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Skin spots on 'Cripps Pink' and 'Elstar' apples are identical

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Summary

Brown spots have recently been reported on the surfaces of 'Cripps Pink' apples, grown in Marsillargues, France. Preliminary observations suggest the symptoms on 'Cripps Pink' apples resemble those reported earlier on 'Elstar' apples and then referred to as 'Elstar skin spots'. Elstar skin spots occur particularly in coastal production areas of northern Europe, and in rainy seasons. The objective of this study was to establish more definitively whether the skin spots observed on 'Cripps Pink' are identical to those reported on 'Elstar'. Hence, the morphological and anatomical characteristics of 'Cripps Pink' skin spots were assessed in more detail. The skin spots on 'Cripps Pink' develop on the non-blush side of the fruit. High resolution scanning light microscopy revealed a network of cuticular microcracks in both symptomatic and non-symptomatic regions of an affected fruit. When fruits with microcracks were dipped in a 1:1 chloroform:methanol mixture, the width and depth of the microcracks increased, indicating that the microcracks had previously been partially filled with wax. The microcracks on a symptomatic surface were wider and deeper than those on a non-symptomatic surface. After the cuticular wax had been extracted (as above), the microcracks on the symptomatic surface were found to be partially infiltrated with the fluorescent dye acridine orange; but not so the microcracks on a non-symptomatic surface. The fruit skin of symptomatic apples had a higher rate of water loss than that of non-symptomatic apples. Microscopy revealed that the epidermal and some of the hypodermal cells beneath the microcracks within a symptomatic area were brown and their cell walls lignified. We infer from these observations that the skin spots on 'Cripps Pink' are identical to the well-known skin spots on 'Elstar' apples.

Keywords: *Malus* × *domestica*, microcracks, cuticle, wax, transpiration, lignin, cell death

Introduction

Surface blemishes/disorders compromise the appearance of many fruit crop species including apples. Russeting (Faust and Shear, 1972a) and skin spots (Grimm et al., 2012) are typical examples of surface disorders of apples. Both disorders cause cosmetic damage without affecting the nutritional value of the fruit. However, since consumer preferences are generally based on external appearance, fruit with such surface disorders is typically diverted from the high-value table-fruit market, to the lower-value processing market. This is usually accompanied by severe economic loss for the grower.

Russeting is known to be triggered by environmental factors such as surface wetness during the early stages of fruit development (Faust and Shear, 1972a, b; Winkler et al., 2014; Khanal et al., 2021). In addition, the susceptibility to russeting differs markedly among apple cultivars indicating that the genotype is also important (Khanal et al., 2013; Khanal et al., 2020a). Susceptibility to skin spots is similar to russeting, in that skin spot incidence is also determined by a mix of both environmental and genetic factors. Moreover, surface wetness is also causal for skin spots, particularly if occurring late in fruit development (Winkler et al., 2014). Till now, skin spots have been reported exclusively in the apple cv. Elstar, and especially when it is grown in coastal areas of northern Europe (Grimm et al., 2012). Recently, however, 'Cripps Pink' apples have been found to develop symptoms that are strikingly similar to the 'Elstar skin spots'. The objective of this study was to establish whether the skin spots of 'Cripps Pink' are histologically and physiologically identical to those described for 'Elstar'.

Materials and methods

Plant materials

'Cripps Pink' apples (*Malus* × *domestica* Borkh.) grafted on 'M9' rootstocks were grown in a commercial orchard near Marsillargues, France (lat. 43°40'54" N, long. 4°10'44" E) according to current regulations of integrated fruit production. The trees were 10 to 20 years old. Fruit were harvested at commercial maturity and stored for periods of maximum two months in a cold room (2.5 °C and 95% RH).

Macroscopy and microscopy of symptomatic fruit

Fruit with and without typical skin spot symptoms were sampled. Macrographs were prepared using a camera (EOS 550D, lens EF-S 60 mm f/2.8 Macro USM; Canon, Tokyo, Japan) mounted on a photostand. Subsequently, fruit were observed under a digital scanning light microscope (VHX-7100; Keyence Corporation, Osaka, Japan). Images of microscopic cracks ('microcracks') of symptomatic and non-symptomatic regions of the fruit surface were taken. Thereafter, cuticular wax was extracted by dipping in a 1:1 v:v chloroform:methanol mixture and fruit surfaces re-inspected.

Cross-sections of the skin were prepared from symptomatic and non-symptomatic areas of the skin using a razor blade. Sections were inspected under a light microscope (40×, BX-60; Olympus, Hamburg, Germany) under transmitted white light. Potential impregnation of the cell wall with lignin was investigated by incubating cross-sections for 10 min in 2% (w/w) phloroglucin prepared in 95% (v/v) aqueous ethanol. Thereafter, sections were transferred to a microscope slide, a droplet of concentrated H_2SO_4 was added and viewed in transmitted white light (40×, BX-60; Olympus).

Acridine orange assay before and after wax extraction

Fruit with or without symptoms were selected. Cuticular wax was extracted on a subsample of fruit by dipping in chloroform/methanol (1:1 v/v) for 30 s. The dewaxed and the non-dewaxed fruit were incubated for 10 min in 0.1 % aqueous acridine orange (AO; Carl Roth, Karlsruhe, Germany) containing a silicone surfactant 0.025 % (w/v) (Break-Thru S 240; AlzChem, Trostberg, Germany). Thereafter, fruit were rinsed in deionized water, dried using soft tissue paper and ob-

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served under a fluorescence binocular microscope (MZ10F; Leica Microsystems, Wetzlar, Germany). Intact cuticles are impermeable to the fluorescent tracer AO and hence, dye penetration is limited to any imperfections in the cuticle. Solutions containing silicone surfactants have surface tensions sufficiently low to overcome capillary exclusion and infiltrate even very small pores such as open stomata and lenticels (KNOCHE, 1994; KHANAL et al., 2020b). Calibrated images were taken under incident bright light and under incident fluorescence light (GFP-plus filter, 480-440 nm excitation, ≥510 nm emission, Leica; Camera DP73, Olympus).

Transpiration

Transpiration from symptomatic and non-symptomatic regions of the fruit skin was quantified using excised skin segments (ES) mounted in stainless steel diffusion cells (GEYER and SCHÖNHERR, 1988; KNOCHE et al., 2000). Fruit with and without skin spots were selected and the skin segments excised using a razor blade. The cut surface of the ES was blotted dry using soft tissue paper. The ES were mounted on diffusion cells using a high-vacuum grease (Korasilon-Paste; Kurt Obermeier, Bad Berleburg, Germany; KNOCHE et al., 2000). The gap between the base and the lid of the diffusion cell was sealed using clear transparent tape (Tesa film; Beiersdorf, Norderstedt, Germany). The diffusion cell was then turned upside down and the inner chamber of the cell was filled with deionized water through a port in the base using a fine syringe. Subsequently, the port was tape-sealed. This setup restricted water loss from the diffusion cell to the surface of the ES exposed in the lid of the diffusion cell (diam. 14 mm). The diffusion cells were positioned upside down on a metal grid in a polyethylene box containing dry silica gel and equilibrated overnight. The distance between the silica and the cuticle surface was less than 2 mm. The purpose of the dry silica gel was to maintain a constant low relative humidity (RH) inside the box throughout the experimental period.

The changes in mass of diffusion cells was recorded by repeated weightings. The rate of water loss (F; g h^{-1}) was calculated as the slope of a linear regression fitted through the cumulative mass loss data vs. time. The permeance (P; m s^{-1}) was calculated according to the following equation.

$$P = \frac{F}{(\Delta C \times A)}$$

In this equation, A is the area of the transpiring surface, i.e., the surface area of the ES exposed in the diffusion cell (m^2) and ΔC the difference in water vapor concentration (g m^{-3}) between the inside of the diffusion cell and that above the dry silica gel. The water vapor concentration above dry silica is practically zero (Geyer and Schönherr, 1988), that in the diffusion cell in equilibrium with liquid water and hence, at saturation. Thus, ΔC equals the water vapor concentration at saturation (20.59 g m^{-3} at 23 °C).

Results

Skin spots on 'Cripps Pink' developed on the non-blushed surface of the fruit. The blushed surface was generally free of skin spots. Skin spots developed in all regions of the fruit skin (pedicel end, center and calyx end) (Fig. 1a-d). The spots were light- to dark-brown in color with some grey area or line in the center (Fig. 1e).

High resolution scanning light microscopy revealed a network of microcracks that surrounded groups of epidermal cells in both symptomatic and non-symptomatic regions of the fruit surface (Fig. 2a, b). There was no difference in mesh width of the microcrack network between symptomatic and non-symptomatic regions of the fruit surface. However, microcracks in symptomatic regions were wider and deeper and, hence, more gaping than those in non-symptomatic regions (Fig. 2c, d). The cells underlying widely gaping microcracks were brown, causing a 'dot matrix printer' type appearance of a skin spot on a microscopic scale. There was no browning in regions with less gaping microcracks.

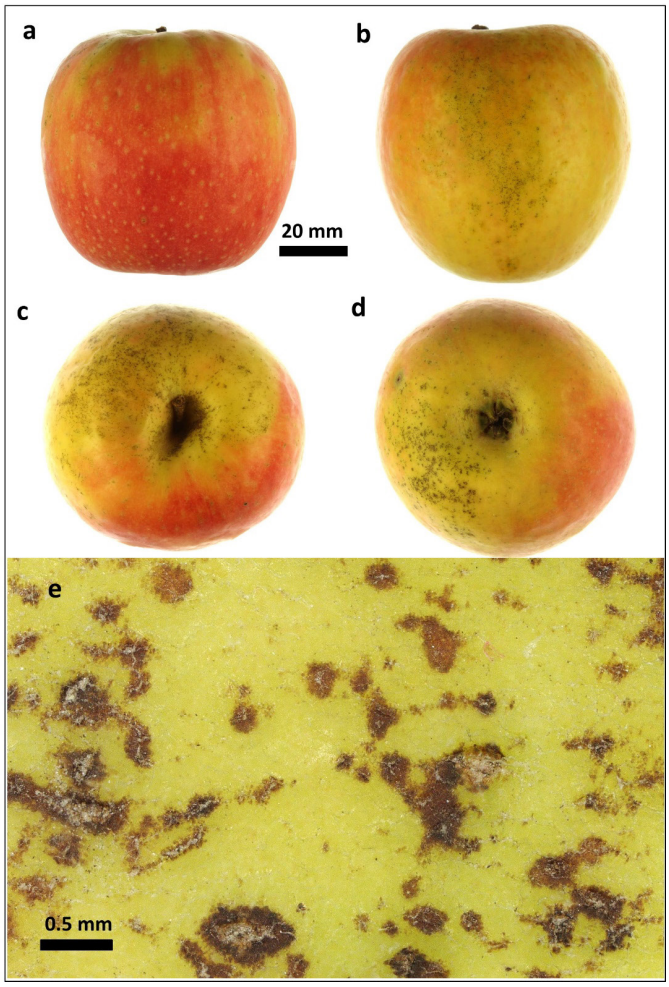

Fig. 1: Macroscopic view of mature 'Cripps Pink' apples without (a) and with (a, c, d) skin spots. Skin spots occur in the cheek (b), pedicel (c) and calyx (d) regions of the fruit, but not on the blush side. (e) Microscopic view of a surface affected by skin spots. Scale bar in (a) to (d) 20 mm, in (e) 0.5 mm.

When the fruit surface was dipped in $CHCl_3$/MeOH, microcracks deepened indicating that wax had originally filled the microcrack but had been extracted (Fig. 3a, b). Again, microcracks above skin spots were markedly deeper and more gaping than those in regions without skin spot symptoms (Fig. 3c, d).

There was essentially no AO infiltration even in the presence of silicone surfactant (at 0.025%) in either symptomatic or non-symptomatic fruit surfaces (Fig. 4a-d). In symptomatic surfaces there were markedly more microcracks and more binding of AO to the microcracked surface as indexed by red fluorescence (Fig. 4d). Only occasionally and in the presence of 0.025% silicone surfactant, did the AO infiltrate some lenticels (Fig. 4e). When wax was extracted using $CHCl_3$/MeOH, AO (with 0.025% silicone surfactant) infiltration through microcracks increased slightly. The increase in infiltration was larger in symptomatic than in non-symptomatic surfaces (Fig. 4f-i). Furthermore, in symptomatic surface, a mesh of microcracks became visible with microcracks exhibiting a yellow greenish fluorescence indicating that AO had infiltrated microcracks that had been plugged with wax prior to extraction.

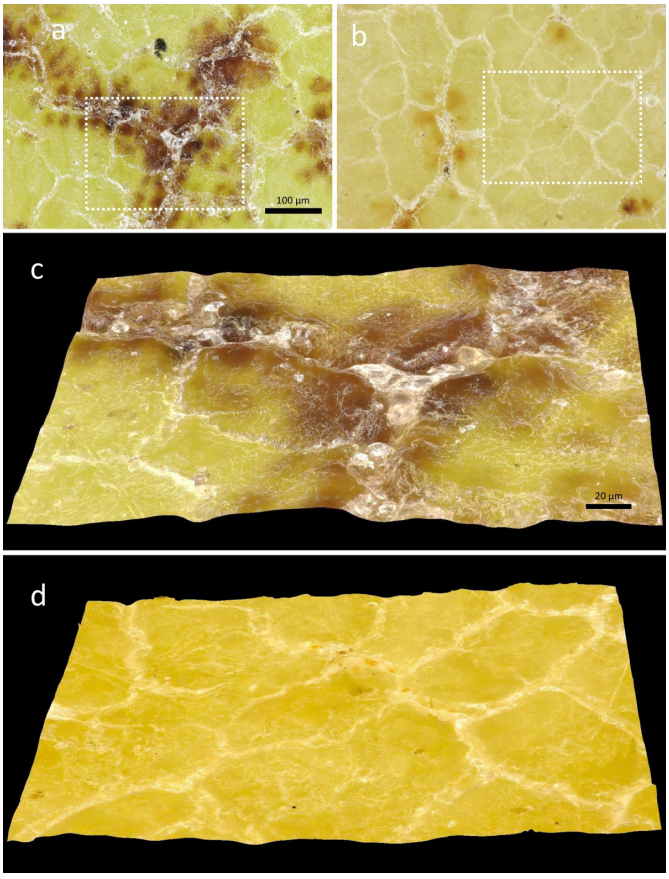

Fig. 2: Micrograph of fruit surface of mature 'Cripps Pink' apples with (a) and without skin spots (b). Magnified 3D view of the dashed region in a and b with skin spots (c) and of a corresponding region without skin spots (d). Scale bar in (a) and (b) 100 µm, in (c) and (d) 20 µm.

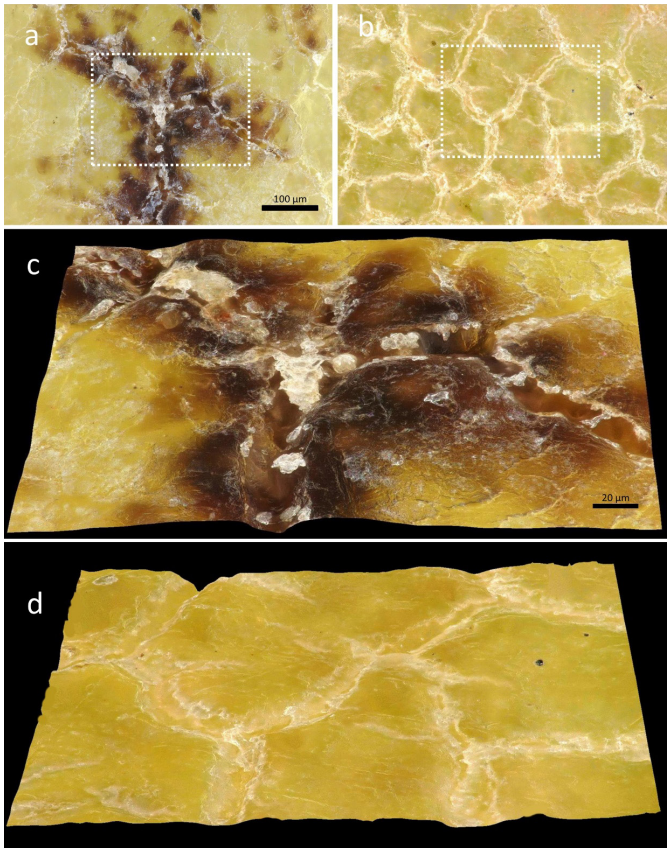

Fig. 3: Micrograph of fruit surface of mature 'Cripps Pink' apples with (a) and without skin spots (b) after extraction of epicuticular wax. Magnified 3D view of the dashed regions in a and b with skin spots (c) and of a corresponding region without skin spots (d). Scale bar in (a) and (b) 100 µm, and in (c) and (d) 20 µm.

Cross-sections through symptomatic surfaces revealed browning of protoplasts and cell walls of epidermal and occasionally also of outer hypodermal cells immediately below a skin spot (Fig. 5a). Cell walls of cells within a skin spot stained with phloroglucin/H_2SO_4 (a pink coloring of the cell walls as pointed by arrows) indicating lignin deposition in the cell walls (Fig. 5b).

Cumulative water loss (transpiration) from ESs excised from symptomatic and non-symptomatic surfaces increased linearly with time (Fig. 6). Transpiration through the symptomatic surface was 1.9-fold greater than that through a non-symptomatic surface (Fig. 6). Permeances (*P*) for water vapor diffusion of the symptomatic and non-symptomatic surface were $7.6 \pm 0.5 \times 10^{-5}$ m s^{-1} and $4.0 \pm 0.3 \times 10^{-5}$ m s^{-1}, respectively.

Discussion

From the results of our study, we conclude that skin spots on 'Cripps Pink' are identical to those described previously in 'Elstar' (Grimm et al., 2012). First, the distribution of symptoms over the fruit surface was similar. In both cases, it was the non-blushed region of the surface that was most affected. The blushed side typically is the sunny side that has a shorter wetness duration than the shaded side. Second, a microscopic view revealed that a skin spot was composed of a number of tiny brown spots - similar to the image from a dot-matrix printer. In both apple cultivars, the spots were the result of cell death, as indicated by the coagulated cytoplasm in the affected epidermal and hypodermal cells. Third, in both cultivars the tissues affected by a skin spot were sealed off by the deposition of lignin. Fourth, skin spots were always associated with microscopic cracks. There was clear evidence that a shallow microcrack did not trigger skin spot development but a gaping microcrack that traversed the cuticle was associated with a skin spot. The lack of infiltration of microcracks with acridine orange even in the presence of a silicone surfactant on mature 'Cripps Pink' apple fruit can be attributed to the deposition of wax in the microcracks. Wax deposition in microcracks has often been documented for apple fruit and may result in a healing effect that at least partially restores the cuticle's barrier properties (Roy et al., 1999; Curry, 2005, 2009; Knoche and Lang, 2017). Fifth, extended periods of surface wetness and high humidity environments are conducive to microcracking (Knoche and Grimm, 2008; Khanal et al., 2021). Microcracking triggers russeting when this occurs early in the season, but late in the season it triggers skin spot formation in 'Elstar' (Winkler et al., 2014). Wet and humid conditions are often met with in the coastal regions of northern Europe where they have also been shown to be causal for 'Elstar skin spots' (Winkler et al., 2014). They also occurred in 2022 in southern France in a 'Cripps Pink' orchard (V. Mathieu, personal communications). Sixth, symptomatic fruit skins of both 'Elstar' and 'Cripps Pink' are more permeable to water vapor than non-symptomatic fruit skins. These observations combine to suggest most strongly that the etiologies of skin spots in 'Cripps Pink' and in 'Elstar' are identical.

Based on the findings reported in this study and also in our earlier study, extended periods of surface wetness result in the formation of microcracks in the cuticles of both 'Elstar' and 'Cripps Pink' apples (Winkler et al., 2014; Khanal et al., 2021). A mismatch between fruit volume and surface area growth on the one hand, and cutin and

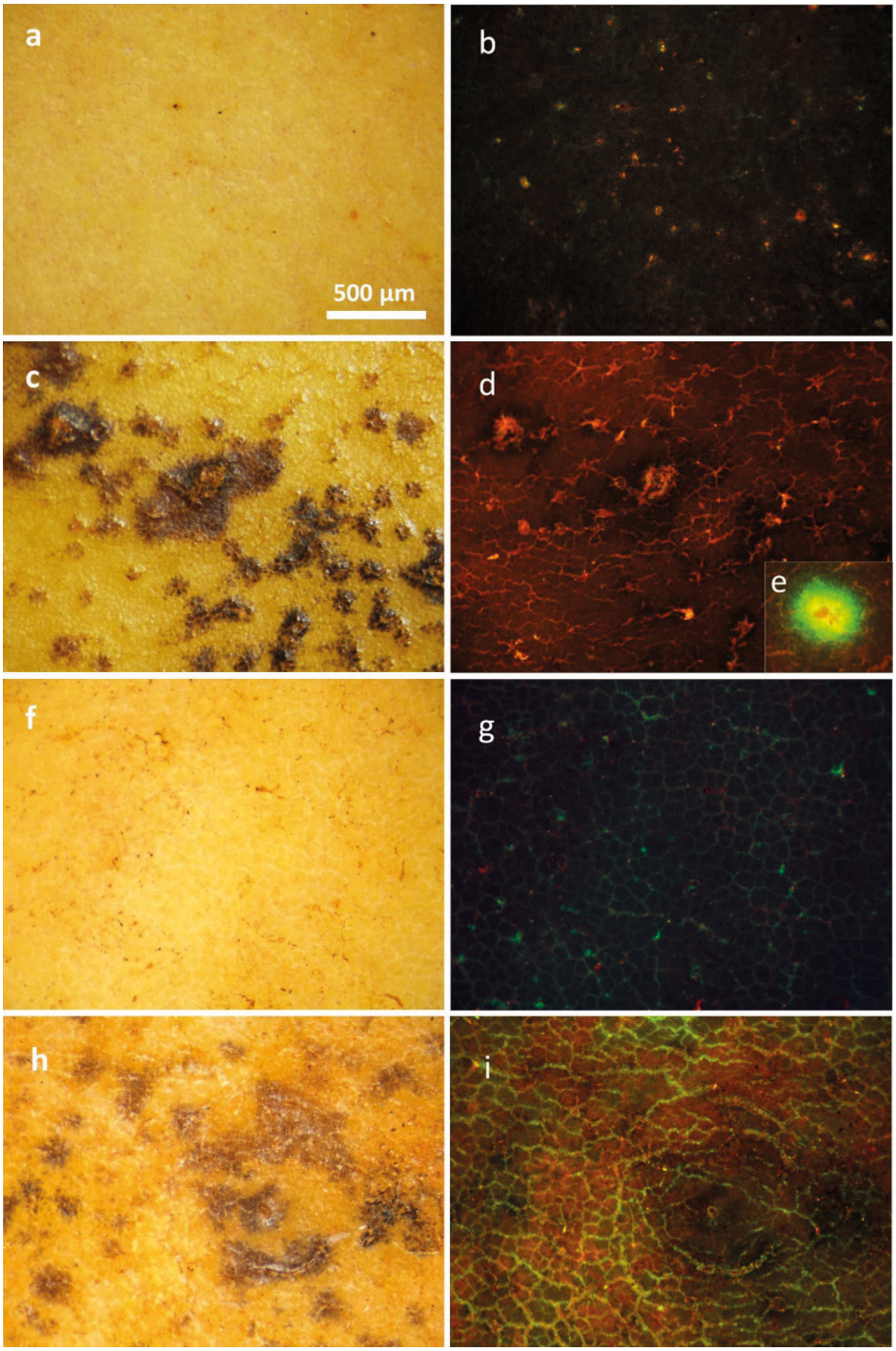

Fig. 4: Micrographs of the surface of mature 'Cripps Pink' apple without (a, b, f, g) or with (c, d, h, i) skin spots and before (a-e) and after extraction of epicuticular wax (f-i). Fruit were incubated in the fluorescent tracer acridine orange (AO) and viewed in incident bright (a, c, f, h) or fluorescent light (b, d, e, g, i). Inset (e) shows representative image of an open lenticel that was infiltrated with AO. Scale bars in (a) 500 µm and representative for all images of the composite.

wax deposition on the other, allows microcracks to deepen, and so, eventually, to traverse the cuticle (KNOCHE et al., 2018). Exposure of the underlying tissue to atmospheric oxygen and the possible induction of reactive oxygen species (BLOKHINA et al., 2003) then cause localized cell death in the immediate vicinity of the microcrack. In the dead and dying cells, the cytoplasm coagulates and turns brown, a skin spot develops as multiple cells in a region turn brown. The affected tissues are isolated from the surrounding healthy tissues by of cell wall incrustation with lignin.

From the sequence of events reported here, it is clear that measures to prevent skin spot formation in these two apple cultivars must focus on maintaining the fruit surfaces as dry as possible. We suggest that to speed fruit drying after rainfall, management measures might include row orientation (in line with the prevailing wind to maximize air movement), maintaining an open tree architecture (canopy management) and minimizing canopy rainwater capture by keeping the understorey grass short (reducing orchard RH).

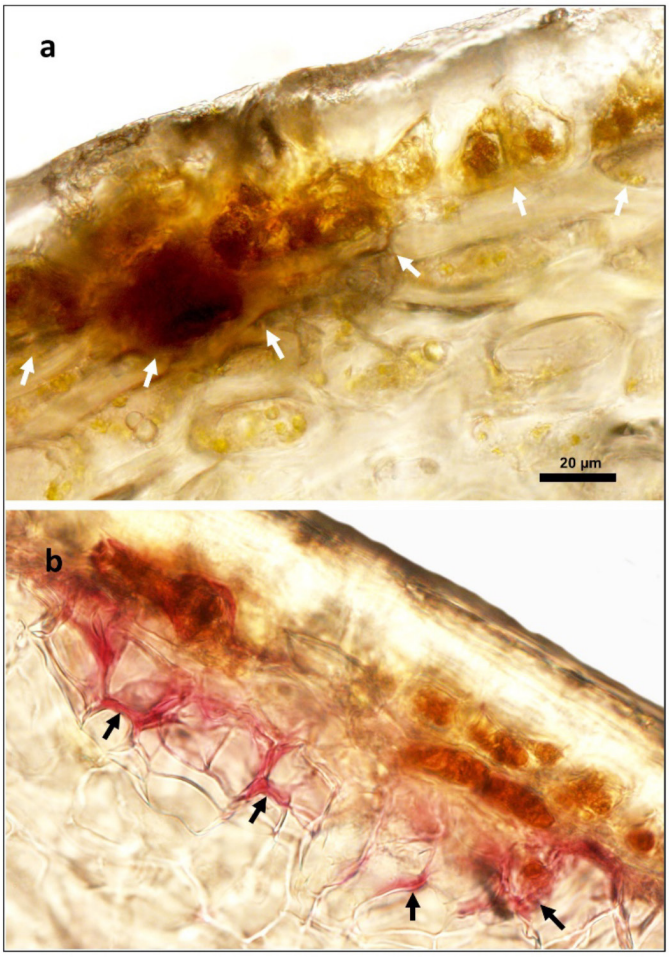

Fig. 5: Micrographs of cross-sections through the skin of a mature 'Cripps Pink' apple affected by skin spots. Sections were viewed and photographed in transmitted white light before (a) or after (b) staining for lignin with phloroglucin/H_2S0_4. Scale bar in (a) 20 µm and represents also for (b).

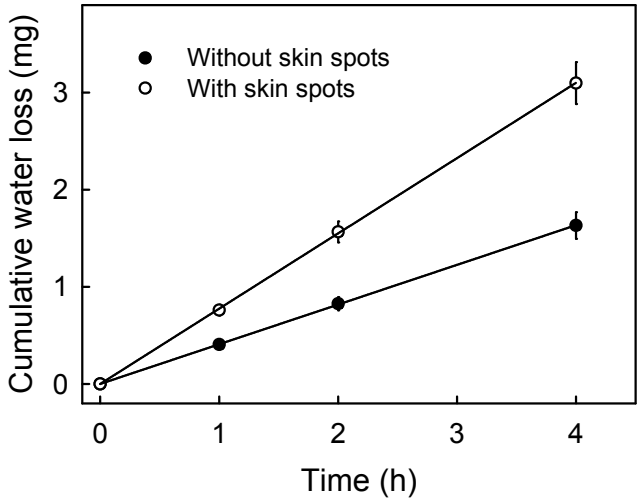

Fig. 6: Cumulative water loss (transpiration) through the fruit skin of mature 'Cripps Pink' apples with and without skin spots.

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Conflict of interest
No potential conflict of interest was reported by the authors.

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