Notas Científicas

Necrotrophic fungi associated with epidermal microcracking caused by chilling injury in pickling cucumber fruit

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Abstract – The objective of this work was to visualize the association between microcracking and other epidermal chilling injury symptoms, and to identify rots in cucumber fruit (*Cucumis sativus* L.) by scanning electron microscopy (SEM). Depressed epidermal areas and surface cracking due to damages of subepidermal cells characterized the onset of pitting in cucumber fruit. The germination of conidia of *Alternaria alternata*, with some of them evident on the fractures in the cultivar Trópico, occurred after damaging on the epidermis. Before, the chilling injury symptoms became visible, *Stemphylium herbarum* conidia germinated, and mycelium penetrated through the hypodermis using the microcracks as pathway. In the cultivar Perichán 121 the fungus was identified as *Botrytis cinerea*.

Index terms: *Alternaria alternata*, *Botrytis cinerea*, *Cucumis sativus*, *Stemphylium herbarum*, cryoscanning electron microscopy, pitting.

Fungos necrotróficos associados à microfissura epidérmica causada pelo frio em pepinos em conserva

Resumo – O objetivo deste trabalho foi visualizar a associação entre microfissuras e outros sintomas na epiderme, induzidos pelo frio, e identificar as podridões de pepino (*Cucumis sativus* L.) por microscopia eletrônica de varredura. O início do desenvolvimento da lesão em pepino é caracterizado por depressões epidérmicas e pelo fendilhamento superficial, provocado pelo colapso das células subepidérmicas. A germinação dos conídios de *Alternaria alternata*, localizados nas fendas de pepino cultivar Trópico, ocorreu após o início do desenvolvimento dos sintomas dos danos, causados pelo frio, na epiderme do fruto. A germinação dos conídios de *Stemphylium herbarum* e a penetração do micélio na hipoderme pelas microfissuras ocorreram antes de os sintomas dos danos causados pelo frio se tornarem visíveis. Na cultivar Perichán 121 observou-se o fungo *Botrytis cinerea*.

Termos para indexação: *Alternaria alternata*, *Botrytis cinerea*, *Cucumis sativus*, *Stemphylium herbarum*, microscopia eletrônica de varredura, lesão.

Chilling injury (CI) develops in cucumber fruit stored at temperature below 13ºC, and is commonly followed by alternaria rot (Snowdon, 1991; Saltveit, 2004), proportional to the length of storage and to the cultivar sensitivity (Eaks & Morris, 1958; Fernández-Trujillo & Martínez, 2006). The association between chilling injury and alternaria rot is well established in tomato fruit, and the fungus was isolated from pitted tissues and from water-soaked areas (Snowdon, 1991). *Stemphylium herbarum* (anamorph of *Pleospora herbarum*) has been reported as causing disease in several species of plants (Snowdon, 1991), including cucumber (Neergaard, 1945). *Botrytis cinerea* is a common postharvest pathogen on cucumbers in cool temperate countries (Snowdon, 1991)

In cucumber fruit, CI symptoms are tissue collapse, pitting, translucent water-soaked spots and water-soaked areas in the mesocarp (Saltveit, 2004). Pitting in cucumber fruit has been associated with a combination of factors, as epidermal cracks, the collapse of inner tissues including the hypodermal tissue (parenchyma), and the deposit of mucilage (Tatsumi et al., 1987;

Fernández-Trujillo & Martínez, 2006). Cryogenic scanning electron microscopy (cryo-SEM) has been used to observe sound epicarp fruit tissue, bacteria, fungal infection processes, and other surface organisms (Echlin, 1992; Roy et al., 1994, 1996; Fullerton et al., 1999). Pickling cucumber is used for fresh consumption in Spain and Russia, but whole pickles can be pasteurized. Skin damages at harvest were hypothesized to be an important factor of pickling cucumber deterioration (Cook et al., 1957). Holding cucumbers at 4.4ºC for one to three days or longer, before processing, reduces the quality of whole fresh dill pickles (Cook et al., 1957). A better understanding of the relative importance of microcracking and the visualization of the disorders and decay could be useful in designing better methods for improving cucumber fruit quality.

The objective of this work was to visualize the association between microcracking and other epidermal chilling injury symptoms, and to identify rots in cucumber fruit by scanning electron microscopy (SEM).

Two sets of experiments with different pickling cucumber (*Cucumis sativus* L.) cultivars with very soft-spined fruits were carried out. In the first experiment, perlite-grown cucumbers the cultivar Trópico F1, cultivated in a glasshouse in Cartagena (Murcia, SE, Spain), during winter or spring season, were used as described by Fernández-Trujillo & Martínez (2006). In a second experiment, cucumber fruits of the cultivar Perichán 121, which had been grown in similar conditions, were purchased from a leader Spanish supermarket, with a previous storage less than 1–2 days from harvest, to offering to consumers at 6ºC. A data logger (Hobo Pro H08-032-08) measured air temperature and relative humidites (RH).

In this first experiment, fruits were sealed in 20±2 µm thick, non-oriented macroperforated (32 holes of 1.2 mm diameter per square dm) cast polypropylene film. Weight loss in cucumbers stored at 4ºC is about 0.1% (w/w) per day using this package (Fernández-Trujillo & Martínez, 2006).

In the winter experiment, three replicates of five fruits per bag were stored for up to 4 days at 4±0.2ºC, then transferred without opening the perforated bags to 20±1ºC and 75±5% RH for 4–8 days of commercial shelf life period. Before examination, they were stored at 4ºC for more 1–2 days.

In the spring season, three replicates of five fruits per bag were stored for up to 12 days at 4±0.5ºC and 95% RH, before examination, to avoid the appearance of severe CI symptoms. Cucumbers of the second experiment, purchased from the supermarket, were packed in macroperforated polypropylene bags (20 µm thick, 6 perforations of 5 mm Ø per square dm), with three fruits each, within a molded plastic tray to avoid mechanical damage. The perforations were only on the adaxial face of the package. The fruits were stored for 0, 9, 16 and 23 days at 6±0.2ºC and 98% RH.

Cryo-SEM was used to study epidermal disorders, microscopic CI symptoms and fungi (Echlin, 1992; Fernández-Trujillo & Martínez, 2006). Cucumbers were examined with a magnifying glass to select interested areas. Epidermal samples (1–2 mm thick) were excised with a razor blade; the juice exudates were removed by blotting and placed on a flat specimen holder with adhesive (1:1 mixture of Colloidal Graphite G303 and Sakura Tissue-Tek 4583). The details of sampling and of the subsequent cryoscanning electron microscopy have previously been reported (Fernández-Trujillo & Martínez, 2006).

Microorganisms were identified according to somatic and reproductive structures using both a stereoscopic and an optical microscope. Fungi were classified to genera level according to Barnett & Hunter (1999). The species *S. herbarum* and *A. alternata* were identified with the same protocol, as described above, and the complete handbook of Neergaard (1945). *B. cinerea* was identified according to Snowdon (1991). Fungi were also identified and photographed by cryoscanning electron microscopy.

Healthy pickling cucumber fruits (at harvest) were usually covered with a smooth waxy cuticle and had homogeneous surface with stomata (Figure 1 F). Epidermal cells close to the stomata were elevated and covered with a smooth wax layer. Marked epidermal cells were less observed than those by Reina et al. (2002), because they used fixation and critical point drying with CO_2.

The characteristic trichomes of cucumbers, popularly known as spines, showed craters of less than 350 µm diameter when trichomes were detached. Hypodermal cells just beneath the epidermal cells were flattened, while the typical cells deep within the parenchyma tissue were bigger and rounded. Healthy parenchyma tissue showed turgid or empty cells, depending on the cutting method, or tissue leakage that may remain on the cuts after blotting the tissue before the cryo-SEM analysis.

Microscopic CI symptoms which facilitate fungal growth were epidermal microcracks, expanded cracks of 45–250 µm associated to stomata and lenticels collapse, and epidermal collapse, leading to massive epidermal necrosis (Figure 1 A and B and Figure 2 B, C, E and F).

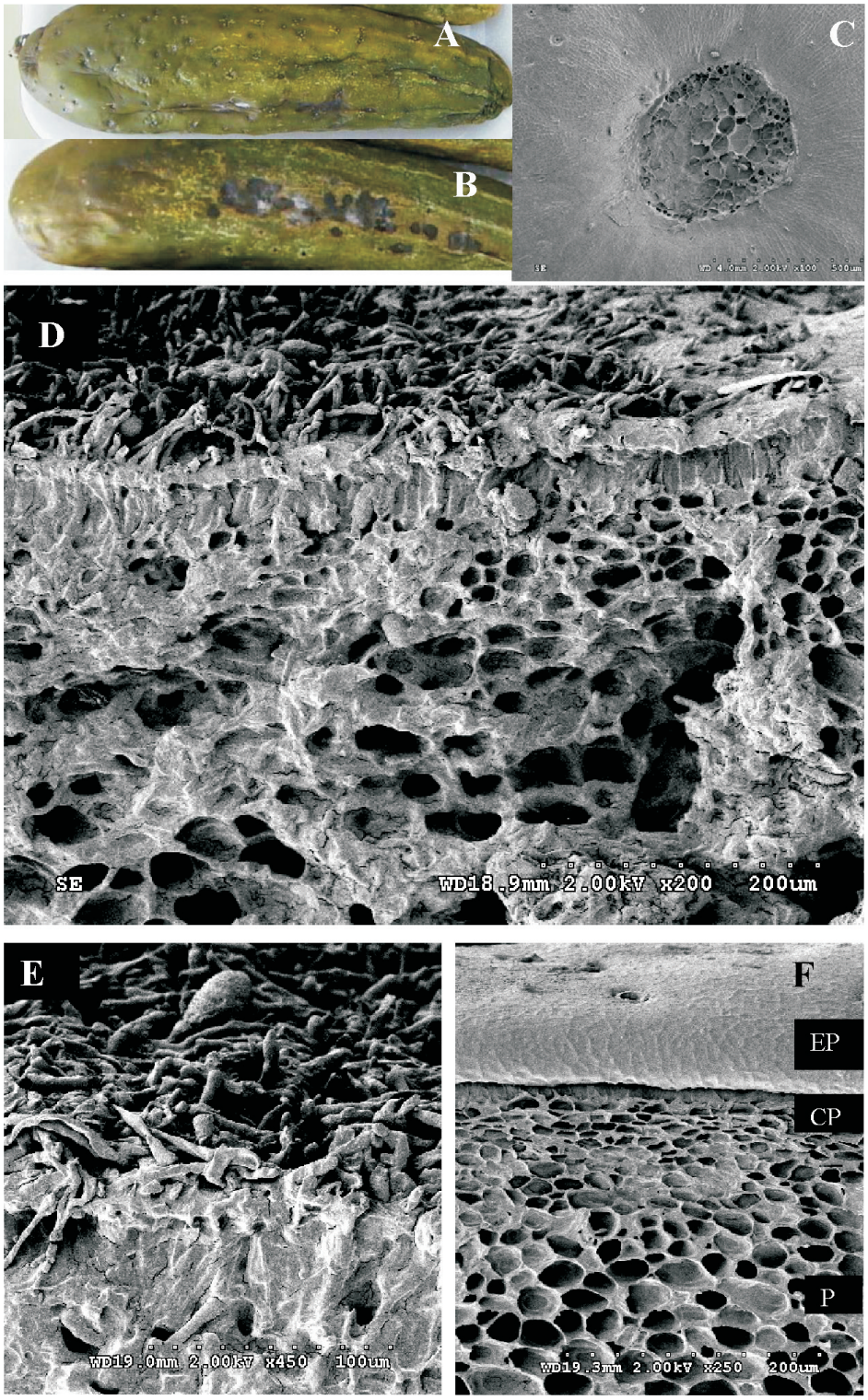

Figure 1. Microscopic chilling injury symptoms and growth of *Alternaria alternata* on fractures of chilling injured cucumber fruit cultivar Trópico. A: surface view of the sinking of trichomes of cucumber epidermal tissue; B: external symptoms in rotted fruit; C: sinking and expansion of the trichome with conidia inside; D: conidia and conidiophores of *A. alternata* on epidermis showing a cross-section of fruit affected; E: detail of conidium and conidiophores of *A. alternata*; F: sound cross-section of cucumber including epidermis (EP), compact parenchyma (CP) and parenchyma (P) tissue.

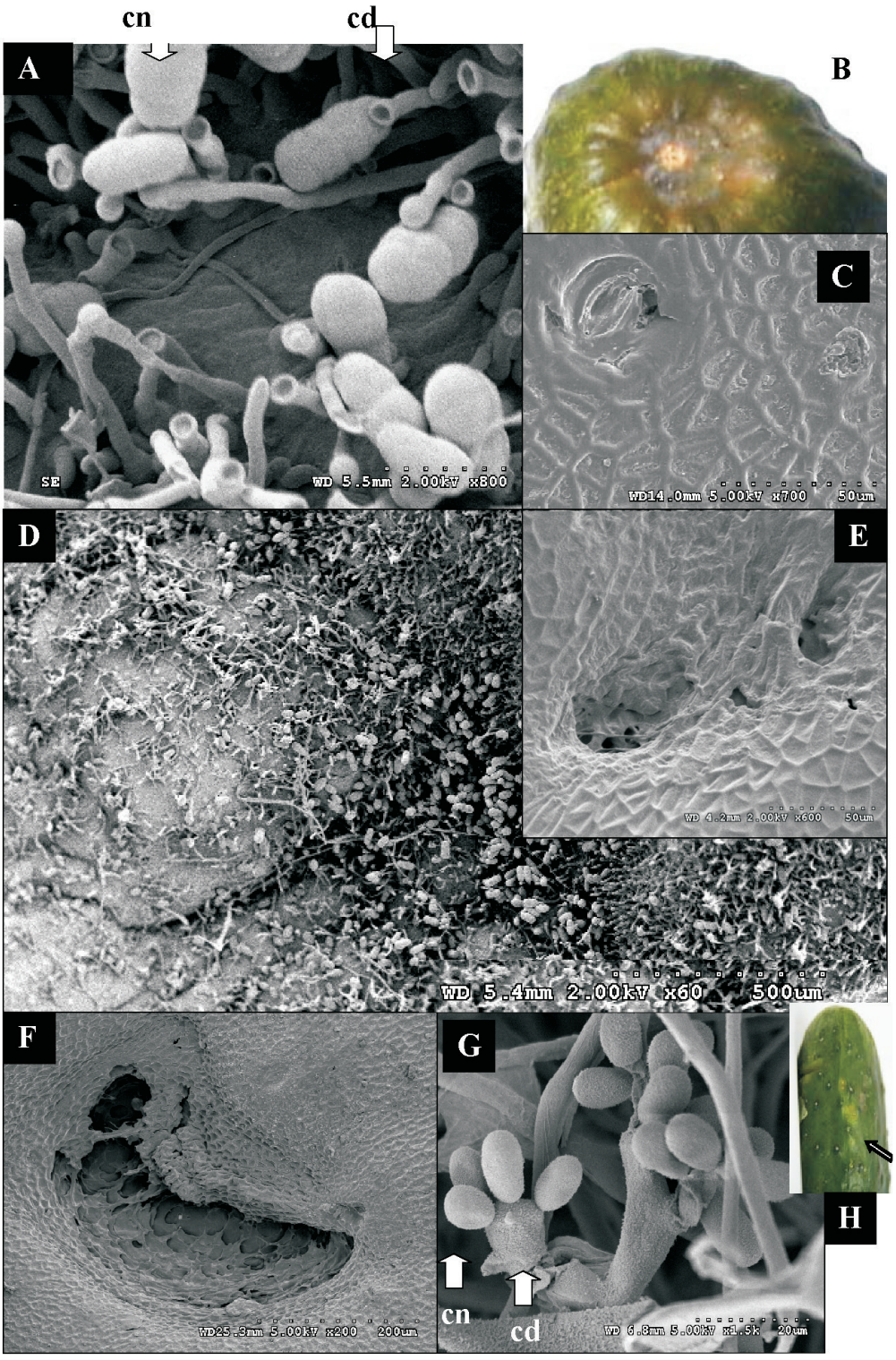

Figure 2. A: conidia (cn) and conidiophores (cd) of *Stemphylium herbarum*; B: macroscopic symptoms at the distal area of the cucumber fruit cultivar Trópico, also showing necrotic tissue; C: stomata with microcracks; D: growth of *S. herbarum*; E: sunken stomata with microcracks; F: expanded crack caused by chilling injury; G: conidia and conidiophores of *Botrytis cinerea*; H: growth of *B. cinerea* on chilling injured cucumber fruit cultivar Perichán 121.

In the cultivar Trópico, the first visible CI symptoms at 4ºC (microcracks and depressed stomata) appeared after 4–5 days at 4ºC plus the additional 4 days at 20ºC and 75% RH (winter season), or after 6–9 days at 4ºC (spring season). In the early stages of CI, when no decay was detected, crater sizes were about 30 µm diameter, while lenticels were of 20 µm diameter. The spring season resulted in slight pitting and a burst in decay (*A. alternata*, *S. herbarum*), which showed a broad range depending on many factors (0 to 45% fruit). In the cultivar Perichán 121, *B. cinerea* emerged from the hypodermal tissue and sometimes used stomata breakage, and the period of induction of CI was 4–5 days at 4ºC (Figure 2 G and H). Sinking of the spines, negligible at harvest, covered a wider skin area, also allowing fungal growth due to the presence of superficial conidia (Figure 1 A and C). These results agree with those reported by Fernández-Trujillo & Martínez (2006) and Rhee & Iwata (1982), suggesting an important contribution of microcracking to the extent of external CI symptoms and decay.

Flattened (brown) and sunken tissue as a result of CI was detectable by magnifying glass, even before necrotrophic fungi as *A. alternata* or *S. herbarum* developed (Figure 1 B, D and E and Figure 2 A and D). At this point, the decay was not evident to human eye. The limits of diameter for the visual detection of pitting in sunken epidermal areas were established by Tatsumi et al. (1987) at 70–130 µm Ø. The conidia of *A. alternata* or *S. herbarum* were concentrated in the epidermal microfractures of type II pitting (affecting both epidermal and hypodermal cells), with a density of around 250 conidia of 35 µm Ø per mm^2). Their corresponding hyphae were of 4 µm Ø deep in the parenchymatous tissue. The brown and depressed tissue developed decay caused by *A. alternata* even before the symptoms were evident. In the cultivar Perichán 121, stored for 9 days at 6ºC, fruit surface presented pitted tissue, and the first injured cells and areas of collapsed tissue were respectively 85–250 mm or 200–350 mm below the fruit surface. Chilling injury was more severe in fruits stored during 23 days, and *B. cinerea* developed during the first day of shelf life at 20ºC (Figure 2 G and H). Sometimes, unidentified coccus-bacterium of 2–3 µm Ø were also located close (or not) to some stomata, probably a typical microbial community on fruit surface (Reina et al., 2002).

Microcracking was not detected in sound cucumber fruit. However, the brushing and blotting to remove water condensed on the fruit surface probably covered some of the stomata. Usually, sound fruit had no cracked areas, although cracks and microcracking are common in stored fruit (Lurie et al., 1997). However, this effect was limited, because the fruit surfaces were similar in fresh and refrigerated fruit.

Other artefacts could have been associated to cucumber tissue leakage after cutting or blotting, resulting in transformation of ice crystals, after introduction into the cryogenic accessory, probably turning later into tissue fractures of circular and regular shapes (Echlin, 1992). These regular shapes were rarely detected in fresh fruit, but can be found in freeze dried tissue.

Acknowledgements

To the Ministry of Education and Science of Spain and Fundación Séneca de la Región de Murcia, for financial support; to A. Alcolea (SAIT-UPCT), for the cryo-SEM assistance; to M.C. García-Romero and J.M. Mercader, for technical assistance; to Plásticos del Segura, for providing macroperforated films.

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Received on October 3, 2006 and accepted on February 8, 2007