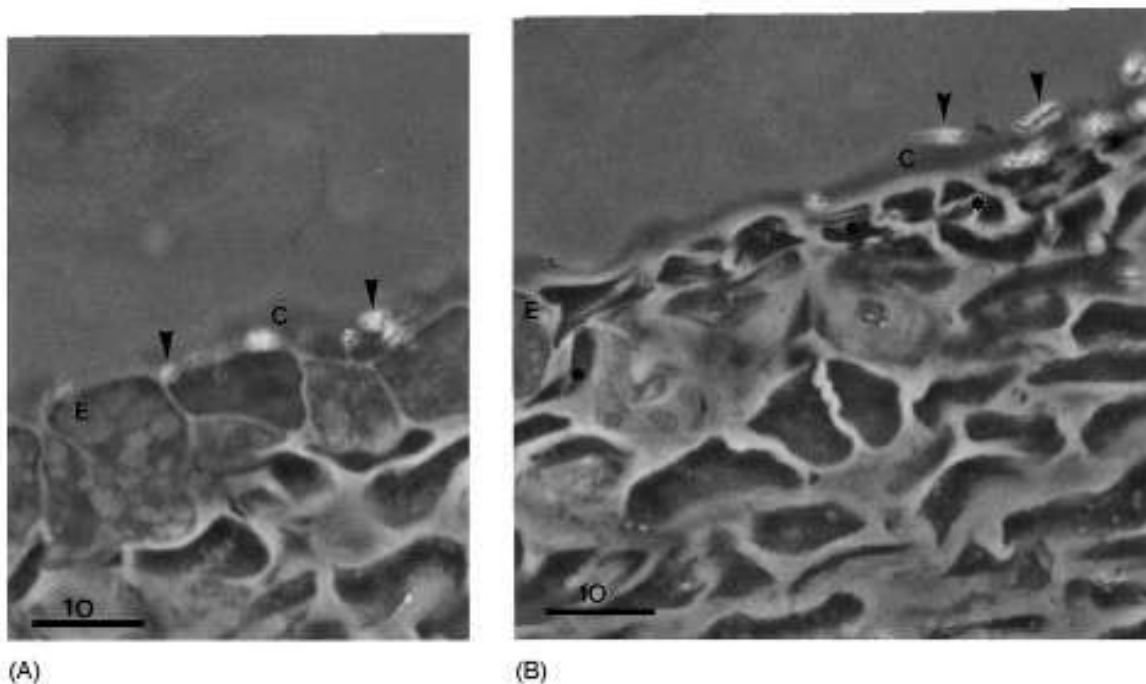


Fig. 6. External bodies associated with the beginning of epidermal cell disorganisation. (A, B) Thin external threads (arrows) seem to cross the cuticle (C) (using LM). (B) The thread-like bodies are inside the cuticle and cell wall (arrows), close to the epidermal cells that start to be disorganised (\*).

#### 4. Discussion

In a previous study (Medeira *et al.*, 1999), it was observed that the collapse and flattening of parenchymal cell layers of flavedo increased in parallel with the epidermis, extending between apparently healthy zones, and being the most evident alteration in pitted areas.

The serial and exhaustive sectioning of the flavedo with the first signs of the disorder revealed the connection between the small damaged areas of the epidermis and the collapsed parenchyma.

Some histological aspects of this disorder resembles the damage process described in several ‘peel-pitting’ disorders in *Citrus* (Vercher *et al.*, 1994; Agustí *et al.*, 2001), or even in other injury processes like chilling injury in egg plant (Rhee and Iwata, 1982), or in post-harvest injury in lemon (Obenland *et al.*, 1997). In all these cases, the excessive loss of water have been invoked.

In contrast to other pittings, in ‘Encore’ mandarin, the first lesions were observed in hot weather, generally by early September and on the outermost part of the rind of fruits most exposed to solar radiation. It is possible that under high temperatures and direct solar radiation, over a long period, some structural alterations of cuticle may occur. Small discontinuities in cuticle were observed, even in apparently healthy flavedo.

Vitor *et al.* (2000) verified that rindstain in ‘Encore’ mandarin may be the result of an oxidative stress induced in epicarp by high light intensity. The same authors verified a significant decrease in the number of spots when a shade net of 50% of solar radiation was applied from the first stages of fruit development.

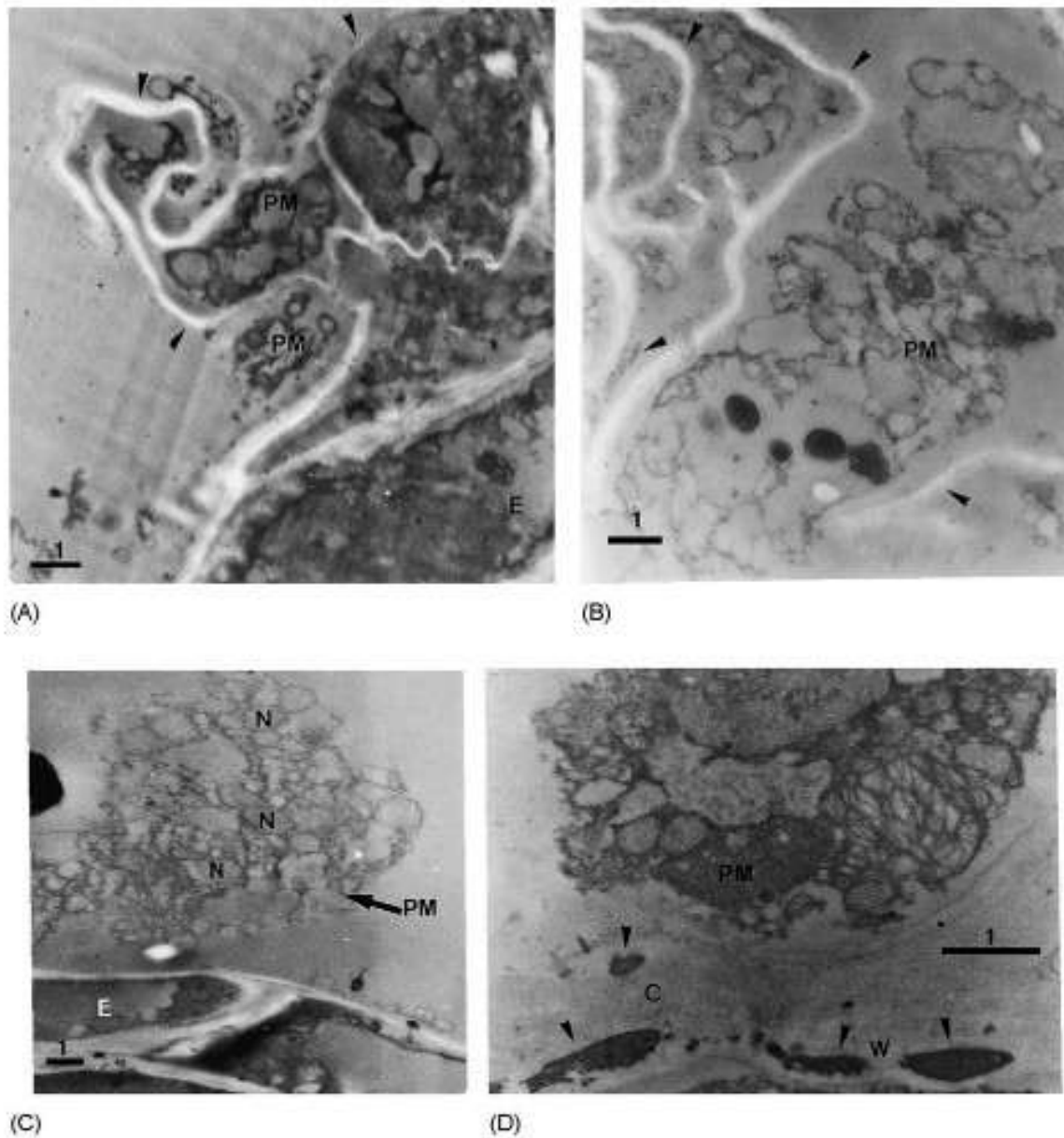


Fig. 7. Cross-sections of the flavedo in the same area of Fig. 6. External bodies on the epidermal cells (arrows), corresponding to the thread-like bodies observed using LM (using TEM). (A, B) Highly vesiculated protoplasmic masses (PM) being released on epidermal cells (E) from bodies (arrows) of different sizes and shapes with thin walls. (C) A multivesiculated plurinucleated (N) protoplasmic mass (PM) on the epidermal cells, suggesting a plasmodial formation. (D) A portion of a plasmodial-like mass (PM) adherent to the cuticle (C). Dense and vesiculated protoplasmic masses seem to go through the cuticle (arrows) and cell wall (W).

The individual alteration of 'Encore' epidermal cells concerning loss of membrane integrity and accumulation of large amounts of osmiophilic material in the cytoplasm may result from degrading processes. Essential oils and other lipids released from their compartments, become toxic to the cells (Heinrich, 1970).

Our observations suggest that the degradation of epidermal and sub-epidermal flavedo cells were associated with plasmodial-like organisms adherent to the cuticle epicarp at beginning of the injury process.

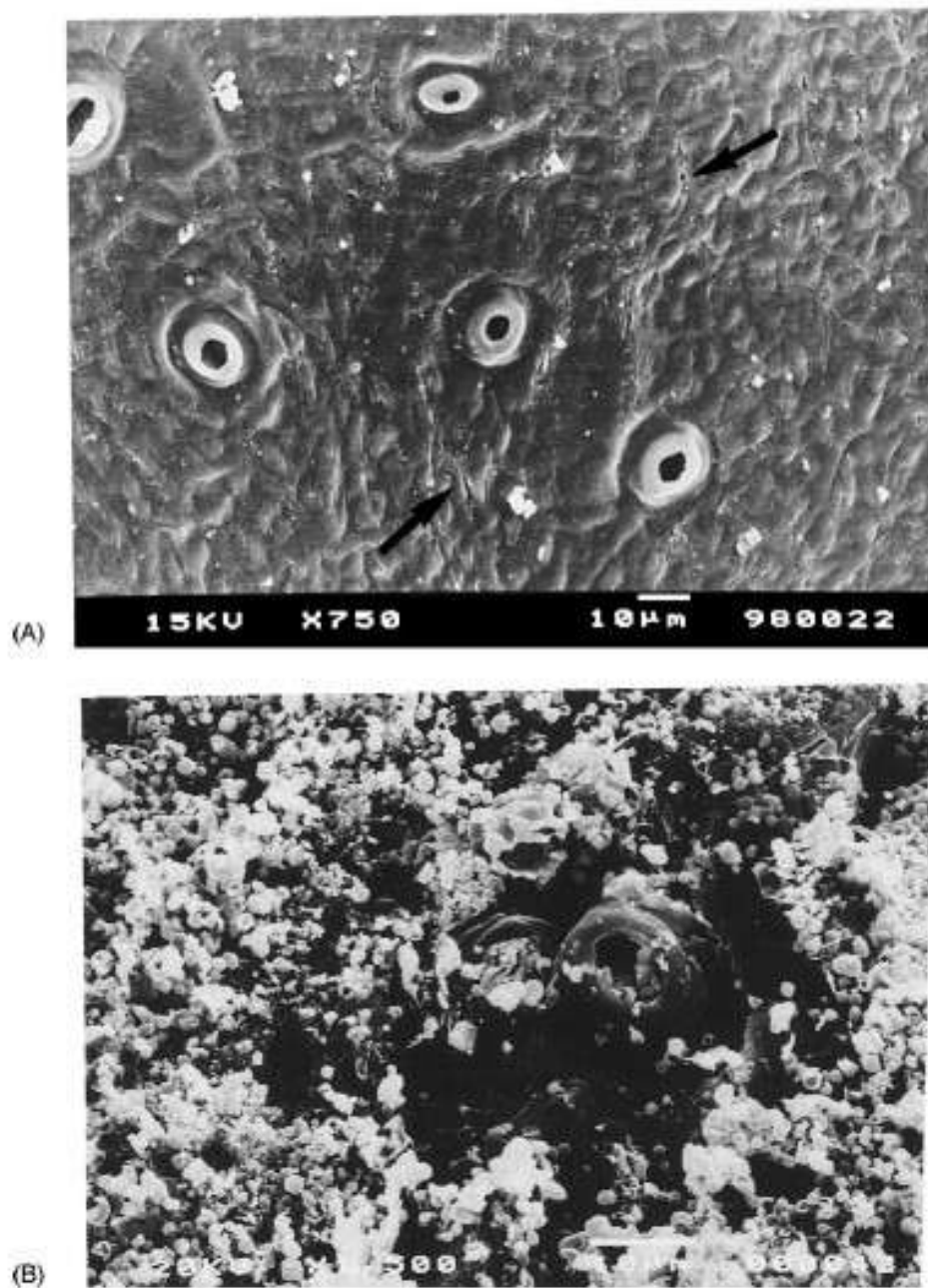


Fig. 8. (A) External surface of apparently healthy flavedo (using SEM). Minute discontinuities (arrows) in the epicuticular waxes of the cuticle. (B) External surface of the flavedo in a spotted zone covered by numerous 1–4  $\mu$ m aggregated bodies.

The minute disruptions of rind cuticle become shelters for resting forms of amoeboid and plasmodial-like organisms, providing good environmental conditions for their maintenance and germination.

The aggregates of bodies observed by SEM on epicuticular waxes correspond to plasmodial-like organisms observed by TEM on flavedo sections. The external morphological aspect and dimensions of the bodies observed by SEM are in agreement with the sectioned material observed by TEM. The presence of these organisms seems to be related with the disorder expression.

Mechanical factors or other external factors may be injurious or may induce irreversible physiological alterations in epidermal cells, thus becoming more vulnerable to biont penetration into the epicarp.

## Acknowledgements

The authors are grateful to Eng. G. Passos for photographic assistance, to Mr. O. Chaveiro for help with electron microscopic equipment and to Commandant Melo Sampaio for providing the orchard facilities. This research was supported by a grant from FCT—Fundação para a Ciência e Tecnologia (PRAXIS XXI program/project 3/3.2/hort/2156/95).e

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