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# Ultrastructure of the onset of chilling injury in cucumber fruit

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## Summary

The onset of the symptoms of disorders provoked by low temperature storage or chilling injury (CI) in pickling cucumber fruit (*Cucumis sativus* L. cv. 'Trópico' and in the cv. 'Perichán 121') and associated rot were monitored by cryoscanning electron microscopy. Fruit were stored at 4ºC for different times (4 to 12 d) and samples were transferred to 20ºC every 2-3 d (cv. Trópico). In a second experiment, fruit cv. 'Perichán 121' were stored at 6ºC. Macroscopic CI symptoms included small-flattened areas with sunken but externally sound tissue. Later, the damage was manifested as pitting and decay due to the presence of necrotrophic fungi (*Pleospora herbarum*, *Alternaria* sp.) on the surface of the broken areas, and *Botrytis cinerea* in cv. 'Perichán 121'. The period of induction of CI becoming apparent lasted about 4 d at 4ºC followed by a phase of slow increase of around 4-5 d prior to an exponential increase in CI. Micro fractures of 45-250 µm length developed in CI tissue around the stomata of 20 µm Ø. The first response to chilling was the sinking of stomata accompanied by 10 µm Ø fractures with collapse of hypodermal cells. These small fractures expanded into a sink of around 40 µm Ø and more than 50 µm depth. Refrigerated tissue had visibly collapsed in 4-6 d, starting with a small brown area indicating collapse of parenchymatous cells, followed by sinking and collapse of epidermal cells, particularly around the micro fractures. The pitted tissue showed flattening of the cell walls, plasmalemma and middle lamella region, with severity increasing with increasing depth in the parenchymatous tissue. Translucent water soaked areas were also located depth in the mesocarp tissue with similar cell damages.

## 1. Introduction

Below 7-13ºC cucumber fruit is very sensitive to chilling injury (CI), which is the main physiological disorder that limits the use of refrigeration to extend its shelf-life. The breakdown of the tissue provides a suitable environment for the growth of saprophytic pathogens that colonize chilling injured fruit (HAKIM et al., 1999; KANG et al., 2002). The main symptoms of CI are tissue collapse, pitting, translucent water-soaked spots and water-soaked areas in the mesocarp (FUKUSHIMA et al., 1977; HAKIM et al., 1999; KANG et al., 2002). Extensive decay (i.e. *Alternaria* sp.) is known to develop when chilled cucumbers are removed from low-temperature storage (ABE, 1990). Cucumbers chilled at 0ºC have vertical fine wrinkles and/or shallow pitting, while fruit at 5ºC have deep pitting and/or surface depressions (FUKUSHIMA et al., 1977). FUKUSHIMA and TSUGIYAMA (1977) reported plasmalemma leakage in cucumber fruit stored at 0ºC and membrane breakage in fruit stored at 5ºC. The severity of pitting is also inversely related to the relative humidity of the storage atmosphere and the growing temperatures (ABE, 1990; HAKIM et al., 1999; KANG et al., 2002), although the primary effect of low temperature on pitting is not a dehydration dependent process (JACKMAN et al., 1989).

Cryogenic scanning electron microscopy (cryo-SEM) avoids typical problems that arise with other methods in high water content tissues, such as extraction by chemical fixation or solubilization of fats and carbohydrate, and structure collapse, shrinkage and distortion, (ECHLIN, 1992). For this reason cryo-SEM is a suitable tool to observe CI symptoms (RHEE and IWATA, 1982; TATSUMI et al., 1987), sound epicarp fruit tissue, fungal infection processes, surface organisms, and disorders (ECHLIN, 1992).

Pitting in cucumber fruit is associated with a combination of factors such as epidermal cracks, the collapse of inner tissues including the hypodermal tissue (parenchyma), and the deposition of mucilage (TATSUMI et al., 1987). The cracks in the cuticle and the sinking of epidermal cells near the stomata lead to an enhanced transpiration rate (TATSUMI et al., 1987). As regards tissue destruction, ABE (1990) classified pitting as type I (affecting parenchymatous cells), or type II (affecting both epidermal and hypodermal cells). Typical symptoms of CI in different fruit tissue including cucumber have been characterized previously (ABE, 1990; JACKMAN et al., 1989; LURIE et al., 1997; RHEE and IWATA, 1982; TATSUMI et al., 1987). The first goal of the present study was to visualize the development of CI of the epicarp tissue and cells (including cell wall and plasmalemma) of cucumber fruit using cryo-SEM. Different storage conditions and two cultivars were used to provoke several CI symptoms.

## 2. Material and methods

### 2.1. Greenhouse and plant material

Two sets of experiments with different cucumber (*Cucumis sativus* L.) cultivars were carried out. These cultivars used for fresh consumption in Spain and Russia were parthenocarpic, pickling types with very soft-spined fruits. In the first experiment perlite-grown cucumbers cv. 'Trópico F1' were used. In the second, cucumber fruit of cv. 'Perichán 121', which had been grown in similar conditions, were purchased from a local supermarket when fruit remained refrigerated less than 1 d at 5-8ºC. In the first experiment, cucumber fruit were grown in a glasshouse during the winter and spring seasons in Cartagena (Murcia, SE Spain) as described by GÓMEZ et al. (2003). The two successive crops considered in the first study were transplanted in January and April (referred to as winter and spring seasons respectively).

The cucumber canopy cv. 'Trópico' F1 (Nunhem Seeds, The Netherlands), in rows, covered about 140 $m^2$ of ground, with a plant density of 2.08 plants $\cdot$ $m^{-2}$. The plants were grown on perlite (type K-13) in polyethylene bags using an average of 8 $dm^3$ per plant. The plants were irrigated with a nutrient standard solution (pH = 5.5 to 6.0, EC = 2.2 mS $\cdot$ cm $^{-1}$) of the following composition (in mmol $\cdot$ $L^{-1}$): 13 $NO^{3-}$, 1.7 $H_2PO^{4-}$, 0.5 $NH^{4+}$, 7.5 $K^+$, 3.5 $Ca^{2+}$, 2.0 $Mg^{2+}$. Micronutrients used were 110 mg : $L^{-1}$ of a commercial micronutrient complex containing 5.77 ppm Fe, 0.055 ppm Co, 0.66 ppm Cu, 0.44 ppm B, 1.87 ppm Mn, 0.66 ppm Zn and 0.055 ppm Mo. The open-drainage rate for programming the fertigation was about 30% of the solution used (i.e. we assumed that 70% is used for the plant). The crops were managed as usual in commercial greenhouses under fertigation, pruning and climate control as described by GÓMEZ et al. (2003).

### 2.2. Harvesting and chilling injury studies

In the first experiment, the fruit development periods, corresponding to the time taken to reach the commercial maturity stages, ranged from 12 to 28 d after anthesis, although it usually lasted 12-15 d in the two harvest dates considered in both seasons. Fruit were packed in polyethylene bags to reduce dehydration, transported within 20 min to the laboratory, and stored for 1-2 h at 6ºC ± 1ºC in a domestic refrigerator. Then fruit with an equatorial caliber of 140-160 mm and minimum color of 125-128º (hue angle) and 31-34% lightness were selected and randomly divided into bags of five fruits each (n=5 replicates). These were sealed in 20 ± 2 µm thick, non-oriented macroperforated (32 holes of 1.2 mm diameter per square dm) cast polypropylene film (Plásticos del Segura, Murcia, Spain). These bags increase the relative humidity (RH) around the fruit up to 95-99% without changing the gaseous atmosphere, which is useful for differentiating CI effects from moderate to severe dehydration symptoms. In this first experiment, three replicates of five fruit each harvested in the winter were stored for up to 4 d at 4 ± 0.2ºC, then transferred without opening the perforated bags to 20 ± 1ºC and 75 ± 5% RH for 4-8 d in order to allow chilling injury to develop. Before examination they were stored at 4ºC for 1-2 d more. This experiment was designed to visualize the severe CI that can be delayed or masked by refrigeration according to the results found by previous authors (ARTÉS et al., 1998; FUKUSHIMA et al., 1977; HAKIM et al., 1999). This experiment was also conducted to optimize the cryo-SEM techniques for CI studies. In the spring season three replicates of five fruit per bag were stored for up to 12 d at 4 ± 0.5ºC and 95% RH before examination to avoid the appearance of severe CI symptoms. Plastic bags remained inside a 360 L cabinet with a continuous humidified air flow of 250 L:h$^{-1}$ supplied by bubbling air in water using a membrane compressor (Schego Optimal, Germany). This experiment uses modified atmosphere packaging for retail display, because RH levels surrounding the fruit are higher and fluctuate less than in macroperforated bags (FERNÁNDEZ-TRUJILLO and ARTÉS, 1998).

In the second experiment, cucumbers cv. 'Perichán 121', a winter line of cucumber developed by Agrícola Perichán SAT (Mazarrón, Murcia, SE Spain) were used. The fruit were purchased packed in macroperforated polypropylene (20 µm thickness, 6 perforations of 5 mm Ø per square dm) bags of three fruits each within a molded plastic tray to avoid mechanical damage. The perforations were only on the adaxial face of the package. The fruit were stored for 0, 9, 16 and 23 d at 6 ± 0.2ºC and 98% RH. The RH and temperature used represent the subsequent storage of cucumber fruit in domestic refrigerators after purchase.

Weight loss was evaluated immediately after storage or after the additional shelf-life period of 4 d at 20ºC. CI symptoms evaluated visually on a 0 to 4 scale according to the mean diameter of the pitted areas (0=sound; 1-2, very slight to slight, Ø between 0 and 5.5 mm or 5.5<Ø<15 mm; 3-4, moderate to severe, 15<Ø<25 mm or Ø>25 mm). A similar scale was used for scoring shriveling symptoms. The fungi were identified according to conventional methodology (ARTÉS et al., 1998).

### 2.3. Cryo scanning electron microscopy

Cryo-SEM, also known as low temperature SEM (ECHLIN, 1992), was used to study epidermal disorders and microscopic CI symptoms. Cucumbers were examined with a magnifying glass to select areas of interest. Control fresh fruit (sound, non-refrigerated) or refrigerated fruit were allowed to equilibrate at room temperature before cryofixation to avoid water condensation. Samples of the fruit epidermis were obtained for SEM by dissecting sections (3-5 mm$^2$ by 1-1.5 mm thick) from different epicarp areas (the distal and proximal areas, and the middle, depending on the CI symptom studied) with a razor blade. Tissue leakage was removed by blotting. The cucumber tissue samples were placed on a flat specimen holder with a 1:1 mixture of Colloidal Graphite G303 (Agar Scientific LTD., Essex, England) and Tissue-Tek 4583 glue (Sakura, Zoeterwoude, The Netherlands), skin (with or without ground tissue) side up. The fresh samples were frozen with nitrogen slush. A nitrogen gas flow was used to remove possible water vapour inside the equipment (CRAIG and BEATON, 1996). Once frozen, the sample was transferred to the preparation chamber (PolarPrep 2000 cryo transfer system) under vacuum with a Polaron PP7480 Polarprep control unit and a PP7483 Polaron gas control unit (Quorum Technologies, East Sussex, UK). Here the superficial ice was removed from the surface of the specimen by sublimation when heating the sample with a resistor from (-140ºC) to -85ºC for 5 min (vacuum pressure around 0.08 mm Hg).

Then the sample was cooled again to -140ºC and coated with a gold-palladium alloy (80:20) in a Polaron PolarPrep 2000 during 180 s at 10 mA in order to improve the specimen conductivity. Cooling was used to avoid the accumulation of electrical surface charges and therefore increasing the quality of the final image. The coating thickness (8-10 nanometers) was monitored by Polaron FF7690 film thickness monitor. The valve gate between the preparation chamber and the microscope chamber was opened and the sample holder was mounted for observation on the cold stage inside the Hitachi S-3500 N microscope chamber (Hitachi, Ratingen, Germany). To ensure the correct insertion of the holder in the chamber an infrared chamberscope was used. The accelerating voltage for sample observation ranged from 2 to 15 kv with a cold stage at -140ºC and a cold trap at -190ºC to avoid contamination by aerial particles (both temperatures were maintained with liquid $N_2$). Levels of magnification ranged from x40 to x6000 (usually less than x1300). Working distances ranged from 3.9 to 30 mm, depending on the detail required. Generally the working distance was close to 10±3 mm. Three samples were mounted per holder. Images were exported to JPG format at 640 x 480 pixels and 256 colours (black & white image) or BMP format (2560 x 1920 pixels).

## 3. Results and discussion

### 3.1. Onset of weight loss and macroscopic CI symptoms and associated decay

During cold storage weight loss increased linearly in winter harvested 'Trópico' cucumber fruit from the first experiment by around 0.1% (w/w) every day ($r^2$=0.98) reaching, for example, around 0.56±0.1% after 6 d at 4ºC (data not shown). The 4 d shelf-life periods increased weight loss by about 1.2% (before 6 d of storage) or more (around 2%) above this period probably because of the onset of CI. In spring harvested 'Trópico' cucumber fruit from the first experiment, dehydration mainly affected the fruit with slight symptoms (pulp whitening and pulp dryness) with dehydration indices of around 25%, 40% and 60% after 4, 9 or 12 d at 4ºC plus the additional shelf-life periods. The first visible chilling injury symptoms at 4ºC developed after 6-9 d (in fruit from the first experiment harvested in the spring) and were represented by slight pitting and a burst in decay, which showed a broad range depending on many factors (0% to 45% fruit). Decay was mostly due to *Alternaria* sp., which usually accompanies CI (ARTÉS et al., 1998), though species of *Pleospora herbarum* (perfect state teleomorph of *Stemphylium botryosum*) also developed. In cv. 'Perichán 121', *Botrytis* sp emerged from the hypodermal tissue and sometimes used stomata breakage. This indicated a very short period of induction for the first visible CI to be seen after 4-5 d at 4ºC under the macroperforated film (i.e. high

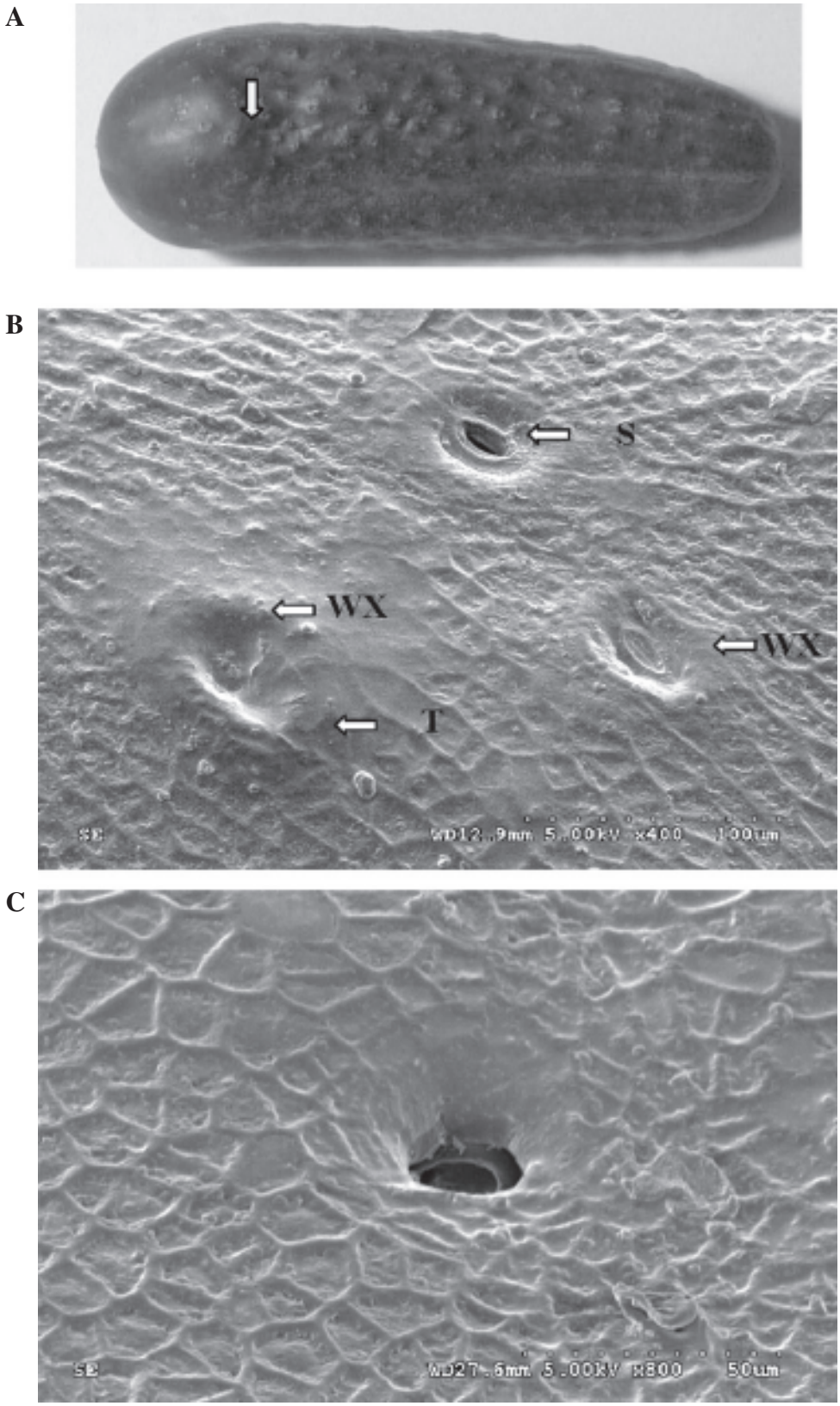


**Fig. 1:** First chilling injury symptoms on the epidermis of cucumber fruit cv. 'Trópico'. A. Macroscopic aspect of chilling injured fruit. B. Sound epidermal tissue including stomata covered by epicuticular wax (WX), active stomata (S) and broken trichome covered by continuous epicuticular wax (T). C. Epidermal depression surrounding sunken stomata.

RH). Additionally, the shelf-life period used in winter harvested fruit from the first experiment favored better observation of the symptoms that usually develop slowly at low temperatures (ARTÉS et al., 1998). In contrast, the colonization of the pitting by decay was very rapid,

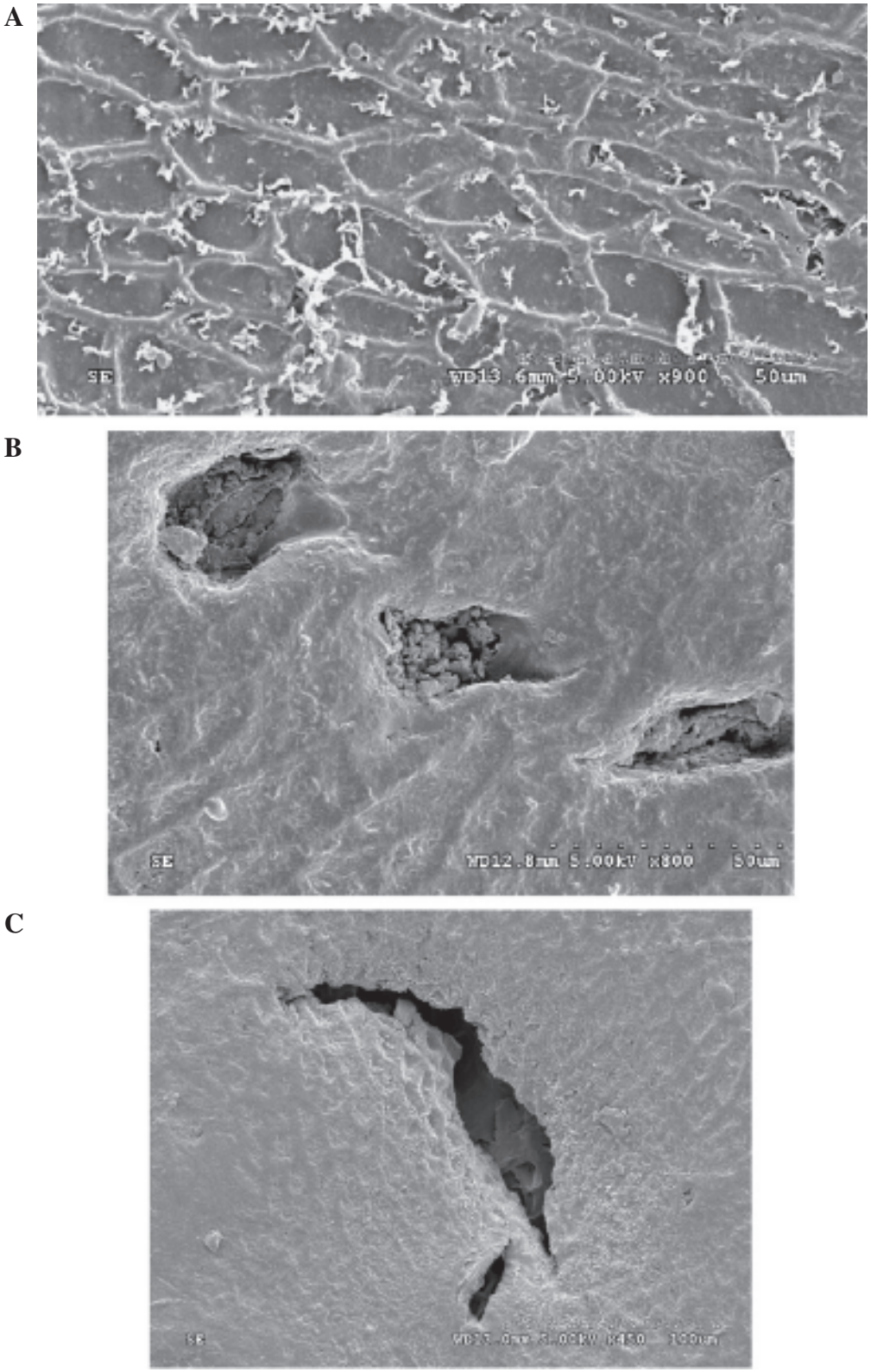


**Fig. 2:** Chilling injury symptoms on the epidermis of cucumber fruit cv. 'Trópico'. A. Epidermal cells of sound tissue with epicuticular wax. B. Micro fracture with some conidia inside C. Crack with a half-moon shape.

reaching almost 100% of the pitted fruit, which also agrees with the onset of CI in tomato fruit (ARTÉS et al., 1998). This period might be as short as 3 d at 5ºC (TATSUMI et al., 1987) or longer. In this experiment micro cracks and depressed stomata appeared after 4-5 d at 4ºC plus the additional shelf-life period. The more evident macroscopic CI symptoms include sunken but intact tissue, flattened tissue as a form of pitting, and *Alternaria* sp. decay on flattened or pitted areas.

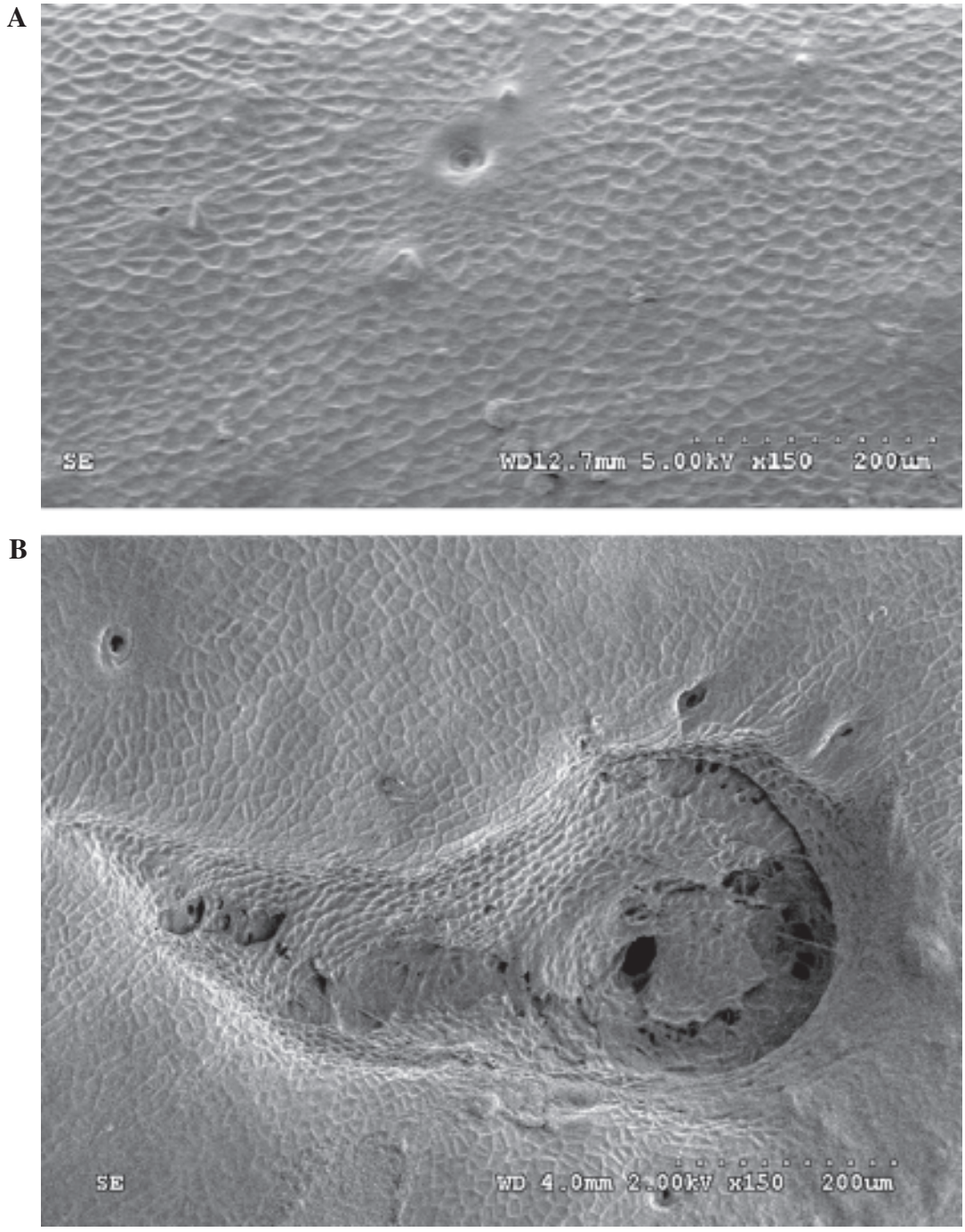


**Fig. 3:** A. Sound cucumber fruit cv 'Trópico' tissue with stomata seen from the surface covered by epicuticular wax, stomata and broken trichome covered by continuous epicuticular wax. B. Massive sinking (craters) of epidermal tissue surrounding stomata.

### 3.2. Development of chilling injury as monitored by cryo-SEM

The first symptoms of epidermal deterioration, which are not observed visually, were cracks in the cuticle and sinking of the hypodermal and epidermal cells near the stomata. Microscopic symptoms of the extent and severity of the different disorders detected are described below, irrespective of the experiment considered.

#### 3.2.1. Onset of CI by observing depressed epidermal area including micro cracks and sunken trichomes

Sound fruit had homogeneous surface (Fig. 1 B), with stomata frequency depending on the maturity stage of the tissue (Fig. 1 B). Epidermal cells close to the stomata were elevated and covered with a smooth wax layer resembling an extinct volcano structure of 40-

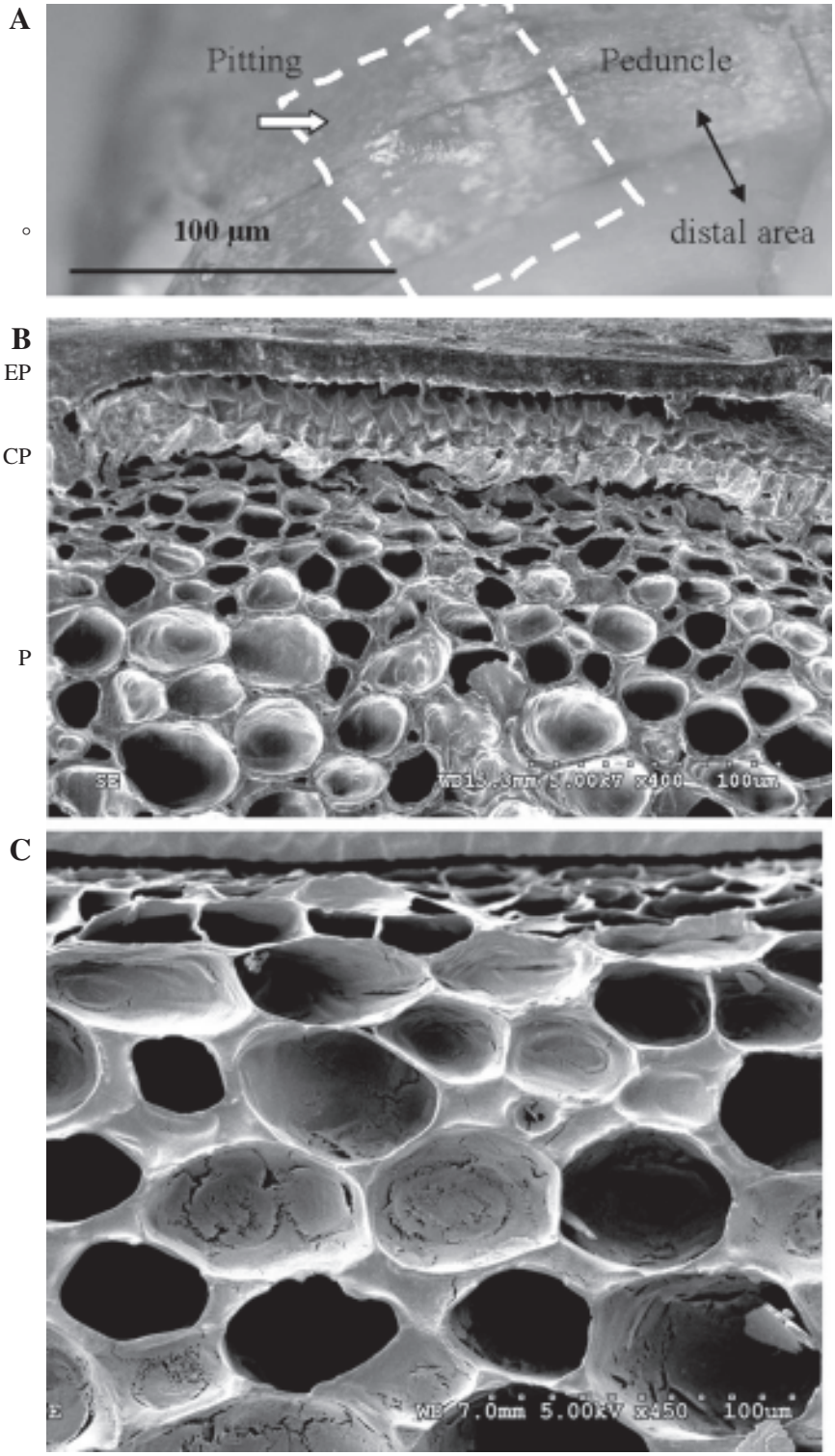


**Fig. 4:** Chilling injury on the distal zone of cucumber fruit cv. 'Perichán 121' stored in macroperforated films. A. Cross-section with light brown epidermis and pitting after 9 d at 6ºC. B. Cross section of fresh and sound epidermal tissue. Epidermal cells (EP), compact parenchyma (CP) and parenchyma (P). C. Pitted hypodermis after 23 d at 6ºC.

60 µm Ø (Fig. 1 B). The characteristic trichomes of cucumbers popularly known as spines were not observed in physiological mature cucumber fruit from the cultivars studied, although the fruit showed the characteristic crater of less than 350-400 µm diameter when trichomes were detached. A top view of the epidermal tissue with very slight CI symptoms (Fig. 1 A) pointed to structural breakdown with void cells underneath the stomata. Stomata started to sink as a result of low temperature storage (Fig. 1 C). Sound tissue (Fig. 2 A) rarely had the small holes of 10 µm Ø that expanded to become a sink of around 40 µm Ø and more than 50 µm deep (Fig. 2 B), although cracks and micro cracking are common in stored fruit (LURIE et al., 1997; ARTÉS et al., 1998).

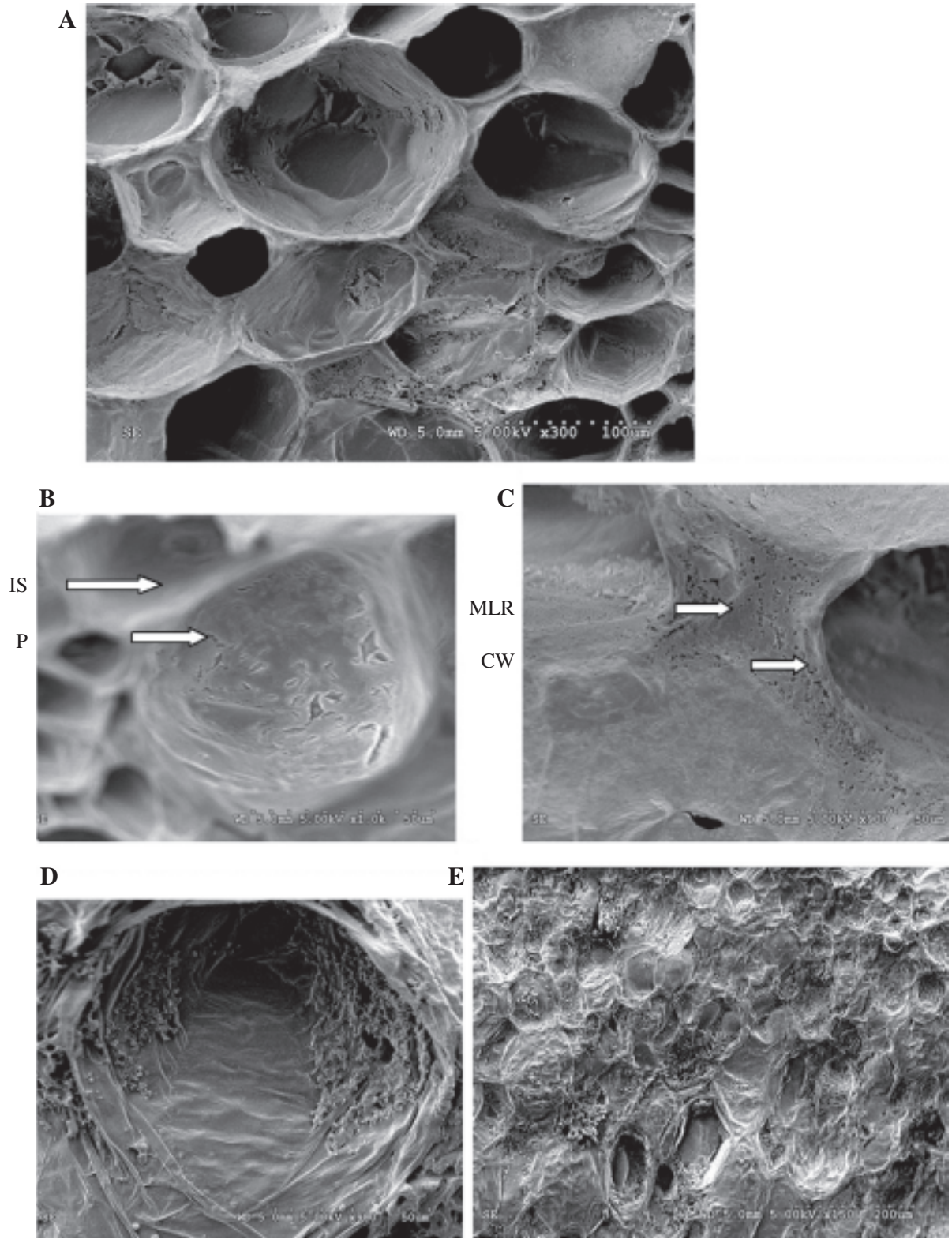


**Fig. 5:** Details of chilling injury in cv. 'Perichán 121' after 9 d at 6ºC. A. Hypodermal tissue with chilling injured plasmalemma. B. Detail of parenchymatous cell deterioration caused by pitting. Intercellular space (IS). C. Cell wall deterioration. Cell wall (CW), middle lamella region (MLR). D. Chilling injured plasmalemma. E. Aspect of a water-soaked cell of the spongy mesocarp (5 mm below the fruit surface).

The limit of pore diameter for the visual detection of pitting in sunken epidermal areas was established by TATSUMI et al. (1987) at 70-130 µm Ø, while micro cracks that appeared in epidermis suffering CI had 45-250 µm length. Then, the irregular shapes (waning moon, mussel) of the micro cracks can be detected sometimes by optical microscopy. These micro cracks were frequently associated to stomata collapse (Fig. 2 C), and were more evident in cv. 'Trópico' than in cv. 'Perichán 121' as a result of the shelf-life periods used

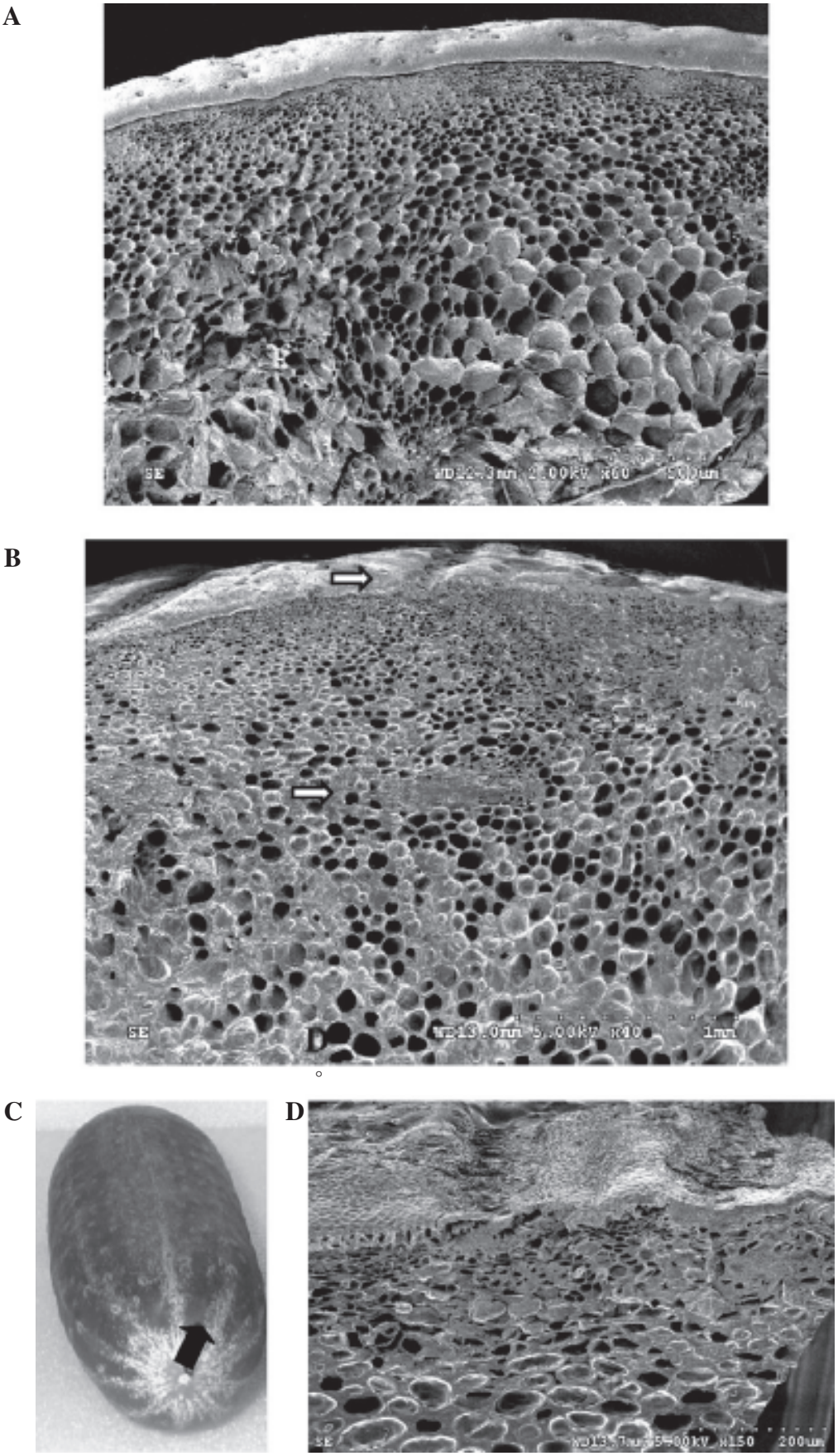


**Fig. 6:** Collapse of parenchymatous cells of cucumber fruit cv. 'Trópico'. A. Top and cross section of sound tissue. B. Top and cross-section of with arrows indicating epidermal pitting and collapsed parenchymatous cells. C. Macroscopic symptoms (black arrow). D. Detail of pitted tissue.

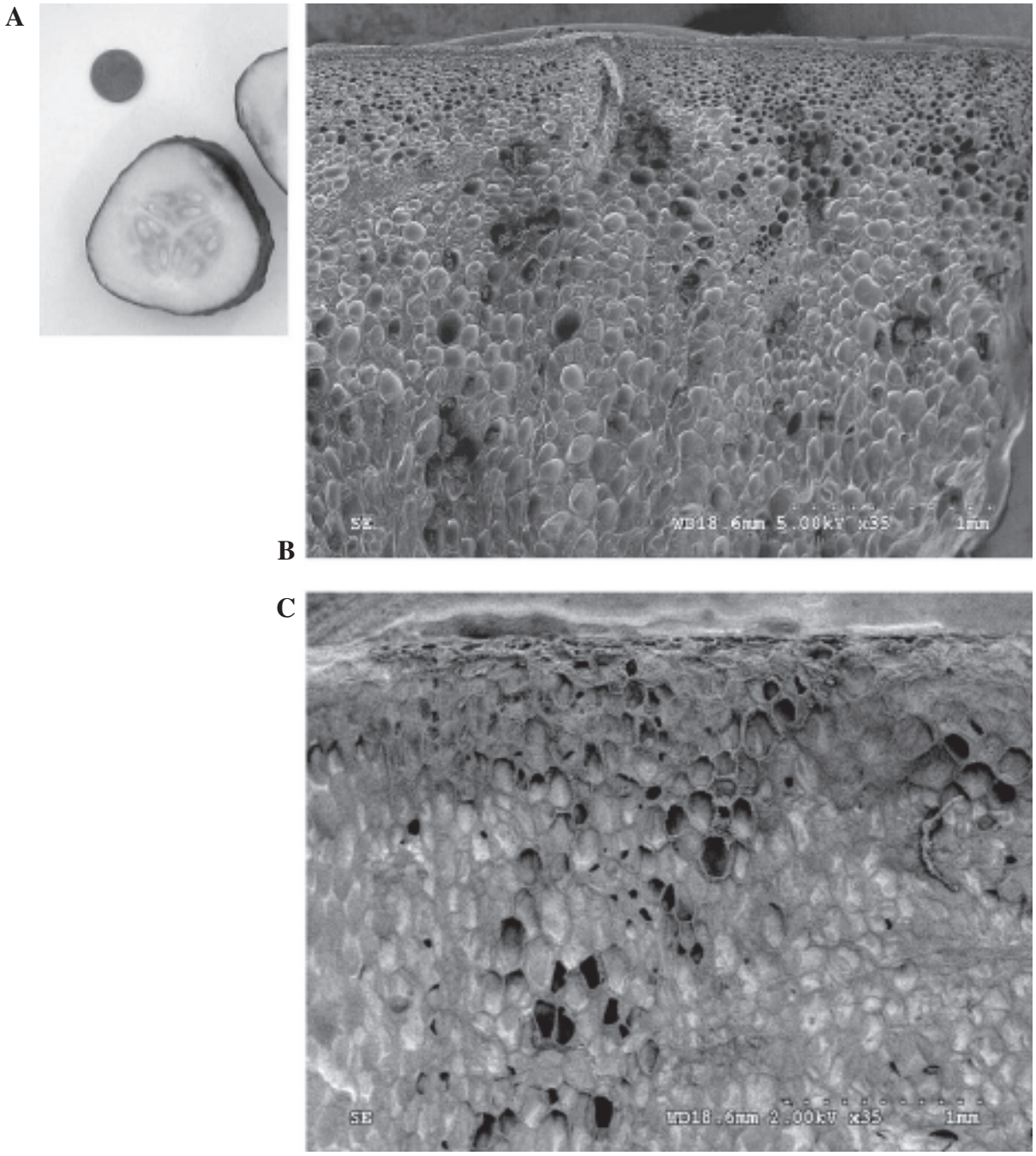


**Fig. 7:** Necrotic tissue of cucumber fruit cv. 'Trópico'. A. Surface view of different degrees of necrotic tissue in the distal area of cucumber fruit. B. External aspect of the necrotic tissue. C. Detail of necrotic tissue by cryo-SEM, with epidermal and parenchyma cells affected.

after refrigeration in the former cultivar. Sometimes fruit epidermis can develop epidermal cracking as a result of a sudden increase in the water content of the soil, atmospheric humidity or temperature (OPARA et al., 1997); in some cases, the cracks may be lignified. Smaller micro cracks were or not near to each other depending on nearby stomata densities. An extensive collapse joining nearby cracks (Fig. 2 B) in one was generally the result of hypodermal chilling injury. These results matched with the initial CI symptoms around the stomata observed in cross-sections of parenchyma cells of pitted cucumber (RHEE and IWATA, 1982), and in cold stored mango and avocado (BOWER et al., 2003).

The final step of pitting included massive sinking of the fresh sound epidermal tissue (Fig. 3 A) that led to irregular craters including a group of stomata and cells beside the stomata of 300 µm Ø or more, and up to 660 µm in length (Fig. 3 B). However, this was not very common in these experiments, in contrast with the results obtained by FUKUSHIMA et al. (1977) at 5ºC, indicating a probable cultivar effect on pitting.

Another CI symptom was the sinking of the basal cells of epidermal tissue from which trichomes (spines) of cucumber cv. 'Trópico' were dislodge. The sinking were observed visually even in non-refrigerated tissues due to dehydration. Craters with a neat circular shape of 350-400 µm Ø included collapsed and empty epidermal cells, and CI expanded this diameter up to 500 µm (data not shown). More probably, this was a result of the combined effect of CI and severe dehydration in this area (TATSUMI et al., 1987).

### 3.2.2. Onset of CI as monitored by cryo-SEM in the cross-section of the fruit

In sound tissue, cell walls plus plasmalemma were clearly differentiated from the internal content and no damages were observed in the cultivar 'Perichán 121' (Fig. 4 B). Pitted tissue (Fig. 4 A) resulted from the flattening of the cell wall, middle lamella region and plasmalemma (Fig. 4 C, 5 A to 5 C). The deeper the hypodermal cells, the higher the severity of the disorder. In the cv. 'Perichán 121' stored for 9 d at 6ºC, the fruit surface presented pitted tissue, and the first injured cells and areas of collapsed tissue respectively were 85-250 µm or 200-350 µm below the fruit surface (Fig. 4 C). CI was more severe in fruits stored 23 d, and *Botrytis* sp. developed during the first day of shelf-life at 20ºC. After 23 d at 6ºC, CI tissues had injured cells even in the first layer of hypodermal cells (30-50 µm below the epidermal cells) or massively collapsed tissue. The appearance of chilling injured cells several layers below the fruit surface has been reported in cucumber (FUKUSHIMA et al., 1977) and eggplant (ABE, 1990) pitting.

In fresh sound cucumber tissue cv. 'Trópico', the overall appearance of the parenchyma tissue is that of a compacted mass of turgid of empty cells (Fig. 6 A), depending on the cutting method or the tissue leakage that may remain on the cuts after blotting the tissue before the cryo-SEM analysis. Healthy hypodermal cells just beneath the epidermal cells were flattened, while the typical cells deep within the parenchyma tissue were bigger and rounded. However, parenchymatous cells suffering CI collapsed about 100-200 µm below the epidermis, causing a subsequent epidermal depression (Fig. 6 D) and later a massive epidermal collapse (around 20-30 µm deep) (Fig. 6 B). The kind of pitting found here, resulting in destruction of both epidermal and parenchymatous cells (Fig. 6 B and 7 A), has been named as type II (ABE, 1990), in contrast with type I pitting, when only epidermal cells are destroyed.

When epidermal CI was evident and severe in a small-flattened area (Fig. 7 A), epidermal cells beneath the epidermis were also collapsed (Fig. 7 A and 7 C), as occurred in tomato fruit (LURIE et al., 1997). The flattened areas were characterized by a shapeless mass of parenchyma cells compared with sound tissues (Fig. 7 B), and included the epidermis of 6-20 layers and the fruit surface (Fig. 7 C). The flattened tissue was frequent in the skin of the cucumber apex (Fig. 6 C). and was subsequent to pitting, or the appearance of translucent water-soaked areas that advanced from the mesocarp to the cucumber fruit surface and resulted in a massive tissue damages (water soaked blemishes), as reported in melons (XU et al., 1990) and cucumbers (KANG et al., 2002). Mesocarp tissue (spongy endocarp with a translucent aspect located about 5 mm below the fruit surface) observed by cryoSEM in the cultivar 'Perichán 121' had tissues showing a damaged cell wall, middle lamella and plasmalemma (Fig. 5 D and 5 E), which is affecting these areas more severely than layers above the cross-sections. Water evaporation of intercellular water and transpiration, though limited in this experiment by the macroperforated packaging used, would raise the tonicity of the remaining solution and reduce the rupture of the protoplasts, inducing water-soaking at an early stage of CI (TATSUMI et al., 1987).

## 4. Conclusions

The visualization and extent of CI symptoms can be monitored by cryo-SEM. The breakage of the plasmalemma, cell wall and middle lamella zone in hypodermal cells preceded the collapse of hypodermal tissues and the appearance and expansion of epidermal micro cracks. The severity of CI increased with increasing depth in the parenchymatous tissue, leading to translucent water soaked areas when affected the mesocarp tissue. The sinking of epidermal tissues, particularly around the stomata and trichomes, and CI-associated rots were later events in the development of the disorder. Packing cucumbers in perforated films at higher RH seems to induce less severe CI symptoms as observed by decreased tissue collapse and better cell wall integrity. However, possible cultivar- and season-related effects on the period required for inducing the first irreversible CI symptoms cannot be ruled out.

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