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Histology of hazelnut *(Corylus avellana* L.) kernel affected by brown spots in kernel cavity physiopathy

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Abstract

Cotyledon tissue changes have been studied during hazelnut cv. Negret kernel growth in order to define the developmental steps of a seed alteration called «brown spots in kernel cavity», an inner brown cavity that devalues the kernel. Seed histology has been studied by both light and scanning electron microscopy. The affected zone shows empty cells while increasing suberization can be observed in limiting cells between affected and healthy zones. The brown spots in kernel cavity cells loose their isodiametric shape until tissue is destroyed by lysis and the inner cavity is formed.

Key words: quality, ultrastructural analysis, light microscopy, scanning electron microscopy.

Resumen

Histología del grano de avellana *(Corylus avellana* L.) afectado por la fisiopatía denominada «cor-negre» («brown spots in kernel cavity»)

Se han estudiado los cambios tisulares durante el desarrollo de los frutos de la variedad de avellano 'Negret', con el fin de describir las etapas de aparición de la fisiopatía denominada 'cor-negre', consistente en la formación de una cavidad de color marrón en el núcleo de la avellana que motiva su depreciación. Los estudios histológicos se han llevado a cabo mediante técnicas de microscopía óptica y microscopía electrónica de barrido. La zona afectada presenta células vacías, mientras que las células que delimitan las zonas alteradas y sana van suberificándose como respuesta protectora a la afección. Las células de la zona de 'cor-negre' van perdiendo su forma isodiamétrica hasta que el tejido se destruye por lisis y da lugar a la formación de cavidades internas del grano.

Palabras clave: calidad, microestructura, microscopía óptica, microscopía electrónica.

Introduction

Spain is the fourth most important hazelnut producer in the world, after Turkey, Italy and the US, with a production of 16,200 tons of grain and a surface area dedicated to this crop of 28,189 ha (MAPA, 1999). Around 97% of the national surface area with this crop can be found in the Tarragona province, where it represents an important source of income in several municipalities (Tous and Romero, 1997).

The hazelnut kernel is used as a human food product either in the raw form, toasted, fried or salted, or as one of the ingredients of several manufactured food items, together with cocoa in chocolates or to make turrons (traditional Spanish sweets), ice cream, cakes, drinks, in sweetmeats, etc. (Tous and Romero, 1997). The presence of brown spots in the kernel cavity devalues the final product and means that the whole kernel cannot be used as it tends to split more easily during industrial processing.

Brown spots in the kernel cavity are an alteration that affects the inside of the kernel and appears simultaneously to filling of the hazelnut kernel cavity (Romero *et al.*, 1997). It is an increasingly important commercial problem that affects the main varieties of hazelnut, and is also observed in the harvests in other countries such as Turkey, Italy and the US. In Spain, this condition can affect between 13% and 30% of the fruit produced (Romero *et al.*, 1997), although some later stu-

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dies by the same authors seem to show that in the specific case of the Negret variety in Catalonia, brown spots in the kernel cavity can affect up to 97% of the fruit, mainly in higher regions (Romero *et al.*, 2001).

The causes of this alteration are still unknown and there is very little information about this. In Switzerland, Keme and Messerli (1976) detected the presence of polyphenol-oxidase in affected hazelnuts and observed that this alteration appeared more frequently in Spanish commercial batches. In Italy, Rivella (1984) described hidden damage only visible when the hazelnut is split, making it difficult to eliminate affected fruit from commercially classified batches. For fruit of the Negret variety grown in Tarragona, Romero *et al.* (2001) observed a strong influence of climate on the appearance of this condition. The main causal factor appears to be accumulated heat units in the final stages of kernel formation (middle of July); while mean relative humidity appears to have a crossed effect. The appearance of «brown spots in the kernel cavity» increases in warmer regions and decreases in colder regions.

Similar alterations have been described in the potato (*Solanum tuberosum* L.) (Van Denburgh *et al.*, 1986; Mc Cann and Stark, 1989; Rex and Mazza, 1989; Olsen *et al.*, 1996), Japanese radish (*Raphanus sativus* L.) (Kano and Fukuoka, 1996), watermelon (*Citrullus vulgaris* Schrad.) (Kano, 1993; Schultheis and Dufault, 1994), chicory (*Cichorium intybus* L.) (Den Outer, 1989), apples (*Malus sylvestris* Mill.) (Recasens, 1997) and peanut (*Arachis hypogea* L.) (Netsangtip *et al.*, 1987; Keerato-Kasikorn *et al.*, 1987; Rerkasem *et al.*, 1988). There is a wide range of causes for these alterations but due to a lack of sufficient research none of these can be related to the specific case of hazelnut.

The main reason for carrying out this study is the lack of any published histological study to date of this condition in affected hazelnut tissues.

Material and methods

Samples

Hazelnuts of the 'Negret' variety were used since this is the most important variety in the Tarragona region (78% of the cultivated surface area). These were collected from experimental farms in the municipalities of La Selva del Camp and Constantí, both in the Tarragona province.

Sampling was done weekly (50 fruits/tree and 20 trees in each plot) from 22 June to 26 August of the years 1995, 1996, 1998 and 1999. The kernels were separated manually from the shells in the laboratory. A total of 40 kernels were collected per tree and their length, weight and the presence of brown spots in the kernel cavity were determined. The other 10 kernels were fixed for 24-48 h to be later analyzed in a histological study with a FAA solution, composed of 10% formol at 40%, 80% of absolute alcohol and 10% of glacial acetic acid. Afterwards, the tissue was conserved in 70º alcohol until analysis. All samples were processed as described by Durfort (1994). However, given the very few published studies on histological techniques in nuts in general, and especially in hazelnuts, we describe here the protocols followed to section and stain the tissues.

Histological sections

For their inclusion in paraffin, the samples were rinsed with abundant running water (2-3 h) to wash off the FAA. They were then dehydrated at room temperature in serial concentrations of aqueous ethanol solutions (70, 96 and 100%), 6 h per bath changing the alcohol every 3 h. Samples were then placed in an intermediate bath of toluene and absolute alcohol (1:1) for 2 h and 30 min, replacing the dehydration alcohol by toluene (organic solvent of paraffin), in a 20-30 min bath. Finally, the samples were transferred to a paraffin and toluene bath for 4-5 h and a final bath of melted paraffin (Panreac, melting point of 51-53ºC for clinical diagnosis) and for 24 h in a furnace at 60ºC in cubic moulds of vegetable parchment. Before making the histological sections, samples were left to cool for at least 24 h.

Longitudinal and cross-sections of the samples of a mean thickness of 10 µm were made with a rotary microtome (omS Reichert), using a stainless steel knife.

The slices were placed in a hot water bath (40-45ºC) with Mayer albumin, to make them easier to spread and stick to the slides. After 6-12 h in a furnace at 40ºC the staining protocol was started. The sections were first deparaffinised with two successive toluene baths (10 min each bath) the toluene was eliminated and the material was rehydrated, using a decreasing series of aqueous ethanol solutions (100, 75, 50% and distilled water), for 5 min in each one.

For observations with the scanning electron microscope, after deparaffinising the sections these we-

Table 1. Dimensions of hazelnut kernel the first day of appearance of brown spots on the internal cavity

	Length (mm)	Weight (g)	Date	Days after setting
Mean	16.3	1.25	16 July	162
Minimum	14.5	0.83	8 July	154
Maximum	17.9	1.70	30 July	176
Standard deviation	± 0.86	± 0.26	—	± 9.4

Measurements are means from data recorded in 1995, 1996, 1998 and 1999.

re coated with a gold layer (thickness of 300 Å), using a Polaron E-5000 vaporiser.

Staining

A first complete staining was done with Picrofuschin to establish the amount of tissue affected and the cellular structure of the affected parts. The dye was prepared with 1 g of fuschin acid (DC Panreac) with 10 ml distilled water and 100 ml picric acid in saturated solution (DC Panreac). The sample was bathed in the dye for 30 min and then washed with absolute alcohol, 3 times for 5 min. Finally, the sample was transferred to a xylene bath for 10 min and mounted in Canada balsam.

Also, a selective stain was applied consisting of Sudan IV, in order to confirm the presence of suberin defining the region affected by brown spots in the kernel cavity. The dye was prepared with 0.5 g Sudan IV (DC Panreac) and 100 ml 70% alcohol, shaking well and leaving to rest at 30ºC overnight, filtering the saturated solution the following morning. The sample was submerged for 20 min in dye, washed rapidly with alcohol 2-3 times and mounted in glycerine.

Optical and electronic microscope

A Leitz Dialux 20 EB, optical microscope from the Centro Mas Bové of the IRTA, with lens of different magnifications (×2.5, ×10, ×25, ×40 y ×100), eyepiece of 10x and white light was used. The most representative samples were photographed with a Wild Photoautomat MPS 45 camera, attached to the microscope.

A scanning electron microscope Hitachi Scanning model S-2300, of the science-technical services of the Universidad de Barcelona was used, operating with a voltage of 15 to 20 kV.

Results and Discussion

«Brown spots in kernel cavity» in the hazelnut is a condition that appeared between 154 and 176 days after setting (Table 1), when the kernel measured approximately 14.5 to 17.9 mm long (in the case of the Negret variety), that coincides with the final growth phase of the kernel (the first fortnight in July).

Macroscopic description

On a macroscopic level, the condition manifested initially with a light-colored spot with a watery and glassy appearance, which became darker as the kernel matured to reach a dark brown color. In the initial stages, this stain appeared on the inside of the cotyledons and not directly where these met. It was also observed that in most affected kernels the first manifestation of brown spots in the kernel cavity was associated with the appearance of a small crack around the site of closure of the two cotyledons (Fig. 1).

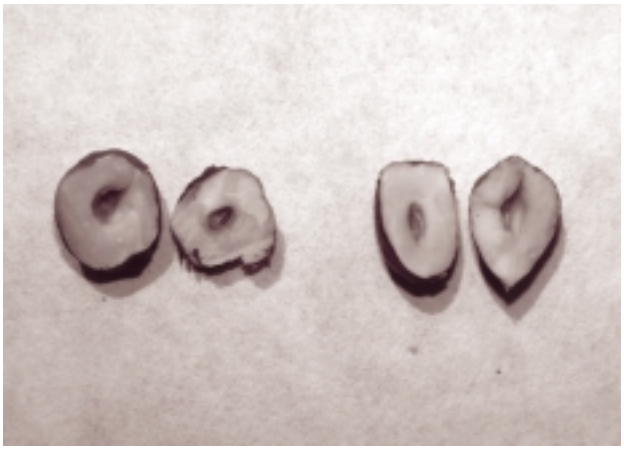

Figure 1. Hazelnut kernel affected by brown spots on internal cavity.

On observing the cotyledons individually, the first stage of the affection was distinguished by the appearance of a ring with a different color to the rest of the tissue which, seen in profile, presented a small central depression. The longitudinal section of the cotyledons showed the real extent of the affection, and the depth of tissue affected by the stain could be seen to have increased.

The brown spots usually appeared in the middle of the kernel interior, although they could also be found

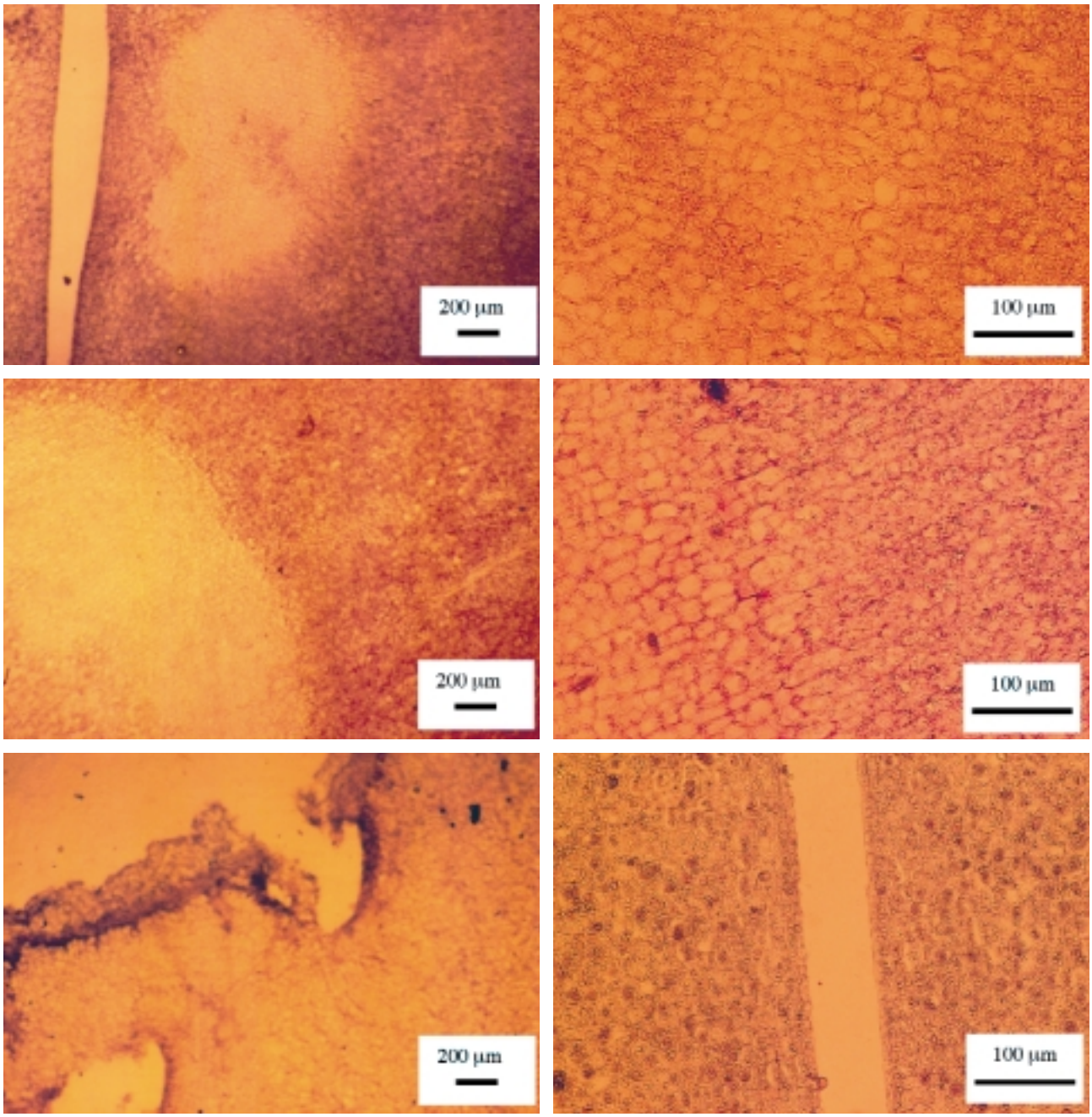

Figura 2. Micrographs of representative sections of hazelnut cotyledons, with differing degrees of affection by brown spots in internal cavity. Sudan IV stain. (a) The first area to be affected is the interior of the cotyledon near to the zone of closure. (b) Detailed image of the limiting zone between healthy tissue (right) and affected tissue (left), with incipient suberization. (c) Affected tissue (left), second stage of the condition. (d) Detailed image of the zone limiting healthy tissue (right) and affected tissue (left), with intense suberization. (e) Tissue intensely affected by brown spots on internal cavity. (f) Healthy hazelnut tissue.

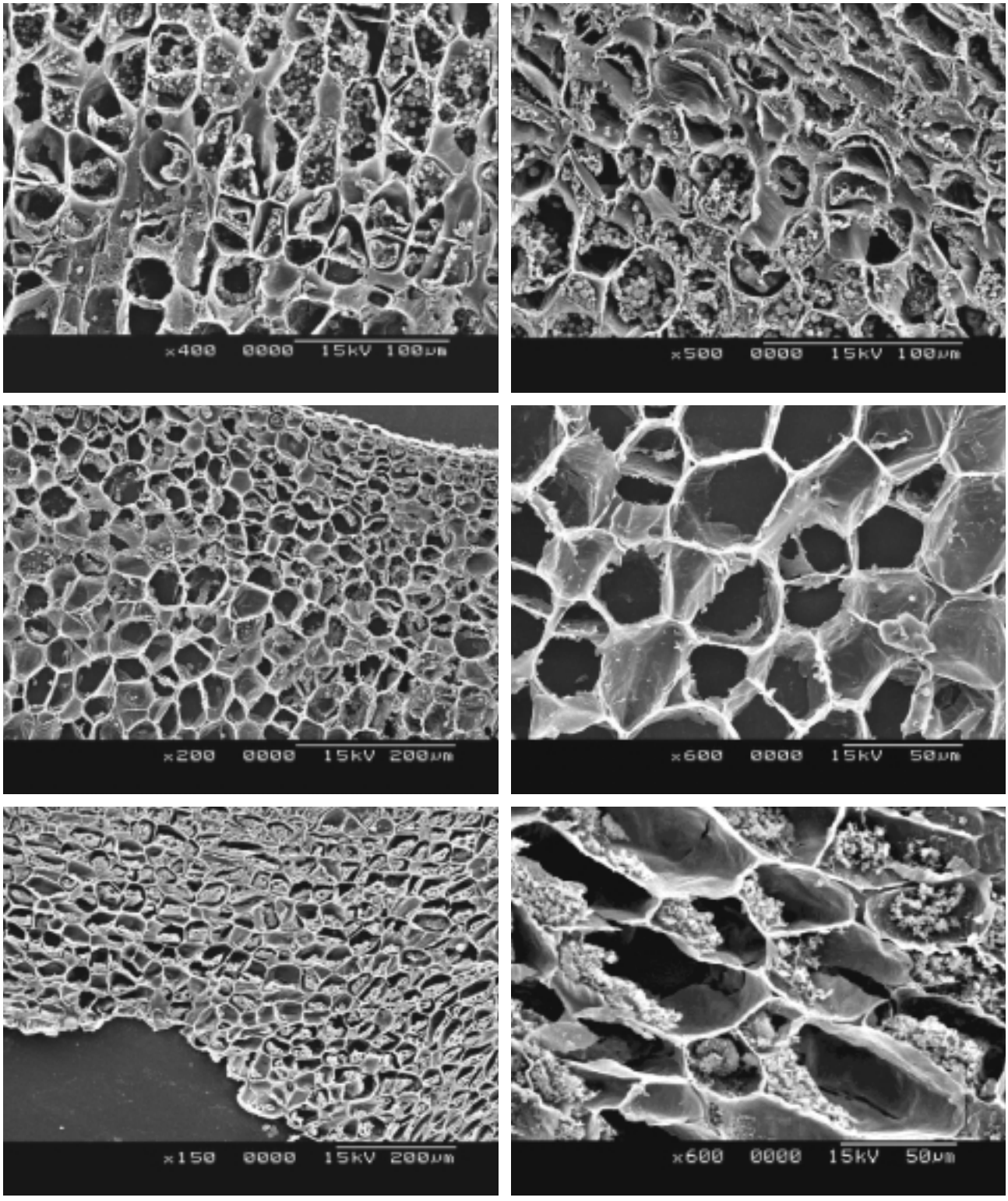

Figura 3. Micrographs of sections representative of hazelnut cotyledons, with different degrees of affection by internal brown spots. Scanning electron microscopy: (a) Affected cells that lose their cell contents. (b) Cells from the area limiting healthy tissue (bottom left) and affected tissue (top right). (c) Totally empty cells (bottom left) and cells that are emptying (top) because of the disease. (d) Detailed picture of the totally empty cells. (e) Internal cavity formed by the disease (bottom left) and strongly affected nearby cells. (f) Detailed picture of cells with a content displaced towards the affected zone.

slightly displaced towards to end away from the embryo. On the other hand, both cotyledons from the same kernel could be affected, only one, or both but to a different extent.

After the stain had turned completely brown, small holes appeared which, finally, form the central brown cavity, approximately 21 days after appearance of the stain or spots (in Tarragona this occurs in the first week of August). Afterwards, the physiopathy did not seem to change.

Microscopic description

Under the microscope, the central brown cavity was observed to present empty cells and was limited by suberized cell walls.

As described previously, the inside of the cotyledon was affected (Fig. 2a and 2b). The cells situated between this stain and the site of cotyledon closure presented little lipid synthesis and although not initially affected, the disease advanced towards this area and the cells finally became empty.

Between the affected zone and parenchymatic cells of the cotyledon interior, suberin was deposited as a protective response of the affected tissue (Fig. 2c and 2d). This was deposited more and more and the cells finally became empty and the tissue was profoundly altered (Fig. 2e and 2f).

We must bear in mind that suberization in the epidermis of stems with secondary growth is a normal process and depends on the presence of a suberogenous or phelogenous cambium. In this case, secondary suberin deposits in the cell wall alter the cell contents owing to the anoxia produced by impermeabilization of the cell wall.

Loss of the cell contents can occur due to internal plasmolysis (Fig. 3a and 3b), until only one wall remains, which, in later stages gradually looses its structure (Fig. 3c and 3d) causing folding and rupture of the affected tissue, finally forming small holes that produce the internal cavity of the kernel (Fig. 3e).

Cell contents of cells not affected but close to the inner brown cavity, were displaced towards to affected zone (Figure 3f).

The changes described are similar to the internal browning of chicory (*Cichorium intybus* L.), where the affected cells suffer plasmolysis, changing their shape from round to oval, followed by collapse and cell death (Den Outer, 1989).

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