Environmentally Friendly and Safe Technologies for Quality of Fruits and Vegetables

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Environmentally Friendly and Safe Technologies for Quality of Fruits and Vegetables

Editor
Carla Nunes

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The papers contained in this book report some of the peer reviewed Proceedings of the International Conference "Environmentally friendly and safe technologies for quality of fruit and vegetables", but also other papers related with the subject were included. The manuscripts were reviewed by the Editor and Editorial Board, and only those papers judged suitable for publication were accepted. The Editor wish to thank to all the reviewers and authors for their contribution.

Authors are responsible for content and accuracy of their papers.

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SECTION 1. NON-DESTRUCTIVE METHODS FOR MEASURING QUALITY OF FRUITS AND VEGETABLES

01. X-RAY COMPUTED (MICRO)TOMOGRAPHY FOR DETECTING INTERNAL DEFECTS IN FRUIT

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Abstract

Internal disorders in horticultural products are not revealed by external visual symptoms. Non-destructive and non-invasive monitoring techniques are required to detect the occurrence and investigate development of internal disorders. X-ray CT allows visualization and analysis of plant materials with a resolution down to a few micrometers, and without sample preparation or chemical fixation. X-ray tomography is based on X-ray radiography: an X-ray beam is sent on a sample and the transmitted beam is recorded on a detector. The level of transmission of these rays depends mainly on the mass density and mass absorption coefficient of the material. The resulting image is superimposed information (a projection) of a volume in a 2D plane. The classical way to retrieve 3D information is to perform a large number of radiographs while rotating the sample between 0° and 180°. The filtered back-projection algorithm can then be used to reconstruct the volume of the sample from these radiographs. Compared to two-dimensional radiography used in medicine and linescan radiography applied on grading machines, X-ray computer tomography (CT) is the most powerful technique from the horticultural research point of view, since two and three dimensional images can be reconstructed from the accumulated data to study internal physical and physiological processes. Most internal disorders like woolliness in nectarines, hollow heart in potato, core breakdown in pears, watercore in apples and spongy tissue in mango affect the density and water content of the internal tissue and, hence, are detectable by means of X-ray measurements. For *in vivo* observations, high resolution sub-micron tomography has up to recently only been achieved on relatively dry -or hard- biological samples, such as plant seeds. We have shown, however, that by means of synchrotron radiation X-ray sources, it has now become feasible to image plant tissues at a resolution of less than one micrometer.

Internal Disorders and Fruit Microstructure

Fruit and vegetables cover 16.6% of the EU production of agricultural food[1]. Fruit are, after milk and leading over cereals, the most important food item consumed in the EU (over 400 g capita^{-1} day^{-1}). Yearly the production amounts to 38.3 million tons of fruit and 66 million tons of vegetables. Fresh apple and pear fruit are produced in many countries of the EU, with main production in France, Spain, Italy, Germany, Poland, Austria, Belgium and The Netherlands. Exports (3 billion EUR in 2005) are growing for both fruit and vegetables, but imports are more than four times higher and are competing strongly on the home market.

Fruit are an important category of biological food products with high water content and a cellular microstructure. Their visual, textural and nutritional quality has direct economical impact for fresh consumption as well as processing. A fruit consists of different tissue types such as the epidermis (with cuticle), cortex parenchyma tissue, core tissue and vascular tissue; each with different microstructural composition. From the macro- to the nanoscale, physical properties of fruit are affected by the type of tissue, the geometric properties of the cell, the presence of an adhesive middle lamella between individual cells, the cellular water potential, the mechanical properties of the cell wall, the presence of intercellular spaces

1 http://ec.europa.eu/agriculture/capreform/fruitveg/presentations/fresh_en.pdf

and sub-cellular features such as the plasma-membrane, plasmodesmata and aquaporins. Degradation of the fruit microstructure after harvest quickly leads to interior quality defects. The amount of loss due to internal disorders that develop during storage varies from year to year, but for some cultivars this is always significant. Peak losses have been recorded as high as 20 to 30% of the fruit in some years and locations. Several disorders may occur that reduce the commercial value of the fruit:

Pome fruit are often stored for up to 10 months at a low temperature (typically around 0 °C) in combination with a reduced O_2 and increased CO_2 partial pressure (so-called "Controlled Atmosphere (CA) storage") to reduce their respiration rate, and, hence, extend their storage life. However, the optimal gas composition is critical, as too low an O_2 partial pressure in combination with too high a CO_2 partial pressure may lead to physiological disorders and off-flavours. In fact, under suboptimal conditions some fruit may develop browning and core breakdown. This storage disorder is characterised by the development of brown internal tissue which will further develop into cavities so that the fruit can no longer be commercialised. This disorder has been monitored nondestructively by means of MRI and X-ray CT (Lammertyn *et al.* 2003). How it relates to microstructure is unknown.

The incidence of senescent breakdown in apple, with symptoms similar to browning varies from year to year, apparently being affected by preharvest growing conditions. This disorder is related to the age of the fruit, and occurs more often in large, over-mature apples. Late harvest, delay in cooling and storage at temperatures above those recommended favor the occurrence. Incidence of scenescent breakdown usually indicates that storage life has passed.

Water core is a disease that appears as hard glassy regions near the core in apples. Apples that have been exposed to high temperatures and sunlight near maturity are more susceptible to develop water core. Water core does not develop in storage and may even disappear when originally present in a mild form.

Bitter pit symptoms are mainly found in the cortex apple tissue near the skin and appear first as soft, brown areas, which eventually become desiccated due to the collapse of surrounding cells, forming a dry cavity or 'pit'. The disorder is due to preharvest factors including fruit mineral status and climate. The microstructure of fresh pome fruit (apple and pear) has recently been investigated by means of light microscopy (Schotsmans *et al.* 2004), scanning electron microscopy and confocal microscopy (Veraverbeke *et al.* 2001) and -very recently- by X-ray computed microtomography (Mebatsion *et al.* 2006; Mendoza *et al.* 2007; Verboven *et al.* 2008).

Internal disorders in fruit can cause extreme losses during the storage season. Since fruits normally have to be cut to detect internal disorders, they are often only observed upon quality inspection of the whole batch after shipping. This typically leads to refusal and subsequent destruction of the whole batch which may cause large financial losses. Using new microstructure sensors and improved understanding of the effect of microstructure on the disorder, their occurrence may be detected earlier or even in advance, resulting in more high quality fruit.

X-ray Imaging

Principles of X-rays

WC Roentgen discovered a new kind of radiation in 1895. The nature of the radiation was at first unknown, therefore they were called X-rays. X-rays are a form of electromagnetic radiation in the range of wavelengths between 10 and 0.01 nanometers, corresponding to a frequency of 30 PHz to 30 EHz and energies in the range 120 eV to 120 keV. Therefore, X-ray photons are highly energetic and have enough energy to break up molecules and hence damage living cells.

When X-rays hit a material, some are absorbed and others pass through. The higher the energy the more X-rays will pass through (Table 1). This property of X-rays enables us to "see inside" things.

Table 1. X-ray transmission through material.

Energy carried by each photon	Wavelength (1pm = 10-12 m)	Thickness of material to halve number of photons			
		Concrete	Lead	Human Tissue	Aluminium
1keV	1240 pm	0.87 μm	0.117 μm	1.76 μm	2.17 μm
100keV	12.4 pm	17.3 mm	0.110 mm	38.6 mm	15.1 mm
10MeV	0.124 pm	132 mm	12.3 mm	298 mm	111 mm

Source: http://www.arpansa.gov.au/radiationprotection/basics/xrays.cfm

X-rays are commonly produced by accelerating electrons through a potential difference and directing them onto a target material (for example the metal tungsten). The electrons that hit the target release X-rays as they slow down (braking radiation or bremsstrahlung). The X-ray photons produced in this manner range in energy from near zero up to the energy of the electrons. When incoming electrons cause electron transfer in the atomic shell of target atoms, X-ray photons of a specific energy (a characteristic X-ray) are produced (Fig 1).

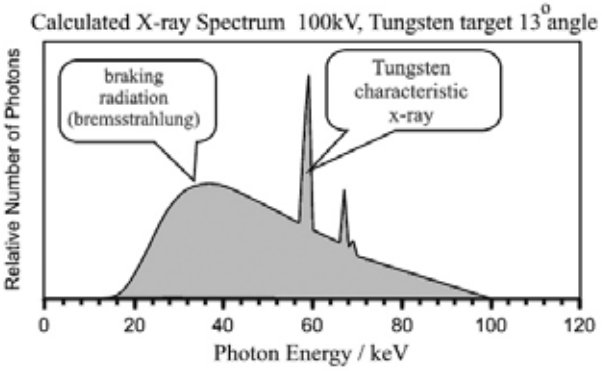
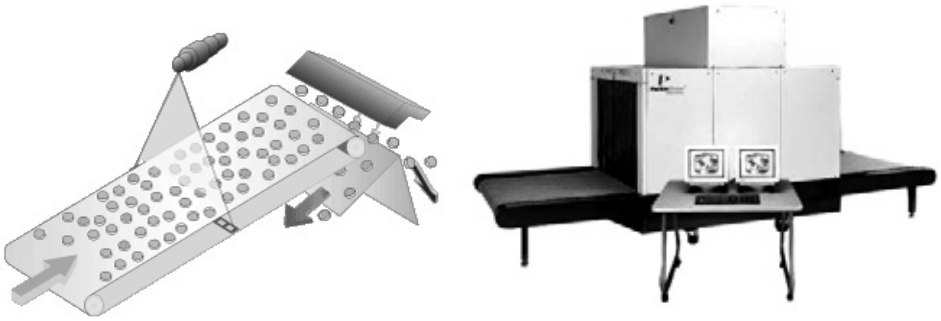


Fig 1. Plot of the number of photons against the photon energy for a tungsten target in a X-ray tube (Source: http://www.arpansa.gov.au/radiationprotection/basics/xrays.cfm).

After passing the material, the X-rays enter crystal scintillators and are converted to flashes of light. The flashes of light are detected and processed electronically. A "single slice" CT has a row of these detectors positioned opposite the X-ray tube and arranged to intercept the fan of X-rays produced by the tube. A "multi-slice" scanner has several rows of these small scintillator detectors.

X-ray Radiography

When a single image of the transmitting X-rays is taken of the material, the method is called radiography. An X-ray beam is sent on a sample and the transmitted beam is recorded on a detector (mainly film or CCD based detector for radiography). According to the Beer–Lambert law, the ratio of the number of transmitted to incident photons is related to the integral of the absorption coefficient μ of the material along the path that the photons follow through the sample. The absorption coefficient μ is linked to the density, the atomic number and the energy (when the beam is monochromatic) by using an empirical law. The resulting image is thus a superimposed information or projection of the material volume in a 2D plane (Salvo *et al.* 2003). Different types of X-ray radiography scanners are given in Fig 2.



(a) (b)

Fig 2. Types of X-ray scanners that use a radiography method. (a) a X_ray sorter distinguishes foreign material from the product by a difference in density, in addition to differences in size and shape (Source: BEST NV, Heverlee, Belgium). (b) Airport carry-on baggage scanner identifies distinct items and attributes them to 'Organic', 'Inorganic' and 'Metal' categories (Photo courtesy of PerkinElmer Detection Systems).

X-ray Computed Tomography

X-ray CT is a relative new technique developed in the late 1970's, which enables the non-destructive visualisation of the internal structure of objects. These first, mainly medical, CT scanners had a pixel resolution in the order of 1mm. In the 80's, after some technological advances towards micro-focus X-ray sources and high-tech detection systems, it was possible to develop a micro-CT (or μCT) system with nowadays a pixel resolution 1000 times better than the medical CT scanners. By using projection images obtained from different angles a reconstruction can be made of a virtual slice through the object. When different consecutive slices are reconstructed a 3D visualisation can be obtained (Fig 3).

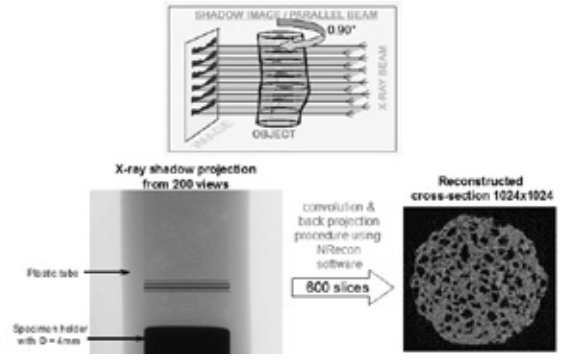


Fig 3. Example of X-ray CT of an apple cylinder in a plastic tube. The cylinder is rotated and projection images are taken. A back propagation algorithm is used to reconstruct the 3D image of the internal structure of the apple sample.

Next to the μCT technology new in-situ stages (rotatable support platforms for the samples) are developed which increase the possibilities of the tomographic systems. An example of such an in-situ stage is the environmental stage (or cooling stage) which is an interesting tool in the study of fresh food products.

μCT as a non-invasive technique has been applied to the study of the internal 3D structures of several food products, e.g, marshmallow, aerated chocolate, chocolate muffin (Lim & Barigou 2004). Kuroki *et al.* (2004) obtained 3D spatial information about gas-filled intercellular spaces in cucumber fruit. Babin *et al.* (2005) studied the microstructure of cellular cereal products captured by synchrotron radiation μCT. Leonard *et al.* (2008) used μCT on processed banana. Synchrotron radiation μCT has been applied by KU Leuven with

success to more difficult products such as apple and pear, with resolutions below 1 micron (Mendoza *et al.* 2007; Verboven *et al.* 2008).

A new challenge in technology is the nano-CT system opening up a new era in X-ray imaging with a spatial resolution in the range of hundreds of nanometers. Proceeding to submicron pixel sizes requires increased performance of the X-ray source, rotation stage and X-ray detector. The fact that the object can be scanned under normal environmental conditions without any coating, vacuum treatment or other preparation techniques makes it an interesting tool as such as well as a reference to interpret the micorstructure measurements with other methods.

Examples of X-ray CT scanners are given in Fig 4.

(a) (b)

Fig 4. X-ray CT scanners. (a) High resolution X-ray CT (to 1 micron image pixel size) (Skyscan 1172, Skyscan, Belgium), (b) Full body medical CT scanner (Siemens, http://www.medical.siemens.com/).

Synchrotron X-ray CT

Visualization by X-ray microtomography of plant tissues in their natural state has been difficult, because of the low contrast and limited resolution (Westneat *et al.* 2003; Kuroki *et al.* 2004; Kim *et al.* 2006; Rau *et al.* 2006; Mendoza *et al.* 2007). Important structural features such as small voids between cells, vascular capillaries or cell walls could therefore not be visualized, rendering incorrect connectivity information (Fig 5A,B).

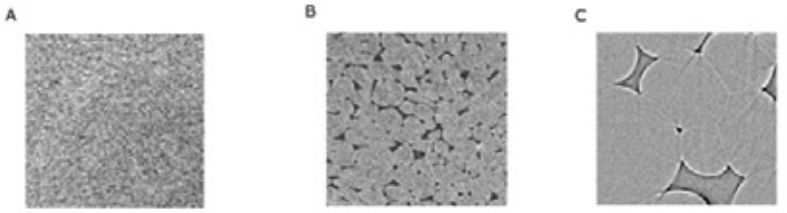
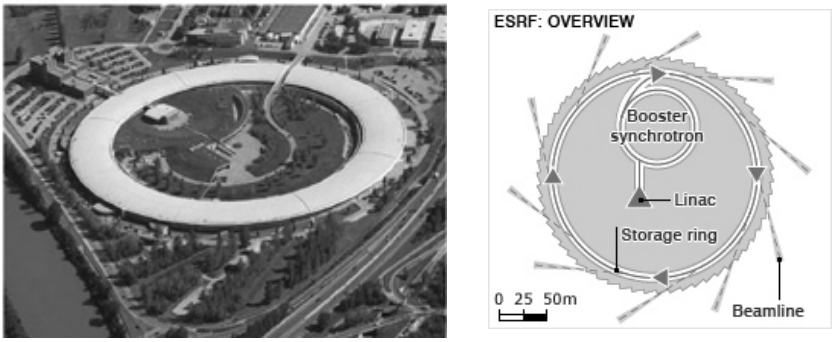


Fig 5. (A) Microfocus X-ray CT of pear at 9.5 μm pixel size, image size 256*256 pixels. (B) High resolution X-ray CT of pear at 4.8 μm pixel size, image size 256*256 pixels. (C) Synchrotron X-ray CT (phase contrast) of pear at 0.7 μm pixel size, image size 256*256 pixels. Dark areas indicate air-filled pores, lighter colored zones are cells.

High resolution tomography using synchrotron radiation offers a means to explore at sub-micrometer resolution 3-D fruit tissues with high water content in their natural state. As explained by Salvo *et al.* (2003), synchrotron radiation X-ray tomography has important advantages over X-ray tube tomography of conventional X-ray CT equipment. X-ray tube tomography produces a divergent beam; therefore the resolution is limited by the beam angle and the required field of view. The beam angle may also produce artefacts in the reconstructed images. The parallel beam produced by synchrotron radiation with good spatial coherence makes a quantitative reconstruction, free of geometrical and beam hardening artefacts, possible.

These conditions can only be achieved at large scale facilities that produce high energy flux sources and long distances between source and tomography set-up (in the order of 100 m). The facility at ESRF (Grenoble, France) was accessible to the authors on the basis of a successful research proposal (Fig 6).



(a) (b)

Fig 6. Synchrotron X-ray facility at ESRF, Grenoble. (a) Aerial view of the facility, (b) Schematic picture of the facility: electrons are fired into a linac, or straight accelerator. They're boosted in a small ring before entering the storage ring 850 m in circumference. The superfast particles are corralled by a train of magnets. Energy lost by turning electrons emerges as very intense X-rays (http://news.bbc.co.uk/2/hi/science/nature/7501420.stm).

Phase contrast imaging with synchrotron X-rays has been developed for edge enhancement on tomographs with low absorption mode contrast (Davis *et al.* 1995; Cloetens *et al.* 1999). High resolution phase tomography of biological tissues at a pixel size close to 1 micrometer has only recently been achieved (Plouraboue *et al.* 2004; Thurner *et al.* 2005), but required sample preparation to improve contrast on the images. For *in vivo* observations, high resolution phase tomography has so far been applied to relatively dry- or hard- biological samples, such as plant seeds (Stuppy *et al.* 2003; Cloetens *et al.* 2006), as well as wet, soft samples that are more prone to damage by the X-rays (Cloetens *et al.* 2006; Verboven *et al.* 2008). Figure 5C demonstrates the power of the technique for imaging cells, cell walls and pores in pear fruit tissues.

Applications of X-ray Imaging for Internal Fruit Quality Assessment

X-ray Sorting Based on X-ray Radiography

Jiang *et al.* (2008) presented an application of X-ray scanners quarantine inspection of fruits, to prevent propagation of foreign insect pests in imported fruits. The X-ray imaging system consisted of a microfocus X-ray source and a line-scan sensor camera, both of which are controlled by a desktop computer. The properties of the system are a divergent beam and polychromatic radiation, therefore quantitative information on the absorption coefficients (and therefore density) of the material is difficult to retrieve. The line detector was 512 mm wide and allowed for a pixel resolution of 1280 pixels. The energy levels that were used were 40–90 kV voltage. Online fruit inspection images of 80×960 pixels were possible at a conveyer belt speed of 1.2 m min^{-1}, including on-line image processing. To accurately determine whether a fruit has signs of insect infestation, the authors have developed an adaptive image segmentation algorithm based on the local pixels intensities and unsupervised thresholding algorithm. Experimental results revealed that the effect of sub-image size and interpolation grid size has little effect on the computational time when the interpolation grid size is greater than 8×8 pixels. The adaptive thresholding algorithm was stable judging from the insignificant difference of threshold value maps created using various sub-image size

and interpolation grid size. The algorithm resolved the frequent problem of segmenting object from X-ray image using global thresholding approach. However, false identification remained an issue (Fig 7).

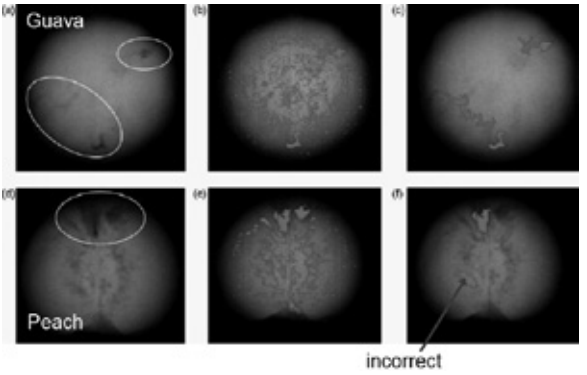


Fig 7. (a) X-ray image of a guava with pest infection, (b) segmented spots after adaptive thresholding of (a), (c) morphological filtering of (b) with three iterations, (d) X-ray image of a peach with pest infection, (e) segmented spots after adaptive thresholding of (d), and (f) morphological filtering of (e) with three iterations (Source: Jiang *et al.* 2008).

CT Imaging of Internal Disorders in Fruit

Most internal disorders like woolliness in nectarines, hollow heart in potato, watercore in apples and spongy tissue in mango affect the density and water content of the internal tissue and, hence, are detectable by means of X-ray measurements (Brecht *et al.* 1991; Tollner *et al.* 1991; Thomas *et al.* 1993; Sonego *et al.* 1995; Schatski *et al.* 1997; Barcelon *et al.* 1999). Lammertyn *et al.* (2003a,b) used X-ray CT to study the development of core breakdown disorder in 'Conference' pears (*Pyrus communis*). With image processing of X-ray tomography slices of pears, we were able to measure non-destructively the breakdown development (in terms of area percentage of affected and unaffected tissue as well as the cavity and core area per slice) during storage measured on actual slices with an underestimation of 12% (Fig 8). MRI was proposed as a better method to follow core breakdown during postharvest storage (Lammertyn *et al.* 2003b). The advantage of X-ray CT is, however, its better resolution over MRI.

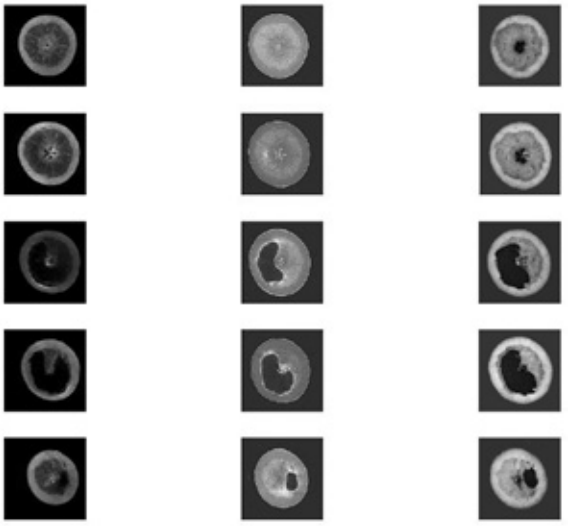


Fig 8. Comparative overview of the corresponding X-ray CT scans, MRI images and actual photographs of core breakdown of pear tissue (Lammertyn *et al.* 2003b; used with permission from Elsevier Science Ltd.).

3-D Imaging of Fruit Microstructure

Cylindrical samples of 5 mm diameter and 1 to 2 cm length were removed from the different tissues of apple 'Jonagold' and pear 'Conference' using a cork bore in the radial direction on the equator of the fruit. The samples were mounted in a Polymethyl methacrylatetube and covered with polymer foil to avoid dehydration. The experiments were conducted at beamline ID19 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The system provided a field of view of 1.43x1.43 mm^2 and, at best, an image pixel size of 0.7 μm. 3-D stacks of 2048x2048x2048 pixels were obtained. Volume renderings and quantitative measurements on the sample were obtained by 3-D image segmentation and isosurface representations with Amira (Mercury Computer Systems, Chelmsford, MA) and dedicated software written for virtual tissue generation (Mebatsion *et al.* 2009).

Figure 8 shows the microstructure of different tissues of 'Jonagold' apple and 'Conference' pear obtained by synchrotron radiation X-ray imaging. The dermal tissue presents a dense assembly of cells with little or no voids. Both fruit are aerated by voids in between the cells of the parenchyma that makes up the bulk of the cortex tissue of the fruit. The vascular tissue in the mature fruit contains empty xylem vessels surrounded by dense tissue without any air voids. The parenchyma in the pear fruit consists of smaller cells and air voids than in apple.

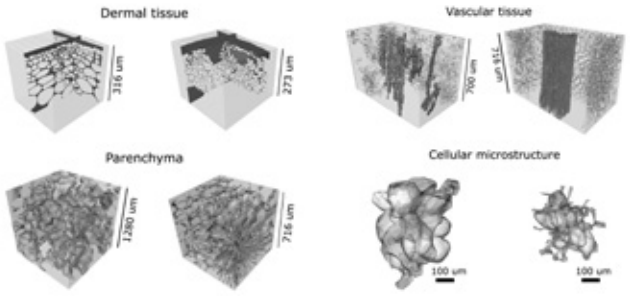


Fig 9. 3-D microstructure of apple 'Jonagold' (left) and pear 'Conference' (right) obtained from synchrotron radiation X-ray tomography. Air-filled pores are colored in blue, cells in yellow, cell walls and sclereids in brown. Details of the results are given in Verboven *et al.* (2008).

The structure of the voids is significantly different in the two fruit. In apple voids have the size of the cells, while in pear the voids are small channel-like structures. The total fraction of voids is also significantly larger in the 'Jonagold' apple than in the Conference pear. Verboven *et al.* (2008) used the microstructure characteristics presented in Fig 3 to interpret the apparent gas exchange properties of the two fruit in relation to storability and internal disorders.

Conclusions

Internal defects in fruits can today only be detected destructively when it is too late; economic risks are therefore high. Early detection of interior defects and/or the aspects that initiate them can prevent severe economical losses. Thereto the role of the fruit microstructure must be understood and measurement of the relevant features online during preservation and transformation of fruits should be targeted. With respect to the first objective, it was demonstrated that the method of synchrotron radiation X-ray tomography provides a suitable tool to probe the microstructure of *in vivo* tissues. To develop fast and cheap online measurement technologies (such as X-ray radiography, nuclear magnetic resonance relaxometry and diffusometry, diffuse spectroscopy, or optical coherence tomography), the presented method serves as a reference.

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02. NON-DESTRUCTIVE DETECTION OF INTERNAL DEFECTS IN APPLE FRUIT BY TIME-RESOLVED REFLECTANCE SPECTROSCOPY

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Abstract

In the present research Time-resolved Reflectance Spectroscopy (TRS) technique was tested to detect some internal defects in different apple cultivars. The absorption (μ_a) and the reduced scattering (μ_s') coefficients were measured at different wavelengths in 'Braeburn' (mealiness), 'Granny Smith' (internal browning) and 'Fuji' (watercore) apples. Afterwards, each fruit of 'Granny Smith' and 'Fuji' were cut and evaluated for internal defects. 'Braeburn' apples were submitted to sensory analysis (firm, crispy, mealy and juicy), percent juice and relative intercellular space volume (RISV) determinations. In addition, for 'Granny Smith', the colour of the pulp was measured. 'Granny Smith' sound fruits on average showed μ_a750=0.029 cm^{-1} and μ_s'750=12.2 cm^{-1}; with the development of internal browning, μ_a750 increased to values >0.04 cm^{-1} and μ_s'750 decreased to values <10 cm^{-1} in severely affected fruit. Non-mealy 'Braeburn' apples were characterized by significantly lower μ_s'790, μ_s'912 and higher μ_a912 than mealy ones. Sensory mealiness was positively related to μ_s'790 and μ_s'912 and negatively to μ_a912. The regions of 'Fuji' apples affected by watercore showed significantly higher μ_a790 and lower μ_s'790 values than the healthy regions. Results confirm the suitability of the TRS technique to inspect apples for internal defects, highlighting the need of determining the TRS threshold values as well as the number of measurement points specific to the cultivar and disorder.

Keywords: absorption coefficient, internal browning, mealiness, scattering coefficient, watercore

Introduction

Under specific conditions, apples (*Malus domestica* Borkh.) may develop internal physiological disorders which are only visible after cutting. Among them, internal browning may show symptoms at different locations, related to different factors, such as diffuse flesh browning, radial browning (related to senescent breakdown) and brown heart (related to CO_2 injury). Watercore develops in some cultivars at harvest, often on the sunny side of the fruit; the affected areas look glassy due to the presence of water instead of air in the intercellular spaces. The water-soaked tissue is usually located around the vascular bundles or the core area. Mealiness is an internal quality defect which develops during storage and shelf life, when fruit flesh softens more by the weakening of middle lamella than of cell wall, producing more cell separation than cell breakages. The intact cells are responsible for the dry feeling in the mouth during mastication associated to the mealy texture (Harker *et al.* 2002).

Time-resolved Reflectance Spectroscopy (TRS) is a non-destructive optical technique by which the optical parameters of absorption coefficient (μ_a, related to chemical composition) and reduced scattering coefficient (μ'_s, related to physical parameters) are estimated. The technique explores a banana-shaped volume of tissue at a depth of 1-2 cm depending on the distance between the injecting and collecting optical fibres (Cubeddu *et al.* 2001).

TRS has been successfully used to detect some internal defects in pears, kiwifruits and apples. In 'Conference' pears brown heart was detected by using μ_a690 and μ_a720, while the presence of translucent tissue, related to overripening and bruises, was detected by μ'_s690 (Eccher Zerbini *et al.* 2002). In kiwifruit, by measuring μ'_s630, it was possible to distinguish the sound region of fruit from that affected by *Botrytis*,

the latter characterized by the higher translucency, which corresponded to a lower μ'_s630 (Eccher Zerbini *et al.* 2008). As for apples, Vanoli *et al.* (2007) found, for 'Jonagored', that μ'_s780 increased with increasing mealiness while firmness, per cent juice and sensory crispness were negatively correlated to μ'_s780. To identify mealiness in 'Golden Delicious' and 'Cox' apples, Valero *et al.* (2005) studied classification models based on TRS measurements at 672, 750, 818, 900 and 950 nm, with model performances ranging from 47 to 100% of correctly identified mealy versus non-mealy apples.

This work aims at applying TRS as a nondestructive method to inspect apples for internal defects, by studying the relationships between reduced scattering and absorption coefficients and mealiness in 'Braeburn', watercore in 'Fuji', and internal browning in 'Granny Smith' apple.

Material & Methods

Two batches of 'Granny Smith' apples from Laimburg (Bolzano province, Italy) with different sensitivity to internal browning were examined after storage at the end of May 2000. Twenty fruits per batch were used. On the equator (the largest transverse circumference) of each fruit, eight equidistant reference points (A–H) were marked. 'Braeburn' apples harvested on 20 September, 2000 in Toggenburg (Bolzano province, Italy) and stored in normal atmosphere (NA) and ultra-low oxygen (ULO) till the end of March. Thirty fruits stored in NA (mealy) and sixteen stored in ULO (non-mealy) were used. On the equator of each fruit two equidistant reference points were marked. 'Fuji' apples were harvested in Laimburg (Bolzano province, Italy) on 7 November, 2000 (corresponding to the late harvest date), transported to CRA-IAA in Milano. On the equator of 30 fruits four equidistant reference points (A-D) were marked.

The TRS measurements were carried out using the system described in Eccher Zerbini *et al.* (2002) at 670 nm ('Fuji'), 750 nm ('Granny Smith'), 790 nm ('Braeburn' and 'Fuji'), and 912 nm ('Braeburn'). For each of the reference point, four measurements were performed using two optical fibers along the axial direction with an acquisition time of 1 s and averaged. For all the apple cultivars, the average of all points/fruit were computed before submitting the optical data to ANOVA.

'Granny Smith' fruit were cut equatorially, the equatorial section of each fruit was photographed, and the presence and position of disorder in the internal tissue were recorded. Browned fruit were divided into: 'brown core' (BC) when internal browning affected only the core and the flesh was healthy, and 'brown pulp' (BP) when the disorder affected either only the pulp or both the pulp and the core. Colour of the pulp was measured on all the browned apples and on five out of the twenty healthy (H) ones (randomly chosen) at the positions correspondent to the maximum and minimum μ_a750 measured values within the same fruit at a distance of 15 mm from the skin, with a Minolta Chroma Meter CR-300. 'Braeburn' apples were submitted to sensory analysis using a panel of eight semi-trained judges evaluating the intensity of the firm, crispy, mealy, and juicy sensory attributes on an unstructured graphic scale with anchors near the extremes ("low", "high"), to measurement of percent juice (Eccher Zerbini *et al.* 1999) and of relative intercellular space volume (RISV, Baumann & Henze,1983). As for the 'Fuji', the equatorial section of each fruit was photographed and the presence (W: watercore) or absence (H: healthy pulp) of watercore for each measurement point was coupled to its own μ_a670, μ_a790 and μ'_s790; the presence of affected tissue close to the measurement point (HW) was also considered.

Statistical analyses were performed using the SAS/STAT (SAS Institute Inc., Cary, NC, 1999) software package. For all the cultivars, data of TRS measurements were submitted to analysis of variance (PROC GLM) considering the internal disorder as factor. Sensory, percent juice and RISV data of 'Braeburn' apples were submitted to analysis of variance (PROC GLM) considering mealiness as factor. Colour data of 'Granny Smith' apples were submitted to analysis of variance considering the browned region as a factor (PROC GLM). Means were compared by Tukey's test at $P \leq 0.05\%$. Correlations between optical and sensory data ('Braeburn') and optical and colour data ('Granny Smith') were studied using the PROC CORR procedure. Significance of *P-value* and *r* are indicated as follows: ns=not significant;* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Results

Internal Browning

The μ_a750 increased with the development of internal browning, with H fruits showing the lowest values of μ_a750 and BP ones the highest. On average, μ_s'750 was higher in H and BC than in BP apples (Table 1). Considering the pulp colour, H fruit had the highest hue and the lowest a*, b* and C* values (Table 1).

Table 1. Mean values of μ_a750, μ'_s750 and colour parameters of the pulp in healthy (H) and browned (brown core, BC; brown pulp, BP) 'Granny Smith' apples.

	H	BC	BP	Sign.	Tukey
Optical properties					
μa750 (cm^{-1})	0.029	0.033	0.039	***	c b a
μ's750 (cm^{-1})	12.22	11.78	10.86	***	a a b
Colour parameters					
L*	80.95	79.47	77.21	**	a a b
a*	-4.90	-3.70	-2.13	***	c b a
b*	15.60	18.10	19.03	***	b a a
C*	16.38	18.49	19.15	***	b a a
Hue (degree)	107.48	101.43	96.33	***	a b c

The μ_a750 was positively correlated to a*, b* and C* and negatively correlated to hue and L*, while μ_s'750 was significantly correlated only to a* and L* (Table 2).

Table 2. 'Granny Smith' apples: correlation coefficients of μ_a750 and μ'_s750 with colour parameters.

	μ_a750	μ'_s750
L*	-0.659 ***	0.543 **
a*	0.867 ***	-0.573 **
b*	0.669 ***	-0.221
hue	-0.855 ***	0.502
C*	0.606 **	-0.158

The μ_a750 was able to distinguish H fruit from BP ones, as the former showed μ_a750 values below 0.030 cm^{-1} and the latter values above 0.033 cm^{-1}. When internal browning affected only the core region of the apple, μ_a750 ranged from 0.030 to 0.035 cm^{-1} (Fig 1).

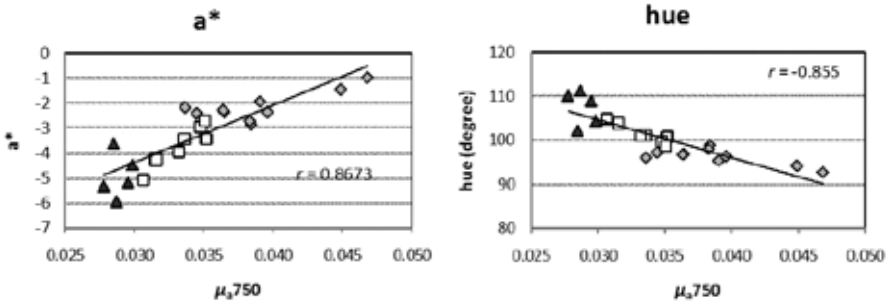

Fig 1. 'Granny Smith' apples: correlations of μ_a750 with a* (left) and hue (right) (H, triangle; BC, square; BP, diamond).

Examples of results of TRS measurements on the eight points around the equator of H, BC and BP fruit are reported in Fig. 2. Point F of μ_a graph for the BP panel has a low value due to an axial zone of pulp with no browning.

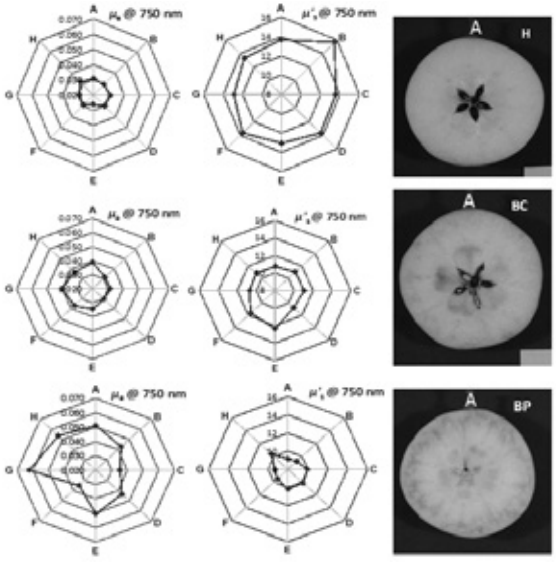

Fig 2. 'Granny Smith' apples: μ_a750 (left) and μ'_s750 (centre) as a function of the position around the equator in a healthy fruit (H), in a fruit affected by brown core (BC) and in a fruit affected by brown pulp (BP). The equatorial sections of fruit are shown in the panels (right). Units for absorption and scattering are cm^{-1}.

Mealiness

Non-mealy 'Braeburn' apples were characterized by significantly lower μ_s'790, μ_s'912 and higher μ_a912 than mealy ones (Table 3).

Table 3. Mean values of μ_a790, μ'_s790, μ_a912, μ'_s912, scores of sensory attributes per cent juice and RISV in non-mealy and mealy 'Braeburn' apples.

	non mealy	mealy	Sign
Optical properties			
μ_a790 (cm^{-1})	0.037	0.037	ns
μ_a912 (cm^{-1})	0.091	0.084	***
μ'_s790 (cm^{-1})	16.41	20.13	***
μ'_s912 (cm^{-1})	16.26	19.47	***
Sensory attributes			
firm	83.9	48.3	***
juicy	81.9	49.6	***
mealy	38.4	70.6	***
crispy	83.0	48.8	***
Quality parameters			
% juice	33.1	17.6	***
RISV(%)	20.7	17.1	***

Mealy apples had significantly lower percent juice and RISV than non-mealy ones, and were described at sensory analysis as less firm, less juicy, less crispy and more mealy than non-mealy fruit (Table 3).

The μ_a790 did not correlate with any of the sensory attributes and quality parameters, while μ_a912 was positively correlated to firmness, crispness, juiciness and percent juice and negatively correlated to mealiness and RISV. The μ_s'790 and μ_s'912 were positively correlated to mealiness and RISV, while they were negatively correlated to the other sensory attributes and percent juice (Table 4). For μ_s'790 and μ_s'912 values above 19 cm^{-1} and for μ_a912 values below 0.09 cm^{-1} mealy, non-crispy and non-juicy apples were found (Fig 3).

Table 4. 'Braeburn' apples: correlation coefficients of μ_a and μ'_s at 790 and 912 nm with sensory attributes and quality parameters (sensory attributes and %juice n=46; RISV n=20).

	μ_a790	μ_a912	μ'_s790	μ'_s912
firm	0.114	0.781	-0.750	-0.742
juicy	0.057	0.746	-0.758	-0.747
mealy	-0.042	-0.720	0.713	0.717
crispy	0.148	0.706	-0.732	-0.734
%juice	0.095	0.759	-0.833	-0.867
RISV	-0.087	-0.664	0.687	0.647

significance of *r*: grey:***; pale grey**

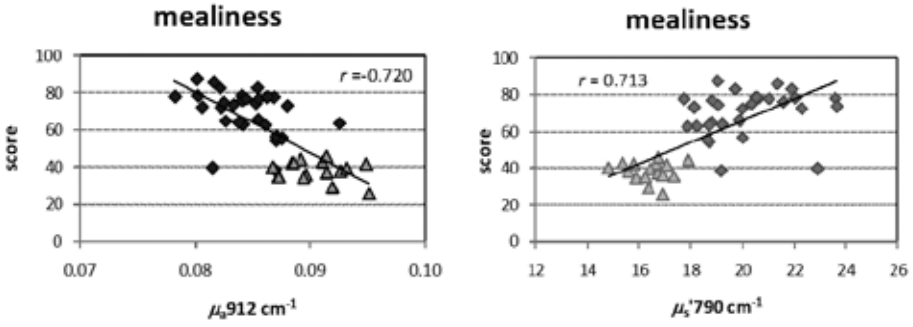

Fig 3. 'Braeburn' apples: correlations of μ_a912 (right) and of μ'_s790 (left) with mealiness (mealy, diamond; non-mealy, triangle).

Watercore

Healthy 'Fuji' pulp was characterized by significantly lower μ_a790 and higher μ_s'790 than zones affected by watercore. If the tissue affected by watercore was near the point of measurement of the optical properties, μ_a790 was on average slightly higher and μ_s'790 slightly lower than the healthy points (Table 5). Examples of TRS results on the four points around the equator of H, HW and W fruit are shown in Fig 4.

Table 5. Mean values of μ_a670, μ_a790 and μ'_s790 in 'Fuji' apples (H, healthy; HW, watercore near the position of measurement; W, watercore).

	H (n=42)	W (n=33)	HW (n=5)	sign	Tukey
μ_a670 (cm^{-1})	0.079	0.096	0.112	*	a a a
μ_a790 (cm^{-1})	0.049	0.059	0.053	***	b a ab
μ'_s790 (cm^{-1})	8.99	8.21	8.68	***	a b ab

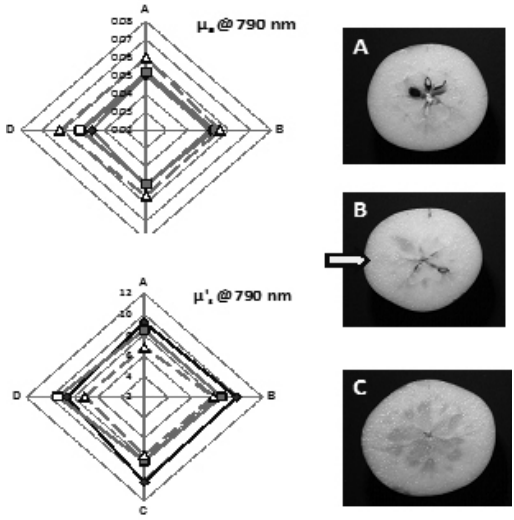

Fig 4. 'Fuji' apples: μ_a790 (top column left) and μ'_s790 (bottom column left) as a function of the position around the equator in an healthy fruit (diamond, H), in a fruit with watercore near the position of measurement (square, HW) and in a fruit affected by watercore (triangle, W). The equatorial sections of fruit are shown in the panels on the right column.

Discussion

Using TRS it was possible to detect some of the internal disorders of apples related to changes in colour (browning) or texture (mealiness, watercore) of the pulp. It was confirmed that internal browning is more related to the absorption coefficient as shown by the high correlation coefficients with colour parameters. In fact, using μ_a750, healthy fruit could be distinguished from those affected by internal browning both in the core and in the pulp, whereas using μ'_s750 fruit affected by internal browning in the core could be classified as healthy.

The positive correlation between μ_s' and mealiness, already found in 'Jonagored' apples (Vanoli *et al.* 2007) was confirmed. The threshold value above which only mealy 'Braeburn' apples are found is μ_s'790>19 cm^{-1} vs μ'_s780>11 cm^{-1} found by Vanoli *et al.* (2007) for 'Jonagored', underlining the need of determining the TRS threshold values specific to the cultivar. Moreover, in this work, high correlations were also found between mealiness and the optical properties measured at 912 nm, near the absorption peak of water.

Tissue affected by watercore can be distinguished by the healthy ones using both μ_a790 and μ_s'790. Differently from what found in pears (Eccher Zerbini *et al.* 2002) and kiwifruits (Eccher Zerbini *et al.* 2008), where using the absorption coefficient measured near the chlorophyll peak (690 and 630, respectively) it was possible to detect translucent tissue, in this experiment no relationships were found between μ_a670 and watercore.

Our results stressed that to make certain the detection of the defect, the number of measurement points has to be suited to the localization and distribution of the affected tissue. In fact, to detect internal defects involving all the pulp, such as mealiness, two measurement points were sufficient, while to detect defects involving only part of the pulp, such as watercore, four points at a distance of 90° were not enough.

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03. ON THE APPLICATION OF SPATIALLY RESOLVED REFLECTANCE AND DIFFUSE LIGHT BACKSCATTERING GONIOMETRY TO THE PREDICTION OF FIRMNESS IN APPLE 'BRAVO DE ESMOLFE'

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Abstract
In this study we have made exploratory tests on a set of 40 apples (*Malus domestica* Borkh.) 'Bravo de Esmolfe', using spatially resolved reflectance (SRR) and diffuse light backscattering goniometry (DLBG). The objective was to test the potential of DLBG for firmness prediction, as compared with SRR, whose potential has been already proved in the literature. SRR is performed with a red diode laser and a CMOS camera. DLBG uses the same laser shining on the apple and a photomultiplier tube collecting the light reemitted from a small area, at angles ranging from 90 deg (tangent to the surface) to 180 deg (normal to the surface). From the measurements several parameters have been calculated (e.g. decay exponent for SRR profiles, anisotropy factor for the DLBG angular distributions) and Partial Least squares (PLS) models for the prediction of firmness were build. The model based on DLBG variables (only) and on SRR variables (only) gave similar results. From here we conclude that, within the obvious statistical limitations of the test, DLBG seems to match the potential of SRR for firmness prediction. The possibility of combining both measures in one model is also discussed.

Introduction
Several optical non-destructive optical techniques (NDOT) for fruit quality assessment are based on the fundamental phenomenon of light propagation, absorption and scattering in random media. Traditional approaches like Near Infra Red Spectroscopy (NIRS) rely on the physics of this process but the analysis is rather heuristic, taking the spectra "as they are" and relating them directly with the fruit physiological attributes of interest. Nevertheless, it is always important to consider the basic aspects of the theory, trying to understand if a more deep knowledge of the optical processes may lead to improved methods in NDOT for fruit quality assessment. The above argument serves the purpose of remembering that in general the propagation of light in a complex media is described by the specific intensity $I(r, \hat{s})$ [units: $Wm^{-2}sr^{1}Hz^{-1}$], where r stands for the position of a given point in space and $\hat{s}$ is a specific direction for energy flow. In other words, the intensity of light in a given point depends on the spatial location of that point and on the direction considered for energy flow. The specific intensity depends further on the wavelength considered. Thus, the most general approach in NDOT would have to discriminate spectral, spatial and angular variations of the light signal.

The investigation of the angular dependency has not attracted attention in NDOT, since all the current methods integrate the angular components or, at least, choose one. The reasons for the lack of data on angular measurements are multiple: i) angular measurements (goniometry) are slow and time-consuming; ii) there are no commercial systems available; and iii) if the conditions for the validity of the so-called "diffusion approximation" (DA) are valid, the angular distribution of light inside the tissue is expected to be nearly isotropic and that emerging from the tissue expected to be Lambertian [dependence of intensity on $\cos(\Theta)$, Θ being the angle between the normal to the surface and the direction of observation]. Hence, angular data would be expected to provide poor additional information. We have overcome the two first

issues by using a light scattering goniometer set up in our laboratory and used already in previous studies (Pinto *et al.* 2007). The answer to the third objection is also twofold: i) there is no evidence that DA is always valid; ii) even when DA is valid the assumption of Lambertian radiance emerging from the tissue may be false (see Li *et al.* 2000 and also our results below).

The objective of this work was then to investigate the potential of angularly resolved light measurements as a possible NDOT for fruit firmness assessment. The technique we will be employing was named Diffuse Light Backscattering Goniometry (DLBG). In this technique a laser beam is incident upon the fruit and the light re-emerging from a nearby small surface is measured as a function of the angle between the normal to the surface and the direction of observation. In parallel to DLBG we have performed measurements of Spatially Resolved Reflectance (SRR), whose applicability for fruit firmness prediction been proven in the literature (Lu 2004). SRR results will be used for comparison with DLBG results. The preliminary results presented here are only indicative, since the reduced number of samples (40 apples) is far from the minimum statistically acceptable when working with biological material. However, the comparison with SRR on the same conditions compensates in part for the sample small size.

Material & Methods

Fruit

Forty 'Bravo de Esmolfe' apples (*Malus domestica* Borkh.) were bought in a local supermarket and kept under shelf-life conditions (70% RH, 23±2 ºC) in dark. In each measurement session, ten apples were followed optically and at the end used to perform destructive measurements of firmness. The four measurement sessions spanned a period of 20 days.

Spatially Resolved Reflectance

A diode laser (Hitachi HL6314MG, Hitachi, Tokyo, Japan, housed in a Thorlabs TCLD M9-TEC mount and coupled to a Thorlabs laser diode controller LDC 205 and to a TED 200 temperature controller, Thorlabs, Newton, USA) emitting at 635 nm was coupled to an optical fiber of 1 mm diameter whose other end was held in contact with the apple skin (this insures that all re-emitted photons travel through the interior of the fruit). The light halo on the apple surface was imaged through an 8 bit CMOS camera (BCi4 CMOS Camera, C-Cam Technologies, Belgium) and appropriate lens. A neutral density filter wheel (Edmund Optics M54-080) was placed in front of the camera and for each halo three photos were taken with different attenuation factors (exposure and gain always kept at the same level). The lowest attenuation provided detail on the most exterior areas of the halo (and the image was saturated on the center of the halo), while the highest attenuation provided detail on the center of the halo (loosing definition in the exterior areas). The intermediate attenuation served to provide a bridge between the extreme attenuation photos. Dark photos (laser off) were subtracted in order to eliminate the ambient and shot noise. The photos were further analyzed through the Matlab software (MATLAB 7.7 (R2008b)®, The MathWorks, Inc., Natick, USA, 2008).

Diffuse Light Backscattering Goniometry

The same diode laser, with horizontal polarization, was sent through an opaque tube of 8 mm diameter whose other end was held in contact with the apple skin (this insures that all re-emitted photons travel through the interior of the fruit). The light halo on the apple surface was blocked through the application of opaque adhesive tape, except in a small square area of 1 cm^2 adjacent to the tube. The angular dependence of the light re-emitted by this area was measured by a light scattering goniometer. This was constituted by a motorized rotational stage (Newport URS100PP rotation stage and Newport ESP 300 motion controller, Newport Corp., Irvine, USA) attached to a rotating arm holding a polarizer and a collecting lens. The arm rotates between 0 º (normal to the halo) and 90 º (tangent to the halo) in steps of 5 º, collecting the light re-emitted at different angles. The lens (74-UV, Ocean Optics, Dunedin, USA) focuses the light on

an optical fiber coupled to a photomultiplier tube (PMT) (H5784-20, Hamamatsu, Shizuoka, Japan). The polarizer was used in two positions: vertical and horizontal polarization. The signal from the PMT was low-pass filtered (SR650 dual channel filter, Stanford Research Systems, Sunnyvale, CA, USA) and acquired by a digital oscilloscope (Picoscope 3206, Pico Technology, Cambridgeshire, UK). Dark measurements (laser off) were subtracted in order to eliminate the ambient and instrumental noise. All the system is automated and controlled through a PC via the Labview software (Labview 7, Austin, USA, 2004).

Fruit Attributes

Firmness was measured destructively immediately after the optical assay in two opposite sites along the fruit equatorial line by puncture, after skin removal, with a fruit pressure tester (FT 327, Italy).

Results

Spatially Resolved Reflectance

The results of SRR are depicted in Fig 1. The three profiles obtained with the three attenuations were normalized through a Matlab script. Basically, it was applied to two pictures at a time and looks for a pixel range where the counts of both pictures are in the range [*p*; 255-*q*], where *p* and *q* are "safety" constants that prevent distortions in linearity due to proximity to the noise floor (*p*) or to the saturation level (*q*). The ratio count (Photo 1)/count (Photo 2) was approximately constant under the above condition and that constant was used to normalize the counts of Photo 1 to those of Photo 2. The process was then repeated for Photo 3. A typical final result is depicted on Fig 1g (conversion pixel/distance also made). Noteworthy, the profiles did not obey the classical result from the diffusion approximation (DA) (Contini *et al.* 1997). In Fig 1g the DA best fit was the line that fails most of the experimental data. The experimental points (dense dots, defining a broad line) were much better fitted by a simple exponential decay (line passing through the points).

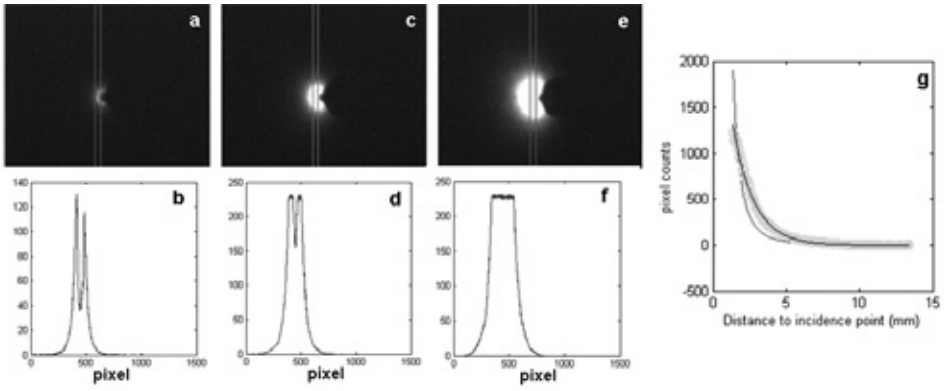

Fig 1. Results of SRR. a) image of the light halo (and of the optical fiber tip) in one of the pears with the highest attenuation; b) intensity profile corresponding to the box represented in a); c) same as a) with intermediate attenuation; d) same as b) with intermediate attenuation; e) same as a) with lowest attenuation; f) same as b) with lowest attenuation; g) full profile for the left wing, obtained after merging the three preceding profiles (detail of the merging procedure in the text).

Diffuse Light Backscattering Goniometry

The results of DLBG are depicted in Fig 2. The phase functions presented are simply proportional to the PMT output at the different angles of measure. The phase functions were not found to follow the uninteresting

Lambertian cosΘ rule. A heuristic fit of the type $a+\cos^b\Theta$ was tried in order to explore the variability of the phase functions. Finally, the polarization of the re-emitted light was calculated by $P=(I_H-I_V)/(I_H+I_V)$, where I_H and I_V are the intensities of the horizontal and vertical polarization signals respectively.

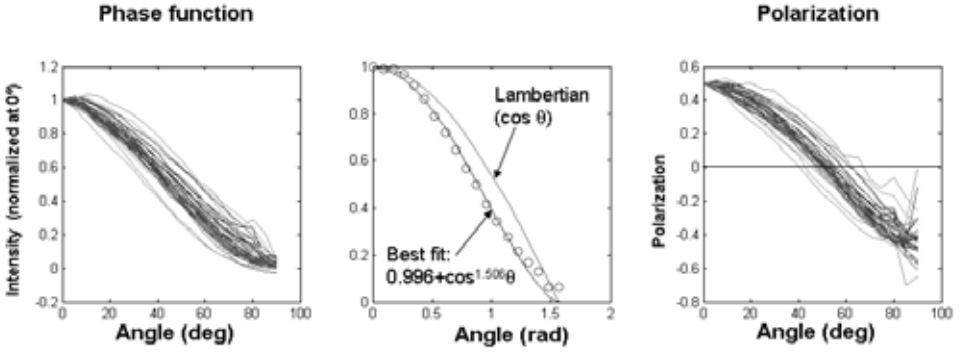

Fig 2. Results of DLBG. Left: phase functions (=angular distribution of the re-emitted light) for all 40 apples (vertical polarization measurements shown). Center: experimental points for one apple (dots). It can be seen that a Lambertian phase function does not fit the results. A heuristic fit of the type $a+\cos^b\theta$ was adopted. Right: polarization curves for the 40 apples (see explanation in the text).

Discussion

Spatially Resolved Reflectance

From each apple we could obtain three profiles. Referring to Fig. 1a and to the center of the halo, located by the fiber tip, we may define the up, down and left profiles. To each of these profiles we have made a fit of the type A*exp(-Bx). We have also segmented the profiles, looking for sections more sensitive to firmness variations; and we have used normalized and non-normalized profiles. The parameters A and B for all these profiles were used as model parameters. We have also integrated the profile along the radial distance, creating a total reflectance variable, also used as a model parameter. Finally, we have used the error on fit itself also as a model parameter. On the whole, 52 variables were created from the initial photos. We have also added the variable shelf-life days. Some of the variables are highly redundant. However, at this preliminary stage we did not perform any type of refinement. We have simply ran a PLS model with the firmness values as the Y block (40×1 matrix) and the model parameters described above as the X block (40×52 matrix). The PLS calculations were performed with the PLS Toolbox 5 (Eigenvector Research, Wenatchee, USA, 2008) for Matlab. Results of the model are shown in Fig 3.

Diffuse Light Backscattering Goniometry

The model variables for DLBG are: the 19 I_H values (19 angles), the 19 I_V values, the 19 polarization values, the anisotropy factors for the H and V polarizations, g_H and g_V ($g=\int I(\Theta)\cos(\Theta)d\Omega/\int I(\theta)d\Omega$, where $I(\Theta)$ represents the scattered intensity and $d\Omega$ the infinitesimal solid angle), the coefficients a and b of the fit $a+\cos^b\theta$ (V and H polarizations) and the coefficients c and d for a second fit of the form $I(\theta)=cI_0(\theta)-dI_1(\theta)$ (V and H pols.), where $I_0(\theta)$ and $I_1(\theta)$ are the Bessel functions of the first kind of orders 0 and 1 respectively. The total number of variables is thus 68, including the extra variable shelf-life days.

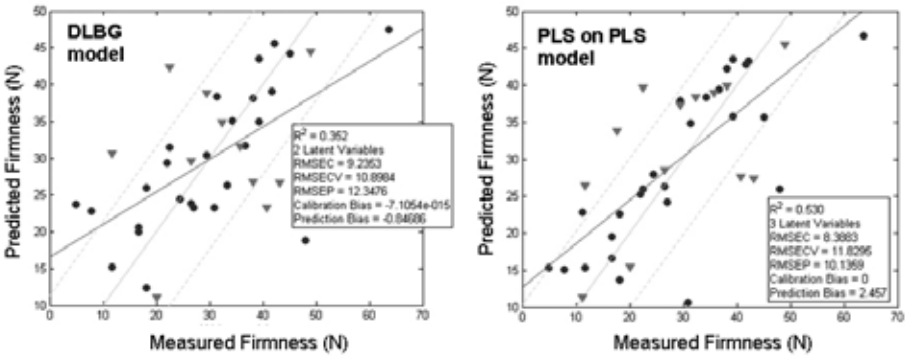

Fig 3. Results of the PLS models. Calibration samples are marked as circles and validation samples as triangles. Left: predicted *vs.* measured values of firmness through the application of a PLS model based on the variables measured in DLBG. Right: the same, but for the "PLS on PLS" model (details about this model in the text).

Results of PLS Model

From the 40 apples, 27 were used for calibration and 13 for prediction. The results for the PLS model based on the SRR measurements and for the PLS model based on the DLBG measurements were very similar and we depict in Fig 3 (left) only the results of the DLBG results. The fact that both models behaved similarly means that the potential of DLBG for firmness prediction should be comparable to that of SRR. And the key point is that the potential of SRR has already been demonstrated (Li *et al.* 2000). This conclusion, however, is severely limited by the small number of samples in this experiment. Further investigation is needed. However, DBLG seems to pass on a first coarse scrutiny.

Refinement of the Model: "PLS on PLS"

Merging all the variables and building a PLS model with all the 52+67 variables ("full model") did not improve the results. The Principal Component Analysis (PCA) of the full model (not shown) showed that the variations in the SRR variables and DLBG variables are mainly independent, since the principal component 1 is more related with goniometric variables and principal component 2 with the SRR variables. However, we did succeed in improving the prediction performance building a "PLS on PLS" model. In this model we have used the predictions of the SRR, DLBG and SRR+DLBG models as input variables. We have also included, as before, the variable "days in laboratory". Thus, the X matrix is simply a 27×4 matrix. The results for this model are presented in Fig 3 (right), for comparison with the DLBG model. Almost all of the parameters describing model performance improved in PLS on PLS (values for the models DLBG vs. PLS on PLS: RMSEC: 9.2 vs. 8.4 (N); RMSECV: 10.9 vs. 11.8 (N); RMSEP: 12.3 vs. 10.1 (N); R^2: 0.35 vs. 0.53; slope of tendency line for external predictions: 0.44 vs. 0.59 (N/N)).

Conclusions

Diffuse Light Backscattering Goniometry (DLBG) is a technique where the fruit is illuminated by a light beam and the angular dependence of the light re-emitted (and thus backscattered) to the exterior is measured by an optical detector rotating on a goniometer arm. As a first approach to the problem one could think that the angular pattern of the backscattered light should obey roughly a Lambertian law ($\cos\theta$), with no interest for any type of fruit internal quality prediction. However, our measurements have shown that this is not the case: the angular pattern of the backscattered light diverges clearly from the Lambertian. Also, there is a considerable variation from fruit to fruit. The question is then to know if these variations

may be correlated with fruit firmness. We have then performed a preliminary scrutiny of the potentialities of DLBG. We have used only forty 'Bravo de Esmolfe' apples, which is a small number to draw significant conclusions. However, we have used an auxiliary measurement to improve the significance level of our test: we have made parallel measurements of Spatially Resolved Reflectance (SRR), which has been proven in the literature to have good correlation with firmness (Lu 2004). Partial Least Squares (PLS) models for firmness prediction based on DLBG variables (only) and on SRR variables (only) gave similar results. From here we conclude that, within the obvious statistical limitations of the test, DLBG seems to match the potential of SRR for firmness prediction in this apple variety.

Finally we have tried to improve the prediction level by merging all the DLBG and SRR variables. This produced no effect. However, a second round "PLS on PLS" model having for variables the predictions of the individual models showed a clear improvement, indicating a possible way to optimize prediction models based on more than one measurement technique.

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04. HOW TO ANALYSE NON-DESTRUCTIVE DATA FOR BIOLOGICAL VARIATION

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Abstract

Biological variance is omnipresent. In animals, in humans, in biology, in sociology, medicine, you name it. In fact, life would be utterly boring without biological variation. Also in agricultural and horticultural produce, the ubiquitous variation causes a lot of trouble in dealing with the product in the supply chain. Basically, the majority of troubles and problems in the food production and supply chain is in one way or another related to the presence of variation between entities like individuals, up to batches, pallet loads, orchards and harvests, and down to cells and organelles. Many modern measuring techniques make it possible to analyse product entities without destroying the samples. These gathered, so-called longitudinal data, offer many advantages for extracting information. By using these techniques, it becomes possible to follow individual units (batches, fruit etc.) in time, and estimate the kinetics of change in (any) properties on an individual level. Destructively obtained data (cross-sectional data) can only be analysed at the level of mean values, neglecting completely the information on variance contained in data. Explained parts of data analysis con increase from 60-70% obtained on cross-sectional data to well over 90% obtained on longitudinal data, with the quantification of the biological variance present. The analysis of longitudinal data, however, requires a special approach and the use of special analysing techniques. The benefits of longitudinal data and their analysis using mixed effect non linear regression for extracting information on maturity and biological variance within a batch, is highlighted based on a large number of examples, already published or in preparation, covering the colour and firmness of nectarines, water loss in plums, mandarins and melons, firmness in Near Isogenic Lines of melons, colour of apples in storage and during growth. More and more papers are published that prove the usefulness for both theory and practice of the applied techniques and viewpoints on biological variation.

Introduction

More and more, experimental data are gathered using non-destructive measuring techniques (so-called longitudinal data). By using these techniques, it becomes possible to follow individual units (batches, fruit etc.) in time, and follow the kinetics of change in (any) properties on an individual level. Much more information can be extracted from this type of data than is possible on destructive data (so-called cross-sectional data), where one inevitably has to deal with mean values in some form. To gain information on the variance in properties between individuals, the analysis of longitudinal data, however, requires a special approach and the use of special analysing techniques.

In this paper, the benefits of longitudinal data and their analysis using mixed effect non linear regression for extracting information on maturity and biological variance within a batch, is highlighted based on a large number of examples, already published or in preparation, covering the colour and firmness of nectarines, water loss in plums, mandarins and melons, firmness in Near Isogenic Lines of melons, colour of apples in storage and during growth.

The increasing number of recent publications applying the same or similar technology indicates that the method is very useful in gaining more insight in the processes occurring in the produce.

Statistical Basics

All measured data contain variation. Where that variation resides in the data is not always clear. In many regression analyses applying basic statistical knowledge and procedures, it is explicitly assumed that the residual variation (**ε**) in the measured property is additive and distributed according to a Gaussian (normal) distribution. In Eq 1 an example is shown for a simple exponential behaviour including an asymptotic value y_{min}. Exponential behaviour is a direct consequence of first order reaction, and is frequently encountered in agricultural and horticultural research.

$$y = (y_0 - y_{min}) \cdot e^{-kt} + y_{min} + \varepsilon \qquad \textbf{Eq 1}$$

The fundamental basis of the presented technique is that the residual variation (**ε**) can be split up in two parts: the variation caused by the measuring technique and/or equipment (the technical error ε_t) and the residual variation caused by the biological system of growth and harvest. Due to differences in location (due to e.g. different exposure to light), and the inevitable small variations in weather conditions (microclimate), soil structure and fertilisation, plants and plant parts do not grow and ripen in the same time. Harvest procedures are another source of variation, and added to previously described factors (sources of variation) will result in considerable variation in the state of maturity or time of development. That really represents the biological variation present in any batch of produce, whether still on the plant or post harvest. So, in short, the biological variation can in fact be expressed as a difference in time of development (biological variation or biological shift factor Δt). That also means that the initial condition (y_0) is bound to be different for each individual fruit or entity in a batch.

In a mathematical sense, the biological variation in the equation (e.g. Eq 1), should be put exactly there where it belongs: at the time variable (t). When the pre-exponential factor in Eq 1 (y_0-y_{min}) is converted into a exponential form as shown in Eq 2, the resulting expression is (Eq 3):

$$\Delta t = -\frac{\log\left(\frac{y_0 - y_{min}}{y_{ref} - y_{min}}\right)}{k} \qquad \textbf{Eq 2}$$

$$y = (y_{ref} - y_{min}) \cdot e^{-k(t+\Delta t)} + y_{min} + \varepsilon_t \qquad \textbf{Eq 3}$$

where y_{ref} is a chosen value (within the range of change observed) as a reference point to express the biological shift factor Δt. Both the biological shift factor Δt and the technical error ε_t in Eq 3 are normally distributed random variables with a mean and standard deviation. Applying different temperatures during storage, the rate constant k depends on the applied temperature (according to Arrhenius' law). As a consequence, the estimated biological shift factors will also show a different value, even for the same fruit. To compare biological shift factors at different temperatures, one can convert them to a dimensionless expression by multiplying with the actual rate constant at that particular temperature: $\Delta t^* = \Delta t^* k$.

Modern measuring techniques are, in general, most of the time accurate and reliable. That means that the technical error is relatively small. If not, the technique of measurement will never provide informative data and will in the long run disappear completely.

So, Eq 3 expresses the exponential behaviour including (or pointing to) the variation in the data. Of course for more complex formulations of the exponential behaviour, or for completely different type of behaviour, the deduction has to be done anew. The line of reasoning, however, remains exactly the same. Some data were generated using Eq. 1 with the input values as shown in Table 1 and 2 that will be used to explain and elucidate the technology.

Table 1. Data used in examples, input, traditional analysis, structured analysis.

Parameter	Meaning	Input	Traditional		Mixed effect	
			value	St.Err.	value	St.Err.
y_{min}	y value at + infinite time	0	-0.822	5.525	-0.002	0.438
k	rate constant of the process	0.1	0.096	0.020	0.099	0.002
$m_{\Delta t}$	mean maturity mother popul.	0	-1.982	0.281	-0.862	1.331
$\sigma_{\Delta t}$	st. dev. maturity mother popul.	5	-	-	5.122	
R^2_{adj}		-	0.576		0.992	

Table 2. Additional input information.

y_{ref}	# initial y value	50
n_{time}	# number of times in a time series	21
n_{rep}	# number of repetitions	15
ε_t	# real measuring (technical) error	2

Data analysis

Traditional Approach

In Fig 1, an example is shown for the traditional representation, not using the individual information (Left). As a modeller and data analyst, one gets not very happy with this type of data. Analysing the data shown in Fig 1, using the traditional approach (classical non-linear regression), the results are not really satisfying. The explained part is about 58%, while the standard errors are large (Table 1). The estimated values are for themselves not that bad compared to the values used for simulating the data. The estimated behaviour is drawn as a solid line in Fig 1 Left.

Structured Approach using Full Benefits of Longitudinal Data

The main question is how to improve the analysis, in terms of reliability, accuracy and information extraction. Assuming that data were gathered using non destructive techniques using the same individuals repeatedly over time (longitudinal data), not only the measured values at the measuring time but also a code or number for the individual fruit are known. Fruit do mature and senesce in time, but the initial condition, where the fruit started at time zero will be and has to be identical for all points in time. When the data are highlighted as individual fruit, the representation is much more understandable (Fig 1 Right).

The same initial condition for each fruit also means that the biological shift factor, that is the shift in time to put each individual line over one another (Fig 1 Right), is identical for all data points in time for that one particular individual. Applying this knowledge about individuals, and assuming the rate constant to be the same for all fruit, one can estimate that common rate constant and one biological shift factor for each individual using standard non linear regression analysis. However, also more realistic and sophisticated methods were developed where some parameters in the model are assumed to be common for all individuals (fixed effects) whereas others vary randomly over the individuals (random effects). These so-called mixed effects regression automatically does just that: estimating some parameters in common (fixed effects) while some parameters are estimated for each individual (random effects). Of course any other factor in the data set can be used for other applications. Procedures of mixed effects estimation are available in the better statistical packages like SAS and S-Plus. A package that is freely available on the internet is R (R Development Core Team 2005). The deeper concepts of mixed effects analysis are described in a simple way by De Ketelaere *et al.* (2006).

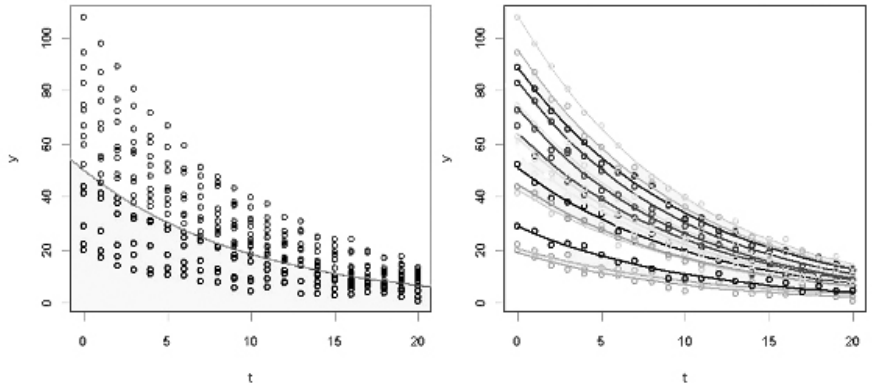

Fig 1. Simulated data according to an exponential behaviour. Left: unstructured, Right: structured per individual.

The results of mixed effects non linear regression analysis of the same data are also shown in Table 1. The explained part (R^2_{adj}) is very high, well over 99% and the standard errors of estimates are low, while the estimated values are more close to the input values.

The estimated biological shift factors can be analysed separately on normality, e.g. using the Shapiro Wilk test. They also can be used to convert the calendar time (as measured) into a biological time (general state of development / maturity / senescence) by adding the individual values of Δt to value of the time points: bt=t+Δt. Especially for graphical representation of the behaviour of all individuals, that is most useful and appealing. In Fig 2 the measured data are plotted against the biological time for all the individuals in the batch.

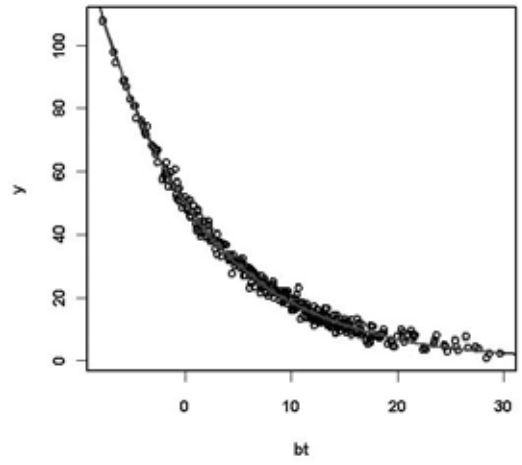

Fig 2. Measured data points (dots) and simulated data (line) versus biological time, based on values from Table 1.

Examples from Practice

Studies dedicated to unravel the behaviour of biological variance were started some 12 years ago (Tijskens & Wilkinson 1996). During the last 5 years, however, the technology was developed and refined and at an increasing rate, studies were published that applied this technology in one way or another (see References).

On every occasion, it is amazing to find out the benefits of the combination of non-destructive (longitudinal data) and mixed effects analysis. The obtained explained parts were always very high, well into to 90% (about 3211 observations over 540 individuals), frequently above 95 to 98%. In the next sections some examples will be presented to provide a feeling for its potential for agricultural and horticultural research.

Colour of Apples in Storage

The colour of horticultural produce very often changes upon storage according to a logistic behaviour. In a recent report, Tijskens *et al.* (2008) described the change in a*-value of 'Granny Smith' apples according to this model. In Eq. 4, the function is shown. The full experimental setup, and the details of the analyses can be found in the original paper.

$$col = \frac{col_{max} - col_{min}}{1 + e^{-k_c \cdot (col_{max} - col_{min}) \cdot (t + \Delta t)}} + col_{min} \text{ with } \Delta t = \frac{1}{k_c} \cdot \frac{\log\left(\frac{col_{max} - col_0}{col_0 - col_{min}}\right)}{(col_{max} - col_{min})} \qquad \textbf{Eq 4}$$

It was found that not only the initial condition in colour (col_0 or the related biological shift factor Δt), but also the lower (green) asymptote (col_{min}) depended on the individual apple. The results of the analysis were so reliable that a small effect of chilling injury could be noticed and modelled. The kinetic constants (rate constants and activation energies) were found to be generic for all three orchards. In Fig 3 the raw data versus calendar time and the standardised colour behaviour versus biological time are shown for storage at 10 °C.

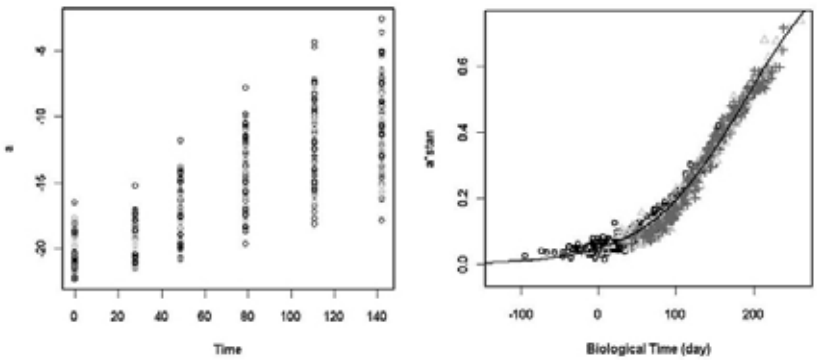

Fig 3. Colour (a*-value) of 'Granny Smith' apples from 3 orchards in Slovenia, stored at 10 °C (Nobs=529, Ngroups=90). Left: Measured data versus calendar time, Right: Standardised data versus biological time.

Firmness of Near Isogenic Lines of Melons

Data on non-destructively measured firmness of near isogenic lines (26 lines in 2 consecutive seasons) was analysed using the biological shift factor systems according to an exponential model (first order kinetics Eq 1-3). Although the lines and the individuals in a line were greatly different with respect to size and shape, for each season the rate constant could be estimated in common (fixed effects) using mixed effects non linear regression analysis, while the biological shift factor and the asymptotic value were estimated for each individual fruit (random effects). The obtained explained parts were 97% (2005: 533 observations in 63 groups) and 91% (2006: 829 observations in 76 groups). A clear effect of the isogenic lines could not be detected. In Fig 4 the raw data, indicating the variation in a boxplot, and the standardised colour behaviour versus biological time are shown.

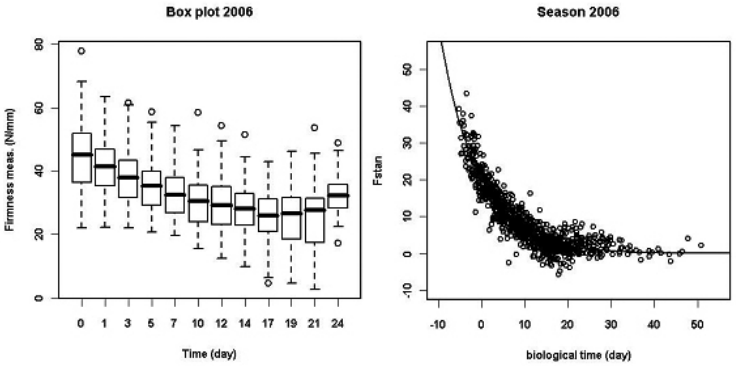

Fig 4. Firmness of NILs of melons, season 2006 (Nobs=829, Ngroups=76). Left: box plot of raw data, indicating the range and variance, versus time, right: standardised firmness versus biological time.

Colour and Firmness of Nectarines

The absorption of fruit flesh of nectarines (strongly related to the chlorophyll content of the fruit flesh) was measured using Time Resolve reflectance Spectroscopy using laser light at 670 nm. The absorption coefficient was determined at harvest (Tijskens *et al.* 2007) and in a separate experiment during storage (Tijskens *et al.* 2006a). The TRS absorption coefficient μ_a was analysed according to a logistic behaviour (Eq 4) using mixed effect non linear regression analysis. The kinetic rate constant could be estimated in common (fixed effects), while the biological shift factor was estimated separately for each fruit (random effects). The obtained explained parts were well over 96% for storage at 20 °C and around 85% at 10 °C. The difference in reliability is probably due to the increasingly relative importance of the technical variation (ε_t). The estimated biological shift factor for the stage of development expressed as light absorption, was used as an estimate for the biological shift factor of the same fruit, but now expressed as firmness. Destructively measured firmness data were analysed using simple non linear regression analysis, but including the variation in stage of development as estimated from the light absorption coefficient, only determined at harvest. In Fig 5 some results are shown for raw data and generic firmness behaviour for one cultivar stored at 20 °C.

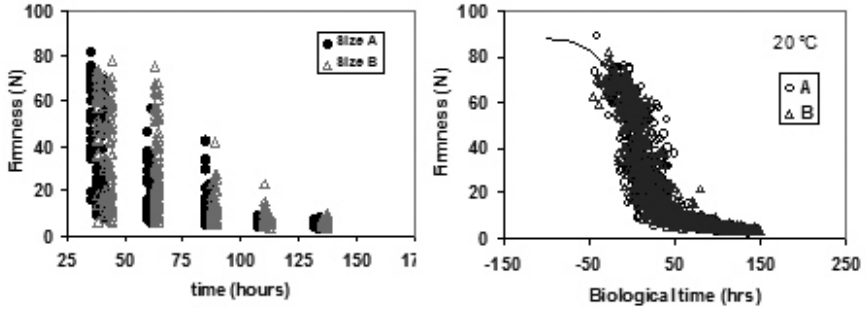

Fig 5. Firmness of nectarines (cv 'Spring Bright', season 2004) at 20 °C. Left: raw data versus calendar time after harvest, right firmness versus biological time, estimated on the biological shift factor of flesh colour (Nobs=1738).

Water Loss

Weight of produce can easily be measured non-destructively, with almost no technical variation. Applying this measuring system repeatedly on individual fruit makes it possible to include the variation in the analysis. Moreover, it makes it possible to pinpoint where the variation resides. Applying mixed effect non linear regression analysis based on an exponential behaviour, which is a massive simplification of the generally applied diffusive model (Eq 5), extremely high explained parts where obtained with R^2_{adj} well over 99% (Tijskens *et al.* 2010), irrespective of size, volume, area or even skin resistance.

$$WL = WL_r \cdot (e - 1^{-k \cdot t}) \qquad \textbf{Eq 5}$$

The variation in water loss in plums, melons and mandarins could be completely attributed to the fraction of weight, i.e. water available for transpiration. Based on these results, it was concluded that the generally diffusive approach, applied for the past 50-60 years, is not valid (enough) and that more dedicated research should be conducted to unravel the real mechanisms and processes ruling this water loss, by applying mixed effect analysis on dedicated longitudinal data. In Fig 6 some results are shown for melons and plums.

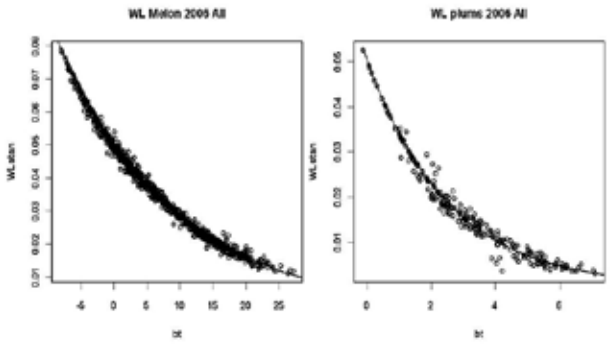

Fig 6. Behaviour of individually measured weight loss of melons (Nobs=834, Ngroups=77) and plums (Nobs=240, Ngroups=60) for one season versus biological time (t+Δt). The standardised weight loss is expressed as the fraction of weight that still can be lost (WL_r-WL).

Conclusions

Non destructive measuring techniques make it possible to gather longitudinal data, measuring the same individuals over time. Applying mixed effect regression analysis using the coded individuals as random factor, more reliable analyses can be obtained. Usually the obtained explained part (R^2_{adj}) increases from about 40 to 60% to more than 90%, sometimes to more than 99%. A clear picture can be obtained where the major impact of the omnipresent biological variation resides in the product or in the mechanism. The remainder of the variation, that is that part of the variation in the data that is not explained by the model or the technical variation, can be more clearly extracted and if necessary analysed further.

In short, the combination of non-destructive measuring techniques, longitudinal data and mixed effects analysis is a very powerful combination to increase the knowledge of produce and procedure to grow and store product in a fashion dedicated to maintain quality and health promoting compounds.

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05. NIRS DETECTION OF MOLDY CORE IN APPLES

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Abstract
Moldy core of apples is undetectable until the fruit is cut or bitten into, it can therefore pose serious problems to both producer and consumer. Removal of diseased fruits prior to storage would be most desirable. The objective of this study was to evaluate the ability of VIS-NIR mini-spectrometers to detect moldy core in apples, on line. An apparatus which is qualified for online NIRS (near infrared spectrometry) measurements was developed based on off-the-shelf mini-spectrometers. 'Top Red' apples, were collected from several orchards before and during the commercial harvest, and were stored at 0°C pending the tests. The data were analyzed by chemometric procedures, specifically, by partial least squares regression (PLSR), and were classified by means of canonical discriminant analysis. The canonical variables were represented by the latent variables of the PLS models, which were based on the spectra. The accuracy of the classification results was high when the moldy fraction threshold was set at 5%; in such a case the mold covers only the seed carpals of the fruit, where it might remain without really damaging the fruit. Improvements should aim to reduce errors in classifying low-level damage, and also in misclassifying some healthy fruits. The rate of testing (1 s per fruit) is acceptable for quality control purposes, but should be accelerated for future packing-line implementation.
Keywords: *Alternaria alternata,* Fruit Quality, Near Infrared Spectroscopy

Introduction
Generally, mechanical and manual sorting are based on external indices and lack the ability to determine essential internal quality attributes. Non-destructive, rapid evaluation methods are in growing demand by growers, packinghouse operators and consumers. Moldy core of apples is undetectable until the fruit is cut or bitten into and can pose as such a serious economic problem to producer and consumer alike. The disease can be caused by a number of saprophytic fungi, of which the most prevalent are *Alternaria alternata* and *Cladosporium herbarum*. Infection occurs through the style of the flower and the disease develops in the orchard within fruit of open calyx cultivars, such as 'Red Delicious'. The infection, which spreads out from the seed cavity during fruit development, can be either dry (*A. alternata*) or wet (*C. herbarum*). At harvest, the former is the predominant cause, but during storage the latter develops more rapidly (Reuveni *et al.* 2002). Removal of diseased fruit prior to storage would be most desirable. Consequently, a reliable, non-destructive method for detecting and grading such fruit would be useful for the industry. The objective of this study was to evaluate the ability of CCD based hyper-spectral spectrometers to detect on-line moldy core in apples. Several types of spectrometers were tested in stationary and conveying conditions.

Near infrared spectroscopy (NIRS) is a non-destructive technology, which could supply rapid, quantitative evaluation of quality indices. Using statistical regression analysis, it can be used to determine ingredients such as water, sugars, dry matter, starch etc. The ability to detect internal disorders by NIR has been demonstrated in several studies (Upchurch *et al.* 1997; Clark *et al.* 2003). The commercial NIR instrument such as the F5 (Sacmi, Italy) is quite expensive. The objective of the present study was to evaluate the ability of a high speed, low cost CCD based spectrometer to detect moldy core in apples.

Materials & Methods

A preliminary study was conducted to test the ability of two spectrometers to detect internal decay caused by *A. alternata* in a stationary condition. The first was Liga (MicroParts, Germany) based on 64 diode array *InGaAs* in the range 1100-1750 nm, and the second S2000 (Ocean Optics, USA), operating in the range of 530 -1100 nm. The spectral analysis of these measurements showed promising results for the S2000. Based on these results, a new instrument was developed, consisting of a rotating table, the S2000 spectrometer, special cells to support the fruit and an illumination unit (Fig 1). The device is controlled by a PC. Specially designated software was developed for data acquisition and online analysis. A cylinder of white Teflon installed into the rotating plate is used as a reference background for transmittance spectra.

'Top Red' apples were picked from several orchards before and during commercial harvest. Some samples were stored at 0°C until used for testing. Twenty-one batches varying in sample size from 40 to 200 apples and totalling 955 fruit were examined with the NIR equipment. The fruit were scanned in transmittance mode with an integration time of 80 ms. The complete scan time was 1 s per fruit. Each intact apple was scanned three times in three placement positions, differing by 120° round the equator, relative to the stem axis. After scanning, apples were halved through their equator and the cut surface was photographed with a digital camera (Fig 2). The photograph was analyzed using an image analysis program, which evaluated the decay level as the ratio between the decayed area and the total area of the cut surface of the fruit.

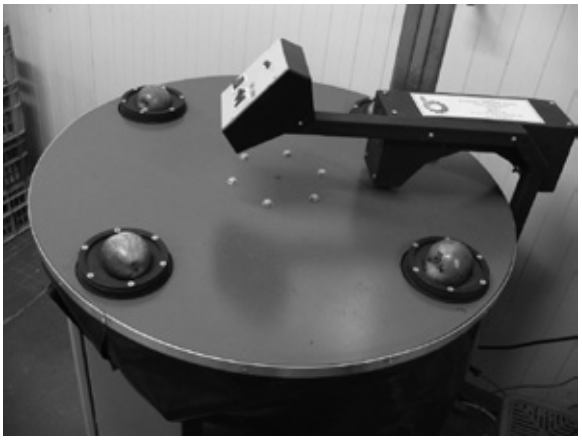

Fig 1. Overview of rotating table with special cells and illumination device for NIR transmittance measurements in motion.

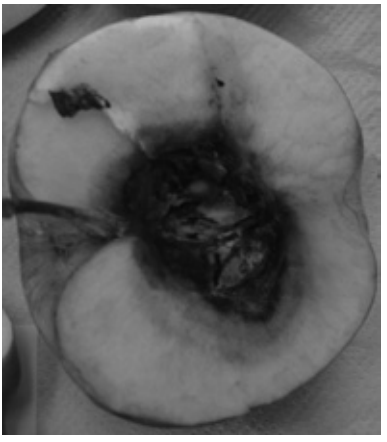

Fig 2. Cut apple with view of advanced moldy core.

The spectral data were analyzed by chemometric procedures and Partial Least Squares (PLS) regression procedure was used for model development. PLS ensures that the loadings and scores reflect variance related to the varying constituent concentrations, by incorporating concentration information into the data reduction step (Martens and Naes, 1989). The raw transmittance spectra and manipulated spectra were analyzed with MATLAB © software. Comparisons were drawn between PLS performance related to treatment of the raw spectra T (transmittance), the first derivative of T (D1T), Log (1/T) and its second derivative (D2(Log(1/T)).

The optimal value for k, the number of factors to be used in the analysis, was obtained by predicting independent sets of samples for different values of k and determining the value of k, for which the root-mean-square prediction error was at a minimum or not significantly different from the minimum. This error is called the Standard Error of Prediction (SEP), also known as root-mean-square prediction error, and has been described and used as a measure of standard deviation for total bias (Biddy & Toutenburg 1977). The

SEP was estimated by cross-validation: one sample was removed from the calibration set, a model was developed by applying PLS to the remaining samples, and the decay level of the initially removed sample was predicted. This process was repeated for every individual sample in the calibration set.

Outliers were rejected according to statistical criteria (Rousseeuw & van Zomeren 1990). In these procedures, the leverage and the residuals of the multilinear regression defined the relevant zone for outlier data points. The spectra were also used to classify the samples into groups of fruit with different decay levels. Classification was performed by means of Canonical Discriminate Analysis. First, the canonical variables were represented by the latent variables of the PLS models that had been built from the spectra. The first N canonical variable calculated for each sample, was then used in discriminate analysis to assign samples to their respective groups. The canonical variables were used as independent variables and the decay level ranges were used as the class labels. Classification was done with the Maximum Likelihood method. One-third of the data were used for training the classifier, with the remainder used for validation (Duda *et al.* 2001).

Results & Discussion

Transmittance spectra for 'Red Top' apples are presented in Fig 3 showing the differences between rotten and healthy apples. For each spectrum, the level of decay is marked as the ratio of diseased to total area of the cut surface, indicating that 0.0 is a healthy fruit. However, as expected, results of whole batch spectra revealed a lot of overlapping, necessitating the application of chemometric methods for analysis.

The results of the SEP and number of factors obtained from the PLS regressions are presented Table 1.

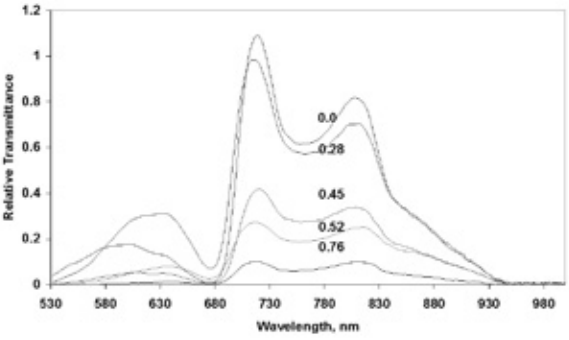

Fig 3. Example of transmittance spectra for 'Red Top' apples with different levels of moldy core. Numbers indicate area of fruit flesh affected by moldy core.

Table 1. PLS analysis of different models for spectral treatment.

	SEP	SEC	Latent factor	r
T	0.053	0.048	10	0.77
T (D1T)	0.052	0.049	4	0.77
Log (1/T)	0.052	0.046	10	0.79
D_2Log(1/T)	0.056	0.052	2	0.72

The table includes results calculated for the raw spectra (T), their first derivatives (D_1T), absorbance (Log (1/T)) and second derivatives (D_2Log(1/T)). It shows that from the point of view of SEP the Log (1/T) model has an advantage. However, the low number of latent factors for D_1T implies the robustness of this model. Example of PLS prediction results is shown in Fig 4.

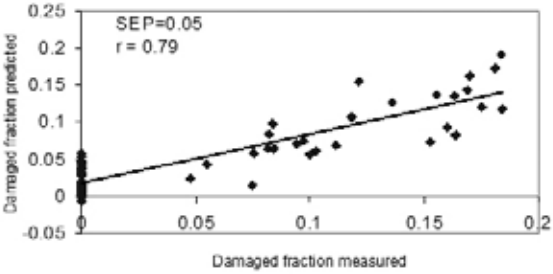

Fig 4. Typical prediction results for detection of moldy core in Top Red apples for Log (1/T) using replicate averages of 3 positions for each fruit.

The results of discriminate analysis are presented in a confusion table (Table 2), with groups of 0, 0-0.1, 0.2-0.3 and 0.3-1.0 levels of moldy core. The diagonal elements, from upper-left to lower-right, describe the accuracy of the classification results and should ideally reach the total number of samples. The off-diagonal elements, ideally, should be zero, and the higher the value of the off-diagonal elements, the greater will be the detection error. The results show a high classifying efficiency for all groups (86-100%).

Table 2. A confusion table depicting results of cluster analysis for one of the batches.

NIR results	Laboratory results				
	0	0-0.1	0.1-0.2	0.2-0.3	0.3-1
0	132	5	0	0	0
0-0.1	12	36	7	0	0
0.1-0.2	0	1	55	2	0
0.2-0.3	0	0	1	13	0
0.3-1	0	0	0	0	18
sum	144	42	63	15	18
error	12	6	8	2	0
Efficiency (%)	92	86	87	87	100

Conclusion

A newly developed NIR system showed promising results for detecting moldy core in ‹Red Top› apples. Improvements need to be made for decreasing errors with regard to low levels of decay as well of misclassifying healthy fruit. The testing rate (1 fruit per s) is acceptable for quality control purposes but will have to be increased for future online implementation.

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06. CHLOROPHYLL FLUORESCENCE IMAGE ANALYSIS FOR NON-DESTRUCTIVE MONITORING OF PHYSIOLOGICAL CHANGES IN FRESH AND FRESH-CUT PRODUCE

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Abstract

Chlorophyll a fluorescence analysis (CFA) is a sensitive indicator of the stability and efficiency of photosynthesis. This fundamental physiological process closely reflects the effects of internal (maturation, aging, senescence etc.) and external (salinity, drought, heat, cold stress etc.) factors on the physiological capacity or vitality of plants. In contrast to many other techniques, CFA can be actually applied remotely and non-invasively. Hence, this method has found widespread applications in plants science. During recent years, CFA has been accepted as a valuable tool to study the metabolic activity of vegetables and salad greens, and some fruits in postharvest. A number of different fluorescence parameters can be used to comprehensively characterize various aspects of photosynthetic performance. For this purpose, commercial chlorophyll fluorescence imaging (CFI) systems may be advantageous because they provided information on both spatial and temporal dynamics of photosynthesis. This yields information on various physiological aspects that are affected by postharvest handling and processing. CFI can, thus, help to objectively, rapidly and non-destructively evaluate and characterize the internal quality of green produce and its changes at many steps of the entire postharvest chain. For a proper application of CFA, a comprehensive knowledge of the physiological background of chlorophyll fluorescence and fluorescence analysis is helpful. Hence, a brief introduction of the physiological basics will be given here. Furthermore, information about alternative approaches to analyse fluorescence data as well as the current technical standard of an available CFI device will be provided. Finally, recent applications of this technique should illustrate the use of this method in fresh food quality research.

Introduction

Effective monitoring of product quality and safety has become an essential demand for growers, packers, distributors, wholesalers and retailers; pre harvest and during the entire postharvest handling chain. For this purpose, instrumental measurements may provide a means to rapidly, objectively and non-destructively characterize and quantify produce defects, overall quality changes and safety problems (Abbott 1999). Many techniques and methods have been developed, improved or adapted for application in this field; most of them are focused on imaging and analysing the optical produce properties. Methods currently investigated or yet in use are conventional RGB image analysis, UV/VIS and NIR-spectroscopy, fluorescence spectroscopy, X-ray- and NMR tomography, and chlorophyll a fluorescence analysis (Abbott 1999). All these techniques rely on the evaluation of physiological or biochemical capabilities of the investigated product. Furthermore, a high actual or potential metabolic activity of a produce also implies a high internal quality.

In chlorophyll-containing, green tissues of fruits and vegetables, photosynthesis is a metabolic pathway of extraordinary importance, comprising many different highly regulated and complex biophysical and biochemical reactions. Hence, measuring the photosynthetic activity and/or its changes offers a promising tool to quantify or predict produce quality and to characterize plant responses to different stressors (von Willert *et al*. 1995). Because photosynthesis is closely integrated into the metabolic network of plant tissue it sensitively responds to many internal (maturation, aging, senescence etc.) and external (salinity, drought, heat, cold stress etc.) stimuli potentially affecting the physiological capacity or vitality.

Chlorophyll a fluorescence analysis (CFA) has meanwhile been widely accepted as an elegant and comprehensive method to indicate photosynthetic activity (von Willert *et al*. 1995; Maxwell & Johnson 2000). As a sensitive indicator of the stability and the efficiency of photosynthesis, CFA has also been applied to studying the metabolic activity of vegetables and salad greens, and some fruits in postharvest during recent years (De Ell *et al*. 1999; Herppich 2002). In contrast to many other techniques, CFA can be applied truly remotely and non-invasively. For this purpose, chlorophyll a fluorescence image analysis (CFI), provided by recently commercially available systems, is advantageous over spot measurements with "normal" glass-fibre fluorometers (von Willert *et al*. 1995) because imaging reveals both the potential spatial and temporal dynamics of photosynthesis (Nedbal *et al*. 2000).

For the comprehensive characterization of the various aspects of photosynthesis, the stability and efficiency of the photosynthetic apparatus and the degree of activation, protection and damage, a large number of different fluorescence parameters are available (Baker *et al*. 2007). Although there are also different approaches to measure and to analyse chlorophyll fluorescence data available, for the sake of simplicity and due to its high flexibility, only the 'conventional' fluorescence analysis according to Schreiber *et al*. (1986) using the 'PAM-fluorometry' will be dealt with in the following.

In general, for a proper application of CFA a comprehensive knowledge of the physiological background of chlorophyll fluorescence and fluorescence analysis is indispensable. Hence, a brief introduction of the physiological basics of CFA and the techniques of CFI will be given. Finally, recent applications of CFI should illustrate the current application of CFI in fresh food quality research.

Chlorophyll Fluorescence – Some Basics

In the chloroplasts of green plant tissues, the chlorophyll a molecules are incorporated at fixed positions in specialized protein complexes, the photosystems (PSI and PSII), and several antenna and light harvesting complexes (LHC). Only outer antenna complexes and LHC also contain chlorophyll b. The chlorophylls, which are the most important light harvesting pigments are able to absorb photons in the wavelength range of 400 to 480 nm (blue) and 630 to 700 nm (red). They are accompanied by smaller amounts of carotenoids, either also functioning in photosynthetic energy absorption, as structural or as photoprotective pigment molecules. The photosystems, along with others protein complexes are located within the thylakoids, the inner chloroplastic membrane network. PSII and PSI, some other proteins and electron transport metabolites form the photosynthetic electron transport chain, which primarily helps to convert the absorbed light into metabolically usable energy (ATP) and reduction equivalents (NADPH/H+). These equivalents are mainly used photochemically in the biochemical reactions of photosynthesis for the assimilation of CO_2 into sugars.

On the other hand, in photosynthetically active plants, chlorophyll a molecules of PSII may also radiatively emit part of the absorbed energy as red fluorescence light of - somewhat longer wavelength (mainly between approximately 660 nm and 760 nm) and, hence, lower energy. Total fluorescence can make up 30 to 50% of the total absorbed light energy. However, due to effective re-absorption by other chlorophyll molecules, net-fluorescence will be only 2 to 3% under normal conditions. Furthermore, only the energy equivalent to red is used photochemically. In contrast, the energy of "blue" photons in excess of the "red portion" can be dissipated as heat by chlorophyll molecules. Furthermore, in a concerted action with carotenoides and, especially, xanthophylls, absorbed energy in excess to what can be used photochemically may be safely dissipated as heat as part of complex photosynthetic protection mechanisms.

If in a stress situation (e.g. heat, cold, frost, drought, anoxia, salinity, light,…) light energy supply exceeds its demand in biochemistry, the above mentioned series of protective mechanisms are further activated and energy flow into heat and, to a smaller extent, into fluorescence increases. Because fluorescence light is relatively simple to measure, it may be used as an indicator of many disturbances in photosynthesis.

Fluorescence Transients

However, modern fluorometers do not only record the continues steady-state fluorescence. Based on the so-called Kautsky-effect (cf. von Willert *et al*. 1995) and in combination with the saturation-pulse technique (Schreiber *et al*. 1986), information on the maximum and the actual activity, and the integrity of PSII as well as on the relative contribution of photochemical and thermal (non-photochemical) energy dissipation can be obtained. For a comprehensive analysis, the produce must be dark-adapted for a certain time, which "switches off" non-photochemical energy dissipation and sets the photochemical reactions of CO_2 assimilation to a "quiescent state". Illumination with short weak photosynthetically inactive light flashes (approx. 5 μmol m^{-2} s^{-1}) induces the initial fluorescence (F_0) arising from chlorophyll a molecules of PSII antenna complexes (von Willert *et al*. 1995). A short (1 s) saturating light pulse (SP), which rapidly excites all chlorophyll molecules, elicits a maximum fluorescence signal (Fm). Because any photochemical or non-photochemical energy dissipation is prevented, fluorescence is the only pathway of de-excitation.

If the object is afterwards irradiated with a continuous actinic light, driving photosynthesis, the fluorescence signal changes in a characteristic pattern, the so-called Kautsky-curve (von Willert *et al*. 1995). The initial rise from F_0 to the fluorescence peak F_p reflects a complex series of reactions including primary charge separation and electron flow in PSII and in the entire photosynthetic electron chain. The decline of the fluorescence signal from F_p to the terminal steady-state fluorescence (F_t) is partially governed by the initial induction and the final fine tuning of all photochemical processes such as the assimilation of CO_2. Furthermore, the activation of the non-photochemical mechanisms further leads to the quenching of fluorescence. If, in the steady state with fully running photosynthesis, a saturation pulse is given, an intermittent maximum fluorescence (F_m') signal can be recorded. This F_m' is smaller than F_m because of the consisting non-photochemical energy dissipation. On the other hand, the fluorescence rise from F_t to F_m' points out the part of potential fluorescence that is currently quenched by photochemistry. Finally, the fluorescence after switching off the actinic irradiation or after rapidly darkening the object yields F_0', the dark fluorescence emitted from antenna chlorophylls in the 'light-adapted' state.

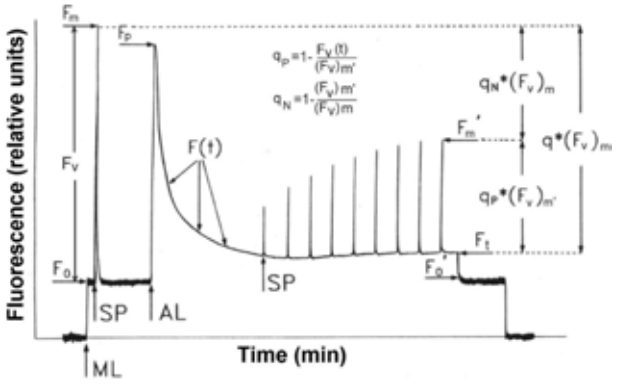

Fig 1. Example of a typical fluorescence transient as recorded with a PAM-fluorometer (ML = measuring light, SP = saturating pulse, AL = actinic light). Additional information is given in the text.

Chlorophyll Fluorescence Parameter and their Meaning

From all these fluorescence signals, quite a (still increasing) number of useful, more or less well defined parameters have been proposed and may be derivated by simple calculations. Very easy to measure is the ratio of the variable fluorescence F_v ($F_v = F_m - F_0$) and F_m of dark-adapted samples. F_v/F_m is an indicator of the potential maximum photochemical quantum efficiency of PSII, and is thus, with some precautions, a valuable tool to determine both capacity and stability of photosynthesis (Krause & Weis 1991; von Willert *et al*. 1995) and its direct response to internal and external constraints.

When a saturation pulse is applied on irradiated samples, the measured fluorescence signals, F_t and F_m', can be used to estimate the fraction of the maximum photochemical quantum efficiency of PSII that is still operating. This actual photochemical quantum efficiency, calculate as $(F_m'–F_t)/F_m'$, is often termed $\Delta F/F_m'$ but also a confusing wealth of other designations can be found (Y, Φ_{PSII}, F_q'/F_m',…). It has been shown that, as the ratio of used to absorbed energy, $\Delta F/F_m'$ is directly proportional to the linear electron flow through PSII. Hence, it can be used to estimate the electron transport rate (ETR) by multiplying it with the amount of absorbed photons I_{abs} (the product of the incident photons and the absorption coefficient) and the relative distribution of photons between PSII and PSI as $ETR = \Delta F/F_m' * I_{abs} * 0.5$. Here, the (over) simplification is generally accepted that the absorption coefficient is 0.84 and the distribution is equal. Under some precautions, ETR is a valuable indicator of the gross photosynthesis.

The comparison of the variable fluorescence measured on a irradiated sample and that of a dark adapted one allows the estimation of the activity of non-photochemical mechanisms because they are the reason for the reduction of F_m to F_m'. Accordingly, a non-photochemical quenching coefficient (Schreiber *et al*. 1986) is defined as $q_N = 1- F_v'/F_v = 1-(F_m'-F_0')/(F_m-F_0)$. Likewise, the activity of photochemical quenching may be estimated from the rise of the fluorescence signal from F_t to F_m', which reflects the transient closure of all (still open) PSII reaction centres, i.e. the transient inhibition of photochemistry. Hence, the photochemical quenching coefficient q_P is defined as $q_P = \Delta F/F_v = (F_m'-F_t)/(F_m-F_0)$. In a different approach based on the Stern-Volmer-equation, non-photochemical quenching (NPQ) is calculated as $NPQ = (F_m-F_m')/F_m'$ (cf. Klughammer & Schreiber 2008). NPQ is directly related to the total amount of quenchers involved in the non-photochemical protection mechanisms, while q_N closely reflects their actual functioning.

Besides many other more or less meaningful fluorescence parameters, calculation of complementary PSII quantum yields has become very popular during recent years (Klughammer & Schreiber 2008). Similar to the terms for dissipation of absorbed energy as heat $(D = 1-(F_m'-F_0')/F_m')$, photochemistry $(P = \Delta F/F_v)$ and the unexplained excess (E = 1-D-P) proposed by Demmig-Adams *et al*. (1996), more recently quantum yields of photochemical energy conversion $(YII = \Delta F/F_v)$, and of regulated $(YNPQ= F/F_m' – F/F_m)$ and non-regulated non-photochemical energy loss in PSII $(YNO = F/F_m)$ have been developed (Klughammer & Schreiber 2008). The latter approach may simplify the measurements because it does not include the assessment of F_0 and, most important, F_0'. However, it, of course, still demands the determination of F_m, which can be complicated in the field and under conditions of long-term stress effects e.g. due to long-lasting NPQ components (Herppich 2000).

Chlorophyll Fluorescence Image Analysis

CFI was performed with an open system (FluorCAM 640MF, PSI, Brno, Czech Republic) measuring sequences of fluorescence images according to user-defined settings of time intervals and irradiance. Recommended maximum sample size is 10 cm×13 cm. Two sets of 345 orange LEDs (λ_{max} = 620 nm) are used to provide the weak measuring flashes as well as continuous actinic irradiation. Short-term saturation light pulses (max. 2500 μmol photons m^{-2} s^{-1}) are generated by a halogen lamp (24 V, 250 W) equipped with an electronically controlled shutter. Synchronously with the measuring light flashes, a 12-bit CCD camera (with F1.2/2.8-6 mm objective and short-pass filter) records fluorescence images (512x512 pixel) with a maximal frequency of 50 images s^{-1}. Measurements are controlled and data are analysed by a WinXP compatible software (FluorCam 6; PSI). With this system, all the above mentioned fluorescence signal can be obtained. For practical reasons in many investigations, a short standard protocol was used including dark-adaptation (10 min) and illumination of the sample with the measuring light to induce F_0, and then F_m was elicited by a 1 s-saturation pulse. After a short relaxation phase of 20 s, actinic light was provided for additional 30 s followed by a 5 s dark period in which the short-term relaxation of F_0 could be obtained. This protocol was used because only a proper Kautsky-effect can truly indicate the vitality of the sample. Even in a fully inactive e.g. dehydrated sample, fluorescence rises in response to a saturation pulse, simply because of the increased light absorption and, hence, fluorescence emission.

CF applications

Pigment Changes in Ripening Sweet Pepper Fruit

For green-ripe sweet pepper cultivars further fruit development, as indicated by chlorophyll degradation and capsanthin accumulation, i.e. the colour change from green to red may reduce produce quality. To optimize postharvest quality maintenance, the dynamics of chlorophyll degradation and carotenoid synthesis and their variation by various storage conditions was non-destructively investigated on green-ripe and full-ripe marketable fruit of the sweet pepper cultivar Kárpia F1. CFI was applied to analyse chances in the photosynthetic activity (F_v/F_m) of whole fruit, while the accumulation of capsanthin was evaluated by the analysis of digital RGB-images. CFI enables the characterisation of the chlorophyll content dynamics independent of the variation in surface colour.

In sweet pepper fruit, stored at 20 °C, capsanthin synthesis is a rapid process, which can be completed within one day (Fig 2). In contrast, chlorophyll degradation occurs more or less continuously and is completed only 4 to 5 d after the fruit was totally red coloured. Digital and chlorophyll fluorescence imaging showed that in ripening sweet pepper chlorophyll degradation and capsanthin accumulation occurred independent but effectively at room temperature in packed or unpacked fruit. Thus, low storage temperature but not modified atmosphere packaging can affect ripening and hence, changes in both pigments (Zsom *et al.* 2010).

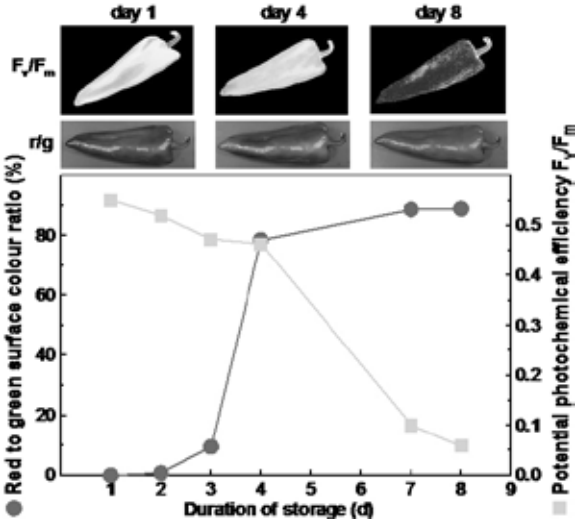

Fig 2. Comparison of the dynamic changes in chlorophyll and carotenoid content of sweet pepper fruit during ripening.

Effects of Continuous Weak Illumination on Packed Fresh Salad Quality

Packaging can maintain the quality of lightly processed fresh salads, reducing mechanical damage, water loss and respiration, and inhibiting browning and microbial decay due to a passively established low O_2 and high CO_2 content of the air (**m**odified **a**tmosphere **p**ackaging, MAP). Lightly processed products retain their physiological activity even in such a specific environment. Illumination and temperature within a display cabinet interactively affect this activity. Optimised illumination may improve quality maintenance of packed photosynthetic active salads, reducing senescence and decay processes. The effects of radiation and temperature on the physiological activity of corn salad and Arugula leaves within the packages can easily and non-invasively be determined using CFI.

The results of long-term (10 d) storage experiments at 20 °C (room temperature) indicated that illumination may better retain salad quality than storage in darkness. It also helps to control the gas composition within the packaging. Even low light intensity effectively improved quality maintenance of the products. Higher photon fluence rates are not necessary but may increase temperature within the

packaging, further reducing product quality. In contrast, illumination, optimised in quality and quantity, can help to retain value-adding substances and reduce senescence of fresh packed products (Fig 3).

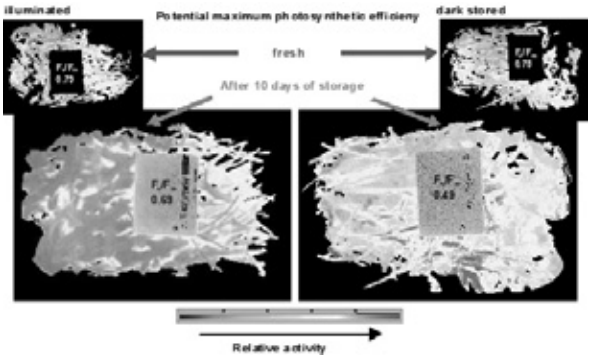

Fig 3. Effect of illumination on potential photosynthetic efficiency of PSII as an indicator of the mean quality of packed Arugula during a 10 d storage at 20 °C.

Evaluation of Heat Treatment Effects

To ensure the distribution of high quality fresh minimally processed products, several preservation methods are currently applied to extend the shelf-life. This may included hot water treatment, which, among other effects, may help to superficial disinfect the produce. However, the window between best effects of the treatment and product damage is often very small. CFI may help to comprehensively characterize the temperature effect on green fruits and vegetables (Schlüter *et al.* 2009).

Even short applications of high, sublethal temperatures (here 45 °C) seriously but reversibly inhibited metabolic activity (F_v/F_m) of intact fresh lamps lettuce leaves (Fig 4). Although applications of 10 and 30 s affected F_v/F_m only temporary and to a minor extent (< 15%), temperature affects the photosynthetic activity, and its ability to recover from heat stress declines with the duration of heat treatment. Furthermore, a small increase of sample temperature to 50 °C resulted in a very pronounced (50%) but still partially (39%) reversible inhibition of F_v/F_m at a 10 s exposure. Any longer treatment causes complete and irreversible damages to the photosynthetic apparatus. Hence, CFI sensitively monitors the short and long-term heat effects and may, thus, help to optimize application of this postharvest treatment.

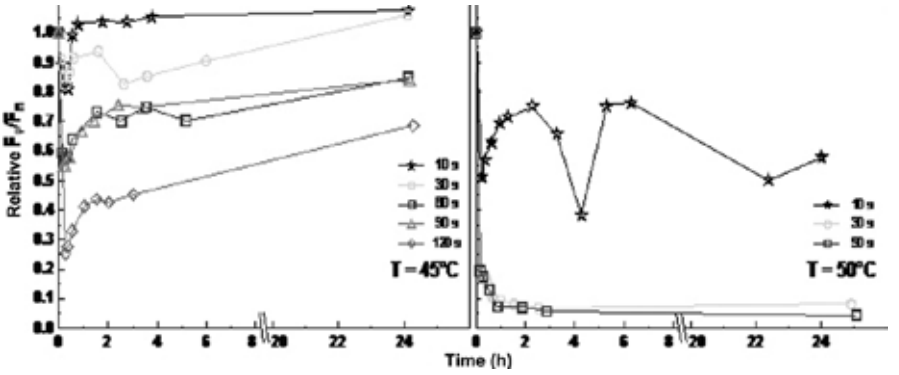

Fig 4. Maximum photochemical efficiency of lamb's lettuce after thermal treatment at 45 °C (a) and 50 °C (b).

Conclusions

Chlorophyll fluorescence analysis is a powerful tool to determine the preharvest and postharvest internal quality of green photosynthetic active produce. However, despite the relative ease of the measurement,

knowledge of the physiological base, and of the actual limits of interpretation of the results are necessary. Chlorophyll fluorescence imaging effectively detects heterogeneity in photosynthetic responses and monitors their local and temporal dynamics.

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07. SHELF-LIFE OF RIPENING 'ROCHA' PEAR: EFFECTS OF THE ORCHARD IRRIGATION REGIME ON QUALITY AND FLUORESCENCE PARAMETERS

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Abstract

The effect of the orchard irrigation regime on the on-shelf ripening of *Pyrus communis* L. var. 'Rocha' fruit was studied and the use of chlorophyll *a* fluorescence as a tool to assess the maturation of fruit was evaluated. Minimal (F_o) and maximal (F_m) fluorescence and maximal photochemical efficiency of PSII reaction centers (F_v/F_m) were measured in pear grown in irrigated (Ir) and non-irrigated (NIr) orchards and stored for two months in cold under normal atmospheric conditions, after which fruits were removed from the refrigerated chambers and held at room temperature (approx. 20 ºC) for 14 days (d). Measurements were made on the day of removal from refrigerated storage (t=0) and after 4, 7, 10 and 14 d at 20 ºC. Total chlorophyll content (Chl a+b) of pears skin was measured as well as the pulp firmness and the total content of soluble solids (SSC). At 0 d at room temperature, Ir fruit, although with a lower chlorophyll content, was firmer than NIr fruit but after 14 d at room temperature no differences were observed between them. No significant differences were generally observed in SSC either between Ir and NIr fruit or during ripening. All fluorescence parameters decreased in Ir and NIr fruit, with F_o and F_v/F_m lower in Ir fruit after 2 months of cold storage while in F_m no significant differences were observed between the irrigation treatments. The correlation of firmness and chlorophyll content with SSC was always weak in both irrigation regimes. In contrast, the correlation between firmness and chlorophyll content was always strong as well as the relationship between firmness and the fluorescence parameters. The maximal fluorescence (F_m) was the parameter that had the best correlation with fruit firmness. The irrigation regimes applied to 'Rocha' pear orchards seems to influence the ripening of 'Rocha' pear fruit with a more steeply ripening of the fruit grown in irrigated orchards characterized by a greater loss of firmness and chlorophyll fluorescence.
Keywords: Chlorophyll fluorescence, fruit ripening, orchard irrigation regime, shelf-life

Introduction

The adoption of a proper irrigation strategy is crucial for sustainability since it could save water and reduce irrigation costs (Behboudian & Mills 1997). However, post-harvest fruit quality must also be taken into account when defining the irrigation regime. Visual fruit sorting in packing houses do not consider intrinsic characteristics such as fruit firmness and sugar content. On the other hand, the techniques to measure those characteristics are destructive. Special attention has been given to the application of chlorophyll fluorescence as a non-destructive method to evaluate the quality of fruit (Song *et al.* 1997). Although it has been shown that the application of this technique is species and variety dependent, the influence of fruit growth conditions on the relation between fluorescence and fruit quality is unknown. The variety 'Rocha' of *Pyrus communis* L. is a Portuguese pear produced mainly in the western region of Portugal. The aim of this study was to understand how the ripening of 'Rocha' pear fruit is influenced by the orchard irrigation regime and to determine possible relationships between chlorophyll fluorescence parameters and fruit quality.

Materials & Methods

'Rocha' pear fruits was collected from irrigated (Ir) and non-irrigated (NIr) orchards and stored at 0 ºC under normal (0.035% CO_2+21%O_2) atmospheric conditions. In Ir orchards each trees received 600-850 liters of irrigation water per year, while in NIr orchards the trees received only rainfall water. During the 2004 fruit growing season (May to July) NIr orchards received only approximately 3% of the water supplied to the Ir orchards. Fruit chlorophyll *a* (Chl *a*) fluorescence was measured with a pulse amplitude modulation fluorometer (PAM 101 Chlorophyll Fluorometer, Germany). Fruit was adapted to dark at room temperature for one hour before the values of minimal (F_o) and maximal (F_m) fluorescence and maximal photochemical efficiency of PSII reaction centers (F_v/F_m) were determined. Firmness (F) and the total soluble solids content (SSC) were respectively determined with a penetrometer FT 327 (TR di Turoni & C.s.n.c., Italy) and a refractometer PR-32 (Atago, USA.). ANOVA, regression and correlation analysis were performed using Origin 7.5 (OriginLab Corporation, USA). Significant correlations were established when $p<0.05$.

Results

At time 0 no significant differences were observed in the SSC and F_m between Ir and NIr. On the contrary, Ir fruit was slightly firmer and had lower F_o and total chlorophyll content and higher F_v/F_m than NIr fruit (Fig 1,2). During ripening, as expected, firmness decreased in fruit from both irrigation regimes. The decrease of firmness on the first 4 d of ripening was more pronounced in Ir fruit (83%) than in NIr fruit (61%) but no differences were found afterwards (Fig 1A).

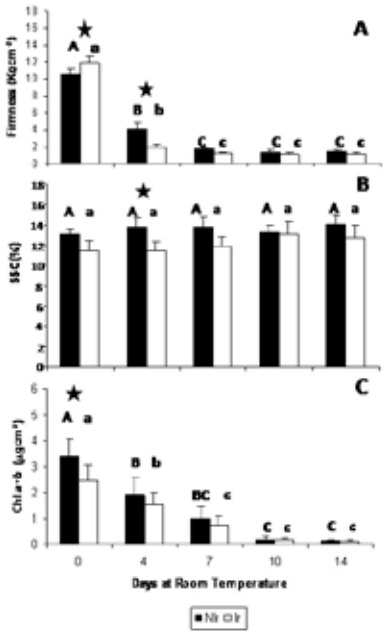

Fig 1. Fruit firmness (1A), total content of soluble solids (SSC) (1B), chlorophyll content (Chl a+b) (1C) of 'Rocha' pear fruit grown in irrigated (Ir) and non-irrigated (NIr) orchards during 14 d ripening at room temperature after two months of cold storage. Different letters indicate significant differences between d at room temperature in each irrigation regime; differences between Ir and Nir are marked with an asterisk (*) ($p<0.05$) (mean + sd, n=5).

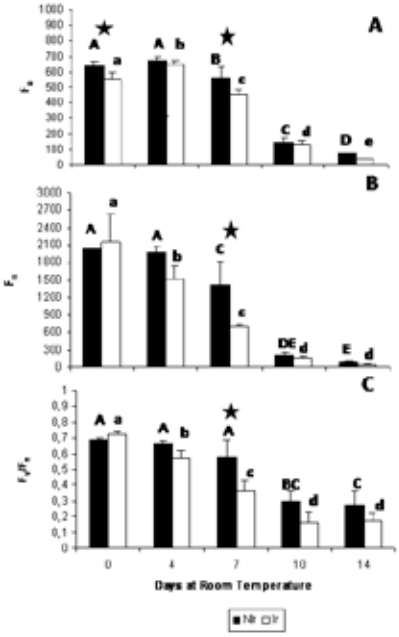

Fig 2. Minimal fluorescence (Fo) (2A), maximal fluorescence (F_m) (2B) and maximal photochemical efficiency of PSII (F_v/Fm) (2C) of 'Rocha' pear fruit grown in irrigated (Ir) and non-irrigated (NIr) orchards during 14 d ripening at room temperature after two months of cold storage. Different letters indicate significant differences between d at room temperature in each irrigation regime; differences between Ir and Nir are marked with an asterisk (*) ($p<0.05$) (mean + sd, n=5).

No significant trend of SSC variation was found during ripening. Although mean daily values of SSC were slightly higher in NIr (13.02-14.01%) than in Ir (11.84-12.70%) only at day 4 the difference was significant (Fig 1B). The decrease of chlorophyll content (Fig 1C) along ripening was similar in both irrigation treatments. However, in the first 4 d at room temperature, the decrease of these pigments was more pronounced in NIr fruit (44%) than in Ir fruit (35%). In both irrigation treatments, the decrease of the total chlorophyll content during ripening seems to follow the softening of the fruit pulp. All fluorescence parameters decreased significantly with the progress of fruit ripening independently of the orchard irrigation regime (Fig 2). The main differences between the irrigation regimes were found after 7 d (d) of ripening, when F_m and F_v/F_m already decreased, respectively, 67% and 49% in Ir fruit but only 31% and 16% in NIr fruit.

The SSC (Fig 3A) almost did not change as firmness decreased. Chlorophyll content of Ir fruits was relatively unchanged in the firmness range 14 - 4 kg cm^{-2} while in NIr fruits it showed a decreasing trend starting at the highest firmness measured (Fig 3B). Fruit firmness had a non-linear relationship with chlorophyll fluorescence parameters in both orchard irrigation regimes (Fig 4A-C). The maximal fluorescence (F_m) (Fig 4B) was the parameter that had the best correlation with fruit firmness (around 0.86 in NIr fruit and 0.9 in Ir fruit), followed by F_o (Fig 4A) and F_v/F_m (Fig 4C). The lowest correlation coefficients were found between F_o and firmness of Ir fruit (0.74) and between F_v/F_m and fruit firmness of NIr fruit (0.78).

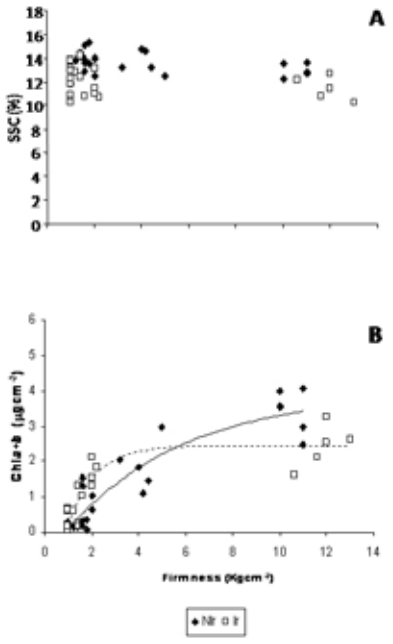

Fig 3. Correlation of total soluble solids content (SSC) (3A) and chlorophyll content (Chla+b) (3B) with firmness (F) of 'Rocha' pear fruit grown in irrigated (Ir) and non-irrigated (NIr) orchards during ripening at room temperature after two months of cold storage. Points represent measurements taken during 14 d at room temperature (n=25).

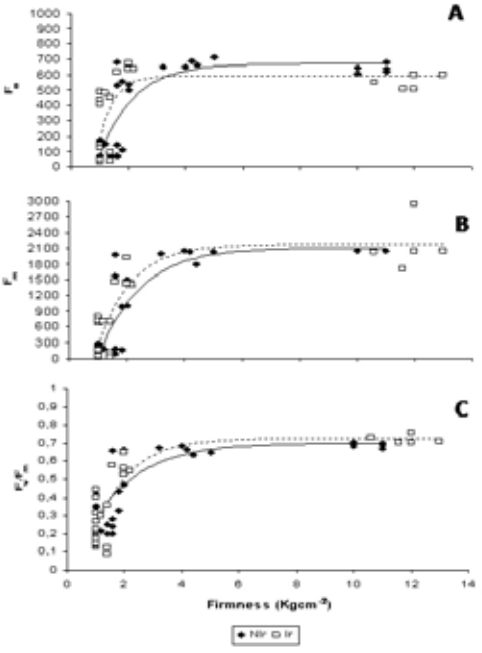

Fig 4. Correlation of minimal fluorescence (F_o) (4A), maximal fluorescence (F_m) (4B) and maximal photochemical efficiency of PSII (F_v/F_m) (4C) with firmness (F) of 'Rocha' pear fruit grown in irrigated (Ir) and non-irrigated (NIr) orchards during ripening at room temperature after two months of cold storage. Points represent measurements taken during 14 d at room temperature (n=25).

Discussion

In spite of the significant initial decrease of firmness, F_v/F_m remained relatively constant after 4 d at room temperature, suggesting that it is not related to early changes in the ripening process. However, in pear, SSC (Fig 1B) almost did not change during ripening, suggesting that it is not a good indicator of maturation, which is also supported by the weak correlation of this parameter with firmness (Fig 3A). As expected, the chlorophyll content of pear skin seems also to be related with fruit maturation, as suggested by the strong (r=0.91) non-linear correlation observed between this parameter and firmness (Fig 3B). Fruit firmness had a non-linear relationship with chlorophyll fluorescence parameters in both orchard irrigation regimes (Fig 4). Cavaco *et al.* (2009) also found that the correlation between firmness and light reflectance at some wavelength bands was markedly different depending on ripening stage. The maximal fluorescence (F_m) (Fig 4B) was the parameter that had the best correlation with fruit firmness (around 0.86 in NIr fruit and 0.9 in Ir fruit), followed by F_o (Fig 4A) and F_v/F_m (Fig 4C). Similar results were obtained by Bron *et al.* (2004) in 'Golden' papaya fruits. The present results indicate that the irrigation regimes applied to 'Rocha' pear orchards influences the shelf-life of 'Rocha' pear fruit, with steeper ripening of the fruit grown in irrigated orchards, characterized by a greater loss of firmness and chlorophyll fluorescence. The chlorophyll fluorescence is a potential tool to assist the quality evaluation and pear fruit maturation. The relationship between chlorophyll fluorescence and quality indexes is cultivar dependent and despite the good results that have been obtained, some problems still remain to be solved. The influence of a large number of environmental parameters during growth and storage, picking time and storage duration can also affect the relationship between chlorophyll fluorescence and quality attributes of fruits.

Acknowledgments

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SECTION 2. PRE-HARVEST FACTORS AFFECTING POSTHARVEST QUALITY AND SAFETY

08. ADVANCES IN PRACTICAL MANAGEMENT OF PEAR RIPENING CAPACITY AND POSTHARVEST DECAY

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Abstract

Most pear (*Pyrus communis*) cultivars are harvested when mature but do not have the capacity to ripen to a buttery-juicy texture without further postharvest conditioning, either with cold temperatures ("temperature conditioning"), ethylene exposure ("ethylene conditioning") at 20 °C, or a combination of the two. It has been assumed that the length of temperature conditioning required to induce ripening capacity is characteristic of the cultivar, and that temperature conditioning is most efficient at normal storage temperatures (0 to -1 °C). At these temperatures, 'Beurré Bosc' pear is expected to need 15 days (d) of conditioning, 'Doyenné' du 'Comice' 30 d, and 'Beurré d'Anjou' 60 or more days. We found that as fruit maturity in the orchard advances, the length of conditioning needed at -0.5 °C decreases linearly. Furthermore, temperature conditioning is satisfied more rapidly as conditioning temperature is increased, up to 10 °C. Combining ethylene exposure and temperature conditioning at 10 °C allows rapid development of ripening capacity, facilitating early marketing of pears with excellent eating quality. With respect to postharvest decay of pears, we have found that postharvest fungicide or biocontrol treatments are relatively inefficient when applied more than 3 weeks after fruit wounding which occurs at harvest, yet pears are typically stored for longer periods before postharvest treatment. Enhancement of fruit calcium through foliar applications during the growing season can reduce the rate of increase in decay risk with delay in postharvest treatment. Application of fungicides regarded as "reduced-risk" by US EPA in the 1-3 weeks before harvest can further reduce decay increase with postharvest treatment delay, and the combination of calcium enhancement plus pre-harvest fungicide treatment can substantially reduce decay risk.
Keywords: fruit calcium, pear ripening, reduced-risk fungicides, temperature conditioning

Introduction

Pear Ripening Capacity

Most pear (*Pyrus communis*) cultivars require postharvest conditioning in order to become uniformly able to respond to warm temperatures (15-25 °C) by softening to a buttery-juicy texture (ripening capacity). Conditioning can be provided by exposure to cold temperatures (temperature conditioning), exposure to ethylene (ethylene conditioning, typically at 20 °C), or a combination of these two types of conditioning (Villalobos & Mitcham 2008). In the pear industry of the USA, two key assumptions have influenced producer practices with regard to conditioning: (1) that the length of temperature conditioning required to induce ripening capacity is characteristic of the cultivar, and (2) that temperature conditioning is most efficient at normal storage temperatures (0 to -1 °C). At these temperatures, 'Beurré Bosc' ('Bosc') pear is expected to need 15 days (d) of conditioning, 'Doyenné du Comice' ('Comice') 30 d, and 'Beurré d'Anjou' ('Anjou') 60+ d. Although there has been some recognition that maturity can influence the duration of the temperature conditioning requirement (Chen & Mellenthin 1981), the precise relationship has not been determined. The objectives of our work were to (1) determine the relationship between fruit maturity at harvest and the length of postharvest temperature conditioning at -0.5 °C for 'Bosc', 'Comice', and 'Anjou' pears; (2) compare the efficacy of temperature conditioning at various temperatures between -0.5 and 18 °C; and (3) identify optimum (fastest) conditioning protocols combining temperature and ethylene conditioning that can be useful as guidelines for early marketing of these three pear cultivars.

Pear Postharvest Decay

Postharvest application of a fungicide or biocontrol agent has long been a key practice in management of postharvest decay of pears intended for long-term cold storage (>3 months). However, treatment with fungicides or biocontrol agents becomes increasingly less effective as the length of time between fruit wounding and natural inoculation at harvest and application of postharvest treatments increases; treatments applied 4-6 weeks after harvest have sharply reduced efficacy compared to treatments applied promptly after harvest (Sugar & Basile 2008). Despite this constraint on treatment efficacy, a large portion of the pear crop is stored for much longer periods before receiving postharvest treatment. The objective of our study was to evaluate pre-harvest applications of calcium and a reduced-risk fungicide in relation to delayed application of a postharvest fungicide.

Materials & Methods

Pear Ripening Capacity

'Bosc', 'Comice', and 'Anjou' pears were harvested at the onset of fruit maturity, locally defined as when average fruit firmness values first fall below 71.2, 57.8, and 66.7 Newtons (N), respectively, and then weekly for 4-7 weeks. Harvested fruit were placed in storage chambers at either -0.5, 5 or 10 °C, then samples were removed after 5, 10 or 15 d ('Bosc'); 5, 10, 15, 20, 25 or 30 d ('Comice'); or 10, 20, 30, 40, 50 or 60 d ('Anjou'). The samples were held in air at 20 °C for seven days, then ripeness was determined by firmess testing using a penetrometer (Fruit Texture Analyzer, Güss, South Africa). Fruit were considered ripe if the average firmness value was below 26.7 N (Bosc), 22.2 N ('Comice'), or 17.9 N ('Anjou'). Additional sets of fruit from the first harvest of 'Comice' and 'Anjou' were treated with 100 ppm ethylene in a sealed room at 20 °C for 24 or 48 h, then moved to normal air at -0.5, 5, or 10 °C. Samples were then removed after 5, 10 or 15 d ('Comice') or 10, 20, 30, 40, 50 or 60 d ('Anjou'). Ripeness was evaluated after seven days at 20 °C as described above. In all experiments, treatments were replicated four times, each replicate representing distinct areas in the source orchard.

Pear Postharvest Decay

'Bosc' pear trees in a 0.5 ha block were either sprayed with solutions of calcium chloride (3.6 g Ca L^{-1}) three times at 2-week intervals beginning in mid-summer, or received no calcium treatment. Calcium-treated or untreated trees subsequently received either fungicide treatment with pyraclostrobin + boscalid (Pristine®, BASF) one week before harvest, or no fungicide treatment. Four replicate trees in a randomized complete block design received each combination of either calcium or no calcium and either fungicide or no fungicide. At maturity, 140 fruit were harvested from each replicate of each treatment combination, and all fruit were wounded with a finishing nail (2 mm diameter×2 mm deep) to simulate stem punctures. Natural populations of pathogenic fungi present on fruit surfaces at wounding were the only source of inoculum for subsequent infections. Twenty wounded fruit from each replicate of each treatment combination were immediately treated (0 d between wounding and treatment) with either water or a solution of thiabendazole (TBZ) at 569 mg L^{-1} applied as a line-spray as the fruit moved across rolling brushes. All fruit were then stored at -0.5 °C. Twenty wounded fruit from each replicate of each orchard treatment combination were removed from storage and treated as above after 1, 2, 3, 4, 6 and 8 weeks of storage. Decay at wounds was evaluated at 12 weeks after wounding.

Results & Discussion

Pear Ripening Capacity

'Bosc' and 'Comice' pears required approximately 15 and 30 d at -0.5 °C, respectively, to develop ripening capacity only when harvested at the onset of harvest maturity. The duration of postharvest temperature conditioning at -0.5 °C necessary for 'Bosc' and 'Comice' pears to develop ripening capacity decreased with

later harvest (Fig 1, 2). The number of days required to induce ripening capacity decreased in a linear fashion, allowing prediction of the conditioning requirement based on the numbers of days after the onset of maturity when the pears were harvested (Sugar & Basile 2009). For 'Bosc' and 'Comice' pears, the slope of the line describing the relationship of harvest date to conditioning duration at -0.5 °C was approximately 0.6. Therefore, for 'Comice' pears the number of days of conditioning at -0.5 °C needed to induce ripening = 30 – (0.6×days after onset of maturity); for 'Bosc' pears, the number of days = 15 – (0.6×days after onset of maturity).

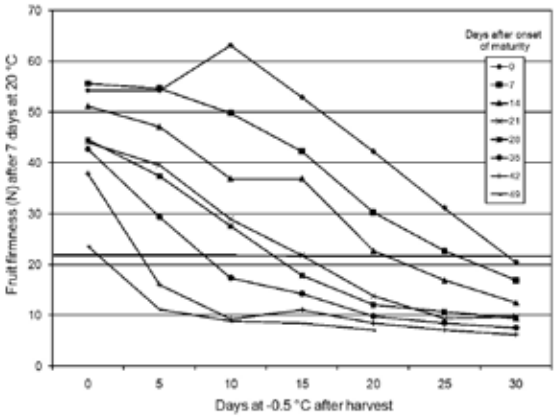

Fig 1. Relationship of fruit maturity at harvest and the duration of postharvest conditioning at -0.5 °C needed to induce ripening capacity in 'Comice' pears. Horizontal line at 22.2 N indicates maximum fruit firmness for a buttery-juicy texture.

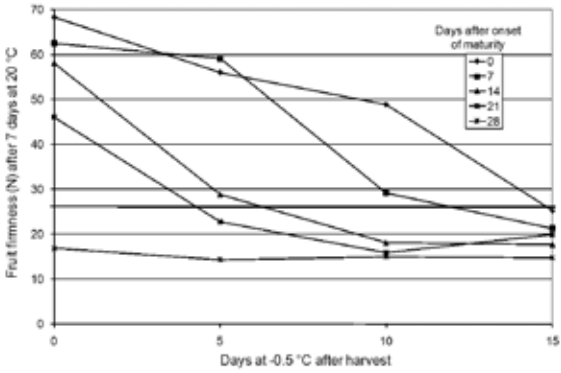

Fig 2. Relationship of fruit maturity at harvest and the duration of postharvest conditioning at -0.5 °C needed to induce ripening capacity in 'Bosc' pears. Horizontal line at 26.7 N indicates maximum fruit firmness for a buttery-juicy texture.

This information should be useful to pear producers in determining the minimum duration of conditioning required based on the actual harvest date, in order to deliver pears capable of ripening. 'Anjou' pears did not develop ripening capacity within 60 d at -0.5 °C, except for the latest harvest date.

Ripening capacity developed in a shorter period of time when fruit were conditioned at warmer temperatures. 'Bosc' pear fruit from the first harvest required approximately 9 d to develop ripening capacity at 5 °C and 5 d at 10 °C. For 'Comice' pears, the optimum temperature for developing ripening capacity appeared to be 10 °C (Fig 3). 'Comice' fruit from the first harvest required 18 d to develop ripening capacity at 5 °C and 12 d at 10 °C. 'Anjou' pears developed ripening capacity after 30 d at 5 °C and 17 d at 10 °C.

We previously found that 'Bosc' pears exposed to 100 ppm ethylene for 24 h did not require further temperature conditioning in order to develop ripening capacity (Sugar & Basile, unpublished). We also identified a protocol for early development of ripening capacity in 'Comice' pears involving 48 h in 100 ppm ethylene followed by approximately 15 d conditioning at -0.5 °C (Sugar & Basile 2006). In the present study, for

both 'Comice' and 'Anjou' pears, the duration of conditioning required decreased with increasing exposure to ethylene from 0-48 h, and with increasing temperature during conditioning from -0.5 – 10 °C (Fig 4, 5). Early marketing of these pear cultivars can be facilitated by ethylene treatment followed by very brief conditioning at 10 °C (Table 1).

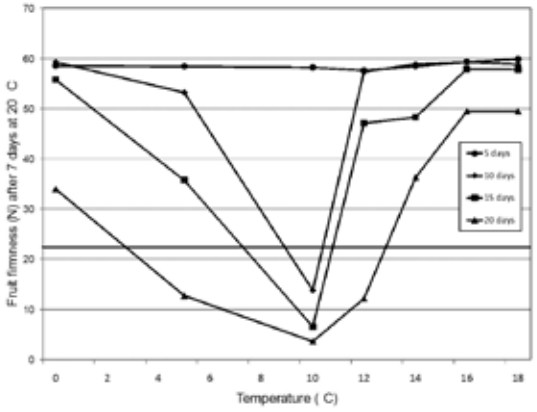

Fig 3. Effects of conditioning temperature and exposure duration on induction of ripening capacity in 'Comice' pears. Horizontal line at 22.2 N indicates maximum fruit firmness for a buttery-juicy texture.

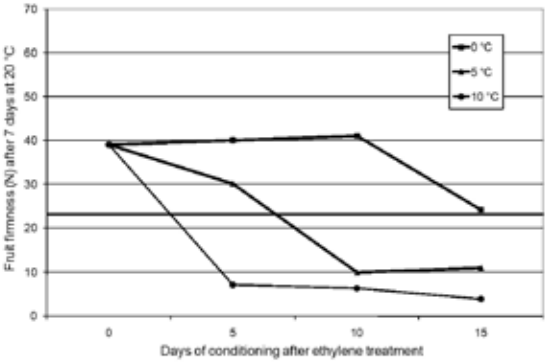

Fig 4. Effects of temperature on conditioning of 'Comice' pears following 24 h in 100 ppm ethylene at 20 °C. Horizontal line at 22.2 N indicates maximum fruit firmness for a buttery-juicy texture.

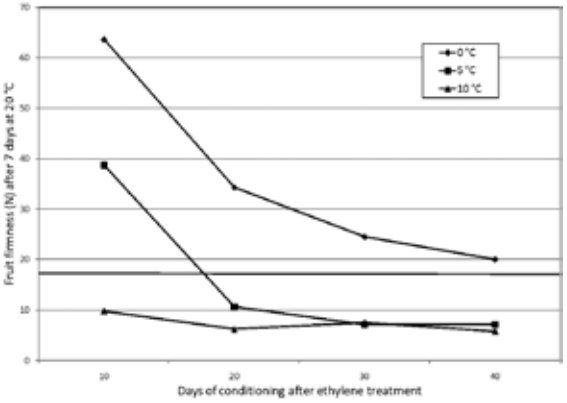

Fig 5. Effects of temperature on conditioning of 'Anjou' pears following 24 h in 100 ppm ethylene at 20 °C. Horizontal line at 17.8 N indicates maximum fruit firmness for a buttery-juicy texture.

Table 1. Approximate number of days of conditioning at various temperatures needed to induce ripening capacity in 'Bosc', 'Comice', and 'Anjou' pears harvested at the onset of fruit maturity, either without ethylene treatment or following 24 or 48 h in 100 ppm ethylene at 20 °C.

	Days of conditioning needed to induce ripening capacity								
	No ethylene			24 h ethylene at 20 °C			48 h ethylene at 20 °C		
	-0.5 °C	5 °C	10 °C	-0.5 °C	5 °C	10 °C	-0.5 °C	5 °C	10 °C
'Bosc'	15	9	5	0	0	0	0	0	0
'Comice'	30	18	12	15	7	3	7	2	2
'Anjou'	>60	35	17	44	18	<10	38	12	<10

Pear Postharvest Decay

The incidence of decay at wounds in 'Bosc' pears increased with increasing delay in postharvest application of TBZ (Fig 6). The decay was predominantly caused by *Penicillium expansum*. In fruit that had received foliar calcium chloride applications during the summer prior to harvest, the rate of increase in decay incidence with delay in postharvest application of TBZ was lower. The rate of increase in decay incidence with delay in postharvest application of TBZ was further decreased by application of pyraclostrobin + boscalid one week before harvest. The rate of decay increase was lowest in fruit that received the combination of summer calcium chloride and pyraclostrobin + boscalid one week before harvest. These results indicate that treatments prior to harvest can substantially reduce the need for prompt postharvest fungicide treatment, and further suggest that such pre-harvest treatments can be important components of an integrated strategy to manage decay without postharvest fungicide applications.

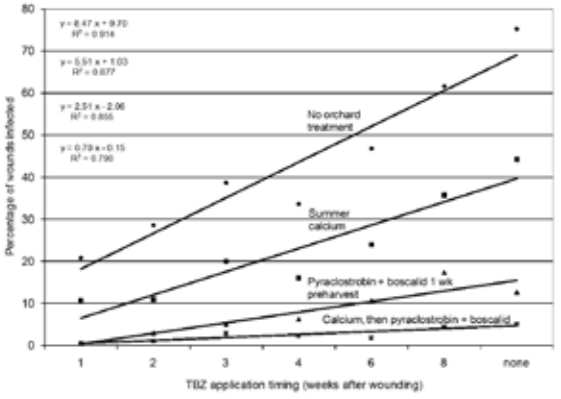

Fig 6. Incidence of decay at wounds in 'Bosc' pears in relation to orchard calcium and fungicide treatments and delay in postharvest application of thiabendazole fungicide (TBZ).

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09. PREHARVEST FACTORS AFFECTING FRUIT QUALITY AND SAFETY

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Abstract

Quality of fruit and vegetables is defined at harvest, and postharvest technologies only ensure that the rate at which products' quality decline is minimized. Fruit growers are faced with the problem of producing enough quantities to be profitable but also to produce with high intrinsic quality. For consumers, flavor and safety are the most important characteristics of quality. The main factors that affect flavor quality are the genotype (cultivars/rootstocks), maturity at harvest, environment and cultural practices and the cross effect between them. Pest and diseases control with pesticides, the use of growth regulators to improve quality and consumer acceptance also play an important role in cultural practices and are firmly related to safety. Some examples of preharvest factors influencing quality and safety of fruits will be discussed, given that postharvest quality is based both on better flavor and safer products, as well as in long shelf life and high acceptability. In the future, producers will have to identify all factors that influence composition and quality and to use environmental friendly technologies that are able to increase postharvest quality.
Keywords: cultural practices, environment, flavor, genotype, safety

Introduction

Quality of fruit and vegetables is defined at harvest, and postharvest technologies only ensure that the rate at which products' quality decline is minimized. Producers must ensure that they deliver the highest quality into the supply chain. This can only be achieved through continuous consolidation and vertical integration among producers and marketers (Hewett 2006). Fruit growers are faced with the problem of producing enough quantities to be profitable and simultaneously, to produce with high intrinsic quality. Most are certified under one of the on-farm assurance systems, like GLOBALGAP, that ensure that Good Agricultural Practices are undertaken and that relationship between consumers, farmers and marketers is strengthened.

For consumers, flavor and safety are the most important characteristics of quality. Kader (2008) in a recent review emphasizes the importance of flavor quality of fruits and vegetables to increase their consumption. Consumers' demand drives organic production expansion and as such, in most western countries, organic retail sales continue to rise. Organic versus conventional production system inputs may affect phytochemical and nutrient content, the ripening pattern and the marketing and food sensory qualities (Perkins-Veazie & Lester 2008). These changes require new postharvest assessments.

From the scientific point of view, in the last decade quality of fruit and vegetables has been the center of attention; ISAFRUIT is one of the EEC projects that focus on fruit quality, involving 200 researchers from 60 Research and Development Institutions. Commercially, packinghouses started to use nondestructive methods, like NIR spectrometry, to measure intrinsic quality (Solid Soluble Content, SSC) and research groups are developing sensor technologies, like E-nose and E-tongue, which may soon be available.

Some examples of preharvest factors influencing quality and safety of fruits will be discussed, given that postharvest quality is based both on better flavor and safer products, as well as in long shelf life and high acceptability.

Quality and safety

Taste and smell related to fruit's composition (mainly sugar, acid and phenolic content) are of the outmost importance in fruit quality (Kader 2008). Appearance, textural and nutritional quality are other quality

components also important to consumers but repeated purchases are dependent upon flavor quality. The main factors that affect flavor quality are the genotype (cultivars/rootstocks), maturity at harvest, environment and cultural practices and the cross effect between them. Pest and diseases control with pesticides, the use of growth regulators to improve quality and consumer acceptance also play an important role in cultural practices and are firmly related to safety.

Genotype

The genotype has more importance to flavor than any other factor, in other words fruit quality is foremost an inherent cultivar trait. For example, Hakala *et al*. (2003) compared mineral composition and vitamin C of six strawberry cultivars produced both through normal farming and organically cultivated; they found that genotype and origin had a larger effect than cultural practices. Breeding programs focus now on quality, developing new cultivars with higher sugars, moderate acids, lower phenolics compounds and higher vitamins content. This is done through conventional methods or using molecular markers to indentify quantitative traits loci (QTLs or candidate genes) for physical and chemical components of fruit quality. An increasing number of QTLs have been identified in apple, melon, peach, almond, apricot, cherry, strawberry, grape, raspberry, cocumber and tomato, that will permit identifying important enzymes involved in flavor pathways. In some cases, like tomato, introgression lines obtained through crosses between tomato and a wild relative, allowed the study of quantitative traits loci and the creation of new varieties by introducing exotic traits. Another step of biotechnology is the design of transgenic fruits and vegetables with higher quality characteristics, like the genetic modified strawberry or the table grape with the defH9-iaaM auxin-synthesizing gene (Mezzetti *et al.* 2004; Costantini *et al.* 2007) but society does not seem to be ready to accept them (Hewet 2006).

Rootstock is a factor of lesser importance than cultivar; nevertheless in citrus it may affect many quality characters. According to Castle (1995) citrus is a crop with minor canopy management and fruit quality depends on plant water relations and sugar transport, whereas in deciduous fruit trees fruit quality relies on factors related to crop load and canopy management.

Kader (2008) rates maturity (after genotype) as the second most important factor influencing flavor quality of fruits and vegetables. In order to provide better tasting fruits and vegetables, optimizing maturity /ripeness stage in relation to flavor should be a goal.

Environmental factors

Orchard design (spacing and row orientation), training systems (Y, V-trellis or vertical axes) with well spaced branches and improved light distribution maximize yield and fruit quality. In fruit trees, yield is related to the total amount of sunlight intercepted, for example in apples, this relation is linear below 60 % of light interception (Lakso 1994). Establishing open and well exposured tree canopies, through cultural practices, as pruning, is essential for obtaining a high potential yield. Red color in some fruits, like apple and pear, requires direct incident light. Solar radiation in combination with cool nights is reported to promote *de novo* synthesis of anthocyanins in fruits (Tromp & Wertheim 2005). Temperature and other weather constraints (rain distribution, frost, hail and wind) greatly affect quality. In warm climates, fruits have more SSC and earlier ripening. Topography and site exposure affect both harvest date and SSC, for example, in Cova da Beira – Portugal, a region of cherry production, earlier cherry maturation was found in south-eastern exposures orchards and SSC was higher in cherries from the western exposure orchard (Costa 2006).

Cultural practices

Trees nutritional status and soil management are accountable for a great number of postharvest disorders, therefore an optimum mineral balance has to be found in each case. High levels of nitrogen and irrigation result in fruits with less sugar and vitamins, with poor texture and more prone to disorders and diseases.

Color is affected by high levels of nitrogen or by nutrients imbalances, for example, in citrus, delayed time to color break, reduced color at harvest, thick rind and less juice result from heavy nitrogen and low phosphorus fertilizations (Ritenour *et al.* 2002). Another well known example is the greater N:Ca and K:Ca ratios in albino strawberry fruits when compared to colored fruits. Ca and K increase firmness and storage capacity and lack of calcium in the fruit is known to be the main reason for several problems. Since there are numerous factors affecting fruit's calcium accumulation (Faust 1989) calcium sprays may not always work. This is due to interactions with other nutrients and with water status, crop load and fruit size. Regulated deficit irrigation (RDI) improves water use efficiency and fruit quality, mainly SSC, and it is increasingly adopted in different fruit crops (Behboudian & Mills 1997).

Pesticides and growth regulators sprays are of major concern as they affect quality and safety, therefore new technologies and new pesticides are under development to reduce residues level. Still, fruit industry uses several growth regulators with different goals, as to increase fruit set in pears, for thinning apples, to prevent fruit drop, to increase fruit size or to increase firmness or color. Concerning pesticides, some diseases have origin in latent fungal infection of fruit in the orchard (*Stemphylium vesicarium, Phytophthora* spp*., Venturia pirina, Mucor piriformis, Nectria galligena, Alternaria alternata, Pezicula malicorticis, Pezicula alba).* In Portugal, *Stemphylium* in pears is an increasing concern resulting in postharvest losses. Weather conditions, cultivar and physiological fruit condition (as fruit maturity at harvest) are relevant to disease development. Thus, new fungicides and treatments which can protect fruit after harvest are under development. In summary, "orchard health" depends not only on weather conditions but also on cultural practices and optimal integrated crop management is determinant to reduce pesticide sprays.

Some examples of cultural practices that increase fruit quality without chemicals are based on crop manipulation, like ringing and scoring (Goren *et al.* 2004) or light manipulation. Reflective ground covers, which help trees to harvest light, improve red skin coloration and advance maturity, fruit size and SSC. Alternatives to chemical thinning can be shading with nets, as shown by recent studies in Swiss and Italy with apple trees (Widmer 2007; Domingos 2008) or mechanical thinning through devices that are now under development (Shupp *et al.* 2008).

It is not easy to assess the differences in fruit nutrient contents when comparing different production systems. Bourne & Prescott (2002) summarized several studies comparing inorganic and organic fertilizers: results were too variable to provide definitive conclusions. The most common problem relates to consider, the same cultivar, same soil, microenvironment, orchard age, fruit size position in the canopy in both production systems (Perkins-Veazie & Lester 2008). In a particular study done over ten years, comparing the influence of organic and conventional crop management practices on the content of flavonoids in tomato, Mitchell *et al.* (2007) found that the levels of flavonoids increased over time in samples from organic treatments, whereas the levels of flavonoids did not vary significantly in conventional treatments. It seems that there is a trend toward higher levels of phenolic antioxidants, ascorbic acid and soluble solids in organic foods, however, more research examining relationships between agricultural production systems and nutrient content is needed.

In the future, producers will have to identify all factors that influence composition and quality and to use environmental friendly technologies that are able to increase postharvest quality.

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10. AMMONIUM FERTILISERS WITH NITRIFICATION INHIBITORS IMPROVE THE NUTRITIONAL QUALITY OF HORTICULTURAL CROPS FOR INDUSTRIAL PROCESSING

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Abstract
Nitrogen management in plant nutrition is an agronomic and environmental key factor. Under soil conditions the main form of N available for the plant is nitrate. Nitrification inhibitors reduce the nitrification rate and increase the ammonium absorbed by the plant. This effect reduces the risk of groundwater pollution by nitrates. Nitrate may be dangerous for human health and can increase oxalate concentration and decrease vitamin C (vit C) content in vegetables. This paper presents the results of three field trials on tomato, spinach and broccoli, all of them for industrial processing (spinach and broccoli for frozen industry and tomato for tomato paste). In the three trials a conventional ammonium plus nitrate fertilization was compared with the application of ammonium plus nitrate plus nitrification inhibitor, using similar rates of N. All of the trials were conducted in Badajoz (Spain) during 2006 and 2007. In the spinach trial the ammonium increase (with the nitrification inhibitor) reduced nitrate and oxalate in leaves and increased vit C. Greater yields were obtained with mixed fertilization (nitrate plus ammonium plus nitrification inhibitor). In the Broccoli trial there was no differences in yields but using the nitrification inhibitor, a slightly increasing of vit C content was obtained. The trial performed with fertigated industrial tomato shown that the use of nitrification inhibitor increased yield on 8% and also increased the lycopene concentration (also colour a/b ratio) from 32 mg kg^{-1} (conventional fertilization) to 45 mg kg^{-1}. These results presented shows that important nutritional parameters, as nitrates, oxalates, vit C or lycopene are influenced by the quantity and characteristics of the N fertilizer applied, and that a mixed nutrition nitrate plus ammonium with nitrification inhibitors, can increase vit C contents and reduce nitrates or oxalates.
Keywords: broccoli, industrial tomato, nitrate, Nitrification inhibitor, oxalate, spinach, vitamin C

Introduction
Nitrogen is an essential plant nutrient but it is involved in environmental problems associated with crops fertilization. Groundwater pollution due to nitrates is increasing in all Europe. Also high nitrate concentration in waters is one of the factors that produce the eutrophication of superficial waters.

For animal and human health nitrates *per se* are not toxic, but its metabolites and reaction products such as nitrite (NO_2^-), nitric oxide, or N-nitrous compounds have adverse implications as, methaemoglobinaemia or certain carcinogenesis (Lee 1970; Farré & Oliver 1982; Hartman 1983; Fraser 1985; Forman *et al.* 1988; Bruning-Fann *et al.* 1993; EFSA 2008). In spite recent research shows positive aspects of antimicrobials as nitrites and nitrates within the blood vessels (EFSA 2008), the main scientific opinion is that nitrate must be controlled and reduced in vegetables and water. Several authors (Hall 2002; Palaniswamy *et al.* 2002; Glaxo-SmithKline 2006) refer the relationships between high intake of oxalate and the formation of kidney stones, as an inadequate absorption of calcium (Okutami & Sugiyama 1994; Ahmed & Johnson 2000). The low consumption of oxalate is particularly important in patients with intestinal problems like Bowel Syndrome (SBS) or patients with a high tendency to form kidney stones. Overall estimates of kidney stones due to calcium oxalate represents between 75 and 90% of the total (Hall 2002; Glaxo-SmithKline 2006). As example in the United States more than 12% of the population is affected by this pathology clinic (Glaxo-

SmithKline 2006). In animal nutrition the high concentration of oxalates in pasture crops is harmful because of its ability to precipitate with calcium, which produces hypocalcaemia in animals (Ramos *et al.* 1998).

Nitrogen fertilization influences the accumulation of nitrate and oxalate in foods (Elia *et al.* 1998; Zhang *et al*. 2005). In field trials with spinach, Elia *et al.* (1998) concluded that the increase of N fertilization increase the concentration of nitrate and oxalate in leaves. In the same trial the increase of ammonium fertilizers reduce the concentration of harmful compounds. Baku & Gawish (1997) showed that the use of ammonia fertilisers with a nitrification inhibitor reduce the accumulation of nitrate in lettuce and spinach, lowering the total and free oxalate, and producing a slight increase in vitamin C (vit C). This result is due to one of the possible routes of formation of oxalate and breaking of the link C2/C3 in ascorbic acid (Libert & Franceschi 1987). Vit C is the main soluble antioxidant present in plants and is essential for humans and animals (Tedone 2005).

An interesting technique to improve the efficiency of N fertilisers is the control of N chemical cycle in the soil. When urea or ammoniac fertilisers are applied to the ground they are quickly transformed to nitric forms by the action of the *Nitrosomona* spp. bacteria (part of the process known as nitrification). In the nitrate form, N can be easily absorbed by the plant, because it is very soluble and mobile in the soil solution. Salt-washing irrigations, rainfalls or inadequate irrigation management can produce important losses by leaching.

Nitrification inhibitors are added to any type of fertilizers and delay the bacteria oxidation of NH_4^+ to NO_2^- (Prasad & Power 1995), due to the competition for the active site in the enzyme AMO (McCarty 1999). Nitrification inhibitors increase ammonium-N and decrease nitrate-N in soils, as a result they also decrease N losses (Fettweis *et al.* 2001). Nitrogen use efficiency is improved and the doses and number of applications can be reduced, producing an economical and environmental benefit (Trenkel 1997). In Europe the most spread nitrification inhibitor is 3,4-dimethylpyrazol phosphate (DMPP) developed by BASF during the late 90's. DMPP is a high efficiency molecule, with a soil optimal activity at 1 kg ha^{-1}, and without negative effects in the crops and the environment (Zerulla *et al.* 2001).

The objective of this work was to study the effects of different N treatments (with and without nitrification inhibitors) on the yield and the nutritional quality of tomato, spinach and broccoli for industrial processing.

Material & Methods

The trials were conducted in the farm "El Bercial" (Badajoz, Spain), which belongs to the Agro Technology Center of Extremadura (CTAEX). Soils have pH=7, the texture is sandy-clay-loam, have low organic matter content (0.9%) and low electrical conductivity, low levels of total N, high available P (Olsen method), medium-low calcium and potassium, and medium-high magnesium.

Spinach trial

The trial was developed in a plot of 2400 m^2 with 5 treatments×4 replications. The variety used was 'El Paso' (Syngenta) planted on March 20 at 0.25×0.08 m seeding spacing. The treatments were: a control without N fertilization (T1); a conventional N fertilization (100 kg ha^{-1}) applied in two fractions (T2); a single application of a granular fertilizer (70% of N as ammonium and 30% of N as nitrate) with a nitrification inhibitor (3,4-Dimethylpyrazole phosphate, DMPP) at 100 kg ha^{-1} (T3); a similar treatment to T3 but applied in two fractions (T4); and two applications of a 100% ammonium fertiliser plus the nitrification inhibitor DMPP, at 100 kg N ha^{-1} (T5).

Tomato trial

Plants of industrial tomato 'UG 8168' were transplanted to field at May 16 with a fertigation system for each treatment. The harvesting was conducted in late August when fruits reached the commercial maturity

(85% of fruits with optimal red colour). Five different treatments, with 4 repetitions, were established: (T1 and T2) - conventional fertilization using the recommended dose of N (T1=150 kg N ha^{-1}) and a reduction of 20% (T2=120 kg N ha^{-1}); (T3 and T4) - an initial base fertilization with N was done and then the remaining N was applied by fertigation; (T5) - all the nutrients was applied by fertigation. In T3 and T5 150 kg N ha^{-1} and in T4 120 kg N ha^{-1} was applied. Fertigation program was adjusted to nutritional requirements of this crop throughout its growing cycle.

The effects on yield were assessed by measuring the total yield, the commercial yield, and firmness of the fruits, average weight and the percentage of damage fruits. In each repetition, 4 kg of tomatoes was sampled and processed at the pilot plant of CTAEX. Soluble solids, juice pH, viscosity, colour and the concentration of lycopene, vit C and nitrate in the fruit was determined.

Broccoli trial

'Monaco' variety was used, at a density of 35000 plants m^{-2}. The treatments were: conventional nitrogen fertilization carried out in 2 applications (T1) with 2 formulations of mixed ammonium/nitrate plus nitrification inhibitor (DMPP): the first of them at the same dose of N and the second reducing 25% the N applied, both in a single application at the start of the crop cycle. Plants were transplanted on August 30 and three harvests were done during November (20, 23 and 29).

The marketable yield, number of broccoli heads per ha, and their average weight and diameter were registered. The concentration of nitrates and vit C was done in the broccoli heads. At the beginning and end of the harvest dates the concentration of N-NO_3^- and NH_4^+-N in the soil were measured.

Results & Discussion

Spinach trial

The results were greatly influenced by the fertilization treatments (Fig 1A). The yield of spinach plants grown without N (T1) was 3 times lower than plants of T3 treatment (N plus N fertilizer with DMPP); the other treatments (T2 and T4) presented intermediate values. Nitrate content in leaves had a high variability (coefficients close to 100% in some cases), however the highest values was obtain in plants fertilised with nitrate (and the lowest values with ammonium (T5- 100% ammonium fertilization plus DMPP). The values were below the maximum limit (2 g nitrate kg^{-1}) proposed by the Regulations Nº 1822/2005 of the European Union Journal (9/11/2005) for spinach to frozen.

The oxalic acid content in leaves was distributed according to this order (Fig 1B): T2>T3>T1>T4>T5. The inverse order was obtained in the concentration of vit C (Fig 1C). The highest content of vit C was obtained in the treatments with ammonium plus DMPP (T3, T4 and T5). In T1 and T2 treatments, oxalic acid to ascorbic acid ratio was 39 while in T3 and T4 treatments was 22/1, and in T5 treatment was 18/1. These results were in accordance with those obtained in spinach (Zhang *et al.* 2005), in cabbage (Elia *et al.* 1998) and in *Portulaca oleracea* L. (Palaniswamy 2002).

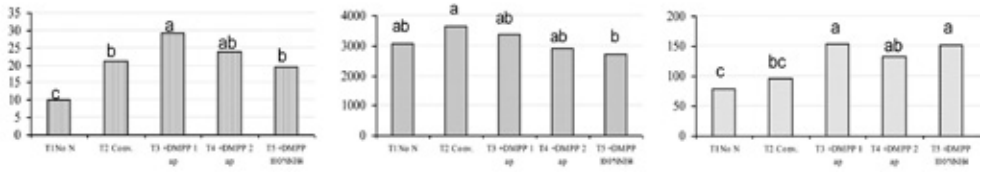

Fig 1. A - total yield (t ha^{-1}) and some nutritional parameters of spinach leaves for each treatment: B - oxalic acid (mg kg^{-1}) and C - vit C (mg kg^{-1}). Different letters in the same column indicate significant differences ($P<0.05$).

Broccoli trial

Fertilization with nitrification inhibitors slightly increased crop production. The ammonium fertilization had effects on the yield classes and anticipates the harvest date. The nitrate content of broccoli was very low and similar in all treatments (data not shown). However, in previous trials conducted with fertigated broccoli, Egea *et al.* (2003) found levels of nitrates 100 times lower in ammonium+DMPP treatments. Moreover, treatments with nitrification inhibitors presented decreases in the nitrates content of final product. There were no statistical differences in the concentration of ascorbic acid, however in treatment with a lower contribution of N plus nitrification inhibitor (T3) ascorbic acid content (257±62 mg kg^{-1} fresh weight) was higher compared to T1 (184±35 mg kg^{-1}) and T2 (183±44 mg kg^{-1}) treatments.

Tomato trial

Production in T1 was abnormally low compared to other treatments which had similar values (Table 1). The results shown that the dose of 120 kg ha^{-1} was sufficient for this crop, considering the high N-mineral in soil at the start of the trial. T3, T4 and T5 treatments (with nitrification inhibitor DMPP) had slightly higher production (Table 1). Colour and lycopene content of tomatoes were affected by N-fertilization treatments (Table 1). Lycopene is one of the main antioxidants in food, along with vit A and C, and is one of the causes of red colouring in fruits (Dumas *et al.* 2003). Antioxidants are important for preventing cardiovascular diseases or cancer (Agarwal & Rao 2000; Dumas *et al.* 2003). In order to improve the colour of the tomato paste it is important to get a high content of lycopene in fruit, which is principally affected by environmental issues (temperature and sunlight) but also by fertigation. The review done by Dumas *et al.* (2003) indicates that the information about the effects of N fertilization on antioxidants contents of tomato is scarce and may provides contradictory and inconclusive results. In the present study the treatments had a clear effect on the content of lycopene (Table 1), but not on the concentration of ascorbic acid. Higher doses of N reduced the content of lycopene (T1 compared with T2 and T3, T4) but if N in ammonium form was used (with nitrification inhibitors; T3 and T4) there was an increase in lycopene content. In any case, these results should be supported in the followings campaigns.

Table 1. Marketable yield and industrial parameters analyzed in tomato trial.

Treatments	Yield	°Brix	pH	Viscosity	Firmness	Colour	Lycopene	Vit C	NO_3
	t ha^{-1}			cm $30s^{-1}$	g	a/b	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
T1	58.4^{b}	4.6^{a}	4.4^{a}	26.7^{b}	4723^{a}	1.99^{b}	17.9^{c}	86.7^{a}	<5
T2	79.1^{a}	4.6^{a}	4.4^{a}	33.1^{ab}	4356^{a}	1.99^{b}	32.7^{bc}	83.2^{a}	<5
T3	84.2^{a}	4.7^{a}	4.4^{a}	33.8^{ab}	4546^{a}	2.09^{a}	30.9^{bc}	72.7^{a}	<5
T4	87.5^{a}	4.8^{a}	4.4^{a}	30.6^{ab}	4134^{a}	2.09^{a}	40.5^{ab}	68.6^{a}	<5
T5	82.6^{a}	4.8^{a}	4.4^{a}	34.9^{a}	4496^{a}	2.03^{ab}	45.8^{a}	73.7^{a}	<5

(T1 and T2) - Conventional fertilization (T1=150 kg N ha^{-1}) and (T2=120 kg N ha^{-1}); (T3=150 kg N $ha^{-1}$and T4=120 kg N ha^{-1}) - an initial base fertilization with N was done and then the remaining N was applied by fertigation; (T5=150 kg N ha^{-1}) - all the nutrients was applied by fertigation. Different letters in the same column indicate significant differences $P<0.05$.

Conclusions

The three trials presented shown that the quantity and characteristics of the N fertilizer applied influenced in the concentration of important nutritional substances as ascorbic acid (vit C) or lycopene and over anti-nutrients as nitrate or oxalate. Mixed nutrition nitrate plus ammonium with nitrification inhibitors increase the ammonium presence in the soil and its absorption by the plant. This effect induces modifications in N metabolism. These trials shown that in field conditions ammonium fertilisers plus DMPP increased vit C in spinach and broccoli plants, and lycopene in tomato plants, compared to conventional fertilization. Besides, the increase of lycopene let to reddish tomatoes which are more profitability for the growers.

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11. VARIATION IN APPLE COLOUR AND MATURITY. CAUSES AND SIMILARITIES OVER ORCHARDS, MANAGEMENT, CULTIVARS AND STORAGE

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Abstract
The colour of apples (flesh colour or skin colour) was assessed using the same individual apples repeatedly in time at three different locations, in several seasons for five different cultivars. Two experiments were conducted in the orchard, one experiment during postharvest storage. The same logistic model was applied to analyse the data, separate for each location and cultivar. Non linear mixed effects regression analysis allows to extract not only information on the kinetic parameters like reaction rate constant and potential greenness, but also on the variation present in the data. The rate constant of the decolouration process was found to be largely the same for all combinations (with one exception). The variation in biological shift factor, as an expression for maturity, seems to be independent of orchard location and only slightly dependent on orchard management procedures. The main differences observed are in the potential greenness of the apples (col_{min}) that vary considerably between successive seasons and between cultivars. The applied technology provides the necessary tools to analyse the effects of season and orchard management, for all locations in the study. It opens wide alleys to investigate more dedicated the effects of weather, season, management and orchard location in growing apples with a constant quality (colour) over the seasons, locations and management procedures.

Introduction
Non destructive measurement of apple colour, skin colour or flesh colour using continuous wave or laser light, allows the assessment of colour aspects, in terms of colouring compounds, of the same individual apples both during growth as during storage. These so-called longitudinal data can be analysed using mixed effect non linear regression analysis. The technology and the benefit of this type of analysis has been reported in quite a number of recently published papers. One of the advantages of mixed effects analysis is that information is obtained on the variation in colour status or maturity in a batch of individuals.

In this paper an overview will be presented of the magnitude of variation in colour aspects of apples, both during growth (where does variation come from?) and during storage (how does variation develop further?). Apples from three different regions and seasons of growth, of different cultivars and grown with different orchard management were separately analysed using the mixed effect technique. The results with respect to kinetics of change in colour, as well as to the magnitude and dynamics of change of the variation will be compared.

The main conclusion seems to be that the orchard (type of planting, soil) and its management (fertilisation, crop load) were the main causes of variation in maturity within a season, while the weather conditions seem to determine the variation over the seasons.

Materials & Methods
Three completely independent projects were carried out on apples of different cultivars.

1. 'Granny Smith' apples were harvest in Slovenia (1997), harvested at 2 stages of maturity. The apples

were individually labelled and the skin colour regularly measured (Minolta CR-200, Minolta Co., Japan) during storage at 1, 4 and 10 °C. The full details of setup and analysis are described in Tijskens *et al.* (2008). Results were expressed in the $L^*a^*b^*$ system.

2. Apples (cvs 'Elstar', 'Pinova' and 'Topaz') were grown in the same orchard in Germany in seasons 2004, 2005 and 2006. The orchard was situated on a hill slope. Six trees were selected uphill, six downhill. Excluding the margin rows, ten fruits from each tree were selected arbitrarily and individually labelled for identification. Flesh colour was measured regularly using a portable miniaturised spectrophotometer (Zude & Herold 2002; Truppel 2003; Herold *et al.* 2005) during development at the tree. Flesh colour was expressed as Red-edge, the wavelength with the steepest slope near the chlorophyll absorption peak around 690 nm. Details of setup and analysis are described in Herold *et al.* (2005) and Tijskens *et al.* (2006). More detailed information on the measuring technique can be taken from Zude (2003, 2006).

3. In two consecutive seasons (2001 and 2002) apple trees (cv. 'Golden Delicious') were fertilised at two levels of N-fertilisation, allowing a high, medium or low crop load. Allowing only fruit with a diameter of 15 mm, fruits were thinned by hand to the chosen crop load of 70, 50 or 28 fruits per tree. 4 fruit per tree were chosen and labelled. At regular times during the development of the apples at the tree, skin colour was measured using a Minolta CR-200 Chroma Meter. Fruit chromaticity was expressed in $L^*a^*b^*$ system. Colour measurement started approximately 1 month before the predicted technological maturity of fruits until harvest when the intensive chlorophyll degradation is expected to begin.

The colour model

The behaviour of colour, whether expressed as a*-value or red-edge wavelength, can be described by a logistic function. Expressing this function in the notation for biological shift factor gives Eq1.

$$col = \frac{col_{max} - col_{min}}{1 + e^{-k_c \cdot (col_{max} - col_{min}) \cdot (t + \Delta t)}} + col_{min} \qquad \textbf{Eq 1}$$

In case of application of a*-value (experiments 1 and 3), it is an increasing sigmoidal, in case of red-edge (experiment 2) a decreasing. The only difference is the sign of the rate constant k_c (positive respectively negative). The biological shift factor Δt is expressed relative to the midpoint of the logistic function. Details on the applied mechanism and the deduction of the model can be found in Tijskens *et al*. (2008).

Results & Discussion

The quite different experimental design of three independent experiments makes it difficult to compare results on kinetics and biological variation. These differences in setup have to taken into account at every comparison. Nevertheless, comparing the results of analyses and the behaviour of apples over season, regions and growing conditions may indicate where which effects are important.

Kinetic Behaviour

The applied model (Eq 1) is derived from a reaction mechanism of chlorophyll degradation (Tijskens *et al*. 2008). That ensures that the equation reflects a fundamental process related to chlorophyll decay and that the model is generic in nature. In Fig 1 some examples are shown for the colour development versus biological time ($t+\Delta t$). As a consequence, the rate constants should be comparable. Of course, the different temperatures during the experiments have to be taken into account, and also the possibility of a different level of chlorophyll degrading enzymes, affecting the rate of the process, in the produce from different cultivars, seasons and regions. The rate constants for location Potsdam, for location Maribor (2002) and for location South Slovenia are quite comparable, considering that the reference temperature for the latter is set at 10 °C.

The rate constant for location Maribor (2001) however is about 10 times higher. The climatic conditions were very different in these seasons: the season 2001 was very wet, while the early season 2002 was very dry, followed by an intense wet period. Possibly, the weather conditions induced this large discrepancy. Nevertheless, a rate constant 10 times higher is very unusual.

Another strange point in these data is that the mean biological shift factors (Δt in Table 1) are so different: in the orchard (location Potsdam and Maribor) is around -20 days, while in the storage experiments the mean biological shift factor is -190 days.

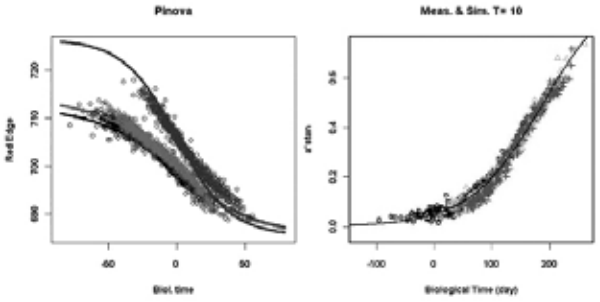

Fig 1. Colour development versus biological time. Left: Postdam data for cv. 'Pinova' in three seasons, right: South Slovenia data cv. 'Granny Smith' from all three orchards stored at 10 °C for the two moments of harvest. Symbols measured, lines simulated.

Even considering the lower temperatures affecting the biological shift factor, reflected in the dimensionless biological shift factor ($\Delta t^* = k_t \cdot \Delta t$ see Table 3), this difference is huge. This could indicate that the colour decay mechanism at the orchard is different than at storage.

Table 1. Overview of the separate result.

Location	Year	Cultivar	col_{min}	Δt	k_c	σ (Δt) *Fruit*	σ (Δt) *Tree*	N_{obs}	N_{gr}	R^2_{adj}
Slovenia South	1997	Granny Smith	-21.498	-189.69	0.00060[a]	41.007	na	3211	540	0.97
Potsdam	2006	Elstar	717.41	-13.53	0.00213	5.540	2.130	661	120	0.97
Potsdam	2005	Elstar	715.78	-22.51	0.00140	6.245	6.889	720	120	0.98
Potsdam	2004	Elstar	711.10	-37.68	0.00223	7.296	5.426	752	120	0.98
Potsdam	2006	Pinova	726.45	-12.00	0.00096	4.968	6.728	711	120	0.97
Potsdam	2005	Pinova	713.96	-34.66	0.00140	7.353	1.629	721	120	0.97
Potsdam	2004	Pinova	711.47	-44.94	0.00181	9.887	2.122	776	120	0.97
Potsdam	2006	Topaz	730.29	-15.06	0.00078	6.398	3.011	723	120	0.98
Potsdam	2005	Topaz	721.13	-7.70	0.00096	6.714	3.184	721	120	0.97
Maribor	2001	G. Delicious	-16.82	-38.74	0.01069	15.266	na	945	190	0.95
Maribor[b]	2002	G. Delicious	-24.00[c]	-33.69	0.00224	10.156	na	1774	299	0.94
[a]					at 10 °C					
[b]					applying an exponential model, rate constant corrected					
[c]					fixed					

Apparently the harvesting is more traumatic than usually expected. It is apparently not related to the measuring technique: at the Potsdam orchard, fruit flesh colour was measured as red-edge and at the South Slovenia and Maribor orchards, skin colour was measured as a*-value.

The initial conditions of col_{min} are of course different for each cultivar and measuring system.

Biological Shift Factor

The estimated values for the biological shift factor were analysed per appropriate series, on mean value (Δt_{mean}) and standard deviation (σ). Also the normality of the distributions was tested using the Shapiro Wilk test (pvalue). The results are shown in Table 2. In case of the 'Granny Smith' experiments (South Slovenia), the distribution could not be proven to be normal: all pvalues except for the orchard Blanca (BL) at commercial harvest (CM) where below the usually applied limit value of 0.05. The mean values nicely show the difference in harvest date: not exactly 10 days between early harvest (EH) and commercial harvest (CM), but quite close. That indicates that the harvest date was not completely correctly estimated for each orchard. It also indicates that this technology, when equipment and procedures are made practical, can provide a better estimate of the optimal or commercial harvest date. The standard deviations (σ) are high compared to the other two experiments. That is most probably related to the different storage temperatures applied in this experiment.

Table 2. Results of the analysis of the biological shift factor Δt relative to their own mean.

Region	Season	L	H	pvalue	Δt_{mean}	σ	N_{obs}
S. Slovenia	1997	AS	CM	0.0015	-22.28	28.42	90
S. Slovenia	1997	AS	EH	0.0002	-30.73	32.08	90
S. Slovenia	1997	BL	CM	0.1941	14.66	39.02	90
S. Slovenia	1997	BL	EH	0.0000	2.98	39.95	90
S. Slovenia	1997	KK	CM	0.0069	20.42	36.15	90
S. Slovenia	1997	KK	EH	0.0003	14.95	40.88	90
Region	Season	Cultivar		pvalue	Δt_{mean}	σ	Nobs
Potsdam	2004	Elstar		0.0979	-37.68	8.94	120
Potsdam	2005	Elstar		0.6973	-22.51	9.19	120
Potsdam	2006	Elstar		0.1033	-13.53	5.76	120
Potsdam	2004	Pinova		0.0250	-44.93	9.77	120
Potsdam	2005	Pinova		0.1951	-34.66	7.25	120
Potsdam	2006	Pinova		0.2175	-11.99	8.26	120
Potsdam	2005	Topaz		0.0011	-7.70	7.26	120
Potsdam	2006	Topaz		0.0000	-15.05	6.97	120
Region	Season	Nitrogen level	crop load	pvalue	Δt_{mean}	σ	N_{obs}
Maribor	2001	60	Lo	0.0013	-32.62	7.34	32
Maribor	2001	60	Med	0.0042	-32.43	7.71	33
Maribor	2001	60	Hi	0.0708	-31.89	7.01	31
Maribor	2001	105	Lo	0.0232	-33.73	6.44	29
Maribor	2001	105	Med	0.0003	-36.16	5.73	32
Maribor	2001	105	Hi	0.0008	-35.08	5.19	33
Maribor	2002	60	Lo	0.0499	-31.35	11.52	50
Maribor	2002	60	Med	0.8289	-32.33	11.93	51
Maribor	2002	60	Hi	0.7832	-35.07	8.56	50
Maribor	2002	105	Lo	0.5354	-34.06	9.02	49
Maribor	2002	105	Med	0.3574	-33.63	10.05	50
Maribor	2002	105	Hi	0.0996	-35.77	9.12	49

Table 3. Overview of the dimensionless biological shift factor.

Location	Year	CV	Δt	k_c	Δt^*
S. Slovenia	1997	Granny Smith	-189.69	0.0006	-0.1138
Potsdam	2006	Elstar	-13.53	0.0021	-0.0288
Potsdam	2005	Elstar	-22.51	0.0014	-0.0315
Potsdam	2004	Elstar	-37.68	0.0022	-0.0840
Potsdam	2006	Pinova	-12	0.0010	-0.0115
Potsdam	2005	Pinova	-34.66	0.0014	-0.0485
Potsdam	2004	Pinova	-44.94	0.0018	-0.0813
Potsdam	2006	Topaz	-15.06	0.0008	-0.0117
Potsdam	2005	Topaz	-7.7	0.0010	-0.0074
Maribor	2001	G. Delicious	-38.74	0.0107	-0.4141
Maribor	2002	G. Delicious	-33.69	0.0022	-0.0755

A lower temperature not only decreases the rate of the colouration process, but also increases the observed variation. That is reflected in the dimensionless biological shift factor ($\Delta t^* = kt \cdot \Delta t$).

In case of the Potsdam experiments, only the distribution of the biological shift of three series could not be proven to be normal: both series of cultivar 'Topaz', and 'Pinova' in 2004.

The standard deviation of the biological shift factor (σ) is low and almost the same for all 8 series.

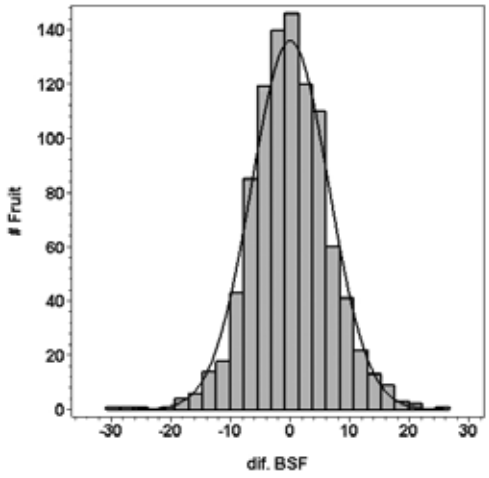

Fig 2. Estimated Biological Shift Factor Δtdif excluding the effects of tree, combined for all seasons and cultivars of the Potsdam experiments.

Moreover when only considering the variation over individual apples, leaving out the variation observed for the trees, the combined biological shift factors were highly normal distributed with a p-value of 0.75. In Fig 2 the distribution is shown.

For the Maribor experiments, there is a clear difference in season: in 2001 the standard deviation of the biological shift factor is of the same magnitude as the experiments in Potsdam. In 2002, the standard deviations are much higher. Clear effects of crop load can not be discerned, while a higher level nitrogen fertilisation seems to induce a lower standard deviation. However, more experiments at various levels and combinations are needed to validate these indications.

Variation in Colour Range

The data on the three cultivars location Potsdam were reanalysed, now applying the biological time (estimated values for the biological shift factor added to the calendar time) as independent variable. The rate constant was estimated in common (fixed effects) while the higher asymptote (Col_{min}) was estimated at random for the three seasons. In Table 4 the results are shown. The explained parts are again very high, but somewhat lower than in the separate analysis. That indicates that the data can indeed be pooled over season, at least for a single location and that the mechanism of colour change is indeed generic. The rate constants are similar but slightly different. The main difference is found in the mean value for col_{min} and especially in the variation over col_{min}. The number of seasons is, however, too low (only 3 seasons) to draw reliable conclusions.

Also in the experiments at the orchards in South Slovenia and Maribor 2001, a variation in the value of col_{min} had to be included to achieve a reliable analysis. The location of the apples at the tree determines the (continuous) effect of light and weather intensity. These differences are reflected in the potential greenness of the apples.

Table 4. Results of the analysis versus biological time for location Potsdam per season.

Cultivar	k_c	col_{min}	σ (col_{min})	R^2_{adj}	N_{obs}
Elstar	0.0019	714.52	2.647	0.956	2133
Pinova	0.0013	717.56	6.333	0.967	2208
Topaz	0.0009	725.74	4.556	0.981	1444

Conclusions

The mechanism of colour change is generic for all three independent experiments. With some exceptions, the rate constant of colour change seem also to be generic in nature, with a very similar value for all cultivars, orchards, and management procedures. Where the exceptions relate to is not known. The data sets are too limited (sic!) to extract that information. The variation in biological shift factor seems to be independent of orchard location and only slightly dependent on orchard management procedures. The main differences observed are in the potential greenness of the apples (col_{min}) that vary considerably between successive seasons and between cultivars.

As the main conclusions, the applied technology provides the necessary tools to analyse the effects of season (weather, temperature, rainfall etc) and orchard management quite well, for all locations in the study. It opens wide alleys to investigate more dedicated the effects of weather, season, management and orchard location in growing apples with a constant quality (colour) over the seasons, locations and management procedures.

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12. FOLIAR FERTILIZATION WITH POTASSIUM, MAGNESIUM AND CALCIUM AND POSTHARVEST FRUIT QUALITY IN PLUMS (*PRUNUS DOMESTICA* L)

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Abstract

Foliar applications of fertilizers are an important part of standard fertilization programmes in apples to optimize the content of nutrients important to growth and fruit quality. In an experiment in plums (*Prunus domestia* L.) a foliar fertilization programme was compared to traditional application of fertilizers on the soil. In another experiment extra magnesium, potassium and the combination of both magnesium and potassium were compared to control trees given a standard foliar fertilization programme. Yield and fruit size was registered. Analyses included fruit quality factors, content of major nutrients in fruits and leaves as well as storability. The effects of the different programmes on yield, fruit size and most fruit quality factors were not significant. Fruits from trees given foliar fertilizers had, however, higher content of nitrogen and significantly lower contents of the other major nutrients. The application of extra potassium and magnesium did not increase the level of these elements, but a significant reduction in the Ca-content was observed. Accordingly a higher K:Ca-ratio was observed in potassium and magnesium sprayed fruits. Even though no differences in fruit firmness were observed between the treatments at harvest, after two weeks of storage the plums from potassium sprayed trees were significantly softer than the controls.
Keywords: fruit analyses, fruit quality, K:Ca ratio, leaf analysis, nitrogen, shelf life

Introduction

It is well known that a good nutritional status is important to grow high quality fruit for the fresh fruit market. In apple growing foliar fertilizers are commonly used to improve the level of important nutrients like nitrogen, calcium, potassium and magnesium (Bertchinger *et al.* 1997). This practice is less common in plum growing.

Calcium is important in cell structures and firmer fruits are obtained by extra calcium treatments (Burns & Pressey 1987). In plums the positive effects on fruit quality and storability by applying calcium as a foliar fertilizer in the growing season, has been shown by Vangdal & Børve (2001) and Plich & Wojcik (2002). Alcatraz-Lopez *et al.* (2003) found that calcium and magnesium applications improved fruit firmness. Magnesium alone had no effect, while a tendency of firmer fruit was observed when the plums were treated by calcium only. The uptake of calcium is influenced by the availability of magnesium and potassium. Hence the ratios between the contents of these nutrients indicate the storability of fruit. (Holland 1980; Huguet 1980; Tomala 1997; Zavalloni *et al.* 2001). High (K+Mg):Ca or K:Ca-ratios are not desirable. However, magnesium is important in photosynthesis and general growth and potassium is important to fruit quality (colour and acidity) (Ystaas & Frøynes 1995). Foliar sprays with calcium are recommended in Norwegian commercial plum growing. In years with heavy crop postharvest sprays with nitrogen (urea-solutions) is recommended. Otherwise few foliar fertilizers are used. In this work the effects of foliar applications of magnesium and potassium on fruit quality in plums were studied.

Materials & Methods

Orchard management

Two separate field experiments were performed in the experimental orchard at Bioforsk Vest Ullensvang in Lofthus, situated in the fjord districts in Western Norway in 2007 and 2008. Eight year old trees of the cultivars 'Avalon', 'Excalibur', 'Reeves' and 'Victoria' were pruned with a vertical axis. Planting distance was

2×4.5 m. A 1 m wide stripe along the tree rows was kept free of weeds by herbicides. Plant protection was made according to standard programme in the area. Fruit thinning was done by hand whenever necessary in June (6 weeks after petal fall) to approx. 20 fruitlets per meter branch length.

Experimental Design

Plum trees of the cultivars 'Avalon', 'Excalbur' and 'Victoria' were fertilized according to a programme including foliar application of N, P, Ca, K, Mg, Cu, B, Mn, S and Zn as shown in Table 1. In addition the trees were given 250 kg/ha 6-5-20 N-P-K mineral fertilizer on the soil in the spring. Control trees were given only mineral fertilizer on the soil. The amount of 6-5-20 N-P-K fertilizer was, however, increased by 60 kg/ha to 310 kg/ha. The total amount of N applied should be approximately the same with and without foliar fertilization programme. The experiment was designed as a randomised block design with 3 replicates and two trees in each plot.

Table 1. The foliar fertilization programme used in the experiments (kg pr ha).

Stadium	Week	N	P	Ca	Other nutrients
D / 54	16	3 kg urea			1 kg Cu
E2 / 57-58	18	3 kg urea	1 kg MAP[1]		2 kg S + 1 kg Solubor
End of flowering	21	1 kg urea	1 kg MAP		2 kg S + 5 kg $MgSO_4$
After flowering	24	1 kg urea	1 kg MAP	2.5 kg $CaCl_2$	0.5 L Mantrac + 0.2 L Zintrac
	27			2.5 kg $CaCl_2$	
	30			2.5 kg $CaCl_2$	
	31		3 kg MKP[2]		
	33			2.5 kg $CaCl_2$	
Post-harvest	38	5 kg urea	1 kg MAP		5 kg $MgSO_4$ + 0.5 L Mantrac

[1] Monoammoniumphosphate; [2] Monopotassiumphosphate

In another experiment trees of the cultivars 'Avalon', Excalibur' and 'Reeves' were sprayed with extra magnesium and potassium (Table 2) and compared to controls given the foliar fertilization programme shown in Table 1. The experiment was designed as above.

Table 2. Treatments in the experiment with extra foliar application of magnesium and potassium (in addition to the programme shown in Table 1).

Treatment	Week	Magnesium	Potassium
1. Control			
2. 2×Mg	24 and 27	5 kg $MgSO_4$	
3. 2×K	29 and 32		3 kg MKP
4. 2×both Mg and K	24 and 27 (Mg) 29 and 32 (K)	5 kg $MgSO_4$	3 kg MKP

Harvest and Analyses

In late August leaf samples from each plot were collected and analysed by standard methods for N, P, K, Ca and Mg content.

The plums were harvested in week 35-37. Every five days mature plums were picked and brought to the laboratory for analyses. The total weight of the plums and average fruit weight was registered. 3 parallell samples of 10 fruits each were analysed. Trained judges estimated ground colour according to a scale from 1 (=green) to 9 (=yellow) and blush colour as per cent of the fruit surface covered. Fruit firmness was measured by a Durofel Instrument (Copa Technolgie S.S. / CTIFL, France) with a 0.25 cm^2 plunger (Planton 1992). Soluble solids content was measured by refractometer and titratable acidity by titration of diluted juice samples with NaOH-solution to pH=8.1. Fruit

samples from each plot were analysed for content of N, P, K, Ca and Mg by standard methods. Fruit samples of 10 fruits each were stored at 2 and 20 °C for 3 weeks. Every week three samples of each cultivar, treatment and storage temperature were analyzed as described above.

Statistical Analyses

For the statistical analyses of fruit quality average value of three samples of three pickings were used. The data were analysed by ANOVA and paired t-tests using Minitab® and Excel® statistical programmes.

Results & Discussion

Soil analyses showed that the status of the major nutrients in the orchard was within the range recommended in Norwegian plum growing. The leaf analyses showed no statistically significant differences between the treatments (data not shown). However, the tendencies were similar to that observed in the fruit analyses (see below).

Effects of a Foliar Fertilization Program

The effects of the foliar fertilization program on yield, fruit size and fruit quality were not significant. However, the foliar sprayed trees tended to have larger crops and smaller fruits. The fruit analyses showed an increase in N-content and a decrease in the contents in all other measured elements. The decrease was significant for phosphorus, magnesium and calcium. As foliar nitrogen application did not increase fruit weight, the decrease in other nutrients was not a dilution effect. The foliar fertilization programme included application of P, Ca, Mg and K. Still the content of these elements decreased. As the decrease in Ca-content was stronger than the decrease in K-content, the fruits from trees given a foliar fertilization programme tended to have higher K:Ca-ratio.

Table 3. Yield, fruit size and colour of plums treated with a foliar fertilization programme compared to soil fertilization only. Average of three cultivars and two years.

Treatment	Yield (tons/ha)	Fruit weight (g)	Blush colour[1]	Ground colour[2]
Control	11.6	49.7	54	6.9
Foliar fertilizing programme	13.5	47.5	54	6.4
P-value	n.s.[3]	n.s.	n.s.	n.s.

[1] per cent fruit surface covered; [2] Scale from 1 = green to 9 = yellow; [3] n.s. = not significantly different (P>0.05)

Table 4. Firmness and content of soluble solids and titratable acidity in plums treated with a foliar fertilization programme compared to soil fertilization only. Average of three cultivars and two years.

Treatment	Firmness (DUROFEL units)	Soluble solids content (%)	Titratable acidity (%)	SSC: acidity ratio
Control	62	15.2	1.62	9.38
Foliar fertilizing programme	63	15.3	1.62	9.44
P-value	n.s.1	n.s.	n.s.	n.s.

[1] n.s. = not significantly different (P>0.05)

Table 5. Content of major nutrients as percent of dry weight in fruits of plum trees treated with a foliar fertilization programme compared to soil fertilization only. Average of three cultivars and two years.

Treatment	N	P	K	Mg	Ca	K:Ca
Control	0.78	0.17	1.38	0.067	0.123	11.2
Foliar fertilizing programme	0.85	0.13	1.18	0.052	0.088	13.4
P-value	n.s.[1]	0.03	n.s.	0.04	0.03	n.s.

[1] n.s. = not significantly different (P>0.05)

Effects of Extra Magnesium and Potassium Application

The extra applications of magnesium, potassium or both did not have significant effects on yield and fruit size. The extra Mg and K treatments tended to give fruit with less blush colour. The plums from trees given both extra Mg and K had significantly less blush colour than plums from control trees.

The fruit analyses did not show increases in Mg and K-contents in plums from trees given extra Mg and K. The plums from trees sprayed with potassium had, however, significantly less magnesium than plums from control trees and trees given only magnesium. And the lowest K-content was observed in plums from trees given extra magnesium. The strongest effect of extra Mg and K-application was found in the Ca-content. The decrease in Ca-content was significant in plums trees given additional magnesium and potassium compared to plums from control trees. As a result of the decrease in Ca-content the K:Ca-ratio tended to be higher in plums from Mg and K-treated trees. Plums from trees given extra potassium had significantly higher K:Ca-ratio compared to controls.

During ripening the plums soften, and the limiting factor of storability or shelf life is the firmness (Vangdal *et al.* 2007). Even though no significant change in firmness due to treatments was observed at harvest, after two weeks of storage the potassium treated plums were significantly softer than control plums.

Table 6. Yield, fruit size and colour of plums when treated with extra magnesium and potassium as foliar fertilization. Average of three cultivars and two years.

Treatment	Yield (tons/ha)	Fruit weight (g)	Blush colour$_1$	Ground colour$_2$
1. Control	13.7	58.5	55 b[3]	6.5
2. 2 × Mg	16.9	59.0	55 b	6.7
3. 2 × K	15.7	59.2	53 ab	6.2
4. 2 × both Mg and K	12.3	57.1	51 a	6.1
P-value	n.s.[3]	n.s.	0.05	n.s.

[1] per cent fruit surface covered; [2] Scale from 1 = green to 9 = yellow; [3] n.s. = not significantly different (P>0.05). Numbers followed by the same letter within a column are not significantly different (P>0.05).

Table 7. Firmness and content of soluble solids and titratable acidity in plums treated with extra magnesium and potassium as foliar fertilization. Average of three cultivars and two years.

Treatment	Firmness (DUROFEL-units)	Soluble solids content (%)	Titratable acidity (%)	SSC: acidity ratio
1. Control	67	15.9	1.47	10.82
2. 2 × Mg	67	15.5	1.43	10.84
3. 2 × K	67	15.4	1.41	10.92
4. 2 × both Mg and K	66	15.9	1.48	10.74
P-value	n.s.[1]	n.s.	n.s.	n.s.

[1] n.s. = not significantly different (P>0.05)

Table 8. Content of major nutrients as percent of dry weight in fruit of plum trees treated with extra magnesium and potassium as foliar fertilization. Average of three cultivars and two years.

Treatment	N	P	K	Mg	Ca	K:Ca ratio
1. Control	0.72	0.16	1.38	0.069 b[1]	0.122 b	11.31 a
2. 2 × Mg	0.73	0.15	1.16	0.070 b	0.079 a	14.68 ab
3. 2 × K	0.73	0.15	1.34	0.048 a	0.076 a	17.63 b
4. 2 × both Mg and K	0.72	0.16	1.32	0.057 ab	0.093 ab	14.19 ab
P-value	n.s.[2]	n.s.	n.s.	0.02	0.02	0.04

[1] Numbers followed by the same letter within a column are not significantly different (P>0.05); [2] ns = not significantly different (P>0.05)

Table 9. Firmness (DUROFEL-units) in plums treated with extra Mg and K as foliar fertilizers and stored for two weeks. Average of two temperatures (2 and 20 °C), three cultivars and two years.

Treatment	Weeks in storage		
	0	1	2
1. Control	67	68	55 b
2. 2 × Mg	67	67	53 b
3. 2 × K	67	64	51 a
4. 2 × both Mg and K	66	68	52 ab
P-value	n.s.[1]	n.s.	0.05

Conclusions

A foliar fertilization programme in plums had no significant effects on yield, fruit size and quality compared to control trees were all fertilizers were applied on the soil; Plums from trees given foliar fertilizers tended to have higher content of nitrogen and lower contents of the other major nutrients. The decrease was significant in P, Mg and Ca; Extra magnesium and potassium had no significant effects on yield, fruit size and fruit quality factors, except blush colour. Extra potassium application reduced the amount of blush colour; The extra application of magnesium did not increase the magnesium level in the fruits. However, a significant reduction in calcium was observed in plums from Mg treated trees; Additional application of foliar potassium did not increase the K-levels. The extra K-sprayed trees had fruits with significantly less Mg, Ca and higher K:Ca-ratio. The K-treated plums softened faster than control fruits and accordingly had a shorter shelf life than unsprayed plums.

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13. EFFECTS OF NITROGEN FERTILIZATION ON FRUIT QUALITY DURING STORAGE OF SPANISH 'PIEL DE SAPO' MELON

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Abstract

The aim of the work is the evaluation of the effect of nitrogen fertilization on fruit quality during postharvest conservation at low temperatures. Melon cultivation was fulfilled at open air with a plastic mulch and drip irrigation (100% of crop evapotranspiration). Nitrogen fertilization was applied at 0, 50, 100 and 150 kg N ha^{-1}. Melon fruits were harvested at 48-50 d (days) after fruit set, and stored at 9±1 ºC and 85 % RH during 10, 20 and 30 d. After each period of conservation colour, firmness, soluble solids, vitamin C and total nitrogen contents from pulp tissue were analysed. The % fruit weight loss was determined too. No influence from the level of nitrogen fertilization on the quality parameters analysed has been detected. As expected, only total nitrogen content in fruit was affected by the different fertilization treatments. During conservation fruit firmness gradually diminished, % weight loss gradually augmented and at the end of storage vitamin C content decreased. The whole set of results showed that melon plant cultivation could be carried out with low levels of nitrogen fertilization without negatively affecting quality during postharvest, but a compromise must be found in order to avoid a negative effect on fruit production.

Keywords: conservation, fertilization, fruit quality, melon fruit

Introduction

Melon is one of the most demanded and consumed fruits in the developed countries. Spain is the main European producer of melon, with 46% of the total European production and a high level of exports. It produces more than one million tonnes per year, generating an income higher than 6 billion euros (FAOSTAT 2006). The largest areas dedicated to melon crops are concentrated in the southern half of Spain and more than 13,000 ha (33% of the national area) are found in Castilla – La Mancha region, with a total production of 365,000 t (MAPA2006).

Melon quality is defined by several parameters, in particular by those referred to pulp tissue such as colour, firmness, sweetness, although the fruit size and shape are important too. Another important quality parameter is one related to compounds that contribute to human health such as vitamin C (Odet 1991; Lester 1996). Fruit quality must be accomplished during growth and ripening on the vine because after harvest, during postharvest life, the quality can only be preserved. Quality parameters mainly depend on genotype but environmental factors such as cultivation conditions play an important role too. Among the latter ones management of fertirrigation is crucial for assuring fruit yield and quality. Irrigation and nitrogen fertilization are two important preharvest factors influencing melon production and quality (Lester *et al.* 1994; Yasuo *et al*. 2000). This crop has critical periods of growth when irrigation is necessary for optimal yield and quality (Hartz 1997) and, as in other horticultural crops; nitrogen is a dominant nutrient in growth development, productivity and longevity (Huett 1996). In particular the effect of nitrogen fertilization on melon quality has been scarcely studied until very recently (Ferrante *et al.* 2008).

The aim of this work has been evaluating the effect of nitrogen fertilization at 0, 50, 100 and 150 kg N ha^{-1} dose on melon quality during the storage of fruits at low temperatures after harvest. The interest of this objective is the optimal management of nitrogen fertilization in order to obtain the best postharvest fruit quality and at the same time avoiding environmental contamination by excessive doses of application of nitrogen to the fields.

Material & Methods

Plant Material and Experimental Design

This conservation assay was performed with Spanish 'Piel de Sapo' melon (*Cucumis melo* L., var. *saccharinus* cv. 'Sancho'). Melon plants were grown during spring and summer seasons of 2007 in the Spanish province of Ciudad Real, in the Agronomic Experimental Station 'Entresierra' (3º 56' W – 39º 0' N, 640 m altitude).

The cultivation design of the plantation was fulfilled in randomized split-plots with four replications, where the nitrogen fertilization was the only factor of variation. This fertilization was accomplished with four treatments: 0, 50, 100 and 150 fertilization units (FU, kg N ha^{-1}). Each elemental plot (126 m^2) included 7 planting rows with 8 plants per row placed 1.5 m apart, with a distance of 1.5 m between plants. The irrigation system consisted of one drip line per crop row and emitters of 2.0 l h^{-1}, 0.5 m apart. Irrigation water treatment was 100% of the crop evapotranspiration (Ribas *et al.* 1995). The cultivation was performed with transparent plastic mulch. As nitrogen source ammonium nitrate was applied at 34.5 % with the irrigation water from 13th June until 31st August, with 120 kg P_2O_5 as phosphoric acid.

Fruits were tagged at the moment of fruit set and harvested when they reached the commercial ripening stage, 48-50 d after fruit set. Immediately after harvest, fruits were transported to the research centre CEBAS-CSIC, located in Murcia, and subjected to low temperature storage. The conservation conditions were 9±1 ºC of temperature and 85% of relative humidity. After 10, 20 and 30 d of conservation samples were collected for their analysis. Also samples at day of harvest (day 0) were previewed. Samples were made of 3 fruits per each level of nitrogen fertilization, and pulp tissue was collected of each fruit for analytical determinations of fruit quality and total nitrogen content.

Fruit weight was measured at harvest and at the end of the experiment in order to assess weight loss. The following quality parameters were determined: colour, total soluble solids (TSS) content and firmness, according to methods described by Flores *et al.* (2007). Colour was expressed as L (luminosity), C (chroma or colour purity) and Hue angle (tone or true colour) parameters. Firmness and TSS results were expressed in Newtons (N) and ºBrix respectively. Ascorbic acid (ASC) was determined according to Wimalasiri & Wills (1983) with the modifications introduced by Egea *et al.* (2007). For the extraction 10.0 g of frozen pulp was dissolved into 10.0 mL 5% metaphosphoric acid. The results of total ASC content were expressed as mg 100 g^{-1} FW.

The experimental results are shown as the mean of determinations made for each sample. A factorial analysis of the variance (ANOVA) was applied to the results and when significance was revealed a test of Tukey was applied ($p \leq 0.05$).

Results

No significant differences has been observed with regard to the evolution of colour parameters and TSS content in relation to nitrogen level in fertilization treatments and period of conservation of the fruits. The colour parameters values were around 66±0.69 for L, 12.34±0.82 for C and 108.3±0.58 for Hue angle for every sample, and for TSS the average value of all samples was 11.6±0.75 ºBrix.

Total nitrogen content in pulp tissue gradually increased as nitrogen level in fertilization became higher, with significant differences among every level of nitrogen fertilization assayed (Fig 1A). The period of storage of fruits at low temperature seemed to have a negligible influence on the evolution of this parameter (Table 1). Total ASC content was not affected by nitrogen level of fertilization, but period of storage did

have an influence, since it significantly decreased after 30 days (d) of storage of the fruits (Fig 1B, Table 1). Also no significant influence of nitrogen level of fertilization has been observed on pulp firmness (Table 1).

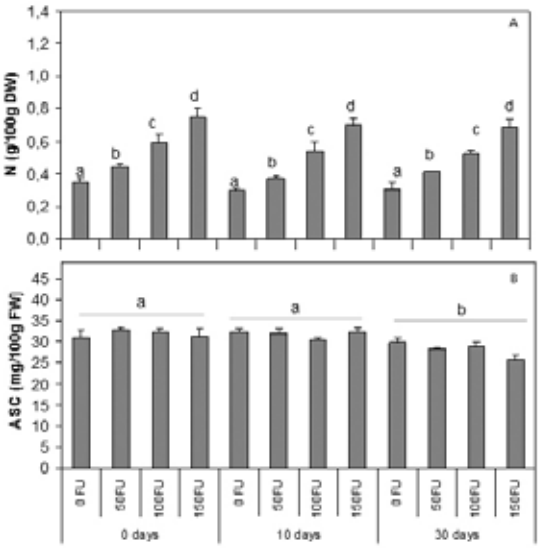

Fig 1. Total nitrogen (N) and ascorbic acid (ASC) contents in pulp tissue from 'Piel de Sapo' melon fruit in function of nitrogen fertilization treatment of plants and period of storage at 9 ºC of fruits.

Table 1. Statistical F and p-value of ANOVA test performed on total nitrogen (N) and ascorbic acid (ASC) contents, and firmness in pulp tissue, and fruit weight loss, in function of period of storage (days), applied fertilization units (FU), and the interaction between both factors (D*FU).

	Total N		ASC		Firmness		Weight Loss	
	F	p-value*	F	p-value*	F	p-value*	F	p-value*
Days	1.60	0.22	5.15	0.02*	22.33	0.00*	110.00	0.00*
FU	37.39	0.00*	0.30	0.83	0.99	0.61	2.59	0.60
D*FU	0.06	0.99	0.49	0.81	1.86	0.99	1.39	0.21

*p-values lower than 0.05 mean significant differences.

However, the evolution of this parameter is greatly influenced by the period of conservation of fruits at low temperatures (Fig 2, Table 1). In this case, pulp firmness gradually decreases along the period of storage, with significant differences among each date of sampling ($p \leq 0.05$). The opposite occurred with fruit weight loss evolution, which significantly augmented with the period of conservation at 9 ºC ($p \leq 0.05$) (Fig 3). Also it was observed that fruits from plants with higher degree of nitrogen fertilization showed lower weight loss, mainly in samples with longer periods of storage, but differences among different fertilization treatments were not significant ($p \leq 0.05$) (Table 1).

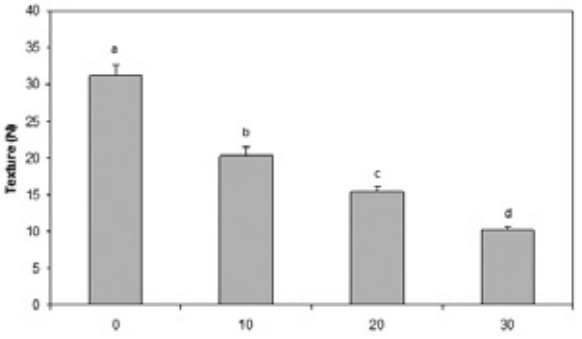

Fig 2. Pulp firmness evolution of 'Piel de Sapo' melon fruit during storage at 9 ºC.

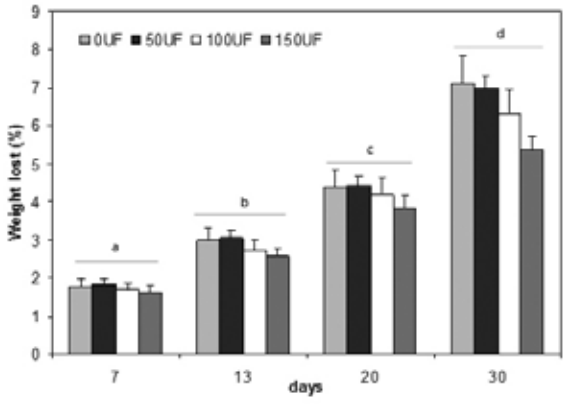

Fig 3. Percentage of fruit weight loss of 'Piel de Sapo' melon fruit during conservation at 9 ºC in function of nitrogen fertilization treatment of plants and period of storage at 9 ºC of fruits.

Discussion

Among quality attributes of 'Piel de Sapo' melon fruit, pulp colour, firmness and sugar content are the most important ones to determine the degree of acceptance of the fruit by consumers. The results from this experiment show that nitrogen fertilization did not affect pulp colour, contrary to results presented by Ferrante *et al.* (2008) in similar experiments with netted cantaloupe melons (*C. melo* L. var. *reticulatus*). These authors found that fruits from plants with the highest N fertilization dose showed an increase in lightness and yellowness. The minimum value of pulp firmness to be considered acceptable by consumers is 20 N. This parameter has not been found to be affected by nitrogen fertilization, although in other fruits it seems to be inversely correlated to the level of nitrogen applied in fertilization (Hernandez-Fuentes *et al.* 2003). However, storage strongly affected pulp firmness, as this parameter gradually and significantly decreased with longer periods of storage, already reaching values below 20 N from 20 d. Content of TSS did not vary with nitrogen dose in fertilization, a result in agreement with other authors. Kirnak *et al.* (2005) reported that nitrogen levels in fertilization of plants generally had little or no effect on TSS of fruits. Total nitrogen content in fruit increased with fertilization levels; these results support the hypothesis stated by Ferrante *et al.* (2008) who suggest that at high fertilization levels, the nitrogen content is not a growth limiting factor and therefore an accumulation dynamic situation takes place. Some studies have shown a negative correlation between nitrogen fertilization and ASC content (Lee & Kader 2000), while others found no effect of this first parameter on the latter one (Ferrante *et al.* 2008). In this study, ASC remained

steady among the different fertilization treatments applied. Also weight loss was not significantly affected by fertilization treatment, in spite of the fact that samples with longer periods of storage seems to suffer lower weight loss when plants were treated higher doses of nitrogen.

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14. CONSEQUENCES OF IRON DEFICIENCY ON FRUIT QUALITY IN CITRUS AND STRAWBERRY

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Abstract
Iron deficiency (iron chlorosis) is an important nutritional disorder in several plants, including fruit trees and strawberry. Iron chlorosis does not result from a small level of iron in soils but rather from impaired acquisition and use of this metal by plants. Calcium carbonate, present in great amounts in calcareous soils, and the resulting large levels of bicarbonate ions, are the main causes of iron deficiency. Countries in southern Europe, such as Portugal, Spain, Italy and Greece, have large areas of calcareous soils with established orchards, where iron chlorosis is a major factor that limits yield and profit for the farmer. Iron chlorosis affects several metabolic processes and leads to nutrient imbalances in sensitive plants. Decreased yield and poor quality of fruit resulting from the iron deficiency justify the development of methods to diagnose and correct this disorder. No single approach has been found to solve iron chlorosis satisfactorily, making it one of the most complex nutritional deficiencies known. In this chapter we describe some aspects of the effects of iron availability on quality of strawberry and citrus fruit.
Keywords: Iron deficiency, fruit quality, orange, strawberry, tangerine

Introduction
Iron deficiency leads to a decrease in the concentration of photosynthetic pigments in leaves, usually referred to as iron chlorosis. The symptoms occur primarily in young leaves and became apparent as an interveinal chlorosis with the appearance of a fine reticulation (Abadía 1992).

Iron chlorosis is a major limiting factor in fruit trees established on calcareous soils in Mediterranean areas, due to limitations on iron absorption, long distance transport and/or utilization by plants. Iron chlorosis affects several metabolic processes, leads to nutrient imbalances in plants (Sanz *et al.* 1995; Belkhodja *et al*. 1998; Pestana *et al.* 2004, 2005), and is responsible for significant decreases in yield, fruit size and quality (Álvarez-Fernández *et al.* 2006). Iron chlorosis can also lead to a delay in fruit ripening in orange and peach (Sanz *et al.* 1997; Pestana 2000; Pestana *et al.* 2001a, 2002). In *Citrus* spp., El-Kassas (1984) reported a negative effect of iron chlorosis on gross yield and fruit quality, resulting in smaller fruit that were more acidic and contained less ascorbic acid. In peach, changes on chemical composition were reported, affecting organoleptic and nutritional properties, although the external aspect of the fruits remained unaltered (Álvarez-Fernández *et al.* 2003).

In a review, Tagliavini *et al.* (2000) summarized the economical impact of iron chlorosis in kiwi, peach and pear orchards established on calcareous soils in Italy, Spain and Greece and concluded that yield losses were directly related to the intensity of iron chlorosis, and that a significant proportion of peaches and kiwifruit were unsuitable for the market. However, Sanz *et al.* (1997) found that iron chlorosis only affected peach quality when visual symptoms were obvious, corresponding to a severe deficiency.

Strawberries are also affected by iron chlorosis, and they are a more convenient test plant, as they grow faster and occupy a smaller area than trees. They can also be grown in hydroponic systems so that the nutritional status can be manipulated more easily.

In this chapter we review some aspects of the effects of iron availability on quality of strawberry and citrus fruit.

What Are the Effects of Iron Chlorosis in Citrus Fruit Quality?

In a citrus orchard established on a calcareous soil, foliar treatments with iron (+Fe) increased both fruit quality and size in 'Encore' tangerine trees and 'Valencia Late' orange trees (Table 1).

Table 1. The effect of iron chlorosis (-Fe) on yield of tangerines and oranges (Pestana *et al.* 1999, 2002). (+Fe) - Trees sprayed with Fe.

	Fresh weight	Diameter	Juice content	Maturation
	g fruit^{-1}	mm	ml	Index
Tangerine 'Encore'				
-Fe	66 b	55 b	33 b	4.5 b
+Fe	107 a	65 a	51 a	6.7 a
Variation	*-38* %	*-15*%	*-35*%	*-32*%
Orange 'Valencia Late'				
-Fe	121 b	62 b	59 b	9.1 b
+Fe	157 a	68 a	80 a	10.9 a
Variation	*-23* %	*-9* %	*-26*%	*-17*%

Maturation Index is calculated by the ratio between total soluble solids and titrable acidity. For each species, means in a column followed by the same letter are not significantly different at 5% (Duncan test).

Non-sprayed trees had smaller fruits (a decrease between 9 and 15%), less fresh weight (a decrease between 23 and 38%) and less total juice content (a decrease between 26 and 35%) compared to treated trees. No differences were found for total soluble solids, but the maturation index was smaller in fruits from chlorotic trees (Table 1). The application of Fe to orange trees thus enhanced fruit quality and advanced the ripening process, as in citrus the acidity (mainly citric acid) declines and the sugar concentration (expressed as total soluble solids) increases (Spiegel-Roy & Goldschmidt 1996). In conclusion, addition of iron resulted in fruits with a greater diameter, representing a gain of more than 35% in gross income to the farmer (Pestana *et al.* 2001a).

Which Are the Residual Effects of Iron Pools in Orange Trees?

Iron chlorosis is a complex process in fruit trees, as the development of this nutritional imbalance in one year may affect the reproductive cycle in the following years. Furthermore, iron chlorosis is also associated with other nutritional imbalances such as: P, Mg, K and Zn deficiencies (Pestana *et al.* 2001b, 2002, 2004, 2005). Together, these elements negatively affect fruits characteristics and cause a delay in fruit ripening. Accordingly, a prolonged period of chlorosis is supposed to induce a depletion of carbohydrate and Fe reserves, and consequently affect fruit production (Álvarez-Fernández *et al.* 2006).

In another field experiment, orange trees established on a calcareous soil were sprayed with (+Fe) or without iron (-Fe) during one growing season (Year 1). One year later (Year 2), the same trees were studied, but no iron was applied in both treatments. Fruits were collected at the end of the foliar treatments (Year 1) and again one year later (Year 2) to assess the residual effects of iron sprays on quality parameters. In the first harvest season (Year 1), the fruits of treated trees were larger (greater diameter), heavier (greater fresh mass) and matured earlier (maturation index) than those of chlorotic trees (Table 2).

Table 2. Fruit quality of oranges collected in two consecutive years (Year 1 and Year 2) in the same orchard. (+ Fe) - Trees sprayed with iron only in Year 1; (-Fe) – Untreated trees (Pestana *et al.* 2002).

		Fresh weight	Diameter	Juice content	Maturation
	Fruit harvest	g fruit^{-1}	mm	ml	Index
- Fe	Year 1	184 b	72 a	89 b	9 a
	Year 2	143 c	65 b	67 c	6 b
	Variation	*-22 %*	*-10 %*	*-25 %*	*-37 %*
+ Fe	Year 1	206 a	76 a	98 a	9 a
	Year 2	193 a	74 a	95 a	10 a
	Variation	*-6 %*	*-5 %*	*-3 %*	*6 %*

Means in a column followed by the same letter are not significantly different at 5% (Duncan test).

One year later (Year 2), the fruits of treated trees still had greater diameters, and more juice and fresh weight than those of untreated trees. Although the positive impact of iron sprays in Year 2 was smaller than in Year 1, it is clear that fruit size and quality in Year 2 were dependent on the nutritional status of the tree in the previous year. In conclusion, foliar applications with Fe made in Year 1 had a positive effect on fruit quality one year later.

Does Iron Chlorosis Affects Internal Quality of Strawberries Fruits?

Strawberry (*Fragaria ananassa* Duch.) quality can be defined by texture, taste (soluble sugars and organic acids) and colour (anthocyanin content) of the fruit at harvest (Kafkas *et al*. 2007). Although the main constituents of strawberries during maturation are well known, very few studies have concentrated on the impact of nutritional disorders on these parameters. In spite of a similar external appearance, fruits grown in the absence of Fe had changes in internal quality parameters associated with a delay in fruit ripening, namely smaller sugar and anthocyanin contents (Table 3).

Iron deficiency did not significantly affect the relative proportions of each type of anthocyanin (Pestana *et al.* 2010). However, chlorotic fruits had smaller total anthocyanins content, comparatively to fruits of green plants (Table 3).

It is curious to note, however, that parameters related to health-promoting compounds can be enhanced in chlorotic plants. Strawberry fruits are an excellent source of ascorbic acid, and its concentration was greatest in fruits collected from chlorotic plants (an increase of 28%).

Table 3. Total content of sugars, ascorbic acid and anthocyanins of juice of fruits from strawberry plants grown with (+Fe) and without iron (-Fe) (Pestana *et al.* 2010).

	Total	Total	Ascorbic	Antioxidant activity		
	sugars	anthocyanins	acid	DPPH$^{\bullet}$	ORAC	TEAC
	mg g^{-1} FW	μg g^{-1} FW	mg 100 g^{-1} FW	IC_{50}	μM Trolox ml^{-1} juice	IC_{50}
-Fe	11 b	431 b	37 b	260 b	27 b	272 b
+Fe	14 a	651 a	29 a	359 a	48 a	341 a
Variation	*-21 %*	*-34 %*	*+28 %*	*-28 %*	*-44 %*	*-20 %*

For each column, means with the same letter are not significantly different at 5% (Duncan test). FW–fresh weight; DPPH$^{\bullet}$ - 1,1-diphenyl-2-picrylhydrazyl radical; ORAC - Oxygen Radical Absorbance Capacity; TEAC - Trolox Equivalent Antioxidant Capacity; IC_{50} - sample concentration scavenging 50% of free radicals.

Fruits from non-chlorotic plants had more capacity to scavenge peroxyl radicals, while fruits from chlorotic plants had more capacity to scavenge DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals (reflected as smaller values) than green plants. The greater antioxidant activity measured by these two methods followed the increase observed in ascorbic acid concentrations (Pestana *et al.* 2010).

The delay observed in fruit ripening due to Fe deficiency was probably related to the different biomass allocation in chlorotic plants (Saavedra *et al.* 2009). Strawberry plants with symptoms of iron chlorosis produce fruits with similar weight but with less intense colour and poor organoleptic characteristics (Pestana *et al.* 2008).

Conclusions & Outlook

Undoubtedly, there has been a major improvement in the understanding of lime-induced iron chlorosis over the last 20 years. Nevertheless, several aspects remain unclear, especially those related to fruit trees grown under field conditions. Our results have indicated that iron chlorosis affects compounds related to flavour and healthfulness. Citrus and strawberries are classified as non-climateric fruits, so the nutritional imbalance induced by Fe deficiency may affect not only the harvest date but also fruit storage and commercialization. Further studies should focus on iron management to increase strawberry quality even if this leads to a reduction in berry size and yield. The use of an integrated management system to correct iron chlorosis should consider economic, ecological and social aspects. Orchard management techniques are sustainable only if they represent an advantage for fruit growers, and the studies on iron chlorosis should include the effects on fruit quality and yield. Iron chlorosis causes fruit quality losses in citrus orchards that may be prolonged for at least another year. The impact of controlling iron chlorosis prior to fruit formation on fruit yield and quality also deserves further investigation.

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SECTION 3. QUALITY MANAGEMENT OF FRUIT AND VEGETABLES

15. QUALITY OF TWO TOMATO CULTIVARS GROWN UNDER DIFFERENT AGRONOMICAL CONDITIONS

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Abstract

Quality of two tomato cultivars ('Dundee' and 'V1') produced in greenhouses, under different agronomical conditions during a season, was studied. The main goal was to measure quality of tomato fruits produced under different environmental conditions, and to understand their influence on final quality. To achieve this objective several physical and chemical general quality parameters were evaluated and to study nutritional quality, antioxidants (lycopene, beta-carotene, vitamin C) and total sugars (fructose and glucose) were analyzed. Analyses of variance were performed considering the factors "Harvest time", "Cultivar" and "Agronomical conditions" that correspond to the different conditions inside greenhouses. The MANOVA statistical analysis revealed that all the factors considered were significant as well as their interactions. The factor "Harvest time" was the most important to explain the differences. The 'V1' fruits produced in the metallic greenhouse without additional CO_2 had higher and more homogeneous weight values and also higher skin firmness. The colour coordinate a* was generally lower for fruits grown in traditional greenhouse. 'Dundee' fruits reached the highest SST medium value of 6.37 ºBrix. Biosynthesis of lycopene and vitamin C was affected by agronomical conditions and also predetermined by cultivars. 'Dundee' cultivar seems to be more sensitive to agronomical conditions than the 'V1'. Beta carotene content was mainly due to genetic factors. The 'V1' exhibited higher values of beta carotene for all the greenhouse conditions.

Keywords: antioxidants, physical-chemical analysis, quality parameters, tomato

Introduction

Traditionally fruit consumers prefer products that exhibit an adequate ripeness stage, freshness, good flavor and aspect, and after these aspects they consider nutritive value and price (Shewfelt 1993). However, nowadays consumers are becoming more informed about nutritional quality of food and its significance in health maintenance.

The importance of antioxidant intake on human diet as a prevention strategy of developing some types of cancer and coronary heart disease is a fact of common knowledge (Sabio *et al.* 2003). Tomatoes are consumed all over the world and can provide an important amount of total antioxidants in the human diet, mainly lycopene with unique antioxidant properties (Calvo & Santa-María 2008). The antioxidant content of tomato mostly depends on genetic, environmental and ripening factors (Martinez-Valverde *et al.* 2002).

Quality characteristics of tomato depend upon cultivar, agronomical conditions, ripening at harvest and storage conditions, mainly if considering sugar content, acidity and flavour used by consumers to define quality (Nuez 2000). However, colour and firmness are the characteristics more appreciated by consumers when buying tomato.

The interest of studying different agronomical conditions is due to technical changes introduced by growers from the south of Portugal, Algarve, in order to produce during all year: greenhouses of wood structure, covered with PE were changed by new greenhouses with metallic structures, equipped with heating and hydroponic systems. Sometimes, in order to improve yield, the addition of CO_2 in the inside atmosphere is also used (Islam *et al.* 1996; Reinert *et al.* 1997).

The main goal was to measure quality of tomato fruits produced under different environmental conditions, and to understand their influence on final quality. To achieve this objective were evaluated physical and chemical parameters and studied nutritional quality antioxidants (lycopene, beta-carotene, vitamin C) and sugars (fructose and glucose).

Material & Methods

Plant Material and Experimental Design

Two tomato (*Lycopersicum esculentum* Mill) cultivars, 'V1' (Hazera ©) and 'Dundee' (Ruitter Seeds ©) both suitable to hydroponic greenhouse cultivation, were grown in the region of Algarve under different greenhouses: A - traditional greenhouse with wood structure; B- metal greenhouse, with heating system (day temperatures of 25 ºC and night of 12 ºC), and C- metal greenhouse with the same temperatures and addition of CO_2 (500 to 700 ppm).

Fruits were harvested during the usual season at mature light red stage, considered adequate to commercial purposes. Five harvest dates were considered (2nd May, 17th May, 5th June, 19th June and 5th July), and 20 fruits of each modality were randomly picked up on each day. Tomatoes were carefully accommodated inside isothermal boxes and transported to the Post-harvest and Technology Laboratory at the University of Évora. For nutritional evaluation samples of 5 fruits of each modality were harvested at mature light red stage at middle season and kept at -80 ºC until analysis.

The experimental design was factorial: "Cultivar" ('Dundee' and 'V1'), "Agronomical conditions" (A, B and C), and "Harvest time" (1st, 2nd, 3rd, 4th and 5th harvest day).

Physical and Chemical Analysis

Samples of 10 fruits of each modality were submitted to physical analysis. Tomato weight was measured by using a centesimal balance (Mettler Toledo PB 1502). External color (L* a* b* coordinates color space) was measured using a colorimeter Minolta CR-300, and two measurements per fruit were made along the equatorial axis. Textural characteristics of epidermis (skin) and mesocarp (pulp) were evaluated with a Texture Analyser TA-HDi using a 3 mm diameter cylindrical probe until a maximum deformation of 10mm; each test was performed three times in the equatorial region of each fruit. To evaluate the epidermis, the maximum force (MFSkin) was considered and the firmness of the pulp was measured as the stable force after skin rupture.

Total soluble solids (TSS) was measured twice on juice from each tomato, using a digital refractometer Atago (ATAGO, Inc. Kirkland, WA, USA) and results were expressed as ºBrix. Titratable acidity (TA) was measured on juice from each tomato, using a Crison Compact Titrator, with NaOH 0.1N, to pH 8.2 (ISO 750 – 1981), and results were expressed as citric acid percentage.

Antioxidants (Lycopen, Beta-carotene and Vitamin C), Fructose, Glucose and Total sugars were determined at the laboratory of INTAEX (Junta de Extremadura) Three repetitions of each sample were performed.

The glucose, fructose and total sugar contents were measured according to the method described by Lozano *et al.* (2007) by making up 2 g of homogenate to 10 mL with deionised water, passing it through a 0.45 μm filter and injecting it into an HP 1050 chromatograph (Agilent Technologies, Inc., Palo Alto, USA) using a Zorbax NH2 5 μm 4.6×250 mm column and a refractive index detector. Calibrations were carried out for each sugar (D(+)-anhydrous glucose Merck 8337.0250, D(-)-fructose Fluka 47739, sucrose Sigma S5016).

The total amount of lycopene and beta-carotene were determined by high performance liquid chromatography (HPLC), using 5 g of fruit homogenate with an HP 1100 chromatograph, diode-array detector (DAD) (Agilent Technologies, Inc., Palo Alto, USA.) equipped with a 10-μm Lichrosorb RP-18 column (4.6×250 mm) and using acetone (solvent A, HPLC grade from Merck) and water (solvent B) as

eluents. An isocratic elution was used: first, 10 min with a solvent composition of 75% A/25% B and, then, 20 min with 95% of solvent A/5% of solvent B. By comparing the retention times of the two pigments in the extract mixture with those of their respective standard compounds (Sigma), lycopene and beta-carotene were identified. Qualitative analysis of lycopene was also carried out by comparing the UV-visible spectrum of the obtained sample with that of the standard compound.

Vitamin C was extracted from 9 g of fruit homogenate with EDTA/H_3PO_4 (85%) solution, and was determined by HPLC in an HP 1050 chromatograph, diode-array detector (DAD) (Agilent Technologies, Inc., Palo Alto, USA), equipped with a 5 μm Agilent Zorbax SB-C8 column (4.6x250 mm) at 30 °C. The eluent was 50 mM acetic/acetate pH=4.

Data Analysis

Data were analyzed by ANOVA (MANOVA), for physicochemical values considering three variables "Harvest time", "Cultivar" and "Agronomical conditions". When necessary mean comparisons were performed using Tukey test for $p<0.05$. For antioxidants and sugars, two variables, "Cultivar" and "Agronomical conditions", were considered. For all the statistical analysis Statistica 6.0 program was used.

Results & Discussion

Cultivars behaved differently under the different agronomical greenhouse conditions. However, 'V1' tomato fruits produced in the metallic greenhouse without added CO_2 showed higher and more homogeneous weight values and also better resistance to manipulation during postharvest, due to higher skin firmness values (Table 1).

Table 1. Analysis of variance of general quality parameters considering three factors "Harvest time", "Cultivar" and "Agronomical conditions" for $p<0.05$.

Parameters	Factors	F	p
Weight	Harvest time	*67.029*	0.000
	Cultivar	*19.732*	1.3E-05
	Agronomical conditions	*8.857*	0.0002
Colour a*	Harvest time	*49.227*	0.000
	Cultivar	*54.186*	2.21E-12
	Agronomical conditions	*71.026*	1.64E-25
Skin Firmness	Harvest time	*70.229*	0.000
	Cultivar	*35.149*	9.3E-09
	Agronomical conditions	*3.798*	0,024
Pulp Firmness	Harvest time	*74.487*	0.000
	Cultivar	*19.648*	1.36E-05
	Agronomical conditions	*42.134*	1.19E-16
TSS	Harvest time	*173,676*	0,000
	Cultivar	*9.741*	0.002
	Agronomical conditions	*13.248*	6.33E-06
TA	Harvest time	*50.102*	0.000
	Cultivar	*34.367*	1.3E-08
	Agronomical conditions	*33.220*	1.3E-13
TSS/TA	Harvest time	*70.221*	0.000
	Cultivar	*97.375*	8.2E-20
	Agronomical conditions	*19.279*	1.5E-08

The colour coordinate a* (green-red) was generally lower for those fruit grown in the traditional greenhouse.

The evaluation of quality was mainly supported by values of TSS, TA and TSS/TA. The ratio TSS/TA often had values higher than 10, referred by Kader *et al.* (1978) as an indicator of good quality. However 'V1' presented those values more often. Cultivar Dundee reached the highest medium TSS value of 6.37 ºBrix. Results of TA were higher than that of 0.32%, referred by the same research team as a value of good quality.

In general, the results of Manova for all the instrumental parameters revealed that the factors considered were significant (Table 1), as well as their interactions (data not shown), and "Harvest time" was the one that most contributed to the differences in most cases.

Biosynthesis of lycopen and vitamin C in both cultivars were affected by agronomical conditions and also predetermined by genetic characteristics of cultivars. The 'Dundee' exhibited better results of these constituents, mainly for fruits produced inside metallic greenhouses (Fig 1). These greenhouses maintain

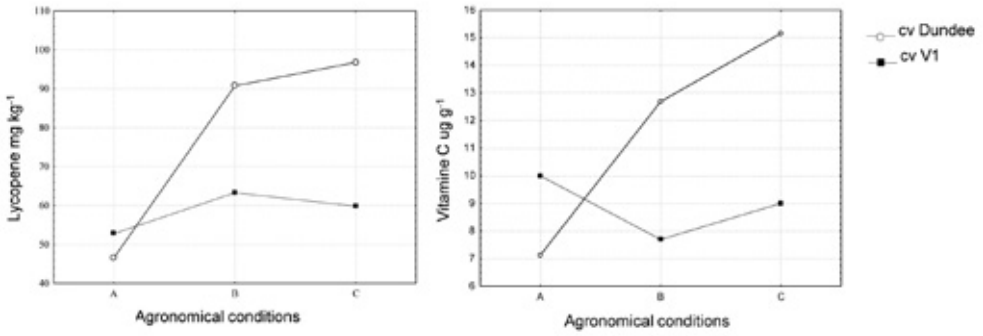

Fig 1. Means of lycopene and vitamin C content of 'Dundee' and 'V1' from different agronomical greenhouse conditions.

more stable and higher inside temperatures. According to Martinez-Valverde *et al.* (2002), lycopene synthesis is favoured at temperatures between 16 and 21 ºC and inhibited at temperatures above 30 ºC. On the other hand, Dumas *et al.* (2003) affirmed that since the antioxidant content of tomatoes may depend on genetics factors the choice of varieties cultivated may affect the results at harvest. The 'Dundee' seems to be more sensitive to agronomical conditions than the other cultivar.

Beta-carotene revealed a different behaviour than lycopene and vitamin C, being determined mainly by genetic reasons and not affected by agronomical conditions (Table 2). The 'V1' exhibited higher values of beta-carotene for all the greenhouse conditions. Both cultivars had a different response to temperature, light and CO_2. For instance 'Dundee' had higher Beta carotene content in fruits produced under metal greenhouse with CO_2 and 'V1' had better results without it (Fig 2).

Low values of vitamin C were obtained for fruits of both cultivars grown in the traditional greenhouse. Temperatures acted as a limiting factor on vitamin C production. Adequate regimes of temperatures allow reaching the potential level of vitamin C. Under greenhouse conditions, seasonal variation on vitamin C content of 'Jumbo' tomato ranged 70 to 230 mg kg^{-1} fwt at the mature-green stage, and was directly correlated with the temperature variations (Liptay *et al.* 1986). From the ANOVA results it can be said that vitamin C content is influenced by the cultivar, the agronomical conditions, and their interaction (Table 2).

Table 2. Analysis of variance of some chemical quality parameters considering two factors "Cultivar" and "Agronomical conditions" for $p<0.05$.

Chemical parameters	Factors	*F*	P
Lycopene	Agronomical conditions	*46.219*	0.000
	Cultivars	*50.102*	0.000
	Interaction	*22.729*	0.000
Beta-carotene	Agronomical conditions	*2.724*	0.106
	Cultivars	*12.082*	0.005
	Interaction	*0.140*	0.871
Vitamin C	Agronomical conditions	*6.888*	0.010
	Cultivars	*12.692*	0.004
	Interaction	*13.406*	0.001

The higher concentration of total sugars was found for 'Dundee' tomatoes produced in metallic greenhouse with CO_2. For cultivar V1 sugar concentration did not show any sensitivity to CO_2 level (Fig 2).

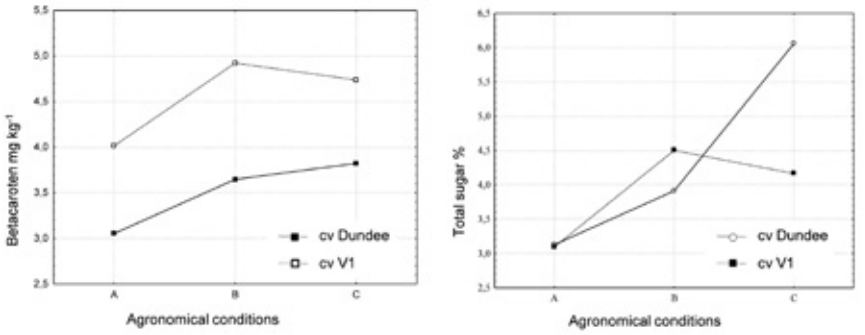

Fig 2. Means of beta-carotene content and total sugars of 'Dundee' and 'V1' obtained from different agronomical greenhouse conditions.

The lowest concentrations of antioxidants and sugars in the fruits produced under the traditional greenhouse can be justified by temperatures not properly maintained and less light intensity.

Excess of vegetative growth can cause a decrease on Beta carotene and vitamin C because it is necessary that radiation reaches the fruits to improve synthesis of these compounds (Dumas *et al*. 2003). Vitamin C produced in tomatoes 'V1' was higher in those from traditional greenhouse because plants had less leaves, so solar radiation could reach the surface of those fruits. The usual technique of taking off some leaves in order to promote aeration inside the plantation should be promoted to increase also the production of Beta-carotene and vitamin C, mainly with cultivars that tend to have too many leaves.

The different agronomical factors studied influenced the quality of tomato and both the metallic and heated greenhouses exhibited good conditions for improving quality.

Acknowledgements

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16. DESERT PLANTS WITH MEDICINAL VALUE

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Abstract

Treatment of cancer with chemotherapy has two main problems: toxicity to normal cells and failure to kill cancer cells. Cancer cells are characterized by uncontrolled cells proliferation and unlimited life span. Development of anti-cancer drug should involve the search for compounds capable of halting cell proliferation and/or leading to cell death. Combination of both types of drugs will make efficient chemotherapy. The compounds selected in this study are unique in their mode of action: they activate the protein procaspase-3, a critical enzyme in cell death process known as: apoptosis or programmed cell death. Although programmed cell death occurs naturally, too much or too little apoptosis cause diseases. Not enough apoptosis cause cancer. Apoptosis involves a cascade of enzymes (caspases) that are made as latent zymogens (pro-enzymes); procaspases activated following apoptotic death stimuli, lead to cleavage of cellular proteins, cleavage of DNA and cell death. The enzyme caspase-3 acts in a point of no return in this cascade. As such, compounds that will activate caspase-3 will be considered as potential anti-cancer drugs. In practice, for a compound to be considered as a potential lead drug, it should be a small molecule, stable, selective, and able to penetrate cellular membranes effectively. Following screening of plant extracts against several cancer models and through development of an assay that can detect compounds which activate caspase-3, several extracts capable of killing cancer cells by activating caspase-3 were identified.

Introduction

Cancer is one of the leading causes of death. In the United States there are 1.4 million cases of cancer per year and 0.5 million cases of death. Cancer of the prostate, breast, lung and colon are the most frequent type of cancers. onventional treatment work by killing the cancer cells with a dose of chemo-or radiotherapy. New generation of anti-cancer drug will be based on the knowledge regarding how cells kill themselves- a process called apoptosis or programmed cell death (Weil *et al.* 1996). Unlike cell death that occurs during and after tissue injury, apoptosis occurs in healthy tissue and does not trigger inflammation. Although programmed cell death occurs naturally, too much or too little cause disease. Excessive apoptosis may underlie the nerve damage in diseases such as Parkinson's and Alzheimer's. Not enough apoptosis, on the other hand, causes cancer. Too little apoptosis leads to too many cells; compounds capable of activating cell death will enable the reduction of cell number. The goal is a drug that triggers apoptosis in cancer cells but not in normal cells. At the heart of the conserved biochemical pathway that mediates the highly ordered process of apoptosis are a family of cysteine proteases, termed 'caspases' (cysteinyl aspartate-specific proteinases). Caspases are produced as precursor molecules that require processing into two subunits to produce a fully active enzyme (Thornberry & Lazebnik 1998). On the basis of primary structure, proapoptotic caspases can be divided into two classes, class I including caspases that contain a long amino-terminal prodomain, and class II with a short or absent prodomain. One of the key regulatory steps for apoptosis is the activation of caspases, leading to the characteristic morphological changes associated with apoptotic cells including chromatin condensation, DNA fragmentation into nucleosomal fragments, nuclear membrane break down, externalization of phosphatidylserine and formation of apoptotic bodies that are readily phagocytosed. The mechanism of caspase activation is poorly understood but recent studies demonstrate that procaspases can be activated through dimerization/oligomerization. Caspases are present as inactive pro-enzymes, most of which are activated following cleavage at a specific aspartate cleavage site and assembly of their active subunits. Caspase-8, caspase-9 and caspase-3 are situated at

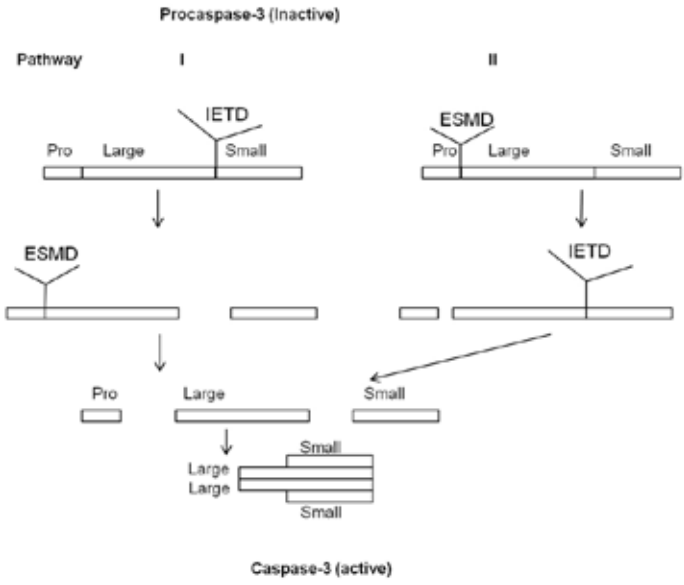
pivotal junction. Caspase-3 appears to amplify the caspase-8 and caspase-9 signal into a full commitment to disassembly. We have recently shown that p53-null T lymphoma cell lines, but not normal T cells, are highly sensitive to inhibition of gene/protein expression. These lymphoma cells seem to be primed for apoptosis but protected by an unknown labile protein (Ofir *et al.* 1999, and unpublished results). Some protein inhibit caspase activity: when they are not produced, apoptosis begins. The hope is to find drugs that will inactivate such inhibitors of apoptosis only in cancer cells.

Natural products research continue to be an invaluable source of leads, with examples such as antibiotics, analgestics such as epibatidine, the cholesterol-lowering drug mevinolin and the anticancer agent taxol. Purification of the active constituents of natural products enable to define their chemical structure and make it possible to chemically modify them to improve their efficacy and their safety. According to recent studies on apoptosis in plants it became clear that the process of apoptosis is conserved also in plants and as such it is reasonable to assume that there are activators/inhibitors of apoptosis in plants. It has been shown recently that natural compounds like resveratrol (Jang *et al.* 1997; Szende *et al.* 2000), wheat bran (Jenab & Thompson 2000), the Chinese herb *Tripterygium Wilfordii* hook (Lee *et al.* 1999), agents found in plant extracts like mistletoe and *Semicarpus anacardium* (Thatte *et al.* 2000) induce apoptosis. The progress in defining, at the molecular and cellular levels, the mechanisms of pathology in plants and the finding of caspase- like enzymes in plants (Korthout *et al.* 2000) make the search for caspase activating compounds in plant less dependent on serendipity than in earlier times.

In the present study we identified plant extracts capable of activating the protein procaspase-3.

Two pathways for processing of procaspase-3 are described (Scheme 1): I. Cleavage between the large and small domains following by cleavage between the pro domain and the large domain. II. Cleavage between the pro domain and the large domain following by cleavage between the large and the small domains. Among the active extracts described in this project, one extract uses pathway II and three use pathway I for the activation of procaspase-3.

Scheme 1: Two pathways for processing procaspase-3



Results

In this section results will be presented showing that the selected active plant extract activate the enzymatic activity of caspase-3. The parameters tested were: enzymatic activity, DNA ladder, Western blotting and processing of procaspase-3 *in situ (*in the test tube) and in cellular extracts. In addition, the processing of procaspase-3 synthesized in reticulocytes lysate from the cDNA of procaspase-3 was also tested.

The active plant extracts were identified by the following symbols: RO4 and RO39, are aqueous plant extracts; RO139, RO144 and RO197 are plant extracts prepared in ethanol.

Cytotoxicity of Plant Extracts in Various Tumor Models

Cell lines representing various tumor models were incubated with plant extracts and cellular viability was measured; results are summerized in Table 1.

Table 1. Viability of human tumor cell lines following incubation with plant extracts.

Plant Extract	Human tumor models				
	Lymphoma	Prostate cancer	Melanoma	Breasts cancer	Ovarian
RO4	+	+	-	-	-
RO39	+	-	-	-	-
RO139	+	+	+	+	-
RO144	+	+	+	-	-
RO197	+	+	+	+	-

+ more than 50% killing; –less than 50% killing.
Viability was defined as cell survival above spontaneous death. Viability was measured with the reagant XTT (XTT as a tetrazolium salt react with mitochondtrail enzymes that are active only in alive cells).

Apoptosis of Human and Mouse Hematopoietic Cell Lines Following Incubation With Plant Extracts

Cell were incubated with plant extracts and the apoptotic cells were identified by FACS analysis. The results are summerized in Table 2.

Table 2. FACS analysis of leukemic cells following incubation with plant extracts.

Plant Extract	Apoptotic cells (%)			
	Mouse cell line		Human cell line	
	5 h	16 h	5 h	16 h
Control	0.5	2	1	1
RO4	2	47	0	58
RO39	0.5	6	1	10
RO139	2	56	0	0
RO144	22	33	0	13
RO197	0	26	1	25

Caspase-3 activation Following Incubation of Tumor Cell Lines With Plant Extracts

Initial screening for proapoptotic activity in plant extracts lead to the identification of 5 extracts containing agents capable of inducing caspase-3 enzymatic activity. The results of caspase-3 activity, as measured in cellular extracts following the incubation of mouse leukemic cells with plant extracts, are summarized in Table 3 and Fig 1.

Table 3. Caspase-3 activity following incubation of mouse leukemic cells with plant extracts*.

Plant Extract	Caspase-3 activity (pmol/min/ug) of protein
RO4	11
RO39	11
RO139	78
RO144	99
EtOH	0

*mouse leukemic cells were incubated with plant extracts for 6 h Caspase-3 activity was measured in cellular extracts using a colorimetric substrate according to the manufecturers instructions.

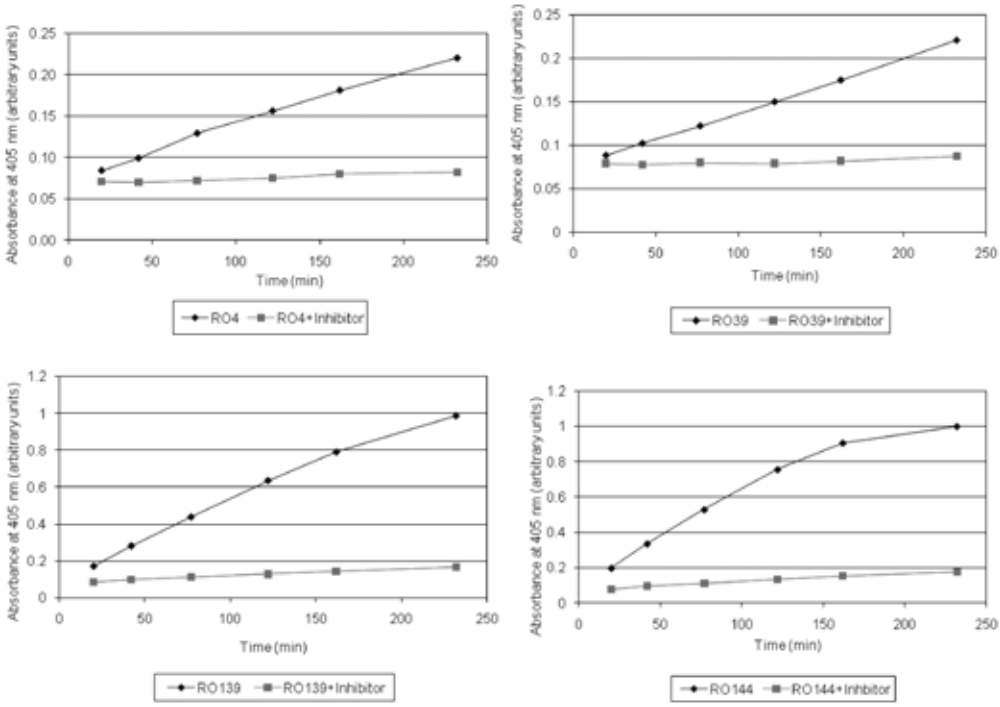


Fig 1. The inhibitor DEVD-CHO inhibits Caspase-3 activity induced by plant extracts. Leukemic mouse cells were induced to undergo apoptosis by treatment with a plant extract. Cellular extracts prepared in lysis buffer (50 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4) were incubated with the colorimetric substrate DEVD-pNA. Cleavage of the substrate by the active caspase-3 lead to changes in the absorbency at wavelength 405nm as measured along the experiment (every 15-30 min). Caspase-3 activity is expressed as arbitrary units of absorbance at 405 nm. In order to show the specificity of the cleavage, the enzymatic reaction was performed also in the presence of the caspase-3 specific inhibitor DEVD-CHO (0.1μM).

One of the hallmarks of apoptosis is the unique fragmentation of DNA into a ladder pattern. Caspase-3 activation following incubation of mouse leukemic cells with RO139, RO144 and RO197 resulted in DNA fragmentation (data not shown).

Processing of Procaspase-3 *in Vitro* Following Incubation of Cellular Extract With Plant Extracts

One of the goals of the project was to find a compound capable of directly activating procaspase-3. We developed an assay, which will enable us to follow in vitro the processing of the inactive procaspase-3 into

an active enzyme. A cellular lysate prepared from untreated cells was incubated with plant extract for 90 min at 37 °C. According to the Western blotting shown in Fig 2, the extracts RO39, RO197, RO144 lead to the conventional processing of procaspase-3 (scheme 2 pathway I): 32kDa----->20kDa----->17kDa (lanes 1,3,4) while RO4 (lane 2) uses an alternative path (scheme2 pathway II): 32kDa----->29kDa----->17kDa. A similar processing mode is used by RO4 when incubated with procaspase-3 produced in a reticulocyte lysate (Fig 3 lane 1; the procaspase-3 protein is the translation product of the cDNA of procaspase-3). Heat inactivation of RO4 abolished its capability to process procaspase-3 (data not shown), indicating that RO4 contain an active protease.

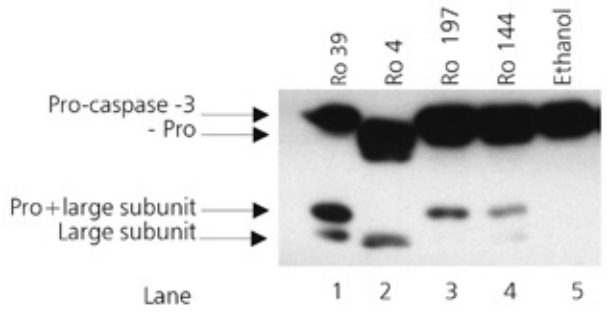


Fig 2. Activation of procaspase-3 following incubation of plant extracts with naïve cellular extract. Western blot analyses of the activation of procaspase-3. Procaspase-3 is detected in naïve cellular extract (extract prepared from untreated U937 cells) as 32kDa protein by antibody directed against the large subunit. The presence of large sububit indicates activation and is detected following treatment with RO39, RO4, RO197, and RO144, lanes 1,2,3,4, respectively. Treatment with RO39, RO197 and RO144 generated the intermediate pro+large subunit as well (lanes 1,3,and 4, respectively). Treatment with RO4 generate an intermediate consisit of large and small subunit (-pro).

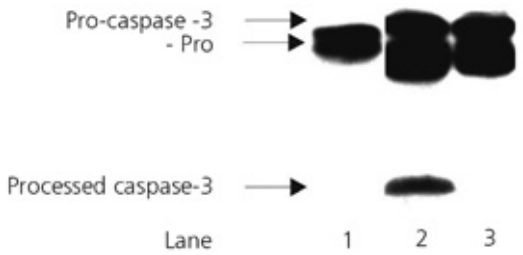


Fig 3. Procaspase-3 protein translated in reticulocytes lysate is processed following treatment with plant extracts. Western blot analyses of translation product of procaspase-3 cDNA incubated with caspase-3 activator plant extract C8 (lane 2) or with RO4 (lane 1). Detection was performed with anti caspase-3 antibodies.

Conclusion

The extracts RO4, RO39, RO139, RO144, RO197 directly induce caspase-3 processing and enzymatic activity. It is suggested that each extract contain at least one active compound that can be lead for anti cancer drug.

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17. INVESTIGATION ON THE ESSENTIAL MINERAL ELEMENT CONTENTS OF CULTIVATED AND WILD BLUEBERRY FRUITS IN LATVIA

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Abstract

Wild blueberry (*Vaccinium murtillus*) is one of the most popular wild-harvested fruit in Latvia, traditionally used in folk-medicine and food. Unfortunately there are wide fluctuations in yields. The recent years mark a tremendous boom in high-bush blueberry (*Vaccinium corymbosum*) cultivation in Latvia. As the total berry production increase, blueberries have found a place in a daily intake as excellent source of antioxidants, dietary fibber, vitamin C and minerals. Thus, the chemical composition of *Vaccinium* spp. has important implication on human health. The aim of this study was to compare the contents of twelve biologically essential elements (N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, Mo, B) in berries of two *Vaccinium* species: *V. myrtillus* and *V. corymbosum*. Together, 48 (plant and berries) samples were collected from 3 main high-bush blueberry producing sites and 5 woodland areas during summer 2008. A comparison of two species showed similar concentrations for macroelements N, P, and Mg in fruits. N and K were the predominant minerals in blueberries. It should be stressed that wild blueberries had higher level of K (on average 98.77 mg 100 g^{-1} fresh fruit). The data indicated statistically significant differences in microelements composition of wild and cultivated blueberry fruits. The highest concentrations of Fe, Mo and B (0.53, 0.01 and 0.14 mg 100 g^{-1} FW, respectively) were found in high bush blueberries, while the highest Mn and Zn concentrations (1.53, 0.13 mg 100 g^{-1} FW, respectively) were found in wild blueberries. Plant leaf tissues analyses supported these differences. The present study shows that fruits of both *V. murtillus* and *V. corymbosum* are valuable sources of Mn (66.55% and 49.86% from recommended daily dose, accordingly) in human nutrition. The content of Fe, Cu, Mo and B in 100g fresh fruits of both blueberries also contributes from 3.75 to 20.50% of daily micronutrient requirement.

Keywords: *Vaccinium myrtillus*, *Vaccinium corymbosum*, mineral composition

Introduction

Wild blueberry (*Vaccinium murtillus*) is one of the most popular wild-harvested fruit in Latvia, traditionally used as healthy food as well as in folk-medicine. Unfortunately there are wide fluctuations in yields from year to year. The recent years mark a tremendous boom in high-bush blueberry (*Vaccinium corymbosum*) cultivation in Latvia. As the total berry production increase, blueberries have found a place in a daily intake as excellent tasty source of antioxidants, dietary fibber, vitamin C and minerals. Increased consumption of fruits and vegetables can help replace foods high in saturated fats, sugar and salt and thus improve the intake of most micronutrients and dietary fibre (Ekholm *et al*. 2007). Daily consumption of fresh fruits and vegetables (>400 g d^{-1}) is recommended to help prevent diseases such as cardiovascular diseases and certain cancers (WHO 2003). Thus, the chemical composition of *Vaccinium* spp. has important implication on human health. Many minerals are essential for normal metabolic functions and are required components in a balanced diet (Grusak & DellaPenna 1999). While nutritional research on blueberries has been done on low-bush, high-bush and rabbit-eye blueberry fruits in the main blueberry production countries (Dekazos 1978; Bushway *et al.* 1983; Rupasova *et al.* 2007), there are practically no information on mineral element content of blueberry crop in Latvia. Many external factors as growth environment (soil, geographical conditions), cultivation and fertilization practices are widely diverse in different blueberry production countries and could contribute to the mineral composition of fruits.

The aim of this study was to detect and compare the contents of twelve biologically essential elements (N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, Mo, B) in berries and plant leaf tissues of two *Vaccinium* species: *V. murtillus* and *V. corymbosum*.

Materials & Methods

The present study was carried out on wild blueberry (*V. murtillus*) and highbush blueberry (*V. corymbosum*) crops in different regions of Latvia. Together, 48 (leaf and berries) samples were collected from 3 main high-bush blueberry producing sites and 5 woodland areas during summer 2008. Berry and leaf materials were collected at each site as a composite sample from an area of about 10×10 m.

The plant material was oven-dried at 60 °C and ground. Then the plant samples were dry-ashed in concentrated HNO_3 vapours and re-dissolved in HCl solution (HCl - distilled water mixture 3:100). The levels of Ca, Mg, Fe, Cu, Zn, and Mn were measured by atomic absorption spectrophotometer (Perkin Elmer Analyst 700, acetylene-air flame); those of N, P, Mo, B by colorimetry, S by turbidimetry, and K by flame photometer (Jenwey PFP7, air-propane butane flame) (Rinkis *et al*. 1987). Mineral elements content in berries were expressed as mg 100 g^{-1} fresh fruit. All chemical analyses were done in the Laboratory of plant mineral nutrition of the Institute of Biology, University of Latvia.

The levels of statistical significance were determined with MS Excel 2003. T-test "Two-Sample Assuming Unequal Variances" ($p<0.05$) was used to compare mean element concentrations in *V. murtillus* and *V. corymbosum* fruits and leaves.

Results

To characterize the mineral content of cultivated and wild blueberry fruits, the levels of 12 biologically essential elements were estimated. Mean macro- and micronutrient concentrations, as well as concentration range are shown in Table 1. A comparison of two species studied showed similar concentrations for the macroelements N, P and Mg in fruits. Statistically significant differences ($p< 0.05$) were noted for K, Ca and S. The data indicated that nitrogen and potassium were the major mineral constituents in both blueberry fruits tested. The richest source of K and Ca (on average, 98.76 and 17.97 mg 100 g^{-1} fresh fruit) in this study was *V. murtillus*, while the highest S (22.93 mg 100 g^{-1} fresh fruit) contents were found in *V. corymbosum* fruits.

Table 1. Mineral composition of *V. corumbosum* and *V. murtillus* fruits in Latvia, 2008.

Element	*V. corumbosum*		*V. murtillus*	
	Range	Mean ± SE	Range	Mean ± SE
Macroelements (mg 100 g^{-1} fresh weight)				
N	88.0 – 97.6	93.87 ± 2.97 a[1]	72.0 – 160.0	97.49 ± 7.40a
P	17.6 – 19.2	18.67 ± 0.53 a	12.8 – 38.4	16.67 ± 0.56a
K	73.6 – 92.8	82.13 ± 5.64 a	64 – 192.0	98.77 ± 3.91b
Ca	11.2 – 14.4	12.80 ± 0.92 a	12.8 – 54.4	17.97 ± 1.25b
Mg	8.0 – 9.6	8.53 ± 0.53 a	6.4 – 30.4	10.20 ± 0.50a
S	22.4 – 24.0	22.93 ± 0.53 a	12.8 – 32.0	17.81 ± 1.20b
Microelements (mg 100 g^{-1} fresh weight)				
Fe	0.512 – 0.560	0.533 ± 0.024a	0.240 – 0.720	0.410 ± 0.01b
Mn	0.640 – 1.440	1.147 ± 0.131a	0.240 – 4.640	1.531 ± 0.21b
Zn	0.075 – 0.115	0.097 ± 0.021a	0.096 – 0.320	0.132 ± 0.04b
Cu	0.058 – 0.072	0.067 ± 0.008a	0.050 – 0.147	0.062 ± 0.02a
Mo	0.006 – 0.011	0.009 ± 0.003a	0.001 – 0.005	0.002 ± 0.001b
B	0.128 – 0.144	0.136 ± 0.008a	0.080 – 0.128	0.102 ± 0.001b

[1]Means with different letters in a row were significantly different (t-Test, $p<0.05$)

Statistically significant differences ($p<0.05$) were found in microelement (except Cu) composition of wild and cultivated blueberry fruits. The highest mean concentrations of Fe, Mo and B (0.53, 0.01 and 0.14 mg 100 g^{-1} fresh fruit, respectively) were found in high-bush blueberry, while the highest Mn and Zn (on average, 1.53 and 0.13 mg 100 g^{-1} fresh fruit, respectively) concentrations were found in wild blueberry. In general, plant leaf analysis supported these differences (Table 2).

Table 2. Mineral element concentrations in V.corumbosum and V.murtillus leaf samples in Latvia, 2008.

Element	*V. corumbosum*		*V. murtillus*	
	Range	Mean ± SE	Range	Mean ± SE
	Macroelements (% dry weight)			
N	1.0 – 1.35	1.18 ± 0.08a	0.8 – 1.5	1.08 ± 0.05a
P	0.14 – 0.15	0.14 ± 0.01a	0.08 – 0.17	0.12 ± 0.01b
K	0.58 – 0.74	0.69 ± 0.04a	0.64 – 1.0	0.77 ± 0.03a
Ca	0.42 – 0.65	0.49 ± 0.01a	0.56 – 1.12	0.84 ± 0.05b
Mg	0.15 – 0.22	0.17 ± 0.02a	0.17 – 0.54	0.32 ± 0.03b
S	0.23 – 0.31	0.25 ± 0.02a	0.12 – 0.18	0.15 ± 0.01b
	Microelements (mg kg^{-1} dry weight)			
Fe	68.0 – 102.0	85.5 ± 7.4a	54.0 – 128.0	81.9 ± 6.8a
Mn	176.0 – 360.0	224.0 ± 45.4a	130.0 – 1720.0	814.4 ± 146.4b
Zn	10.1 – 11.0	10.6 ± 0.2a	14.0 – 26.0	18.6 ± 1.0b
Cu	4.8 – 32.0	13.1 ± 6.4a	3.6 – 6.4	5.0 ± 0.2b
Mo	0.9 – 1.4	1.1 ± 0.1a	0.2 – 0.3	0.2 ± 0.01b
B	15.0 – 48.0	36.8 ± 7.4a	15.0 –30.0	22.7 ± 1.2b

[1]Means with different letters in a row were significantly different (t-Test, $p<0.05$)

Contribution of *V. murtillus* and *V. corymbosum* fruits as a dietary source of mineral elements was estimated from our study results. The potential contribution of 100 g of wild and cultivated blueberry fruits to the Recommended Dietary Allowances (RDA) (USDA RDA chart 2004) for mineral elements is presented in Table 3. Wild and cultivated blueberry fruits supplies 66.55 and 49.86%, respectively, of the adult daily requirement for Mn. The content of Fe, Cu, Mo, and B in 100 g fresh fruits of both blueberries studied also contributes with 3.75 to 20.50% of the daily micronutrient requirement. From macronutrients, only K in *V. murtillus* fruits was in appreciable amounts (3.95% of the RDA). One hundred grams of fresh wild and cultivated blueberry fruits may supply also a few percent of RDA for P, Ca, Mg, S and Zn.

Table 3. Contribution of 100 g of blueberry fruits to the Recommended Dietary Allowance (RDA) for adults per day.

Element	RDA*, mg	% of RDA supplied by 100 g blueberries	
		V. corumbosum	*V. murtillus*
P	700	2.67	2.38
K	2500	3.29	3.95
Ca	1000	1.28	1.80
Mg	420	2.10	2.43
S	850	2.70	2.10
Fe	8	6.67	5.12
Mn	2.3	49.86	66.55
Zn	11	0.88	1.20
Cu	0.9	7.47	6.92
Mo	0.045	20.50	3.75
B	1.5	9.07	6.78

* USDA RDA chart (2004)

Discussion

The relationship between food and health becomes increasingly significant as consumers now demand healthy, tasty and natural foods that have been grown in uncontaminated environments. Numerous studies have shown that among horticultural crops fruits are important source of dietary nutrients, especially with respect to minerals (Grusak & DellaPenna 1999). Among berry fruits, blueberries are considered to be not only an excellent source of phenolic compounds and vitamins, but also a valuable source of Mn, K, Cu (Bushway *et al.* 1983; USDA National Nutrient Database for Standard Reference 2006).

Our research revealed statistically significant differences between *V. murtillus* and *V. corymbosum* results for K, Ca, S, Fe, Mn, Zn, Mo, B in fruit samples and P, Ca, Mg, S, Mn, Zn, Cu, Mo, B in leaf samples. Cultivated high-bush blueberry fruits had higher content of S, Fe, Mo and B while wild blueberry fruits showed the highest levels of K, Ca, Mn and Zn. The sequences with regard to the content of macro- and microelements in *V. corymbosum* fruits were N > K > S > P > Ca > Mg and Mn > Fe > B > Zn > Cu > Mo. The order of macro- and micronutrient concentrations in *V. murtillus* was K = N > Ca = S > P > Mg and Mn > Fe > Zn > B > Cu > Mo.

The fruit mineral nutrient concentrations found in the Latvia studied species were similar or considerably higher (P, Ca, Mg, Fe, Mn) than values reported for high-bush blueberriy and rabbit-eye blueberry fruits (Eitenmiller *et al.* 1977; Dekazos 1978; USDA 2006; Rupasova *et al.* 2007). It should be mentioned that low-bush blueberries (*Vaccinium augustifolium*) are particularly high in Mn as well as Ca content. Reported mean values for Mn content (2.60 mg 100 g^{-1} fresh fruit) in low-bush blueberry fruits (Bushway *et al.* 1983) were almost twice higher than our Mn results for high-bush blueberries. To the author's knowledge, there are scarce comparable data in the literature which show the detailed mineral content of *V. murtillus*. Macronutrient content in wild blueberries analyzed was higher but, Mn concentrations were significantly lower than reported values for *V. murtillus* in Finland (Ekholm *et al.* 2007)

The nutritional significance of fruits as dietary source of minerals is related to the contribution it makes to the Recommended Dietary Allowance (RDA). The present study shows that fruits of both *V. murtillus* and *V. corymbosum* are excellent sources of Mn (66.55% and 49.86% from recommended daily dose, accordingly) in human nutrition. The content of Fe, Cu, Mo, and B in 100 g fresh fruits of both blueberries studied also contributes from 3.75 to 20.50% of daily micronutrient requirement. It should be stressed that cultivated high-bush blueberries had almost 5 times higher concentrations of Mo in their leaves and fruits, apparently due to use of Mo containing fertilizers. Therefore 100 g of high-bush blueberry fruits could provide 20.5% of RDA.

From macronutrients only K in *V. murtillus* fruits was stated in appreciable amounts (3.95% of the RDA). One hundred grams of fresh wild and cultivated blueberry fruits may supply also a few percent of RDA for P, Ca, Mg, S and Zn. The availability of Ca in the body to great extent depends on calcium to phosphorous ratio. The recommended optimal Ca:P ratio in the diets is 1.0 to 1.3 (Calvo & Park 1996). In our study such ratio was characteristic for wild blueberry fruits.

As a conclusion, the present study reveals, that *V. murtillus* and *V. corymbosum* differ in their elemental composition. Cultivated high-bush blueberry fruits had higher content of S, Fe, Mo and B while wild blueberry fruits showed the highest levels of K, Ca, Mn and Zn. Both blueberries studied could be qualified as good source of microelements: excellent source of Mn and valuable source of Fe, Cu, Mo, and B in human nutrition.

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18. TOMATO FRUIT QUALITY AS AFFECTED BY RIPENING ON- AND OFF-VINE

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Abstract
In order to extend postharvest life and marketable period, tomato fruits are mainly harvested at the mature green stage and they ripen off vine. However, most of the consumers are convinced that on vine ripened tomatoes are of superior quality. The objective of this study was to investigate the quality of tomato fruit (cv. 'Belladona') in relation to their ripening on vine and off vine. During the 10 days of ripening period, color parameters (Lightness, Hue angle and Chroma) were recorded daily. At the end of this period firmness, soluble solids content (°Brix), pH, titratable acidity, dry matter, nitrates, lycopene, total carotenoids, ascorbic acid, total soluble phenols and DPPH radical scavenging activity were determined. Tomatoes ripened off vine had significantly lower L* and Hue values from the 5th day of ripening and thereafter, indicating more red color than tomatoes ripened on vine. However, there were not significant differences in firmness among fruits of both ripening conditions. Also, the results showed that ripening conditions did not affect soluble solids content, pH, dry matter, SSC/acidity ratio, ascorbic acid, total soluble phenols as well as DPPH radical scavenging activity. On the contrary, tomatoes ripened on vine had significantly higher nitrates and titratable acidity and lower lycopene and total carotenoids than tomatoes ripened off vine.
Keywords: antioxidants, maturity, postharvest ripening, tomato

Introduction
Tomato is one of the most popular vegetables worldwide (Frusciante *et al.* 2007), with advantages the high nutritional value and the availability year-around (Wold *et al.* 2004). The beneficial effect of tomato consumption on human health is correlated with the high content of lycopene, ascorbic acid and other phenolic compounds with strong antioxidant activity (Raffo *et al.* 2006).

The appropriate stage for tomato, a climacteric fruit, harvesting varies from mature green to red and it is depending on the market requirements (Wold *et al.* 2004). In the case of market place far away from production area, the harvest at the mature green stage is critical to avoid overipening before purchase by the consumers (Arias *et al.* 2000). However, most of the consumers are convictive that on vine ripened tomatoes are of superior quality than the fruits which were harvested at the mature green stage and ripened off vine during postharvest handling (Arias *et al.* 2000).

The results for the effect of ripening conditions (on or off vine) on quality of tomatoes are reversely. Better quality (flavour, aroma) for tomatoes ripened on vine was observed previously (Kader *et al.* 1977; Arias *et al*. 2000) as well as higher content of ascorbic acid, lycopene and β-carotene (Kader *et al*. 1978; Arias *et al*. 2000). On the contrary, Giovanelli *et al.* (1999) reported significantly higher antioxidant content (lycopene, β-carotene, ascorbic acid and total phenolics) for tomatoes harvested at the mature green stage and full ripened off vine. Moreover, it has been reported that the ripening conditions (on or off vine) of tomatoes did not affect the ascorbic acid content (Arias *et al.* 2000) and the total antioxidant activity (Wold *et al.* 2004).

The objective of this study was to investigate the quality (color, firmness, chemical composition) of tomato (cv. 'Belladona') as affected by ripening on vine and off vine.

Materials & Methods

Tomatoes (*Lycopersicon esculentum* cv. 'Belladona') were grown in a glasshouse in the experimental farm of the Aristotle University of Thessaloniki, Central Macedonia, Greece under usual production practices. On 9th of May green mature fruits were tagged and half of them were harvested and placed into the laboratory to simulate the postharvest handling of fruits, while the other half remained on the vine. Tomatoes on and off vine were allowed to ripen for 10 days, while temperatures (minimum, maximum) in the glasshouse and in the laboratory were recorded. During this period the color of the selected fruits was measured daily with a chromameter (Minolta CR-200, Japan) and color changes were quantified in the L*, a* and b* color space. Hue angle and chroma values were calculated from a* and b* values (McGuire 1992).

When fruit were ripe, their firmness was recorded using a 3 mm probe of a Chatillon penetrometer. Furthermore, fruit of each replication were macerated in a blender for compositional analysis. Dry matter content was determined after drying about 40 g of the blended material in an oven set at 70 °C for 72 h. Soluble solids content (SSC) was measured in the blended material by a digital refractometer Atago PR-1 (Japan). The pH and titratable acidity were determined in a 50-ml filtrate obtained from a blend of 10 g blended material in 100 mL deionised water. The filtrate was titrated to pH 8.2 with 0.01 N NaOH. Ascorbic acid was extracted in 1% oxalic acid and measured by using Reflectoquant ascorbic acid test strips in an RQflex reflectometer (Merck, Germany). Nitrate content was determined colorimetrically, as described by Cataldo *et al.* (1975). Lycopene and total carotenoids were extracted in acetone and the absorbance was measured at 503 and 445 nm, respectively. Total soluble phenol content was determined according to Scalbert *et al.* (1989) with gallic acid used as a standard. DPPH radical scavenging activity was determined using a modified method of Brand-Williams *et al.* (1995) as described by Koukounaras *et al.* (2007). The standard curve was developed with ascorbic acid.

The experimental design was a completely randomized one with three replications, each one consisting of two fruit. Data for color was subjected to analysis of variance and mean separation was conducted by LSD at 0.05 level.

Results & Discussion

Both on- and off-vine tomatoes showed a great alteration in color parameters (a decrease of L* and Hue and an increase of C*) during ripening. However, off vine ripened tomatoes had significantly lower L* and Hue values from the 5th day and thereafter, as well as, significantly higher C* values from the 5th till to the 9th day (Fig 1). These results indicate that tomato fruits ripened off vine had more red color at the end of the 10 days period. The above result is a consequence of the significantly higher (by 73.4%) content of lycopene in off vine ripened tomatoes (Table 1). It is well known that the lycopene biosynthesis is inhibited at high temperatures (>38 °C) (Cheng *et al.* 1988). In the glasshouse the maximum temperature for some days was higher than 38 °C, while in the laboratory the maximum temperature was 22.5 °C (Table 2).

Table 1. Ascorbic acid, lycopene, total carotenoids, total soluble phenols and DPPH radical scavenging activity of tomato fruits as affected by ripening conditions (on- and off-vine).

Ripening conditions	Ascorbic acid (mg 100 g^{-1} f.w.)	Lycopene (μg g^{-1} f.w.)	Carotenoids (μg g^{-1} f.w.)	Phenols (GAE g^{-1} f.w.)	DPPH (AEAC 100 g^{-1} f.w.)
On vine	14.17 a[z]	16.98 b	21.77 b	0.40 a	24.20 a
Off vine	12.38 a	37.73 a	37.73 a	0.45 a	17.47 a

[z] Each value is the mean of the three replications

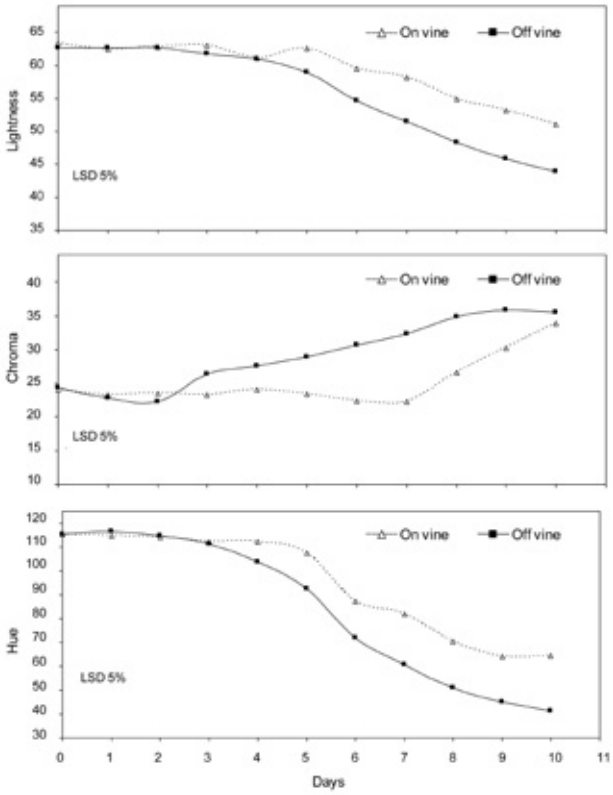

Fig 1. Color parameters (Lightness, Chroma and Hue angle) of tomato fruits as affected by ripening conditions.

Table 2. Minimum, maximum and mean temperature into glasshouse and laboratory during ripening period of tomatoes.

	Glasshouse Temperature (°C)			Laboratory Temperature (°C)		
Day	Minimun	Maximum	Mean	Minimun	Maximum	Mean
1st	15	37	26.5	21	21	21.0
2nd	12	40	26.0	20	21	20.5
3rd	12	37	24.5	21	21	21.0
4th	11	38	24.5	20	21	20.5
5th	15	41	28.0	21	21	21.0
6th	11	38	24.5	20	22	21.0
7th	12	35	23.5	20	22	21.0
8th	16	44	30.0	21	22	21.5
9th	14	43	28.5	21	23	22.0
10th	14	44	29.0	22	23	22.5

Firmness of the fruits ripened on and off the vine were not significantly different (0.99 and 1.09 kg, respectively). On the contrary, significantly higher firmness for on vine tomatoes as compared to off-vine ripening has been reported by Arias *et al.* (2000).

The nitrates and titratable acidity were significantly higher for the fruits ripened on vine (Table 3). On the contrary, no significant effect on acidity for on- and off-vine fruits was observed by Arias *et al.* (2000) and Picha (1986).

Table 3. Dry matter, nitrate, SSC, pH, titratable acidity and SSC/acidity ratio of tomato fruits as affected by ripening conditions (on- and off-vine).

Ripening conditions	Dry matter (%)	Nitrate (mg kg^{-1} f.w.)	SSC (%)	pH	Acidity (% citric)	SSC/Acidity
On vine	6.65 a[z]	114.73 a	5.57 a	3.87 a	0.29 a	19.18 a
Off vine	6.37 a	91.99 b	4.63 a	3.94 a	0.25 b	18.61 a

[z] Each value is the mean of the three replications.

The ripening conditions did not affect soluble solids content, pH, dry matter, SSC/acidity, ascorbic acid, total soluble phenols as well as DPPH radical scavenging activity (Tables 1, 3). Other authors (Arias *et al.* 2000; Wold *et al.* 2004) reported the same results for ascorbic acid and the total antioxidant activity.

Also, we found that the SSC/Acidity ratio was similar for both fruits ripened on- and off-vine (Table 3). It is well known that the sugars/acid ratio is a basic parameter for the quality classification of fresh tomatoes (Kader *et al.* 1977).

From the above results it is evident that the ripening mechanism was not significantly affected by the ripening conditions, although differences in color due to differences in lycopene content were detected, which could be attributed to differences of temperatures between the two ripening conditions.

In conclusion, on-vine ripened tomato fruits had less red color, lower lycopene content and total carotenoids, higher nitrates and acidity than the off-vine ones and similar firmness, dry matter, soluble solids, pH, ascorbic acid, antioxidants and SCC/acidity ratio.

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19. QUALITY CHANGES DURING STORAGE OF APRICOT (*PRUNUS ARMENIACA* L.) CV. 'BELIANA' AND 'LINDO' TREATED WITH CALCIUM

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Abstract
Calcium salts have been successfully used to maintain firmness and to slow down ripening processes in some fruit. This work studies the effect of postharvest calcium chloride applications on the quality preservation of apricot (*Prunus armeniaca* L.) cv. 'Beliana' and cv. 'Lindo' during storage. Harvested apricots were dipped in 0, 1, 3 or 5% $CaCl_2$ solutions for 2 min. Fruit were left to dry for 1 h at ambient temperatures and then stored at 3 ºC. Fruit were analysed after 0, 6, 14, 21 and 28 d storage for weight loss, firmness and soluble solids content (SSC). A taste panel was performed at the beginning and at the end of the experiment. Weight loss increased through storage in both cultivars. 'Lindo' did not show differences among treatments in weight loss, but 'Beliana' had higher weight loss in fruit treated with 3% $CaCl_2$ followed by 5%. Firmness decreased through storage without differences among treatments in 'Beliana', except after 5 d where all treatments where firmer than control. The 'Lindo' apricots treated with 3 and 5% $CaCl_2$ showed higher firmness values than the other treatments through storage, except at 28 d where only the 5% $CaCl_2$ had significantly higher firmness. 'Beliana' did not show differences in SSC among treatments. However, 'Lindo' had lower SSC when treated with 1% $CaCl_2$. When tested after 27 d storage, panellists preferred fruit from the 1% $CaCl_2$ treatment followed by the control for both cultivars. It seems that $CaCl_2$ treatments with concentrations over 3% are prejudicial for apricots. Concentrations between 1 and 3% should be assayed, since 1 and 3% look to be beneficial for the different quality parameters.
Keywords: *Prunus armeniaca*, quality, storage

Introduction
Apricot fruit are highly perishable. Their quality is linked to some attributes like appearance, texture, flavour and nutritional value, which are all determined by the ripening stage at harvest and storage conditions. The most important chemical changes during apricot ripening are in the sugars, acids and pigments levels, since these properties contribute to the organoleptic characteristics for optimal consumption (Amoros *et al.* 1990). Firmness is also an important attribute to indicate the ripening stage of fruit (Souty *et al.* 1990,1995).

One of the major problems of apricots is rapid postharvest softening. It is well known that calcium plays a significant role in maintaining quality in a number of different fruit (Hopkirk *et al.* 1990). The pre and postharvest application of calcium salts has been used successfully in many fresh fruit to maintain firmness and to slow down ripening processes (Souty *et al.* 1995; Antunes *et al.* 2003).

This research studies the effects on 'Beliana' and 'Lindo' apricot fruit (*Prunus armeniaca* L.) quality during storage after postharvest application of $CaCl_2$ at concentrations up to 5%.

Material & Methods
Apricot fruit (*Prunus armeniaca* L. cv. 'Beliana' and cv. 'Lindo') were harvested with firmness 20 N and SSC 12% for 'Beliana' and firmness 12 N and SSC 14% for 'Lindo' cultivars. Fruit were dipped in 0, 1, 3 or 5% $CaCl_2$ solutions for 2 min and were left to dry for 1 h at room temperature, and then placed in trays and stored at 3 ºC. After 0, 6, 14, 21 and 28 d, 10 fruit per replication were removed from storage and used for analyses.

Soluble solids content (SSC) was measured with a digital Atago refractometer (Model PAL-1, Atago Co. LTD, Japan). Firmness was recorded with a Chatillon Force TCD 200 and Digital Force Gauge DFIS 50 penetrometer fitted with a conical plunger of 6.5 mm diameter and 2.4 mm height. Maximum penetration depth was 12.6 mm. Weight loss was expressed as a percentage of the initial fruit weight. The taste panel consisted of 25 panellists.

Statistical analysis were carried out with the SPSS 16.0 software (SPSS Inc.). Two-way analyses of variance (ANOVA) and Duncan's Multiple-Range Test ($P<0.05$) for comparisons among treatments over time were conducted.

Results & Discussion

Firmness was higher in 'Beliana' than in 'Lindo' apricot fruit, and it slightly decreased during storage for 'Beliana' and remained almost constant for 'Lindo' (Fig 1). Increased concentrations of calcium chloride were efficient in maintaining fruit firmness, this effect was more pronounced in 'Beliana' than 'Lindo'. The effect of calcium on firmness decreased after 21 d storage.

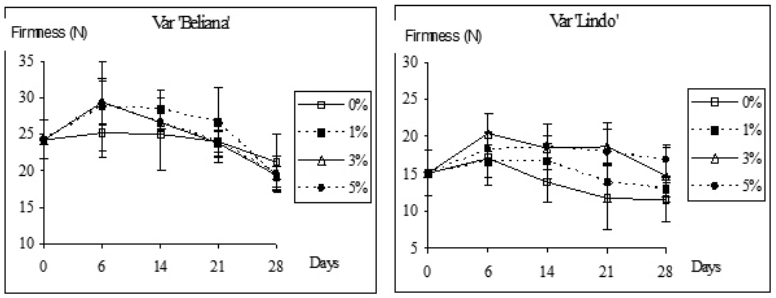

Fig 1. Firmness of apricot fruits during storage at 3 °C, after postharvest dip in water with 0, 1, 3 or 5% $CaCl_2$ for 2 min.

Calcium applications made both pre and postharvest to fruit tissues, can delay softening rates and ripening, by maintaining cell wall integrity and importantly cell cohesion (Knee & Bartley 1981; Roy *et al.* 1994).

Souty *et al.* (1995), Tzoutzoukou & Bourakis (1997) and Antunes *et al.* (2003) also found beneficial effects of calcium application to apricot fruit of some cultivars on their storage life capacity.

The soluble solids content were lower in 'Beliana' apricots after harvest than in 'Lindo', but at the end of the storage period they reached similar values (Fig 2).

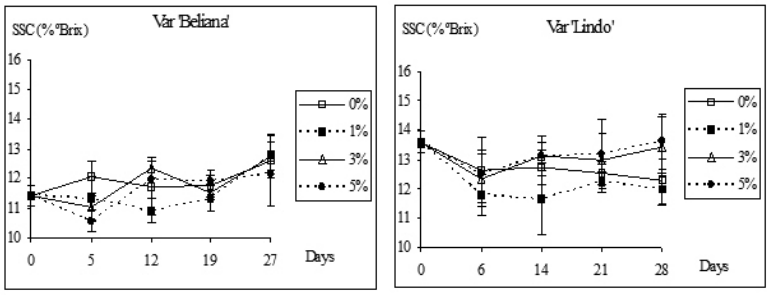

Fig 2. Soluble solids content of apricot fruits during storage at 3 °C, after postharvest dip in water with 0, 1, 3 or 5% $CaCl_2$ for 2 min.

It seems that 'Lindo' apricots were already eating-ripe at harvest but 'Beliana' were not. 'Beliana' did not show differences in SSC among treatments, but 'Lindo' had a lower SSC when treated with 1% $CaCl_2$.

Weight loss increased with storage time in both cultivars (Fig 3). Weight loss was higher in 'Lindo' than

in 'Beliana' throughout storage. In both cultivars fruit treated with 3 or 5% $CaCl_2$ lost more weight than the control or 1% $CaCl_2$ treatments, although the differences were smaller in 'Beliana'.

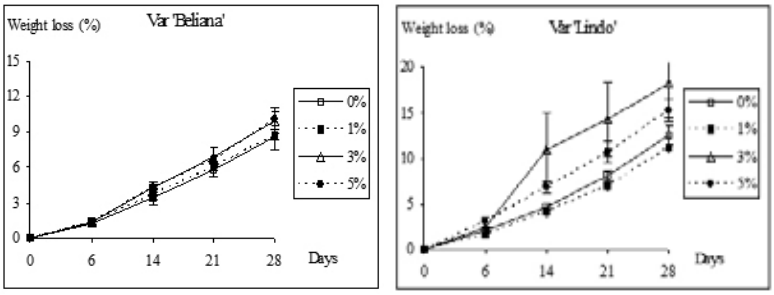

Fig 3. Weight loss of apricot fruits during storage at 3 °C, after postharvest dip in water with 0, 1, 3 or 5% $CaCl_2$ for 2 min.

When tested after 27 d storage, panellists preferred the 1% $CaCl_2$ treatments followed by the control in both cultivars (Fig 4). Panellists preferred the 1% treatment followed by control in both cultivars in terms of appearance, while for flavour the preference values were still acceptable for the 3% $CaCl_2$ treatment until the end of storage. Generally, fruit from the 3 and 5% treatments were rejected. Apricots of both cultivars treated with 3 or 5% $CaCl_2$ showed, at the end of the experiment, brown spots on the skin (data not shown).

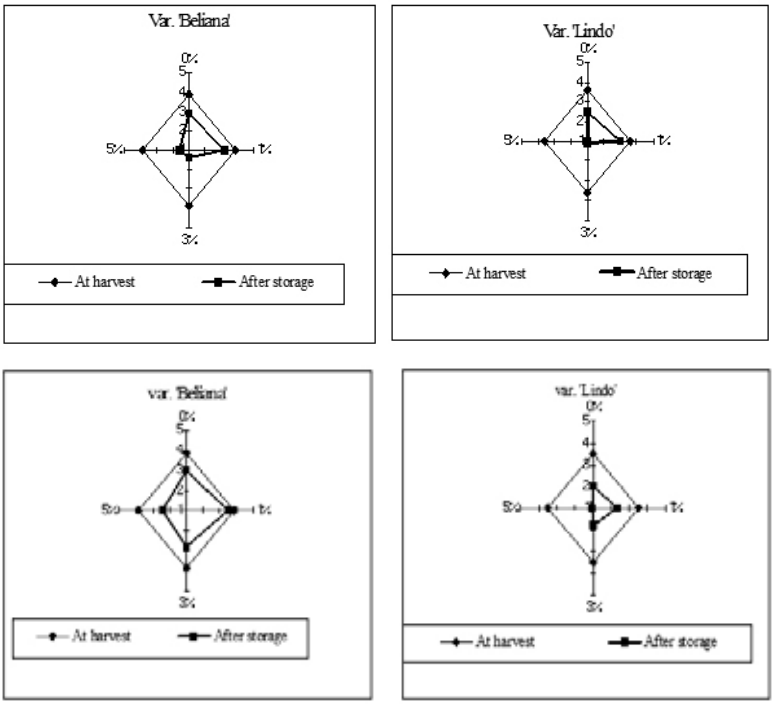

Fig 4. Appearance (A) and flavour (B) of apricot fruits stored at 3 °C, after postharvest dip in water with 0, 1, 3 or 5% $CaCl_2$ for 2 min, as evaluated by panellists at harvest and after 27 d storage.

Souty *et al.* (1995) and Antunes *et al.* (2003) already reported a detrimental effect from high $CaCl_2$ concentration postharvest dips on apricot fruit (4% and 3-5%, respectively).

This current work suggests that postharvest dipping of apricot fruit in 1% $CaCl_2$ increases potential storage life, but levels over 3% are prejudicial. Concentrations between 1 and 3% should be assayed.

Acknowledgements

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20. WATERCORE DISSIPATION IN 'FUJI' APPLES AT DIFFERENT HOLDING TEMPERATURES

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Abstract
Fruit with high levels of watercore are rejected during market quality control inspections because affected apples can develop alcoholic off-flavours and internal browning symptoms. In 2007 and 2008 late harvest 'Fuji' apples with a high incidence of watercore were kept at 1 °C (2008), 3 °C (2007), 6 °C and 10 °C in air. Some fruit at 10 °C were also treated with 1-MCP and Ethephon. Changes in flesh firmness and watercore were followed during 51 d storage. Fruit at 1, 3 °C and 1-MCP treated fruit at 10 °C showed no clear change in flesh firmness during 51 d storage while fruit at 6 °C softened between 0.7 and 0.9 kg/cm^{-2} and untreated fruit at 10 °C lost around 1.0 kg/cm^{-2}. Watercore in 'Fuji' apples with or without 1-MCP or Ethephon treatments at 10 °C dissipated to acceptable levels after around 16 to 22 d, while apples at 6, 3 and 1 °C showed acceptable watercore at 26, 29 and 51 d respectively. When 'Fuji' apples have high levels of watercore at-harvest, one option is to treat them with 1-MCP and store at 10 °C. The watercore will reduce rapidly but fruit flesh firmness will generally be maintained at acceptable levels.
Keywords: 1-MCP, fruit firmness, *Malus domestica*, physiological disorders

Introduction
Apples with watercore accumulate sorbitol-rich solutions within the fruit flesh intercellular spaces. This physiological disorder occurs while fruit are still on the tree and symptoms appear in the fruit flesh as hard and glassy water soaked areas (Dart & Newman 2005). Watercore reduces the market and storage options available for the fruit as affected apples can develop an alcoholic taste and internal breakdown symptoms especially when stored under controlled atmosphere conditions (Argenta *et al.* 2001). Consumers can also be confused by the unusual and curious symptoms and more severely affected apples are normally rejected at official quality control inspections. Watercore incidence varies with season and cultivar, however, in 'Fuji' apples watercore can dissipate during storage (Brackmann *et al.* 2001) and it is usual for apples with high watercore levels to be held for a period to allow the watercore to dissipate before the fruit are marketed. However, the rate of watercore dissipation is not known and not quantified in relation to either: the storage temperature; other postharvest treatments like 1-MCP and Ethephon applications; or changes in the key fruit quality parameters such as flesh firmness.

Material & Methods
Two experiments were conducted in 2007 and 2008 with late-harvest high watercore 'Fuji' (mutant 'Kiku') apples from the Competence Centre for Fruit Growing, Ravensburg, Southern Germany. Fruit were sorted and randomly allocated into 1 (2008), 3 (2007), 6 and 10 °C storage temperature treatments. In 2008, 1-methylcyclopropene (1-MCP) and Ethephon were also applied to fruit at 10 °C. Fruit samples were regularly taken over a 51 d storage period to determine watercore and fruit flesh firmness. Treatment lots comprised 100 fruit in 2007 and 60 fruit in 2008, with individual fruit as replications.

Apples were cut transversally through the equatorial region and watercore incidence scored against reference photographs from 0 to 5 (0 = no watercore and 5 > 40% of the cut flesh area, Fig 1). Apples with an average watercore score of < 2.5 were considered commercially acceptable.

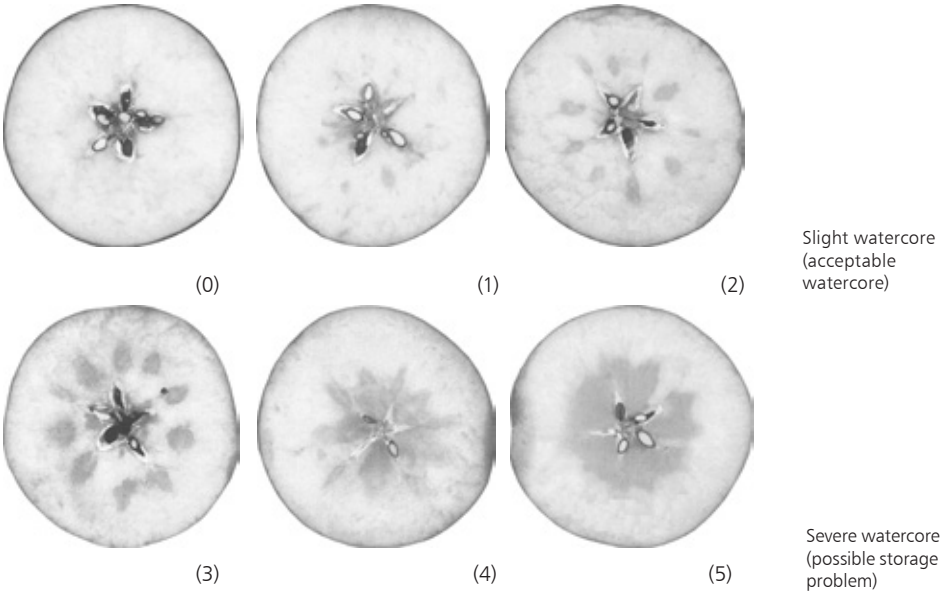


Fig 1. Reference table for watercore occurrence with a range of 0 to 5 (0 represents fruit without watercore and score 5 indicates 40% of flesh with watercore (maximal score).

Flesh firmness was measured from the equatorial region of the apple between the green/yellow (shaded side) and red blush (sun exposed side) the skin was removed and a fruit texture analyser (Güss, South Africa) with an 11 mm diameter tip used to determine fruit flesh firmness as kg cm^{-2}. Flesh firmness readings are an average of 3 repetitions each of eight fruit.

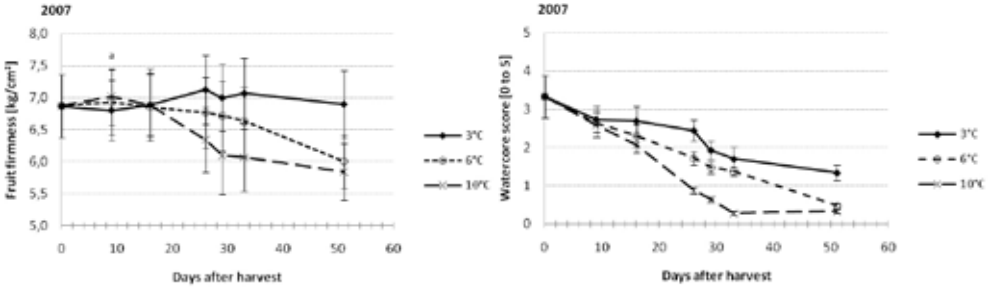
The experimental design was completely randomised and the standard deviation was calculated.

Results & Discussion

Fruit held at 1, 3 and 10 °C plus 1-MCP showed no clear changes in flesh firmness during 51 d storage, while fruit held at 6, and 10 or 10 °C plus Ethephon softened between 0.7-0.9 kg cm^{-2} and by around 1.0 kg cm^{-2} respectively (Fig 2A, 3A). These results confirm the potential of 1-MCP to maintain the flesh firmness in apples (Watkins 2008), and even though the 1-MCP treated fruit were held at 10 °C the softening rate was similar and slightly slower than fruit held at 1 °C without 1-MCP (Fig 2A, 3A).

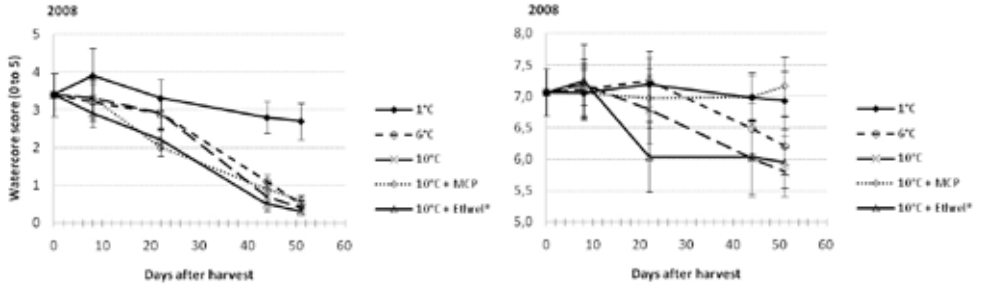
'Fuji' apples at 10 °C showed acceptable watercore levels (< 2.5) after 16 to 22 d and the 1-MCP or Ethephon treatments had no additional inhibitory or promoting effect. Apples at 6 °C had acceptable levels of watercore after 16 d in 2007 and 26 d in 2008. Fruit held at 3 °C achieved this level after 29 d in 2007 (Fig 2B), apples held at 1 °C achieved this level after 51 d in 2008 (Fig 3B). The reason for the differences in watercore dissipation between the two years is not clear, but comparing both years, a general slower ripening progress of the apples was observed in 2008 (Kittemann 2008). Storage temperature has a clear effect on watercore dissipation rates but the rate varied between the two years. Brackmann *et al.* (2001) also found watercore dissipated during storage. However, as the 1-MCP and Ethephon treatments did not influence the rate of watercore dissipation, this suggests ethylene induced effects are not involved in watercore dissipation and other processes such as transpiration could be, as also suggested by other workers (Marlow & Loescher 1984; Ferguson *et al.* 1999; Yamada *et al.* 2004).

When 'Fuji' apples show high levels of watercore at-harvest one option is to treat them with 1-MCP and then store at 10 °C. This will rapidly dissipate the watercore but maintain fruit flesh firmness within generally acceptable levels.



(a) (b)

Fig 2. Reductions in flesh firmness (A) and watercore dissipation (B) for 'Fuji' apples held at three different temperatures in 2007 (..... approx quality inspection acceptance limit in watercore). The error bars represent SD, n=100 (watercore), n=24 (fruit firmness).



(a) (b)

Fig 3. Reductions in flesh firmness (A) and watercore dissipation (B) for 'Fuji' apples held at three different temperatures in 2008 (..... approx quality inspection limit in watercore index). The error bars represent SD, n=60 (watercore), n=24 (fruit firmness).

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21. EFFECT OF POSTHARVEST ELICITORS ON 'FUYU' PERSIMMON QUALITY DURING COLD STORAGE

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Abstract

To induce resveratrol synthesis and reduce skin browning in 'Fuyu' persimmons, fruit were treated directly after harvest with the following elicitors: UV irradiation, ozone, phosphonate, or acibenzolar-S-methyl and then kept for three months at -0.5 °C in air storage at around 95% relative humidity. Traces of trans-resveratrol were detected in the skin of the persimmons at harvest, but after treatment and cold storage this compound was not found. After shelf-life (storage plus 5 d at 20 °C), the soluble solids content (SSC), reducing and non-reducing sugars and total polyphenols were not significantly influenced by any of the elicitor treatments. Fruit treated with ozone showed a higher reducing sugar content but developed skin damage, flesh browning and small internal cavities. These results were correlated with the skin colour. Fruit treated with acibenzolar-S-methyl had the highest flesh firmness and total polyphenol content. However, this treatment had a higher skin browning index after shelf-life.

Keywords: acibenzolar-S-methyl, irradiation, ozone, phosphonate, UV

Introduction

'Fuyu' persimmon is the main non-astringent cultivar grown in Brazil. Most of Brazil's persimmon production is consumed directly in the domestic market, but recently a part is also being exported. Increased export opportunities have created a need for extended storage life, to allow for the long distance sea transport required to supply 'out of season' fruit to the northern hemisphere. However, improved postharvest practices and storage techniques are required in order to meet these increased export demands. Furthermore, fruit quality requirements are changing as consumer acceptance is now linked not only to the fruit's visual appearance and sugar content but also to its functional or nutraceutical properties. Resveratrol (*trans*-3,5,4´-trihydroxystilbene) is a biologically active phenolic compound and phytoalexin, and is produced *de novo* or formed in response to abiotic stress or pathogen attack (Grimmig *et al.* 2002). Resveratrol effectively acts as a free radical scavenger, and as been shown to inhibit low-density lipoprotein (LDL) oxidation (Pinto *et al.* 1999), to provide cardio-protection and induce vasorelaxation (Wallerath *et al.* 2005), to protect neurons (Zhuang *et al.* 2003) and to inhibit platelet aggregation and cancer activity (Fremont 2000).

This study investigates the effect of postharvest elicitors on nutraceutical properties, skin browning and general fruit quality of 'Fuyu' persimmon.

Material & Methods

'Fuyu' persimmon fruit were harvested in 2005 at commercial maturity stage from an orchard located at Farroupilha, Rio Grande do Sul, Brazil, and immediately transported to the storage facilities at the University of Santa Maria where they were sorted to remove damaged, blemished and unripe fruit. After grading, samples were dipped for 3 min in water (control) or treated with solutions containing phosphonate (P_2O_5 and K_2O at 1.27 g L^{-1} and 1.18 g L^{-1} respectively) or acibenzolar-S-methyl at 50 mg L^{-1}. The UV-C (2.4 kJ m^{-2}) treatment was applied with 25 cm between the source and the fruit surface using a wavelength of 254 ηm at

30 ºC. For the ozone treatment, fruit were exposed to 0.03 μL L^{-1} ozone during storage and as an untreated control, fruit were cold stored in air. After the experiment treatments were applied, the persimmons were kept at -0.5°C for three months and then 5 d shelf-life at 20 °C.

After shelf-life (storage plus 5 d at 20 °C) fruit were analyzed for: skin color using a Minolta CR-300 colorimeter and expressed in L*a*b* values. Skin browning was scored in four categories: (1 = no symptoms; 2 = < 20%; 3 = 20-50%; and 4 = > 50%). A browning index was calculated as the average browning score (Neuwald 2008) and the percentage of decayed fruit based on a visual evaluation. A small portion of the skin was removed from two opposite sides of the equatorial region of the fruit for phenolic compound analysis. Flesh firmness was measured on two opposite sides of each individual fruit after peel removal using a penetrometer (11 mm diameter tip). Soluble Solids content (SSC) was determined with a hand-held refractometer from a juice sample. Reducing and non-reducing sugars were determined by the method of Somogyi & Nelson (Nelson 1944). The analysis of trans-resveratrol was carried out in a HPLC and expressed in μg $100g^{-1}$ following the method as described by Sautter et al. (2008). Total polyphenol concentration was determined by the colorimetric method of Folin–Ciocalteu and expressed in mg gallic acid equivalents L^{-1} (Sautter *et al.* 2008).

The skin browning index data were normalized using an arc sine transformation. ANOVA was performed and means were compared using the Tukey test at a 5% significance level.

Results & Discussion

Traces of trans-resveratrol were detected at-harvest in the skin of 'Fuyu' persimmon (data not shown) but none was detected after cold storage in all treatments (Table 1).

Table 1. Contents of trans-resveratrol and polyphenol in the skin and reducing and non reducing sugars in flesh of 'Fuyu' persimmons after 3 months of cold storage at -0.5°C plus 5 d shelf-life at 20 ºC.

Treatments	Resveratrol (μg 100 g^{-1})	Polyphenols (mg L^{-1})[2]	Reducing sugars (g 100 mL^{-1})	Non reducing sugars (g 100 mL^{-1})
Air control	Nd[1]	3495.3 ab[3]	15.0 a	0.0 b
Irradiation UV-C	Nd	3373.6 b	16.1 a	0.6 ab
Ozone	Nd	3511.0 ab	14.9 a	1.3 a
Phosphonate	Nd	3633.8 ab	16.9 a	0.0 b
Acibenzolar-S-methyl	Nd	4069.2 a	14.3 a	0.1 b

[1] Nd = Resveratrol was not detected after shelf-life; [2] Values for total polyphenols in mg of gallic acid; [3] Values followed by the same letter vertically do not differ by the Tukey test at 5%

Table 2. Quality parameters of 'Fuyu' persimmons after 3 months of cold storage at -0.5°C followed by 5 d shelf-life at 20 °C.

Treatments	Skin browning	Colour			Firmness	SSC[1]
	(0 - 3)	L*	a*	b*	(N)	(°Brix)
Air control	1.6 b[2]	37.5 ab	19.9 ab	16.8 ab	26.2 bc[2]	15.7 a
Irradiation UV-C	2.1 a	36.6 ab	17.6 b	15.0 bc	26.5 bc	15.8 a
Ozone	2.2 a	35.2 b	16.4 ab	13.2 c	18.1 c	14.7 a
Phosphonate	1.6 b	38.8 a	20.0 b	17.3 ab	36.8 ab	15.9 a
Acibenzolar-S-methyl	2.3 a	38.8 a	22.5 a	18.8 a	54.5 a	15.3 a

[1] SSC = Soluble solids content; [2] Values followed by the same letter vertically do not differ by Tukey test at 5%

These results agree with Neuwald *et al.* (2008) who found no differences in the SSC of 'Fuyu' persimmon after various storage treatments. Sautter *et al.* (2008) evaluated 'Gala' apples after postharvest elicitor treatments and also found no differences in reducing sugars.

UV-C resulted in typical fruit damage (Fig 1A, B) but there was less severe skin browning compared with the ozone treatment, probably because the mechanism that triggered the injury occurred just before storage. The abiotic stress, caused by UV irradiation, disturbs the cellular homeostasis to increase hydrogen peroxide (H_2O_2) levels (Mittler 2002) which stimulates FAL and increases the permeability of plasma membranes to Ca^{2+} (Cassells & Doyle 2003), to trigger the phenylpropanoid pathway. Ultraviolet irradiation at low doses can trigger repair mechanisms in the cell, and induce the biosynthesis of phytoalexins that are active for periods ranging from hours to days (Shama 2007). UV-C and UV-B are elicitors of stilbenes, in particular the phytoalexin resveratrol in grapes, (Adrian *et al.* 2000). However, in this work UV-C did not stimulate the synthesis of resveratrol in persimmon (Table 1). Skin browning may be a consequence of a too higher UV-C dose (Fig 1A, B) when polyphenols condense or oxidize to form browning compounds, but not changing the total polyphenol content (Table 1). Similar skin browning symptoms were also found in grapes following UV-C (Nigro *et al.* 1998). In our results, UV-C light was correlated with skin colour (Table 2) and skin browning (Table 1). There is evidence that the UV-C treatment increased fruit metabolism as shown in the subtle elevation of the reducing sugars content (Table 1).

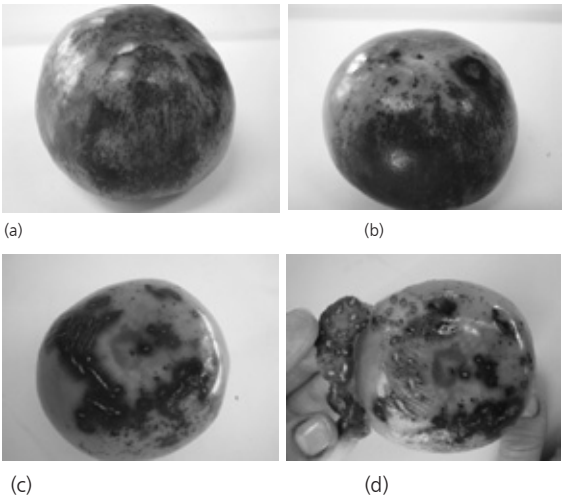

Fig 1. Skin damage caused by irradiation treatment (A, B), ozone showed typical skin damage (C) flesh browning and small internal cavities (D).

The fruit treated with ozone showed typical skin damage with flesh browning and small internal cavities (Fig 1C, D). Oxidative stress stimulates phenylalanine ammonia-lyase (FAL), to trigger the formation of polyphenols and subsequent oxidation can occur enzymatically by polyphenoloxidase to give a brown colour (Cassells & Doyle 2003). Sensitivity to ozone differs among different plant species and even cultivars. When ozone enters the plant tissue through stomata, the first contact is with the apoplastic fluid. In an aqueous media, O_3 produces H_2O_2, and subsequently OH^-, by the Haber-Weiss reaction (Mano 2002). The guard-cells have a number of stress indicator proteins such as G proteins and these are involved with oxidative stress response signalling. This stress signal response is complex and unknown, but it is mediated by abscisic acid (Joo *et al.* 2005). Without this first defence, ozone can breakdown the cell wall and plasma membrane by active free radicals (oxygen species) generally leading to the hypersensitive response and cell death (Langebartles *et al.* 2002). The damage observed in our experiment may have occurred by the effect of ozone on the cell wall and plasma membrane, causing cells to collapse and form cavities. In this case there is evidence of increased metabolism as shown by the higher reducing sugars content (Table 1).

Fruit treated with phosphonate showed no significant differences in any of the parameters (Table 1, 2). However, fruit treated with acibenzolar-S-methyl had the highest flesh firmness (Table 2), although this is contrary to results found in apples where it accelerated fruit softening (Sautter *et al*. 2008). The acibenzolar-S-methyl treatment had the highest total polyphenol content (Table 1) but also the highest skin browning index after storage plus 5 d shelf-life at 20 °C.

Conclusions

The elicitors acibenzolar-S-methyl, UV-C irradiation, ozone and phosphonate did not stimulate the synthesis of trans-resveratrol in persimmon. The combination of acibenzolar-S-methyl postharvest dips maintained higher flesh firmness and total polyphenol content during air storage, but resulted in skin browning after shelf-life. Future work should explore the use of postharvest elicitors under CA conditions, to try to reduce the amount of skin browning and also to evaluate the effect on decay control.

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SECTION 4. ENVIRONMENTALLY FRIENDLY AND SAFE METHODS TO CONTROL POSTHARVEST LOSSES

22. NEW DEVELOPMENTS IN ALTERNATIVE METHODS TO CONTROL POSTHARVEST FRUIT DECAY

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Abstract

Public concern in food safety and the increase of pathogen resistant populations has enhanced the interest in developing methods to control postharvest fruit decay alternative to fungicides. According to their nature alternative methods can be classified as biological, chemical or physical. This article reviews research on alternative postharvest disease control methods and explores new possibilities of research to improve their efficacy.

Keywords: alternative methods, biological control, chemical control, integrated approach, physical methods, postharvest control

Introduction

Postharvest decay of fruits and vegetables may reach very important values depending on species, harvest methods, storage, transportation, etc., representing up to 25% of the total production in developed countries and 20-50% in developing countries. Therefore working with efficient methods to reduce losses caused by postharvest pathogens remains a priority.

Postharvest losses can be reduced by preventing fruit infections avoiding fruit by damages, carefully handling, applying correct sanitation procedures and using fungicides. The repeated and continious use of fungicides has led to the development of fungal strains resistant to many fungicides. In addition, the growing concern for human safety and environmental protection, the imperative of sustainable agriculture and development of integrated crop management and organic production have resulted in the need to find other methods to control postharvest decay. According to their nature these alternative methods can be classified as biological, chemical or physical.

The purpose of this manuscript is to review the research work in alternative environmentally friendly and safe approaches to control postharvest diseases of fruits.

Biological Control in Postharvest

Over the past 20 years biological control of postharvest diseases using microbial antagonists has emerged as an effective strategy to control the major postharvest decays of fruits and several reviews have been published (Janisiewicz 1988; Wilson & Wisniewski 1989; Wisniewski & Wilson 1992; Janisiewicz & Korsten 2002; Droby *et al.* 2009; Nunes *et al.* 2009; Sharma *et al.* 2009). During this period several programs worldwide have been carried out to develop microorganisms with antagonistic activity in several fruits, using different strains of bacteria, yeast and filamentous fungi (Table 1).

Postharvest environment represents a particular advantage to develop biological control. Injuries made during harvest and transportation to packinghouse can be protected from wound pathogens with only a single application of the biocontrol product directly to infection site (harvested fruit), using the existing facilities (Janisiewicz & Korsten 2002). During storage, fruits are kept in a constant physical environment, which can be controlled to favour the antagonist growth. The high value of the commodities in postharvest makes the application of a biocontrol fungicide more justified than in the field.

Table 1. Biological control agents of fruit postharvest diseases.

Biocontrol Agent	Disease	Fruit	Ref
Bacteria			
Bacillus amyloliquefaciens	*Colletotrichum musae, Fusarium moniliforme*	banana	Alvindia & Natsuaki 2009
Bacillus licheniformis	*Botryosphaeria* spp., *C. gloeosporioides*	mango	Govender *et al* 2005
Bacillus pumilus	*Penicillium digitatum*	citrus	Huang *et al* 1992;
Bacillus subtilis	*Botrytis cinerea, C. gloeosporioides, Monilinia fructicola, P. digitatum, P. expansum*	apple, avocado, citrus, stone	Korsten *et al* 1995; Sholberg *et al* 1995; Fan *et al* 2000; Leelasuphakul *et al* 2008
Pantoea agglomerans	*B. cinerea, P. digitatum, P. expansum, P. italicum, Monilinia* sp., *Rhizopus stolonifer*	citrus, pome, stone	Nunes *et al* 2001a,2002; Teixidó *et al* 2001; Bonaterra *et al* 2003;
Pantoea ananatis	*P. expansum*	pome	Torres *et al* 2005
Pseudomonas cepacia	*B. cinerea, P. digitatum, P. expansum, Monilinia* sp.	pome, lemon, stone	Janisiewicz & Roitman 1988; Smilanick & Denis-Arrue 1992; Smilanick *et al* 1993
Pseudomonas glathei	*P. digitatum*	citrus	Huang *et al* 1995
Pseudomonas syringae	*B. cinerea, P. digitatum, P. expansum, P. italicum*	citrus, pome	Janisiewicz & Marchi 1992; Bull *et al* 1997; Nunes *et al* 2007a
Serratia plymuthica	*P. digitatum*	orange	Meziane *et al* 2006
Yeast			
Candida famata	*P. digitatum*	citrus	Arras 1996
*Candida guilliermondii**	*B. cinerea, P. digitatum, P. italicum, Geotrichum candidum, R. stolonifer*	apple, citrus, peach	Chalutz & Wilson 1990; McLaughlin *et al* 1992
Candida oleophila	*B. cinerea, G. candidum, P. digitatum, P. expansum*	apple, citrus, stone	Droby *et al* 1998; 2002b
Candida sake	*B. cinerea, P. expansum, R. stolonifer*	kiwifruit, pome	Viñas *et al* 1998; Cook *et al* 1999
Candida saitoana	*B. cinerea, P.digitatum, P. expansum*	apple, citrus	El-Ghaouth *et al* 2000
Cryptococcus laurentii	*Aspergillus niger, B. cinerea, P. expansum, R. stolonifer*	grape, pome, strawberry	Roberts 1990; Lima *et al* 1998
Kloeckera apiculata	*A. niger, B. cinerea, R. stolonifer*	apple, grape, peach	McLaughlin *et al* 1992
Metschnikowia andauensis	*B. cinerea, P. digitatum, P. expansum, P. italicum, R. stolonifer*	citrus, pome	Manso & Nunes 2010
Metschnikowia fructicola	*Alternaria* spp., *A. niger, B. cinerea*	grape	Kurtzman & Droby 2001; Karabulat *et al* 2003
Metschnikowia pulcherrima	*B. cinerea, Monilinia* sp., *P. expansum*	apple, grapes	Piano *et al* 1997; Janisiewicz *et al* 2001
Pichia anomala	*B. cinerea, Botryodiplodia theobromae, C. musae, F. moniliforme*	apple, banana, guava	Jijakli & Lepoivre 1998; Lassois *et al* 2008; Hashem & Alamri 2009
Rhodotorula glutinis	*B. cinerea, P. digitatum, P. expansum*	apple, oranges	Zheng *et al* 2005; Zhang *et al* 2009
Filamentous fungi			
Aureobasidium pullulans	*B. cinerea, M. laxa, P. expansum*	apple, peach, strawberry	Adikaram *et al* 2002; Bencheqroun *et al* 2007; Zhang *et al* 2010
Epicoccum nigrum	*M. laxa*	peach	Madrigal *et al* 1994
Muscodor albus	*B. cinerea, M. fructicola, P. digitatum, P. expansum*	apple, lemon, peach	Mercier & Jiménez, 2004; Mercier & Smilanick 2005
Trichoderma asperellum	*Thielaviopsis paradoxa*	pineapple	Wijesinghe *et al* 2010

* *Candida guilliermondii syn.: Pichia guilliermondii, Debaryomyces hansenii*

Selection of a Postharvest Biocontrol Agent

The first step to develop a biocontrol system is the isolation and screening of a biological control agent (BCA). The fructoplane has been an excellent source of antagonists to control postharvest fruit pathogens. Different strategies have been used to screening BCAs, but since the antagonists are applied in consumable products often they are evaluated on wounded fruits instead of on *in vitro* studies (Nunes *et al.* 2009). The development of a BCA for postharvest diseases is an interactive process with several steps, including tests reflecting packinghouse conditions, enhancement of biocontrol activity, scale-up the production and development of a formulated product of the BCA.

Mode of Action of Postharvest Biocontrol Agents

The mode of action involves a complex interaction between host, pathogen, BCA and environment, comprising process of antibiosis, nutrient and space competition, induced resistance, parasitism and lytic enzymes production. Often more than one mechanism is present.

Competition for nutrients and/or space is reported has the major mode of action of postharvest BCAs (Droby *et al.* 1989; Janisiewicz *et al.* 2000; Nunes *et al.* 2001b; Bencheqroun *et al.* 2007). This hypothesis is supported by the fact that biocontrol activity of an antagonist depends on their concentration in the wound. So it can be considered if a BCA rapidly grows by depleting the available nutrients in the wound, it will prevent the possibility of the pathogen to use these nutrients to germinate and initiate the infection process. In most reports on biological control of postharvest diseases a quantitative relationship has been demonstrated between the BCA concentration in the wound and its efficacy (Vinãs *et al.* 1998; Nunes *et al.* 2002, 2007a).

Antibiosis by antibiotic production has been suggested in part as mode of action of bacteria. However leads us the debate if an antibiotic-producing microorganism should be used in postharvest phase, due to the concern of introducing an antibiotic into food and the possible development of a pathogen resistance. Concerning to parasitism and lytic enzymes production by BCAs, few reports are available, as the attachment of *Pichia guilliermondii* to the mycelium of the pathogen and subsequent changes in hyphae (Arras *et al.* 1998) and changes and degradation of the hyphae and cell walls of *Botrytis cinerea* due to the production of exo-beta-1,3-glucanase by *Pichia anomala* (Jijakli & Lepoivre 1998). Induced resistance in fruits has been observed in the presence of some BCAs, such the increase of ethylene production, phenylamina ammonia lyase (PAL) activity, phytoalexin biosynthesis, accumulation of chitinase and β-1,3-glucanase in fruits (Droby *et al.* 2002b).

Volatile compounds are suggested as a new mode of action and refers the use of antimicrobial volatiles produced by BCAs, and has been introduced as a better alternative because there is no contact with the food and less manipulation of the commodities would be involved. Good candidates for biocontrol by biofumigation are *Muscodor albus* (Mercier & Smilanick 2005) and *Bacillus subtilis* JA (Chen *et al*. 2008).

Enhancement, Development and Commercial Application of Biocontrol

The effectiveness of a BCA when applied alone has been a limitation of its use since is inconsistent and has a narrow range of activity either on fruits and/or diseases. Taking into account the mode of action, the importance of a rapid colonization of fruit wounds by the BCA and the interactions in microbial communities, different approaches could be used to improve and develop new biocontrol systems: (i) BCAs mixture that could increase the spectrum of activity, as the antagonist action will result from the action of a community of microorganisms; (ii) manipulation of nutritional environment in order to make it advantageous to the BCA and/or limited to pathogens; (iii) pre-harvest application that allow the BCA to have longer interact with the pathogen, and colonise tissues before the arrival of pathogen, such as latent infection and incipient infections; (iv) genetic manipulation of BCA, as insertion of genes or over-expression of endogenous genes responsible for antifungal activity or, insertion of genes for better utilization of

available nutrients; (v) production process to improve the ecological fitness of BCA and formulation to enhance viability, efficacy and shelf-life of BCA formulated cells, and (vi) integration with physical and/or chemical methods, (will be discussed in this review) taking advantage of the additive or synergistic effects in order to improve the efficacy of each method.

From an industry point of view, a BCA should be able to be produced at a large-scale in a short period of time and as a cost-effective process using as a growth medium by-products from food industries (Hofstein & Chapple 1998). Recently Manso *et al.* (2010) reported high biomass productivity of *Pantoea agglomerans* PBC-1 using by-products from carob industry as carbon source. Other by products, such molasses, malt extract, dry beer extract have been used (Abadias *et al*. 2003). To be commercialized a BCA has to be developed as a formulated product. Postharvest BCAs have been formulated mainly as a refrigerated liquid (Costa *et al.* 2001; Abadias *et al.* 2003), a solid formulation using freeze-drying (Abadias *et al.* 2001) and wettable refrigerated powder (Janisiewicz & Jeffers 1997). All these techniques of formulated cells have particularly effects on cells viability.

Several microorganisms have been reported as BCAs against fruit postharvest diseases however only a few biological products are available in the market: Aspire™ (*Candida oleophila*, Ecogen Inc, USA), Bio-save™ (*Pseudomonas syringae*, Jet Harvest Solutions, USA), Shemer™ (*Metschnikowia fructicola*, Bayer Crop Science, AG), YieldPlus™ (*Cryptococcus albidus*, Anchor Yeast), Avogreen (*Bacillus subtilis*, RE at UP, South Africa) are commercialized in various countries. In Europe there are three more products: Candifruit™ (*Candida sake*, Sipcam-Inagra, Spain), Pantovital (*Pantoea agglomerans*, Biodurcal, Spain) and Boni-Protect® (*Aureobasidium pullulans*, Bio-protect, Germany). The search for new antagonists should be permanent as in chemical industry the search for new molecules are constant, as well the objective to broaden the use of BCAs to different diseases and commodities.

Physical Control

Physical treatments when applied alone have the advantage of produce no residues, providing an environmentally friendly means of postharvest pathogen losses control. The most important and developed physical methods are: heat and UV-C.

Heat Treatments

Heat treatments for control postharvest diseases may be applied to fruit by hot air (curing) or hot water.

Curing treatment is applied by holding fruits at high temperature (30-40 ºC) and high relative humidity (>90%). Can be applied in a cycle of 2-6 days at approximately 30-38 ºC (Fallik *et al.* 1995; Plaza *et al.* 2003), in a shorter period, 0.5-24 h, at higher temperature, >40 ºC (Nunes *et al.* 2007b), or as intermittent treatment with more than one cycle. Pérez *et al.* (2005) effectively controlled decay in citrus applying a curing treatment of 2 cycles of 18 h at 38 ºC with an intermediate period of 6 h at 20 ºC. The effect of curing has been studied in different fruits (Fallik *et al.* 1995; Cook *et al.* 1999; Casals *et al.* 2010; Wang *et al.* 2010) and extensively in citrus fruit (Ben-Yehoshua *et al.* 1987; Rodov *et al.* 2000; Plaza *et al.* 2003; Pérez *et al.* 2005; Nunes *et al.* 2007b). In fact the most common use of curing to control decay is in citrus, and was first reported in the first half of 20th Century. Commercial use is not frequent as is expensive, at low temperature required long time and at higher temperature have some risks, such heat phytotoxicity and weight loss.

Hot water treatments can be applied by brief dippings (0.5-5 min) in water at 45-55 ºC or by hot water drench (20-60 s at 55-65 ºC) (Palou *et al.* 2002a; Smilanick *et al.* 2003; Torres *et al.* 2007). A more recent technique is the use of hot water sprays at 50-70 ºC during 10-60 s over rotating brushes (Porat *et al.* 2000; Karabulat *et al.* 2002). Among heat treatments the use of hot water is preferred because water is a more efficient heat transfer medium, easier to use, require shorter period of treatment, technology is cheaper and is easier to combine with other alternative methods. Hot water has been tested either alone or in combination with other alternative methods in a huge range of type of fruits: table grapes (Gabler *et al.*

2005), cherries (Karabulat *et al.* 2004a), stone fruit (Karabulat *et al.* 2002), litchi (Olesen *et al.* 2004) and again in more extend in citrus (Porat *et al.* 2000; Palou *et al.* 2002a; Torres *et al.* 2007).

The effect of heat is direct to the pathogen, by inhibition of spore germination and mycelium development, and indirect by induction host resistance to the pathogen. The inhibition of pathogens is affected by different factors. For example Barkai-Golan (2001) reported that germinated spore are more sensitive than ungerminated spores; in other work Sommer *et al.* (1967) showed that *Monilia fructicola* is more sensitive than *Botrytis cinerea*, B. *cinerea* than *Rhizopus stolonifer*, and *Penicillium expansum* is more tolerant than these species. Plaza *et al.* (2003) reported a 90% reduction of green and blue mould decay in oranges treated with curing at 33 ºC for 65 h, but total control of both pathogens was observed with treatments at 40 ºC for 18 h (Nunes *et al.* 2007b). Apart from species, physiological state, temperature and duration of treatment, the effect of treatment depends in other factors, such, moisture content, metabolic activity, age of inoculums, etc. (Ben-Yehoshua & Porat 2004).

The effect of heat treatment in induction of resistance by the host has been attributed to physical changes in the epiticular surface of fruits, such closing cuticle fractures by melting natural peel waxes, the enhancement of antifungal activity, induction of heat-shock proteins and pathogenesis-related (PR) proteins.

UV-C Illumination

Application of UV-C at low doses (180-280 nm) is known to reduce postharvest decay in several fruits (Stevens *et al.* 1996; Nigro *et al.* 1998; Marquenie *et al.* 2003; Cia *et al.* 2007). Despite the fact that UV-C direct inhibit the pathogen by DNA damages the major mode of action of UV-C is elicitation of resistance in fruits (Droby *et al.* 1993; Shama & Alderson 2005). As heat treatments, UV-C at 248 nm has shown to elicit production of phytoalexins (Kim *et al.* 1991), lignin-like compounds on fruit peel and PR proteins (Pombo *et al.* 2009), induction of PAL or peroxidase enzymes (Droby *et al.* 1993; El-Ghaouth *et al.* 2003). Other beneficial effects in fruit quality induced by the abiotic stress caused by UV-C should be taking into account, since all these changes in chemistry of fruits could enhance their nutraceutical value (Cisneros-Zevellos 2003). Erkan *et al.* (2008) reported the increase of antioxidant enzyme activity in strawberry after treatment with UV-C, and Dong *et al.* (1995) the increase of anthocyanins in apples.

Shama & Alderson (2005) made a compilation of several works in UV-C treatments, and reported treatment doses of UV-C to control decay in a range of 0.5 to 15 kJ m^2 depending on fruit and pathogen. However due to undesire effects such UV-C phytotoxicity the intensity of the treatment should be carefully monitored.

The use of UV-C treatment in fruits could become a commercial practice. There are some patents and commercial prototypes, however more research is needed since the response of the product to UV-C treatments is specific for each commodity and depends among other factors, on the level of maturity, temperature of storage and the side of fruit that have been exposed to the illumination.

Other Physical Methods

Pulsed light, ionizing radiations and microwaves are other physical methods to control postharvest decay. Pulsed light is a relatively recent technology, using short time pulses of intense broad spectrum rich in UV-C, with only a few reports of its use in postharvest (Lagunas-Solar *et al.* 2006). Ionizing radiation has a relatively short wavelength and high energy that allow high penetration and effectiveness. To be applied in fruits ionizing radiation can be produced by Gamma rays, X-rays or electrons beams. The use in fruits and vegetables was approved by United States Food and Drug Administration (US-FDA) at doses up to 1,000 Gy. As it was previously discussed for physical treatments, the effect of ionization radiation depends on type of radiation and energy level, type of fruit, maturity stage, temperature, etc. (Kader 1999). The effects of these treatments are also direct and indirect depending on the type of radiation; however it is not clear for each treatment which is the most important effect. The application of ionization radiation has limitations, mainly due to the acceptance of the consumers and the high cost of equipments and operations.

Chemical Control

There are several chemical alternative control methods to postharvest fungicides, however to be considered as a safe alternative should have minimal toxicological effects.

GRAS Substances

Generally Regarded as Safe, GRAS, is a classification of the US-FDA and is recognized among experts as safe under the conditions of its intended used.

Bicarbonate and carbonate salts are widely used as food additives with no restrictions for many applications by European and USA regulations. It has been found to have antimicrobial activity. Among them, to control postharvest decay, focus have been made to sodium bicarbonate (SBC; $NaHCO_3$) and sodium carbonate (SC; Na_2CO_3), especially to control postharvest decay of citrus fruit (Smilanick *et al.* 1995, 1997, 1999), but also in other fruits such grapes (Gabler & Smilanick 2001), melon (Aharoni *et al.* 1997), banana (Alvindia & Natsuaki 2007), etc. The mode of action of these salts is unclear but appears to be primarily fungistatic and not very persistent (Smilanick *et al.* 1999). SBC and SC are inexpensive, readily available, and can be used with a minimal risk of injury to the fruit, and when solutions are heated their activity is greatly enhanced.

Ethanol occurs in many food products and additives, and it was approved for use as a disinfectant or sanitizer USDA. Ethanol, as liquid or vapour treatment, has been reported to be effectively to control postharvest decays especially of table grapes and against several pathogens such *B. cinerea*, *Alternaria alternata*, and *Aspergillus niger* (Karabulat *et al.* 2004a,b; Gabler *et al.* 2005), but also to control postharvest diseases of stone and citrus fruit (Smilanick *et al.* 1995; Margosan *et al.* 1997).

Ozone (O_3) was declared as GRAS for food contact in 1997 and since that the interested in its application has been increased. Ozone is a natural substance in the atmosphere and one of the most potent sanitizers against a wide spectrum of microorganisms (Khadre *et al*. 2001). O_3 can be easily produced *in situ* and applied as a gas (continuous or intermittent exposure) or dissolved in water without leaving any residue since their product of degradation is oxygen. To control postharvest decays, O_3 has been applied in citrus, stone, strawberry and table grapes (Pérez *et al.* 1999; Palou *et al.* 2001, 2002b; Smilanick *et al.* 2002; Gabler *et al.* 2010). However there are several reports demonstrating the lack of effect of ozone (Palou *et al.* 2001). The application of ozone in air has some risks for human health, so requires some protective measures to workers.

Natural Compounds

Plants produce a wide range of secondary metabolites (e.g. essential oils, alkaloids, phenols, flavonoids) biologically actives with antifungal properties and with low toxicity to mammals and safe for environment. Some of them are recognize as GRAS compounds. Among secondary metabolites, flavour compounds have distinctive properties to be used in postharvest, such volatility, low- water solubility and easily adsorption. Special interest has been addressed to essential oils. The activity of essential oils in control postharvest pathogens in vitro was reported in different studies (Arras & Usai 2001; Plaza *et al.* 2004a; Viuda-Martos *et al.* 2007) however their effects rarely have been demonstrated in fruit at concentrations similar to *in vitro*. In general, the essential oils that showed greatest antifungal postharvest activity were from thyme (thymol and carvacrol), cinnamon and clove. Interest has been driven to the natural compounds and essential oils of citrus fruit, as will be discussed in a manuscript in this book by Rodov *et al.* The commercial application could be interesting however much more investigation is needed: the mode of action of essential oils is not fully explained but has been attributed to the damage of the membrane structure (Moleyar & Narasimham 1987), and many problems of phytotoxicity are associated.

Chitosan is a soluble form of chitin, normally obtained from crustacean shells, with antifungal properties and the capacity to induce resistance in plants at low concentration (approximately 1%). Chitosan has been showed to control decay in apple, table grape, strawberry, banana and citrus (El-Ghaouth *et al.* 1992;

Capdeville *et al.* 2002; Chien *et al.* 2007; Romanazzi *et al.* 2007). The primarily antifungal mechanism of chitosan is believe to be the direct effect in pathogen, although some works report the induction of host resistance (Capdeville *et al.* 2002).

Integrated Control

This review reported the extensive research and significant progress made worldwide in the last decades in postharvest diseases of fruits. There are already several non-fungicidal methods at a commercial stage with ability to control of several diseases in different crops. However to be commercially successful, any product/technology to be used in the postharvest phase have to control diseases in more than 95%. Therefore one approach to use these methods as an alternative to synthetic fungicides is an integrated strategy, taking advantage of the additive or synergistic effects of different treatments in order to overcome the performance and improve the efficacy of each one.

Some examples are the combination of biocontrol agents with physical and/or chemical treatments. Positive synergistic effect occurred with the combination of *P. agglomerans* followed by curing treatment at 33 ºC for 6 h in controlling green mould in lemons (Plaza *et al.* 2004b). Application in citrus fruit of *P. agglomerans* after treatment with solutions of heated sodium bicarbonate allowed a control similar or superior to imazalil (Torres *et al.* 2007; Usall *et al.* 2008). Enhancement of biocontrol activity was achieved in pome fruits combining L-serine, L-aspartic acid or ammonium molybdate with *Candida sake* (Nunes *et al.* 2001b, 2002), in papaya using sodium bicarbonate and *Candida oleophila* (Gamagae *et al.* 2004). Other compounds such sugar analogs, calcium salts, organic acids have been combined with biological methods to manage postharvest decay control (Janisiewicz *et al.* 1998; Karabulut *et al.* 2001; Nunes *et al.* 2001b; Ippolito *et al.* 2005). A result of this integrated approach of biocontrol systems is the development of a second generation of products such "Biocoat" whose main components are *Candida saitoana* and chitosan or "Biocure" also with *C. saitoana* and lysozyme. Both products contain other additives such sodium bicarbonate (Wisniewski *et al.* 2007).

Another important approach has been the combination of different physical and/or chemical treatments, in most of the cases including heat, specially the use of heated chemical solutions. As already reported the heating solution of sodium carbonate or bicarbonate significantly enhanced their effectiveness (Smilanick *et al.* 1997; Palou *et al.* 2002a). The use of heated ethanol has been tested with success for control postharvest decay in citrus, peaches, table grapes and sweet cherry (Smilanick *et al.* 1995; Karabulat *et al.* 2004a,b; Gabler *et al.* 2005) in a concentration between 10-30%. The combination of these two treatments allowed a reduction in concentration of ethanol and temperature improving not only the efficacy of treatments but also safety issues and minimizing injuries of the product. The efficacy of organic acids was also enhanced in the control of *P. expansum* on apples and *P. digitatum* on oranges when applied as heated solutions (Salazar *et al.* 2007)

Several works report other combinations using alternative methods, including UV-C, white light and heat (Marquenie *et al.* 2003), X-ray and sodium bicarbonate (Palou *et al.* 2007), chitosan and ethanol (Romanazzi *et al.* 2007), plant extracts, chitosan and heat (Win *et al.* 2007) etc.

Conclusions

In this review several safe and environmentally friendly treatments alternative to conventional fungicides were reported as effectively to control postharvest decays in fruits, and, in general, with effectiveness comparable to fungicides. Research in biological control has made important advances allowing the development of some commercial products, showing a promising and new alternative system. Physical methods have several advantages such a total lack of residues, presenting often direct and indirect effect, and some of them have a simple and inexpensive application. Chemical methods use natural or synthetic compounds with low toxicity, like GRAS compounds. They are in general inexpensive, readily available, and

suitable for the postharvest handling practices. Although the high efficacy of the treatments the specificity host/pathogen, the cost, the lack of curative effects and the high variability represent a restriction to their implementation. To overcome these shortcomings, the combination of these methods as an integrated strategy demonstrates to be viable approach alternative to synthetic fungicides.

In the future the commercial use of these methods will be very important thus organic producing are growing worldwide, since the use of synthetic fungicide is not allowed. Furthermore, nowadays some supermarkets already demands for fruits and vegetables free of residues of postharvest products, because the chemical residues are more likely to be present when fruits will be consumed. Research should be driven to large scale trials, to understand the host/pathogen interaction and validate the viability of the implementation of these practices in packinghouses.

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23. STUDY OF MODES OF ACTION OF THE BIOCONTROL AGENT *METSCHNIKOWIA ANDAUENSIS* PBC-2

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Abstract

Metschnikowia andauensis NCYC 3728 (PBC-2) is an effective antagonist against the postharvest pathogens *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolofiner* on pome fruits, however its mode of action is unknown. The ability of this strain to produce inhibitory compounds in 4 distinct media (PDA, NYDA, YPDA, CJA) at 3 temperatures (1, 25, 30 ºC), was investigated. It was also assayed the competition for iron in media with different iron concentrations and characterized the capability of PBC-2 produce and secrete fungal cell wall lytic enzymes, like chitinase, protease, and glucanase in a culture media with fungal pathogen cell wall as unique carbon source. *M. andauensis* PBC-2 did not show any inhibition zone to cope pathogens in any of the tested media. The results obtained in this study suggest that the production and secretion of lytic enzymes is not the main or more important mode of action of the new biocontrol agent PBC-2, since the production of chitinase was observed only past 5 and 7 d of incubation, and the production of β-1.3-glucanases and proteases was not observed, which mean that the biocontrol agent PBC-2 have more than one mechanism of action.
Keywords: biocontrol agent, inhibitory compounds, iron competition, lytic enzymes, mode of action

Introduction

Fruits and vegetables are highly perishable products, especially during the postharvest phase, when considerable losses, due to microbiological diseases, disorders, transpiration and senescence, can occur. Traditionally, postharvest diseases are often controlled by the application of synthetic fungicides, however and during the last decade the application of microorganisms for the biocontrol of postharvest diseases has received increasing attention (Droby 2006; Nunes *et al.* 2009). Several yeasts and bacteria have been shown to protect against a number of postharvest pathogens on a variety of harvested commodities (Janisiewicz *et al.* 1994; Chand-Goyal & Spots 1996; Ippolito *et al.* 2000; Nunes *et al.* 2001; Kurtzman & Droby 2002; Vero *et al.* 2002). Antagonistic yeasts have received particular attention, as their activity usually does not depend on the production of antibiotics or other toxic secondary metabolites, which could have a negative environmental or toxicological impact (Sipiczki 2006). Different species of genus *Metschnikowia* have been described as an effective biocontrol agents (Kurtzmann & Droby 2002; Spadaro *et al*. 2002; Karabulut *et al* 2004; Kinay & Yildz 2008). The yeast *Metschnikowia andauensis* NCYC 3728 (PBC-2) (Nunes & Manso 2010), used in this study was evaluated with success against *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer* in pome and *Penicillium digitatum* and *Penicillium italicum* in citrus fruits.

The knowledge of the mechanisms involved in biological control play an essential role in the development and registration of a biocontrol agent and in maximizing the efficacy of this control system (Janisiewicz *et al.* 2001). Attempts to characterize those mechanisms has resulted in a variety of studies like antibiosis with volatile and non-volatile compounds, competition for nutrients and space, induction of resistance in fruits (Calvente *et al.* 1999; Janisiewicz *et al.* 2000; Poppe *et al.* 2003; Santos & Marquina, 2004; Saravanakumar *et al.* 2008). The direct interaction of the biocontrol agent with the pathogen, for example, by the involvement of fungal cell wall degrading enzymes, like chitinase, protease and glucanase, is also suggested to play a role in the mechanisms of action (Berto *et al.* 2001; Castoria *et al*. 1997; Mahadevan *et al*. 1997; Saravanakumar *et al.* 2009).

The aim of this study was to determine the mechanisms of biocontrol activity by *M. andauensis* PBC-2. The ability of this strain to produce *in vitro* antagonism, siderophes and produce and secrete fungal cell wall lytic enzymes was investigated.

Material & Methods

Biocontrol Agent

Metschnikowia andauensis NCYC 3728 (PBC-2) previously isolated from the carposphere of 'Bravo de Esmolfe' apples from Portugal and characterized as a biocontrol agent for postharvest diseases of apples and citrus was used in this work. It was stored as a cell suspension in 20% (v/v) glycerol at -80 ºC. When required, *M. andauensi*s PBC-2 was streaked on NYDA medium (8 g L^{-1} nutrient broth; 6 g L^{-1} yeast extract; 10 g L^{-1} glucose; 15 g L^{-1} agar) and incubated at 25 ºC.

Pathogens

P. expansum, *R. stolonifer* and *B. cinerea* strains used were isolated from decayed pome fruits, and selected for their high level of aggressiveness on 'Red Delicious' apples. All the strains were maintained on PDA medium (Potato Dextrose Agar) at 4 °C.

In Vitro Antagonism

The ability of the yeast to inhibit growth of *P. expansum*, *B. cinerea*, *R. stolonifer* was tested on dual cultures on different culture media, at different temperatures. A mycelial disk (diameter 5 mm) from 10 days (d) old cultures of each pathogen, was placed at the center of Petri dishes containing the following media, PDA, NYDA, YPDA (10 g L^{-1} yeast extract; 20 g L^{-1} peptone; 20 g L^{-1} glucose; 15 g L^{-1} agar), and CJA (20 g L^{-1} citrus juice by-products with 15 g L^{-1} agar). For each medium tested, the yeast, produced in the respective liquid media, was streaked at two sides of the pathogen. Plates were incubated in different temperatures, 1, 25 and 30 ºC. Control plates without antagonist were performed for each pathogen, in each media at all temperatures. After 15 d of incubation diameters of pathogens colonies towards the antagonist were measured in each case and compared to colony diameters in control plates. Three plates constituted a single replicate and the experiment was replicated twice.

Competition for Iron

Petri dishes containing a medium for siderophore production (sucrose 25 g L^{-1}; ammonium sulphate 4 g L^{-1}; potassium dibasic phosphate 3 g L^{-1}; citric acid 1 g L^{-1}; magnesium sulphate 0.08 g L^{-1}; zinc sulphate 0.002 g L^{-1}; agar 20 g L^{-1}) were amended with different concentration of iron (0, 10, 100 and 500 μM of ferric chloride). An agar disk (diameter 5 mm) from 10-d old cultures of *P. expansum* or *B. cinerea* was placed at 30 mm from the edge plate. A single streak of *M. andauensis* PBC-2 was inoculated 40 mm from the pathogen. Plates were incubated at 25 ºC and the development of both organisms was observed.

Fungal Cell Wall Production and Induction of Lytic Enzymes

P. expansum strain was grown on YES broth (sucrose 150 g L^{-1}; yeast extract 20 g L^{-1}) at 25 ºC, for 7 d. Obtained mycelium was dried with sterile filter paper and ground in a sterile mortar in the presence of liquid nitrogen to get a fine powder. The mycelial powder was suspended in 5 M NaCl, sonicated for 5 min and centrifuged at 5724×*g* for 10 min. The supernatant was discarded and the pellet was washed 3 times with distilled water. Fungal cell wall were dried in Petri dishes at 60 ºC for 3 h.

Flasks containing yeast nitrogen base medium (YNB) supplemented with previously prepared fungal cell wall at 1 g L^{-1}, were inoculated with PBC-2 fresh cells at an initial concentration 1×10^5 cfu mL^{-1} and incubated at 25 ºC, 150 rpm. Samples were withdrawn aseptically at different times and filtrated by 0.45 μm (Millipore). Filtrates were stored at -20 ºC, until enzymes determination. Experiment was conducted in three replicates.

Enzymes Assays

β-1,3 glucanase activity was assayed in 62.5 μL of culture filtrate and 62.5 μL of 0.05 M acetate buffer, pH 5, containing 1% laminarin. The reaction was stopped by adding dinitrosalicylic reagent (DNS) and heating the tubes for 5 min at 100 °C. The net increase of reducing sugar in the reaction mixture was determined by comparing the measured optical densities (540 nm) with those on a standard curve prepared with glucose. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 μmol reduction group min^{-1}.

Chitinase activity was assayed in 90 μL of culture filtrate and 10 μL potassium phosphate buffer, pH 6.1, containing 0.18 mM nitrophenyl N-acetyl β-D glucosamide. The reaction was stopped by adding 10 μL NaOH 1 M. The formation of *p*-nitrophenol was followed by absorbance at 405 nm. An increase of 0.01 units of absorbance corresponded to the formation of 1 unit (U) of enzyme.

Protease activity was measured in a reaction mixture contained 100 μL of culture filtrate, 100 μL 0.05 M acetate buffer, pH 5 and 100 μL azocasein 1%. The reaction was stopped adding 400 μL TCA 10% and after centrifugation 500 μL of NaOH 525 mM was added to the supernatant and the optical density was measured at 450 nm. An increase of 0.01 units of absorbance corresponded to the formation of 1 unit (U) of enzyme.

Protein quantification was determinate using the kit BCA from Pierce, following the fabricant instructions.

Results

In Vitro Antagonism

M. andauensis NCYC 3728 (PBC-2) did not show any inhibition zone to cope pathogens. In YPDA and CJA media, pathogens developed without sporulation. Optimal growth of pathogens, in the absence or in the presence of the biocontrol agent, was observed in PDA medium (Fig 1). Growth rate of all pathogens was higher at 25 °C, when compared to 1 and 30 °C. Although with some differences in growth between the media, the pathogens in the presence of the biocontrol agent, developed without inhibition, when compared with the pathogen inoculated separately.

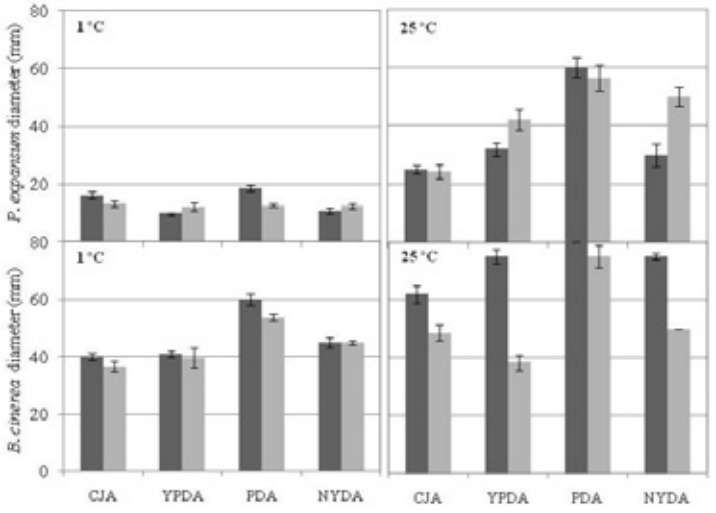

Fig 1. Diameter development of *P. expansum* and *B. cinerea* grown in 4 different culture media, *in vitro* assays, incubated at 1 and 25 °C. ■ Control treatment, inoculation only the pathogen. ■ Simultaneous inoculation of the pathogen and *M. andauensis* PBC-2. Bars represent standard deviation.

The high diffusion of pulcherrim produced by the biocontrol agent was observed in PDA and NYDA (data not shown).

Competition for Iron

At the tested conditions no inhibition zones were observed in the presence or in the absence of iron. *M. andauensis* PBC-2 produced pale pink to dark red colonies under different iron conditions. The intensity of the colour increased with increasing iron concentrations. The antagonistic yeast produced wider pigmented halos in the medium without iron and an increase in ferric chloride concentration decreased the pigmented halo (Fig 2).

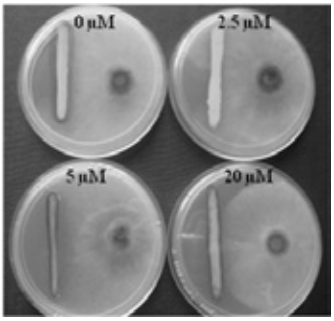

Fig 2. Dual cultures of *M. andauensis* PBC-2 and *B. cinerea* in media with different concentrations of ferric chloride (0, 2.5, 5, 20 μM).

Fungal Cell Wall Production and Induction of Lytic Enzymes

The biocontrol agent PBC-2 showed a slight growth in the YNB media supplemented with *P. expansum* cell wall. Growth was more evident after 5 d of incubation even so no more than 10 fold of the initial population was achieved.

Enzymes Assays

The production of glucanase and protease was not observed at the tested conditions. The maximal level of protein was reached after 5 d of incubation (Fig 3), reaching 113 μg mL^{-1}, after which the production of chitinase increased until the 7th day (Fig 4).

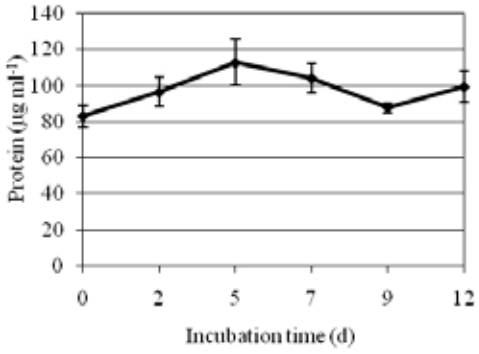

Fig 3. Time course of changes in concentration of protein during the culture of the biocontrol agent PBC-2 in YNB media supplemented with fungal cell wall. Bars represent standard deviation.

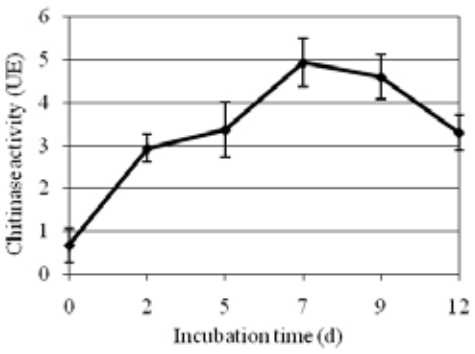

Fig 4. Time course of changes in chitinase activity during the culture of the biocontrol agent PBC-2 in YNB media supplemented with fungal cell wall. Bars represent standard deviation.

Discussion

Metschnikowia andauensis NCYC 3728 (PBC-2), used in this study was evaluated with success against *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus stolonifer*, in pome and *P. digitatum* and *P. italicum* in citrus fruit. However the mechanism by which the yeast reduces decay is not known. In the present work, *in vitro* antagonism, competition for iron and production of extracellular lytic enzymes, was investigated as possible modes of action. No inhibition of any of the tested pathogens was observed in the 4 different media, at the 3 temperatures studied. Results obtained suggest that in our experimental conditions, the production of inhibitory compounds is not the mode of action of this biocontrol agent. Numerous reports in the literature describe the inhibition of postharvest diseases by antibiotics-producing microorganisms, especially in the case of bacteria. *Bacillus subtilis* and *Pseudomonas cepacia* are known to kill pathogens by producing the antibiotic iturin (Gueldner *et al.* 1988). *Pseudomonas cepacia* inhibited the growth of postharvest pathogens like *B. cinerea* and *P. expansum* in apple by producing an antibiotic, pyrrolnitrin (Janisiewicz *et al.* 1991). *Aureobasidium pullulans* produces aureobasidin A, an antifungal cyclic depsipeptide antibiotic that inhibit the development of *P. digitatum*, *P. italicum*, *P. expansum*, *B. cinerea* and *Monilinia fructicola* (Liu *et al.* 2007). However, at present, antibiotic-producing antagonists are not likely to be registered for postharvest use on food products (Nunes *et al.* 2009; Sharma *et al.* 2009). Since the antagonistic activity of *M. andauensis* PBC-2 did not seem to be related to inhibitory substances, competition for iron was tested as a possible mechanism. Iron is essential for the fungal growth and pathogenesis and iron sequestration by non-pathogenic microbes, could be exploited in novel systems for biological control of postharvest pathogens (Calvente *et al.* 1999; Zhang *et al.* 2007). The yeast *M. andauensis* PBC-2 produced a pigmented zone around them, in the media without iron or with low concentrations of this micronutrient. Increasing the ferric chloride concentration the pigmented halo diminished, but the pale pink colour of the colonies became red. Contrasting with the observed by Sipiczki (2006), Saravanakumar *et al.* (2008) and Vero *et al.* (2009), in the present work, no inhibition in the pathogens development was observed, in the presence or in the absence of iron in the media (Fig 2). In the mentioned studies, higher inhibition halos by the antagonist strains in front of the pathogens were observed in lower iron amendments, suggesting the depletion of the micronutrient by the biocontrol agent under low iron conditions. Nevertheless, the colour change of the *M. andauensis* PBC-2 colonies observed with the increase of iron concentration is in agreement with previous findings of Sipiczki (2006), who demonstrated the iron competence between strains of *M. pulcherrima* and pathogenic fungi, suggesting the iron immobilization from the medium and the formation of a red, insoluble pigment called pulcherrimin. By the formation of this insoluble complex, it was suggested that iron remained in the medium but was inaccessible.

Microbial antagonists produce lytic enzymes such as glucanases, chitinases, and proteases that help in the cell wall degradation of the pathogenic fungi (Castoria *et al.* 2001). In the present study, the production of proteases and β-1,3-glucanase was not observed at the tested conditions. The highest level of protein was reached after 5 d of incubation and 2 d later the chitinase activity achieved its maximum level. Chitin, the unbranched homopolymer of N-acetyl glucosamine in a β-1,4 linkage, is a structural component of cell walls in most of the fungi, chitinases hydrolyze this polymer and have been implicated in biocontrol processes (Castoria *et al.* 2001). Although reported in many works, as an important role in the biocontrol activity, the chitinase productivity differs greatly between biocontrol agents. Saravanakumar *et al.* (2009) found a higher production of chitinases by yeast *M. pulcherrima* strain MACH1 in PDB and YPD in the presence of pathogen cell wall indicated the induction of chitinases by biocontrol yeast. Vero *et al.* (2009) demonstrated that *A. pullulans* strain ApB produced both chitinase and β-1,3-glucanase at 5 °C, in the presence of *P. expansum* cell walls in minimal medium and in apple juice. The production of these enzymes at 25 °C by an *A. pullulans* isolate has previously been reported by Castoria *et al.* (2001).

The current study demonstrated that *M. andauensis* PBC-2 did not inhibit pathogens development, through the production of inhibitory substances or by iron competition. The determination of lytic enzymes

revealed that this strain, at the tested conditions, did not produced and secrete β-1,3-glucanase and proteases, nevertheless it showed a modest chitinase activity, which can be consider an advantage, once the biocontrol agent PBC-2 have more than one mechanism of action.

Further studies should be performed in order to clarify the modes of action involved in the antagonistic activity of the biocontrol agent *M. andauensis* PBC-2. Competition for nutrients, other than iron, as well as, the ability of the biocontrol agent, to induce host plants to produce antioxidants and synthesis of pathogenesis-related proteins, should be examined.

Acknowledgements

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24. IS IT POSSIBLE TO IMPROVE BIOCONTROL AGENTS TO PRACTICAL APPLICATIONS? THE *PANTOEA AGGLOMERANS* CPA-2 EXAMPLE

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Abstract
A major hurdle in exploitation of biocontrol agents is the limited tolerance of fluctuating environmental conditions practically and the difficulties in developing a shelf-stable formulated product as effective as fresh cells. Most of microorganisms are very sensitive to drying processes involved in formulation and biological control is usually limited by the narrow range of conditions below microorganisms are able to survive, establish and effectively control pests and diseases. *P. agglomerans* cells grown at low water activities using NaCl exhibited osmotic adaptation and also demonstrated thermotolerance and desiccation tolerance after spray drying, freeze drying and fluidized bed drying. Different formulation strategies of *P. agglomerans* cells were tested in order to improve survival under field conditions and efficacy in controlling postharvest rots, including lyophilised cells, osmotic adaptation by NaCl treatments and additives. In general, osmotic adapted and lyophilised *P. agglomerans* cells showed greater survival rates than non-osmotic adapted or fresh cells when these bacterial treatments were sprayed at field conditions. However, this superiority was only found when additive Fungicover was added to suspension treatments. The improved formulation of *P. agglomeran*s provided an effective control for oranges against natural postharvest pathogens infections and *P. digitatum* artificial infections. These results allowed us to conclude that it is possible to improve environmental stress tolerance and ecological competence of *P. agglomerans* by integrating certain formulation strategies. Enhancing stress tolerance and formulation strategies could be appropriate approaches to obtain consistency and broaden the spectrum of use of biocontrol agents.
Keywords: biological control, citrus, fluidized bed drying, improving environmental stress resistance, pre-harvest treatments, spray drying

Introduction
Biological control of postharvest diseases of fruits has advanced greatly during the past decade. Concerns regarding human health and environmental risks associated with chemical residues in foods have been the main driving force of the search for new and safer control methods. Among the proposed alternatives, the use of naturally occurring antagonistic microorganisms has been the most extensively studied. Several microbial antagonists, based on either yeast or bacteria were developed and commercially tested. However the success of these products remains limited and just a few microorganisms are commercial available to control postharvest decay of citrus and pome fruits such as Bio-Save 10 (*Pseudomonas syringae* ESC-10, Jet harvest solutions, USA), Shemer (*Metschnikowia fructicola*, Bayer Crop Science, AG), Boniprotect (*Aureobasidium pullulans*, Bio-protect, Germany) and Candifruit (*Candida sake* CPA-1, Sipcam Inagra, Spain).

There are several reasons of the limited number of commercial available biocontrol agents, such as the limited tolerance of fluctuating environmental conditions practically and the difficulties in developing a shelf-stable formulated product that is as effective as fresh cells. This is because of the simultaneous exposure of the microorganism to environmental stress conditions such as low a_w (dehydration) and high temperatures. Thus, improvement in physiological quality of the biocontrol agents during production which can enable survival and activity under such environmental conditions are an important challenge for exploitation and potential suitability in commercial conditions.

It has been demonstrated with both food-borne pathogens (O'driscoll *et al.* 1996; Mattick *et al.* 2001; Greenacre *et al.* 2003) and probiotics (Ananta & Knorr 2003; Prasad *et al.* 2003) that adaptation to hostile environmental conditions also has the potential to alter cellular physiology such that the organism becomes more resistant to further stress. In the case of osmotic stress, the significant changes reported in bacteria include the accumulation of compatible solutes such as amino acids and sugars (Csonka 1989).

Subjection to a mild stress makes cells resistant to a lethal challenge with the same stress condition. Preadaptation to one particular stress condition can also render cells resistant to other stress imposing conditions: this phenomenon is known as cross protection (Sanders *et al.* 1999).

Detailed studies have shown that the strain CPA-2 of *Pantoea agglomerans* - previously classified as *Erwinia herbicola* - is an effective antagonist to the major postharvest fungal pathogens of pome and citrus fruits (Teixidó *et al.* 2001; Nunes *et al.* 2001, 2002; Usall *et al.* 2008) and it is in commercialization process in Spain as a solid formulation named Pantovital by BIODURCAL S.L. Commercial and technical formulations of these two biocontrol agents are been developed in the Postharvest Pathology, IRTA, Lleida, Catalonia.

Before our studies, there were very few reports describing the physiological osmotic stress responses in biological control agents, and all of them have been on filamentous fungi (Hallsworth & Magan 1996; Pascual *et al.* 2000) or yeasts (Teixidó *et al.* 1998a,b; Abadias *et al.* 2001). During 12 years our group has focussed part of the research in improving biocontrol agents (*C. sake* and *P. agglomerans*) behaviour in front stress conditions achieving interesting results that allow enhance biocontrol treatments at field conditions, improve biocontrol agents behaviour during formulation process and broaden their spectrum of action. The main results with osmotic adaptation achieved with *P. agglomerans* are summarized in this chapter.

Improving Low Water Activity Tolerance by Osmotic Treatments

The improvement of tolerance to low water activity (a_w) and desiccation in *P. agglomerans* cells subjected to mild osmotic stress during growth was studied using different solutes to change a_w of growth media. It was shown that cells grown in media at low a_w using NaCl exhibited osmotic adaptation in solid media at low a_w obtaining high production level and maintaining biocontrol efficacy (Teixidó *et al.* 2006). Osmotic-adapted cells also demonstrated thermotolerance (Teixidó *et al.* 2005).

The role of different compatible solutes in adaptation of the bacterium to osmotic stress was determined and this study suggested that glycine-betaine and ectoine play a critical role in environmental stress tolerance improvement (Teixidó *et al.* 2005; Cañamás *et al.* 2007).

Formulation Process Enhancement

P. agglomerans cells grown at low water activities using NaCl not only exhibited osmotic adaptation but also demonstrated better desiccation tolerance after spray drying, freeze drying and fluidized bed drying than non-modified cells.

Spray Drying Process

Cells grown in NaCl modified media which showed the best low a_w adaptation were tested in spray-drying trials to check desiccation tolerance. *P. agglomerans* was grown on unmodified, and NaCl 0.98, 0.97 and 0.96 a_w modified basic media for 24 and 48 h at 30 ºC and 150 rpm agitation on a rotary shaker. Harvested cells were resuspended in $MgSO_4$ 10% suspension to obtain an initial concentration of $1x10^{10}$ cfu mL^{-1}. These suspensions were then incubated for 30 min at room temperature, constantly shaken to allow cell adaptation and then spray-dried at an inlet temperature of 140 ºC and a delivery rate of 500 mL h^{-1}. The powder obtained was rehydrated with reconstituted nonfat skimmed milk (10%), which acted as a rehydration medium. Used methodology was optimized by Costa *et al.* (2002).

Significant differences between growth treatments were found with respect to the survival of spray-dried cells (Data not shown). The best survival was achieved with cells grown for 48 h in NaCl 0.97 medium

(29%), followed by cells grown for 48 h in NaCl 0.98 (23%). The survival rate of cells grown in unmodified medium was always less than 7%. In the case of NaCl, the survival of pre-stressed cultures improved when cells were incubated for 48 h instead of 24 h. The opposite tendency was observed with control cells. Our research demonstrated that the treatments that showed the best adaptation to low a_w also presented better survival during the spray-drying process than control cells. These results confirm others obtained by Prasad *et al.* (2003), who found that when pre-stressed with either heat (50 ºC) or salt (0.6 M NaCl), *Lactobacillus rhamnosus* HN001 showed a significant improvement in viability compared with a non-stressed control culture after storage at 30 ºC in its dried form. The drying technique applied in this case was fluidised bed drying.

Although desiccation improvement of modified NaCl cells is clear, it is not sufficiently good in practical terms to consider spray-drying as an appropriate strategy for dehydrating this biocontrol agent.

Fluidized Bed Drying Process

The effect of osmotic treatments on the viability of cells after fluidized bed drying process was studied. Assayed treatments were the same described above in the previous section, using NaCl to adjust a_w (0 g L^{-1} NaCl (0.99 a_w)- P, 35 g L^{-1} NaCl (0.98 a_w)-P980 or 53 g L^{-1} NaCl (0.970 a_w)-P970) place another treatment with 25 g L^{-1} NaCl (0.988 a_w)- P988. For an optimal level of growth in P, P988, P980 and P970 treatments, incubation times were 20, 20, 22 and 30 h, respectively. For P980 and P970 cultures were also obtained after 48 h of incubation, corresponding this time to an optimal level of osmoresistance of *P. agglomerans* cells (P980+48 h and P970+48 h treatments) as it had been demonstrated by Teixidó *et al.* (2006).

Results pointed out those treatments which were effective to improve survival of *P. agglomerans* cells during drying process. The best survival values of *P. agglomerans* cells were achieved with osmotic treatments when cells grown at a_w of 0.98 and 0.97 for 48 h using NaCl to adjust a_w (Fig 1). These treatments also shown the highest viabilities following spray-drying process in studies carried out by Teixidó *et al.* (2006). However, P988 osmotic treatment was chosen for further experiments because it was a cheaper and optimized production treatment. Although P980+48h and P970+48h treatments gave higher viabilities than P988 osmotic treatment, they showed lower levels of biomass production. Other authors such as Prasad *et al.* (2003) obtained also higher viabilities in osmotic shocked cells of *Lactobacillus rhamnosus* HN001 (DR20) than non-adapted after drying in fluidized bed dryer.

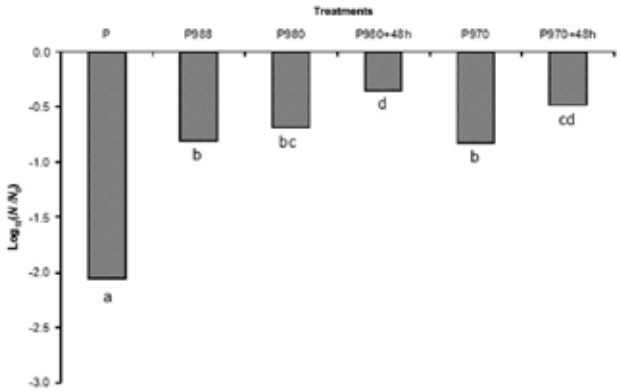

Fig 1. Survival of non-osmotically (P treatment) and osmotically adapted cells of *P. agglomerans* after dehydration in a fluidized bed dryer for 20 min at 40 ºC. Levels of viability after drying process were expressed as logarithmic value of survival fraction $\log_{10}(N\,N_0^{-1})$. Results are the means of at least two independent fluidized bed drying trials with two independent replications per trial. Columns with different letter are statistically different according to Duncan's multiple range test at $P<0.05$.

Freeze Drying Process

Significant differences in the viability of *P. agglomerans* cells after freeze-drying and shelf life period were observed depending on the presence or absence of NaCl (25 g L^{-1} P988) on growth medium (Fig 2).

Just after drying process, survival of *P. agglomerans* cells grown in unmodified or NaCl-amended medium was 90.2 and 100% respectively. Differences among both treatments increased along the storage or shelf life of formulated products and after 6 months of storage at 4 °C, cells grown in NaCl medium maintained 100% of viability and the ones grown in basic medium showed less than 25% of cell survival. It is also remarkable that cells grown in 25 g L^{-1} NaCl-medium showed viabilities higher than 80% after one year of storage. Survival and shelf life achieved are reasonably good for commercial application.

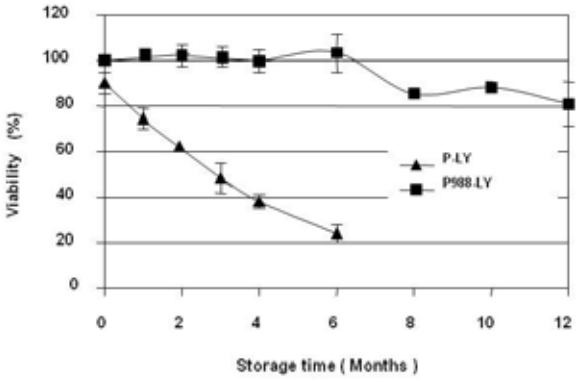

Fig 2. Survival of non-osmotically P-LY and osmotically P988-LY adapted cells of *P. agglomerans* after freeze-drying and shelf life of dehydrated product stored at 4 °C during one year. Results are means of four independent samples and vertical bars indicate standard errors.

Preharvest Treatments Enhancement

Osmotic adapted cells described above were used in preharvest treatments in order to control the main postharvest diseases on citrus fruit.

In the first experiment (Cañamás *et al.* 2008a), it was observed that *P. agglomerans* cells (osmotic-adapted and non-adapted) were unable to maintain a stable population on the fruit surface and consequently, preharvest applications resulted ineffective against both naturally and artificially inoculated *P. digitatum*. This low survival rates revealed the sensibility of *P. agglomerans* cells to environmental field conditions. These results also led us to conclude that it is necessary a minimal antagonist population levels on fruit surfaces in order to guarantee competition with pathogens for sites and nutrients, and to subsequently obtain an efficient control. The establishment of bacterial populations on plant surfaces is a critical phase in disease control. Microbes can be inactivated by several environmental factors, including sunlight, temperature, humidity, leaf surface exudates and competitors.

The effect of main environmental factors on *P. agglomerans* cells, such as relative humidity and solar radiation was studied and different formulation strategies were used in order to enhance survival on fruit surface under field conditions and subsequently enhance biocontrol efficacy (Cañamás *et al.* 2008a).

Osmotic adapted *P. agglomerans* cells, especially when these cells grew at 0.98 a_w were more resistant than non-adapted cells when both were applied in oranges and stored in chambers at a low Relative Humidity (43%). However, the minimum values of relative humidity registered during the first field assay were between 15 and 50%. These extreme values may restrict the survival and growth of *P. agglomerans* cells. The relative importance of each of these factors depends on why and where a particular product is used. In applications in foliar environments (which were our case), solar radiation, and especially the ultraviolet (UV) portion of the spectrum, is probably the main important factor affecting the persistence of microbial insecticides (Rhodes 1993; Filho *et al.* 2001). This detrimental effect of UV radiation was also

checked in a laboratory assay in which a major decrease in *P. agglomerans* population was observed during exposure to sunlight.

In laboratory studies, different additives, such as summer oils, alginate, glycerol and food additives at different concentrations were tested mixed with *P. agglomerans* in order to study its compatibility. The non-toxic ones (Citroline, Summer oil, Alginate, Sunspray, Glycerol, Siapton and Fungicover) were added to the biocontrol agent, sprayed on detached oranges and left outdoors. Population dynamics of the antagonist on fruit surface was determined along the time. Fungicover (FC) was the most effective additive for improving the adherence and persistence of *P. agglomerans* cells on oranges and it was also compatible with the antagonist. Firstly, adherence was improved by Fungicover since the *P. agglomerans* cell population just after application and drying (0 h), was greater than when cells were only sprayed with water. It was also visually observed that spreading, wetting and dispersion were also clearly improved. This could have been due to the fact that the additive Fungicover contains fatty acid derivatives in an alcohol solution. These components could have reduced the surface tension of the cell suspension and thereby improved the spread and wetness of the spray over the plant surface (Burges 1998). On the other hand, the persistence of *P. agglomerans* cells was also improved outdoors under springtime environmental conditions in the presence of Fungicover at 5%. It has not been possible to exactly elucidate the mechanism(s) by which this additive was able to protect the antagonist population. The additive Fungicover could also have protected *P. agglomerans* cells from solar radiation as sunscreen, physically reflecting and scattering, or selectively absorbing radiation, converting short wavelengths to harmless longer ones (Jones & Burges 1998).

Fungicover is an edible film-forming compound for fruits and vegetables to reduce weight loss, delay senescence, improve natural brightness and reduce physiological disorders. It also reduces droplet size and improves uniformity of distribution on the surface to be protected. This additive did not show any fungicidal effect on *P. digitatum* (Cañamás *et al.* 2008a).

In this study it has also been demonstrated that inoculum formulation can influence the persistence of *P. agglomerans* cells. Bacterial treatments prepared with lyophilised (LY) *P. agglomerans* cells become more resistant to environmental conditions than fresh cells (SH), as Stockwell *et al.* (1998) observed when the bacterial antagonists *Pseudomonas fluorescens* A506 and *Erwinia herbicola* C9-1R were applied under field conditions.

Different strategies could be used to improve *P. agglomerans* cell survival on oranges under non-controlled environmental conditions and all them were applied in two representative field trials on 'Lane late' and 'Valencia' oranges, in order to evaluate the effectiveness of different bacterial formulations of *P. agglomerans* applied at preharvest for controlling postharvest decays caused by natural infection and also by artificial infection (*Penicillium digitatum*) (Cañamás *et al.* 2008b).

Population dynamics of *P. agglomerans* during trials (under field conditions and at postharvest) are shown in Fig 3. In both experiments greater adherence and persistence of populations were observed when the biocontrol agent cells were sprayed using the additive Fungicover, being the population level similar to that treatment applied at postharvest.

Results for the efficacy of preharvest treatments for artificial infection by *P. digitatum* are shown in Fig 4. In experiment 1 (Fig 4A) the P988-LY+FC and P988-SH+POST treatments were significantly more effective than the other preharvest treatments and non differences were found between both after 15 d. In experiment 2 (Fig 4B) and after 15 d of storage, all the preharvest treatments with the additive Fungicover showed effective control against *P. digitatum* with decay values of below 12.5%. Treatment P988-LY also showed effective control with 12.8% decay. However, only treatments P988-LY+FC, P98-LY+FC and P97-LY+FC with decay values of between 7 and 5.3%, exhibited levels of control on *P. digitatum* no statistically different to the antagonist postharvest treatment P988-SH+POST.

The protective effect of the additive Fungicover was again confirmed by results and this effect varied according to whether or not the *P. agglomerans* cells had been osmotic adapted and if the cells had been

lyophilised or not. It is therefore likely that adding Fungicover to formulations could protect cells against field conditions. At the same time, it was possible to observe that populations of osmotic adapted cells showed a higher level of survival than non-adapted cells when the additive FC was used. Furthermore, the positive effect of applying lyophilised *P. agglomerans* cells instead of fresh cells was also evident when treatments were combined with Fungicover.

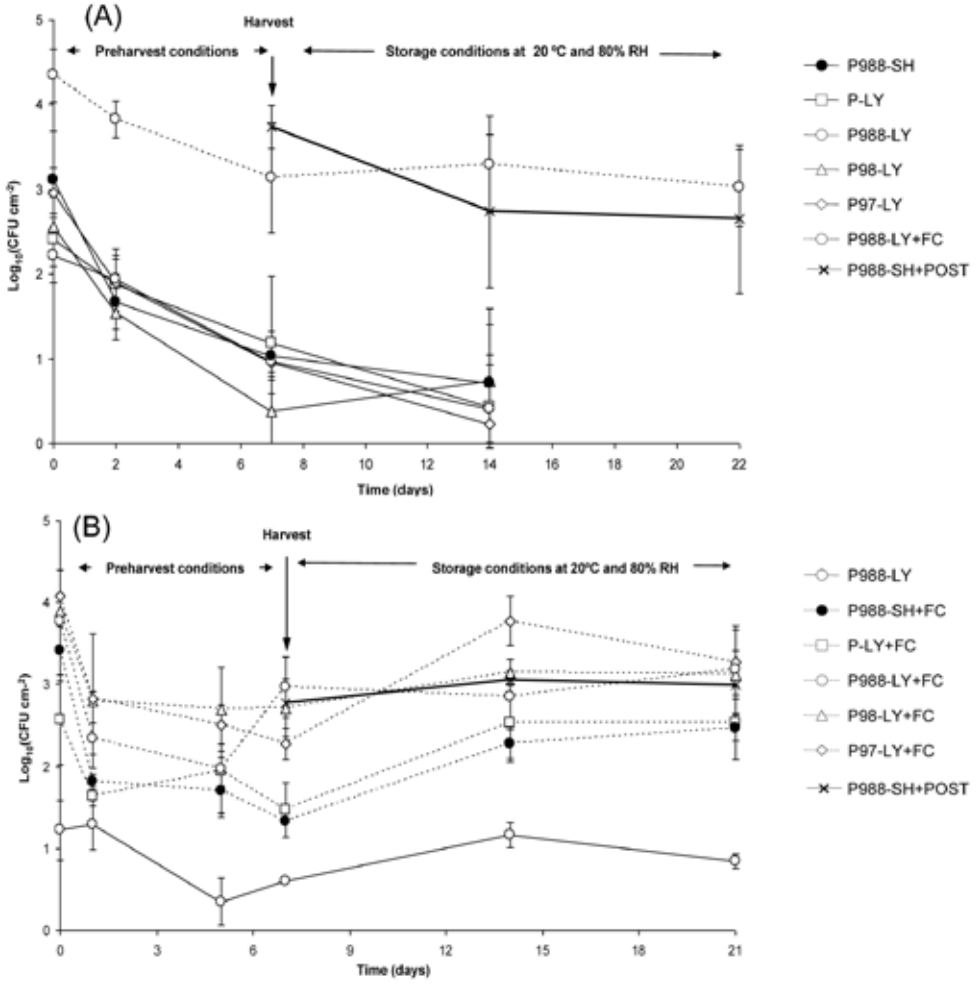

Fig 3. Population dynamics of *P. agglomerans* treatments during field and storage conditions. Bacterial treatments were prepared from lyophilized (LY) or fresh (SH) and from non-adapted (P) or osmotic adapted *P. agglomerans* inocula in presence of 25 g L^{-1} 0.988 a_w (P988), 35 g L^{-1} 0.98 a_w (P98) or 53 g L^{-1} 0.97 a_w (P97) of NaCl in the medium, respectively). The additive Fungicover (+FC) was used in some treatments at a concentration of 5% in order to check its adherence and persistence effect on the populations of *P. agglomerans* cells. An adequate volume of non-adapted or osmotic adapted *P. agglomerans* inocula for each bacterial treatment was mixed into 30 litres of water in a plastic recipient to obtain a final concentration of 2×10^8 cfu mL^{-1}. Bacterial treatments were sprayed onto orange fruits cv Lane Late (Experiment-1A) or Valencia late (Experiment-2B) one week before harvest. Treatment P988-SH+POST was applied at postharvest dipping oranges in a solution at 1×10^8 cfu mL^{-1} before the storage period. Results are means of four independent samples and vertical bars indicate standard deviations.

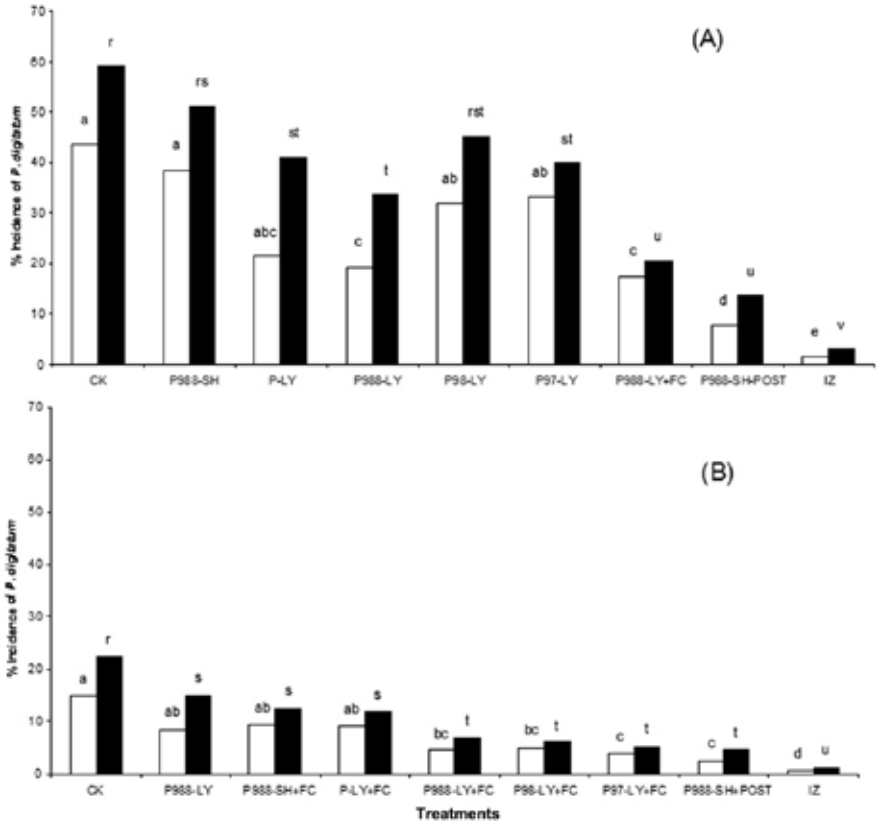

Fig 4. Effectiveness of preharvest treatments against artificial infection of the fungal pathogen *P. digitatum* compared with postharvest treatments: "*P. agglomerans* and commercial fungicide Imazalil (IZ) at 1.125 g/L". Fruits were stored for 15 d at 20 °C and 85% RH. The incidence of decayed fruits was scored after 7 (white bars) and 15 d (black bars) of storage and expressed as % decay produced by *P. digitatum* on orange cultivars, Lane late and Valencia late in Experiments 1(A) and 2 (B), respectively. Different letters in the bars indicate significant differences between means according to a Duncan's Multiple Range Test ($P<0.05$).

Results indicated that there was a close relationship between the population level associated with a given treatment under field conditions and the level of control achieved by this treatment during storage. Moreover, only bacterial treatments, which were prepared from lyophilised and osmotic adapted cells, showed a level of control comparable to postharvest treatments with the biological control *P. agglomerans* when they were applied with additive Fungicover. These preharvest treatments were also associated with population levels that were higher under field conditions. These findings were in concordance with those of Tian *et al.* (2004) who found that only *Rhodotorula glutinis* and *Cryptococcus laurentii*, whose populations remained at high and stable levels, significantly reduced fruit decay during storage at 25 °C.

We conclude that survival and stability of *P. agglomerans* populations could be maintained under field condition by integrating certain formulation strategies: adding additives, ecophysiological osmotic adaptation and lyophilisation. Thus, it has highlighted that it is very important to optimize both, distribution of the biological control agent on the host surface and survival under field conditions.

The results presented in this chapter are an example that the induction of stress adaptation responses is a

useful and practical tool, that could broaden new possibilities for improving performance of biocontrol agents to other hosts and diseases and it could improve their antagonistic activity in a wide range of conditions.

Acknowledgements

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25. INHIBITION OF POSTHARVEST *PENICILLIUM* MOLDS OF ORANGES BY ANTIFUNGAL HYDROXYPROPYL METHYLCELLULOSE-LIPID EDIBLE COMPOSITE FILMS AND COATINGS

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Abstract
New hydroxypropyl methylcellulose (HPMC)-lipid edible composite films and coatings containing low-toxicity chemicals with antifungal properties were developed. Tested antifungal chemicals were mainly salts of organic acids, salts of parabens, and other compounds, most of them classified as food additives or generally recognized as safe (GRAS) compounds. Stand-alone edible films were used for *in vitro* evaluation of their antifungal activity against the pathogens *Penicillium digitatum* and *Penicillium italicum* by disk diameter tests. Selected edible coatings containing food preservatives were tested *in vivo* on 'Valencia' oranges to determine their curative (coated after fungal inoculation) and preventive (coated before fungal inoculation) activity to control citrus postharvest green and blue molds, caused by *P. digitatum* and *P. italicum,* respectively. Film disks containing parabens and the organic acid salts potassium sorbate (PS) and sodium benzoate (SB) were the most effective to inhibit both *P. digitatum* and *P. italicum.* The use of mixtures of organic acid salts did not provide any additive or synergistic effect for *in vitro* pathogen inhibition when compared to the use of single chemicals. On 'Valencia' oranges, the curative activity of coatings with food preservatives was higher for blue mold than for green mold. Coatings containing the mixture SB + PS and SB and sodium propionate (SB + SP) reduced the incidence and severity of blue mold by 85 and 95%, respectively. PS- and SB-based coatings controlled green mold more effectively than coatings formulated with other food preservatives. Fruit coated before inoculation did not show any incidence or severity reduction of both green mold and blue mold (preventive activity). The antifungal curative action of the coatings was fungistatic rather than fungicidal.
Keywords: citrus, food additives, hydroxypropyl methylcellulose, *Penicillium digitatum*, *P. italicum*

Introduction
Postharvest diseases of citrus are mainly produced worldwide by the pathogens *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, which cause green and blue molds, respectively (Eckert & Eaks 1989). Consumer concerns about prolonged and extensive use of chemical fungicides such as imazalil, sodium ortho-phenyl phenate, or thiabendazole to control citrus postharvest decay are leading researches to look for alternative non-contaminant methods that do not generate harmful fruit residues and respect the environment. Several physical, chemical, or biological alternative methods and combinations have been assayed against both green and blue molds (Porat *et al*. 2002; Palou *et al*. 2008). Polysaccharides, proteins, lipids, and resins are mainly used to form edible coatings. Plasticizers and emulsifiers are usually added to increase the flexibility and surface tension between aqueous and lipid phases in the formulations that combine lipids and hydrocolloids (Nisperos-Carriedo 1994; Pérez-Gago & Krochta 2001). Edible films and coatings may incorporate food additives and other substances to enhance flavor, color, and texture, control microbial growth, and improve general coating performance (Cuppet 1994). Antimicrobials can be added to edible coatings to retard the growth of bacteria, yeasts, and molds during storage and distribution of fresh or minimally processed products. These compounds

include natural substances or generally recognized as safe (GRAS) compounds such as organic acids and their salts, parabens, bacteriocins, or chitosan (Matamoros-León *et al*. 1999; Chung *et al.* 2001; Min & Krochta 2005). For instance, it has been reported that the addition of potassium sorbate (PS) to edible coatings controlled microbial proliferation on strawberries (García *et al.* 1998; Park *et al.* 2005), the addition of acetic, citric, and sorbic acids effectively controlled the pathogen *Salmonella montevideo* on tomatoes (Zhuang *et al.* 1996), or the addition of parabens reduced coliform bacteria on citrus fruit (McGuire & Hagenmaier 2001). However, very few researches have focused on the development of new edible composite coatings with the addition of antifungal compounds as a new technique to control the major fungal postharvest diseases of fresh citrus fruits. The objectives of this study were to develop new hydroxypropyl methylcelullose-lipid edible composite films containing food additives with antifungal properties, evaluate the *in vitro* activity of selected films against *P. digitatum* and *P. italicum*, and evaluate the curative and preventive activity of selected HPMC-lipid edible composite coatings to control green and blue molds on artificially inoculated 'Valencia' oranges.

Table 1. Composition of HPMC-lipid edible composite films containing antifungal food additives and *in vitro* antifungal activity of films against *Penicillium digitatum* and *Penicillium italicum* at different inoculum concentrations.

HPMC-lipid films with food preservative	Molecular formula	Food preservative (%, wb)	Beeswax-shellac (%, db)	Length of inhibition zone (mm)[x]					
				P. digitatum inoculum concentration (spores mL^{-1})			*P. italicum* inoculum concentration (spores mL^{-1})		
				10^3	10^4	10^5	10^3	10^4	10^5
Organic acid salts									
Potassium sorbate	$C_6H_7O_2K$	2.0	25-25	16.8 e	15.8 e	17.3 ef	9.2 c	6.6 e	5.9 cd
Sodium benzoate	$C_7H_5O_2Na$	2.5	25-25	12.8 de	11.3 d	7.2 c	9.8 c	3.9 cd	2.9 b
Calcium propionate	$C_6H_{10}O_4Ca$	1.0	50-0	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Calcium formate	$C_2H_2O_4Ca$	1.0	50-0	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Organic acid salts (mixtures)									
Potassium sorbate (PS) + sodium propionate (6% SC)[y]		1.5 + 0.5	25-25	16.6 e	12.0 d	4.3 b	0.0 a	0.0 a	0.0 a
Sodium benzoate + potassium sorbate (8% SC)		2.0 + 0.5	25-25	13.6 de	10.6 cd	9.1 cd	11.0 c	4.5 cd	4.3 c
Sodium benzoate + sodium propionate (8% SC)		2.0 + 0.5	25-25	10.2 cde	7.8 b	0.7 ab	2.8 b	1.1 b	0.0 a
Parabens									
Sodium salt of methyl paraben	C_8H_7NaO3	1.5	50-0	22.1 f	24.2 f	21.1 f	18.3 d	18.5 g	19.9 f
Sodium salt of methyl paraben		1.0	50-0	22.3 f	21.9 f	17.4 ef	15.8 d	14.8 f	15.8 e
Other compounds									
2-deoxy-D-glucose	$C_6H_{12}O_5$	0.5	25-25	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Controls[z]									
Control (8% SC)			25-25	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC)			45-5	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC)			25-25	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC)			50-0	0.6 ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a

[x] Values are measurements of length (mm) of inhibitory zones around film disk (from the perimeter of the film disk until the edge of the inhibited area). Values within columns followed by unlike letters are different by the Fisher protected LSD test ($P<0.05$) applied after an analysis of variance of the square root of the inhibition zone plus 0.5. Nontransformed data are shown.

[y] SC = solid concentration.

[z] HPMC-lipid films without food preservatives at 6 or 8% SC and different percentages of beeswax-shellac.

Material & Methods

Preparation of Films and Coatings

To prepare emulsions, an aqueous solution of hydroxypropyl methylcellulose (HPMC, 5% w/w), the corresponding food preservative, beeswax (BW), glycerol, stearic acid, and water were mixed. Shellac solution was added to the HPMC dispersion. The samples were homogenized (90 °C) with a high-shear probe mixer and cooled. The emulsions were degassed and films were cast onto smooth plates and allowed to dry. For each emulsion, three to five films were prepared. Most of the antifungal chemicals tested were classified as food additives or GRAS compounds. The lipids (BW and shellac) were used at 50% (dry basis, db) and the ratios of HPMC-glycerol (2:1) (db) and lipids-stearic acid (5:1) (db) were kept constant throughout the study. The solid concentration (SC) in the samples was 6 or 8% (wet basis, wb). A large number of emulsion formulations were prepared, but only those capable of forming homogeneous films and coatings were used in this study (Table 1).

Determination of *in Vitro* Antifungal Activity. Disk Diameter Test

P. digitatum and *P. italicum* grown in Petri dishes on potato dextrose agar (PDA) for 7-10 d were used. High-density conidial suspensions were prepared by measuring the spore concentration with a haemacytometer. The antifungal activity of edible films was evaluated through the disk diameter test (adapted from that described by Min & Krochta (2005). Film disks (16 mm diameter) were aseptically transferred to dichloran rose-bengal chloramphenicol agar (DRBC) plates previously inoculated with 100 µL of conidial suspension of the corresponding pathogen. Plates were refrigerated at 4 °C for 3 h to allow diffusion of film ingredients and then incubated at 25 °C for 5 d. For each fungal species, inoculum densities of 10^3, 10^4 and 10^5 spores mL^{-1} were used. For each pathogen, inoculum density and film, three agar plates (replicates) were prepared. After incubation, the length of the inhibition zone around the film disk (from the perimeter of the film disk until the edge of the inhibited area) was measured with a digital caliper. Four measurements were performed for each plate.

Evaluation of the Curative and Preventive Activity of the Coatings

Oranges (*Citrus sinensis* [L.] Osbeck) 'Valencia' were used. For determination of curative activity, each fruit was inoculated with *P. digitatum* and *P. italicum* (inoculum density of 10^5 spores mL^{-1}) at opposite sides of the equator, incubated at 20 °C for 24 h, and coated by immersion (15 s at 20 °C) with the HPMC-lipid edible composite. To test preventive activity, the fruit were coated and inoculated with the pathogens about 24 h later. Inoculated but uncoated fruit were used as controls. Each treatment was applied to three replicates of 20 fruit each. Disease incidence (%) and severity (diameter of the infected area in mm) were determined after 7 d of incubation at 20 °C and 90% RH. In a second experiment, 'Valencia' oranges were treated with selected coatings and incubated at 20 °C for up to 21 d.

Statistical Analysis

Specific differences between means were determined by Fisher's protected least significant difference test (LSD, $P<0.05$) applied after an analysis of variance (ANOVA). For pathogen inhibition and disease incidence data, the ANOVA was applied to square root and arcsine transformed values, respectively.

Results & Discussion

In Vitro Antifungal Activity

Among all organic acid salts added into HPMC-lipid films, only films containing PS or sodium benzoate (SB) clearly inhibited the growth of both *P. digitatum* and *P. italicum* plated at 10^3 to 10^5 spores mL^{-1} on DRBC agar (disk diameter test, Table 1). In food systems, PS is one of the most widely used compounds to prevent the growth of molds and thus to extend produce shelf-life (Jarret *et al.* 2005). The antimicrobial activity of PS against *P. digitatum* and *P. italicum* has been observed in both *in vitro* and *in vivo* studies. For instance,

both pathogens were inhibited in PDA when PS was added at a concentration of 0.15-0.20 g L^{-1} (Matamoros-León *et al.* 1999). Films with a mixture of PS and sodium propionate (SP) significantly inhibited ($P<0.05$) the growth of *P. digitatum* on DRBC agar at all inoculum concentrations tested. As expected, *P. digitatum* at 10^3 spores mL^{-1} was more intensely inhibited (16.6 mm) than at 10^4 (12.0 mm) or 10^5 spores mL^{-1} (4.3 mm). The inhibition ability was therefore greatly dependent on the pathogenic inoculum density. In contrast, *P. italicum* was not inhibited at all by films formulated with this a mixture of PS and SP. Films containing a mixture of SB and PS significantly inhibited ($P<0.05$) the growth of *P. digitatum* and *P. italicum* on DRBC agar (Table 1). Jarret *et al.* (2005) observed that the combination of sorbates with either benzoates or propionates may be used to effectively inhibit microorganisms using lower concentrations of each preservative. In this work, however, the use of the mixtures PS + SP, SB + PS, and SB + SP incorporated to HPMC-lipid films was not additive or synergistic with respect to the use of these salts alone. On the other hand, films with sodium salt of methyl paraben (SMP) effectively inhibited both *P. digitatum* and *P. italicum* at all inoculum densities (Table 1). For both pathogens, an increase of SMP concentration in the film from 1.0 to 1.5% (wb) did not significantly increase the inhibition zone. Films containing 0.5% of 2-deoxy-D-glucose did not inhibit the growth of *P. digitatum* and *P. italicum* (Table 1).

***In Vivo* Curative and Preventive Activity**

HPMC-lipid edible composite coatings containing food preservatives showed an important curative activity on 'Valencia' oranges The reduction of incidence and severity of both *P. digitatum* and *P. italicum* with respect to the control treatment was significant ($P<0.05$) for most of the tested coatings. However, coatings containing PS, SB, or their mixtures controlled both *P. digitatum* and *P. italicum* more effectively than coatings prepared with the rest of organic acid salts or 2-deoxy-D-glucose. The SMP-based coating reduced the incidence and severity to values similar to those obtained with coatings formulated with organic acid salts (Fig 1). Some differences were observed between these results and those obtained in the *in vitro* tests, probably due to the complex interactions between host, pathogen and environment that occur during *in vivo* disease development. Likewise, it is probable that notable variations on the growth of green and blue molds *in vitro* and *in vivo* resulted from differences on the rate of release of food preservatives from films located on agar medium and coatings located on the fruit rind. PS and SB are generally recognized as safe compounds by regulations all over the world and they are therefore widely used as food preservatives with a broad-spectrum activity against a variety of yeasts and molds (Jarret *et al.* 2005). The effectiveness of aqueous solutions of PS or SB against postharvest green and blue molds of different citrus species and cultivars has been reviewed (Palou *et al.* 2008). An additional assay was conducted to study the performance of these coatings on 'Valencia' oranges incubated at 20 °C for up to 21 d. It was found that, after 7 d of incubation, these coatings greatly reduced the incidence and severity of green and blue molds if compared to uncoated fruit (Fig 2). However, these reductions were significantly lower ($P<0.05$) as the time of incubation increased, especially in the case of blue mold. After 14 and 21 d at 20 °C, the incidence of green mold on coated fruit was about 40 and 60%, respectively, and the incidence of blue mold was as high as 74 and 96%, respectively (Fig 2). Therefore, the inhibitory activity of the coatings was not very persistent on 'Valencia' oranges and their antifungal curative action was fungistatic rather than fungicidal. Coating the fruit 24 h before fungal inoculation did not significantly reduced the incidence and severity of both green and blue molds (data not shown). Thus, the tested coatings showed no preventive activity against the pathogens.

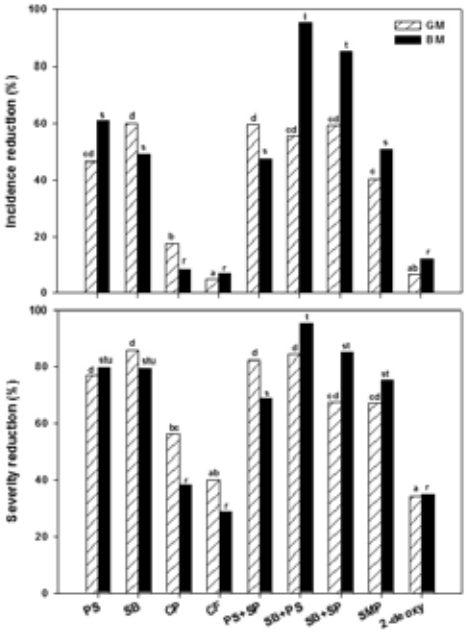

Fig 1. Incidence and severity reductions of green mold (GM) and blue mold (BM) with respect to control fruit (inoculated but uncoated) on 'Valencia' oranges artificially inoculated with the pathogens, coated 24 h later with HPMC-lipid edible composite coatings containing food preservatives, and incubated for 7 d at 20 ºC and 90% RH. For each mold, columns with different letters (a-d, r-u) are significantly different according to Fisher´s protected LSD test ($P<0.05$) applied after an ANOVA. Data on disease incidence reduction were arcsine-transformed; nontransformed means are shown. PS = potassium sorbate, SB = sodium benzoate, CP = calcium propionate, CF = calcium formate, SP = sodium propionate, SMP = sodium salt of methyl paraben, 2-deoxy = 2-deoxy-D-glucose.

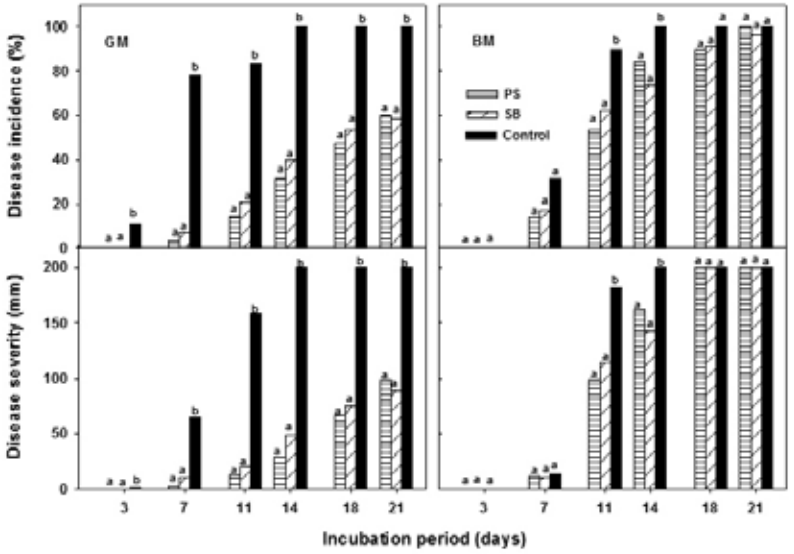

Fig 2. Incidence and severity of green mold (GM) and blue mold (BM) on 'Valencia' oranges artificially inoculated with the pathogens, uncoated (control) or coated 24 h later with HPMC-lipid edible composite coatings containing potassium sorbate (PS) or sodium benzoate (SB), and incubated up to 21 d at 20 ºC and 90% RH. For each incubation period, columns with different letters are significantly different according to Fisher´s protected LSD test ($P<0.05$) applied after an ANOVA. Data on disease incidence were arcsine-transformed; nontransformed means are shown.

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26. ANTIFUNGAL ACTIVITY OF CITRUS ESSENTIAL OIL COMPONENTS *IN VITRO* AND *IN VIVO* AGAINST *PENICILLIUM DIGITATUM* PERS. (SACC.)

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Abstract

In vitro studies were conducted on 37 compounds present in citrus essential oil, to test their activity against *Penicillium digitatum* by three methods: agar diffusion, amended growth medium and vapor assay. The aliphatic alcohols 1-nonanol, 1-decanol and especially 1-octanol exhibited the highest activities, as assayed by all the methods used. The terpenoid compounds perillalcohol, perillaldehyde, citral, terpineol, carveol and citronellol, as well as the reference aromatic compound cinnamaldehyde also exhibited high activity against *P. digitatum*. Neither hydrocarbons nor esters inhibited this fungus. The mode of action of 1-octanol, perillaldehyde, citral, perillalcohol and terpineol against *P. digitatum* was fungicidal, whereas 1-decanol, 1-nonanol, carveol and citronellol were only fungistatic. Application of biocidal formulations comprising 1-octanol and citral either separately or together inhibited decay of *P. digitatum*-inoculated lemons for three weeks after inoculation.

Keywords: antifungal activity, *Citrus*, essential oil components, green mold disease, *Penicillium digitatum*

Introduction

The flavedo of citrus fruit contains large quantities of essential oils compartmentalized inside the oil glands. The composition of citrus peel oils has been studied for many years and the major components are generally established (Shaw 1977; Huet 1991). Chemically the components of essential oils fall into several distinct groups. The terpenoids are the most abundant and are present mostly as monoterpene hydrocarbons, both cyclic (e.g., limonene) and non-cyclic (e.g., myrcene), and as their oxygenated derivatives such as aldehydes (e.g., citral, perillaldehyde), alcohols (e.g., terpineol, perillalcohol), ketones (e.g, carvone), esters (e.g., geranyl acetate). Quantitatively, limonene comprises ~80-95% of the oils of citrus fruits, whereas many of the other compounds are present only as minor or trace constituents. The non-terpenoid compounds include many organic materials, such as aliphatic aldehydes (e.g., 1-octanal or 1-nonanal), alcohols (e.g., 1-octanol, 1- nonanol), esters (e.g., octyl acetate, nonyl acetate), as well as phenolics (e.g., coumarins and psoralens).

The antimicrobial activity of citrus peel oils has been known for a long time (Maruzzella & Liguori 1958; Subba *et al.* 1967; Dabbah *et al.* 1970) and its investigation is continued at present (Romano *et al.* 2005; Sharma & Tripathi 2006, 2008; Viuda-Martos *et al.* 2008; Chutia *et al.* 2009). The subject has been recently reviewed by Fisher & Phillips (2008). Application of natural compounds such as essential oils to control postharvest pathogens attracts attention because of the increasing concern on the health hazards of synthetic fungicide residues. Citrus oils were reported to inhibit *in vitro* the development of the main postharvest citrus pathogen, *Penicillium digitatum* Pers. (Sacc.), the causative agent of the green mold disease (Caccioni *et al.* 1998). However, the interaction of citrus oil with *P. digitatum* is complex. The major compound of citrus oils, limonene, was found to stimulate the pathogen's development (Arimoto 1996; Droby *et al.* 2008). At the same time, products of limonene oxidation and other oxygenated monoterpenes exhibit strong antimicrobial activity (Ben-Yehoshua *et al.* 2008). The monoterpene aldehyde citral was found to be one of the preformed antifungal materials in lemon peel (Ben-Yehoshua *et al.* 1992); its level was suggested to affect the fruit sensitivity to the green mold disease (Rodov *et al.* 1995). Although the *in vitro* activity of several essential oil components against *P. digitatum* was investigated in the past (Moleyar

& Narasimham 1986; Caccioni & Guizzardi 1994; Caccioni *et al.* 1995), the information on this subject is still limited. Moreover, high *in vitro* activity does not guarantee the efficient disease control *in vivo* due to the possible phytotoxicity of the essential oil compounds (Ben-Yehoshua *et al.* 1992; Plaza *et al.* 2004). Applying essential oil compounds within a formulation reducing their phytotoxicity resulted in high microbiocidal efficacy (Ben-Yehoshua 2001; Ben-Yehoshua & Rodov 2006).

The aim of this study was to identify the compounds that would be potentially suitable for use as a postharvest fungicide on citrus fruits. Three different *in vitro* assay techniques were used to evaluate 37 individual components of citrus essential oils for activity against *P. digitatum*. The selected promising compounds were further tested *in vivo* as active ingredients of microbiocidal formulations on *P. digitatum*-inoculated lemons.

Materials & Methods

Most of the essential oil components were supplied by Sigma, Rehovot, Israel. Geraniol was obtained from Frutarom, Haifa, Israel and nootkatone from Aromor, Kibbutz Givat Oz, Israel. Potato dextrose agar (PDA) was obtained from Difco, USA.

The antifungal activity of the essential oil compounds against *P. digitatum* was assayed *in vitro* by three methods: agar diffusion (Maruzzella & Henry 1958), vapor assay (Maruzzella & Sicurella 1960) and amended growth medium ("poisoned food") assay (Grover & Moore 1962). In the first two methods, a 5-mg (if not specified differently) sample of each substance was pipetted onto a sterile 1.3-cm antibiotic assay paper disc which was placed either on the center of the inoculated agar (the agar diffusion assay) or onto the lid of the inverted Petri dish (the vapor assay). The activity was evaluated by visual evaluation of fungal growth and measuring the growth-free zones.

In the amended medium ("poisoned food") assay the samples dissolved in 0.5 mL of acetone, were added to molten PDA to a final concentration of 1 mg mL^{-1}. The medium was inoculated with mycelial discs (8 mm) cut from the fungal agar plate cultures. The antifungal activity was calculated as a percentage of fungal growth inhibition. For any compound that totally inhibited the fungal growth after 7 days fungicidal or fungistatic mode of its action was determined by monitoring the fungal recovery after transferring the mycelial disc to fresh PDA medium. The minimum inhibitory concentration (MIC) of a compound was evaluated by using concentrations from 0.025 to 1.0 mg mL^{-1} PDA.

For *in vivo* tests lemon fruit were washed with tap water, surface sterilized by wiping with 70% ethanol and inoculated with *P. digitatum* (10^4 spores mL^{-1}). After overnight storage at 20 °C, the fruit were treated with l-octanol and/or citral by dipping for a minute in emulsion formulations including 25% ethanol and 2500 or 5000 ppm Tween-20 (Ben-Yehoshua 2001; Ben-Yehoshua & Rodov 2006). After drying the fruit were arranged in four replications of 15 fruit each, and stored at 20 °C in cartons covered with plastic bags. The decay incidence was evaluated daily.

Results

Agar Diffusion Assay

Of the 37 compounds tested 19 showed a zone of total inhibition of *P. digitatum* growth after 48 h incubation (Table 1). The largest zones of inhibition were produced by the primary aliphatic alcohols 1-heptanol, 1-octanol, and 1-nonanol. 1-Octanol was particularly active, and caused almost total inhibition of growth. The effect of l-octanol was similar to that of the reference compound, the synthetic fungicide Imazalil, and exceeded the activity of another reference compound, cinnamaldehyde, known for its antifungal potency (Kurita *et al.* 1981; Moleyar & Narasimham 1986). In contrast, the secondary alcohol 2-octanol was only slightly antifungal. The tertiary terpenoid alcohol linalool showed no activity at all. The primary aliphatic alcohols 1-hexanol and 1-decanol showed zero or low inhibitory activity. The aliphatic aldehydes octanal and nonanal also inhibited fungal growth, but to a markedly smaller extent

than the corresponding alcohols. Aldehydes with main chain of less than seven (hexanal) or more than ten (undecanal and dodecanal) carbon units showed no activity. The most effective terpenoid compounds against *P. digitatum* were perillaldehyde, carvone and citral, but their inhibition activities were much lower than that of 1-octanol. The compounds perillalcohol, terpineol, terpinene-4-ol and carveol exhibited only weak inhibitory activities. Neither hydrocarbons limonene and myrcene nor acetate esters caused any inhibition.

A saturation curve described the correlation between the antifungal activity and the concentrations of citral and 1-octanol (data not shown). The antifungal activity increased with concentration of the compound until a certain saturation level was reached. The compound 1-octanol was much more active than citral in this test, and it totally inhibited the growth of *P. digitatum* at 8 mg per disc, while even 20 mg per disc of citral inhibited growth in an area of only 7.7 cm^2.

Vapor Assay

In the agar diffusion assay the activities of the vapors of the compounds were markedly similar to those of their liquids, although there were some changes in the activity strength (Table 1). The most active compounds were again the aliphatic alcohols 1-octanol and 1-nonanol, especially 1-octanol, with perilladehyde, carvone and citral being the most active of the terpenoid compounds. The vapors of the aldehydes octanal and nonanal showed much lower inhibition activities than the corresponding alcohol vapors However, 1-nonanol was twice as active as a vapor as as a liquid. Other compounds which showed stronger antifungal activity as vapors than as liquids were cinnamaldehyde (1.6-fold), citral (1.7-fold), carvone (1.9-fold), carveol (2.7-fold) and especially perillalcohol (three-fold). The compound nerol was active only as vapor. Other compounds, such as 1-heptanol, were more active as liquids; in particular imazalil was five times active as a liquid than as a gas. The compounds 1-octanol, perillaldehyde and cinnamaldehyde totally inhibited the growth of the fungus, and as vapors they were much more active than imazalil. The vapors of the hydrocarbons d-limonene and myrcene, as well those of the esters had no effect on the growth of *P. digitatum* in this assay.

In most cases, when the vapor of a compound produced a zone of total inhibition then it also inhibited the growth outside this zone (data not shown). An exception to this behavior was imazalil which allowed dense growth outside a sharply delineated zone of total inhibition. With several compounds, notably 1-heptanol, 1-decanol and 2-octanol, although there was no zone of total inhibition, the fungal growth on the plate was generally much less than that on the control (PDA only).

Amended Growth Medium Assay

Four of the primary aliphatic alcohols - 1-heptanol, 1-octanol, 1-nonanol and 1-decanol-totally inhibited the growth of *P. digitatum* in this assay. The corresponding aliphatic aldehydes only partially inhibited the fungal growth (Table 1). Six terpenoid compounds also completely inhibited *P. digitatum* growth; they comprised four alcohols- perillalcohol, terpineol, carveol, citronellol- and two aldehydes, perillaldehyde and citral. The fungus was also totally inhibited by cinnamaldehyde and imazalil. Most of the other compounds tested allowed varying degrees of growth, but less than that in the control (acetone only). Decyl acetate was the only compound tested that actually stimulated *P. digitatum* growth. The use of acetone as a solvent for the tested compounds resulted in only a small inhibitory effect.

The amended growth medium assay was applied to seven compounds - 1-octanol, 1-decanol, perillaldehyde, citral, octanal, cinnamaldehyde and imazalil - to compare their activity against *P. digitatum* spore germination with that against mycelial growth. Most of these compounds totally inhibited spore germination, quite consistent with their effects on mycelial growth. The exception was octanal, which was more active against spore germination than against fungal growth (data not shown). The activities of 1- octanol and citral against germination of *P. digitatum* spores increased with their concentrations,

but 1-octanol was much more active than citral. 1-Octanol not only showed a higher inhibition activity at all the concentrations tested, but also it totally inhibited the fungus growth at 0.6 mg mL^{-1}, whereas total inhibition required citral at 1 mg mL^{-1}.

Compounds that totally inhibited the mycelial growth of *P. digitatum* at a concentration of 1 mg mL^{-1} were further tested to determine their minimum inhibitory concentration (MIC) and to learn if their activity was fungicidal or fungistatic. The most active of the components of citrus peel essential oil was 1-decanol (MIC 0.05 mg mL^{-1} medium), followed by 1-octanol (0.1 mg mL^{-1}) and 1-nonanol (0.2 mg mL^{-1}). Citral, perillalcohol and perillaldehyde, were the most active of the terpenoid compounds (0.4 mg mL^{-1}). None of the natural compounds tested exhibited an MIC close to that of imazalil, which gave total inhibition even at the lowest concentration tested (0.025 mg mL^{-1}). It was found that 1-octanol, citral, perillaldehyde, perillalcohol and terpineol as well as imazalil and cinnamaldehyde were fungicidal, whereas 1-decanol, 1-nonanol, citronellol and carveol were fungistatic.

In Vivo Application

Ninety-seven percent of the inoculated fruit in the control treatment of a water dip rotted six days after their inoculation. Treatment with 25% ethanol without the essential oil compounds resulted in decay incidence of 38% six days after the inoculation and 60% on a day 20. Treatments with 2500 ppm of 1-octanol, citral or their combination reduced the decay of inoculated lemons 20 days after inoculation to 17-20%. None of the fruit in these experiments had visible damage (data not shown).

Table 1. The activity of essential oil components against *P. digitatum*.

Component	AD cm^2	VA cm^2	AGM %	Component	AD cm^2	VA cm^2	AGM %
Aliphatic alcohols				Terpenoid alcohols			
1-Hexanol	0	0	69	Perillalcohol	2	0	100
1-Heptanol	18	0	100	Terpineol	2	0	100
1-Octanol	56	63	100	Terpinen-4-ol	2	0	87
1-Nonanol	14	28	100	Carveol	1	4	100
1-Decanol	1	0	100	Geraniol	0	0	0
2-Octanol	2	0	97	Nerol	0	5	61
6-Meth-5-hepten-2-ol	0	0	33	Citronellol	0	0	100
Aliphatic aldehydes and ketone				Linalool	0	0	79
Hexanal	0	0	30	Farnesol	0	0	11
Heptanal	1	0	20	Terpenoid aldehydes and ketones			
Octanal	8	5	61	Perillaldehyde	18	61	100
Nonanal	5	1	81	Citral	7	12	100
Decanal	3	0	37	Citronellal	0	0	41
Undecanal	0	0	13	Carvone	7	16	83
Dodecanal	0	0	4	Nootkatone	0	0	93
6-Meth-5-hepten-2-one	4	0	17	Terpenoid hydrocarbons			
Aliphatic and terpenoid esters				d-Limonene	0	0	14
Octyl acetate	0	0	2	β-Myrcene	0	0	30
Decyl acetate	0	0	-43	α-Pinene	0	0	0
Geranyl acetate	0	0	6	Controls (reference compounds)			
Neryl acetate	0	0	26	Cinnamaldehyde	30	62	100
Linalyl acetate	0	0	7	Imazalil	52	10	100
Octyl acetate	0	0	2	PDA (control)	0	0	0

Assays used: AD - agar diffusion assay (inhibition area, cm^2), VA - vapor assay (inhibition area, cm^2), AGM – amended growth medium, or "poisoned food" assay (% growth inhibition)

Discussion

Among the 37 tested components of the essential oil of citrus flavedo, the aliphatic alcohols 1-decanol, 1-nonanol and especially 1-octanol, exhibited the highest inhibitory activity against *P. digitatum,* as measured by the three in vitro assays used. The terpenoid compounds perillalcohol, perillaldehyde, citral, terpineol, carveol and citronellol, and also the aromatic aldehyde cinnamaldehyde, which is not a component of citrus oil, exhibited high activity against *P. digitatum.* The hydrocarbons d-limonene and myrcene and the esters that were tested did not inhibit the fungus. 1-Octanol, perilladehyde, citral, perillalcohol and terpineol were fungicidal, whereas 1-decanol, 1-nonanol, carveol and citronellol were only fungisatic. The descending order of antimicrobial activity of the major oil components according to Faid *et al.* (1996) was: phenols > alcohols > aldehydes > ketones > ethers > hydrocarbons. The present results conform to this general pattern, although phenols and ethers were not tested. Esters were found to have low antifungal activity, very much like the ethers. The aromatic compound cinnamaldehyde exhibited high inhibitory activity and is probably ranked near the phenols. The primary aliphatic alcohols were much more active than their corresponding aldehydes, and the primary alcohol 1-octanol showed markedly higher activity than 2-octanol (in the agar diffusion assay).

As for the terpenoid aldehydes: perillaldehyde showed the highest antifungal activity, followed by citral that was fairly potent. In contrast, the antifungal activity of citronellal was low. This is in line with the results of Kurita *et al.* (1981) that suggested that aldehydes which have one or more double bonds conjugated to their carbonyl group have a much higher antifungal activity than those which have not. Moleyar & Narasimham (1986) also reported that the CHO group in conjugation with a carbon to carbon double bond was found to be responsible for the antifungal activity of citral. Similar results were reported for the unsaturated aldehydes 2-hexenal and 2-nonenal, which had a much more potent activity than hexanal and nonanal (Hamilton-Kemp *et al.* 1992). The presence of an α,β unsaturated bond adjacent to the carbonyl moiety enhanced the antifungal activity of these aldehydes (Anderson *et al.* 1994). The effective inhibitory activity of citral against *P. digitatum* and other fungi was reported in several papers (Moleyar & Narasimham 1986; Onawunmi 1989; Ben-Yehoshua *et al.* 1992; Caccioni *et al.* 1995; Rodov *et al.* 1995). In contrast, French *et al.* (1978) reported that citral and nonanal stimulated the germination of *P. digitatum* spores in a water agar medium. However, addition of sucrose to the growth medium caused these aldehydes to be inhibitory. A stimulatory effect of citral on *P. digitatum* growth was reported also by Rodov *et al.* (1995), but only at very low concentrations. Thus, the growth medium used and the concentration of the compound have profound effects on the results.

It is important to note the major differences between the assayed methods used in the present study. In the agar diffusion method the test compound is applied centrally, therefore there is a gradient in concentration of test compound from the center to the edge of the Petri dish. This means that the results depend on the ability of the compound to diffuse in agar, and since agar mainly consists of water, the results greatly depend on the water solubility of the compound. Secondly the inocula used in this method are fungal conidia, so that the effects of the compounds on spore germination are being tested. In the amended growth medium method the test compound is evenly distributed throughout the agar and the inocula are mycelial plugs. These differences in concentration distribution and in the kinds of inocula undoubtedly affect the assay results. 1-Decanol, for example, showed a low activity in the agar diffusion assay, whereas it totally inhibited the growth of the fungi in the poisoned food assay. Moreover, 1- decanol was the most active compound tested, with the lowest MIC in the poisoned food assay, therefore its lack of activity in the agar diffusion assay was probably because of poor diffusion in the agar or poor effectiveness against the germination of *P. digitatum* spores. In fact, many compounds were more active in the amended growth medium assay than in the agar diffusion assay, which hints on distribution problems in the agar diffusion assay, as mentioned above, and/or activity against hyphal growth but not against spore germination. Reduced inhibitory activity against *P. digitatum* spore germination than against hyphal growth was exhibited by 1-octanol and citral.

When the agar diffusion assay was applied to the vapors of the tested compounds, they exhibited a similar pattern of activity levels to that of the liquid phase, although the actual levels were different. The antifungal effectiveness of a vaporized compound depends on its volatility, which determines the amount of vapor in the headspace, and on the ability of the vapor to diffuse in the agar as well as into the mycelium itself. Utama *et al.* (2002) reported that despite the relatively small amount of cinnamaldehyde found in the agar medium, compared with those of water-soluble volatiles, this compound was a strong growth inhibitor of *P. digitatum,* probably because of its high activity or/and because of its hydrophobic nature that may enable it to directly accumulate in the fungus. On the other hand, the inhibitory effect of imazalil in the vapor phase assay was limited by its very low volatility. Some of the compounds (perillaldehyde, cinnamaldehyde, 1-nonanol and carvone) showed increased activity when used as vapors. This could be due to the high volatility of these compounds and/or a better penetration of the vapor than of the liquid into the agar. Alternatively, it could be that the exposure of the compound to air allows oxidation reactions to produce compounds with enhanced antimicrobial activities (Naigre *et al.* 1996).

The present study was aimed to identify a compound that would be suitable for application as a postharvest fungicide on citrus fruits. The results of *in vitro* assays show that 1-octanol has the potential to act as a fungicide: it was most active against *P. digitatum* growth in all the assay methods used, and was shown to have an MIC of 0.1 mg mL^{-1} and a fungicidal mode of action. Citral also exhibited good fungicidal activity. *In vivo* applications of 1-ocnanol or citral or their combinations as active constituents of biocidal formulations were able to effectively inhibit the decay development of lemons inoculated with *P. digitatum*, although total control was not achieved. No phytotoxic effects were observed when the essential oil compounds were applied within formulations comprising ethanol and sufficient concentrations of a food-grade detergent. These encouraging results demonstrate the potential of essential oil compounds as postharvest fungicides.

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27. SUGAR VARIATION IN HEALTHY, BLUE MOLD INFECTED AND *AUREOBASIDIUM PULLULANS* TREATED 'ROCHA' PEAR

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Abstract

Soluble sugars variation in 'Rocha' pear was studied in healthy, infected with *Penicillium expansum* (blue mold) and treated with the antagonist *Aureobasidium pullulans* fruits. Pears from four pickings were analyzed after 1, 3 and 5 months of cold storage (-0.5 ºC, 95% RH). For each storage period fruits were inoculated and kept at room temperature (*ca*. 20 ºC) for 5 d and analyzed. After 1 month of cold storage sucrose increased from the first to the last picking, while fructose, glucose and sorbitol were stable in healthy fruits. After 5 d at room temperature non-inoculated fruits presented fructose and glucose rises and sorbitol decreases for the 4 harvest dates, while sucrose increased in earlier yielded fruits but decreased in the last two pickings. After infection with *P. expansum*, in general, was observed a tendency to decrease in all sugars. The application of the antagonist *A. pullulans* partly reverses such tendency. The antagonist alone causes lower disturbances in sugar contents, except in glucose that may present slight decreases. Considering only the fruits of the commercial harvest date (DC3), sugars tend to increase along cold storage (except sucrose), particularly in healthy fruits and for most cases of both blue mold and antagonist inoculated fruits. On the other hand, the tendency for sugar decrease in *P. expansum* infected fruits, reported for 1 month of cold storage, is still detectable for glucose and sucrose after 3 months, and glucose, fructose and sorbitol after 5 months. After 3 and 5 months of storage, the maintenance of sugar content in fruits of the DC3 inoculated with both blue mold and the antagonist was not as clear as for 1 month. Data suggested that cold storage was beneficial for sugar increase of healthy fruits, except for sucrose. In the DC3 fruits, sugar loss caused by *P. expansum* was higher after 1 month of storage, but the use of the antagonist *A. pullulans* partly reverses that tendency.

Keywords: Antagonism, biocontrol, soluble sugars

Introduction

´Rocha' is a Portuguese pear cultivar that shows important losses caused by postharvest infections with *Penicillium expansum* Link (blue mold), usually initiated in wounds occurring during harvest and packing. Synthetic fungicides are usually used to control pathogens, but its application has become more limited due to health concerns and contamination by chemical residues (Ragsdale & Sisler 1994). Also, many fungicides are loosing their effectiveness due to the development of resistance by many pathogens.

Various control methods have been investigated with promising results but none, alone, was as effective as fungicides. Biological control is an alternative to chemical control that shows promising results in the control of postharvest diseases (Janisiewicz *et al.* 2001). Among the microorganisms tested, the yeast-like fungus *Aureobasidium pullulans* showed antagonistic activity in apple (Ippolito *et al.* 2000) and in 'Rocha' pear (Borges *et al.* 2004) fruits infected by *P. expansum*. In 'Rocha' pears, the highest efficacy was observed at the commercial harvest date, decreasing thereafter during cold storage of fruits. That activity was improvement by calcium treatments and when chemically complemented with low doses of Imazalil (Barreiro *et al.* 2006). Understanding the mode of action of biocontrol agents is important to improve their

performance; competition for space and nutrients, antibiosis and activation of host defenses are the main antagonistic activities exerted (Castoria *et al.* 2001).

Sugars are indicators of metabolic activity in fruits and quantitative changes can result in flavor changes. In pears, sorbitol performs the function of a reducer, regulates coenzymes activity and may play a role in osmoregulation of water stress resistance. Moreover, sorbitol synthesis appears always as an additional metabolic path to sucrose synthesis (Hudina *et al.* 2000).

The objectives of this study were the elucidation of a possible involvement of some sugars in the biocontrol of *P. expansum* by *A. pullulans* and the evaluation of sugars changes in healthy, infected and treated 'Rocha' pears.

Material & Methods

Fruits

'Rocha' pears were grown under a program of Integrated Fruit Production in an orchard of Estação Nacional de Fruticultura Vieira Natividade (ENFVN), Portugal. Fruits were weekly hand-harvested at 4 dates, including commercial one (DC3), and cold stored (*ca.* -0.5 ºC and 95% RH) at COOPVAL until use. Pears were disinfected with 0.5% NaClO solution, water rinsed and air dried prior to the infection assays.

Pathogens

Isolates of *P. expansum* were obtained from infected 'Rocha' pear and maintained on PDA. The pathogen was incubated at 25 ºC for 7 days (d). Spore suspensions (3.1×10^4 conidia mL^{-1}) were prepared as described by Janisiewicz & Marchi (1992).

Antagonist

A. pullulans was isolated from 'Rocha' pear, leaves and fruits in the ENFVN orchard. The antagonist was cultured on PDA at 25 ºC for 7 d. Subsequently the cultures were flooded with sterile distilled water and diluted to a concentration of 3.2×10^8 cfu mL^{-1} for biocontrol assays.

Biocontrol Assays

Infections were performed in 1, 3 and 5 months cold stored fruits, left at room temperature overnight (Barreiro *et al.* 2006). 'Rocha' pears were wounded twice (each wound 1.5 cm distant from the equator of the fruit, with a depth of 4 mm and a diameter of 4 mm) and inoculated with 25 µL of an aqueous suspension of *A. pullulans* and/or 20 µL of an aqueous suspension of *P. expansum*. The following treatments were performed: fruits receiving 25 µL of sterilized water (C), infected with *P. expansum* (P), treated with *A. pullulans* (A), pretreated with *A. pullulans* and infected 3 h later with *P. expansum* (A+P). Fruits were analysed 5 d after the treatments.

Sugar Analysis

Beside the mentioned treatments (C, A, P, A+P), also healthy pears (H) immediately after cold storage were used. In infected fruits, healthy tissues below wounds were collected with a cork borer, weighed, frozen in liquid N_2 and kept at -80 ºC until analysis. The analyses were performed according to the method of Hudina & Stampar (2000). Sugar separation and quantification were performed in 20 µL aliquots, using an HPLC Waters (USA) equipped with a RI Detector (2414 Waters), a Sugar-Pak1 Column (300×6.5 mm, Waters), at 90 ºC, with H_2O (containing 50 mg EDTA-Ca L^{-1}) as elluent and a flow of 0.5 mL min^{-1}. For sugar identification and quantification were used known standards.

Statistical Analysis

Analysis of variance (ANOVA) was applied to the results and a Tukey's test for mean comparison (95% confidence level) was used.

Results & Discussion

The efficacy of *A. pullulans* to control *P. expansum* postharvest infections of 'Rocha' pears was already shown (Borges *et al.* 2004; Barreiro *et al.* 2006). It was found that sugars changed not only during ripening, (when fruits were taken from cold storage and kept at *ca*. 20 °C for 5 d), but also due to pathogen infection and antagonist application.

Results presented in Fig 1 and 2 shows that fructose is the major soluble sugar. In fact, fructose, due to its restricted phosphorylation, is a less active precursor in fruit carbohydrate metabolism than glucose. That facilitates its accumulation in the vacuole of the parenchyma cells in apple (Berüter *et al.* 1997) leading to its high value.

Furthermore, healthy fruits (H) analyzed immediately after 1 month of cold storage (Fig 1) showed a sucrose increase during the picking period (DC1 to DC4), probably a consequence of starch breakdown (data not shown) in the later stages of fruit development. The conversion of starch into sucrose by sucrose synthase, as found in pears (Moriguchi *et al.* 1992), contributes to fruit sweetening (Beruter & Feusi 1997).

Stable values of fructose and sorbitol were observed along the picking dates in H fruits, while glucose presented a slight (non-significant) tendency to decrease.

When H fruits were kept for 5 d at *ca*. 20 °C (C) some changes occurred. In general, glucose, fructose and sucrose (the latter only in DC1 and DC2) increased, while sorbitol decreased. In fact, sorbitol seems to be an important C-source for hexose production during fruit maturation (Berüter & Feusi 1997). Thus, sorbitol oxidation after 5 d at 20 °C (C) could have contributed to fructose and glucose increase, as reported also in apples (Berüter & Feusi 1997; Berüter *et al*. 1997). Along the picking dates, fructose had a slight increase between H and C, while glucose presented increasing differences between H and C going from DC1 to DC4.

In fruits inoculated with *P. expansum* analyzed after cold storage (1 month) followed by 5 d at *ca*. 20 °C (P), the fructose and sorbitol contents (as well as in glucose and sucrose in some DCs) were lower than in healthy fruits under the same conditions (C) (Fig 1). The application of the antagonist together with *P. expansum* (A+P) reverses that tendency for most of the cases where P provoked a decrease compared to C.

Fruits from commercial harvest (DC3), cold stored for 1, 3 and 5 months (Fig 2) showed a decrease of sucrose during storage. These decreases may have contributed to increase fructose and glucose content, as observed along the storage period in all treatments (Berüter & Feusi 1997). Sorbitol also increases. On the other hand, the decrease of sugars in *P. expansum* infected fruits (P) as compared to C, observed after 1 month of cold storage, is still detectable for glucose and sucrose after 3 months and for glucose, fructose and sorbitol after 5 months. In most cases, the sugar content was not influenced by the application of the antagonist followed by the pathogen (A+P), when compared to the C treatment.

In general, as expected, cold storage favored the increase of sugars except sucrose that decreased. Keeping the fruit at 20 °C for 5 d was beneficial for sugar increment in healthy fruits, except for sorbitol that might have been used for sucrose synthesis and afterwards for monosaccharide increment. Late harvest (DC4) was beneficial for glucose content in healthy and infected fruits kept 5 d at room temperature after cold storage, while sorbitol slightly decreased. Fruits inoculated with *P. expansum* only in some cases, showed loss of sugars. However, the application of the antagonist *A. pullulans* previous to the inoculation with *P. expansum* helped (in most cases) the maintenance of sugar levels closer to those of healthy fruits.

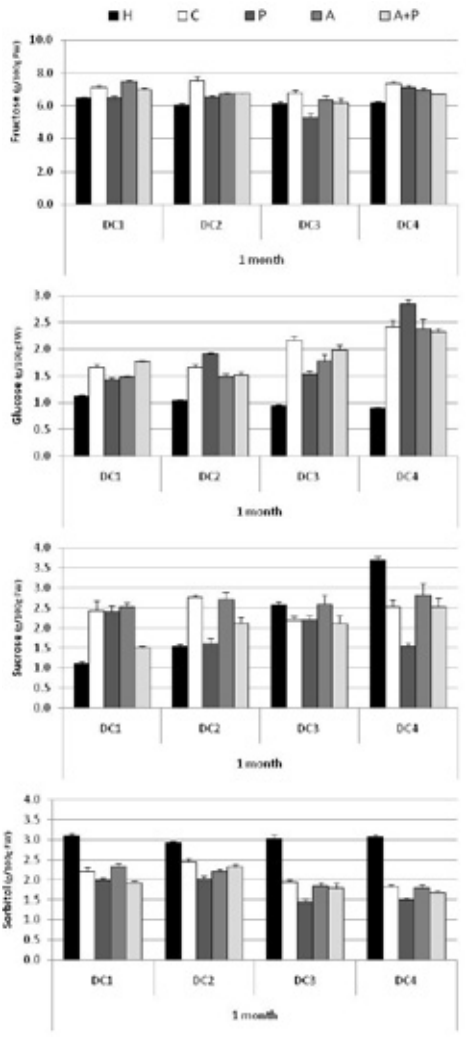

Fig 1. Sugar variation along weekly picking dates (DC1 to DC4) after 1 month of cold storage. Healthy fruits were analyzed immediately after cold storage (H), after 5 d at 20 ºC (C), after infection with *P. expansum* (P), after treatment with *A. pullulans* (A) after treatment with *A. pullulans* followed by infection with *P. expansum* (A+P). Bars represent the mean ± SE (n=10 fruits).

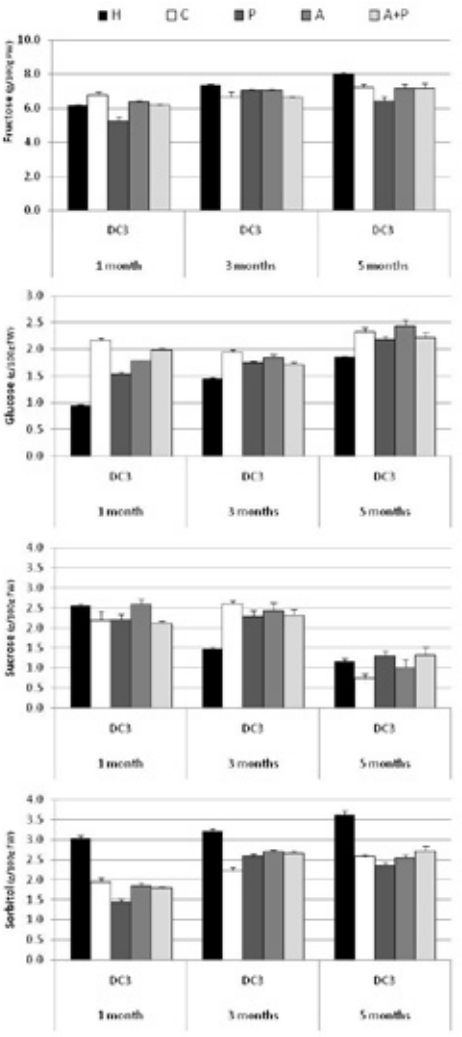

Fig 2. Sugar variation in fruits of the commercial harvest date (DC3) along the cold storage period (1, 3 and 5 month). Healthy fruits were analyzed immediately after cold storage (H), after 5 d at 20 ºC (C), after infection with *P. expansum* (P), after treatment with *A. pullulans* (A) after treatment with *A. pullulans* followed by infection with *P. expansum* (A+P). Bars represent the mean ± SE (n=10 fruits).

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28. EFFECT OF COATING APPLICATION ON THE GAS FLOW AND *P. DIGITATUM* GROWTH IN LEMON CV. 'VERNA'

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Abstract

All citrus fruits are waxed during postharvest, independent of their final destination for reducing the weight loss. Any physical barrier applied on the surface can have a significant influence on the various metabolic pathways acting on the fruit-environment system. The effect of applying a wax coating on the percentage of blocked stomata and the diffusivity of water vapor was studied in lemon fruits cv. 'Verna', healthy and inoculated with *P. digitatum*. In addition, the growth of *P. digitatum* was modelled with the Gompertz equation modified to three parameters for non-linear regression. The analysis of the percentage of blocked stomata based on the respiratory gas exchange in the healthy coated fruit, suggests that the obstruction is transient. The coating application significantly reduced water vapor diffusivity as a result of the reduction in the gas permeability of fruit peel, being this parameter affected by the mould development in the skin fruit surface. Wax coating significantly delayed the lag phase and reduced the rate of relative growth of *P. digitatum*.

Keywords: citric, decay, stomata, water diffusivity, water wax

Introduction

Citrus fruits are waxed during postharvest manipulation for storage and direct commercialization. The waxing process reduces weight loss, increase fruit resistance during manipulation, and gives an intense shine attractive to the consumers. The interface between the fruit and its environment is very complex due to the diversity of flows of matter and energy across the interface, and the major internal metabolic activity. This complexity can be increased by the application of physical barriers on fruit surface and by atmospheric composition that surrounds it, which can influence the different metabolic pathways involved with it. In the closed system fruit-environment it can be defined the input of O_2, output of CO_2 and input and output of H_2O. The flow of CO_2 and O_2 are due to the respiration process, while water flow is mainly caused by dehydration of fruit surface that will depend on environment temperature and relative humidity. The mechanism by which the coating restricts gas exchange depends not only on the properties of coating (composition and thickness) and fruit, but also on it mode of distribution on the fruit surface (Banks *et al.* 1993). For the non-coated fruits lenticels, stomata, scars and injuries are probably the main route of gas exchange. For coated fruits, on the other hand, it is possible that these spaces are filled by coating (Hagenmaier & Baker 1993). The coating also influences in different ways the growth of microorganisms. The wax film in fruit surface forms a barrier to the secondary infections during the large periods of conservation or transport. In many cases, waxed fruits show less percentage of decay and greater shelf-life than non-waxed fruits. The coating also reduces the injuries produced in the fruit surface during manipulation (Ben-Yehoshua *et al.* 1994).

The objective of this work was to evaluate the effect of applying a wax coating on the percentage of blocked stomata and the diffusivity of water vapor in lemon fruits cv. 'Verna' healthy and inoculated with *P. digitatum*; and also modeling the growth of *P. digitatum* with the Gompertz equation modified to three parameters for non-linear regression.

Material & Methods

Plant Material

Lemons cv. 'Verna' were used in the experiments. At the laboratory fruits were washed, dried and maintained at 13 ºC. A lot of eight fruits were used to perform the analysis.

Measurements of Gas Exchange

The respiratory gas exchange was evaluated by head space analysis (Carvalho *et al.* 2002) with a micro gas chromatograph (MicroGC HP M200 Model G2890A). Each analysis was performed during 50 min at 5 min intervals. The quantification was made with the HP EZ Chrom Chromatography Data System. After each analysis the air inside the chamber was refreshed, considering 3% of CO_2 as the limit concentration. All the experiments were made in a thermostatic chamber at 13 ºC and 86.3% RH (10^{-3}M HCl dissolution saturated with KCl).The initial volume of fruit was determine by the water immersion method. With the change in concentration of the respiration gases analyzed in the headspace ($d[x_i]/dt$), the diary flux of each gas through the interface (N*i* kmol *i* kg^{-1} h^{-1}) was determine by the following equation:

$$N_i\Big|_{t_1}^{t_2} = \frac{d[x_i]}{dt} \cdot \frac{P \cdot V_e}{M \cdot R \cdot T} \qquad \text{Eq 1}$$

Fruit Inoculation

The inoculation of *P. digitatum* was made using the strain 2594 provided by the Spanish Type Culture Collection of the University of Valencia. A spore suspension was prepared and adjusted to 1×10^6 spore mL^{-1} using a hemacytometer. Four wounds were made in the fruit equator zone and each wound was inoculated with 20 μL of suspension. The lesion growth was monitored measuring the concentric lesion diameter formed around each wound with a caliper and expressed as mean diameter in cm.

Coating Treatment

The fruits were coated with a commercial water-wax emulsion (Water Wax® - U.E.), supplied by Fomesa: shellac, polyethylene and other components with a wax and coadjutants composition of 18% (w/w). This procedure was carried out in a pilot scale equipment (Ortolá *et al.* 1999) for 30 s at 25 ºC with a turn velocity of 10 rpm for rollers and 50 rpm for brushes. Then fruits were dried in a laboratory dryer (Fito *et al.* 1997) at 25 ºC for 2 min with an air velocity of 1.4 m s^{-1}.

Statistical Analysis

Statistical procedures were performed using a commercial statistical software (Statgraphics plus 4.1, Manugistics, Inc., Rockville, MD, USA.). All data were subjected to analysis of variance, and means were compared using LSD test at $P \leq 0.05$.

Results

Percentage of Blocked Stomata

As O_2 and CO_2 flows in a gas phase mainly through the stomata to the outside of fruit, a relationship was search between the percentage of blocked stomata and coating in healthy lemon fruits. The molar flow of O_2 and CO_2 in the system was calculated by the equation 1. The flow of O_2 and CO_2 that go through the stomata could be expressed in a flow rate (Q_i m^3 m^{-2} s^{-1}) according to the following equation (data not shown):

$$Q_i = \frac{\partial x_i}{\partial t} \cdot \frac{V_E}{S_E} \qquad \text{Eq 2}$$

The opening section of the stomata will vary according to the external (surface evaporation) and internal (CO_2 concentration in the interior of the apoplastic way) conditions. At the same time, when the stomata are blocked by coating, the opening section is highly reduced. In this way, an adimensional parameter for stomata blocking caused by coating (F= Qe / Qne) was defined; being Qe the gas flow rate in coated fruits and Qne the gas flow rate in non-coated fruits. An open stomata will have an F equal to 1 and a stomata totally blocked will have an F equal to 0. Knowing the O_2 and CO_2 flow rate of coated and non-coated fruit, the percentage of stomata blocked by the coat was calculated according the following equation:

$$\% \text{ stomata blocked} = [1 - (Qe / Qne)] * 100 \qquad \textbf{Eq 3}$$

Figure 1 shows that the coating of lemon cv. 'Verna' originated an immediately obstruction of the stomata in the first day, with a 10-15% of blocked stomata. Nevertheless, this obstruction was transient.

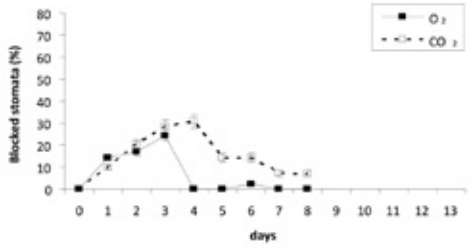

Fig 1. Effect of coating with Water Wax® on the percentage of blocked stomata in lemons cv. 'Verna' at 13 ºC and 86.3% RH.

Water Vapor Diffusivity

As the total mass loss in the closed system is a result of the weight loss experimented by fruit during storage, a molar balance could be thought. Like this, the total molar flow (N_T kmol m^{-2} s^{-1}) diffused in the closed system during the storage period was defined according to the equation reported by Fito (2002). The molar flows (N_i kmol m^{-2} s^{-1}) of O_2 and CO_2 were calculated by the equation 1 (data not shown). The molar flow of water that goes through fruit skin was calculated by the following equation:

$$\frac{dN_{H_2O}}{dt} = \frac{\frac{dM_T}{dt} - 32\frac{dN_{O_2}}{dt} - 44\frac{dN_{CO_2}}{dt}}{18} \qquad \textbf{Eq 4}$$

The total mass flow ($\frac{dM_T}{dt}$, kg m^{-2} s^{-1}) was determined by fruit weight loss, and the molar flow of water ($\frac{dN_{H_2O}}{dt}$, kmol m^{-2} s^{-1}) was calculated with the equation 4 (data not shown). The first law of Fick establish that the gas flow N_i diffused through a barrier is determined by the diffusivity of this gas D (m^2 s^{-1}), and the concentration gradient through the barrier $\partial C/\partial x$ (kmol m^{-3} m^{-1}). The system fruit-environment is stationary, so the concentration gradient is lineal (Banks 1985), and the water vapor that flow through fruit skin (N_{H_2O} kmol m^{-2} s^{-1}) can be calculated by the equation:

$$N_{H_2O} = -c \cdot D_{H_2O} \cdot \left(\frac{x^E_{H_2O} - x^F_{H_2O}}{L} \right) \qquad \textbf{Eq 5}$$

The water molar fraction of the gas phase ($x^i_{H_2O}$ $kmol_{water}$ / $kmol_{Total}$) present in head space (E) and in fruit skin (F) was calculated in function of the water activity (a^i_w adimensional) of salt and the water activity of

fruit skin (0.97, Cháfer 2000), according the Raoult Law (data not shown). In this way, with the molar flow of O_2, CO_2 and water vapor diffused in the system (data not shown) the water vapor diffusivity for the different treatments was calculated. Figure 2 shows that the water vapor diffusivity is reduced after coating (1st day) in both fruits (inoculated and non-inoculated) remaining constant after it. Nevertheless, while in non-inoculated fruits the diffusivity remains constant with time in inoculated fruits a significant reduction with time was observe.

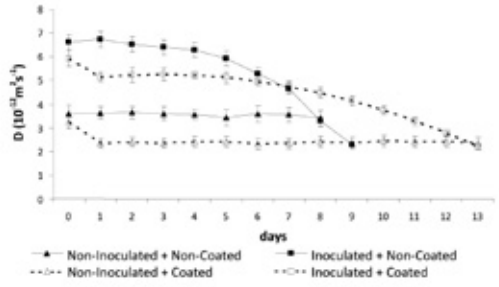

Fig 2. Effect of coating with Water Wax® on water vapor diffusivity in lemon cv. 'Verna' non-inoculated and inoculated with *P. digitatum* (1×10^6 spore mL^{-1}) at 13 ºC and 86.3% RH.

Modeling the Growth of *P. digitatum*

The relative growth values (lesion diameter/fruit diameter) of *P. digitatum* in fruits inoculated with and without coating, were adjusted to a sigmoid line with the Gompertz equation for three parameters by non-linear regression (Sigmaplot version 8.0):

$$y = a \cdot e^{-e^{-\left(\frac{x - x_0}{b}\right)}} \qquad \textbf{Eq 6}$$

The asymptote (a) was considered 1 as it is the maximum growth of the mould, x_0 is the mean point of the logarithmic phase (days), being directly related with the lag phase; and 1/b is the constant that define the logarithmic phase (relative growth velocity of the mould). In Fig 3, the growth of *P. digitatum* in both fruits (coated and non-coated) was compared.

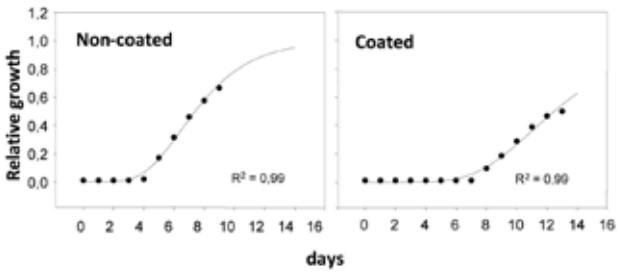

Fig 3. Predicted values (–) versus observed values (●) of the relative growth of *P. digitatum* (1×10^6 spore mL^{-1}) in lemon cv. 'Verna' coated with Water Wax® at 13 ºC and 86.3% RH. Predicted values at 99% confidence level (t-Test).

The coating kept the sigmoid form of relative growth; nevertheless the lag phase was notably extended. The coating also reduced significantly the growth velocity of *P. digitatum* in fruit (0.40 for non-coated fruits and 0.26 for coated fruits); increasing at the same time the x_0 (6.5 for non-coated fruits and 11.1 for coated fruits).

Discussion

According to Ben-Yehoshua *et al.* (1985) the commercial wax is much more efficient than the natural wax obstructing the stomata because flows into the pore as a fluid. The results of this work, also suggest an obstruction of stomata; however this obstruction was transient. This phenomenon could be due to the releasing of wax plates from the pores of stomata in time as a consequence of the action of CO_2 flow and fruit handling. The coating significantly reduced water vapor diffusivity in the non-inoculated fruits during storage (2.9×10^{-12} m^{-2} s^{-1} for coated fruit and 3.6×10^{-12} m^{-2} s^{-1} for non-coated fruit). In coated fruits higher values in inoculated fruits with respect to non-inoculated was also observed, as a consequence of higher weight loss registered in those fruits. Knoche *et al.* (2001), observed a reduction in the conductance of stomata to water vapor in the cuticle membrane of cherry fruit, attributed to the obstruction of stomata by wax, which would reduce the cross-section area available for the dissemination of water vapor. The fruit inoculated and coating take four days more to reach the same value of water diffusivity reach by the same fruit non-coated on day 9 (2.3×10^{-12} m^{-2} s^{-1}), suggesting that coating reduce the velocity of fruit deterioration. The coating significantly delays the lag phase and reduced the rate of relative growth of *P. digitatum*. According to McGuire & Hagenmaier (2001), the effect of wax on mould growth is mainly due to two factors: the natural physical barrier that forms the coat on the fruit surface and the composition of coat that can have a fungistatic effect.

Nomenclature Used

P: total pressure of the chamber (atm)
Va: air volume of the chamber (L)
M: fruit mass (kg)
R: ideal gas constant (atm L $kmol^{-1}$ K^{-1})
T: temperature (K)
V_E: volume of the head space (L)
S_F: surface of the fruit (m^2)
L: thickness of the fruit skin (m)
c: molar density of the gas phase ($kmol_T$ m^{-3})

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29. HEAT TREATMENT EVALUATION ON QUALITY AND SAFETY OF WHOLE TOMATO (*LYCOPERSICUM ESCULENTUM* L.) FRUITS

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Abstract

Quality and safety attributes, like colour (CIELab parameters), texture (Maximum force – MF), total phenolics content (TPC), peroxidase activity (POD), microbial count and moulds & yeasts (Log_{10} cfu g^{-1}) were determined on heat treated mature-green tomatoes at temperature ranging of 40 to 50 ºC and different times. Heat treatments (HT) did not affect significantly ($p>0.05$) the tomatoes colour. In terms of texture, a reduction of 10% MF was observed at 50 ºC_15min. The enzymatic activity of POD increased at all temperatures during the first 30 min at 40 ºC (≈37%), 20 min at 45 ºC (≈120%) and 15 min at 50 ºC (≈43%). In relation with TPC an increase of ca. 20% at 40 ºC_60 min and 50 ºC_15 min was observed, when compared to fresh tomatoes. A total elimination of microbial count and moulds & yeasts was successfully found at 50 ºC and at 45 ºC_20 min. In conclusion, the more effective HT in terms of total elimination of microbial count and moulds and yeasts, without negatively effect on tomatoes quality attributes appears to be at 50 ºC. However, more studies are required to conclude about the efficiency of HT on tomatoes quality and safety during shelf life period.

Keywords: Heat treatment, quality, safety, tomato

Introduction

Tomato (*Lycopersicum esculentum* L.) is a climacteric fruit, and during ripening several important changes occur on physical-chemical quality. Treatments, such as heat treatment (HT), have been investigated for controlling postharvest fruits and vegetables quality for example in grapefruit (Porat *et al.* 2000a), peaches (Zhou *et al.* 2002), asparagus (Siomos *et al*. 2005), pomegranates (Palou *et al.* 2007) and citrus (Porat *et al.* 2000b). HT inhibits biochemical pathways involved in ripening, like chilling injury, external skin damage during storage, control of decay and insects (Lurie 1998; Jacobi *et al.* 2001; Yahia *et al.* 2007). This treatment could substitute a non-damaging physical treatment for chemical prevention if a combination of time and temperature could be found to provide the desired control without significant quality loss in the commodity (Lurie 1998).

The main objective of this work was to evaluate the immediate effects on quality and safety attributes, like colour (CIELab parameters), texture [Maximum force – MF, (%)], total phenolics content [TPC, (%)], peroxidase activity [POD, (%)], microbial count and moulds & yeasts (Log_{10} cfu g^{-1}) of HT on mature-green tomatoes at temperature ranging of 40 to 50 ºC and different times.

Material & Methods

Heat Treatments

Tomatoes (*Lycopersicum esculentum* L.) were purchased on a commercial greenhouse Carmo & Silvério at west region of Portugal. Fruits were harvested at mature-green stage and their classification was performed through external colour evaluation according to USDA standard tomato colour classification (USDA 1991).

Tomatoes were immersed in a thermostatic bath (50 L of capacity) at 40, 45 and 50 °C during different times. After HT the samples were cooled in ice and water bath for 5 min and dried for removing excess of water.

Colour Evaluation

Colour was evaluated with a tristimulus colorimeter (Minolta chroma Meter, CR-300, Osaka, Japan). The instrument was calibrate against a standard white colour tile (L*=97.10, a*=0.19, b*=1.95), using an illuminate C. A CIE colour space co-ordinates, L*a*b* values, was determined. Sixteen measurements were determined for each sample.

Texture Evaluation

Texture was determined by TPA test / penetration test with a Texture Analyzer (TA.HDi, Stable Microsystem Ltd, Godalming, UK), using a 50 N load cell and a cylinder probe with a diameter of 2 mm. The TPA / penetration test was performed at 3 mm s^{-1} of speed and at 7.5 mm of distance penetration. Results were expressed as % of maximum force ($MF = 100 \times \frac{MF}{MF_0}$, where MF is maximum force of heat treated tomatoes and MF_0 maximum force of untreated tomatoes). Sixteen measurements were determined for each sample.

Total Phenolics Content

Total phenolics were determined using the Folin-Ciocalteau reagent (Singleton & Rossi 1965). The percentage of total phenolic content was defined as $TPC = 100 \times \frac{TPC}{TPC_0}$, where TPC is total phenolic content of heat treated tomatoes and TPC_0 total phenolic content of untreated tomatoes. Six measurements for each sample were determined.

Peroxidase Activity Determination

Peroxidase (POD) activity was determined as described in Yahia *et al.* (2007) and was expressed as $POD activity = 100 \times \frac{A}{A_0}$, where A is POD activity of heat treated tomatoes and A_0 POD activity of untreated tomatoes. Six replicates were carried out per each sample.

Microbial Evaluation

Plate count agar was used as the media for total mesophylic counts, incubated at 30 °C for 3 days using NP 4405 (IPQ 2002). Yeasts & moulds were determined using Rose Bengal 200 Chloramphenicol Agar, surface inoculation and incubated at 25 °C during 5 days using NP 3277 (IPQ 1987). Mesophylic, moulds and yeasts counts were expressed as $\log_{10}$ colony forming units per g of sample [Log_{10} (cfu g^{-1})] and three replicates were determined per each sample.

Data Analysis

Data were subjected to analyses of variance (one way ANOVA) using a Statistic v.7.0 Software (StatSoft, Inc., 2004) to determine the effect of heat treatment on tomatoes quality. Significant differences between samples were detected using Scheffé test (significant at $p<0.05$).

Results & Discussion

Colour and Texture Evaluation

Figure 1 shows the values of colour parameter a* tomatoes after heat treatment. Tomatoes a* average value (± standard deviation) at day 0 before treatment was -12.94 ± 0.80. After HT no significant differences were observed on a* colour, only at 40 °C_60 min was denoted an decrease ($p<0.05$) of about 10%. These results indicate that HT used in our studies did not exert any negative effect on colour tomatoes. Similar results were achieved by Nguyen *et al.* (2004) in a study of heat treated cherry tomatoes where the study treatment promoted the delay red colour development without tomato damage.

Tomatoes maximum force (%) was evaluated after heat treatment and results are observed in Fig 2. Maximum force decrease was observed as the intensity of heat treatment increase, nevertheless, firmness of heat treated tomatoes at 40 and 45 ºC was not affected significantly ($p>0.05$), while at temperatures of 55 ºC the treatment was more aggressive to whole tomatoes firmness. For example, after 45 min at 40 ºC and 40 min at 45 ºC a reduction of 7% and 3% was denoted, respectively. Firmness loss of fruits and vegetables could be related to enzymatic activity such as pectin methyl esterase (PME) (Ali *et al.* 2004)) and the firmness retention due to heat treatment can be possible due the inhibition of carbonate-soluble pectin fraction (Shalom *et al.* 1996).

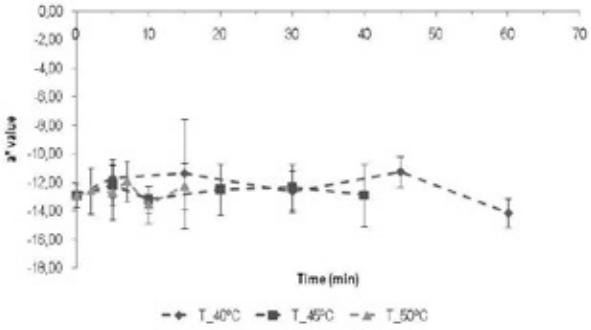

Fig 1. Effect of HT on tomatoes a* colour parameters Bars represent mean ± standard deviation.

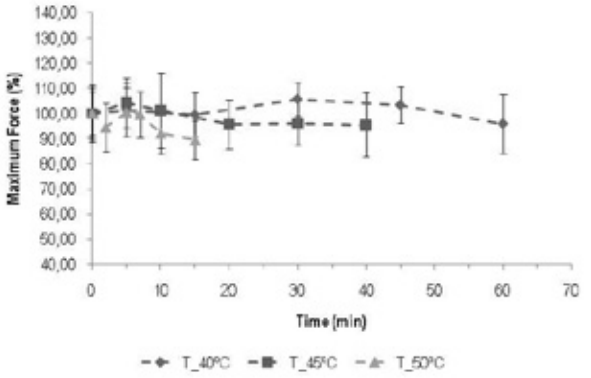

Fig 2. Effect of HT on tomatoes maximum force (%). Bars represent mean ± standard deviation.

Total Phenolics Content and Peroxidase Activity

Figure 3 shows the results of total phenolics content (TPC) of heat treated tomatoes. On the first 15 min of treatment at 40 and at 45 ºC a decrease of this parameter was denoted. On the other hand, after 30 min at 40 ºC an increase ($p<0.05$) of 20% content was observed. During HT at 50 ºC a TPC increase was observed. Hunt & Baker (1980) and Smith (1973) associate the increase of TPC with the augment of enzymatic activity, especially phenylalanine ammonia-lyase (PAL), which plays an important role in phenolic compounds synthesis.

Results of POD activity (%) of heat treated tomatoes are presented in Fig 4. POD activity presented an opposite behavior that was observed on total phenolics content.

At high temperature (50 ºC) and after 10 and 15 min, an increase of 20 and 40% were observed, respectively. At 45 ºC was denoted a significant increase ($p<0.05$) of enzymatic activity. POD activity of heat treated tomatoes at 40 ºC increase in the first 30 min but after 40 min an increase was observed. Peroxidases are ubiquitous enzymes that have diverse biochemical functions in higher plants and are involved in the response of plants to stress (Yahia).

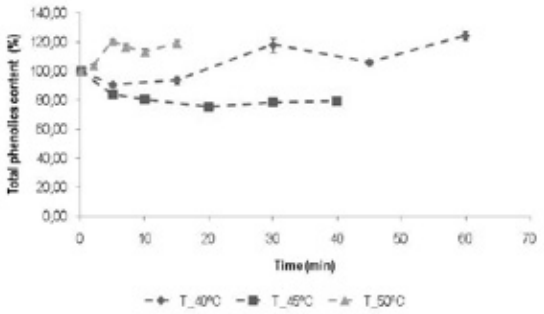

Fig 3. Effect of HT on tomatoes TP content (%). Bars represent mean ± standard deviation.

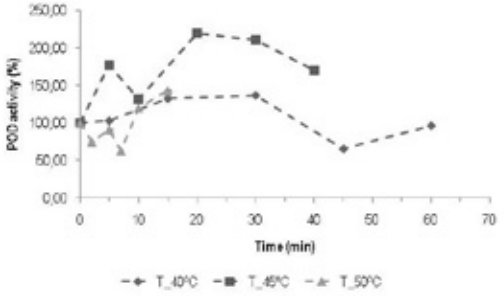

Fig 4. Effect of HT on tomatoes peroxidase activity (POD, %). Bars represent mean ± standard deviation.

Microbial Evaluation

Figure 5 and 6 show the microbial count and moulds & yeasts of heat treated tomatoes, respectively. The initial load of microbial count of untreated tomatoes was 1.82±0.45 Log_{10} cfu g^{-1}. With heat treatment this value decrease in all temperatures with exception at 40 ºC_60 min when was observed an increase of microbial load (0.57 Log_{10} cfu g^{-1}). In a study by Klaiber *et al.* (2005), carrot treated at 50 ºC_10 s a reduction of 2 Log_{10} in microbial load was observed. The initial value of moulds and yeasts of fresh tomatoes was 1.65±0.41 Log_{10} cfu g^{-1}.

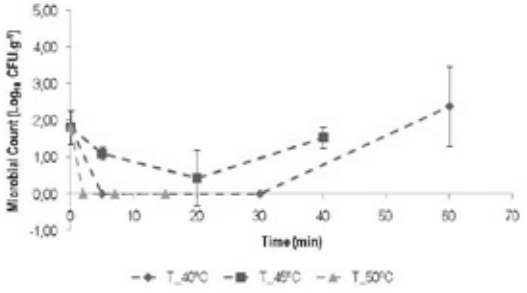

Fig 5. Effect of HT on tomatoes microbial count (Log_{10} cfu g^{-1}). Bars represent mean ± standard deviation.

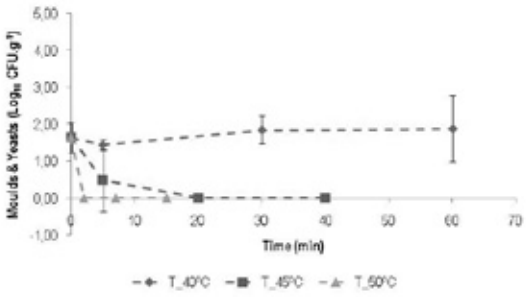

Fig 6. Effect of HT on tomatoes moulds & yeasts (Log_{10} cfu g^{-1}). Bars represent mean ± standard deviation.

Moulds and yeasts are more sensitive to higher temperatures than mesophylic microorganism. Tomatoes treated at 45 and 50 ºC was revealed a reduction in terms of moulds and yeasts load during treatment, being more effective at high temperature and time. After 2 min at 50 ºC a total elimination was denoted in terms of moulds and yeasts on tomatoes treated.

Conclusions

In conclusion, the more effective HT in terms of total elimination of microbial count and moulds and yeasts, without negatively effect on tomatoes quality attributes appears to be at 50 ºC. However, more studies are required to conclude about the efficiency of HT on tomatoes quality and safety during shelf life period.

Acknowledgements

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SECTION 5. NEW APPROACHES TO ENHANCE SAFETY AND QUALITY OF MINIMALLY PROCESSED FRUITS AND VEGETABLES

30. TECHNOLOGICAL INNOVATIONS TO PRESERVE QUALITY AND SAFETY OF FRESH-CUT HORTICULTURAL PRODUCTS

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Abstract

Fresh-cut or minimally processed fruit and vegetables have strongly increased their market share all over the industrialized countries. For that reason this market is currently very competitive, and forces the specialized industry to develop improved sustainable techniques in order to satisfy new consumers' requirements, and guarantee safety as well as nutritional and sensory quality. The most important goal for keeping overall quality of these commodities is the control of microbial spoilage flora improving safety. Every step in the production chain will influence the microbial load. In this way, the implementation of a proper disinfection program should be the main concern. Washing and disinfection is the only step that reduces microbial load throughout the production chain and chlorine is commonly used as an efficient sanitation agent, but the undesirable byproducts generated when it reacts with organic matter, force to find alternatives. Moreover, efficacy of chlorine is limited on some products. For that reason, several ecofriendly innovative techniques as alternative antimicrobial washing solutions (peroxyacetic acid, ClO_2, acidified sodium chlorite, O_3, electrolysed water, etc.), pre-treatment with UV-C radiation or packaging under high O_2 or non conventional gas mixtures (N_2O, noble gases, etc) alone or combined, seem to be promising to preserve overall quality. However, industrial changes for replacing conventional with innovative techniques request a fine knowledge of the benefits and restrictions as well as practical outlook. This work review some recent results obtained with these emergent techniques on quality changes of fresh-cut horticultural products.

Keywords: ecoinnovative techniques, minimal processing, noble gases, ozone, sanitizers, superatmospheric oxygen, UV-C

Introduction

The current worldwide healthier style of life has led to a rising demand of convenient fresh foods, free from additives, with high nutritional value, antioxidant and free-radical scavenging properties. In this way, the suitability of minimally processed or fresh-cut fruit and vegetables offers great advantages for consumers (Artés *et al.* 2009). Although this kind of processing maintains alive the commodities, it destroys plant structure and therefore increases the rate of senescence of tissues and reduces their resistance to microbial spoilage (Artés *et al*. 2007) which also may increase the number of microorganisms, some of which may be potentially harmful to human health (Leistner & Gould 2002). Moreover, shelf-life of fresh-cut horticultural products is affected by pre-processing factors (crop varieties, cultivation conditions, harvesting, ripening stage, handling, transportation), processing factors (precooling, trimming, conditioning, cutting, peeling, washing, disinfecting, draining, rinsing, drying, packaging) and distribution conditions (temperature, relative humidity, atmosphere composition and duration). In order to achieve fresh-cut plant produce with fresh-like quality, safety and high nutritional and sensory quality, the industry needs to implement improved ecoinnovative techniques. Currently, the major preservation techniques applied to prevent or delay overall quality loss are chemical coadjutants like antimicrobial solutions, acidulants, antioxidants, etc., combined with chilling throughout processing and shelf life and modified atmosphere packaging (MAP) until consumption (Leistner & Gould 2002).

The aim of the present work is to review the main emergent techniques which can be used at industrial level for keeping quality and safety of fresh-cut plant commodities.

Antimicrobial Solutions

Washing with chlorinated (NaClO) water is the most common way to minimize the transmission of pathogens by the food industry due to its powerful oxidizing properties and because it is generally effective and comparatively inexpensive (Nieuwenhuijsen *et al*. 2000). Its effectiveness against microorganisms depends on pH, temperature, concentration, organic matter present in the washing water and produce, time of exposure, and initial microbial load (Boyette *et al.* 1993). Their efficacy increases when increasing concentration of available chlorine, but high levels may cause product tainting and sodium residue on the product and equipment (Adams *et al.* 1989).

When NaClO is added to water, it increases pH and generates hypochlorous acid (HOCl), which is the active antimicrobial species. The acid dissociates readily to hypochlorite ions (OCl^-) at high pH, or chlorine gas (Cl_2) at low pH, thus the pH must be kept in the range of 6.5 to 7.5 for HOCl to be stable and efficient (Suslow, 1997). However, NaClO may oxidize food constituents that contain natural organic materials to produce unhealthy byproducts in water, such as trihalomethanes (THM) like chloroform ($CHCl_3$) or bromoform ($CHBr_3$), haloacetic acids (mono, di and trichloroacetic, and mono and dibromoacetic), haloacetonitrils or others, that have known or suspected carcinogenic or mutagenic potential effect (Nieuwenhuijsen *et al.* 2000). Consequently, these concerns have encouraged the search for alternatives to NaClO in water solutions. Among them some emergent sanitizing solutions water have been tested.

Peroxyacetic Acid

Peroxyacetic acid (CH_3COOOH) is a promising sanitizer due to it is dissociated in water in acetic acid and H_2O_2. Its breakdown products, water, O_2 and acetic acid are biodegradable. It is applied for surface cleaning in concentrations ranging from 85 to 300 ppm, and the U.S. Food and Drug Administration has set a minimum of 85 ppm (FDA 1997). Because of peroxyacetic acid tolerance to several factors like temperature, pH (from 1 to 8), and hardness and soil contamination, its current main area of application is in fruit and vegetables processing. For the treatment of plant surfaces, recommended formulations combine 11% H_2O_2 and 15% CH_3COOOH at 80 ppm (Suslow 1997). It has been reported that it was effective for controlling *Escherichia coli* and *Listeria monocytogenes* in fresh-cut products (Rodgers *et al*. 2004). Compared to 150 ppm NaClO, 68 ppm of peroxyacetic acid reduced the psychrotrophic counts by 2 log units and mesophilic counts by 1 log unit in fresh-cut 'Galia' melon after 10 d (d) at 5 °C (Silveira *et al*. 2007). A similar effect of NaClO was found in fresh-cut Tatsoi baby leaves after 9 d at 5 °C where a decrease in the mesophilic and enterobacteria load of 1.2 and 1.0 log cfu g^{-1} respectively without detrimental effect on the sensory attributes was found (Tomás-Callejas *et al*. 2008)

Ozone (O_3)

Ozone is a highly unstable tri-atomic oxygen molecule (O_3) formed by the addition of an oxygen atom ($O^·$) to a molecular diatomic oxygen (O_2) and acts as a strong oxidizing agent effective in destroying microorganisms (Guzel-Seydim *et al.* 2004). O_3 destroys microorganisms by the progressive oxidation of vital cell components, preventing the microbial growth and extending the shelf-life of many fruit and vegetables (Parish *et al.* 2003). Washing with ozonated water has been suggested as an interesting alternative to traditional sanitizers due to its efficacy at low concentrations and short contact times as well as the breakdown to non-toxic products. It is remarkable that the efficacy of ozonated water is closely related to O_3 solubility, which increases as the temperature of water decreases. Kim *et al.* (1999b) reduced between 1.5 and 5.0 log cfu g^{-1} *E. coli* O157:H7, *Pseudomona fluorescens, Leuconostoc mesenteroides* and *L. monocytogenes* counts by using 1.5 ppm of ozonated water (pH = 6, 25 °C) for 15 s. Khadre & Yousef (2001) found effectiveness of O_3 in disinfecting food-contact surfaces. Zhang *et al*. (2005) reported that in fresh-cut celery sticks dipped in 0.18 ppm O_3 water for 5 min browning was inhibited and sensory quality was improved. After 9 d at 4 °C, bacterial population was reduced 1.69 log cfu g^{-1} compared to control

water. Selma *et al*. (2007) treated shredded lettuce with 5 ppm O_3 water for 5 min reaching a reduction of 1.8 log cfu g^{-1} in *Shigella sonnei* counts. Silveira *et al.* (2007) reported a similar sanitizer effect between 150 ppm NaClO and O_3 dips (0.4 ppm, 3 min) on fresh-cut 'Galia' melon after 10 d at 5 ºC.

However O_3 seems to be not always highly successful. Aguayo *et al.* (2003) found no clear sanitizing effect on fresh-cut 'Amarillo' melon after 10 d at 5 ºC and fresh-cut 'Thomas' tomato after 10 d at 5 ºC Aguayo *et al.* (2006). Beltrán *et al*. (2005) found no evidence of browning in fresh-cut potatoes dipped in O_3 water (20 mg L^{-1} min^{-1}) or in O_3 plus peroxyacetic acid (300 mg L^{-1}) after 14 d at 4 ºC. However, the ozonated water alone was not effective in reducing total microbial populations. Rico *et al.* (2006) found that ozonated water (1 mg L^{-1} at 18-20 ºC) reduced in fresh-cut lettuce the enzyme activity and enzymatic browning which also reduced the activity of the texture-related pectin methylesterase that was correlated with a lower crispiness.

Chlorine Dioxide (ClO_2)

ClO_2 is a stable eco-friendly dissolved gas, with a higher oxidation and penetration power than NaClO, being more effective against spores (FDA 1998). With minimal contact time, it is highly effective against pathogenic organisms such as *Legionella, amoebal cysts, Giardia cysts, E. coli*, and *Cryptosporidium* (Xie 2003). The ClO_2 does not ionize to form weak acids (as chlorine) or form carcinogenic by-products like THM. This allows ClO_2 to be effective over a wide pH range. One of the most important qualities of ClO_2 is its high water solubility, especially in cold water. However there are very few reports about the use of ClO_2 in fresh-cut products. Some of them have shown that for apple, lettuce, strawberry and cantaloupe a solution of 5 ppm was effective for inhibiting inoculated *E. coli* and *L. monocytogenes* (Rodgers *et al.* 2004). In fresh-cut faba bean (*Vicia faba* L.) dipping for 2 min in 4 ppm ClO_2 was as effective as 150 ppm NaClO in reducing *enterobacteriacea* and mesophilic counts after 8 d at 5 ºC, decreasing browning and improving sensory quality (Artés *et al.* 2007).

Acidified Sodium Chlorite ($NaClO_2$)

Acidified sodium chlorite (ASC) chemistry is principally that of chlorous acid ($HClO_2$), which forms on acidification of chlorite. Once formed, $HClO_2$ gradually decomposes to form chlorate ion, chlorine dioxide, and chloride ion. It is hypothesized that the mode of action of ASC derives from the uncharged $HClO_2$, which is able to penetrate bacterial cell walls and disrupt protein synthesis by virtue of its reaction with sulfhydryl, sulfide, and disulfide containing amino acids and nucleotides. The undissociated acid is thought to facilitate proton leakage into cells and thereby increase energy output of the cells to maintain their normal internal pH thereby also adversely affecting amino acid transport. ASC in the range 500-1200 ppm has been shown to have stronger efficacy against pathogens and spoilage bacteria than NaClO and does not form carcinogenic products. A 1200 ppm ASC washing treatment showed a good efficiency for inactivation *Salmonella* spp. in bell peppers and tomato (Hun-Gyun *et al*. 2005, 2006). However, ASC in this level can aggravate tissue damage. An ASC pre-washing controlled microbial growth in Chinese cabbage preserving quality (Inatsu *et al.* 2005). Fresh-cut bell peppers washed with 250 ppm ASC registered 2 log reductions after 7 d at 5 ºC in mesophilic, psychrotrophic and *enterobacteriaceae* counts when compared to 150 ppm NaClO at pH 6.5, but a best retention of total antioxidant activity for ASC was recorded (Conesa *et al*. 2007c).

Hydrogen Peroxide (H_2O_2)

H_2O_2 is a powerful bactericide and oxidant. Its efficacy has been demonstrated in extending shelf-life and reducing natural microflora and pathogens in fresh-cut cucumber, zucchini, bell peppers, and melons (Sapers 2003). Although H_2O_2 is permitted for other uses in food processing and packaging because it leaves no potentially harmful residues, it is not yet approved by the FDA as a sanitizing agent for fresh produce. A H_2O_2 solution was promising for sanitation of fresh-cut commodities, although results were inconsistent.

Washing with 5% H_2O_2 was more effective than with 1000 ppm NaClO and Na_3PO_4 in terms of reducing the microbial load on cantaloupe rinds. A H_2O_2 vapour treatment reduced microbial counts, extended shelf life and maintained quality of fresh-cut green bell pepper, cucumber, and zucchini (Sapers 2003). However, browning of shredded lettuce increased after dipping in a H_2O_2 solution (Parish *et al*. 2003).

Organic Acids & Calcium Salts

Organic acids have been largely applied for the prevention of enzymatic and non-enzymatic browning and microbial growth at levels that did not adversely affect taste and flavour of plant commodities (Yildiz 1994). They are more effective for bacteria than for moulds and yeast due to the low pH (between 2.1 and 2.7) at which they are applied. Kim & Klieber (1997) reported that citric acid (10 g L^{-1}) repressed the petiole sprouting (black speck) development of fresh-cut Chinese cabbage and prolonged their shelf life to 14 d at 5 ºC. Fresh-cut 'Amarillo' melon dipped in 0.52 mM citric acid for 30 s before a MAP reached a shelf life of 10 d at 5 ºC with no translucency and discoloration (Aguayo *et al.* 2003). Dipping green celery crescents in a 0.5 M ascorbic and 0.1 M citric acid solution was as effective as 100 mg L^{-1} NaClO for reducing microbial counts and improving consumers acceptability (Gómez & Artés 2004).

Calcium is related to maintain cell wall structure and firmness of plant commodities by combining with pectin to form calcium pectate (Rosen & Kader 1989). Washing with calcium lactate (15 g L^{-1}) at 50 ºC was effective in keeping turgor of cortex tissue cells and reduced the extent of lignification at cutting-edge areas (Rico *et al*. 2007). Aguayo *et al*. (2008) found that fresh-cut melon dipped into $CaCl_2$ (0.5%) solution at 60 ºC for 1 min, or organic acid salts like calcium propionate (0.9%) or lactate (1.4%) were very effective in reducing microbial growth and maintaining firmness during 8 d at 5 ºC.

Electrolyzed Water (EW)

Electrolyzed water has a strong bactericidal effect against pathogens and spoilage microorganisms, more effective than NaClO due to its high redox potential (Izumi 1999; Koseki & Itoh 2001). Hypochlorous acid is present in EW at 6.8 pH and it is generated by electrolysis of NaCl solution, since HCl formed at the anode site neutralizes the NaOH at the cathode site. Few studies have been reported the use of EW in fresh-cut vegetables (Izumi 1999; Koseki & Itoh 2001; Wang *et al*. 2004; Rico *et al*. 2008). EW containing 15 to 50 ppm available NaClO was effective as a disinfectant for fresh-cut carrots, spinach, bell pepper, potato and cucumber, without discoloration and lowering microbial counts from 0.6 to 2.6 log units (Izumi 1999). It was also shown that EW has no impact on the sensory quality of fresh-cut lettuce (Koseki *et al.* 2001). Rico *et al*. (2008) showed that washing with EW containing 60 mg L^{-1} free chlorine (pH 6.5) resulted as effective as chlorine, with good quality retention.

Prepackaging: UV-C Radiation and Intense Light Pulses

The use of non-ionizing and germicidal ultraviolet light (UV) at 190–280 nm (UV-C) could be effective for surface decontamination of fresh-cut products. It has been reported that UV-C affects several physiological processes in plant tissues and damages microbial DNA (Lucht *et al.* 1998). Lado & Yousef (2002) reported that 0.5 to 20.0 kJ UV-C m^{-2} inhibited microbial growth by inducing the formation of pyrimidine dimers which alter the DNA helix and blocks microbial cell replication. The effectiveness of UV-C seems to be independent of the temperature (5 to 37 ºC) but depends on the incident irradiation determined by the structure and surface of the product (Bintsis *et al.* 2000). However, UV-C can change the cell permeability increasing electrolytes, amino acids and carbohydrates leakage, which can stimulate bacterial growth (Artés-Hernández *et al.* 2009). The crucial point is whether a safe dose could be found which would greatly impair pathogen growth without damaging the product (Ben-Yehoshua & Mercier 2005). It has been reported that abiotic stresses, like UV light, may enhance the nutraceutical content of fresh fruit and vegetables (Cisneros-Zevallos 2003). However, more research is needed to optimize its use.

In vitro studies have demonstrated the efficiency of UV-C illumination on microbial inhibition (Abshire & Dunton 1981). Some *in vivo* studies have reported that UV-C inhibited microbial growth, delaying decay and senescence. In zucchini squash slices, UV-C exposition reduced microbial activity and deterioration during storage at 5 or 10 °C (Erkan *et al.* 2001). Civello *et al.* (2006) reported that 4 to 14 kJ UV-C m^{-2} applied to broccoli heads delayed yellowing and chlorophyll degradation at 20°C with an increased in total phenols, antioxidant capacity and flavonoids.

The use of two sided UV-C radiation at 1.18, 2.37 or 7.11 kJ m^{-2}, was effective for reducing the natural microflora of fresh-cut 'Red Oak Leaf' lettuce up to 10 d at 5 °C although 7.11 kJ m^{-2} induced tissue softening and browning after 7 d at 5 °C (Allende *et al.* 2006). Similar results were previously found for one sided UV-C radiation of fresh-cut 'Red Oak Leaf' and 'Lollo rosso' lettuces throughout 10 d at 5 °C (Allende *et al*. 2003ab). Bell peppers sticks from integrated cultivation, showed a high reduction in mesophilic and Enterobacteria counts after 12 d at 5 °C when 2.27 kJ UV-C m^{-2} was applied (Artés *et al.* 2006). Low to moderate UV-C radiation can be effective for sanitizing minimally processed spinach leaves preserving their quality (Artés-Hernández *et al*. 2009).

However, López-Rubira *et al.* (2005) found inconsistent results regarding the effect of UV-C (0.56 to 13.62 kJ m^{-2}) on microbial growth in fresh-cut pomegranate arils stored up to 15 d at 5 °C. Microbial counts were not systematically reduced throughout shelf life and yeast and moulds were unaffected. In the same way, Artés-Hernández *et al.* (2009) reported that despite 4.54, 7.94 and 11.35 kJ UV-C m^{-2} showed an initial reduction in mesophilic and psychrophilic counts on processing day, no residual inhibitory effect was found after 6 to 13 d at 5 and 8 °C compared to a control sanitizing treatment with 150 mg L^{-1} NaClO.

In whole bell peppers harvested from integrated pest management production and 2.27 kJ m^{-2} UV-C illuminated, decay was reduced after 21 d at 5 °C + 5 d at 15 °C (Artés *et al*. 2006). In tomatoes destined for the fresh-cut industry, a pre-treatment of 4 kJ UV-C m^{-2} and CA storage under 5 kPa O_2 + 1 kPa CO_2 at 12 °C for 21 d retarded ripening and kept better firmness and sensory attributes (Robles *et al.* 2007). Doses of 2.15 and 4.3 kJ UV-C m^{-2} retarded the development of decay in strawberry and increased the phenolic content (Erkan *et al.* 2008).

Intense light pulses (ILP) are an innovative decontamination method for food surfaces approved by the US-FDA that could be suitable for sanitizing fresh-cut plant commodities. ILP kills microorganisms using short time (85 ns to 0.3 ms) high frequency pulses (0.45 to 15 Hz) and energy per pulse (3 to 551 J) of an intense broad spectrum, rich in UV-C light (Gómez-López *et al*. 2005). Hoornstra *et al.* (2002) showed a 2 log cfu g^{-1} reduction in aerobic counts on vegetables increasing their shelf-life in 4 d.

Non Conventional Packaging

High Oxygen

An alternative active MAP by the use of superatmopheric O_2 was described as effective to inhibit enzymatic browning, prevent anaerobic fermentation, moisture and odour losses, and reduce aerobic and anaerobic microbial growth (Day 2001). The exposure to superatmospheric O_2 levels may stimulate, have no effect, or reduce respiration rates and C_2H_4 production, depending on the commodity, ripening stage, O_2 level, storage time and temperature, and CO_2 and C_2H_4 levels in the atmosphere (Kader & Ben-Yehoshua 2000).

The combined high O_2 level and 10 to 20 kPa CO_2 may provide adequate suppression of microbial growth and prolong shelf-life of several fresh-cut plant commodities (Allende *et al.* 2004; Conesa *et al*. 2007a,b; Escalona *et al*. 2007). Fresh-cut bell peppers showed that 80 kPa O_2 combined with 15 kPa CO_2 maintained the main sensory quality attributes and inhibited growth of the spoilage microorganisms and *enterobacteriaceae* (Conesa *et al.* 2007a,b). Levels higher than 75 kPa O_2 were needed to reduce *L. innocua* growth on fresh-cut lettuce (Escalona *et al.* 2007). Allende *et al.* (2002), reported yeast counts higher than 5 log cfu g^{-1} after 3 d at 4 °C on mixed salad stored under superatmospheric O_2 MAP.

There are few reports on the effects of elevated O_2 on enzymatic browning. The *in vitro* kinetics of PPO,

main enzyme responsible of browning, with respect to O_2 levels from 5 to 100 kPa, and using chlorogenic acid as substrate, has been examined. The substrate concentration as well as the O_2 level had a clear inhibitory effect on the reaction rate. Moreover, the inhibitory effect of O_2 was more evident at low final product concentration (Gómez *et al.* 2006).

Regarding sensory quality, acceptable scores were found in spinach leaves under 80 to 100 kPa O_2 combined with moderate kPa CO_2 compared to low O_2 and high CO_2, where the spinach was affected by fermentation (Allende *et al.* 2004).

Other Innovative Gas Mixtures

The use of non-conventional gases like Ar, He, Xe or N_2O has been proposed for improving quality of selected fresh-cut plant commodities. It has been shown that MAP enriched in Ar reduced microbial growth and delayed quality loss of fresh broccoli and lettuce (Day 1996). However, it has been found that 90 kPa Ar + 2 kPa O_2 did not delay the accumulation of phenolics in fresh-cut lettuce, or the loss of chlorophyll from broccoli florets beyond that of low O_2 atmospheres made with He or N_2 (Lougheed & Lee 1991). Zhang *et al.* (2008) have found promising results of combined Ar and Xe for extending shelf life of green asparagus spears.

Partial pressures of 50, 80 and 100 kPa N_2O reduced the respiration rates of onion bulbs by 50% after 5 d at 18 ºC compared to air storage, increased the organics acids content and reduced decay (Benkeblia & Varoquaux 2003). Innovative MAP of 90 kPa Ar + 5 kPa O_2 and 90 kPa N_2O + 5 kPa CO_2 were applied to kiwifruit slices at 4 ºC for 12 d and active 90 kPa N_2O kept the best quality of slices delaying firmness losses and browning. Slight modifications in the most important discriminated quality factors for the slices in N_2O and an acceptable quality in Ar after 8 d was found (Rocculi *et al.* 2005). An active 90 kPa He MAP showed a beneficial effect in retaining total chlorophylls and vitamin C content after 8 d at 5 ºC in minimally processed Red chard baby leaves compared to 100 ppm NaClO, at pH 6.5 under conventional MAP, with no differences in development of natural microflora (Tomás-Callejas *et al.* 2009).

Conclusions

Although NaClO is being widely used for assuring safety of fresh-cut plant commodities, improved ecofriendly strategies are promissory alternatives to preserve the high quality and safety demanded by consumers. Some GRAS compounds, ozonated or electrolyzed water, UV-C illumination or non conventional gas mixtures for active MAP can reduce initial microbial load and its development prolonging shelf life. However, in order to facilitate its industrial implementation, the potential and limits of these emergent techniques must be well defined and included in the regulations.

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31. VOLATILE AND QUALITY CHANGES IN FRESH-CUT CANTALOUPE AND HONEYDEW MELONS STORED IN MODIFIED ATMOSPHERE PACKAGING

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Abstract
Modified atmosphere packaging (MAP) in fresh-cut melons has been reported to preserve the visual quality during storage, yet its effect upon aroma and firmness is cultivar-dependent. The main objective of this research was to compare the properties of fresh-cut from a fast senescing cantaloupe with a slow senescing honeydew melon, regarding changes in quality and volatiles, when stored in passive MAP. Fresh-cut cubes of cantaloupe and honeydew melons were packaged in polypropylene trays, over-wrapped with a microperforated film and stored for 14 days at 5 ºC. Three replicate packages of each cultivar were assayed at day 0, 4, 7, 11 and 14 for color, firmness, soluble solid content (SSC), respiration rate and volatile retention, and the experiment was repeated. Volatile compounds were extracted using a relatively recent technique, Stir Bar Sorptive Extraction (SBSE), and quantified via GC-MS. Color and SSC remained constant throughout storage. During the first 4 days of storage, the cantaloupe cubes softened at a higher rate (2.0 N day^{-1}) than honeydew cubes (0.9 N day^{-1}), but firmness remained relatively unchanged thereafter until the end of the storage period. Fresh-cut cantaloupe cubes exhibited a higher respiration rate (17.5-43.4 mL CO_2 kg^{-1} h^{-1}) than fresh-cut honeydew cubes (7.7–30.0 mL CO_2 kg^{-1} h^{-1}), throughout the entire storage period. Esters and aldehydes were the major volatile compounds present in the samples of both cultivars, but cantaloupe yielded higher concentrations of esters and aldehydes, and lower concentrations of alcohols than honeydew. Esters increased their levels during storage, whereas alcohols and aldehydes decreased.
Keywords: *Cucumis melo*, firmness, minimally processed, quality

Introduction
Fresh-cut processing induces a physiological response to tissue due to rupturing, and wounding enhances respiration rate and ethylene production. Color, sweetness and texture, perceived as important quality attributes of fresh-cut melons (Portela & Cantwell 1998) change as well as aroma compounds that are often only released upon cell disruption (Buttery 1993). The understanding of the processes leading to these changes is essential to develop better approaches to improve quality perceived by the consumer and shelf-life (Rico *et al.* 2007; Toivonen & Brummell 2008).

Cantaloupe and honeydew melons are commonly used as fresh-cut fruit. Cantaloupe melons are climacteric, exhibit higher respiration and ethylene production rates, show faster softening and shorter shelf-life than the non-climacteric honeydew melons. In climacteric melon fruits, ethylene regulates rind degreening, aroma formation and abscission, and partly regulates flesh softening (Bauchot *et al.* 1998; Flores *et al.* 2001, 2002). Although the role of ethylene in regulating the expression of genes involved in aroma volatile synthesis has been demonstrated in climacteric fruits such as melons and apple (Defilippi *et al.* 2005; El-Sharkawy *et al.* 2005; Manriquez *et al.* 2006), little is known about the regulation of aroma formation in non-climacteric fruits.

Aroma of fresh-cut melon is made up of a complex mixture of volatile compounds, such as aldehydes, acetate and nonacetate esters, alcohols and sulphur-containing compounds. Our goal was to assess the effect of minimal processing upon the physicochemical quality and aroma volatiles of cantaloupe and honeydew melons.

Material & Methods

Orange fleshed cantaloupes ('Sol Real') and green fleshed honeydews (unknown cultivar) were received and kept overnight at 5 °C, prior to processing. Fruits were washed in cold water, dipped in 100 ppm of sodium hypochloride solution, and allowed to drain. The skin was removed using a CP-44 Melon Peller (Muro Co.), the blossom and stem ends were discarded, placental tissue and seeds were removed and cubes were prepared (ca. 2.5×2.5 cm). The cubes (ca. 175 g) were placed immediately in 250 g polypropylene MAP trays (Green-Tek, USA), and overwrapped with a microperforated film with an O_2 transmission rate of 5200 $cm^3 m^{-1}$ dia^{-1} atm^{-1} (Amcor Flexibles, Europe). Samples were stored for 14 days (d) at 5 °C, and analyzed at days 0, 4, 7, 11 and 14.

Color was measured in the CIE L*C*h color space, with a colorimeter CR-400 (Konica Minolta, Japan), using the illuminant D65. Pulp color of melon cubes was measured twice in each of three cubes samples, from three replicated packages of each cultivar. Mesocarp tissue firmness was measured with an 8 mm-probe hand-held penetrometer (McCormick, FT327, Italy) on three cubes from each replicate. Soluble solids content (SSC, °Brix) was determined on the juice obtained from three cubes using a hand-held refractometer (Atago PAL-1, Japan). In order to monitor the package headspace, a small piece of adhesive rubber strip was attached to the MAP film, and the sampling needle of the gas analyzer (Mocon Pac Check Model 650 Dual Head Space Analyzer, USA) was inserted in each package. The respiration rate was calculated from measurements of CO_2 released to the headspace of a closed system for 2 h. CO_2 was measured with a gas analyzer. For volatile analyses, samples were juiced with a Braun MP80 Juicer (Germany) and the stir bar sorptive extraction (SBSE) technique was used. Stir bars with 10 mm length, manufactured by Gerstel (Muelheim an der Ruhr, Germany), were conditioned at 300 °C for 1 h, and properly re-conditioned between samplings. The stir bars were placed in 10 mL vials containing 1 mL of juice, from each replicated sample, 4 mL of 62.5 % saturated salt solution and 2-methylbutyl 3-methylbutanoate as internal standard, and sealed with a steel cap fitted with a teflon/silicon septum. Extraction was then performed for 1 h at 38.5 °C and 850 rpm, via Variomag Multipoint HP15 stir plate. After said extraction, the volatile compounds were quantified via GC-MS (Agilent 5973 MSD, USA) using a DB5 capillary column (30 m×0.25 mm×0.25 μm, Agilent).

Results

Color and SSC remained relatively constant throughout storage for both melon cultivars (data not shown). SSC ranged between 9.6%±0.2 and 9.5%±0.2 for cantaloupe, and 11.3%±0.5 and 11.5%±0.4 for honeydew.

Initial firmness values were 30.1 N ± 3.6 for cantaloupe and 21.5 N±2.7 for honeydew cubes. Firmness of both fresh-cut cultivars decreased during the first 4 d at 5 °C, and remained relatively unchanged for the remaining storage period. During the first 4 d of storage, cantaloupe cubes softened at a higher rate (2.0 N day^{-1}) than honeydew cubes (0.9 N day^{-1}), and by the end of the 14 d of storage, firmness values were 18.9 N±2.3 for cantaloupe cubes and 15.9 N±2.1 for honeydew cubes (Fig 1).

O_2 levels inside the packages gradually decreased to 11.1% for cantaloupe and 14.2% for honeydew, while CO_2 increased to 11.3% and 7.9% for cantaloupe and honeydew, respectively (data not shown). Fresh-cut cantaloupe cubes exhibited a higher respiration rate (17.5-43.4 mL CO_2 kg^{-1} h^{-1}) than fresh-cut honeydew cubes (7.7–30.0 mL CO_2 kg^{-1} h^{-1}), throughout the entire storage period (Fig 2).

For both cantaloupe and honeydew melons, the total volatile abundance increased during the 14 d of storage. This increase was mostly due to the increase in acetate (Fig 3) and non-acetate esters (Fig 4). Cantaloupe yielded higher concentrations of esters and aldehydes (Fig 5), and lower concentrations of alcohols than honeydew (Fig 6), and sulfur-containing compounds were found to be low in both cultivars (data not shown).

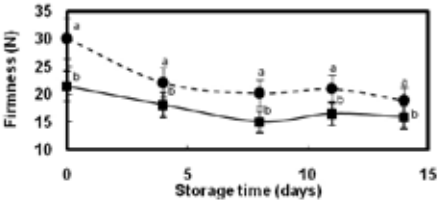

Fig 1. Firmness of fresh-cut cantaloupe (●) and fresh-cut honeydew (■), stored for 14 d at 5 °C. Data points are the average of 18 replicates. Statistical analysis comparing differences between cultivars. Values followed by the same letter are not significantly different ($p \leq 0.05$), by the non parametric Kruskal-Wallis H test.

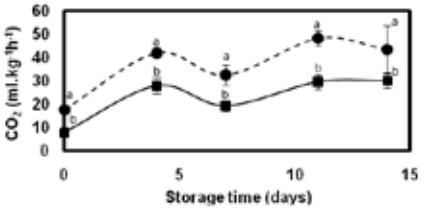

Fig 2. Respiration rate of fresh-cut cantaloupe (●) and fresh-cut honeydew (■), stored for 14 d at 5 °C. Points are the average of 6 replicates. Statistical analysis comparing differences between cultivars. Values followed by the same letter are not significantly different ($p \leq 0.05$), by the non parametric Kruskal-Wallis H test.

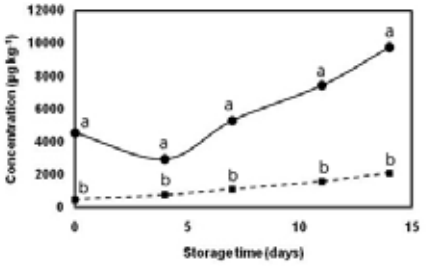

Fig 3. Total concentration of acetate esters in fresh-cut cantaloupe (●) and fresh-cut honeydew (■), stored for 14 d at 5 °C. Points are the sum of different compounds. Statistical analysis comparing differences between cultivars Values followed by the same letter are not significantly different ($p \leq 0.05$), by one way ANOVA.

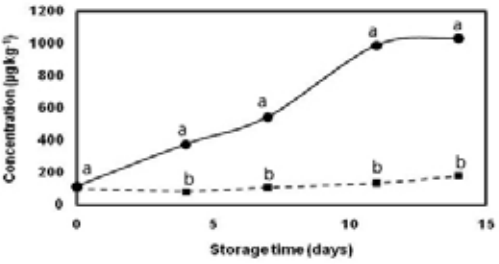

Fig 4. Total concentration of non-acetate esters in fresh-cut cantaloupe (●) and fresh-cut honeydew (■), stored for 14 d at 5 °C. Points are the sum of different compounds. Statistical analysis comparing differences between cultivars Values followed by the same letter are not significantly different ($p \leq 0.05$), by one way ANOVA.

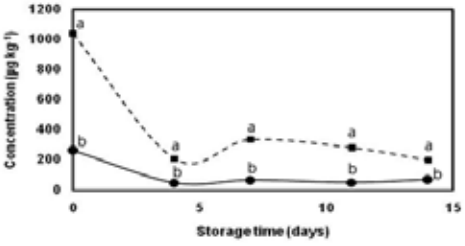

Fig 5. Total concentration of alcohols in fresh-cut cantaloupe (●) and fresh-cut honeydew (■), stored for 14 d at 5 °C. Points are the sum of different compounds. Statistical analysis comparing differences between cultivars Values followed by the same letter are not significantly different ($p \leq 0.05$), by one way ANOVA.

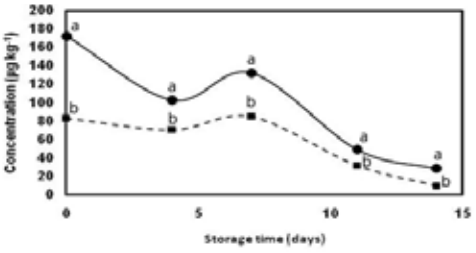

Fig 6. Total concentration of aldehydes in fresh-cut cantaloupe (●) and fresh-cut honeydew (■), stored for 14 d at 5 °C. Points are the sum of different compounds. Statistical analysis comparing differences between cultivars Values followed by the same letter are not significantly different ($p \leq 0.05$), by one way ANOVA.

Discussion

Quality attributes differed between cantaloupe and honeydew cubes. Color parameters (L*, C and h) did not change significantly during storage, and visual differences were only due to the characteristic flesh color of the cultivar. Similarly, little color change was reported in previous studies (Boynton *et al.* 2005; Beaulieu & Lea 2007).

Soluble solid content was higher in honeydew than in cantaloupe cubes (data not shown). Similar results were reported by Saftner *et al.* (2006) with no consistent pattern of change in SSC among any of the genotypes being observed during storage.

Cantaloupe, as expected from its climacteric behaviour, exhibited higher softening rates than honeydew cubes, as previously reported (Saftner *et al.* 2006). Throughout the entire storage period there were significant differences in firmness between cultivars, with fresh-cut cantaloupe exhibiting higher firmness than fresh-cut honeydew.

The respiration rate of fresh-cut cantaloupe was higher than that of fresh-cut honeydew, as previously reported (Saftner *et al.* 2006). Both cultivars showed an increase of CO_2 production between days 0 and 4, unlike the results in cantaloupe by Luna-Guzmán *et al.* (1999) and Aguayo *et al.* (2003), and in honeydew by Saftner *et al.* (2003), according to which the respiration rate of fresh-cut melons decreased in the first two days of storage. An increase in CO_2 production was observed between days 7 and 11. This increase may have been induced by microbial growth, as reported by Aguayo *et al.* (2003), Bai *et al.* (2003) and Saftner *et al.* (2003), although visual microbes were not observed. Levels of O_2 and CO_2 inside the cantaloupe and honeydew packages were similar to those found in cantaloupe by Bay *et al.* (2001) and Aguayo *et al.* (2003) and in honeydew by Bai *et al.* (2003) in which levels of O_2 and CO_2 inside the packages did not reach equilibrium during MAP storage and were not sufficient to inhibit microbial growth.

Preliminary volatile data analysis indicated that volatile compounds discriminate cantaloupe from honeydew melons. There were distinct qualitative and quantitative differences in the aromatic volatiles studied between cultivars (data not shown). The total volatile concentration in fresh-cut cantaloupe was 3-fold that in fresh-cut honeydew. While Saftner *et al.* (2006) reported an increase of total volatile abundance of fresh-cut melons only in the first 2 d of storage under MAP, in this study such an increase was observed throughout the entire storage period. Esters were the major volatiles identified in both cultivars. Among them, butyl acetate, 2-methylbutyl acetate, benzyl acetate and hexyl acetate were the most abundant (Wang *et al.* 1996; Wyllie *et al.* 1996; Bauchot *et al.* 1998; Bai *et al.* 2003; Saftner *et al.* 2003; Aubert & Bouger 2004; Beaulieu 2006b). There was a continuous change in ester balance during fresh-cut storage. Saftner *et al.* (2003) indicated a decrease of acetate esters during fresh-cut melon storage under MAP, and Beaulieu (2006a,b) demonstrated an acetate decrease with concomitant non-acetate ester increase in fresh-cut stored in clamshell containers, however, in this study an increase of both acetate and nonacetate esters levels was observed. Total esters concentration was 67% higher in cantaloupe than in honeydew. Alcohols and aldehydes decreased during storage. Alcohols were more abundant in honeydew than in cantaloupe due to the higher level of (*Z*)-6-nonenol, a flavour-related compound in honeydew melons (Buttery *et al.* 1982). Similar to results generated by Bauchot *et al.* (1998), sulphur-containing compounds, which were believed to have an important role in the overall aroma profile of melon (Kemp *et al.* 1972; Wyllie & Leach 1992), were detected in very small amounts in both cultivars.

Conclusion

For most quality parameters, the discrimination by climacteric and non-climacteric behaviour was better at processing than by the end of the storage period. Regarding aroma development, acetate esters that are often considered the most important flavour-related volatiles in several melon cultivars (Buttery *et al.* 1982; Beaulieu 2006b); increased while a decrease of alcohols and aldehydes was observed during fresh-cut cantaloupe and honeydew storage. In this experiment, MAP provided a mean to reduce respiration. Likely

due to high O_2 availability in MAP, there was an available pool of acetyl-CoA in both cultivars, even after 14 d of storage, which allowed acetate ester formation via alcohol esterification by alcohol acetyltransferase (AAT). Simultaneously, AAT esterified other acyl donors with available alcohols, to form non-acetate esters. These results suggest that MAP retarded the loss of volatile compounds in fresh-cut cantaloupe and honeydew melons, thus maintaining quality during storage.

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32. PHYSIOLOGICAL BASES FOR TEXTURE AND COLOR CHANGES IN FRESH-CUT 'ROCHA' PEAR: IMPLICATIONS FOR THE DEVELOPMENT OF PRODUCTS AND PROCESSES

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Abstract
The physiological bases for the effectiveness of technologies used in fresh-cut fruit were examined in an attempt to foster the shift from a market-pull to a science-push innovation model. Respiration rate of fresh-cut 'Rocha' pear were measured at various oxygen concentrations. Apparent Km values for oxygen uptake were very close to the fermentation thresholds, indicating that it is physiologically impossible to reduce the respiration rate of fresh-cut pear by more than 50% without inducing fermentation. Based on this information, we hypothesize that optimizing oxygen concentration inside packages is of little or no value in fresh-cut pears. Additionally, additives used to reduce enzymic browning and softening may have pleiotropic effects on quality. It was found that pH significantly affected the rate and intensity of browning of fresh-cut pears. Browning was more intense in pears treated at pH 3.0 and less pronounced at pH 7.0, but the softening rate was lower at the latter pH value. Calcium salts also affected texture and color in a salt-specific manner. Larger color changes were observed with propionate and lactate. Although calcium ascorbate reduced color changes, fruit slices treated with this salt were softer than those treated with calcium propionate, lactate or chloride. Based on the physiological information gathered, we will develop and evaluate an integrated process to maximize the quality of fresh-cut 'Rocha' pear throughout the distribution chain.
Keywords: minimally processed, modified atmosphere packaging, pH, *Pyrus communis*, respiration

Introduction
Fresh-cut produce are one of the fastest growing segments of the fresh food market in developed countries, both at retail level and restaurant outlets. The fresh-cut fruit industry is urged to provide products to the growing number of consumers who seek convenience and will not lower their high expectations regarding the intrinsic quality and food safety of fresh-cut fruit. Fruit processing has physiological consequences that impact negatively on the quality of the final fresh-cut product. Loss of cellular compartmentation caused by cutting deeply affects tissue metabolism. Wounding causes an increase in respiration rate, ethylene synthesis and area-to-volume ratio, favors water loss, and destroys structural barriers to the entry of microorganisms (Watada & Qi 1999). Additionally, the metabolic changes induced by cutting can produce undesirable flavor, loss of vitamins, excessive softening and color changes all of which severely reduce shelf life (Wiley 1994). Enzymatic browning and softening are the major limiting factors for fresh-cut pear (Gorny *et al.* 2000), when microbiological stability is secured. Anti-browning additives, often combined with calcium, and modified atmosphere packaging are used to extend shelf-life in fresh-cut pear. A number of studies encompassing packaging films and geometries have assessed O_2 concentration versus produce quality, in order to empirically find the best package (Del Nobile *et al.* 2007; Soliva-Fortuny *et al.* 2007). An alternative approach to the trial-and-error, should data be available, is to deduce optimal packaging geometry and film permeability based on the produce respiration rate (Lakakul *et al.* 1999; Jacxsens *et al.* 2000), as affected by oxygen concentration and temperature.

This research project aims at evaluating the potential of MAP utilization for fresh-cut 'Rocha' pear. The relationship between oxygen concentration and respiration rate was modeled to help design of MAP for fresh-cut pear at temperatures normally found during processing and distribution. The effect of dipping solutions with different pH values and calcium salts in the control of quality loss of pear slices during cold storage has also been studied. The physiological information gathered is useful to develop and evaluate an integrated process to maximize the quality of fresh-cut fruits.

Material & Methods

Plant Material and General Processing

Pears (*Pyrus communis* L. 'Rocha') with a flesh firmness ranging between 38 to 56 N (8-mm probe) were sanitized and hand cut in wedges, without skin removal.

Respiratory Data

The fresh-cut pear wedges (30 to 26 g) were packed in low-density polyethylene (LDPE) (Dow Chemical Company, Midland, MI) pouches that were hermetically sealed using an impulse heat sealer. A range of steady-state O_2 and CO_2 partial pressures was achieved by varying fruit weight (30-260 g), film surface area (450-800 cm^2), and film thickness (27-105 μm) among the pouches. Oxygen and carbon dioxide were measured daily with a gas analyzer. Rates of O_2 uptake and CO_2 production were calculated by mass balance, once steady-state O_2 and CO_2 partial pressures were achieved inside the packages. Three replicates of each combination of film thickness, film area, and fruit mass were stored at 0, 5, 10 and 15 °C.

Respiration rate (R_{O_2}) was described as a function of O_2 partial pressure (pO_2) by a Michaelis-Menten model, as suggested by Lee *et al.* (1991).

Respiratory quotients (RQ) and ethanol as function of steady-state oxygen partial pressure were used to determine the lower limit for aerobic respiration (Joles *et al.* 1994). The sudden increase in RQ or headspace ethanol concentration occurring when pO_2 dropped below a certain level is termed 'fermentation threshold'.

pH Treatments

Pear slices were dipped for 1 min in a cold buffer solution (200 mM sodium phosphate and 100 mM citric acid) at pH 3.0, 5.0 and 7.0, packed in clamshells with normal atmosphere and stored at 4 °C. Six replicated packages for each dipping solution were analyzed at 0, 3, 6, 10 and 13 days (d), for color, firmness, soluble solid content, pH, titratable acidity and *mesophilic* and *psychrophilic* bacterial count.

Calcium Treatments

Fresh-cut pears were dipped in calcium solutions buffered at pH 3 and pH 7, placed in covered trays and stored at 5 °C. Four calcium salts – ascorbate, chloride, lactate and propionate – were dissolved in two buffer solutions with pH 3.0 (100 mM citric acid-100 mM sodium citrate and 100 mM citric acid-200 mM sodium phosphate), and two buffers with pH 7.0 (100 mM Mops-NaOH and 100 mM Tris-100 mM HCl) to give a final concentration of 250 mM (1% Ca^{2+}) of calcium. The experiment was conducted in a randomized complete block design with 30 pear slices per treatment. The two buffer solutions with the same pH were considered as replicates. Data were subjected to two-way analysis of variance, considering pH and calcium salt as factors.

Quality Determinations During Storage Period

Color was measured in the CIE L* C* h color space with a Konica-Minolta CR-400 chromameter (Japan) with a D65 illuminant. Metric hue difference, $\Delta H^* = \sqrt{(\Delta a^*)^2 + (\Delta b^*)^2 - (\Delta C^*)^2}$, where a* and b* are the Cartesian color coordinates and C* is chroma, was used to express changes in color between day 0 and day 6. Firmness was determined using a texture analyzer (TA-XT2 Plus, Texture Technologies, UK) equipped with a

flat plate. Microbiological assessment was attained by enumeration of total plate count of mesophilic and psychrophilic microorganisms (ISO 4833:1991). Soluble solids content (digital refractometer Palette PR-32, Atago, Japan), pH (pH meter Ion 510 Series, Oakton, USA) and titratable acidity (titrator Digitrate, Jencons, UK) were determined in pear juice.

Results & Discussion

Respiration and Modified Atmosphere Packaging

The oxygen uptake rate increased with $pO_{2\ pkg}$ in a manner consistent with saturation kinetics (Fig 1). Michaelis-Menten model was able to explain more than 93% of the total variability of the data and accurately predicted the experimental results (RMSE<0.06). Respiration rates of fresh-cut 'Rocha' pear, as expressed as CO_2 production were similar to the range of value reported for other fresh-cut pear varieties at 0 or 10 °C (Gorny *et al.* 2000). The respiratory quotient (RQ) for aerobic respiration of fresh-cut 'Rocha' pear stored at various temperatures ranged between 1.2 and 1.4 (Table 1), consistent with the usage of organic acids as major respiratory substrates (Fonseca *et al.* 2002). 'Fermentation threshold' ranged between 0.4 and 2.9 kPa (Table 1). At elevated temperatures fermentation occurred at higher pO_2 (Lakakul *et al.* 1999), as observed for 5 up to 15 °C (Table 1).

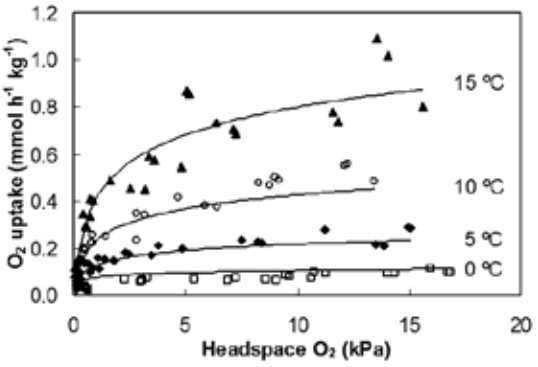

Fig 1. Effect of steady-state O_2 partial pressure and storage temperature on the rate of O_2 uptake of 'Rocha' pear slices in sealed LDPE packages at 0 (□), 5 (♦), 10 (○) and 15 °C (▲) fitted with a Michaelis-Menten model.

Table 1. Estimated respiratory parameters for fresh-cut 'Rocha' pear stored at four temperatures.

Temperature (°C)	R_{O2}^{max} (mmol kg^{-1} h^{-1})	$K_{m,O2}$ (kPa)	RQ	Fermentation threshold (kPa)	Safe working atmosphere (kPa)
0	0.108	0.52	1.4	2.9	None
5	0.224	0.73	1.3	0.4	0.33
10	0.464	1.02	1.2	0.7	0.32
15	0.961	1.43	1.3	0.7	0.73

Respiratory behavior as function of oxygen concentration provides a basis to deduce the potential benefits of using MAP technologies. Low oxygen atmospheres could be useful if a sizeable reduction in respiration rate (e.g. 50%) can be reached without the induction of fermentation (Beaudry 2000). In cases where K_{m,O_2} (apparent K_m - is the $pO_{2\ pkg}$ at half of maximal value of R_{O_2}, kPa) is much higher than fermentation threshold, a reduction in pO_2 slows down metabolic activities without an increase in the RQ and fermentative compounds. The range of pO_2 between K_{m,O_2} and fermentation threshold is termed 'safe working atmosphere' (Beaudry 2000). Fresh-cut 'Rocha' pear had narrow (0.3 to 0.7 kPa) safe working atmospheres at the temperatures tested (Table 1). Low oxygen atmospheres did not offer any reduction in

metabolic activity without the danger of inducing anaerobiosis, especially between 0 and 10 ºC, temperatures normally found during storage and marketing. Gorny (2001) also reported a poor efficacy of modified atmosphere for fresh-cut pear at 0-5 ºC.

Given this physiological behavior, the potential for reducing respiration of fresh-cut via MAP is limited. Efforts in packaging design should, therefore, to directed at avoiding microbial contamination and dehydration, and allow aerobic respiration.

Effect of pH

Color of fresh-cut pear slices was significantly affected by pH of the dipping solutions throughout the storage period (Fig 2a, Table 2). Acidic additives (e.g. ascorbate, citrate) are frequently used during processing of fresh-cut fruit to limit microbial growth. However, dipping in an acidic solution increases pear browning (Sapers & Miller 1998). Enzymatic browning is mediated by polyphenoloxidase (PPO) and optimal pH values for pear PPO are cultivar dependent. Catalytic properties of 'Rocha' pear PPO are yet to be thoroughly characterized but low enzymatic activity at pH 7 is expected, as observed with PPO of 'Bosc' and 'Red' cultivars (Siddiq *et al.* 1994).

A significant effect of pH on firmness was also observed. Higher softening rates were observed at acidic pH, especially during the first 6 to 9 d at 4 ºC (Fig 2b). Similar results observed by Pinheiro & Almeida (2008) in tomato were attributed to enhanced pectin disassembly at lower pH.

Microbial count increased over storage and pears dipped with pH 7.0 solution presented higher counts, although below 10^6 cfu g^{-1} after 10 d in storage (data not shown).

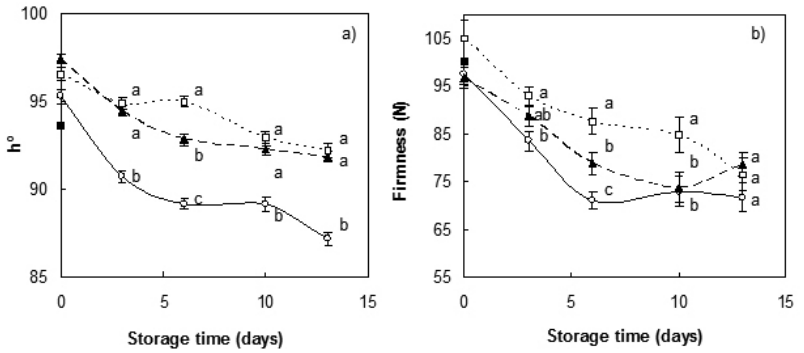

Fig 2. Hue angle (a) and firmness (b) values of fresh-cut pear slices untreated (■) or treated with a solution at pH 3 (○); 5 (▲) or 7 (□), during storage at 4 ºC. Points are average of 36 replicates; bars are standard error.Values followed by the same letter are not significantly different.

Effect of Calcium

Ascorbate was the calcium formulation less effective in maintaining firmness but preserved initial color (Table 2). An interaction between the effects of pH and calcium in color and firmness was observed, as previously reported by Pinheiro & Almeida (2008) for tomato. While color of slices treated with calcium ascorbate or calcium chloride did not differ with solution pH, calcium lactate and calcium propionate clearly promoted browning when dissolved in solutions at pH 3 when compared with solutions at pH 7 (data not shown).

Table 2. Effect of calcium salt and buffer pH on firmness and color of fresh cut pear stored 6 days at 5 ºC. Values are means (n=120 for calcium; n= 300 for pH) ± SE. Values followed by the same letter are not significantly different.

	Firmness variation (%)	Color variation (ΔH*)
Calcium		
Buffer	133.2 ± 3.7 [a]	2.50 ± 0.10 [c]
Ascorbate	93.4 ± 3.3 [d]	0.42 ± 0.03 [e]
Chloride	120.0 ± 3.1 [b]	1.69 ± 0.08 [d]
Lactate	117.5 ± 3.3 [b]	3.26 ± 0.14 [a]
Propionate	106.5 ± 3.6 [c]	3.02 ± 0.23 [b]
pH		
3.0	100.8 ± 2.1 [b]	3.00 ± 0.12 [a]
7.0	127.9 ± 2.2 [a]	1.35 ± 0.06 [b]
Significance (P)		
Calcium	<0.001	<0.001
pH	<0.001	<0.001
pH*Calcium	<0.001	<0.001

Acknowledgments

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33. PHOTOSENSITIZATION AS NOVEL APPROACH TO DECONTAMINATE STRAWBERRY FRUIT SURFACES

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Abstract

The methods recently applied for inactivation of food pathogens are not always efficient, safe for humans, or ecologically friendly. In this context, photosensitization might serve as a promising antibacterial tool. Data obtained in this study indicate that the important food pathogens *Listeria monocytogenes* and *Bacillus cereus* can be inactivated after photosensitization by 6 log *in vitro*. Moreover spores of *B. cereus* as well as biofilms of *L. monocytogenes* are susceptible to this treatment. Decontamination of strawberries from *L. monocytogenes* by photosensitization as well as from aerobic mezophylls reached 3 log. The shelf-life of treated berries in comparison with non-treated control strawberries increased by 40%. No significant changes of antioxidant activity in strawberries was detected.

Keywords: Food decontamination, Non-thermal, Photosensitization

Introduction

Many procedures are required to be taken until food reaches consumers in an acceptable form. Consequently there are many possibilities for food to be lost between harvest and consumption. Food susceptibility to various losses demands novel technologies for its preservation after harvest and during storage. Microbial contamination is one of the major food and food-related surfaces contamination agent. Various microorganisms can cause human diseases under certain conditions. The methods recently applied for inactivation of food pathogens (heat treatment, various chemical sanitizers, irradiation) are not always efficient, safe for humans, or ecologically friendly. Current food treatment methods frequently have associated disadvantages, specifically resulting in unfavourable changes of organoleptic and nutritional characteristics. Moreover, under appropriate treatment conditions some pathogens resist destruction.

Food packaging cannot resolve all of these problems. Foodborne pathogens can easily contaminate packaging surfaces and food surfaces. According to the U.S. Food and Drug Administration, some disadvantages may be addresed using "smart" packaging when volatile compounds indicate packaged food freshness (US FDA 2002).

Photosensitization involves the administration of a photoactive compound that selectively accumulates in the target microorganism. After illumination with visible light plethora, photochemical reactions induce selectively death of microorganism without any harmful effects on surrounding (Luksiene *et al.* 2004; Luksiene 2005).

Material & Methods

For photosensitization experiments in vitro, *Bacillus cereus* ATCC 12826 and *Listeria monocytogenes* $ATC_{L3}C$ 7644 were grown at 37 °C in Luria-Bertani (LB) medium to the mid-log phase (~ 6×10^7 colony forming units (cfu) mL^{-1}, OD_{540}=1) and were harvested by centrifugation (10 min, 5000 g). Cells were resuspended and diluted in PBS to give ~1×10^7 cfu mL^{-1} final concentration. Aliquots (10 mL) of bacterial suspensions were incubated with a chlorophyll-derivative ($7.5x10^{-7}$ and 7.5×10^{-8} M) in darkness at 37 °C. Afterwards 150 μL aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for different time (0-20 min). Light emitting diodes (LED) based light source for (constructed in the Institute of Applied Sciences of Vilnius university) emitted light λ=400 nm with intensity 20 mW cm^{-2} at the surface of samples. Light dose was calculated as light intensity multiplied on time.

Strawberries (*Fragaria ananassa* Dutch.) purchased in a local supermarket were stored at +6 ^{0}C and processed within one day. The samples (strawberries of about 15 g) were soaked in 1.5×10^{-4} M concentration of chlorophyll derivative solution, the control samples – in phosphate-buffered saline (100 mM PBS, pH 7.2). All samples kept in the dark for 5 min and after inoculum decantation, dried in the thermostat at 37 °C. Dried strawberries were placed in the treatment chamber in a sterile Petri dish without cover and exposed to light intensity 20 mW cm^{-2} at λ=400 nm for 30 min. Control samples were not irradiated. After treatment, each sample (also control samples) mixed with 135 mL 100 mM sterile PBS buffer in a sterile 100 BagPage and homogenized 60 s with a BagMixer. Then, 100 μl of appropriate dilutions (0.9 % NaCl) of homogenized strawberries placed on LB agar. All plates were kept in the thermostat for 48 h at 37 °C. The surviving cell populations enumerated and expressed by $\log_{10}$ (cfu/g). For shelf-life studies one part of samples were soaked in 1.5×10^{-4} M chl-derivative solution, the other one – in sterile distillated water. Samples, treated with chl-derivative were illuminated for 30 min at 20 mW/cm^2 (λ=400 nm) and stored with refrigeration at 6 ^{o}C. The control samples were not illuminated. Total antioxidant capacity was measured by FRAP (ferric reducing ability of plasma) method. Precision Celsius temperature sensors (Deltha Ohm, Italy) were used for temperature measurements.

Results

The data depicted in Fig 1 indicate that main pathogens *L. monocytogenes, B. cereus* were susceptible to chlorophyll-based photosensitization and can be inactivated by 7 log *in vitro*. The decontamination of berries inoculated with pathogens by photosensitization seems promising, as about 3 log decrease in pathogen population was observed. Moreover, total aerobic mesophylls on the surface of berries were reduced by 3 log as well (Fig 2).

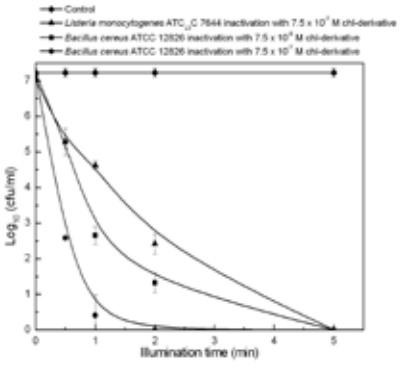

Fig 1. Inactivation of *Listeria monocytogenes* ATC$_{L3}$C 7644 and *Bacillus cereus* ATCC 12826 by chlorophyll-derivative based photosensitization, when different concentration of chlorophyll-derivative was used.

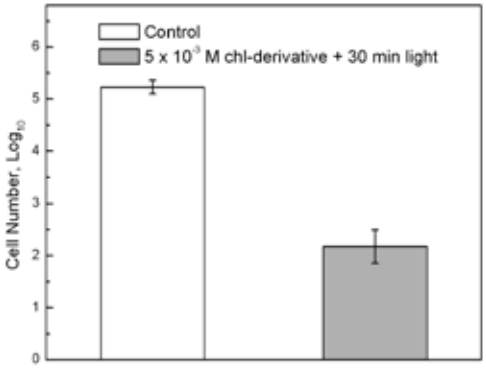

Fig 2. Inactivation of total mesophylls from strawberries by photosensitization with 5×10^{-3} M chlorophyll-derivative.

Data presented in Fig 3 clearly indicate, that photosensitization is non-thermal treatment, as during all illumination time the temperature increase was very slow and never exceeded 27 °C. Afterwards shelf-life of treated berries was evaluated. As depicted in Fig 4, the shelf-life of treated strawberries prolonged about 40% in comparison with control. Data indicate, that no inactivation of antioxidant enzymes was detected under certain experimental conditions (Fig 5).

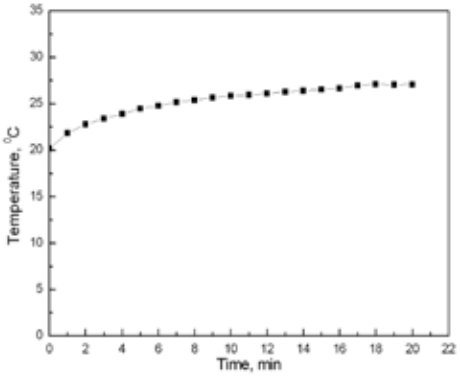

Fig 3. The increase of temperature on strawberries placed in LED-based light source during 20 min of illumination. Thermometer (Delta Ohm, Italy) was used for temperature measurements.

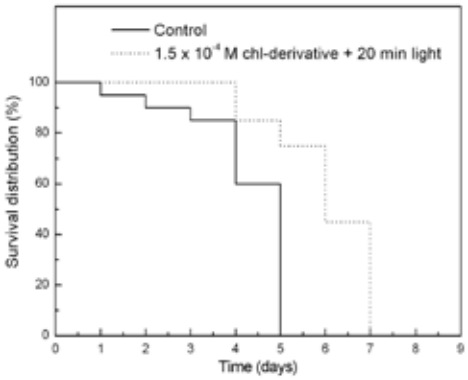

Fig 4. Shelf-life of strawberries after photosensitization with 1.5×10^{-4} M chlorophyll-derivative.

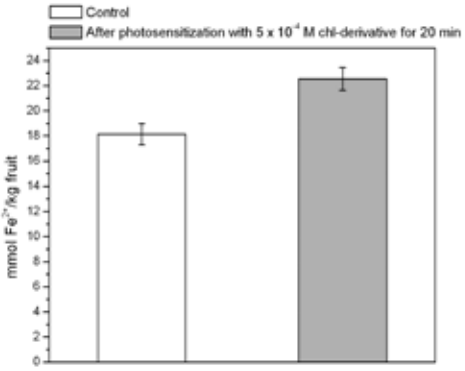

Fig 5. Total antioxidant activity (FRAP) of strawberries after photosensitization.

Discussion

Strawberries are an important, nourishing, and popular fruit worldwide. Unfortunately they are also particularly perishable, and are highly susceptible to mechanical injury and contamination during storage. Strawberries fruits have been reported to contain high phenolic and other antioxidant content (Kähkönen *et al*. 2001). Additionally, they possess very short ripening and senescence periods that aggravate their selling. The loss of strawberries can reach 40% during storage (Satin 1996). The most widely known postharvest treatments to decrease microbial contamination degree and reduce water loss and respiration rate are low temperature and modified atmosphere packaging (Nielsen & Leufven 2008) However, it has been reported that these procedures can also impinge on strawberries quality (Ayala-Zavala *et al.* 2007). According to the data obtained, photosensitization-based treatment can decontaminate surface of strawberries by 3 log, prolong their shelf-life 40%, without any inactivation of antioxidant enzymes.

Acknowledgments

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34. EFFECT OF ELECTROLYZED WATER AS A DISINFECTANT FOR FRESH-CUT FRUIT

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Abstract

In fresh-cut processing the disinfection is the most important step. This process can affect the quality and the safety of the end product as well as its shelf life. Chlorine is the most used disinfectant in fresh-cut industry but due to its association with environmental and health risks, the development of alternative sanitizers is emergent. Electrolyzed water (EW) may be an alternative to the use of chlorine sanitizers and both acidic electrolyzed water (AEW) and neutral electrolyzed water (NEW) can be used with this purpose. In fact, several studies have shown that EW can be effective in reducing pathogenic bacteria on the surface of fruits and vegetables. This manuscript presents a review of electrolyzed water approaches, its antimicrobial activity in fresh-cut produce and the results of a study undertaken in our laboratory to evaluate the disinfection efficacy of AEW and NEW on fresh-cut apples and oranges. Fresh-cut apples and fresh cut oranges were inoculated with a suspension of *Listeria innocua* and fresh-cut oranges with *Escherichia coli* at a concentration of 10^7 cfu mL^{-1} and treated with the different solutions. Untreated fresh-cut fruits were used as control. The best sanitizing treatment for both pathogens and fruits was AEW at 200 ppm of free chlorine applied during 5 minutes.

Keywords: acidic electrolyzed water, apples, *Escherichia coli*, *Listeria innocua*, neutral electrolyzed water, oranges

Introduction

Over the last few years consumers demand for minimally processed fruits and vegetables has increased rapidly. To achieve this growing demand, many emerging food preservation techniques have been introduced and are being studied extensively. Fresh produce can be a vehicle for the transmission of bacterial, parasitic and viral pathogens responsible for human illnesses and a number of reports refer to raw vegetables harbouring potential foodborne pathogens (Beuchat 1996). Chlorine is the most common disinfectant used in fresh-cut industry and it is, normally, applied at a concentration of 50-200 ppm with a contact time of 1-2 min (Beuchat 1998; Abadias *et al.* 2008). Its biocidal activity depends on the amount of free available chlorine in the water that comes into contact with the microorganisms.

In recent years, acidic electrolyzed water (AEW) and neutral electrolyzed water (NEW) have been considered for application as sanitizers. These solutions can be used in food industry to reduce or eliminate bacterial populations on food products, food-processing surfaces and non-food contact surfaces (Hricova *et al.* 2008; Huang *et al.* 2008).

In Japan, the Health, Labor and Welfare Ministry has approved EW as a food additive (Yoshida *et al.* 2004). EW generators have been also approved to be used in the food industry by the U.S. Environmental Protection Agency (Park *et al.* 2002). One of the main advantages of using electrolyzed oxidizing water for disinfection is that it is produced using pure water with no added chemicals except sodium chloride (NaCl). For that reason, it has less adverse impact on environment (Kim *et al.* 2000) and it has no harm to the human body (Mori *et al.* 1997).

The purpose of this article is to review issues related to electrolyzed water in fresh cut produce and report a study undertaken in our laboratory to evaluate the disinfection efficacy of AEW and NEW in fresh-cut apples and oranges.

AEW and NEW Generation and Characteristics

There are many different types of electrolysis equipment used to produce EW but, in general, they can be divided into those that have a diaphragm and produce AEW and NEW and those that do not have a diaphragm and produce only NEW (Hricova *et al.* 2008). In equipment that produces both AEW and NEW, EW is generated by electrolysis of a diluted NaCl solution in an electrolysis cell where anode and cathode electrodes are separated by a non selective membrane (Kim *et al.* 2000). AEW is produced by the anode and it has a strong bactericidal effect on most known pathogenic bacteria due to its low pH (2-4), its high oxidation-reduction potential (ORP >1000 mV) and its content on active oxidizers like hypochlorous acid (Kim *et al.* 2000; Len *et al*. 2000). It is effective in killing food-borne pathogens *in vitro* conditions and in reducing microbial counts and pathogens in vegetables. NEW is generated like AEW, but part of the product formed at the anode is redirected into the cathode chamber. In the case of a neutral solution (pH 8) the main biocidal agents are HOCl, ClO^-, HO_2 and O_2). Because of its neutral pH, NEW is not as aggressive as AEW to the corrosion of equipment or to skin irritation, and is more stable, as chlorine loss is significantly reduced at pH 6-9 (Len *et al.* 2002).

The physical properties and chemical composition of EW vary as they depend on the concentration of NaCl, amperage level, time of electrolysis, or water flow rate (Kiura *et al*. 2002). AEW and NEW used in our laboratory were produced by an EW generator (Envirolyte EL-400, Envirolyte Industries International Ltd., Estonia). A saturated sodium chloride solution was pumped into the equipment and the current passing through the EW generator was set at 20-23 A. The properties of the treatment solutions obtained are shown on Table 1. The sodium hypochlorite solutions were more alkaline than the NEW and AEW solutions. The ORP values were also inferior which indicated that it contained less oxidizers than NEW and AEW solutions. AEW solutions had the ORP higher than NEW and sodium hypochlorite solutions.

By diluting AEW, its pH slightly increases and ORP shows the opposite behaviour. AEW200 has the lowest pH and higher ORP while the most diluted solution, AEW10, has the highest pH and the lowest ORP. When NEW solutions are diluted, both pH and ORP decreases. This way, NEW200 shows superior pH and ORP than NEW100 and NEW10. However, in the case of sodium hypochlorite solutions (SH), dilution decreases the pH and increases ORP values.

Table 1. Physicochemical properties of tested solutions.

Treatment solution	pH	ORP (mV)	Free chlorine concentration (ppm)
AEW200	2.91±0.02	1131±2	201±1.2
AEW100	3.16±0.04	1097±1	101±2.3
AEW10	3.28±0.02	1072±2	10±1.2
NEW200	7.77±0.02	774±5	199±1.5
NEW100	7.71±0.01	743±2	99±1.2
NEW10	7.64±0.01	738±2	10±0.1
SH200	11.12±0.02	571±1	200±2.4
SH100	10.76±0.01	589±1	102±1.8
SH10	9.97±0.03	598±4	10±0.1

Antimicrobial activity

Antimicrobial activity of EW can be influenced by many factors such as type of EW, exposure time, treatment temperature, pH, ORP and amperage or voltage. Additionally, different microorganisms can be more or less sensitive to the effect of EW.

Several studies have revealed that EW is effective in reducing or eliminating pathogenic microorganisms on minimally-processed vegetables (Abadias *et al.* 2008). Izumi (1999) has demonstrated that electrolyzed oxidizing water can be used for disinfecting fresh-cut carrots, bell peppers, spinach, Japanese radish and potatoes. However, its efficacy on fresh-cut fruits as only been reported on fresh-cut apples by Wang (2006).

Assays carried out in our lab were made to determine the effectiveness of AEW and NEW at 200, 100 and 10 ppm of free chlorine, in killing *Listeria innocua* on fresh-cut apples and *Escherichia coli* on fresh-cut oranges. These treatments were studied in two different washing times, 3 and 5 min. The effectiveness of those solutions was compared with distilled water and sodium hypochlorite solutions at the same chlorine concentrations and during the same periods of time.

Reduction values of *L. innocua* are shown in Table 2 and of *E. coli* in Table 3. In general, for both pathogens, exposure time of 5 min allowed a higher microbial reduction than 3 min. The reduction of *L. innocua* was higher in apples than in oranges, but for both fruits the highest reduction values were achieved with AEW200 treatment (1.70 and 1.10 log cfu g^{-1}). The reduction of *L. innocua* with AEW100 was similar to that obtained with the other sanitizer solutions containing 200 ppm of free chlorine, indicating that the application of AEW, during 5 min, allowed the use of treatments with half concentration of chlorine.

In relation to the population of *E. coli,* the highest reduction was also observed in fresh-cut fruits washed with AEW during 5 min (2.1 log cfu g^{-1}), followed by washes with AEW100 for 3 min and NEW200 for 5 min.

Table 2. Reduction of *L. innocua* population on 1 g of fresh-cut apples and oranges treated during 3 or 5 min with the different disinfection solutions.

Treatment	Time (min)	Reduction (log cfu g^{-1}) in fresh-cut apples[a]	Reduction (log cfu g^{-1}) in fresh-cut oranges[a]
AEW200	3	1.37 bc	0.55 cd
	5	1.70 a	1.10 a
AEW100	3	1.16 cd	0.65 c
	5	1.30 bc	0.72 c
AEW10	3	0.71e	0.29 e
	5	0.81e	0.36 de
NEW200	3	1.26 cd	0.66 c
	5	1.12 cd	0.99 b
NEW100	3	0.80 e	0.45 d
	5	1.08 d	0.65 c
NEW10	3	0.69 e	0.15 f
	5	0.69 e	0.35 de
SH200	3	1.16 cd	0.65 c
	5	1.50 b	0.77 c
SH100	3	1.25 cd	0.35 de
	5	1.11 d	0.59 cd
SH10	3	0.81 e	0.20 f
	5	0.62 e	0.15 f
DW	3	0.72 e	0.21 f
	5	0.78 e	0.25 ef

[a]Values are the mean of 3 experiments with 3 replicates each. Within type of fruit, rows with different letters indicate significant difference between means using LSD ($P<0.05\%$).
Initial counts of *L. innocua* were 1.2×10^6 cfu g^{-1} on fresh-cut apples and 4.5×10^5 cfu g^{-1} on fresh cut oranges.

Table 3. Reduction of *E. coli* population on 1 g of fresh-cut oranges treated during 3 or 5 min with the different disinfection solutions.

Treatment	Time (min)	Reduction (log cfu g^{-1}) in fresh-cut oranges[a]
AEW200	3	1.80 ab
	5	2.05 a
AEW100	3	1.11 d
	5	1.36 c
AEW10	3	0.90 e
	5	0.87 e
NEW200	3	1.31 c
	5	1.70 b
NEW100	3	1.34 c
	5	1.56 bc
NEW10	3	0.85 e
	5	0.93 e
SH200	3	1.20 cd
	5	1.45 bc
SH100	3	1.44 c
	5	1.48 c
SH10	3	0.90 e
	5	0.99 e
DW	3	0.70 e
	5	0.85 e

[a]Values are the mean of 3 experiments with 3 replicates each. Different letters indicate significant difference between means using LSD ($P<0.05\%$).
Initial counts of *E. coli* on fresh-cut oranges were 2.6×10^5 cfu g^{-1}

For both pathogens and fruits, the treatments with concentrations of free chlorine of 10 ppm, resulted in microbial reductions similar to those obtained with distilled water, in almost all the tested situations.

The results obtained in our work indicate that AEW or NEW could be used as an alternative sanitizer to sodium hypochlorite. Park *et al.* (2004) has demonstrated that EW water is very effective for inactivating *E.* O157:H7 and *L. monocytogenes* in a wide pH range (between 2.6 and 7.0), if sufficient free chlorine (>2 ppm) is present. In the assay carried out by Abadias *et al.* (2008), NEW with 89 ppm of free chlorine reduced the bacterial population inoculated on lettuce by 1.2 to 1.5 log cfu g^{-1} and 1.4 to 1.7 log cfu g^{-1} for 1 and 3 min treatments, respectively. Park *et al.* (2001) reported that when lettuce was immersed in EW with 45 ppm of free chlorine for 1 min, populations of *E. coli* O157:H7 and *L. monocytogenes* were reduced 2.8 and 2.4 log cfu $leaf^{-1}$, respectively. Koseki (2004) showed that AEW treatment on strawberries, for 10 min, resulted in a reduction of naturally present aerobic bacteria by 1.6 log cfu g^{-1} and coliforms by 2.4 log cfu g^{-1}.

The results obtained during the present work, showed that the higher microbial reductions were achieved when the tested fruits (apple and orange) were treated with AEW, during 5 min, regardless of the chlorine concentrations.

The reduction values obtained with AEW and NEW at 200 and 100 ppm of free chlorine ranged, for *L. innocua,* from 0.7 to 1.70 log cfu g^{-1} on apples and 0.5 to 1.1 log cfu g^{-1} on oranges. For *E. coli* the reductions obtained varied from 0.5 to 1.7 log cfu g^{-1} on oranges. Studies with *E. coli* carried out in our laboratory allowed reductions in other fruits, such apple (Nunes *et al.* 2010) and pears (data not shown), of more than 2.4 log cfu g^{-1} using AEW at 100 ppm of free chlorine during 5 min. These results were also achieved in other works. Pre-cut produces, treated with electrolyzed oxidizing water by dipping, rinsing or dipping/blowing,

showed a bacterial reduction of 0.6-2.6 log cfu g^{-1} (Izumi 1999). Yang *et al.* (2003) reported that fresh-cut lettuce dipped in electrolyzed oxidizing water (pH 7) with 300 ppm of free chlorine, for 5 min, could reduce 2 log cfu g^{-1} of *Salmonella typhimurium*, *E. coli* O157:H7 and *L. monocytogenes* populations. Results presented by other authors have shown that both electrolyzed oxidizing water containing 200 and 444 ppm of free chlorine significantly reduces the populations of *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* on the surfaces of tomatoes without affecting their sensory quality (Bari *et al.* 2003; Deza *et al.* 2003).

The disinfection washing time may differ depending on the treatment solution and on the type of microorganisms present on the fruits. Wang *et al.* (2007) reported that the microbial population reduction achieved with different sanitizers, including electrolyzed water, was higher for contact periods of 5 min than for exposures of 1 or 3 min. On the other hand, some studies revealed that in the case of vegetables, the increasing of washing time above 1 or 2 min had no effect on bactericidal activity (Adams *et al.* 1989; Beuchat *et al.* 1998). In an assay made by Koseki *et al.* (2000) with AEW treatment on lettuce, the length of treatment time did not increase the reduction of aerobic bacteria population. Park *et al.* (2001) found no significant differences in efficacy based on treatment time when treating lettuce with EW. Abadias *et al*. (2008) also reported that different washing times of 1, 3 and 5 min with NEW or with SH does not affect significantly antimicrobial activity on different fresh-cut vegetables. This difference could be due to the fact that reduction in microbial populations on fresh-cut produce is dependent upon the type of produce (FDA 2001).

Chlorine reacts with organic matter and some components from tissues of cut produce surfaces, such as apple juice, could neutralize some of the chlorine before it reaches microbial cells, reducing its effectiveness which does not occur in vegetables (specially in non cut surfaces). Subsequently, increasing the time of exposure increases the antimicrobial activity in apple slices. Chlorine form present in AEW (HOCl) is more stable than in SH (ClO^-), thus chlorine form present in SH solutions reacts quickly and this could be an explanation why increasing washing time does not increase the reduction of the microorganism population, in washings with SH. Anonymous (1997) reported that HOCL is 80 times more effective as a sanitizer than an equivalent concentration of the hypochlorite ion (ClO^-).

Labbé and Garcia (2001) reported that a longer exposure time is necessary to eliminate microorganisms harboured in porous surfaces (as apple tissues) than in nonporous surfaces. Wang *et al*. (2009) reported that different times are required to achieve the same level of inactivation of *E. coli* in fresh-cut cantaloupe melon and in fresh-cut apple. This reduction has a dual-phasic behaviour with a first fast inactivation phase and a much lower inactivation in a second phase, due to a different accessibility of the sanitizer agents to pathogens. As a result, during the first phase, the loosely attached cells on the superficial areas will be quickly removed when compared to the second phase removal.

Conclusions

The possibility of using AEW or NEW instead of chlorine solutions in the fresh-cut industry may help to improve the safety of the products and workers. Using electrolyzed water in the fresh-cut industry has the advantages of lowering adverse impact on the environment and human health, as no hazard chemicals are added during processing. In addition, it is less expensive than other sanitizing techniques because the only expenses are water, sodium chloride and energy.

Acknowledgements

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35. NOVEL APPROACH TO DECONTAMINATE FRUITS AND VEGETABLES: COMBINED TREATMENT OF PULSED LIGHT AND PHOTOSENSITIZATION

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Abstract

Taking into account that in post - modern society the consumption of ready to eat meals is increasing all over the world, development of novel, more effective non-thermal technologies to increase food microbial control and reduce the risk of foodborne disease outbreaks becomes issue with global dimension. This study is concerned with the development of novel approach to decontaminate fruits and vegetables from pathogenic and harmful microorganisms. To decontaminate surface of vegetables from inoculated pathogen in non-thermal conditions 5.4 J cm^{-2} broad spectrum UV (which UV light? A, B or C?) light dose was used and 1.3 log inactivation was achieved. For photosensitization experiments the vegetables inoculated with pathogen were submerged in chlorophyll salt solution (1.5×10^{-4} or 5×10^{-3} M) for 5 min and afterwards illuminated with 20 mW cm^{-2} (λ=400 nm) light up to the total dose 36 J cm^{-2}. Inactivation of 2.4 log was achieved in this case. Combined treatment of photosensitization and pulsed light reduced the level of inoculated pathogen by more than 4 log, the natural contamination (mesophyls) - by 3.8 log. No effects on inactivation of antioxidant enzymes were detected. Moreover, the significant increase of shelf-life of treated vegetables was observed. In conclusion, fast and effective non-thermal technology might be developed for decontamination of fruits and vegetables for freshly cut or ready to eat meals.

Keywords: Food decontamination, non-thermal, photosensitization, pulsed light

Introduction

Despite the extensive efforts and scientific progress, food safety remains a worldwide problem. Frequently only one technology cannot insure efficient microorganisms decontamination and reduce others food losses. To obtain better result combination of two or more technologies (hurdle technologies) is used (Suparlan & Itoh 2003; Özdemir *et al*. 2005).

This study is focused on possibility to combine two light technologies: high power pulsed light and photosensitization for decontamination of freshly cut vegetables.

Material & Methods

For photosensitization and pulsed light experiments in vitro *Bacillus cereus* ATCC 12826 was grown at 37 °C in Luria-Bertani (LB) medium to the mid-log phase (~ 6×10^7 colony forming units (cfu) mL^{-1}, OD_{540}=1) and collected by centrifugation (10 min, 5000 g), resuspended and accordingly PBS-diluted to ~1×10^7 cfu mL^{-1} final concentration. Aliquots (10 mL) of bacterial suspension with appropriate concentration of chlorophyll-derivative (7.5×10^{-8} cfu mL^{-1}) were incubated in the dark at 37 °C. Afterwards 150 μL aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for 60 s. Light emitting diodes (LED) based light source for (constructed in the Institute of Applied Sciences of Vilnius university) emitted light λ=400 nm with intensity 20 mW cm^{-2} at the surface of samples. Light dose was calculated as light intensity multiplied on time. Then, 100 μL of appropriate dilutions (in 0.9% NaCl) of photosensitized bacterial suspension were placed on LB agar and illuminated with pulsed light (60 s, 1400 V, 0.162 J cm^{-2})

Cocktail tomatoes (*Solanum lycopersicum*) were obtained from a local supermarket and stored at

refrigerator temperature (+6 °C). For inoculation experiments, *B. cereus* ATCC 12826, grown at 37 °C in Luria-Bertani (LB) medium until mid-log phase was harvested by centrifugation (10 min, 5000 g), resuspended and diluted with phosphate-buffered saline (100 mM PBS, pH 7.2) to $\sim 1 \times 10^7$ cfu mL^{-1} final concentration. Cell suspension was then mixed with chlorophyll derivative solution to obtain final 5×10^{-3} M photosensitizer's concentration and immediately used for further experiments. The control samples were soaked in PBS. All samples kept in the dark for 5 min and after inoculum decantation, dried in the thermostat at 37 °C for 20 min. Dried examples were placed in the treatment chamber in a sterile Petri dishes without cover and exposed to light intensity 20 mW cm^{-2} at λ=400 nm for 30 min. After photosensitizing, samples were treated with pulsed light (1400V, 1000 pulses, 5.4 J cm^{-2}). Control samples were not irradiated. After treatment, samples (also control samples) mixed with PBS buffer in sterile bags and homogenized with a BagMixer for 60 s. Then, 100 μL of appropriate dilutions (in 0.9% NaCl) of homogenized tomatoes placed on LB agar. All plates were kept in the thermostat for 48 h at 37 °C. The surviving cell populations enumerated and expressed by log (cfu g^{-1}).

For statistical analysis bacterial populations cfu mL^{-1} were transformed into $\log_{10}$ mL^{-1}. Analysis of variance (Anova) was performed ($P < 0.05$). In addition, Bonferroni tests were performed between means. Each experimental point is an average of 3-5 experiments. A standard error was estimated for every experimental point and marked in a figure as an error bar. The data were analyzed with Origin 7.5 software (OriginLab Corporation, Northampton, MA, USA).

Results

Our previous data indicate that food pathogens *B. cereus*, *Listeria monocytogenes* as well as *Salmonella typhimurium* are susceptible to photosensitization as well as to pulsed light and can be inactivated by 6-7 log. These results prompted us to investigate the efficiency of combination of these two antibacterial treatments in different sequences. Data presented in Fig 1 clearly indicate that combined treatment of pulsed light and photosensitization exhibits synergy in inactivation of food pathogen *B. cereus*. It is important to note that sequence of treatments plays crucial role. For effective decontamination algorithm photosensitization must be used before pulsed light. Otherwise, photosensitizer bleached from pulsed light and was unuseful for photosensitization.

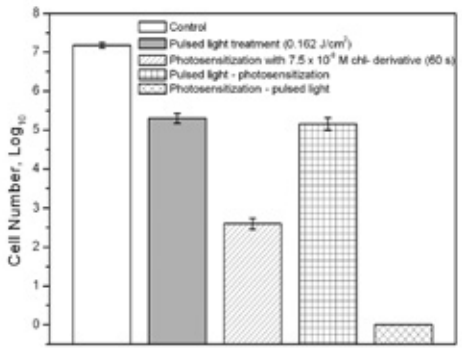

Fig 1. Inactivation of *B. cereus in vitro* by photosensitization, pulsed light and combined treatment.

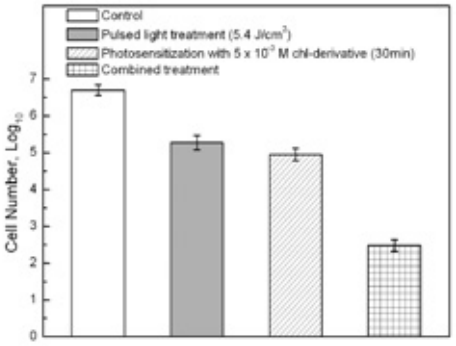

Fig 2. Inactivation of *B. cereus* inoculated on cocktail tomatoes by photosensitization, pulsed light and combined treatment.

Data depicted in Fig 2 show the decontamination of cocktail tomatoes from aerobic mesophyls by photosensitization. It is clear that more than 4 log inactivation is possible to reach, when photosensitization is combined with pulsed light technique. In the next step *B. cereus* was inoculated on the surface of cocktail tomatoes and treated by this combination.

Data obtained indicate that more than 4 log inactivation of *B. cereus* is possible to achieve after combined treatment of photosensitization and pulsed light (Fig 3).

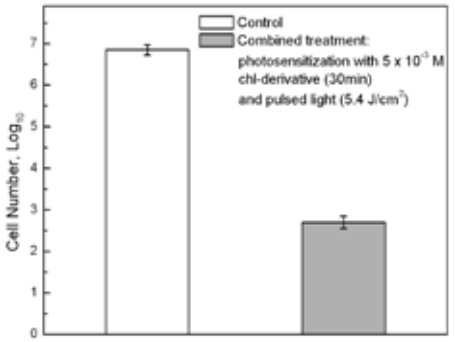

Fig 3. Decontamination of natural contaminants (mesophyls) by combined treatment of photosensitization and pulsed light.

Discussion

The lack of proper sanitation procedures can cost a lot of money for food processing companies. Chemical sanitizers like hypochlorite, iodine, ozone and chloramines are largely used in food industry but these hazardous materials did not ensure its safety and change food chemical parameters. Also, various microorganisms are able to form very resistant forms like biofilms and spores whose are more resistant to chemical treatment then vegetative cells. However, combined treatments of chemical sanitizers and modified atmosphere packaging demonstrate sufficient efficacy on food matrix (Lee & Baek 2008).

Our data in vitro reveals that *B. cereus* decontamination with combined treatment of photosensitization and pulsed light is more effective than individually. Combined treatment reduced the level of inoculated tomatoes by more than 4 log, the natural contamination (mesophyls) - by 3.8 log.

In conclusion, fast and effective non-thermal technology might be developed for decontamination of fruits and vegetables for freshly cut or ready to eat meals.

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36. CRACKED GREEN TABLE OLIVE FROM THE SOUTH OF PORTUGAL: THE INFLUENCE OF DIFERENT BRINING CONDITIONS

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Abstract

In the south of Portugal, cracked green olives represent a traditional way of processing table olives. After harvesting and sorting, the olives are washed, broken and immediately brined (Process A) or submitted to various washing steps with water prior to brining (Process B). The objective of this work was to study the effect of different processing treatments on the evolution of microbiological and physicochemical parameters during the fermentation of cracked green olives at a homemade scale and at different temperatures (25 ºC, room temperature and 18 ºC). Yeasts were found to be the dominant group in fermented cracked green table olives. Process A originated brines without countable Enterobacteria, with higher free acidity and higher phenolic content, when compared to brines resulting from Process B, suggesting that cracking and brining them straight away, represent a preferable way of processing olives which may guarantee the safety of the final product.

Keywords: brines, cracked table olives, *Enterobacteriaceae,* fermentation, yeasts

Introduction

Table olives and olive oil are relevant components of the Mediterranean diet. However, they are also consumed worldwide. The International Olive Oil Council (IOOC 2008) estimates that table olives´ world production reached around 1,823,000 ton in 2006/07 crop year. In the EU, Spain was the leading producer with 499,700 ton, followed by Greece (108,000 ton), Italy (80,000) ton, Portugal (19,200 ton) and France (1,500 ton).

The table olives of greatest importance are the Spanish-type green olives, Greek-type natural black olives and ripe olives produced by alkaline oxidation called Californian style (Garrido-Fernández *et al.* 1997). There are many other traditional or industrial ways of processing table olives according to fermentation conditions (temperature, aeration and salt content) and maturation of fruits (green, turning color or black). Additionally, homemade production of table olives is a widespread practice in Mediterranean rural areas. In some cases, untreated olives are directly brined after harvesting, without NaOH debittering. Once in brine, olives undergo fermentation depending on their variety, temperature, concentration of salt, presence of anti-microbial compounds in fruits and in brines, among others. Fruits are fermented in these solutions until they lose their natural bitterness, at least partially (Garrido-Fernández *et al.* 1997; Panagou *et al.* 2003; Panagou 2006; Arroyo-López *et al.* 2007, 2008a; Hurtado *et al.* 2008). The organoleptic properties of these untreated olives called "naturally green olives" (Garrido-Fernández *et al.* 1997) are different from the lye treated ones, mainly due, to their residual bitterness. Garlic and aromatic herbs are often added, either during fermentation or at the end of the fermentation period.

In the southern part of Portugal (Algarve), one of the most popular table olives are cracked, brined and left to ferment, without NaOH treatment. The production methodology relies on empirical knowledge and the levels of salt and sensorial characteristics are the unique parameters controlled.

The aim of this work was to study the effect of two different traditional ways of processing table olives, produced at a homemade scale, in the microbiological parameters (total viable counts, lactic acid bacteria, yeasts and *Enterobacteriaceae*) and physicochemical parameters (pH, titratable acidity, phenolic content and organic acids) during the fermentation of cracked green table olives. Two different processing methods

were compared: i) Process A where olives were cracked and immediately brined and, ii) Process B where olives were cracked and submitted to washing steps with water before brining. Both cases were studied at room temperature, 25 and 18 ºC.

Material & Methods

Fermentation Conditions

Olives ('Manzanilla' cv.) were hand-picked in the Algarve in October 2008, while their surface colour was green and were transported in the same day to the laboratory in polyethylene bags and kept away from direct sunlight to avoid qualitative losses. On arrival, were selected to remove fruits showing blemishes, cuts and insect damage. After washing with tap water to eliminate dirt, were cracked with a stone machete, washed and placed in screw–capped glass flasks and treated following two different traditional processes: i) Process A: olives were immediately covered with freshly prepared brine (NaCl, 8%, w/v) and ii) Process B: olives were washed every 2 days (d) with water, during one week and brined (NaCl, 8%, w/v) on the day 7. Using this last method, producers can achieve a quicker debitterization of the final product.

Fermentation experiments were carried out, at room temperature, at 25 and 18 ºC, during 25 to 50 d, depending on the temperature. Fermentations were stopped based on the development of the adequate sensorial characteristics and on the overall eating quality. Each treatment was repeated twice.

Microbiological Analysis

Samples of brine were taken under sterile conditions, analyzed immediately after brining (day 1) and at regular time intervals throughout fermentation. One mL of fermenting brine was treated, following the decimal dilution protocol, in sterile ¼ Ringer solution and aliquots were plated, by surface spreading or mixing, in duplicate, on the agar media for the detection and enumeration of microorganisms: a) Plate count agar (PCA) (pH 5) for total viable count, incubated 5 d at 25 ºC; b) Man, Rogosa & Sharpe agar (MRS) (pH 5) with cycloheximide (0.05%) overlaid with the same medium without cycloheximide, for lactic acid bacteria, incubated 5 d at 25 ºC; c) Malt Extract Agar (MEA) (pH 5) for yeasts, incubated 5 d at 25 ºC and d) Violet Red Bile Glucose Agar (VRBGA) (pH 6) and Chromocult Agar for *Enterobacteriaceae* incubated 2 d at 32 ºC. Microbial colonies grown on MRS, MEA and VRBGA were checked regularly for morphological characteristics, Gram staining and catalase activity.

pH and Titratable Acidity

pH brine samples were studied using a pHmetter (Crison, Micro pH2000) and titratable acidity was determined following AOAC's methods (AOAC 942.15 2005).

Determination of Total Phenol Contents

Each brine sample (0.2 mL) was mixed with 1 mL Folin-Ciocalteau's reagent and with 0.8 mL of saturated sodium carbonate solution (7.5%). After incubation at room temperature, the absorbance was read at 765 nm in a spectrophotometer (Genesys 10 UV Scanning, Thermo Electron Corporation, USA). Phenol contents were calculated based on calibration curves of gallic acid and expressed as mg of gallic acid per 100 mL of brine (Huang *et al.* 2006).

Quantification of Organic Acids

Brines were filtered through 0.45 μm filters and the organic acids present (lactic, acetic, malic, citric and succinic) were monitored by HPLC performed with liquid chromatograph equipped with PU-2080 Plus solvent pump (Jasco, Japan), photodiode array detector (PDA) (Jasco, Japan) and Borwin software. A Lachrospher 100 RP-18 with a reversed-phase column (25 cm×4 mm×5 μm) was used throughout this study. Organic acids were detected at 214 nm. The mobile phase was phosphate buffer (KH_2PO_4, 0.2 M) with a pH adjusted

to 2.4 with phosphoric acid, at a flow rate of 0.8 mL min^{-1}. Injection volume was 20 μL, using an injector with a 20 μL loop (Rheodyne, model 7725 (i), Manual Sample injector). Acids were identified by comparison of their retention times with those of pure standards and quantified individually based on standard curves of each standard compounds. The results were expressed as g of organic acid per 100 mL of brine. Each sample was analyzed in triplicate.

Results & discussion

Microbiological Analysis

The production of cracked green table olives in the south of Portugal is a traditional process that has not been previously studied. The microbial populations involved in the process are represented in Fig 1. It should be noticed that the lactic acid bacteria group was not found in the monitored processes (lactic acid bacteria $<$ 10 cfu mL^{-1} of brine). Figure 1 shows the changes in total microbiota, yeasts and *Enterobacteriaceae* in brines of cracked green table olives submitted to two different treatments: Process A: olives were brined immediately after cracking (Fig 1A) and Process B: olives were cracked, washed with water 3 times during 7 d and finally brined (Fig 1B).

During the first 8 d, the total microbiota showed an exponential growth reaching then a stationary phase with counts between 5-7 log cfu mL^{-1} of brine in olives treated by both processes at 25 ºC (Fig 1A1,B1) and at room temperature (Fig 1A3,B3). A similar behavior was observed with yeasts which were present throughout all the experiments. At 18 ºC the total microbiota and the yeast population started an exponential growth after 15 d of the adaptation phase, reaching approximately 5 log cfu mL^{-1} of brine, in Process A (Fig 1A2).

In olives directly brined, at 25 ºC (Fig 1A1) and at room temperature (Fig 1A3) *Enterobacteriaceae* were detected in the beginning of the fermentation and reached levels of 2.5 and 5.8 log cfu mL^{-1} of brine, respectively, after 8 and 10 d. Thereafter, a decline of these bacteria can be observed and no viable counts were found after day 19 at 25 ºC (Fig 1A1) and after day 15 at room temperature (Fig 1A3). A clear decrease of the *Enterobacteriaceae* population was observed in brines prepared according to Process A. Hurtado *et al.* (2008) and Arroyo-López *et al.* (2007) reported an increasing population of *Enterobacteriaceae* in 'Arbequina' table olives and in seasoned 'Manzanilla-Aloreña' table olives, followed by its disappearance, in both cases.

In the case of olives submitted to preliminary washing treatments with water before brining *Enterobacteriaceae* grew since the beginning of the process (0.7-2.8 log cfu mL^{-1} of solution) and survived throughout the fermentation processes, at 25 ºC (Fig 1B1) and at room temperature (Fig 1B3) showing counts between 5.5 and 7.0 log cfu mL^{-1} of brine, at the stationary phase.

When fermentations were performed at 18 ºC, the *Enterobacteriaceae* population was not detected in the case of Process A (Fig 1A2), while in Process B (Fig 1B2) those bacteria increased during the first 10 d followed by a decrease and no viable counts were detected after day 21.

In Process B, counts of *Enterobacteriaceae* were higher except when the fermentation occurred at 18 ºC. Those high numbers of *Enterobacteriaceae* may increase the risk of deterioration due to the production of gas pocket spoilage in the olives' surface and can also originate off flavors (Garrido-Fernández *et al.* 1997).

Yeasts were present throughout all the experiments (Fig 1A1, A2, A3 and B1, B2, B3). At the start of the processes, counts of the yeast population increased from 1.0-2.5 log cfu mL^{-1} of brine (day 1) to maximum values of 5.5-7.0 log cfu mL^{-1} of solution (days 7-9) in both Process A and B (Fig 1) at 25 ºC and at room temperature. Similar yeast profiles have been reported for green and black olives' fermentation by Spanish and Greek researchers (Spyropoulou *et al.* 2001; Tassou *et al.* 2002; Leal-Sánchez *et al.* 2003; Panagou *et al.* 2003; Chorianopoulos *et al.* 2005; Panagou & Katsaboxakis 2006; Arroyo-López,*et al.* 2008a; Panagou *et al.* 2008; Hurtado *et al*. 2008).

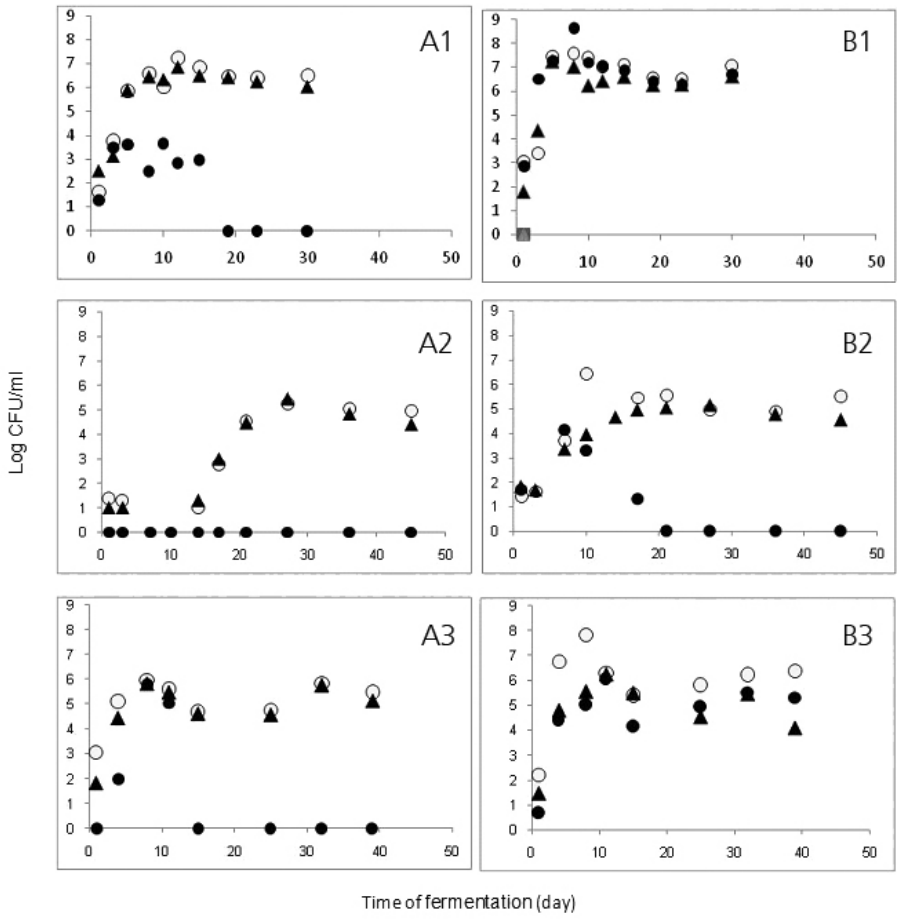

Fig 1. Evolution of microbiota during fermentation of cracked green olives treated according to Process A (immediately brined) (A1, A2, A3) and Process B (washed with water before brining) (B1, B2, B3), submitted to different temperatures, 25 ºC (A1, B1), 18 ºC (A2, B2) and room temperature (A3, B3). Total microbiota (○), yeasts (●) and *Enterobacteriaceae* (▲).

Lactic acid bacteria were not detected in both processes of olive fermentation previously described. The high levels of phenolic contents found in the brines studied in the present work, (especially in Process A), and the NaCl concentration (8%) used, may have interfered with the growth of lactic acid bacteria as described by Medina *et al.* (2008a,b). These authors reported that in the Manzanilla variety of green olives, the presence of substances such as the dialdehydic form of decarboxymethyl elenolic acid, may explain a delay or even lack of lactic acid fermentation during their processings. Additionally, Landete *et al.* (2008) reported that the phenolic compounds sinapic and syringic acids inhibit the growth of *Lactobacillus plantarum*.

Physicochemical Analysis

Fermentations were also monitored through pH and titratable acidity changes represented in Fig 2. The pH values showed an initial drop in the first 8-10 d in all fermentations, at 25, 18 ºC and at room temperature. After this initial decrease, pH continued to drop slowly until it reached a plateau with values of 4.2-4.5 in Process B and of 4.5-4.6 in Process A. The final pH obtained may be slightly high for a fermented product (Garrido-Fernández *et al.* 1997).

The titratable acidity increased during the first 8-12 d in the fermentation where olives were immediately brined (Fig 3). From this point onwards, acidity reached 0.25, 0.20 and 0.35% (w/v), expressed as lactic acid in olives fermented at 25, 18 ºC and at room temperature, respectively. When olives were submitted to a pretreatment with water during the first week before brining, the titratable acidity increased slightly and, after that, remained almost constant during the entire fermentation period, at all temperatures, reaching values from 0.13 to 0.15%, expressed as lactic acid. The washing steps may have caused the removal and loss of nutrients and other substances diffusing from the olives into the brines and the loss of the end products, resulting from the microbes' activity (such as acids).

The diffusion of phenolic compounds from the drupes into the fermentation liquids is represented in Fig 4. After submerging the fruits into the liquids, the concentration of phenolic compounds increased from 0.18-0.30 g L^{-1} to 1.00-1.40 g L^{-1}, after 5-7 d and stayed almost constant in olives directly brined. In the case of olives submitted to a pretreatment with water the level of phenolic compounds increased slowly in the first 5-7 d, due to removals induced by the washings, and slightly increased thereafter reaching 0.30 g L^{-1} in fermentations occurring at 25 and 18 ºC and of 0.40 g L^{-1} at room temperature. The washing treatments in Process B caused a loss of phenolic compounds that diffused from the olives into the washing water, while in Process A brines were richer in phenolics.

The acids studied during this work were lactic, acetic and succinic acids. The organic acids profile found in the brines (room temperature) are represented in Table 1. In Process A, the level of lactic acid increased in the first days and remained almost constant thereafter. In olives treated according to Process B, there was an increase in the concentration of lactic acid. However, the overall concentrations found were lower than those observed in Process A. Acetic acid was produced in Process A, but when Process B was used it was not detected. The differences between Process A and B are explained by the fact that in the second one, the successive water washings may have caused the loss of metabolites, including acids. Low levels of succinic acid were detected, regardless of the processing treatment (Table 1). The presence of the referred acids is also reported in the literature for both green olive and black olive fermentations (Spyropoulou *et al.* 2001; Tassou *et al.* 2002; Montaño *et al.* 2003; Panagou *et al.* 2003,2008; Panagou & Katsaboxakis 2006).

Table 1. Evolution of organic acids during the fermentation of cracked green olives treated according to Process A and Process B, at room temperature. (Organic acids (g 100 mL^{-1} of brine). Values are means ± SD of triplicate assays. ND- Not detected.

Day	Organic acids (g 100 mL^{-1})					
	Acetic acid		Lactic acid		Succinic acid	
	Process A	Process B	Process A	Process B	Process A	Process B
1	0.15±0.090	ND	1.15±0.456	1.12±0.567	0.01±0.004	0.02±0.007
8	0.47±0.004	ND	2.05±0.909	1.31±0.156	0.81±0.005	0.09±0.005
15	1.37±0.001	ND	1.99±0.378	1.02±0.189	0.17±0.099	0.15±0.045
39	2.37±0.034	ND	2.23±0.886	1.13±0.098	0.24±0.048	0.16±0.007

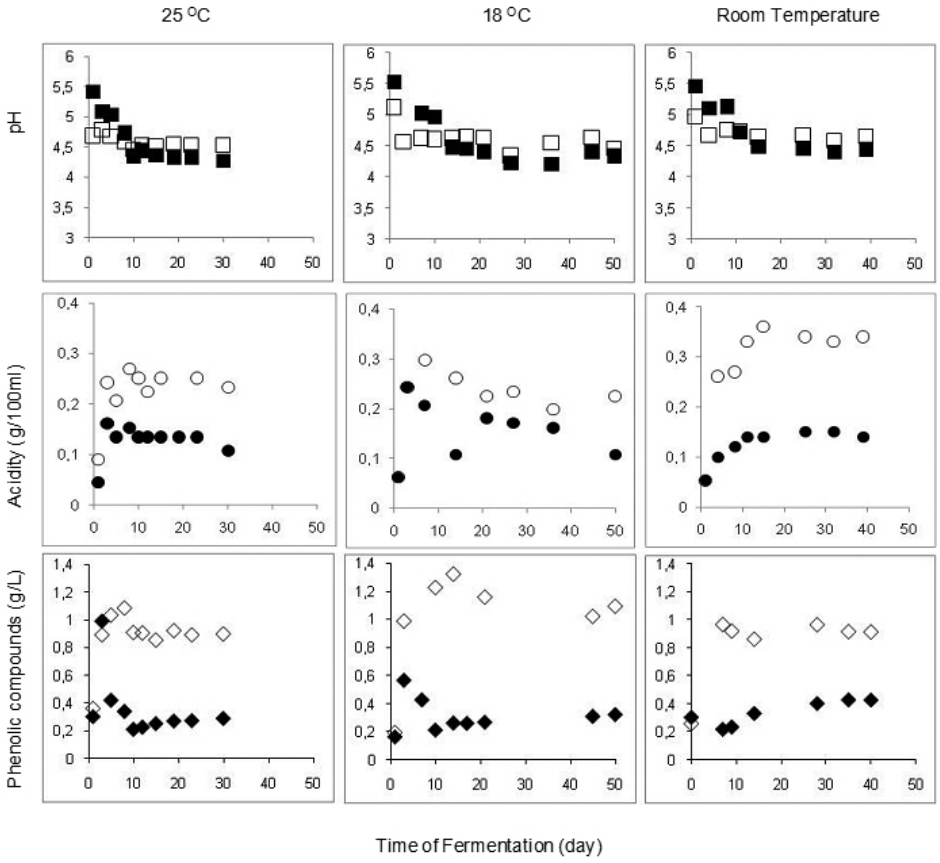

Fig 2. Evolution of brines' pH (□,■), total acidity (% lactic acid) (○,●) and phenolic compounds (◊,♦) during fermentation of cracked green olives treated according to Process A (□,○,◊) and Process B (■,●,♦), at 25 ºC (A), 18 ºC (B) and room temperature (C).

Conclusion

The results presented in the present study are a contribution to understand the fermentation process underlying the production of cracked green table olives. To guarantee the high quality of cracked green table olives, special attention should be paid to the initial brining conditions and the evolution of the fermentation phase. The initial brining conditions affect the pH values, total acidity, phenolic contents, concentration of organic acids and evolution of microorganisms' growth, in particular, of the *Enterobacteriaceae* group and consequently, the safety of the product. Olives immediately brined after cracking originated final products with higher total acidity, higher phenolic contents, higher concentration of lactic and acetic acids, without *Enterobacteriaceae*, suggesting that this process represents a preferable way of processing olives. On the other hand, Process B allowed for a fast debittered final product. However, the loss of acids and phenolic compounds due to the washing steps enable fermentation processes where *Enterobacteriaceae* found conditions to survive and grow, at 25 ºC and at room temperature, representing a risk of spoilage and affecting the safety of the final product. The conditions of the fermentation processes can result in spoilage or survival of pathogens, thereby creating unexpected health risks in this fermented food product.

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37. DECONTAMINATION OF PACKAGING BY ALA-BASED PHOTOSENSITIZATION

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Abstract

This study deals with the development of a novel approach to decontaminate packaging from food pathogens by photosensitization. For this purpose, packaging samples with adhered pathogen were submerged in aminolevulinic acid (ALA solution (3-7.5 mM) for 10 min. Samples were then illuminated with 20 mW cm^{-2} (λ=400 nm) for 5-20 min up to the total exposure of 24 J cm^{-2}. Gram-positive *Bacillus cereus* and Gram-negative *Salmonella enterica* were inactivated, with population reductions of 4.2 and 2.5 log, respectively. Inactivation of Gram-positive *Listeria monocytogenes* biofilms ranged from 1.7-3.1 log. Moreover, our data indicated that the *B. cereus* spores were susceptible to this treatment, with as much as a 3.1 log reduction in spore population observed after ALA-based photosensitization *in vitro* and 2.7 log on the surface of packaging material.

Introduction

Interest in non-thermal processing of food and food-related packaging among scientists, consumers and producers is increasing. This interest is based on the fact that these technologies have minimal impact on the nutritional and sensory properties of foods (Pirttijarvi *et al.* 1996; Wainwright 1998). The aim of this study was to investigate the susceptibility of foodborne bacterial pathogens to novel emerging non-thermal treatment- photosensitization.

Materials & Methods

Bacillus cereus ATCC 12826 and *Listeria monocytogenes* $ATC_{L3}C$ 7644 were grown at 37 °C in Luria-Bertani (LB) medium to the mid-log phase (~6×10^7 colony forming units (cfu) mL^{-1}, OD_{540}=1). Cells were harvested by centrifugation (10 min, 5000 g), resuspended and diluted in phosphate buffer solution (PBS) to give a final concentration of ~1×10^7 cfu mL^{-1}. For *B. cereus* ATCC 12826, spores were prepared by growing the strain for 3 d at 37 °C in brain heart infusion (BHI) broth (Liofilchem) containing (per liter) 0.05 mg manganese until 80-90% sporulation was obtained. Spore suspensions were prepared by washing with sterile distilled water, centrifuging (20 min, 6000 g) and heating to 80 °C. *L. monocytogenes* biofilms were prepared according to the method of Pan *et al. (2006)*. Yellow packing trays cut into 4 cm×8 cm pieces were soaked in 50 mL of suspensions of *B. cereus* ATCC 12826 and *L. monocytogenes* ATCL3C 7644 to ensure pathogen cell adhesion to the packaging surfaces. After inoculation with the pathogens, the packaging samples were dried in a laminar flow hood for 30 min. Samples were incubated in darkness with a 3-10 mM concentration of ALA for different periods (5, 10, 20 min). Control samples were treated with PBS not containing pathogens and incubated under the same conditions.

After incubation with ALA, all packing samples were dried at room temperature for 20 min, placed in the treatment chamber and exposed to light for different times ranging from 5 to 20 min at λ=400nm. The control samples were not illuminated. Then cells, spores or biofilms were washed by mixing with 30 ml PBS separately. Appropriate dilutions of 100 μl (in 0.9 % NaCl) of suspension were placed on LBA plates. The colonies were counted after 24 h incubation at 37 °C. The surviving cell populations were enumerated and expressed as $\log_{10}$ (cfu mL^{-1}) and N/N_0 where N_0 is the number of cfu mL-1 in the untreated culture and N is the number of cfu mL^{-1} in the treated culture. Precision Celsius temperature sensors (Deltha Ohm Italy) were used for temperature measurements.

Results

Data describing ALA-based photoinactivation of *B. cereus* as function of illumination time are presented in Fig 1. Clearly, 20 min illumination was sufficient to inactivate *B. cereus* (4 log population decrease), when with ALA incubation time was 10 min. In order to estimate the decontamination efficiency of ALA-based photosensitization, food packaging material was submerged in *B. cereus* spore solution. Different concentrations of ALA solution (3-7.5 mM) were exploited for experiments. Data shown in Fig 2 show that *B. cereus* spores attached to plastic food-related packaging material and were inactivated by ALA-based photosensitization (2.7 log).

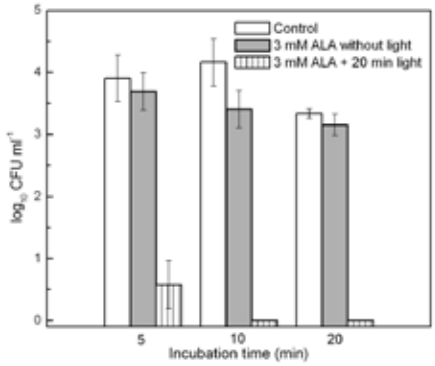

Fig 1. Inactivation of *Bacillus cereus* by 3 mM ALA-based photosensitization onto packaging samples as function of incubation time. Illumination time was 20 min.

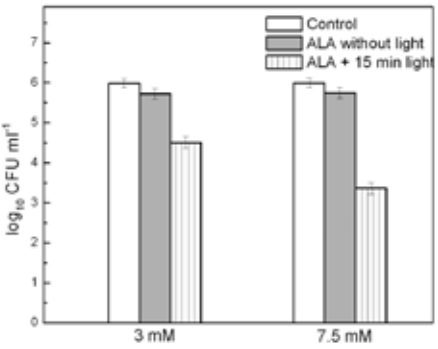

Fig 2. Decontamination of food-related packaging from *B. cereus* spores.

The data, depicted in Fig 3, clearly indicate that the inactivation of *Listeria* cells after photosensitization treatment decreased from 2.3 up to 3.7 log, depending on the ALA concentration used. Our task was to evaluate susceptibility of *Listeria* biofilms to ALA-based photosensitization treatment. For this purpose, bacterial biofilms were adhered on the surface of packaging material. The treatment of biofilm-associated cells by 7.5-10 mM ALA and subsequent illumination reduced significantly the formation of biofilms. Depending on the used ALA concentration (3-7.5-10 mM) inactivation of biofilm-associated cells decreased from 1.7 log to 3.0 log, respectively.

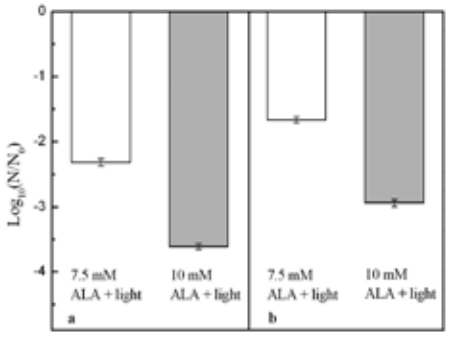

Fig 3. Susceptibility of *Listeria monocytogenes* $ATC_{L3}C$ 7644 to ALA - based photosensitization: cells (a) and biofilms (b) adhered to the surface of packaging material. ALA concentration 7.5-10 mM, illumination time – 15 min, total light dose 18 J cm^{-2}. Control, not treated sample = 0 log (N/N_0).

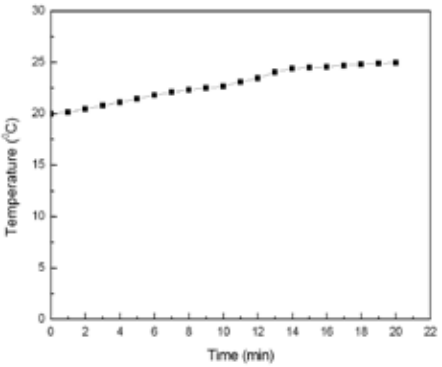

Fig 4. The increase of temperature in the chamber of LED-based light source during 20 min of illumination.

One of our tasks in this study was inactivation by photosensitization in a non-thermal way. For this purpose, precise thermophora were used. Dynamics of temperature inside the chamber was monitered every minute. Data presented in Fig.4 clearly indicate, that the temperature in the chamber of LED-based light source slowly increased up to 24 °C. Some saturation of temperature started from 14 min into the treatment and continued up to 20 min. Even after 20 min, of illumination temperature in the chamber did not exceed 25 °C.

Discussion

Due to very high resistance of bacterial spores to UV (Nicholson 2000), germicidal lamps are insufficient to decontaminate packaging materials. Decontamination of packaging material from *B. cereus* adhered to the surface by this treatment seems promising. More than 4 log inactivation was achieved after ALA-based photosensitization. Moreover, obtained data indicated that the *B. cereus* spores are susceptible to this treatment as well. As much as a 3.1 log reduction in spore population was observed after ALA-based photosensitization *in vitro* and 2.7 log inactivation was observed when spores were placed on the surface of packaging material (Fig 1).

The data presented in Fig 2 demonstrate that inactivation of cells after photosensitization can reach up to 2.3-3.7 log reduction. Inactivation of biofilms by 1.7-3.1 log indicate that this treatment has potential to combat biofilms.

Conclusions

The decontamination of packaging material from adhered *B. cereus* after ALA-based photosensitization reached 4 log. Of importance to note, that spores of *B. cereus* are susceptible to this treatment and can be inactivated by 3.1 log *in vitro* or 2.7 log on the surface of packaging material. Efficient photoinactivation of food pathogens onto packaging materials looks promising and, may be, could serve as a background for the development of a novel non-thermal or hurdle technology for decontamination of foods or food-related surfaces.

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