Leibniz Universität Hannover

Faculty of Natural Sciences

Institute of Horticultural Production Systems

Fruit Science Section

GROWTH RATE AND SURFACE WETNESS ARE CRITICAL FACTORS IN MICROCRACKING, LOSS OF WATER VAPOUR AND RUSSETING OF THE APPLE FRUIT SURFACE

A MASTER OF SCIENCE THESIS

Submitted in partial fulfilment of the requirements for the award of

Master of Science (MSc.) in International Horticulture

By

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DECLARATION

I, Imoro Yahaya, do hereby declare that the work presented in this thesis is my work and that it has not been and will not be submitted for a degree in any other university.

Signature.....

Place, Date..... HANNOVER, 12. 09. 2016

DEDICATION

This work is dedicated to my parents and the entire Batesima family for their immense prayers, support and encouragements which has brought me this far.

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ABSTRACT

Russeting is an economically critical fruit surface disorder in horticultural crops like apple globally. If cuticle of primary fruit surface is failed mechanically and develops cracks, periderm is formed underneath the cracks and replaces the cuticle. This periderm becomes visible on the surface and the surface turns brown. If the cracks are repaired in time then there is no need for periderm formation. Fruit surface growth rate is the primary cause of microcracking in the fruit surface and regarded as a symptom of russeting. Surface wetness is said to increase microcracking and eventual russeting of the fruit surface. The objectives were to quantify; microcracking of the cuticle, fruit surface permeability and fruit surface russeting. Selected portion of the fruit surface was exposed to CPPU solution (20 ml l^{-1}) or liquid water for two days and 12 days respectively and sampled four times after the termination of the treatment. Fruit sampled were analysed for microcracking, permeability and russeting. Microcracking of the fruit surface was quantified using acridine orange infiltration. Permeability was quantified by water loss using excised epidermal segments (ES). Calibrated photographs of the russeted fruit were taken and russeted area quantified by image analysis using Cell P^. At 21 DAFB, AO infiltrated area of the CPPU treated and moisture treated surface of the fruit were respectively 4-fold and 8-fold more than that of the untreated surface. There was no effect of CPPU and moisture on microcracking of the fruit surface at 44, 73 and 100 DAFB. Water loss through the excised ES of CPPU treated and moisture treated surface of the fruit were higher than that of the untreated surface at 21 DAFB. Permeability of CPPU treated and moisture treated surface of the fruit were respectively 1.5-fold and 4-fold higher when compared to the untreated surface at 21 DAFB. The effect decreased when CPPU and moisture were applied at 44, 73 and 100 DAFB. The permeability of CPPU treated and the moisture treated fruit surface were more pronounce in early fruit development with increased AO infiltrated area, but the effect decreased in later stages of development and became similar to the untreated surface as AO infiltrated area decreased with time. There was 10 % and 37 % russeting when the fruit surface was treated with CPPU and moisture respectively at 21 DAFB. There was no russeting (0 %) in the untreated surface of the fruit. Surface moisture had a more severe effect than the effect of increase growth rate. However, surface moisture alone is not enough to induce cracks if fruit surface growth rate is low. Therefore, both factors are critical in microcracking, loss of water vapour and russeting of the apple fruit surface.

Keywords: apple cuticle, microcracking, permeability, russeting, wax.

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LIST OF ABBREVIATIONS

AO	Acridine orange
CM	Cuticular membrane
DAFB	Days after full bloom
ES	Epidermal segment
RH	Relative humidity
SE	Standard errors

1. INTRODUCTION

Russeting is a fruit skin defect of economic concern worldwide in many horticultural crops including apple. In russeting, periderm replaces the primary fruit skin when the cuticle is wounded. The periderm results in brownish and rough appearance of the fruit skin. Commercial apple cultivars differ considerably in their susceptibility to russeting. Some cultivars such as 'Egremont Russet' and 'Karmijn' are highly sensitive to russeting (Khanal et al., 2013a). The fruit surface of these cultivars is almost fully covered by periderm. This is considered by consumers as normal feature of the fruit. Another group of apple cultivars ('Granny Smith', 'Idared', 'Braeburn' etc.) are non-susceptible to russeting (Khanal et al., 2013b) and remain almost russet free even in adverse climate conditions. There are some cultivars which are medium russet susceptible such as 'Elstar', 'Golden Delicious', 'Pinova' etc (Khanal et al., 2013b). In these cultivars adverse climate (high humidity) increase the severity of russeting (Faust and Shear, 1972). Russeted fruit of this group of cultivars loses its fresh market value because the consumers do not buy the russeted fruit thereby posing serious economic loss for the grower.

Formation of microcracks in the cuticle is the first event in the sequence of phenological events of russeting (Faust and Shear, 1972). When the functional barrier of the fruit skin breaks, epidermis and hypodermis are exposed to the external environment. This result in stress on the cellular layer, thus the fruit has to repair or re-establish the barrier loss. Some researchers show that wax is depositing into cracks (Curry, 2009; Roy et al., 1999) thereby healing microcracks. Periderm develops underneath the cracks and repairs the injury only when wax deposition is not sufficient to heal the cracks properly. Growth stress is the primary cause of microcracking (Skene, 1982). The growth stress on the surface is high when the fruit surface growth rate is high. The growth stress on the fruit skin increases strain that results in the failure of the cuticle. This then manifest as microscopic cracks in the fruit skin and considered as symptoms of russet formation (Simons and Chu, 1978; Hatch, 1975). In commercial apple fruit production, different kinds of plant growth regulators are used. The essence of these plant growth regulators includes increasing the fruit volume or changing the fruit shape. One typical example of such fruit growth regulators is Forchlorfenuron commonly called 1-(2-chloro-4-piridinyl)-3-phenylurea; CPPU); a synthetic cytokinin of the phenylurea group (Costa, 1999). Application of CPPU on the fruit skin increases fruit growth rate and

surface expansion rate (Zhang and Whiting, 2011). As the fruit growth rate increases, it generates higher strain in the cuticular membrane (CM) thereby increasing the severity of microcracks (Iwahory et al., 1988). Furthermore, environmental factors such as fruit surface wetness or high humidity are reported to increase microcracking in the cuticle and subsequently russeting of the fruit surface (Winkler et al., 2014; Knoche and Grimm, 2008; Elfving and Allen, 1987; Faust and Shear, 1972). There is no comparative study between fruit surface growth rate and surface moisture in causing russeting of apple. Therefore, in this study we monitor the role of fruit surface growth rate and surface wetness in microcracking of the cuticle, the water vapour permeance of the cuticle and the fruit surface russeting.

2. LITERATURE REVIEW

2.1 Morphology of apple fruit surface

The outer compact covering of the fruit is generally termed as fruit skin. Fruit skin which consists of cuticle as an outermost protective layer followed by epidermis and hypodermis tissue is called primary fruit skin or surface.

2.1.1 Cuticle

The cuticle is a non-living lipoidal polymeric membrane that covers the outermost part of the fruit surface and acts as boundary between the fruit and its environment (Jeffree, 1996). Cuticular membrane (CM) function as a main barrier to water transport and pathogen invasion (Kerstiens, 1996; Post-Beittenmiller, 1996), thus an intact CM is necessary to effectively maintain the fruit surface. The cuticle consists of cutin, wax and polysaccharides.

Cutin: It forms a polymer matrix of the cuticle for the deposition of other components. The cutin is formed by oxygenated C_{16} and C_{18} fatty acid. Common monomers of C_{16} family include 9(10), 16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid. Those of C_{18} family are 18-hydroxy-9, 10-epoxyoctadecanoic acid and 9, 10, 18-trihydroxyoctadecanoic acid (Dominguez et al., 2011). These fatty acids are usually in combination with their monounsaturated homologs and are held together by ester bond (Heredia, 2003).

Wax: This is a homologous series of long chain C_{20} and C_{40} fatty acid (Dominguez et al., 2011). According to Khanal et al., (2013b), apple fruit CM has about 50 % waxes. The major component of the wax of apple fruit surface includes triterpenes (Ursolic acid), alkanes, alcohols, ketones, aldehydes, and esters (Dominguez et al., 2011; Kunst and Samuels, 2003; Belding et al., 1998). In addition to serving as a protective coating, wax also provides mechanical strength and support to the fruit. Waxes occur both epicuticularly as surface deposits on the cutin matrix and intracuticularly embedded within it (Kunst and Samuels, 2003).

Polysaccharides: Common polysaccharides found in cuticle are cellulose, hemicellulose and pectin. They are cell wall polysaccharides of the epidermal cell walls, and incorporated and distributed in the cuticle layers from inside (Dominquez et al., 2011).

They play a significant role in the mechanical strength of cuticles (Lopez et al., 2007).

2.1.2 Epidermis

Epidermis is a single-layered group of cells that lie underneath the cuticle. The epidermal cells are tightly linked to each other and largely provide mechanical strength to the fruit skin (Khanal et al., 2013b).

2.1.3 Hypodermis

The hypodermis lies beneath the epidermis and consists of several layers of collenchyma cells. Mature apple contains about 5 to 8 layers of hypodermal cells. The hypodermis also provides mechanical support to the fruit skin (Khanal et al., 2013b; Evert, 2006).

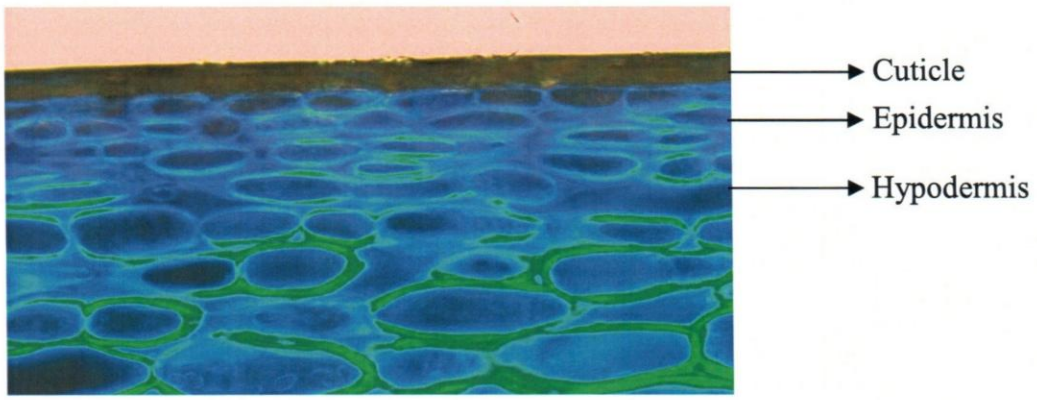

Fig. 1: A cross-section through the primary fruit surface of apple (Khanal et al., 2013b)

2.2 Secondary fruit surface

When the cuticle of primary fruit skin is failed mechanically (cracked), the periderm is developed underneath the cracked cuticle. Subsequently cuticle is replaced by periderm and so periderm is visible on the surface. The fruit skin which is covered by periderm is called secondary fruit skin. The periderm is made up of phellem, phellogen and phelloderm (Sabba and Lulai, 2002; Meyer, 1944; Verner, 1938). The phellem (cork) is located at the outermost portion of the periderm. Phellem is a series of layers generated from the phellogen. Phellem cells later become suberized and die at maturity, forming a protective layer. Phellogen originates from the hypodermis and is a single layer of meristematic cells. Phelloderm is

located beneath the phellogen. It is also derived from the phellogen. The phelloderm is the innermost layer of the periderm.

2.3 Fruit growth and cuticle deposition in apple

Apple fruit growth exhibits a sigmoidal pattern (Knoche et al., 2011; Westwood, 1993). The early (initial) phase of growth is initiated by cell division and cell expansion. But in later phase the growth rate is dominated by cell expansion. The slope of the curve that represents growth rate (surface area versus time) increases rapidly during early stage of fruit growth; reaches peak at about 80 DAFB and decrease continuously thereafter (Knoche et al., 2011). Relative growth rate is the increase in fruit surface area per unit time (day) per unit of existing area. Relative growth rate is quite high during the early developmental stage, but decrease continuously towards fruit maturity (Creasy, 1980).

The synthesis and deposition of CM in apple fruit surface is continuous throughout the growth and development (Knoche et al., 2011). Thus, CM mass per fruit and per unit surface area increases continuously throughout fruit development. However, in some horticultural crops such as sweet cherry, CM deposition occurs only during the early stage of fruit development. CM mass per fruit remain constant during later stages of fruit development. As a result CM mass per unit surface area decreases continuously in later stages of fruit growth (Knoche et al., 2004).

2.4 Strain development in the cuticle

As the fruit develops there is surface expansion. This results in CM stretching leading to strain development. Strain is the deformation of the fruit cuticle. Strain can be elastic or plastic. When this strained CM is excised from the surface, it shrinks. This shrinkage is elastic strain. This elastic strain disappears when stretching forces is removed, so called reversible. Plastic strain is irreversible. Elastic strain released in CM after isolation is much lower in apple (about 7 % at maturity; Khanal et al., 2012; Knoche et al., 2011) than in sweet cherry (95 %; Knoche et al., 2004). This should be due to the following reasons; in apple there is continuous deposition of wax and cutin. That continuous deposition fixes the strain in CM (Khanal et al., 2014). In sweet cherry, wax and cutin deposition takes place only during early stages of development (Knoche et al., 2004).

2.5 Microcracking of the cuticle

When strain of the CM exceeds tolerable limit, CM fails and this leads to cracks and these cracks are termed microscopic cracks. Growth stresses is key to formation of microcracks (Skene, 1982). Plant growth regulators have been applied in apple fruit production mainly to increase fruit size or change its shape. Commonly used plant growth regulars to increase fruit size are cytokinins like Forchlorfenuron (CPPU). Application of CPPU in the early stage of fruit growth and development enhance cell division (Costa, 1999; Famiani et al., 1997). Consequently, fruit surface expansion rate increases due to more cells per fruit; thereby increasing the severity of microcracks (Ginzberg et al., 2014; Iwahory et al., 1988). Also, environmental factors such as surface moisture or high humidity could trigger microcracks (Creasy, 1980; Faust and Shear, 1972). The early stage of fruit development is sensitive to microcracks because expansion rate is quite high.

2.6 Repair mechanism of cracks

Microcracking of the cuticle causes loss of its barrier properties. Some researchers show that wax is depositing on the face of the cracks. That is shown in some electron micrographs (Curry, 2009; Roy et al., 1999). This may be due to low diffusive resistance. This means that when the crack is formed on the surface the distance between the site of synthesis and base of crack is shorter than to the surface. If this is true, then the crack is filled by wax and the protective barrier is re-established. If not, then the fruit needs another mechanism which is periderm formation. But the mechanism of filling cracks by periderm formation is not demonstrated experimentally with sound experimental procedures. There is very limited study on the effect of CPPU in microcracking and subsequently russeting in apple. Even though, there are a number of studies on effect of surface moisture in russeting. But no detail study has been conducted in cracks quantification and water vapour permeance in apple. Also, there is no comparative study between fruit growth rate and surface wetness in causing russeting. Therefore, the research was conducted to monitor the role of fruit surface growth rate and surface wetness in microcracking of the cuticle, the water vapour permeance of the cuticle and the fruit surface russeting.

3. OBJECTIVES AND HYPOTHESES

3.1 Objectives

The major objective of the research was to study the effect of fruit surface growth rate and surface wetness on physical integrity of apple fruit surface. The focus was on

i. Quantifying microcracking of the cuticle.
ii. Quantifying fruit surface permeability.
iii. Quantifying fruit surface russeting.

3.2 Hypotheses

1. High rate of fruit surface growth and surface wetness both increase microcracking of the cuticle, increase water vapour permeability of the fruit surface and results in russeting of the fruit surface.
2. The effect of growth rate and surface moisture is more severe during early stage of the fruit growth.

4. MATERIAL AND METHODS

4.1 Plant material

Fruit of apple (*Malus* x *domestica* Borkh. Cultivar 'Pinova') were obtained from the experimental orchards of Leibniz Universität Hannover at Ruthe (52^0 14/ N, 9^0 49/ E). Fruit were grown in compliance with the European Union regulation for integrated fruit production. Selected portion of the fruit surface were treated with Forchlorfenuron (CPPU) or moisture (see detail below). CPPU or moisture treated fruit were harvested at different stages of growth and transferred to the laboratory. Fruit were processed immediately on the same day of sampling or stored in conventional cold storage (2 °C; 90 – 94 % humidity) for not more than 24 h.

4.2 Monitoring fruit growth

Fruit growth was monitored from full bloom to maturity. Fruit were sampled at two weeks interval from full bloom to maturity. The mass of the fruit was quantified gravimetrically using a digital balance (Sartorius LA820) after the flower remnants were removed. Fruit surface area was calculated from the fruit mass assuming the shape of the fruit as sphere and the density of the fruit equal to 1 g cm^{-3}. This procedure is reliable because the fruit surface area calculated from the measured fruit height and diameter and calculated from the fruit mass resulted in linear relationship with slope equals one and R-square equals one (Khanal, unpublished data).

4.3 CPPU treatment on the fruit surface

Four different stages (21, 44, 73 and 100 days after full bloom; DAFB) of fruit growth were selected. Application of CPPU solution (20 mg l^{-1}) was done by selecting the fruit inflorescence and trimmed to only one fruit and labelled. A small piece of cotton pad was then attached on the cheek region of the fruit by wrapping with a parafilm and the pad soaked with CPPU using a fine syringe. The CPPU was left to dry for two days and the treatment terminated. Fruit were then sampled on the day the treatment was terminated and three subsequent sampling were made. At 21 and 44 DAFB, fruit were sampled at respectively 0, 14, 42, 103 and 0, 14, 36, 89 days after the termination of the CPPU treatment. At 73 and 100 DAFB, fruit were sampled at respectively 0, 11, 46, 70 and 0, 15, 40, 47 days after the termination of the CPPU treatment.

4.4 Moisture treatment on the fruit surface

Four different stages (21, 44, 73 and 100 DAFB) of fruit growth were selected. Moisture treatment was done by selecting the fruit inflorescence and trimmed leaving only one fruit per inflorescence and labelled. Tips of eppendorf tubes (8 mm in diameter) were then fixed on the cheek region of the fruit surface using fast curing silicone glue. After curing of the glue, tubes were filled with water by injecting using a fine syringe via the minute hole prepared on the tip of the tube and sealed with the silicone glue. As the fruit grows its surface expands and this can cause the loosening of the eppendorf tube thereby cause the water to pour out. Therefore, the tubes were regularly monitored to make corrections where necessary, and after 12 days the treatment was terminated. Fruit were sampled on the day the treatment was terminated and three subsequent sampling were made. At 21 and 44 DAFB, fruit were sampled at respectively 0, 14, 42, 103 and 0, 14, 36, 89 days after the termination of the moisture treatment. At 73 and 100 DAFB, fruit were sampled at respectively 0, 11, 46, 70 and 0, 15, 40, 47 days after the termination of the moisture treatment.

4.5 Monitoring microcracking of the cuticle

Microcracks on the surface of CPPU treated and moisture treated and non-treated fruit were monitored by acridine orange infiltration assay. Whole fruit were incubated for 10 minutes in 0.1 % acridine orange solution in 50 mM citric acid buffer solution. Fruit were then rinsed in deionized water and cautiously blotted using soft tissue paper. The fruit were then transferred to the florescence dissecting microscope (Model MZ 10F; Leica Microsysteme GmbH, Germany; camera DP73, Olympus, Hamburg, Germany; Software Cell Sens, Bensheim, Germany) and a portion (2 mm^2) of the CPPU and moisture treated and non-treated surface of the fruit were observed in transmitted and incident blue light (330 to 385 nm excitation wavelength). Three representative images of randomly selected area of the CPPU treated and moisture treated and non-treated surface of the fruit were prepared using software cell sens. The non-treated surface served as control. The images were then calibrated and the acridine infiltrated area quantified using Cell P^. The acridine infiltrated area was then expressed as a percentage of the area observed under the microscope. The number of replication was 10.

4.6 Quantifying permeance

Transpiration from excised epidermal segment (ES) of the CPPU treated and moisture treated and non-treated surface were observed using diffusion cells. Fruit were sampled four times

after the termination of the CPPU and moisture treatment in the same way as stated above. The fruit mass were determined gravimetrically using a digital balance (Sartorius LA820). The ES from the CPPU treated and moisture treated and non-treated surface were excised using a sharp razor blade and mounted in greased stainless steel diffusion cells such that the outer part of the fruit skin were exposed in the 7 mm diameter orifice (Geyer and Schönherr, 1988). The diffusion cells were then filled with deionized water through a minute port in the base and sealed using clear tape, placed upside down in a closed box containing dry silica gel at low humidity (0 %) and stabilised for 1 h (Grimm et al., 2012). Water loss was quantified by repeatedly weighing the diffusion cells using a digital balance (Sartorius CPA225D) at regular intervals (0, 2, 4, 6 and 8 h) during the incubation. The amount of water loss with time (transpiration) was computed as the decrease in the weight of the diffusion cell with time. The rate of water loss was calculated from the slope of a linear regression line by plotting cumulative water loss against time. Permeance (P, m s^{-1}) of the ES to water vapour was then calculated using the formula $P = F/(A \cdot \Delta C)$. Where F (kg s^{-1}) is the rate of water loss; A (m^2) is defined as the area of the orifice of the diffusion cells or the transpiring surface; ΔC (kg m^{-3}) = $C_i - C_o$ is the water vapour concentration between the inside of the diffusion cell (C_i) and the atmosphere in the box (C_o). According to Geyer and Schönherr (1988), C_o above the silica gel in the box is nearly zero. Therefore, the saturation concentration of water vapour (100 % RH) at the respective temperature of 24 °C (from climate data loggers) was 21.8 g m^{-3} (Nobel, 1999). The number of experimental replication was 20.

4.7 Quantifying russeted area of the fruit surface

CPPU treated and moisture treated fruit were allowed to grow on the trees till maturity. Fruit that were treated at 21 days after full bloom were sampled for russeting analysis at 138 and 159 DAFB. A total of 30 to 40 fruit in each stage of the treatment were left on the tree and sampled. Fruit that were treated at 44, 73 and 100 days after full bloom were sampled and calibrated photographs of the treated and non-treated surface prepared for russeting analysis at 159 DAFB. The russeted area was quantified by image analysis using Cell P^. Photographs of the russeted area of the fruit that were taken and calibrated were quantified using a closed polygon tool. The percentage of russeted area of the fruit was then calculated by dividing the russeted area by the treated fruit surface area at the time of sampling and expressed as a percentage.

4.8 Data analysis and presentation

Data were subjected to analysis of variance and presented in figures using SigmaPlot (version 12.5) and tables as means ± standard errors of the means. Where not indicated, the error bars are smaller than the symbol.

5. RESULTS

Fruit growth of the 'Pinova' apple increased continuously in a sigmoid pattern throughout the course of fruit development (Fig. 2). Fruit surface area growth rate rapidly increased during the early stage of fruit growth and development, attained a peak at about 90 DAFB, and then decreased continuously in the later stage of development towards maturity (Fig. 2 inset; upper left corner). The relative growth rate of the fruit surface area was highest (about 0.04 d^{-1}) during the early stage of fruit development from 0 to 30 DAFB, thereafter, it decreased rapidly and continuously and became lowest in the later stage of fruit development until maturity (Fig. 2 inset; lower right corner).

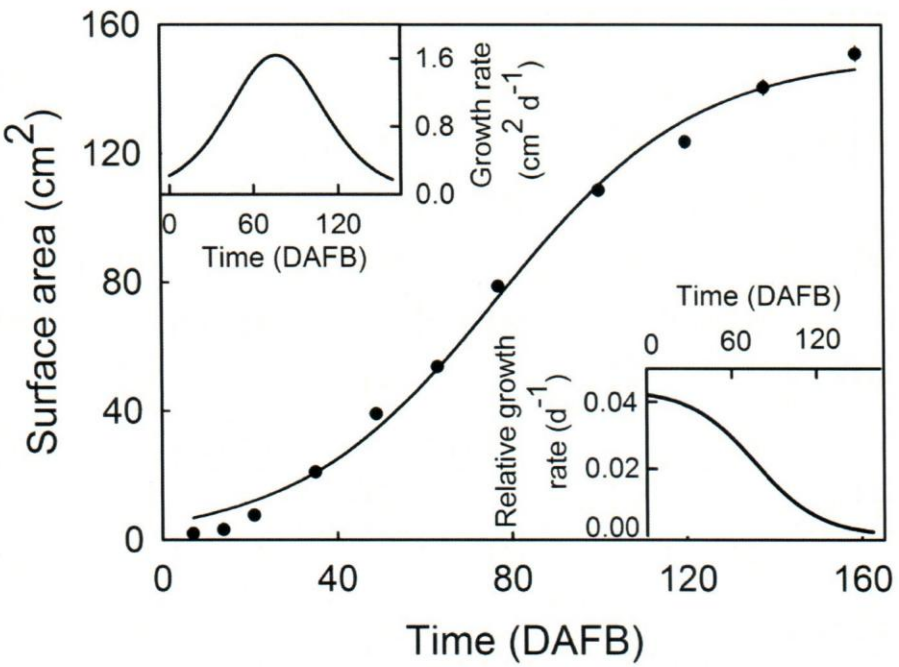

Fig. 2: The increase in fruit surface area with time in days after full bloom (DAFB) of 'Pinova' apple (main graph). The growth rate (inset upper left corner) and relative growth rate (inset lower right corner) of the surface area as affected by the time of developing fruit. Data in figure were presented as means ± SE of the means. Where error bars are not visible, they are smaller than the data symbol.

Application of Forchlorfenuron (1-(2-chloro-4-pyridinyl)-3-phenylurea; CPPU) increased acridine orange (AO) infiltrated area of the treated fruit surface only when applied during early developmental stage (21 DAFB; Fig. 3A). At 21 DAFB, AO infiltrated area of CPPU treated surface was four times higher than that of the untreated surface. AO infiltrated area of the CPPU treated surface of the fruit was not different from the AO infiltrated area of the untreated surface when CPPU was applied at 44, 73 and 100 DAFB (Fig. 3A). Application of

moisture increased AO infiltrated area of the treated surface of the fruit only when applied during early developmental stage (21 DAFB; Fig. 3B). At 21 DAFB, AO infiltrated area of the moisture treated surface was 8-fold higher than that of the untreated surface. AO infiltrated area of the moisture treated surface of the fruit was similar to the AO infiltrated area of the untreated surface of the fruit when moisture was applied at 44, 73 and 100 DAFB (Fig. 3B).

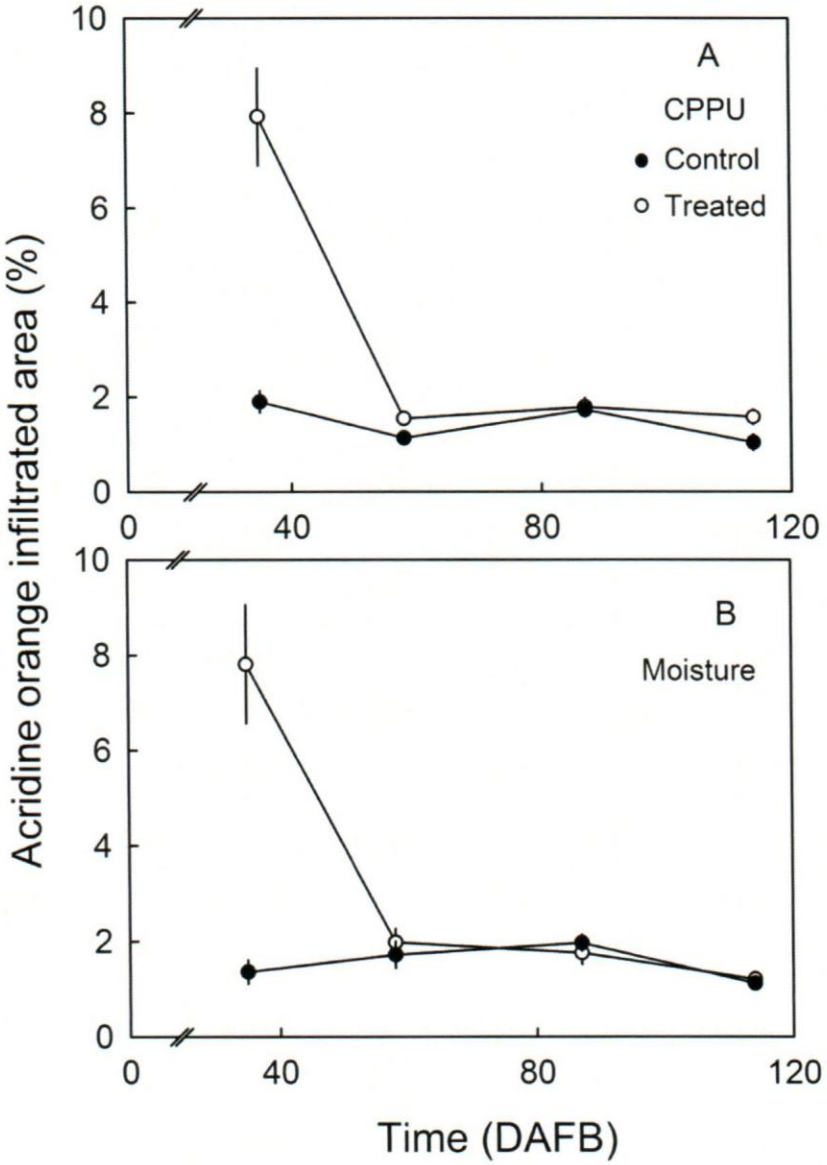

Fig. 3: Effect of CPPU (A) and surface moisture (B) on the acridine orange infiltrated area of the fruit surface as affected by the developmental stages of the fruit. Selected portion of the surface of the growing fruit was exposed to CPPU solution (20 mg l^{-1}) or liquid water (treated) at different stages of the fruit development and harvested and the acridine orange infiltrated area of the treated and non-treated surface was quantified. Values represent mean ± SE of the means (n = 10).

AO infiltrated area of the CPPU treated surface of the fruit decreased continuously with time after removing the CPPU treatment at 21 DAFB; where the effect of CPPU on microcracking was highest (Fig. 4A). When CPPU was applied at 44, 73 and 100 DAFB, there was no effect of AO infiltrated area in microcracking between the treated surface and the untreated surface of the fruit, but decreasing slightly with development time (Fig. 4B, C and D).

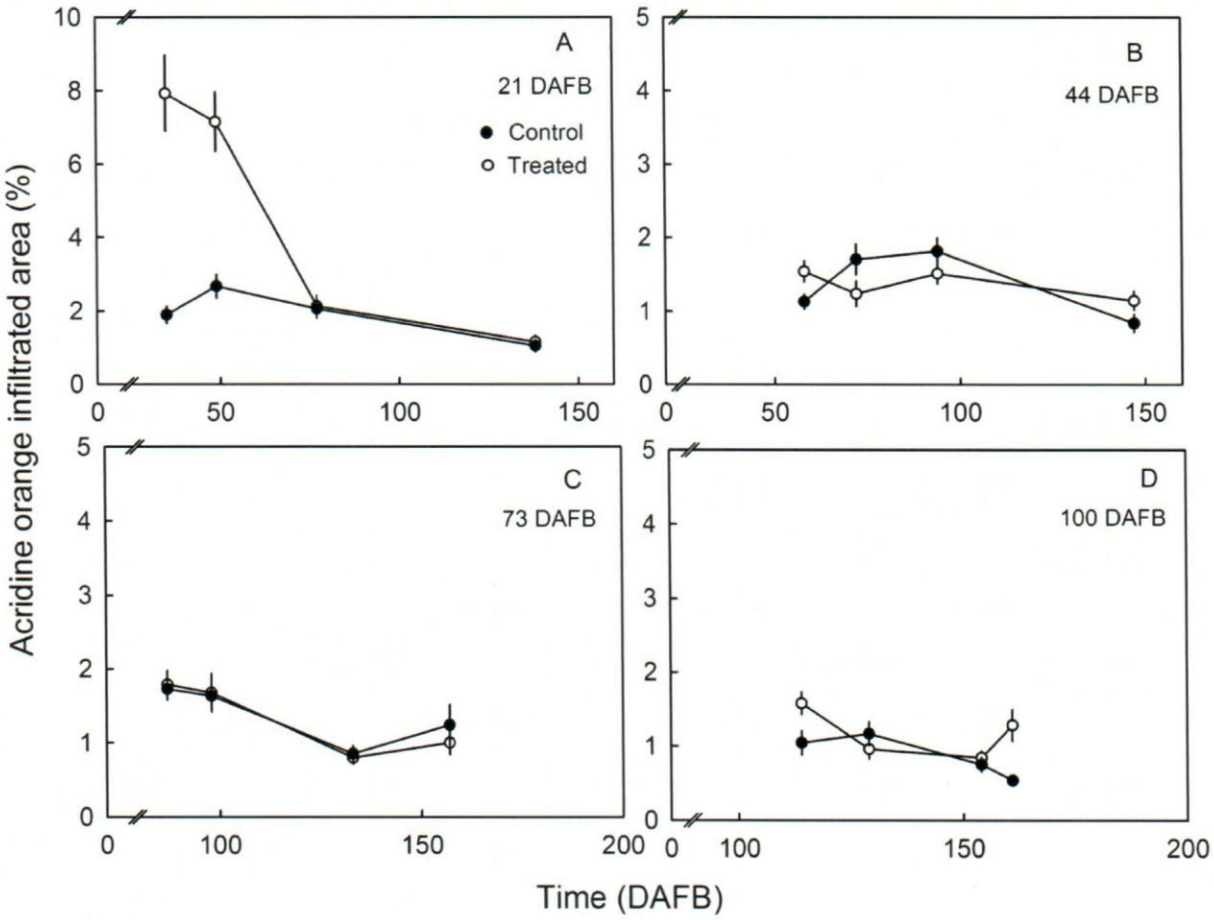

Fig. 4: Change in the acridine orange infiltrated area of the CPPU treated surface of the growing fruit with time after the termination of the CPPU application. CPPU was applied on the selected portion of the fruit surface at 21 DAFB (A), 44 DAFB (B), 73 DAFB (C) and 100 DAFB (D) for two days. Fruit were sampled at various stages of the fruit development where the first sample was taken on the day of treatment termination and the acridine orange infiltrated area of the fruit surface was quantified. Values represent mean ± SE (n = 10).

AO infiltrated area of the moisture treated surface of the fruit at 21 DAFB was highest from 35 to 50 DAFB, and then decreased continuously with time after removing the moisture treatment (Fig. 5A). The AO infiltrated area of the treated and untreated surface of the fruit

remained similar, as there was no effect of moisture at 44, 73 and 100 DAFB (Fig. 5B, C and D).

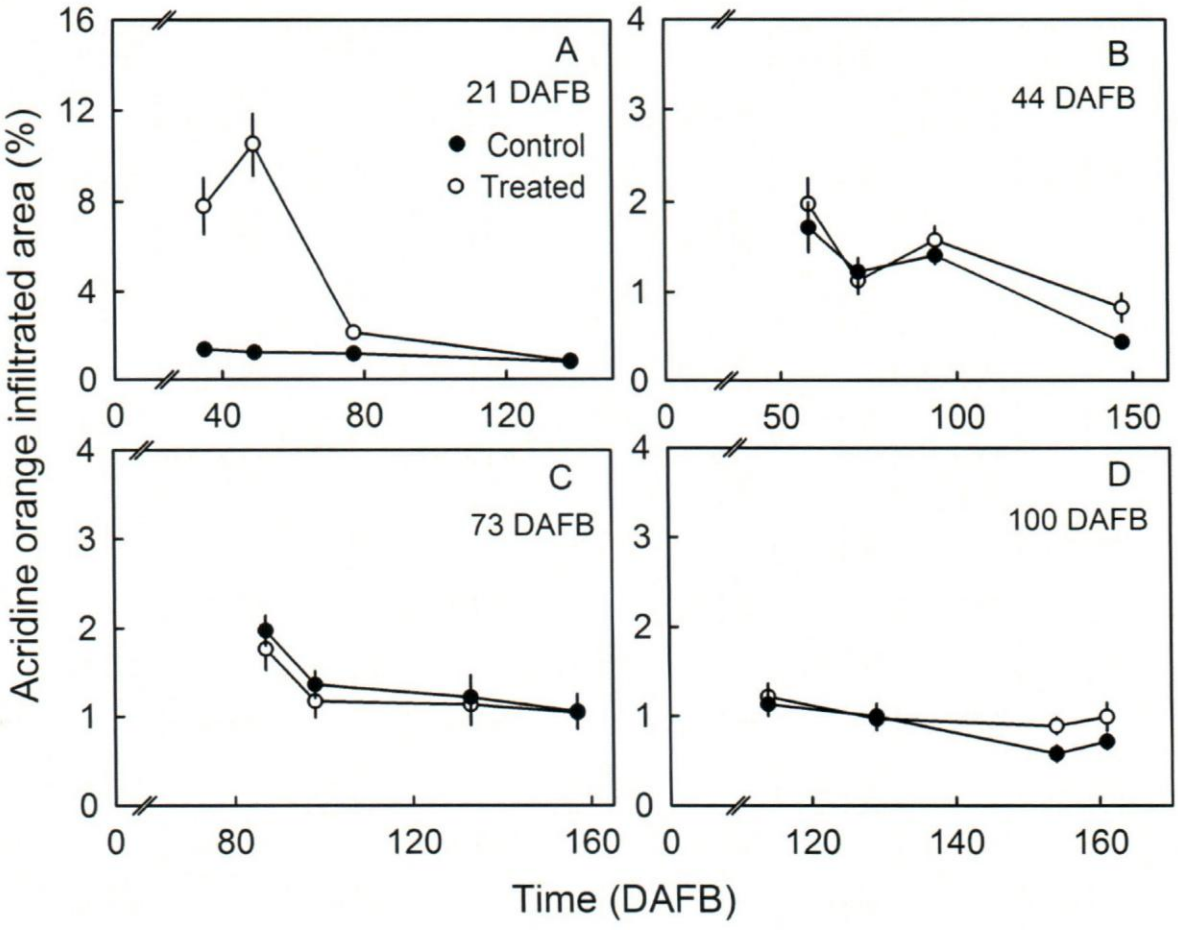

Fig. 5: Change in the acridine orange infiltrated area of the moisture treated surface of the growing fruit with time after the termination of the moisture application. Moisture was applied on the selected portion of the fruit surface at 21 DAFB (A), 44 DAFB (B), 73 DAFB (C) and 100 DAFB (D) for 12 days. Fruit were sampled at various stages of the fruit development where the first sample was taken on the day of treatment termination and the acridine orange infiltrated area of the fruit surface was quantified. Values represent mean ± SE of the means (n = 10).

Water loss from the excised ES of the CPPU treated and control surface of the fruit increased linearly with time in all the sampling (Fig. 6A, B, C and D). The treated surface had higher amount of water loss than that of the control surface in early developmental stage (21 DAFB; Fig. 6A). The effect was similar in the treated and control surface of the fruit at 44, 73 and 100 DAFB (Fig. 6B, C and D).

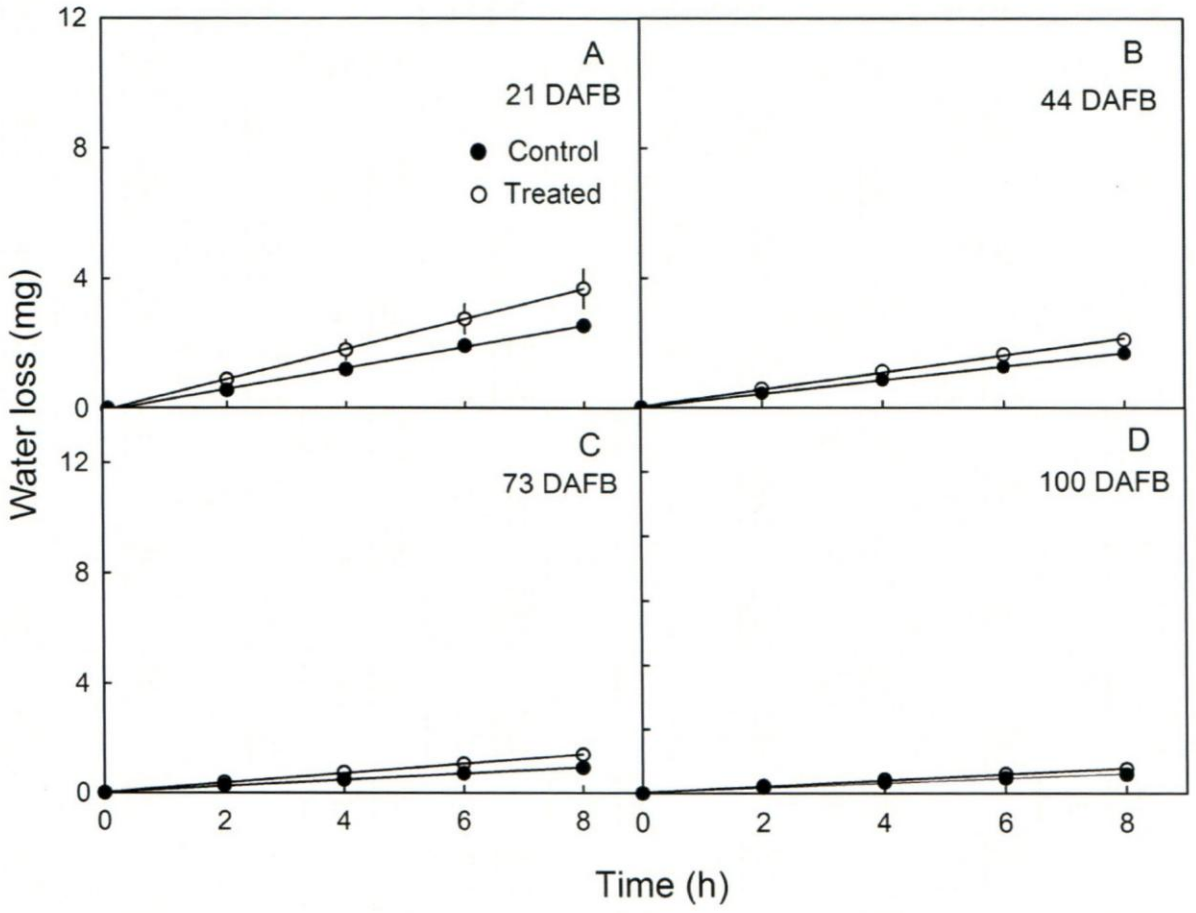

Fig. 6: Time course of water loss through excised epidermal segments (ES) of CPPU treated and non-treated 'Pinova' apple fruit surface. Selected portion of the fruit at various developmental stages (21 days after full bloom (DAFB; A), 44 DAFB (B), 73 DAFB (C) and 100 DAFB (D)) were treated for two days and fruit were sampled and water loss from the treated and non-treated surface was quantified using excised ES (see detail in material and methods). Values represent mean ± SE of the means (n = 20).

Water loss from the excised ES of the moisture treated and control surface of the fruit increased linearly with time in all the sampling (Fig. 7A, B, C and D). The amount of water loss was significantly higher in the treated surface than in the control surface during early developmental stage (21 DAFB; Fig. 7A). The effect decreased and became similar to the control during later developmental stage (44, 73 and 100 DAFB; Fig. 7B, C and D).

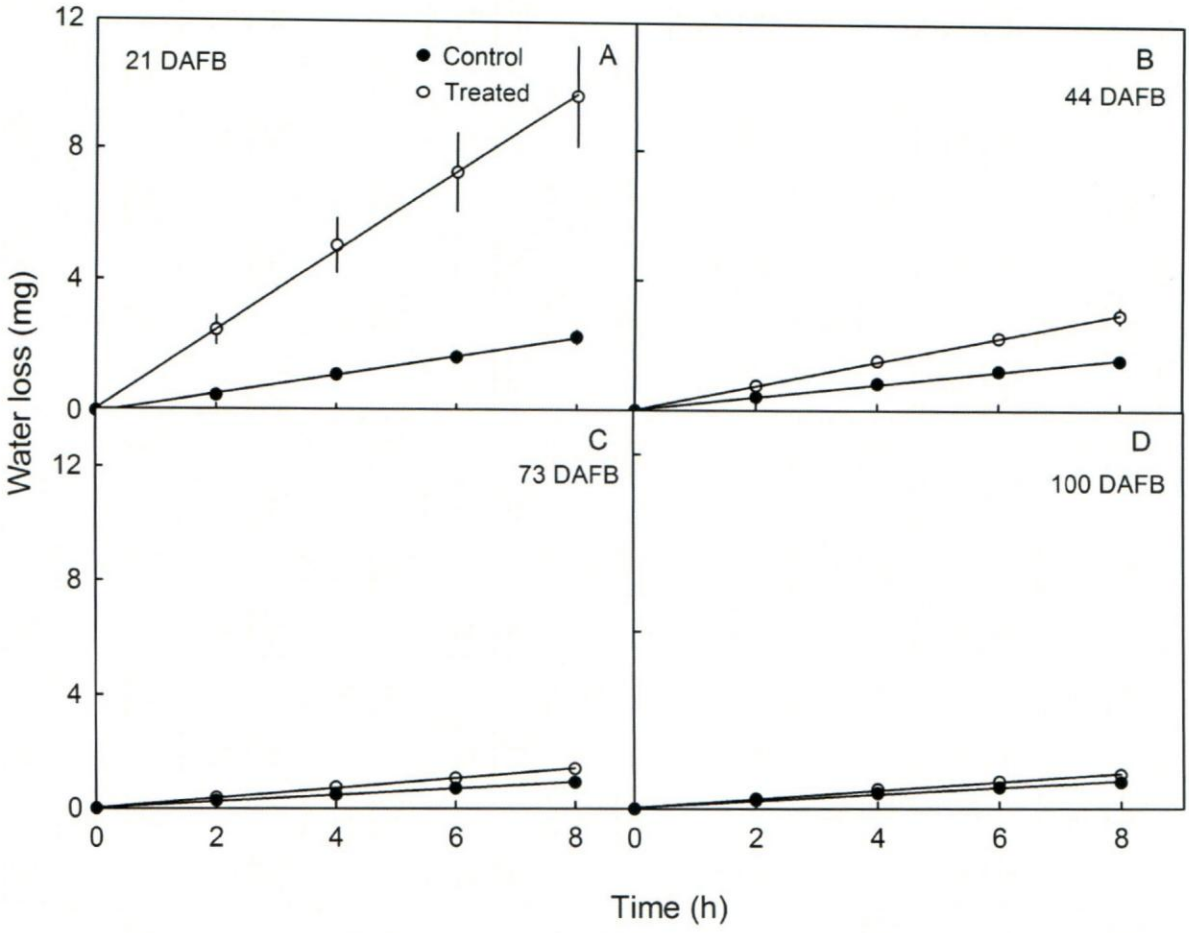

Fig. 7: Time course of water loss through excised epidermal segments (ES) of moisture treated and non-treated 'Pinova' apple fruit surface. Selected portion of the fruit at various developmental stages (21 days after full bloom (DAFB; A), 44 DAFB (B), 73 DAFB (C) and 100 DAFB (D)) were moistened for 12 days and fruit were sampled and water loss from the treated and non-treated surface was quantified using excised ES (see detail in material and methods). Values represent mean ± SE of the means (n = 20).

Application of CPPU increased permeability of the treated fruit surface only during early developmental stage (21 DAFB; Fig. 8A). The permeability at 21 DAFB of the CPPU treated surface was about 1.5 times higher than that of the control surface. The permeability of the CPPU treated surface of the fruit decreased and became similar to the permeability of the control surface when CPPU was applied at 44, 73 and 100 DAFB (Fig. 8A). The application of moisture increased permeability of the treated surface of the fruit when applied during early developmental stage (21 DAFB; Fig. 8B). At 21 DAFB, the permeability of the moisture treated surface was four times higher than that of the control surface. Permeability of the moisture treated surface of the fruit decreased and was similar to the permeability of the control surface of the fruit at 44, 73 and 100 DAFB (Fig. 8B).

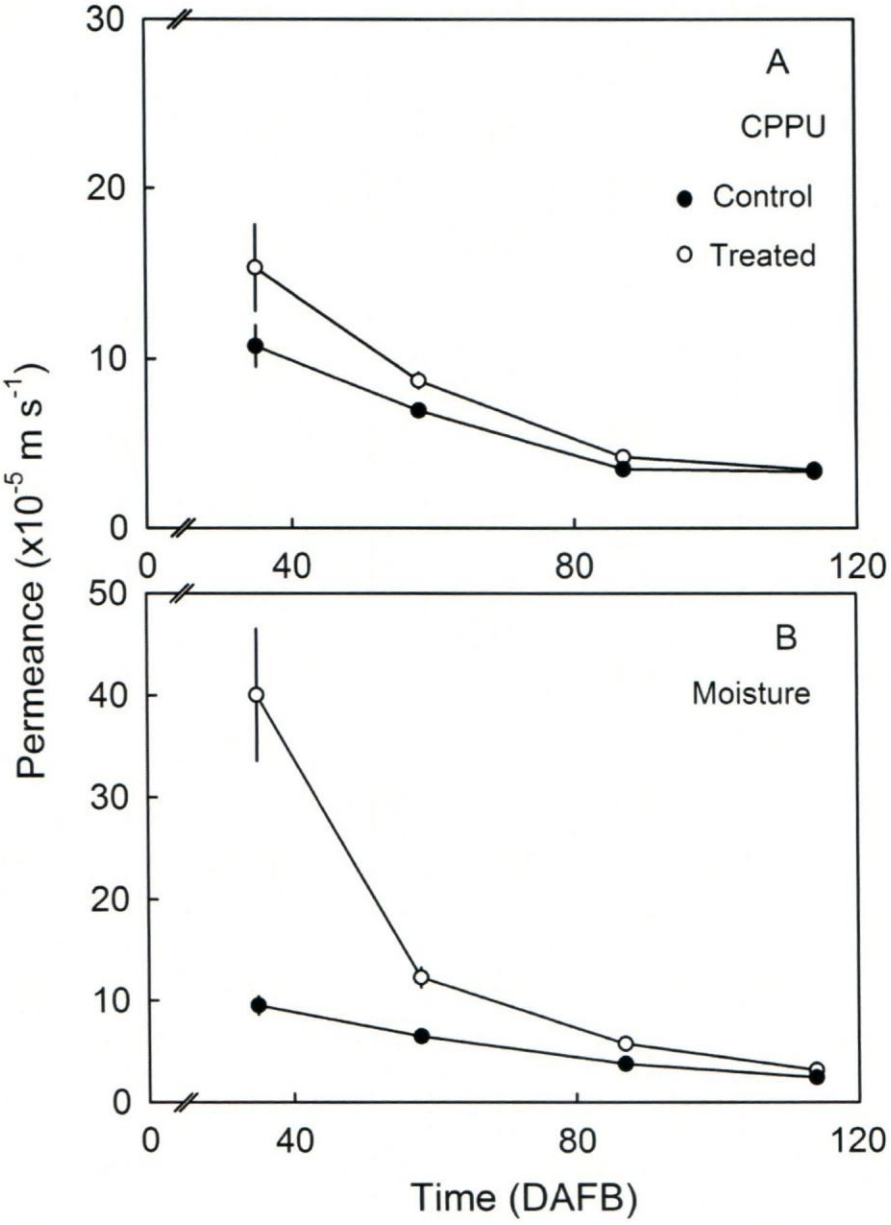

Fig. 8: Effect of CPPU (A) and surface moisture (B) on the permeance of the fruit surface as affected by the developmental stages of the fruit. Selected portion of the surface of the growing fruit was exposed to CPPU solution (20 mg l^{-1}) or liquid water (treated) at different stages of the fruit development and harvested and the permeance of the treated and non-treated surface was quantified. Values represent mean ± SE of the means (n = 20).

Permeability of the CPPU treated surface of the fruit at 21 DAFB was highest from 35 to 50 DAFB, and then decreased continuously with time after the treatment was removed (Fig. 9A). The permeability of the treated and control surface of the fruit remained similar when CPPU was applied at 73 and 100 DAFB (Fig. 9C and D), but not at 44 DAFB (Fig. 9B).

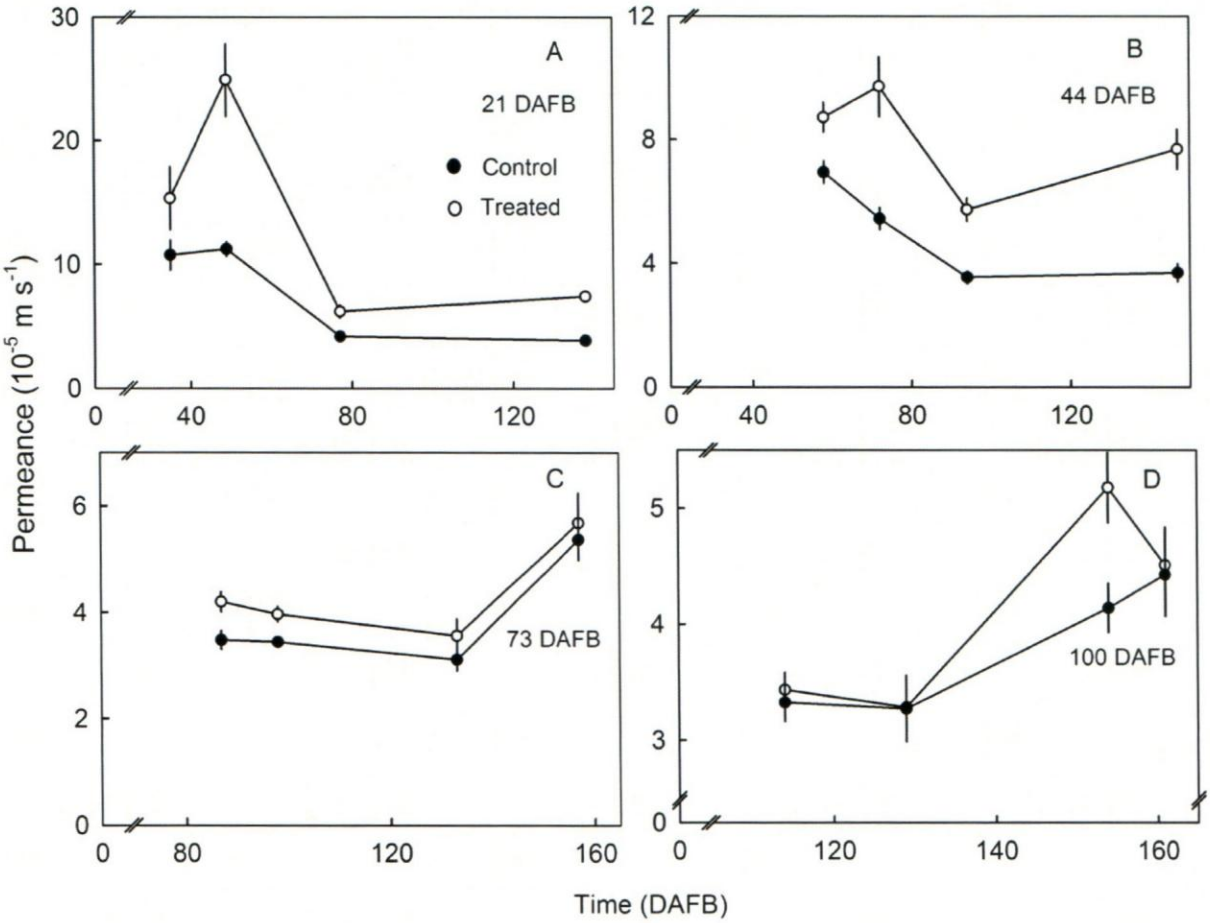

Fig. 9: Change in the permeance of the CPPU treated surface of the growing fruit with time after the termination of the CPPU application. CPPU was applied on the selected portion of the fruit surface at 21 DAFB (A), 44 DAFB (B), 73 DAFB (C) and 100 DAFB (D) for two days. Fruit were sampled at various stages of the fruit development where the first sample was taken on the day of treatment termination and the permeability of the fruit surface was quantified. Values represent mean ± SE of the means (n = 20).

The permeability of the moisture treated surface of the fruit at 21 DAFB was tremendously higher from 35 to 50 DAFB, and then decreased continuously with time after the removal of the treatment (Fig. 10A). The permeability of the treated and control surface of the fruit remained the same at 44, 73 and 100 DAFB (Fig. 10B, C and D).

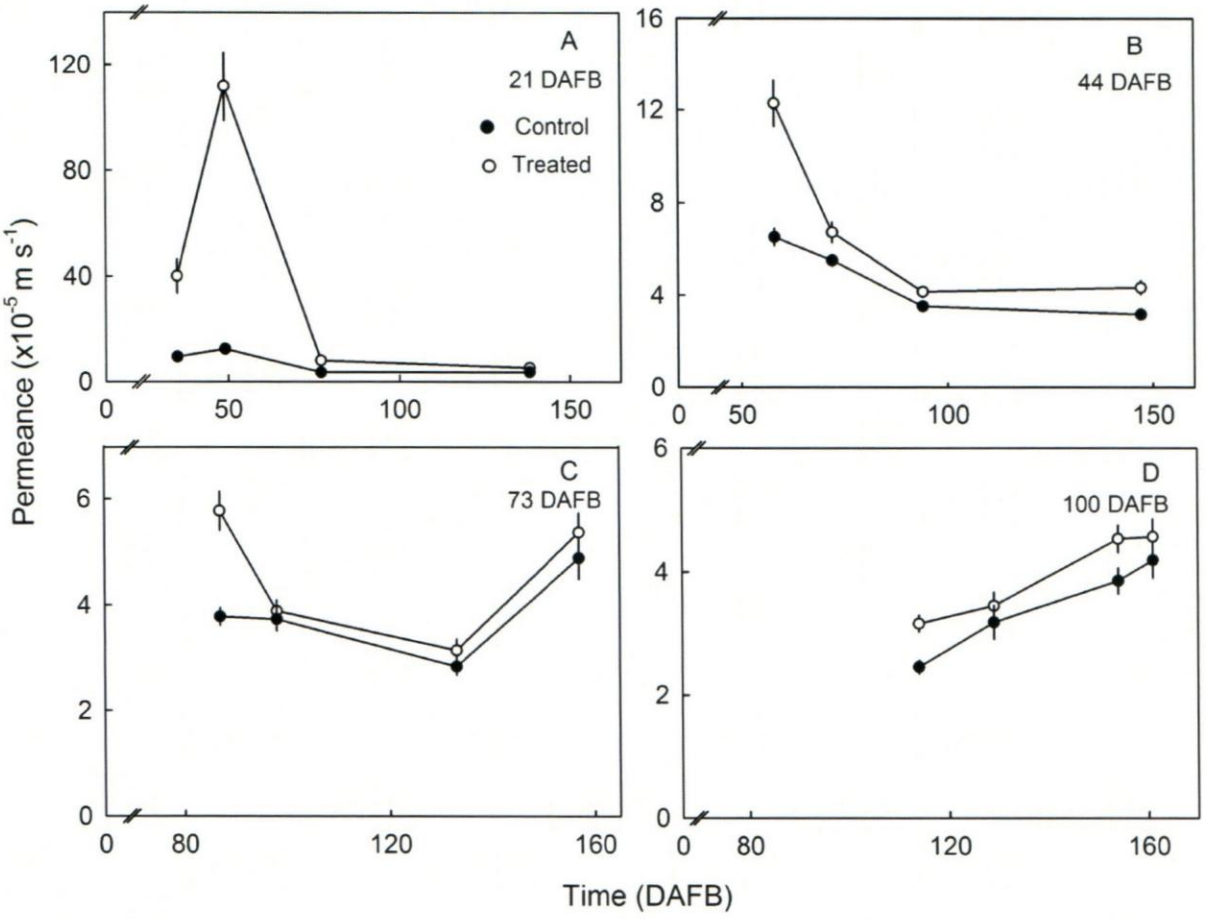

Fig. 10: Change in the permeance of the moisture treated surface of the growing fruit with time after the termination of the moisture application. Moisture was applied on the selected portion of the fruit surface at 21 DAFB (A), 44 DAFB (B), 73 DAFB (C) and 100 DAFB (D) for 12 days. Fruit were sampled at various stages of the fruit development where the first sample was taken on the day of treatment termination and the permeability of the fruit surface was quantified. Values represent mean ± SE of the means (n = 20).

Permeability of the surface of the fruit increased with increasing area of AO infiltration in the treated and non-treated surface in CPPU ($r^2 = 0.68$, $P =< 0.0001$; Fig. 11A) and moisture treatment ($r^2 = 0.88$, $P =< 0.0001$; Fig. 11B). As the AO infiltrated area and the permenace of CPPU and moisture treated surface was very high during the early stage of fruit development, two data points in case of both treatments were very far from the rest. Therefore, the data were re-plotted excluding those two data points in both treatment cases (Fig. 11 insets). Again, there was a significant relationship between permeance of the surface and the AO infiltrated area in CPPU treatment ($r^2 = 0.16$, $P =< 0.031$; Fig. 11A inset) and moisture treatment ($r^2 = 0.20$, $P =< 0.014$; Fig. 11B inset).

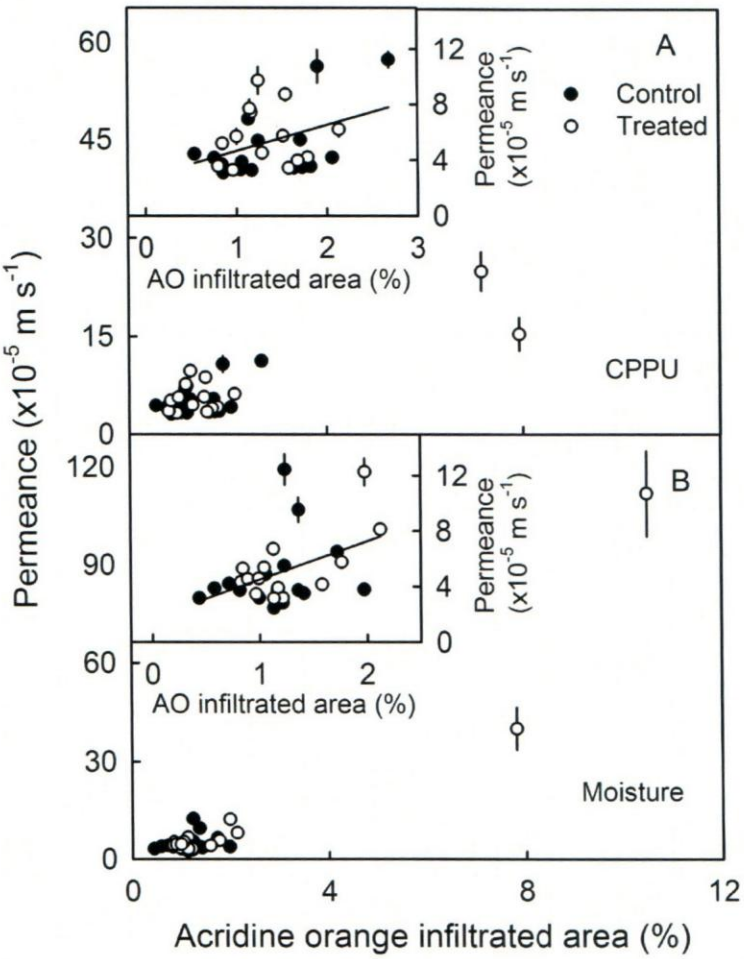

Fig. 11: Relationship between permeance and acridine orange infiltrated area (A) in CPPU and (B) moisture treated fruit skin. Selected portion of the surface of the growing fruit was exposed to CPPU solution (20 mg l^{-1}) or liquid water (treated) at different stages of the fruit development and harvested and the acridine orange infiltrated area and permeance of the treated and non-treated surface were quantified. Values represent mean ± SE (n = 10 and 20; acridine orange infiltrated area and permeance respectively).

Application of CPPU on the growing fruit surface induced fruit russeting when treatment was applied at 21 DAFB (Table 1). Thus, at 21 DAFB, percentage of russeted area of the treated fruit surface when russeting was analysed at 138 and 159 DAFB were 12 % and 10 % respectively. However, there was no effect of the CPPU in inducing fruit russeting when the treatment was applied at 44, 73 and 100 DAFB.

Table 1: Effect of fruit development in CPPU induced russeting in 'Pinova' apple. CPPU was applied at four different stages during the course of fruit development and the russeting on the treated and non-treated surface was analysed at 138 and 159 days after full bloom (DAFB) or only at 159 DAFB.

Treatment time (DAFB)	Russeting analysis (DAFB)	No. of reps (n)	% of fruit with russet	Russeting area (% of treated area)	
				Treated (Mean ± SE)	Control (Mean ± SE)
21	138	23	87	12.1 ± 3.9	0
	159	26	92	10.3 ± 2.6	0
44	159	26	0	0	0
73	159	26	0	0	0
100	159	26	0	0	0

Application of moisture on the growing fruit surface induced fruit russeting when treatment was applied at 21 DAFB (Table 2). At 21 DAFB, percentage of russeted area of the treated fruit surface when russeting was analysed at 138 and 159 DAFB were 34 % and 37 % respectively. But, there was no effect of the moisture in inducing fruit russeting when the treatment was applied at 44, 73 or 100 DAFB.

Table 2: Effect of fruit development in moisture induced russeting in 'Pinova' apple. Moisture was applied at four different stages during the course of fruit development and the russeting on the treated and non-treated surface was analysed at 138 and 159 days after full bloom (DAFB) or only at 159 DAFB.

Treatment time (DAFB)	Russeting analysis (DAFB)	No. of reps (n)	% of fruit with russet	Russeting area (% of treated area)	
				Treated (Mean ± SE)	Control (Mean ± SE)
21	138	30	100	34.3 ± 5.9	0
	159	21	100	37.1 ± 7.3	0
44	159	21	0	0	0
73	159	21	0	0	0
100	159	21	0	0	0

6. DISCUSSION

Our study has demonstrated that 1) high rate of fruit surface growth and moisture on fruit surface both resulted in the physical failure of the fruit surface, 2) the effect of surface growth rate and surface moisture on physical failure of the fruit surface were more pronounced during early stage of the fruit development.

At 21 DAFB, AO infiltrated area on microcracking of CPPU treated surface of the fruit was four times higher than that of the untreated surface (Fig. 3A). We observed that application of CPPU on the fruit surface enhanced fruit growth rate by increasing fruit surface area expansion rate due to rapid cell division in the early stage of development. This subjects the fruit surface to strain resulting in microcracking (Cruz-Castillo et al., 2014; Costa, 1999; Famiani et al., 1997). This finding agrees with Grimm et al., (2012), which demonstrated that AO infiltration is indicator of mass flow through microcracks. We found that water loss via the excised ES of the CPPU treated surface of the fruit was higher than in the untreated surface in early developmental stage (21 DAFB; Fig. 6A). Similarly, at 21 DAFB, the permeability of CPPU treated surface of the fruit was one and half times higher when compared to the untreated surface (Fig. 8A). It may be argued that CPPU had an effect on the relative growth rate in fruit surface area (Fig. 2 inset; lower right corner) in early developmental stage (Ginzberg et al., 2014; Iwahory et al., 1988). This effect may have resulted in increased microcracking (Fig. 3A), hence increased rate of water loss in the CPPU treated surface than in the untreated surface during the early developmental stage. Also, at 21 DAFB, we observed 10 % russeting in CPPU treated fruit surface (Table 1), but there was no russeting (0 %) in the untreated surface of the fruit. In apple, there is a linkage between the rate of strain and relative growth rate of the fruit surface (Khanal et al., 2013b). Relative growth rate in fruit surface area is quite high during the early development (Fig. 2 inset; lower right corner) when fruit is sensitive to russeting (Creasy, 1980). We also found that when the fruit surface was treated with moisture at 21 DAFB, AO infiltrated area on microcracking was eight times higher than in the untreated surface (Fig. 3B). Surface moisture increased the severity of microcracks as depicted in the acridine orange infiltrated area (Fig. 3B). This supports the findings that application of surface moisture promotes microcracking during young fruit development (Khanal et al., 2013b; Considine and Brown, 1981; Creasy, 1980; Faust and Shear 1972). It was evident that the effect of moisture on AO infiltrated area of the fruit surface (Fig. 3B) was more pronounced than the effect of CPPU on AO infiltrated area

of the fruit surface (Fig. 3A) during early developmental stage. Water loss through the excised ES of the moisture treated surface was much higher than in the untreated surface in early developmental stage (Fig. 7A). Permeability of the moisture treated surface of the fruit was four times higher when compared to the untreated surface (Fig. 8B). It is presumed that the cuticular membrane was not sufficiently deposited or developed to restrict moisture uptake/loss (Kerstiens, 1996) during early developmental stage. This could have contributed to altering the physical integrity of the moisture treated fruit surface culminating in high permeability of the fruit surface. Generally, water loss in the moisture treated fruit surface (Fig. 7A) or permeability in the moisture treated surface of the fruit (Fig. 8B) was more severe than the water loss in CPPU treated surface (Fig. 6A) or permeability of the CPPU treated surface of the fruit (Fig. 8A) in early developmental stage. We found 37 % russeting in the moisture treated fruit surface at 21 DAFB (Table 2), but there was no russeting (0 %) in the untreated surface. It is speculated that the moisture may have prevented the deposition of wax on the fruit skin during the early developmental stage. Consistent with Faust and Shear (1972) and Turkey (1969), which reported that moisture play a significant role in russeting during early fruit development. The effect of moisture in inducing fruit russeting was more severe than in the CPPU treated fruit surface during early fruit development.

The effect of CPPU on microcracking of the treated fruit surface was severe only when applied during early developmental stage (21 DAFB; Fig. 4A). But, no effect of CPPU on microcracking of the fruit surface was observed in later developmental stage (44, 73 and 100 DAFB; Fig. 4B, C and D). The probable explanation could be that, the apple fruit surface is characterized by thin cuticular membrane (CM) per unit surface area during young developmental stage and most sensitive to microcracking when exposed to CPPU (Costa, 1999; Famiani et al., 1997). Again, it was observed that the effect of CPPU on permeability of the treated fruit surface was more pronounced only in early fruit development (21 DAFB; Fig. 9A), but the effect decreased when CPPU was applied at 44, 73 and 100 DAFB (Fig. 9B, C and D). Additionally, we found that the permeability of the CPPU treated fruit surface was more severe in early fruit development with increased AO infiltrated area (Fig. 11A). However, the effect of CPPU on permeability of the fruit surface decreased in later developmental stage and was similar to the untreated surface as the AO infiltrated area decreased in the course of fruit development (Fig. 11A inset). Application of CPPU on the growing fruit surface produced russeting when applied at 21 DAFB. But, no effect of CPPU in inducing fruit russeting was observed when applied at 44, 73 and 100 DAFB (Table 1). The

effect of moisture on microcracking of the treated fruit surface was higher when applied at 21 DAFB (Fig. 5A). But the effect of the moisture on microcracking of the treated surface of the fruit and the untreated surface was similar at 44, 73 and 100 DAFB (Fig. 5B, C and D). The results also showed that the effect of moisture on permeability of the treated fruit surface was more pronounced only in early developmental stage (21 DAFB; Fig. 10A), but the effect decreased when the moisture was applied at 44, 73 and 100 DAFB (Fig. 10B, C and D). We also observed that the permeability of the treated fruit surface was more severe in early fruit development with increased AO infiltrated area (Fig. 11B). But, the effect of moisture on permeability of the fruit surface decreased in later developmental stage and was similar to the untreated surface as the AO infiltrated area decreased with time (Fig. 11B inset). This could probably be due to the following arguments; in apple fruit there is continuous synthesis and deposition of newly formed cuticular membrane on the older and more strained CM throughout fruit development (Knoche et al., 2011). The synthesis and deposition increase cuticular membrane thickness with time thereby fixing the strain of the older layers and decrease the permeability of the fruit surface with time (Knoche and Khanal, 2011). Additionally, cutin and wax present in the cuticle of the fruit are continuously deposited in the course of development and may have played a role in fixing the cracks in the fruit surface (Khanal et al., 2014; Curry, 2009; Maguire et al., 1999). The microcracks may have also been repaired by periderm formation so that the permeability of the cracked surface decreases in the course of fruit development (Faust and Shear, 1972). Applying moisture on the growing fruit surface induced russeting at 21 DAFB, but we did not find the effect of moisture in inducing russeting in the treated surface and the untreated surface of the fruit at 44, 73 and 100 DAFB (Table 2).

7. CONCLUSION

Growth rate and surface wetness increase microcracking, permeability and russeting of the apple fruit surface. Surface moisture had a more severe effect in microcracking, permeability and russeting of the apple fruit surface than that of growth rate during early fruit development, even though both factors are critical. There was no effect of surface moisture at late developmental stage where the fruit surface growth rate is low.

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