Master in Advanced Techniques for Research and Development in Food and Agriculture

Escuela Técnica Superior de Ingeniería Agronómica
ETSIA

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Master in Advanced Techniques for Research and Development in Food and Agriculture

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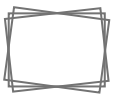

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METHODOLOGICAL SUBJECTS

CHAPTER 1

Techniques in Cellular and Molecular Biology

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1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

In recent years, chromatography has become one of the most powerful and versatile techniques for accomplishing chemical analysis. We can trace the origins of this technique back to the pioneering works of the Russian botanist Tswett who separated pigments present in plant leaves by the end of the 19th century. However, it was not until 60 years after that the basic principles of the technique were established and the technical advances achieved since then (especially in analyte detection) have converted chromatography into what it is today, the most widely used analytical method.

HPLC refers to High Performance Liquid Chromatography (also referred as High Pressure Liquid Chromatography, taking into account the pressure necessary for the system to work). HPLC separations involve a mass-transfer between two phases, the stationary and the mobile phase. In HPLC the mobile phase is always a liquid, while the stationary phase is a solid or a liquid (which coats a solid support) immobilised in a cylindrical column. The mobile phase is forced to pass through the column, and the sample whose components are intended to be separated, is introduced as a solution and it is transported through the column by the continuous addition of mobile phase. This permits the components of the sample to interact with the functional groups in the stationary phase, resulting in relative delays in exit from column (elution) related to interaction intensity of every individual component. It is logical to think that the interaction degree greatly depends on the number of functional groups inside the column, and the latter is related to the stationary phase surface (the higher the surface the higher the number of interactions). So, for maximising the interactions in order to achieve a better separation it is advisable both to use small size particles as stationary phase and pack these particles as much as possible. This, in turn, makes it necessary to operate the system at high pressure in order to keep mobile phase flow, which is what differentiates HPLC from other liquid chromatographies.

1.1 Types of HPLC

HPLC can be conducted exploiting different types of interaction by changing the nature of mobile or stationary phase. Attending the composition of mobile phase, HPLC can be

carried out in isocratic (constant composition) or gradient (variable composition) conditions.

Taking into account the nature of the stationary phase four kinds of HPLC can be distinguished:

- Partition chromatography.
- Adsorption chromatography.
- Ion-exchange chromatography.
- Size-exclusion chromatography.

The two first types only differ in the actual nature of the stationary phase: liquid in the case of partition, and solid in adsorption chromatography.

1.1.1 Partition/adsorption chromatography

This is the most frequently used form of HPLC, especially for non-ionic analytes with relatively low molecular weights (< 3000 amu), and varying polarities. Two modes of operation can be defined regarding the relative polarity of mobile and stationary phase: normal and reversed-phase chromatography.

In normal phase chromatography, the stationary phase is polar in nature, and the mobile phase is non-polar. So, polar compounds will be retained longer than the less polar ones. Reversed-phase chromatography is the opposite of this; i.e. stationary phase is hydrophobic in nature, and mobile phase has a greater polarity. In this case, non-polar compounds will elute later than more polar ones.

Several variants of the basic technique exist that permit, using columns for reversed-phase chromatography, carrying out the analysis of hydrophilic compounds (even ionic species) (Ion Pair Chromatography or Hydrophilic Interaction Liquid Chromatography, HILIC).

1.1.2 Ion-exchange chromatography

The use of this mode of HPLC is practically restricted to ionic or ionisable analytes. Stationary phases are resins modified with cationic or anionic groups. Ionic species in the sample to analyse are retained on stationary phase with different intensity depending on the analyte's nature, charge density in stationary phase, and environment conditions (mobile phase ionic strength and pH, temperature, etc.).

1.1.3 Size-exclusion chromatography

This technique is also called Gel Filtration Chromatography, when mobile phase is an aqueous solution, and Gel Permeation Chromatography, when non-aqueous mobile phases are applied. Despite these names, stationary phases being used are not always a gel. Column packing acts as a molecular sieve in which small molecules penetrate

inside the particle pores, and this retards their elution. Large molecules do not pass through the pores, so they are rapidly washed out, and elute first.

1.2 Instrumentation

The basic HPLC instrumentation includes a pump, injector, detector, and recorder or data system (Figure 1). The process begins by injecting the sample in the column, where separation occurs. Separation of components is achieved by pumping mobile phase through the column. A brief description of some of the main components of the system follows.

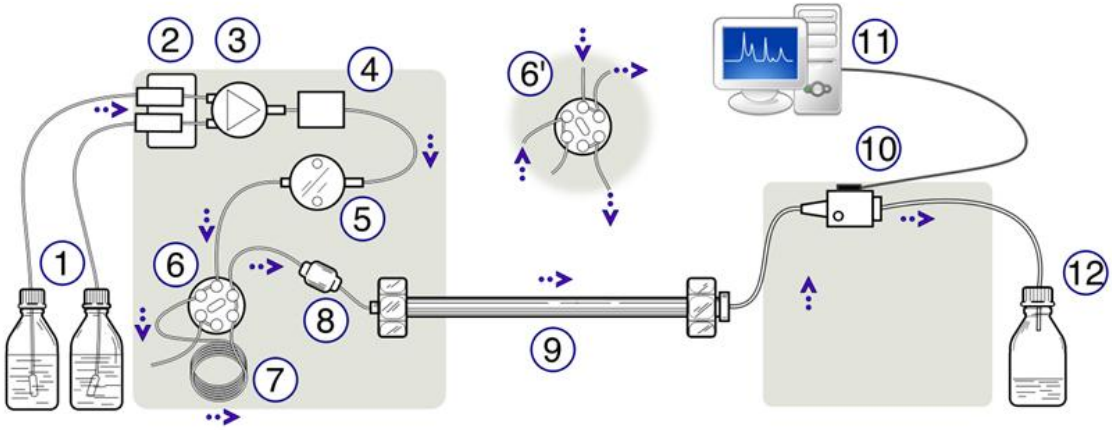

Figure 1. Schematic Diagram of a High Performance Liquid Chromatograph. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column or guard column, (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector. Created by Yassne Mrabet

1.2.1 Pumps

High pressure pumps are necessary to force mobile phase through column packaging. The smaller the particles filling the column are, the higher the pressure to be applied. Depending on the specific application, pumps must reach pressures above 400 atm. The pumping system must be able to provide a stable, and pulsation-free flow (usually in the range 0.01-10 mL/min), and specifications required include high accuracy and reproducibility. Moreover, it has to be constructed with inert and corrosion resistant materials. The HPLC system can operate either at constant pressure or at constant flow, the latter being the most used conditions in analytical applications. Constant flow pumps can be basically of two types: reciprocating piston and displacement pumps.

1.2.2 Injectors

Injectors must be able to introduce the sample in the system without disturbing the existing high pressure conditions. Both manual and automatic injectors exist, and the sample volumes they inject can vary from some nL to several mL.

1.2.3 Columns

The column is the supporting element containing the stationary phase. It must be able to withstand the high pressure developed during the chromatographic operation. Most columns are constructed of stainless steel, although glass, as well as certain types of plastics, is also used, especially for special applications.

The degree to which two compounds are separated is called chromatographic resolution. Two principal factors that determine the overall separation power or resolution that can be achieved by an HPLC column are: mechanical separation power, created by the column length, particle size, and packed-bed uniformity, and chemical separation power, created by the physicochemical competition for compounds between the packing material and the mobile phase. Efficiency is a measure of mechanical separation power, while selectivity is a measure of chemical separation power.

For a given particle size, more mechanical separation power is gained by increasing column length. However, the trade-offs are longer chromatographic run times, greater solvent consumption, and higher backpressure. Shorter column lengths minimise all these variables but also reduce mechanical separation power. For a given particle chemistry, mobile phase, and flow rate, a column of the same length and internal diameter, but with a smaller particle size, will deliver more mechanical separation power in the same time. However, its backpressure will be much higher.

Typical HPLC columns are 10, 15, and 25 cm, although columns with small size are gaining acceptance as ultra fast operation modes have been progressively implemented. Regarding particle size, it can vary from 1 to 10 µm. Apart from packed particle bed, columns can be filled with a polymer with a defined pore size (monolithic columns).

1.2.4 Detectors

Detectors can belong to one of these types:

- Bulk property detectors. These measure properties of mobile phase, as modified, or not, by the presence of analytes.
- Solute property detectors. These measure a property of the analyte that differentiates it from the mobile phase.

Some of the widely used HPLC detectors include: UV/Vis, photodiode array, fluorescence, refractive index, conductivity, light scattering, and mass spectrometry. Today, optical detectors are still the most frequently used in HPLC, although others, like mass spectrometers are gaining acceptance due to the great amount of information they provide. In fact, the use of two coupled detectors (or even more)is not infrequent in order to maximise the information acquired on analytes that are being eluted from column (hyphenated methods).

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2 GAS CHROMATOGRAPHY

2.1 Basis of gas chromatography

2.1.1 Definitions

Gas chromatography (GC) is an analytical technique that separates **gaseous** mixtures into their individual components so that they can be identified and quantified. Liquid or gas-liquid mixtures are previously volatilised within the injector port. The elution is produced by the flow of a **mobile phase** of **inert carrier gas** before entering to the chromatographic column.

The main difference of GC compared with other chromatographic techniques is that the mobile phase only transporting the analyte molecules through the column. However, the mobile phase is not interactioning or reacting with these molecules.

Gas chromatography was used to determine the precise concentration of certain metabolites with physiological interest or as biomarkers of primary and secondary metabolism of plants. This technique is very common for agrifood purposes, for examples in floral development studies, fruit ripening, and studies of senescence of all kind of vegetable tissues. The technique is also used to determine the composition of processed or fresh food, the analysis of pesticides and hormones, etc.

Gas chromatography is an easy-to-use and simple, robust and relatively cheap technique widely used in many applications in the framework of advanced high-throughput screening (advanced phenotyping, metabolomics) for molecular and cell biology analysis. The technique is used for permanent gases (carbon dioxide, nitrogen) but also for simple gas molecules (ethylene, acetaldehyde, ethanol, hydrocarbon products). Although a great deal of the basis of the technique is shared with high performance liquid chromatography, sometimes the techniques are complementary. For example, amino acid analysis can be determined by mean of GC, GC-MS or HPLC-MS with the right preliminary extraction.

2.1.2 Basis components of a gas chromatograph

The essential components of a gas chromatograph are the carrier gas supply systems, the flow/pressure control systems (automatic or manual), the manual and automatic systems for sampling and injection, the injection port (including the system for septum change, the port and the liner), the thermostatised chamber (oven), the column, the detector and the recorder and software for data analysis (qualitative and/or quantitative).

The gas chromatograph can operate in constant flow (balanced with a make-up gas if necessary) or a constant pressure (retention time locking method) at constant temperatures or under temperature ramps. An **electronic pressure control** is essential.

Different extraction systems are essential for the right GC analysis, although the most popularly used are the classical headspace analysis (HS), solid phase microextraction (SPME), extraction by sorption with magnetic stirrer (SBSE), dynamic headspace analysis (DHS) or solid phase extraction (SPE).

A chemical technique known as **derivatisation** is commonly used to volatilise non-volatile compounds (derivates) of liquid samples at the temperature of injection into a reaction's derivate of similar chemical structure (a derivative). Generally, a specific functional group of the compound participates in the derivatisation reaction and transforms the educt to a derivate of deviating chemical properties. The new properties allow quantification or separation of the educt by the GC (modified from Wikipedia).

2.1.3 Innovations

Innovations in gas chromatography are sometimes the combination of improved extraction technologies and detection technologies such as the GC x GC, in order to resolve peak's coelution or reduce the length of analysis. One of the most important ones is fast gas chromatography which is using a thermostatised column system to heat up a column but also to cool down the same column. This system significantly reduces the time of operation and analysis, but a more sensitive detector is required (i.e. analysis round 5 minutes instead of 30 min). Sometimes the technique is not possible coupled with an automatic multisampler.

Other innovations in gas chromatograhpy are the automatic multi fibre exchangers (for SPME), the automatic liner exchangers, the column exchangers (i.e. Dan-switch for capillary flow coupled with a very sensitive electronic pressure control), and the multi-position evaporation station (mVAP) to preconcentrate samples. The goal of these systems is to increase the resolution of the system, to avoid coelutions and to increase sensitivity.

New detectors in the market include the triple quadrupole GC/MS, which is defined as a tandem mass spectrometer consisting of two quadrupole mass spectrometers in series, with a (non mass-resolving) radio frequency (RF) only quadrupole between them to act as a collision cell for collision-induced dissociation (Wikipedia, 2012).

Stir bar sorptive extraction (SBSE) is an extraction technique that utilises glass stir bars coated with polydimethylsiloxane (PDMS) for extraction of organic compounds, in aqueous samples. The same technique for gas samples after forming a headspace is known as HSSE.

Sequential sorptive bar extraction for extraction is also a possibility beyond the typical SBSE using polydimethylsiloxane (PDMS), but new materials are now being tested for selectivity and also a wide range of compounds. On such example is a combination of PDMS with other coatings that can enhance the absorption of polar compounds such as restricted access materials (RAM) SBSE (Mullet and Kwong, 2006). This technique is based on C4, C8, C18 or ion exchange extraction phases located inside the pores of the coating plus the PDMS. Another novel poly(phthalazine ether sulfone ketone) (PPESK) SBSE coating has been described by Guan et al. (2008). Other dual-phase twisters have been proposed such as: PDMS/polypyrrole; PDMS/metachrylate derivates, PDMS/activated carbon or PDMS-ACB; PDMS/poly(vinylalcohol or PDMS/PVA; ethylene glicol(EG)-silicone; polyacrylate (PA) (Barletta et al., 2011; Gerstel website; Pérez et al., 2011).

Sniffing ports coupled to GC-MS also allow the identification of the aromatic notes of certain compounds, particularly if no coelution is present.

2.2 Agrifood applications

The main applications of GC for agrifood purposes comprise analysis of permanent gases (including nitrogen, oxygen, carbon dioxide), and other gases in much lower concentrations in air such as olefins, ethylene, ethanol, etc.

The second main group of applications comprises the analysis of compounds with known volatility such as ethanol, acetaldehyde, esters, ketones, aldehydes, etc. Many of these compounds are to a certain extent responsible for the aroma of fresh produce (fruit, vegetable, flowers) together with fresh or processed animal-derived products (meats, fishes) or vegetable-derived products in a liquid (beer, wine, coffee, tea, oils) or solid stage. Even semi-solid wastes or manures are analysed by GC. Several techniques based on headspace analysis of samples kept in hermetic vials or even containers have been developed. However, some non-volatile compounds such as organic acids, sugars or amino acids can be analysed by GC by using a derivitised form. Examples are given in the course of the analysis of the analytes mentioned above. Additionally, examples of the use of GC-MS and other advances techniques together with advanced techniques such as HSSE or SBSE are given.

Please check the Powerpoint ® presentation for examples.

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3 ELECTROPHORESIS

Electrophoresis is an analytical technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely-used techniques in Biochemistry and Molecular Biology.

When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge, a phenomenon known as electrophoresis. Small, highly charged analytes have greater mobility, whereas large, less charged molecules have lower mobility. Electrophoretic mobility is an indication of an analyte's migration velocity in a given medium. The net force acting on an analyte is the balance of two forces: the electrical force acting in favour of motion, and the frictional force acting against motion. These two forces remain steady during electrophoresis. Therefore, electrophoretic mobility is a constant for a given analyte under a given set of conditions.

Electrophoresis of macromolecules is normally carried out in a porous matrix or gel, which serves as a molecular sieve. The matrix can be composed of a number of different materials, but the most common are agarose, a polysaccharide extracted from seaweed,

and polyacrylamide, a cross-linked polymer of acrylamide. Electrophoresis through agarose gels is the standard method for the separation, identification, and purification of DNA and RNA fragments from about a few hundred to 20 kb (Chawla, 2009). Polyacrylamide gel is the most common matrix for separating proteins, probably due to its versatile applications. Polyacrylamide gels are polymerised from acrylamide monomer, and a cross-linking reagent, usually N,N′-methylenebisacrylamide. The reaction is started with ammonium persulfate (the initiator) and an accelerator, N,N,N′,N′-tetramethylethylenediamine (TEMED). Owing to the fact that oxygen, a radical scavenger, interferes with polymerisation, a proper degassing procedure, to remove dissolved oxygen from acrylamide solutions, is crucial for reproducible gel formation.

The pore size can be exactly characterised by the total acrylamide concentration, T, and the degree of cross-linking, C:

$$\mathbf{T} = [(a+b) \times 100]/V\ [\%] \qquad \mathbf{C} = (b \times 100)/(a+b)\ [\%] \qquad (1)$$

where: **a** is the mass of acrylamide in g, **b** the mass of methylenebisacrylamide in g, and **V** the volumen in mL.

As a general rule of thumb, when C remains constant and T increases, the pore size of the gel decreases. The gel is usually cast between two glass plates separated by a distance of 0.75-1.5 mm. The protein sample is applied to the wells in the top of the gel. A bromophenol blue dye is mixed with the protein sample to both aid sample loading and follow the progress of electrophoresis (Westermeier & Naven, 2002).

Electrophoresis can be performed in two buffer systems: continuous and dis-continuous. A continuous system has only a single separating gel and uses the same buffer in the tanks –electrode buffers- and the gel. In 1964, L. Ornstein and B. Davis proposed a discontinuous system, consisting of two contiguous but distinct gels in order to improve the resolution even using large volume of samples. The two gels are cast with different porosities, pH, ionic strength, and size. The buffer discontinuity acts to concentrate large volume samples in the stacking (upper) gel, and then, proteins, once concentrated in the stacking gel, are separated in the resolving (lower) gel.

3.1 Polyacrilamide gel electrophoresis: SDS-PAGE and native-PAGE

Without any doubt, the **p**oly**a**crylamide **g**el **e**lectrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS-PAGE) developed by Laemmli (1970) is the most common system employed to separate complex protein mixtures. In SDS-PAGE, the protein sample is firstly treated with a reducing agent, 2-mercaptoethanol or dithiothreitol (DTT), to break disulfide bonds, and secondly denatured with SDS, a strong anionic detergent, which disrupts the noncovalent interactions. Most proteins adsorb SDS to a ratio of 1.4 mg SDS/mg protein, which gives the SDS-protein complex a large net negative charge that is proportional to its molecular mass. Thus, charge/size

ratio is equal for all proteins, and separation can be achieved only on the basis of size. Moreover, protein molecular weights can be estimated by running appropriate standard proteins of known molecular weights on the same gel. SDS-PAGE is a low-cost, reproducible, and rapid method for analysing protein purity, for estimating protein molecular weight and the number of polypeptide subunits with a protein (Bollag et al., 1996; Westermeier & Naven, 2002; Chawla, 2009).

Non-denaturing PAGE, also called native PAGE, separates proteins based on their size and charge properties. Since proteins are not treated with SDS or reducing agent, this method is capable of separating molecules which differ by a single unit charge. Native-PAGE should be performed at 0-4ºC to reduce loss of protein activity during the running period (Bollag et al., 1996). Native PAGE is very useful for in-gel catalytic activity assays.

3.2 Isoelectric focusing

In Isoelectric focusing (IEF), proteins are separated by electrophoresis in a pH gradient. Proteins are amphoteric molecules with acidic and basic buffering groups, which become protonated or deprotonated depending on the pH environment (Chawla, 2009). The net charge on a protein is the algebraic sum of all its positive and negative charges. The pH at which a protein carries no net charge (total positive charge equal to total negative charge) is called its ***isoelectric point*** (pI). Below the pI the protein carries a positive charge, and a negative charge at pHs above pI. When protein is placed in a medium with varying pH and subjected to an electric field, it will start to migrate towards the electrode with the opposite charge. During migration through the pH gradient, the protein will arrive at a pH valued of its pI, and stop migrating. If a protein diffuses away, it will become charged again and migrate back to its pI. This is called "focusing effect", which results in very high resolution.

In practice, there are two ways to establish a pH gradient in a gel by amphoteric buffers, the carrier ampholytes, or by immobilised pH gradients in which the buffering groups are part of the gel medium.

3.3 Two-dimensional gel electrophoresis

Isoelectric focusing can be combined with SDS-PADE to obtain very high-resolution separations known as two-dimensional gel electrophoresis or simply 2-D. In this electrophoresis method, proteins are first subjected to IEF using immoblilised pH gradient strips. This gel strip is then placed on top of an SDS-PAGE and electrophoresed to produce a 2-D pattern of spots in which proteins have been separated in the horizontal direction on the basis of their pI, and in the vertical direction on the basis of their size (Berkelman & Stenstedt, 1998; Chawla, 2009). Protein identification is carried out by subsequent MALDI mass spectrometry with peptide mass fingerprinting. The peptide masses of the digested protein are matched with a list of theoretical masses of peptides, which are mathematically derived from the open reading frames of the genome database of a certain organism using bioinformatics (Westermeier

& Naven, 2002). 2D technique has enormous use in proteomics, a word derived from "protein" and "genomics. Proteomics analyse the *proteome*: the composition of all proteins expressed by the genome of an organism.

The proteome is a complex source of biological information that is difficult to analyse. The reasons for this are: (i) protein expression is in constant flux, that is, the proteome is constantly changing in response to internal and external stimuli; (ii) diversity and heterogeneity exist in each proteome, which is largely attributed to (post-)transcriptional and (post-)translational controls; (iii) there is a wide dynamic range of cellular protein expression, which can be as great as six orders of magnitude between the most abundant and least abundant proteins in cells; (iv) the limited dynamic range and detection of separation techniques commonly used in proteomics, such as 2-DE or liquid chromatography (LC)-MS/MS (Ly & Wasinger, 2011).

3.4 Detection of proteins

After electrophoresis, proteins or spots are detected on the gel by using various methods (Coomassie Brilliant Blue, silver staining, zinc imidazol reverse staining, fluorescent staining with RuBPS, etc). The ideal spot detection method in 2D gels should: i) be sensitive enough for low copy number proteins; ii) allow quantitative analysis; iii) have a wide linearity; iv) be compatible with MS; and v) be non-toxic, environmentally friendly and affordable. Unfortunately there is no method that accomplishes all these features (Westermeier & Naven, 2002). In practice the most frequently applied are Coomassie Brilliant Blue, silver and fluorescence staining.

3.5 Western blotting

Western blotting refers to the electrophoretic transfer of the resolved proteins or spots from a polyacrylamide gel to a membrane, like nitrocellulose and polyvinylidene difluoride (PVDF), and their detection with antibody probes. Proteins are transferred from the SDS-PAGE gels, in which all proteins are negatively charged due to the SDS treatment. In an electrical field, these negatively charge proteins migrate towards the positive and become immobilised on the membrane. Protein transfer is usually accomplished by one of two electrophoretic methods: semi-dry blotting and wet blotting. In the former method, the gel and immobilising membrane matrix are sandwiched between buffer-wetted filter papers, and a current is applied for 10-30 min. In wet blotting, the gel-membrane matrix sandwich is submerged in a transfer buffer and current is applied for 90 min to overnight (Bollag et al., 1996). Thus, the membrane has an exact image of the protein or spot pattern of the gel. This blotting is called ***Western blotting***. The entire blot may be stained with a washable anionic total protein dye like Ponceau S to check the quality of protein transfer and to mark the positions of the standards with a pencil. Prior to the addition of antibodies, the membrane is incubated with a blocking agent, such as casein, to prevent unspecific binding of antibodies. Then, the membrane is incubated with the primary antibody, which sticks to the protein of interest and forms an antibody-protein complex. Unreacted antibody is

washed, and the membrane is incubated with a secondary antibody. The second antibody recognises the Fc portion of the first antibody and is coupled to an enzyme or another detectable reagent which produces a coloured product (colorimetric detection) or catalyses a reaction leading to light emission (chemiluminescent detection). Colorimetric detection is typically considered a medium-sensitivity method, about 500 pg of antigen, compared to chemiluminiscent detection, 10 pg of antigen (see Biorad guide).

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4 FLOW CYTOMETRY

Food and bioprocess industries in some instances require fast methods for the detection and assessment of viable cells. Flow cytometry, together with fluorochromes, are very useful tools to fulfil this goal.

Flow cytometry enables to count cells but also to assess their physiological status. It enables to quantitatively measure multiple parameters in thousands of individual cells in very few minutes, while cells flow past a laser beam focussed to the sheath fluid in which the cells are suspended. Results show not only average values of the cell

population, but also its variability, giving an estimation of its heterogeneity and the possible presence of subpopulations. This kind of information cannot be obtained with classical plate counts or biochemical tests.

Flow cytometry started in the 1920s. Former equipments were developed in order to analyse colloidal suspensions, and to detect, count and measure the size of aerosol particles, such as those present in mine powder. In 1970s, large improvements in optics and flourescence allowed for the development of this technique.

4.1 Fundamentals of the technique

A flow cytometer consists of a flow cell, a laser light, detectors and photomultipliers, which transform light photons into an electronic signal (voltage), which can be quantified. It can also be provided with a sorting system for the separation of subpopulations of cells.

The flow cell is a very narrow pipeline, the purpose of which is to force the cells to flow in one single line, one after the other, and pass through the laser beam. Cells are suspended in a sheath fluid, usually a salt solution, and pumped into the flow cell.

The light source is usually a laser. The most common ones are argon lasers of 488 nm. This blue laser excites most of the fluorochromes. Red diode lasers (635 nm) are also used.

The flow cytometer contains lenses and filters to select and direct emission and excitation light. After passing the flow cell, scattered light is gathered through two lenses, one on the way of the laser light, the forward lens, and the other orthogonal to the laser light, the side lens.

Light scattered in narrow angles will be detected by the forward lens and collected into a photodiode. In front of the forward lens, an obscuration bar is placed to stop the laser light. Only light that has been scattered by the cells will avoid the obscuration bar and will be detected. This forward scatter (FSC) is related to the cell size.

Light scattered in wide angles will be detected by the side lens. Side scatter (SSC) gives information on cell complexity and surface structures.

The side lens also detects the fluorescent signals. Between the side lens and the photomultipliers, dichroic mirrors and band pass filters are placed, in order to select and direct the light to the corresponding detector. These detectors gather the light of higher wavelength released by fluorescent molecules present in the cells. These fluorescent substances can be intrinsic (autofluorescence) or intentionally added to the cells (fluorochromes). Common fluorescent light detectors are those for green (FL1, 530 nm), orange (FL2; 585 nm) and red fluorescence (FL3; > 650 nm).

Forward scatter (FSC) is detected with a photodiode, while side scatter (SSC) and fluorescent light are detected with photomultipliers. Both photodiode and

photomultipliers generate an electric signal when the photons impact on them. This electric signal is amplified to a voltage that is proportional to the number of photons. The voltage can be electronically processed. However, cell debris can also generate a voltage. To ignore this voltage, as well as the electronic noise, a threshold has to be fixed. Only signals of voltage higher than this threshold will be recorded.

The results can be analysed in several ways. The most frequently used statistical parameters are total counts, counts in defined gates or regions, mean, median, standard deviation and variation coefficient. Also, there are different ways to show the data: scatter plots, histograms, density plots or tables with statistics.

4.2 Cell sorting

Once a cell subpopulation has been selected through a region of the plot, defined by a combination of parameters, the cell sorter enables to isolate if from the whole sample. There are two different methods to sort cell subpopulations. Sorting can be performed through deflection of electrically charged drops. Flow cell is charged negatively or positively during a very short period when the cell of interest is included in the drop. Then the electrically charged drops are deviated from the main flow stream and collected. The other sorting method is through a piezo-electric system, which deviates a flow collector channel into the flow of the sample, in order to collect the cells of interest. Both methods are quite accurate, but the sorting rate is limited to approx. 300 events per second. Sorted cells can then be examined separately.

4.3 Applications of flow cytometry

Flow cytometry is very useful for counting and analysing cells suspended in concentrations of at least 10^3 cells/mL. Flow cytometry is extensively used in medicine, for counting blood and other cell types. The food industry has also made use of this technique, for example, for counting yeasts in the brewery industry, to detect and count spoilage yeasts in liquid foods (wine, milk, fruit juices, etc.), to check for the viability of starter cultures in dairy products, to detect and count food borne pathogens and spoilage microorganisms or to assess on bacterial spore germination.

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5 SCANNING ELECTRON MICROSCOPY

Electron Microscopes permit the observation and characterisation of materials on a nanometer (nm) scale and were developed due to the limitations of Light Microscopes which are limited by the physics of light to 500x or 1000x magnification and a resolution of 0.2 micrometers.

Electron Microscopes use a beam of highly energetic electrons to examine objects on a very fine scale. This examination can yield information about the topography (surface features), morphology (shape and size of the sample), composition (elements and compounds in the sample their relative amounts) and crystallographic information (how the atoms are arranged).

The Transmission Electron Microscope (TEM) was the first type of Electron Microscope to be developed and is patterned exactly on the Light Transmission Microscope except that a focused beam of electrons is used instead of light to "see through" the specimen. The first Scanning Electron Microscope (SEM) debuted in 1952 with the first commercial instruments around 1965. Its late development was due to the electronics involved in "scanning" the beam of electrons across the sample.

5.1 The electron gun

The first and basic part of the microscopes is the source of electrons. It is usually a V-shaped filament that is wreathed with Wehnelt Cap. Due to negative potential of the Wehnelt Cap, the electrons are emitted from a small area of the filament (point source). A point source is important because it emits monochromatic electrons (with similar energy). The two usual types of electron guns are the conventional electron guns and the field emission guns (FEG).

5.2 Electron-specimen interactions

When an electron beam interacts with the atoms in a sample, individual incident electrons undergo two types of scattering: elastic and inelastic. In the former, only the trajectory changes and the kinetic energy and velocity remain constant (primary or back-scattered electrons). In the case of inelastic scattering, some incident electrons will actually collide with atoms in sample. This interaction makes some incident electrons lose most of their energy in the process (secondary electrons) and displace electrons from their orbits (shells) and places the atom in an excited (unstable) state; excited atoms will relax giving off the excess energy (X-rays, cathodoluminescence and Auger electrons).

5.2.2 Secondary electrons

The secondary electron yield depends on many factors, and is generally higher for high atomic number targets, and at higher angles of incidence. Secondary electrons are

produced when an incident electron excites an electron in the sample and loses most of its energy in the process. The excited electron moves towards the surface of the sample, where it can escape if it still has sufficient energy. Production of secondary electrons is very topography related. Due to their low energy (5eV) only secondary electrons that are very near the surface (<10 nm) can exit the sample and be examined.

5.2.3 Backscattered electrons

Backscattered electrons consist of high-energy electrons originating in the electron beam, which are reflected or back-scattered after interaction. The production of backscattered electrons varies directly with the specimen's atomic number. This differing production rates causes higher atomic number elements to appear brighter than lower atomic number elements.

5.2.4 Relaxation of excited atoms

As was afore mentioned, inelastic scattering, places the atom in an excited (unstable) state. The atom "wants" to return to a ground or unexcited state. Therefore, at a later time the atoms will relax giving off the excess energy. X-rays, cathodoluminescence and Auger electrons are three ways of relaxation. The relaxation energy is the fingerprint of each element.

When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms on the specimen's surface. A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons. The energy of the x-ray is characteristic of the element from which the X-ray was emitted. In practice, EDS X-ray detector is most often used for qualitative elemental analysis, simply to determine which elements are present and their relative abundance.

Cathodoluminescence (CL) is the emission of photons of characteristic wavelengths from a material that is under high-energy electron bombardment.

Auger electrons are electrons ejected by radiationless excitation of a target atom. When an electron from the L shell drops to fill a vacancy formed by K-shell ionisation, the resulting X-ray photon with energy EK-EL may not be emitted from the atom. If this photon strikes a lower energy electron, this outer electron may be ejected as a low-energy Auger electron. Auger electrons are characteristics of the fine structure of the atom and have energies between 280 eV (carbon) and 2.1 keV (sulfur). By discriminating between Auger electrons of various energies, a chemical analysis of the specimen surface can be made.

5.3 Operation

In SEM, a source of electrons is focused in vacuum into a fine probe that is rastered over the surface of the specimen. The electron beam passes through scan coils and

objective lens that deflect horizontally and vertically so that the beam scans the surface of the sample.

SEM works on a voltage between 2 to 50kV and its beam diameter that scans the specimen is 5nm-2µm. The principle images produced in SEM are secondary electron images, backscattered electron images and elemental X-ray maps. Detectors of each type of electrons are placed in the microscope to collect them and the output can be used to produce and image. Secondary and backscattered electrons are conventionally separated according to their energies.

5.4 Advantages and disadvantages

Scanning electron microscopy is suitable for surface topology for every kind of samples (metals, ceramics, glass, minerals, biological samples, plastics, etc). It can also be used for chemical analysis of the sample's surface since the brightness of the image formed by backscattered electrons increases with the atomic number. Backscattered electrons are used to form diffraction images, called EBSD, that describe the crystallographic structure of the sample. In SEM, X-rays are collected to contribute in Energy Dispersive X-ray analysis, which is used for the topography of the chemical composition of the sample.

Consequently, SEM is only used for surface images and both resolution and crystallographic information are limited (because they only refer to the surface). Other constraints are: firstly that the samples must be conductive, so non-conductive materials are carbon-coated; and secondly, that materials with an atomic number smaller than the carbon are not detected with SEM.

5.5 Environmental SEM (ESEM)

Environmental SEM uses differential pumping to permit the observation of specimens at low-pressure gaseous environments (e.g. 1-50 Torr), at high relative humidity (up to 100%) and at higher pressures. In this type of SEM, there is no need for conductive coating; the secondary electron detector operates in the presence of water vapour, and in the microscope's column there are pressure-limiting apertures. The ESEM is ideal for non-metallic surfaces, such as biological materials, plastics and elastomers.

6 DNA SEQUENCE ANALYSIS

6.1 Molecular techniques with relevance for DNA sequence analysis

6.1.1 PCR (polimerase chain reaction)

PCR is an enzymatic method that exponentially amplifies DNA fragments, using two primers complementary to the extremes of the DNA fragment of interest, the enzyme Taq polymerase and dNTPs (deoxyribonucleotides). The amplification requires rapid

changes in temperature between 95ºC for DNA denaturalisation, 50-62 ºC for hybridisation of the primers (Temp. depends on the T_m of the specific primer used) and 72ºC for the DNA synthesis (Fig. 1).

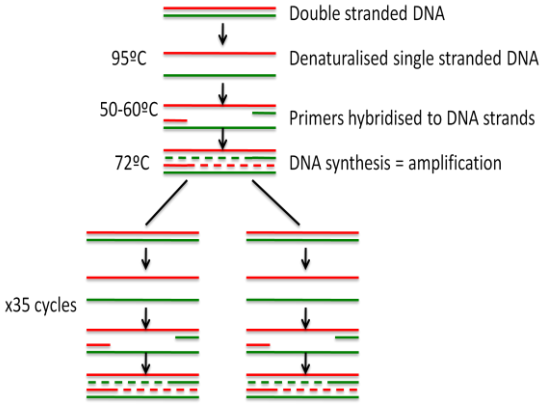

Figure 1. DNA amplification by PCR

6.1.2 DNA gel electrophoresis

DNA fragments can be separated according to their size on gels made out of agarose or polyacrylamide during electrophoresis. PCR products can be analysed on agarose gels stained with ethidium bromide (Fig. 2), while sequencing reactions are run on denaturalising polyacrylamide gels.

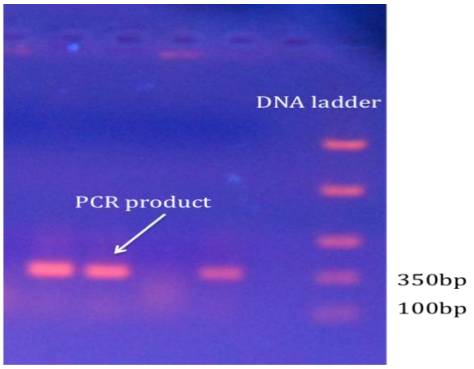

Figure 2. Agarose gel with PCR product

6.2 DNA sequencing methods

6.2.2 Dideoxy sequencing method (F.Sanger and W. Gilbert)

This method is based on the *de novo* synthesis of one DNA strand starting from a primer and using DNA polymerase. The reaction is performed in four tubes containing: primer, dNTPs (A,G,T,C), DNA polymerase and, in each tube, one of the four dideoxy-nucleotides (ddNTPs) in high dilution. During synthesis, the ddNTPs randomly incorporate in the growing DNA strand. Since they are missing the hydroxyl-group at the 3'-end, reaction stops, thus creating a collection of DNA fragments terminating with A,C,T or G. The DNA fragments of different sizes are separated in four different lanes

on the acrylamide gel. The smallest fragments run faster and represent the bases at the 5'-end near the primer (Fig. 3). The dNTPs can be labelled with four different fluorochromes and detected simultaneously by a laser.

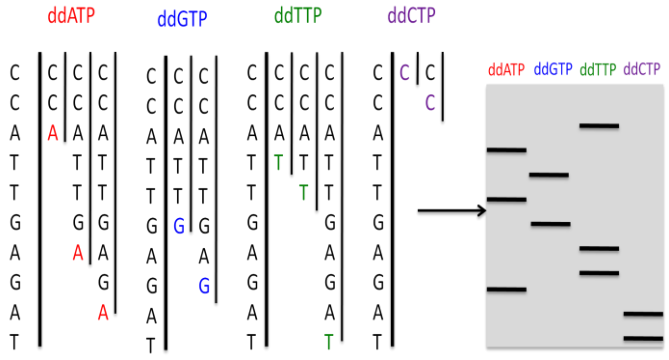

Figure 3. Sanger sequencing

6.2.3 Pyrosequencing (Mostafa Ronaghi and Pal Nyrén 1996)

The method is based on sequencing during DNA synthesis using a primer and DNA polymerase. The dNTPs are added one at a time to the synthesis reaction. For example, the first dNTP to be added is dGTP. If G is incorporated during synthesis, it liberates PPi, forming ATP from AMP. The created energy is used to convert luciferin in oxyluciferin. The light signal is detected in a pyrosequencing machine. Unused dNTPS are eliminated (Fig. 4).

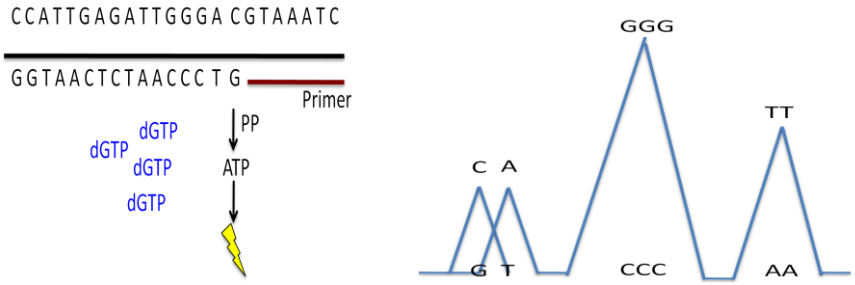

Figure 4. Pyrosequencing

6.2.4 Next generation sequencing - High throughput sequencing

Next generation sequencing permits the rapid and economic sequencing of whole genomes. It includes the following methods

- 454 pyrosequencing (Roche Diagnostics)
- Illumina sequencing (Solexa)
- SOLiD sequencing (Applied Biosystems)

All these techniques are based on 1: fragmentation of DNA; 2: ligation of adaptors to the ends of the DNA fragment; 3: amplification of DNA using as primer a sequence complementary to the adaptor sequence.

In case of 4-5-4 Sequencing (Fig. 5), the amplification of each fragment occurs on the surface of individual micro-beads covered with adaptor sequence in form of emulsion-PCR, creating colonies of identical DNA fragments. The DNA-fragment colonies are than sequenced using the pyrosequencing method.

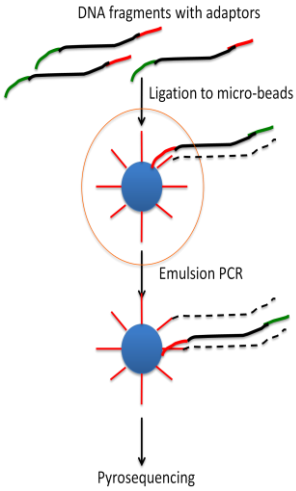

Figure 5. 4-5-4 Sequencing

6.3 DNA sequence analysis by Melting peak analysis and High Resolution Melting (HRM)

6.3.1 qPCR

In qPCR, a specific sequence is amplified during 35-45 cycles from a mixture of DNA fragments using fragment specific primers and the amount of the specific sequence is quantified at the same time. During PCR, a fluorescent compound (i.e. SYBR Green) interlaces within the creating double-stranded DNA. The amount of fluorescence increments exponentially during PCR. A laser detects the amount of fluorescence in each PCR cycle. The beginning of an exponential increase in fluorescence reflects the initial amount of the sequence of interest.

6.3.2 Melting peak analysis

Once qPCR has completed, the PCR products are "melted", rising the temperature from 55 to 95 ºC in small increments. The rise in temperature causes the denaturalisation of the double-stranded DNA and a reduction in fluorescence. A T_m, value is measured (melting temperature) which is sequence specific and thus allows identification of certain DNA fragments. The T_m does not vary in the case of very small differences (SNPs = single nucleotide polymorphism) in the DNA sequence. HRM is a highly sensitive DNA melting analysis, which not only measures T_m but also detects changes in the shape and position of the melting curve caused by small point mutations.

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Online resources:

http://biotech.about.com/od/pcr/a/sequencing.htm

http://pathmicro.med.sc.edu/pcr/realtime-home.htm

http://www.gene-quantification.de/hrm.html

CHAPTER 2

Genomic Tools in Research

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1. BASIC CONCEPTS

1.1 Genome

The genome of a cell is the complete complement of DNA. It includes

–Nuclear chromosomes

–Chloroplasts

–Mitochondria

Genomes are structured in DNA molecules or chromosomes. Chromosomes are called autosomes and sexual chromosomes in those organisms where these distinctions can be made.

Genomes are in most cases encoded by double-stranded DNA. Some viral genomes can be formed by single or double stranded RNA.

Chromosome An organized DNA-protein structure containing part of a eukaryotic genome. Some less precise definitions:

- DNA molecules comprising a genome

Chromosomes can be linear like in eukaryotic nuclear chromosomes, and circular like in bacteria, chloroplast and mitochondria.

1.2 Transposons

Transposable elements are DNA fragments present in the genome in middle to high copy number (hundreds to hundreds of thousands). They originate from viruses that colonized the genome and lost their infection capacity but somehow maintain their proliferation capacity.

There are two types of transposons the so-called type I or retrotransposons. They derive from retroviruses and we can identify a reverse transcriptase in their sequence. They replicate via DNA->RNA-> DNA and reinsertion in the genome. This mode of action (copy and paste) creates an increase of copy number in the genome.

Reverse transcriptase is the enzyme capable of transcribing/copying RNA into complementary DNA or cDNA.

Type II transposons arised from DNA viruses. They replicate by a mechanism of cut and paste in a different position. The enzyme required for transposition is called transposase and in autonomous transposable elements it is encoded by the transposon. In non-autonomous elements, the transposase has been lost during evolution and mobilization requires a trans-acting transposase encoded by another transposable element.

1.3 Microsatellites

Microsatellites are DNA fragments that have sequence repeats. They are also called Simple Sequence Repeats or SSRs or Short Tandem Repeats (STRs). They are useful when surrounded by unique sequences. They are in principle randomly distributed around the genome.

1.4 Gene structure

A gene comprises a regulatory region or promoter. It dictates when, where and how much is it expressed. The promoter is followed by a transcribed region, that is forming the RNA molecule as a result of recruitment of the RNA polymerase to the promoter. We say a gen is expressed when it produces RNA. Thus the product of a gene is a RNA molecule.

A DNA fragment can code for three different reading frame in each direction starting on basepair 1, 2 and 3, (and -1,-2 and -3 in the opposite strand). An open reading frame (ORF) is a fragment, which has the potential to code a protein. There is a debate on the minimal length of coding sequence considered for a sequence to qualify as containing an ORF.

Open reading frames that comprise the origin of a protein usually code for methionine (ATG; AUG in RNA). There are three universal stop codons: TAA, TGA and TAG.

Prokaryotic genes are sometimes arranged in such a way that a single promoter controls several messages. In eukaryotes, a promoter controls only one gene. Furthermore a gene can be interrupted by exons and introns.

The group of fragments that form the final mRNA are the exons and those fragments that are processed during mRNA maturation and cut away are called introns.

2. GENOME MAPPING WITH MOLECULAR TECHNOLOGIES

Getting the sequence of a genome is the first step in genomics, but to do that, having a map is very useful. It tells you where you are in the genome.

Mapping is based on mendelian genetics. Mendelian segregation can be considered as an ideal model that takes into account some assumptions. If these assumptions are correct, then data from segregating individuals should fit to a mendelian segregation. But if they are not, then the assumptions are wrong. And mapping begins.

Mendelian laws describe the results of crossing two pure lines that differ on a single character.

1. Law of uniformity

Crossing two pure lines differing on a single character, results an F1 that should be homogeneous irrespective of the direction of the cross. Differences in a character that can be attributed to a single gene are the result of different alleles. A dominant allele is the one that shows the phenotype in the presence of a second allele. A recessive allele will show its phenotype only when a second allele is not present. Dominance and recessive characteristics are relationships between alleles of the same gene.

2. Law of segregation

In an F2 resulting from a cross of an F1, there is a segregation of the factors (genes) combined in the F1. The F2 comprises ¼ homozygotes similar to one parental, ¼ similar to a second parental and ½ that is similar to the F1 and shows the dominant phenotype.

3. Law of independent transmission and recombination

During the formation of gametes, pairs of factor are independently transmitted, resulting in all possible combinations in a specific relation.

Examples of wrong assumptions, giving wrong results:

1- Crossing A x a gives more than one phenotype in the F1- It indicates that the lines are not pure. There is more than one allele in one of the parentals.

2- The proportion of individuals in the F2 differs from 1.2:1. It means we might be losing one genotype because it is lethal, or we are biasing the process.

3-The combinations appearing have proportions that are different from those expected, with parental phenotypes appearing significantly more often than expected.

In those cases where several genes segregate we can calculate the probability of the different combinations taking into account the percentages in which alleles should appear (3/4 dominant and ¼ recessive). Thus two alleles that are recessive should appear as a combination (aabb) with a probability of ¼+1/4. In those cases when this

prediction is NOT fulfilled, genes are NOT segregating independently. There could be dominance effects between genes known as epistatic interactions.

When two genes are on the same chromosome they are said to be linked. They can separate by a recombination event. Recombination is supposed to be a random even and as such, it is used to measure genetic distance, measured in centiMorgan or cM. One cM corresponds to 1% recombination

The recombination distance between two markers and a third one on the same chromosome is additive. This means we can obtain maps by adding distance between markers. The maximal distance that can be mapped is 50 cM, as longer distances segregate as separate genes.

A molecular marker corresponds to a DNA fragment in one region of the genome.

A marker is polymorphic in a cross or population because we can identify a difference by a given method. A marker can be polymorphic between two individuals, a cross or populations and be fixed (homozygous) and non polymorphic in a different population, cross etc.

A co-dominant marker will produce a pattern giving signal in both parentals, whereas a dominant marker will give signal only in one parental by a pattern of presence/absence.

The degree of linkage of a marker to a gene or chromosome region can be determined by the degree of deviation from a random segregation. A random segregation should give proportions of 1:2:1 for homozygote, heterozygote and homozygote. Differences in proportions obtained in a cross and the ideal one are analyzed by the Chi-square test to estimate deviation from the expected.

Types of molecular markers

Molecular marker types identify DNA mutations that change a base, insert or delete one or more bases

Base changes

The RFLP system (restriction fragment length polymorphism) is based on digesting DNA with a restriction enzyme, that recognizes specific DNA sequences. Thus a single base change might alter in the point of digestion that we can further identify as a new digestion site or a lost site. Restricted (digested DNA) is separated by size on a gel and blotted to a membrane. Fragments are identified with a radioactive probe that recognizes the region studied.

See

http://www.youtube.com/watch?v=CfZkn7D6dro

The system known as CAPS (Cleaved amplified polymorphic sequences) is based on the PCR amplification of a known sequence followed by digestion of the product with a restriction enzyme. The restriction enzyme allows differentiating between two alleles as changes in size are directly detectable on size-based separation procedures.

The modern system of identification of single base changes (Single nucleotide polymorphism o SNP) are basedn on a variety of methods that include CAPS, RFLP, hybridization to a matrix or single base sequencing, where a Sanger-sequencing procedure is developed and single bases incorporated are labeled with different fluorochromes.

Repetitive sequences

Repetitive sequences are in many cases polymorphic and can be detected by PCR using primers designed to anneal in flanking regions of the repetitive element.

The last generation systems like Golden Illumina use hybridization to a matrix of fragments amplified with labeled primers that allow allelic differentiation.

3. PHYSICAL MAPS

Genetic maps are based on percentages of recombination. In contrast, physical maps are based on distances measured in base pairs, and are far more useful.

The first maps developed where based on DNA fragments of defined sizes obtained by restriction digest. The first genomic physical maps of viruses were obtained that way.

The development of Pulse Field Gel Electrophoresis has allowed separating very large DNA fragments cloned in vectors like BACs (Bacterial Artificial Chromosome) or YACs (Yeast Artificial Chromosome).

A collection of genomic DNA fragments of an organism, cloned in a vector is known as genomic library. Genomic libraries can be ordered in contigs. A contig is set of overlapping clones spanning a genome region. Contigs can be developed using markers at the borders of each clone, and lately by digitalized photographic analysis and fragment sorting in silico.

If we obtain a contig spanning a genome fragment we call it minimum tiling path and encompasses the minimal distance to sequence the region.

4. SEQUENCING

From the two differing sequencing technologies developed in the 1980s, Maxam-Gilbert and Sanger, Sanger sequencing became the technology of choice. Sanger sequencing is based on the DNA-polymerase properties that copy DNA fragments in a 5'->3' direction, catalyzing a covalent binding of an OH in a 3' position with a phosphate located in a 5' position of another nucleotide.

The use of a dideoxy nucleotide with an H residue in the 3' causes an irreversible chain synthesis termination. The end of the newly synthesized chain corresponds to the last nucleotide incorporated. This allows using mixtures of dideoxynucleotides that are diluted in such a way that they randomly stop the chain synthesis. The synthesized chains are separated on systems that will discriminate single base sizes. As the dideoxynucleotides are labelled with fluorescent dyes, it allows the automatic reading of the sequence by a laser device.

There are two alternative strategies for sequencing genomes. One is based on sequencing ordered fragments or contigs. The second, called shotgun-sequencing is based on random sequencing of clones followed by in silico assembly of sequenced contigs. In both cases, sequence assembly has as major problem the existence of repeated elements (transposons, retrotransposons, etc). It complicates the true unambiguous determination of some genomic regions where these elements are abundant.

Sequencing can be performed on genomic DNA or using ESTs (Expressed Sequence Tags). An EST corresponds to a RNA that has been transcribed in the sample and retrotranscribed to complementary DNA or cDNA. Sequencing of ESTs allows annotation of exons, introns, regulatory regions and splicing variants with great detail.

Gene annotation is made based on sequence homologies, and it classifies genes according to generic processes like transcription, protein synthesis, signaling, transport etc. In order to perform annotations pairwise alignments are performed between genes or their electronically-determined protein sequence. This is based on the BLAST program that identifies gene homologies with previously annotated genes with a high level of homology.

Newly developed sequencing procedures called Next-Generation Sequencing or NGS sequence millions of reactions on a single shot in parallel. They comprise the so-called Illumina, Ion-Torrent and 454 sequencing. A third generation sequencing is on the way to sequence single cell genomes with high fidelity. The newest generation called PAQ-Bio is already in the market but current error rate is too high to allow proper de novo sequencing.

4.1 Introduction to bioinformatics

BLAST and CLUSTALX

5. PLANT GENOMES

Plant genomes are introduced to review the concepts seen and give an overview applicable to any genome.

Genome sequencing and its interpretation can be made from an evolutionary perspective that allows comparison with previously sequenced genomes and sheds light on newly sequenced fragments via sequence homology and synteny.

Two genes are orthologs if the have a common ancestor sequence, whereas two paralogs are two genes in the same genome that have been produced by gene duplication from a common ancestor.

In closely related plants, we can identify chromosome regions that comprise orthologous genes. In those cases where the gene order is conserved we describe it as regions having synteny or colinearity.

Genome annotation is made by automatic homology sequence annotation, followed in many cases by manual genome annotation and curation. Experts in one type of gene or gene family do this second process.

Genome annotation by functional categories allows having a less dense vision of genomes that includes categories like cell cycle, cell expansion, transport, intracellular transport, signal peptides etc.

6. FUNCTIONAL GENOMICS

Functional genomics is a discipline trying to establish the relationship between genotype and phenotype by massive functional analysis. The first studies in functional genomics were performed using natural variation of characters. In most cases natural variation cannot be explained by a single gene. It is the result of quantitative effects of several loci on a trait. In most cases environmental effects also play a role on the phenotype observed. The number of genes detected will depend on the absolute number of genes in a pathway. The subset of genes identified on a single cross will depend on the specific alleles present in the parentals, but important genes might not be identified if they do not segregate in the cross i.e. they are homozygote in both parentals that share the same alleles.

The sequencing of genomes and EST collections has developed technology allowing estimation of gene expression in parallel for many genes. The microarray system, like northern blotting is based on sequencing of fragments and having them on a physical support like a membrane or a glass. Probes labelled with radioactivity, and lately with fluorescence.

Table 1

Technique	Physical support	Probe	Labelling	Signal
Northern	**Nylon membrane**	**One gene**	**Radiactive**	**Gamma rays**
Microarray	**Glass**	**Library/ genome**	**Fluorescence/ Radiactive**	**Fluorescence**

Microarray experiments are based on hybridizing a glass containing thousands of short sequences corresponding each of them to one gene. This allows exploring differential gene expression in samples. Experiments assess quantitative and qualitative changes in gene expression between two samples that might represent mutant and wild type, or two tissues, or two different physiological responses. It is our imagination and curiosity that will determine what experiment to perform.

Microarrays are designed in such a way that probes will not overlap. This means microarray design should be able to discriminate between paralogs in terms of gene expression. Whitin a microarray part of the experimental design comprises negative and positive controls, clone duplications in certain points that should let assess that hybridization was homogeneous through the glass. Raw data analysis is important to avoid biases.

The major disadvantage of microarray analysis is that it will identify changes on gene expression of those that are represented in the glass, but those transcripts that are rare or very low might not be in. A comprehensive microarray requires a sequenced genome. These type of microarrays should have all the predicted genes in it.

New technologies based on sequencing are becoming the next step in gene expression analysis. SAGE or Serial Analysis of Gene Expression is based on extracting mRNA, making double stranded cDNA, digesting it and ligating small adaptors. Then these fragments are combined in random clones of 25-50 fragments that are sequenced. It allows estimation of gene expression in terms of RNA copies.

The most advanced system is based on massive sequencing allowing identification of gene expressed in a sample and estimate copy number in units that can be of usefor meaningful comparisons. It is generically known as RNA-seq. Bioinformatic analysis of the results can be far more complex than obtaining the sequencing data that is cheap and reliable.

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BLOCK II:

AGRO-FOOD BIOTECHNOLOGY

CHAPTER 3

Plant defence. Biotechnological tools to obtain disease-resistant plants

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1 INTRODUCTION

Since mankind began to "domesticate" plants through the use of agriculture, many advances have been achieved in the control of plant diseases. Through the evolution of our culture technologies during the past twelve thousand years, the principles of management of plants diseases have been built with the progress of our civilisation. The decisions of when, where and what is sown, with the development of specific cultural practices, are based on countless generations of trial and error. Undoubtedly, the successful methods of cultivation were caused by the ability to suppress the development of plant pathogens, even if the farmers had no particular awareness of the underlying biological mechanisms that led to their success. Directly or indirectly, our attempts to manage plant diseases have determined what we eat everywhere in the world.

Nowadays, it is estimated that some 12% of global crop production is annually lost to plant disease. This is because the pathogens seek their own strategies for adapting to new situations, and that genetic adaptability is responsible for the disease. Keeping one step ahead of the pathogens is essential if we want to achieve disease-resistant plants, so we must maintain a close collaboration among technicians in molecular biology and biochemistry, plant pathologists, ecologists and farmers. Therefore, the need for efficient, reliable and affordable disease control measures has never been greater. It is also essential that any new disease control measures maintain crop yield and quality, without harming our fragile environment.

So this course would allow the student to have a general view of the complex interactions that occur between plants and pathogens, the mechanisms of attack by pathogens, the mechanisms of plant defence, the disease resistance signalling, the genetic of resistance, the systemic resistance and the methods to induce resistance in plants in order to have the knowledge to get different ways of dealing with this serious problem to produce crops with durable resistance.

2 LIFE STRATEGIES OF PLANT PATHOGENS

Plant pathogens have developed different mechanisms to attack the plants. First, they must break structural barriers to penetrate inside the cell and overcome defence

reactions, so they can reproduce and infect new cells. Depending on the type of pathogen, their life strategies are different.

- Bacteria enter mainly though stomata or via wounds, and they stay in the intercellular spaces.
- Fungi have mechanisms to enter directly plant epidermis. Biotrophic fungi and oomycetes need living plant cell for surviving, so they can invaginate feeding structures called haustoria.
- Nematodes and aphids feed by inserting a stylet. They also facilitate colonisation by viral, bacterial and fungal pathogens.

These diverse pathogen classes all deliver effector molecules (virulence factors) into the plant cell to enhance microbial fitness.

3 PlANT DEFENCE SYSTEMS

"Plants have evolved with pathogens and insect pests for millions of years. It is therefore not surprising that a particular plant is resistant to most of them" (Keen, 1992).

Plants in natural conditions are subject to a large number of potential enemies (such as bacteria, fungi, viruses, nematodes, herbivores and also other plants). Most plants are resistant to most microbes but how? Unlike animals, which are able to move when they detect the approach of an attack, by their nature plants must develop defence mechanisms against these potential pathogens. These mechanisms (Fig. 1) can be classified into:

- Passive or constitutive
- Active or induce

3.1 Passive resistance

Passive resistance includes:

- Anatomical features: e.g. cuticle, layers of wax and cellulose fibres to prevent pathogen entry. Also each plant cell is surrounded by cellulose wall.
- Preformed inhibitors. Plants produce metabolites toxic to potential pathogens, e.g. phenols, alkaloids, tannins, anti-microbial proteins.
- Signals. Plants have the wrong surface structure/lack of appropriate chemical signals to stimulate pathogen developmental.
- Innate immunity. Plants have an innate defence response that allows them to recognise microbes as 'non-self' and defend themselves.

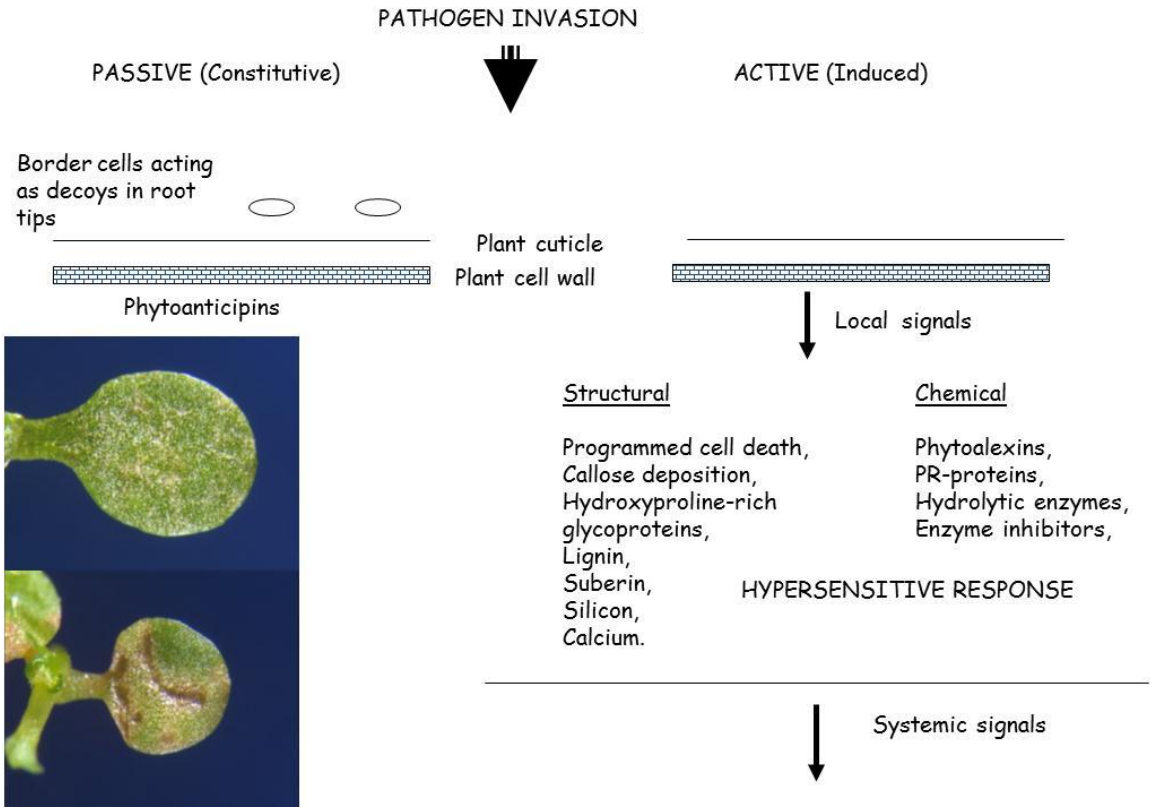

Figure 1. Examples of passive (left-hand side) and active (right-hand side) defence mechanisms in plants (reproduced courtesy of Dr. Matthew Dickinson from Nottingham University, UK).

3.2 Induced resistance

Plants activate a variety of defence responses rapidly at the site of pathogen invasion. The expression of disease resistance is a two phase reaction involving: recognition of the pathogen and response of the host. Among host responses we should highlight the following ones.

3.2.1 Local signals

One of the first responses activated in plants as part of resistance is the production of ion fluxes, reactive oxygen species (ROS), production of nitric oxide (NO) and phosphorylation cascades.

3.2.2 Programmed cell death (pcd)

One of the most visible responses of plants to pathogen attack is the hypersensitive response (HR), which is a localised cell death programme that prevents pathogen spread. This is a temporally and spatially co-ordinated mechanism to limit the amount of host tissue lost to the pathogen. Programmed cell death (pcd) is a general term used to describe this induced cell suicide.

3.2.3 Induced structural barriers

Plants have a number of inducible structural defences. The first of these is cytoskeleton-based to fend off attack from potential pathogens prior to penetration, and involves sensing of the developing pathogen on the surface. At the site of contact, there is accumulation of cytoplasm, along with rearrangements of the plant microtubules in the cytoskeleton. Plant defence proteins such as osmotin and chitinase associate with the actin cytoskeleton to stabilise the actin filaments. Appositions referred to as papillae, consisting of callose (a glucan polymer) and phenolics, may form on the inner surface of cell walls. There is also deposition of hydroxyproline-rich glycoproteins (HGRP), phenolic compounds such as lignin and suberin, and minerals such as silicon and calcium, and these become cross-linked to form an additional barrier against pathogen invasion and ingress. Peroxidase enzymes also have a major role in cell wall strengthening and modify plant cell walls through oxidative cross-linking of two soluble proteins, a hydroxyproline-rich glycoprotein (HGRP) and a proline-rich protein (PRP).

3.2.4 Phytoalexins

They are antimicrobial compounds that include a diverse array of low molecular weight secondary metabolites. They include terpenoids, saponins, indoles, phenolics, flavanoids, and sulphur-containing compounds including inorganic elemental sulphur. Many genes in the pathways to produce phytoalexins, have been shown to be regulated in a co-ordinate fashion in response to pathogen invasion, indicative of an important role in defence.

3.2.5 Pathogenesis-related proteins

Various novel proteins are induced during pathogen attack, known collectively as the pathogenesis-related (PR) proteins. These proteins are expressed at low levels in healthy plants, but certain isozymes are induced only during pathogen attack. Evidence currently suggests that these proteins collectively act as a secondary line of defence against invading pathogens, with a role in degrading the invading organism once it has already been contained.

The proteins induced have been grouped into fourteen PR-classes, although not all are induced in all interactions or in all plant species. The role for many PR proteins has been determined. The chitinases are presumed to hydrolyse chitin in fungal cell walls. Glucanases, proteases and RNases are assumed to have similar roles as hydrolytic enzymes against bacteria, oomycetes, and viruses. Some PR-1 proteins have been shown to inhibit the growth of oomycetes, although their exact biochemical role has yet to be elucidated.

4 GENETIC BASIS OF PLANT-PATHOGEN INTERACTIONS

As we said, plants have evolved the ability to recognise and respond to particular pathogen molecules, leading to rapid activation of defence responses. The induced

responses occur at two levels: recognition of microbe or pathogen associated molecular patterns (MAMPs or PAMPs that include flagellin, lipopolysaccharides, chitin, beta-glucans, ergosterol, bacterial cold shock proteins) and recognition of effectors. Recognition of PAMPS occurs on plant cell surface (apoplast) whilst recognition of effectors occurs normally in the plant cell cytoplasm.

In non-host plants, induction of an innate immune response by non-specific elicitors (MAMPs or PAMPs) may be sufficient - PAMPs triggered immunity (PTI). In host plants, the pathogen may have evolved to evade innate responses or trigger them later in infection, so that they are ineffective, and specific resistance genes may be required for resistance - Effector triggered immunity (ETI).

4.1 Horizontal versus vertical (gene-for-gene) resistance

ETI may involve one or a few genes whose individual effects can be easily detected (gene-for-gene/vertical resistance) or numerous genes with small additive effects (quantitative/horizontal resistance) (Fig. 2).

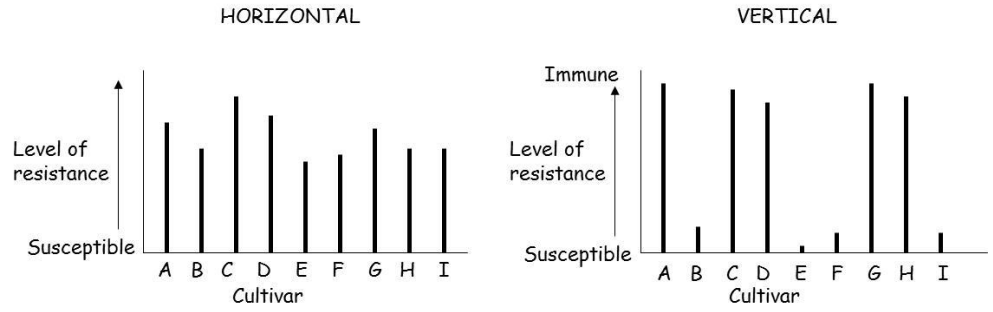

Figure 1. Examples of different cultivars showing two types of resistance horizontal and vertical (reproduced courtesy of Dr. Matthew Dickinson from Nottingham University, UK).

In horizontal resistance, numerous genes have small additive affects so that the resistance varies by relatively small amounts between the different cultivars, each having a moderate level of resistance. On the other hand in vertical resistance controlled by single genes, resistance is either close to complete immunity if the gene is present or complete susceptibility if it is absent.

4.2 Gene-for-gene resistance

The model explains for how the host resistance mechanism works, through a dominant resistance (*R*) gene in the plant encoding a product that recognises a pathogenicity factor (product of a dominant gene) in the pathogen to confer resistance. Support for this model was originally developed by Farrer in the 1890s, who described resistance in wheat against rust as following Mendelian genetics, followed by Biffen in the early 1900s who demonstrated that resistance was a dominant monogenic trait. In the 1940s, Flor, working on flax / flax rust in the USA, and Oort, working independently on wheat

/ wheat smut in the Netherlands, showed that it was possible to discriminate between different genotypes in the pathogen population by using different resistance genes in the plant population.

R genes have been identified in plants for resistance against bacteria, fungi, viruses, oomycetes and nematodes, and a simple model to explain this concept is the elicitor / receptor model. The gene-for-gene theory is based on:

- The pathogen has a gene (avirulence gene or avr) encoding a product 'effector', which is recognised by a 'receptor' encoded by a plant resistance gene (R).
- This recognition process triggers the 'hypersensitive response.
- The plant resistance gene is normally dominant.

4.2.1 The race to clone resistance genes

During the late 1980s, and early 1990s, there was a concerted effort to be the first to clone a resistance gene. Main methods used were transposon tagging or chromosome walking, mainly in tomato and *Arabidopsis*. The first one identified was in 1992 in maize against *Cochliobolus carbonum* (Steve Briggs, Pioneer Hi-Bred). However, this pathogen is a necrotroph and the *R* gene encodes a toxin reductase. The first *R* gene against a biotroph was cloned in 1993 by Greg Martin and Steve Tanksley by chromosome walking: *Pseudomonas syringae* pv.*tomato* (bacterial speck). Within a year, a number of others had been cloned, including tomato *Cf-9* (Jonathan Jones, Norwich), Flax *L*, (Jeff Ellis, Canberra), and Tobacco *N*, (Barbara Baker, California); all these were cloned by transposon tagging.

Now it is possible to clone resistance gene analogues by PCR – approx. 2% (400) of the genes in *Arabidopsis* are *R*-gene like. In addition, synteny between closely related genomes such as rice and sorghum has facilitated the cloning of resistance genes.

The *R* genes cloned to date have been grouped into a number of classes based on sequence relationships. The largest class encodes a nucleotide-binding site plus a carboxy-terminal leucine rich repeat domain (NBS-LRR), and this group makes up at least half (approx 150) of the *R* gene candidates in *Arabidopsis*. The group can be subdivided into a group that possess a Toll/Interleukin-1-receptor homology (TIR) region, and a group that lack this, although some of these may contain coiled coil (CC) domains. Interestingly, the TIR subgroup is very rare in the grasses but is predominant in *Arabidopsis*. The other classes of *R* genes are more diverse.

5 SYSTEMIC PLANT DEFENCE RESPONSES. SIGNALLING

Following the hypersensitive response, salicylic acid (SA) and other potent signalling molecules are synthesised that induce systemic forms of resistance in distal tissues. Wounding and attack by necrotrophic pathogens may also result in jasmonic acid (JA) /

ethylene–based signalling pathways, that can act antagonistically to the SA pathways, and there is evidence of volatile signals being transmitted to adjacent plants.

5.1 Systemic acquired resistance

Systemic acquired resistance (SAR) is one form of inducible resistance activated throughout the whole plant that is long-lasting and can be boosted by repeat infections. It can be effective against organisms other than the one used to stimulate the initial response. SAR has been demonstrated in many plant species, including bean, tomato and *Arabidopsis*, upon induction by bacterial, fungal and viral pathogens. The spectrum of pathogens against which systemic resistance is effective varies between species and it can therefore be considered to provide a characteristic "fingerprint of protection". For example, tobacco cannot be protected against challenge by *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato*. The nature of the resistance has led to the concept that SAR acts to prime host cells for a more rapid future deployment of defences.

Early experiments on systemic acquired resistance in the late 1970s indicated that the addition of salicylic acid (SA) to plants induced the same pathogenesis-related (PR) protein expression fingerprint as occurs in SAR, and gave the same spectrum of resistance. Structurally related chemicals such as 2,6-dichloroisonicotinic acid (INA) and benzo-(1,2,3)-thiadiazole carbothioic acid-S-methyl ester (BTH) have been shown to have the same effects as SA. SA and its conjugated glycoside derivative (SAG) have repeatedly been shown to accumulate to high levels, in both local and systemic tissue, after primary infection of several plant species, and accumulation has also been observed in the phloem. However, it is now believed that the mobile signal through the phloem is methyl salicylate.

There are a number of systemic resistance responses in which *PR* gene activation does not correspond to enhanced levels of SA, and also in which the set of *PR* genes activated are different. Furthermore, these responses also occur in *NahG* transgenic plants (*NahG* encodes the enzyme salicylate hydroxylase, that converts SA into inactive catechol). These include induced systemic resistance (ISR).

5.2 Induced systemic resistance

Induced systemic resistance (ISR) is similar to SAR, in giving protection to normally virulent pathogens. However, rather than induction by phytopathogens, ISR is activated by plant growth promoting rhizobacteria, and it is often PAMPs such as lipopolysaccharides in the bacterial cell wall that are the elicitors of this response. It also appears to provide protection against pathogens such as *B. cinerea* and *Alternaria brassicicola*, for which SAR is ineffective. Evidence suggests that jasmonic acid, methyl jasmonate, ethylene and also abscisic acid play an important role in these additional systemic defence responses.

Some necrotrophic pathogens induce a further form of systemic resistance independent of SAR and ISR.

5.3 Communal resistance

In addition to systemic signalling within plants, plants can communicate with neighbouring plants and induce the activation of defence genes in these. Volatile signals such as methyl jasmonate and methyl salicylate are produced from insect and pathogen infested plants and, in laboratory experiments at least, have been shown to induce defence gene expression. For example, methyl jasmonates released from big sagebrush (*Artemisia tridentate*) following insect feeding were able to induce production of proteinase inhibitors in adjacent tomato plants, and reduce the numbers of insects feeding. Similarly, methyl salicylate released from TMV-infected tobacco plants containing the *N* resistance gene induced *PR-1* expression in adjacent plants and increased resistance to disease. Interestingly, the volatiles produced when herbivorous insects feed on plants can act as attractants for predatory insects that feed on these herbivorous species, and this is being developed into a means of controlling insect infestations on plants.

5.4 Interplay of downstream signalling pathways

There are numerous downstream signalling pathways that lead to systemic defence responses as well as responses in neighbouring plants. How these pathways are integrated and how they result in induction of the different fingerprints of PR proteins, defensins, thionins and proteinase inhibitors that are characteristic of the different mechanisms is complex and not fully understood, but mutational analysis, principally in *Arabidopsis* is helping to position specific genes in signalling cascades and hierarchies.

5.4.1 The EDS1 and NR1 pathways

Mutation analysis has indicated that TIR-NBS-LRR type *R* proteins are strongly dependent on *EDS1* and PAD4 (transcription factors) in *Arabidopsis*, whereas most CC-NBS-LRR type R proteins are dependent on *NDR1*.

5.4.2 The role of NPR1

Studies point to a central modulating role for *NPR1* in both SA-dependent (SAR) and SA–independent (ISR) processes that are clearly influenced by the nature of the input signal. *NPR1* interacts in a yeast two-hybrid assay with two transcription factors, suggesting a direct link between *NPR1* activity and downstream *PR-1* activation. A further gene product identified in the induction of *PR* genes is *SNI1*. This is a negative regulator of transcription, and it appears that *NPR1* works by removing this transcriptional blocker in order to stimulate gene expression.

Induced systemic resistance (ISR) also requires functional *NPR1*, although this mechanism requires JA and ethylene and is independent of SA. This *NPR1*-mediated process is not associated with transcriptional induction of SAR-type genes nor the JA / ethylene resistance response genes generated through necrosis-inducing pathogens, suggesting that either accumulation of JA and ethylene is below the threshold needed to induce expression of these marker genes, or that other as yet unknown genes are induced.

6 BIOTECHNOLOGY IN CROP PROTECTION

Pathogens are constantly evolving to overcome resistance mechanisms, so plant breeders have to continually search for and deploy new resistance genes or new strategies of control to keep ahead of the pathogen and maintain a durable broad spectrum resistance.

There are many biotechnological strategies to active plant defence mechanisms, although some of them keep the active defence pathways in the absence of the pathogen and this could reduce crop yields.

6.1 Manipulation of R gene

Introducing an *R* gene (transgene) thereby confers on the plant the ability to recognise the pathogen and mount an effective defence. E.g. transformation of tomato plants with *Bs2* gene of pepper led to resistance to bacterial spot disease.

6.2 Expressing a pathogen component in the plant

Introducing a transgene in the plant which express elicitor molecules that the plant can recognise and then activate a full defence response. E.g. transgenic tobacco expressing the coat protein gene of tobacco mosaic virus (TMV) was resistant or tolerant to TMV.

6.3 Induce resistance-based on natural defence mechanisms

Over-expression of genes related with defence mechanisms, such as those related with biosynthesis of hormones (SA, JA, ET), master-switch genes (transcription factors, protein kinases), protein related with the pathogenesis genes. E.g. overexpression of *NPR1* in rice conferred more resistance to *Xanthomonas* and *Magnaporthe grisea*; expression of lysozyme in potato increased the resistance to *Erwinia carotovora*.

RNAi is a natural defence mechanism in plants, and a useful tool inhibiting the expression of pathogen genes at both transcriptional and post-transcriptional levels. E.g. transgenic coat protein-protected in papaya gave commercial plants resistant to ringspot virus (PRSV) in Hawaii.

6.4 Induce resistance on the basis of antagonistic microbes

Numerous bacteria and fungi are known to possess an antagonistic capacity against other microorganisms. Such antagonism is manifested in many different ways, including inhibiting the development of the pathogens responsible for causing plant diseases. The genus *Trichoderma* possesses good qualities for controlling diseases in plants caused by pathogenic soil fungi. Different species of *Bacillus subtilis* have been incorporated into some products as biological control, which is effective against a wide range of fungal pathogens.

The biotechnological approaches solve many problems; however it is necessary to weigh the market benefits against the costs involved in the development / registration methods. Although, there are still many dissonant voices against genetically modified plants a promising route is open to achieve increased disease resistance.

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BLOCK III:

TECHNOLOGY AND

ENGINEERING OF PLANT PRODUCTION

CHAPTER 4

Soil Degradation and Regeneration in Semiarid Areas

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1 BASIC CONCEPTS

1.1 Soil functions (Blum and Santelises 1994)

- Ecological functions: - Biomass production (food, energy, etc.); - Buffer capacity to filter/immobilise/transform pollutants; - Ecological habitat and genetic reservoir for plants and animals.

- Functions related to human activities: - Support for industrial and technical structures and other socioeconomic activities such as houses, transport, waste management, etc.; - Source of raw materials (water, clay, sand, minerals, etc.); - Part of our history and heritage (e.g. archaeology).

1.2 Soil degradation

This is a process that decreases the actual and potential capacity of the soil to qualitatively and quantitatively produce goods and services (FAO-PNUMA, 1984). The main types of soil degradation are: biological, physical, chemical (mainly pollution and salinisation).

1.3 Soil desertification

There have been several definitions of soil desertification since 1997 up to today.

- UN Secretariat of the Conference on Desertification (1977): A process that decreases or destroys the biological potential of the soil, which may lead to desertic conditions.

- PNUMA (http://www.pnuma.org/deramb/Chapter12Agenda21.php): A process which decreases soil yield and the value of the natural resources in arid, semiarid, temperate and dry areas, as a result of climate changes and human activities.

- UNCED (1992): Desertification refers to land degradation in drylands (arid, semiarid and dry sub-humid areas) as a consequence of human activities or climatic changes. Here, the term “land” means the terrestrial bio-productive system and “degradation” the diminution or destruction of the biological or economic productivity of the land.

Causes of desertification. Desertification is caused by a combination of factors that change over time and vary by location:

- Indirect factors. Population pressure, socioeconomic and policy factors.

- Direct factors. Land use patterns and practices and climate-related processes.

The **resistance of the land to degradation** includes two concepts:

- Persistence. Capacity of the system to keep system parameters within certain thresholds when environmental factors change.

- Elasticity (resilience). Capacity of the system to recover its former parameters after suffering an impact.

2 EVALUATION OF SOIL DEGRADATION

FAO-UNESCO-PNUMA recommends to measure changes in specific soil parameters to evaluate each soil degradation process:

- Physical degradation of fertility: increase in bulk density (g/cm^3/year); decrease in permeability (cm/h/year).

- Acidification: decrease in cation saturation (%/year).

- Salinisation: increase in electrical conductivity (saturated extract at 25ºC, dS/m/year).

- Sodification: increase in exchangeable sodium (cmolc/kg/year).

- Toxicity: increase in toxic elements (ppm/year).

- Biological degradation of fertility: decrease in humus (%/year).

- Water and wind erosion: loss of soil (t/ha/year).

- Contamination: increase in pollutants (%/year or ppm/year).

3 THE EUROPEAN STRATEGY FOR SOIL PROTECTION

Objectives (http://ec.europa.eu/environment/soil/three_en.htm):

- Identification of the problem. Member States will identify the areas where there is a risk of erosion, decline in organic matter, salinisation, compaction, sealing and landslides. As far as contamination is concerned, they will set up an inventory of contaminated sites.

- Preventing further soil degradation and preserving its functions. Member States must ensure a sustainable use of soil.

- Restoring. Member States will then have to act upon the risks identified by adopting programs of measures for the risk areas, national remediation strategies for the contaminated sites and measures to limit or mitigate sealing.

4 GLOBAL CHANGE AND SOIL DEGRADATION

This occurs because of a general warming of the planet. Therefore all ecosystems functions are affected by the increasing temperatures.

Causes of global change: - Increase in human population; -Increase in demand and consumption of resources; - Changes in technology; -Changes in relation Soil Use/Plant Cover (http://www.pnuma.org/cambio_climatico/index.php).

Soil, as a part of the ecosystems, is affected in several ways. In relation to soil organic matter, for example: - increase in temperature leads to increase in organic matter oxidation (increase in CO2 emissions); - Lower plant cover leads to low inputs of organic matter into the soil; - Drought resistant plant species produce lower biomass; - Lower soil organic matter content leads to low resistance to erosion, lower cation exchange capacity, lower biological diversity, deterioration of soil structure, loss of soil, etc.

5 FACTORS WHICH DETERMINE SOIL DEGRADATION IN ARID AND SEMIARID AREAS

- Climate: aridity, high temperatures, and short-intensive rainfalls.

- Vegetation: low % cover, difficult to recover (slow growth), low inputs of organic matter into the soil, etc.

- Soils and bedrock: soils with not well-developed structure due to low organic matter content. In addition, the presence of easy erosionable rocks, such as marlstones, may favour erosion.

- Landscape: erosion creates non-regular shapes (with high slopes) which increase erosion (above all in areas where soil has not been managed properly).

6 SOIL QUALITY INDICATORS

Soil quality is the capacity of a specific type of soil to function, within natural or managed ecosystem boundaries, sustain plant and animal productivity, maintain or enhance water and air quality and support human health and habitation (NRCS, http://soils.usda.gov/sqi/concepts/concepts.html).

To assess soil quality it is necessary to evaluate physical, chemical and biological processes and their interactions in time, space and intensity. The properties measured are termed indicators of soil quality. The best soil quality indicators are those which integrate the combined effects of several properties and processes.

Soil quality indicators are useful to: - Concentrate the efforts in improving soil properties; - Evaluate management techniques; - Relate the soil resources with other resources (e.g. water resources); - Provide information about tendencies of changes (e.g. global change).

6.1 Types of soil indicators

- Visual indicators: they are useful in field observations but are high dependent of personal opinion (subjective). For example: subsoil exposition, soil colours, rills, pedestals of erosion, waterlogging, etc.

Physical Indicators: they are related to physical soil properties. Some of them can be measured in the field. For example: bulk density, porosity, infiltration, aggregate stability, texture, crusting, compaction, etc.

Chemical Indicators: they are related to chemical soil properties. Some of them can be measured in the field with portable equipment although for more reliable results, lab analyses are needed. For example: pH, salinity, % organic matter, nutrient content, pollutants, etc.

Biological Indicators: they are related to soil biological activity. Most of them should be measured in lab. For example: respiration rate, fungi population, decomposition rate, enzymatic activities, etc.

6.2 Some criteria to select soil quality indicators

An important factor in the use of indicators is to choose a small number of them suitable for each specific case. The criteria to select the most useful indicators are:

1) Which kind of "soil use" do we want to evaluate? For example, it is not the same to evaluate soil pollution as to evaluate soil agricultural uses.

2) Is the information from the indicator adequate for our goal? For example, high calcium carbonate means "good quality" for buffering acidity, but "bad quality" if we are assessing plant nutrition.

3) Which level of precision do we need? Portable equipment for in situ measurements are fast but have lower precision.

4) Is there spatial or temporal variation of the indicator? For example, salinity may appear in spots or showing fluctuations between the driest and wettest seasons of the year.

5) Do the indicator show response after changing the environmental conditions? For example, in soils with high calcium carbonate content acid rain may not cause changes in pH in the short term.

7 BIOLOGICAL AND PHYSICAL SOIL DEGRADATION

This refers to aspects of the deterioration of soil properties related to the activity of micro- and macro-organisms. Microbial activity contributes to the transformation of organic matter, which, in turn, contributes to: (1) It is a source of organic carbon and energy for microorganisms; (2) Formation of humic-clay and organic-mineral complexes (micro-aggregation); (3) Formation of macro aggregates from micro aggregates; (4) Improving of water retention capacity, cation exchangeable capacity, resistance against erosion and water infiltration rate.

In summary, soil organic matter contributes to preserve soil physical, chemical and biological properties.

7.1 Composition of soil organic matter

- Fresh organic matter. Primary sources of organic material inputs are dead roots, root exudates, litter and leaf drop, and the bodies of soil animals such as insects and worms.

- Transformed organic matter. Products that appear due to organic matter decomposition or new products from microbial synthesis. Humic substances: soil humus is fully decomposed and stable organic matter. Humus is the most reactive and important component of soil organic matter and is the form of soil organic material that is typically reported as "organic matter" in soil testing reports.

7.2 Factors influencing soil organic matter transformation

Type and quantity of microorganisms; environmental factors such as temperature and moisture; soil oxygen content; soil nutrient contents; type of soil minerals; pH; anthropogenic activities.

7.3 Soil organic matter and soil structure

Humic substances have a key role in the formation and stabilisation of the organic-mineral complex and formation of micro-aggregates. Labile organic compounds have a key role in the formation of macro-aggregates.

Degradation of the soil structure. The main processes involved in the destruction of soil aggregates are: - breakdown by differential swelling; - breakdown by fast wetting; - mechanical breakdown; - physical-chemical dispersion of soil colloids; - compaction.

8 WATER SOIL EROSION

8.1 Basic aspects

- Factors influencing water soil erosion are: climate; lithology; soils characteristics; topography of the land; vegetation type and cover; anthropic influence.

- The main forms of water soil erosion are: pedestals of erosion; sheet erosion; rill erosion; gully erosion; piping; mass earth movements.

- The mechanisms of water soil erosion are: splash, mainly implied in breakdown of aggregates; transport that carries out the particles.

- The main consequences of water soil erosion are: on site - the reduction in soil quality which results from the loss of the nutrient-rich upper layers of the soil and the reduced water-holding capacity of many eroded soils; off site - the movement of sediment and agricultural pollutants into watercourses can lead to the silting-up of dams, disruption of the ecosystems of lakes and contamination of drinking water.

8.2 Evaluation of water soil erosion

Three types of methodologies are widely employed: (1) Estimation of the current level of erosion; (2) Experimental quantification of the erosion; (3) Mathematical models of erosion.

8.2.1 Estimation of the current level of erosion

Based on the visual identification of forms and evidences of erosion. The goal is to evaluate the intensity of different forms of erosion. Morgan (1997) considers the following factors to evaluate the current erosion: presence of pedestals of erosion; % of root exposition; existence of surface soil sealing; number and size of rills and gullies; type and structure of vegetation cover.

8.2.2 Experimental quantification of the erosion: measures of erosion under field conditions

Three scales for working:

- Plots: sheet erosion and rills (natural and simulated rain).

- Slopes: sheet erosion, rills, gullies and sedimentation.

- Watershed: all of the erosion forms and sedimentation.

8.2.3 Mathematics models to estimate soil erosion

They are used to improve the knowledge about the mechanisms which control erosion processes. In addition, they allow predicting soil loss, evaluating soil management strategies, etc. There are two types:

- Empiric: based on identifying the statistical relationships among parameters. They need a previous existing database. For example: Universal Soil Loss Erosion (USLE).

- Physical: they try to reproduce the erosion processes through mathematical equations, based on physical laws of mass and energy conservation. For example: European Erosion Model (EUROSEM).

8.3 Strategies for soil erosion control

The strategies should focus on controlling the factors which determine erosion:

- Controlling the active factors: - To decrease rain and wind energy; - To decrease impact energy of raindrops (covering the soil); - To decrease energy of water flow (decreasing the slope); - To decrease wind speed (using barriers).

- Protecting the soil by increasing its resistance to erosion: - To improve physical and biological properties; - To increase organic matter content; -To improve structure; - To improve infiltration.

9 SOIL SALINITY. FATE OF SALTS IN SOIL. INDICATORS OF SALINITY.

9.1 Basic concepts

Soil salinisation is one of the main problems in arid zones leading to land desertification.

Soil salinity limits the growing of crops, constrains agricultural productivity, reduces soil quality, can lead to the abandonment of agricultural soils and it also poses a major environmental hazard by degrading the quality of water.

Saline soils have a concentration of salts (more soluble than gypsum) so high that it interferes with the proper growth of non-adapted plants. Salinity is usually evaluated by means of electrical conductivity (EC) of saturation extracts. In the International System of Units EC is expressed as deciSiemens per metre (dS m^{-1}).

Sodic soils have a percentage of exchangeable sodium (ESP) higher than 15% (or sodium adsorption ratio (SAR)>13), which may adversely affect their physical properties and plant growth.

Alkaline soils have a pH value >~8.7, which may adversely affect their physical properties and plant growth.

Soil can be classified according to the EC and EPS as: EC< 2 dSm^{-1}, EPS<15% (no saline, no sodic); EC > 2 dSm^{-1}, EPS<15% (saline, no sodic); EC> 2 dSm^{-1}; EPS >15% (saline-sodic); EC< 2 dSm^{-1}, EPS>15% (sodic).

The Sodium Adsorption Ratio (SAR) expresses the ratio among the concentrations of Na^+, Ca^{2+} and Mg^{2+} in the soil solution (Equation 1). SAR can be used instead of EPS when it is difficult to measure the % of Na^+ in the exchangeable complex (Equation 2).

$$SAR = Na^+ \Big/ \sqrt{\frac{Ca^{2+} + Mg^{2+}}{2}} \quad (1)$$

$$ESP = 100 * (-0.0126 + 0.01475 * SAR)/1+(-0.0126+0.01475 * SAR) \quad (2)$$

9.2 Factors of soils salinity

- Primary (natural) factors for salinity/sodicity: geology and soils; climate (evaporation >> rain); natural infiltration patterns (drainage limitations); saline groundwater; topographic features (low laying areas); distance to the sea.

- Secondary (anthropogenic) factors for salinity/sodicity: inappropriate irrigation methods; poor water quality; insufficient drainage; poor land management; overexploitation of ground-water; clearing of trees; alteration of the natural water balance.

9.3 Fate of salts in soils

Ions can be found in soil in several states: in the form of precipitated salt crystals, dissolved in solution or adsorbed on the exchange complex.

The salt content in any of these three situations is changing continuously as soil moisture changes.

During the drying periods, the crystallisation increases and the salts in solution decrease (concentration of the solution). During the wetting periods, the behaviour is reversed.

9.4 Vegetation in saline areas: plants as bioindicators of soil salinity

Soil salinity must not always be considered a problem of soil degradation. In some native environments, such as salt marshes and salt deserts, salinity is a key factor influencing the organism's distribution. These ecosystems are important to preserve the biodiversity and to support ecological processes implied in nutrients recycling, contributing to the health of the environment. In saline environments vegetation distribution is usually related to salinity and moisture and hence plants can be used as bioindicators of salinity.

We can study plant distribution in saline areas from three different points of view: (1) Edaphic gradients (mainly temporal vs. spatial variation in moisture and salinity); (2) Physiological tolerance vs. interspecific competition of plants; (3) Facilitation processes in plant communities.

10 HYDRIC SOILS. BIOGEOCHEMISTRY IN WATERLOGGED SOILS. ROLE OF WETLANDS IN MITIGATING THE CONTAMINATION: CASE STUDIES.

During flooding or in the absence of oxygen, the oxidised forms of chemical species are converted into reduced forms through microbial mediated processes. Organic matter is the major source of electrons. When it is oxidised, the electrons released are used for reducing reactions. Oxygen is the most preferred electron acceptor for microorganisms. Once the oxygen is depleted in the soil, specialised microorganisms have the capacity to use other electron acceptors, derived from both organic and inorganic sources, for

respiration. The sequence of acceptors is dependent on the electron affinity of the electron acceptor and the energy yield (Equations 3 to 8).

$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$ (Eh from 400 to 500 mV) (3)

$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$ (Eh from 250 to 350 mV, denitrification) (4)

$MnO_2 + 2e^- + 4H^+ \rightarrow Mn^{2+} + H_2O$ (Eh from 200 to 225 mV) (5)

$Fe(OH)_3 + e^- + 3H^+ \rightarrow Fe^{2+} + 3H_2O$ (Eh from 100 to 170 mV) (6)

$SO_4^{2-} + 8e^- + 10H^+ \rightarrow H_2S + 4H_2O$ (Eh from 0 to -150 mV) (7)

$CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$ (Eh from -250 to -300 mV) (8)

Redox potential (Eh) reflects the intensity of reduction (gain of electrons). The Eh decreases when the environment reduces.

Eutrophic water enriched in nitrate is depurated when the NO_3^- is use as electron acceptor and it is reduced to gaseous N_2. However, during the reduction of nitrates N_2O is produced and this is a greenhouse gas. In addition, emissions of CH_4 may occur at very low redox potential, and methane is a greenhouse gas. Hence, wetlands act as green filters against pollution but can also be systems contributing to global warming.

11 SOIL CONTAMINATION. RISK ANALYSIS. SOIL MANAGEMENT AND REMEDIATION IN POLLUTED SOILS. EUROPEAN, NATIONAL AND REGIONAL LAWS FOR SOIL EVALUATION.

Soil contamination has become an important issue worldwide because of its consequences in environmental health, loss of soil productivity and therefore the related socioeconomic impacts. The remediation of polluted soils is then considered a key for the economic reactivation in many sites, however this issue is not easy to achieve since in remediation projects usually meet multiple factors (environmental, technical, legislative and economical) which are site specific.

Nowadays, the public health concern in relation to soil contamination has caught the attention of regulators who have brought the publication of laws, contamination thresholds and legal rules in relation to soil pollution issues and their corresponding risk analysis. In general, laws have included the obligation of affecting the soil reclamation if some previous proposed thresholds are surpassed (Schweizerische Bundesrat, 1998; MHSPE, 2000) or if risk analyses determine an unacceptable risk (BOE 2005). A risk analysis is a study which integrates information about the environment, in order to evaluate the harmful effects of soil and groundwater contamination on human health, ecosystems and goods.

The remediation of a polluted soil has increased then its importance as economical activity and many methodologies are available to reach the goal of “cleaning” the soil. These remediation techniques cover a wide range of processes and engineering

solutions and depend on the type of contaminant, its concentration, the site or the economic evaluations. It is assumed that the cost of the soil remediation projects is usually a limiting factor. The European Environment Agency has estimated the total costs for the cleanup of contaminated sites in Europe to be between EUR 59 and 109 billion (Commission of the European Communities, 2002). In Western Central Europe and South Eastern Europe, there are more than 1.8 million potentially contaminated sites, of which 240,000 are in need of remedial treatment. Potentially contaminating activities have occurred at nearly three million sites (European Environment Agency, 2007).

Traditionally, soil remediation techniques have been differenced between *in situ* (non-excavated soil) and *ex situ* techniques (soil is excavated). Among the latter, the remediation can be achieved "on site" (soil is excavated and remediated in the same site) or "ex site" (soil is excavated and transported long distances to a facility which will carry out the cleansing). The regulators tend to favour *in situ* techniques which imply the non-transport of the soil, trying to achieve a soil disposal as close to the source as possible, with the objective of self-sufficiency at regional scale (EC, 2002; RD 9/2005). The idea of "soil recycling" instead of dig and dump has been included in official regulations such as the Directive 2008/1/EC concerning integrated pollution prevention and control (EU, 2008). The same European regulators proposed a guideline in order to select the most suitable technique in terms of environmental friendly application, new scientific findings, or time consumption. These definitions leave the selection of the remediation technique open depending on the specific site conditions.

Conventional methods to remediate soils are easy to value from an economic point of view since they are based in previously known production rates which allows to predict feasible time scales, although high investments are necessary to achieve them. Moreover, some of these techniques have disadvantages because they do not allow the soil to be reused or they generate wastes which have to be disposed of. For example, thermal desorption generates a "clean soil" in which biological and physical properties have been destroyed; soil washing plants produced a residual cake that has to be disposed in a landfill.

Normalisation and standardisation are key factors in the development of commercial products and services. Standard, defined by ISO (2001) (http://www.iso.org) as "document, established by consensus and approved by a recognized body, that provides, for common and repeated use, rules, guidelines or characteristics for activities or their results, aimed at the achievement of the optimum degree of order in a given context". The need for improving quality assurance has brought the development of some ISO standards in the environmental service companies. In this sense it is possible to carry out environmental inspections of soil, air and water contamination under ISO 17020:2004 (General criteria for the operation of various types of bodies performing inspection). Soil remediation still does not have a standard norm but the future increase of demanding and commercial needs could bring about this issue. For that case, conventional remediation techniques (soil washing plants, thermal desorption, soil

stabilisation plants, etc.) appear easy to normalise since yields and physicochemical parameters are easier to set.

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CHAPTER 5

Evaluation, Management and Reclamation of Soils Affected by Anthropic Activities

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1 THE SOIL: POINT OF CONVERGENCE OF THE DIFFERENT LAND SYSTEMS. FUNCTIONS OF THE SOIL AGAINST CONTAMINATION

Soil is the point of convergence between Atmosphere, Hydrosphere, Lithosphere and Biosphere. It receives influences from each of these systems and responds to them by evolving in a determined manner. It is an open, complex, self-organisational, structural and poly-functional system which acts as a filter through which flows of energy and matter are produced and regulated with the other systems. The soil not only acts as a receiver and transmitter of the contamination but also may influence pollutants behaviour by adsorbing, filtering, transforming-degrading, etc. So, very important functions are: filtration, buffering and the transformation of different types of organic and inorganic substances which reach it. Thus it can mechanically filter gaseous, liquid or solid substances; it can physicochemically buffer them (adsorb them) and can also transform them biochemically and microbiologically by mineralisation and metabolisation.

The soil thus exerts an evident protective function between the atmosphere, biosphere and the hydrosphere. The capacity to exercise these functions is to be found determined very closely by the constituents of the soil and the properties that these confer it.

Buffering *capacity* of *soil* represents the capacity it has to inactivate the negative effects of the contaminants. This can be exercised through several mechanisms: filtration; biotic or abiotic degradation; absorption/desorption; chelation; precipitation/dissolution/coprecipitation/nucleation.

These actions depend on certain properties of the soil: 1.-texture, structure, porosity and permeability; 2.-temperature of the soil. Specific heat capacity; 3 .-Cation exchange capacity; 4.-pH, buffering *capacity* of *soil* and its capacity to neutralise acids; 5.-oxidation-reduction properties; and 6.-microbiological properties.

No matter how favourable the soil's characteristics are, it is evident that its cleansing capacity is not unlimited. It cannot assimilate, immobilise, inactivate and degrade all the contaminants it receives and is therefore at a given moment a "Chemical Time Bomb" (C.T.B.). The C.T.B. depends on three major factors: vulnerability of the soil, entry of chemical compounds, and soil use and management. This fact clashes with the idea of considering the soil as an ideal place to get rid of all manner of waste.

Therefore, soil contamination is a globally recognised environmental problem, like water contamination and air pollution.

2 ACID RAIN. EFFECTS ON SOIL. CAPACITY FOR THE NEUTRALISATION OF ACIDS

Nowadays acid rain is related with industrial activities, principally emissions from thermal electric power stations, and with the emissions produced by the combustion of hydrocarbons containing S, N, Cl, etc. Also, denitrification processes of fertilisers added in excessive doses, and similar natural processes which take place in the zones of mangroves, rice paddies, volcanoes, etc.

It is concentrated in the most economically developed countries, but is progressively extending to other areas.

It is fundamentally constituted by N, S and Cl compounds which in a posterior phase can form nitric, sulphuric or hydrochloric acids.

Although in the atmosphere itself a partial neutralisation by NH_3 can be produced, these compounds are transmitted to the soils. The acidity not neutralised by the treetops enters into the soil through infiltration and runoff.

It has the following effects:

1.-It reduces the content in nutrients as their cycle varies.

2.-It provokes the mobilisation of toxic elements such as aluminium (soluble at pH < 4.2).

3.-Increment in the mobility of heavy metals.

4.-It provokes variations in the composition and structure of the microflora and microfauna.

The acidification is equivalent to:

1.-Decrease in pH.

2.-Decrease in the saturation in bases.

3.-Increase in the proportion of H^+ and Al^{+3} in the exchange complex.

Critical load of acidity (CLA) is the maximum level of acidifying compounds contributed that do not cause chemical changes which are harmful for the long-term structure and functioning of the ecosystem. In addition to the atmospheric, localisation and technological factors, the soils present important differences in the sensitivity to this type of impacts.

The incorporation of nitric, sulphuric or hydrochloric acid to the soil results in an immediate fall in the ion exchange balance, due to an increase in the washing out of basic cations. If said basic ions washed out from the exchange sites during the rain are replaced by others coming from the chemical weathering, the soil pH will not fall. In this way the soil has acted as a strong buffer against the change in pH.

In another way if a soil is really poor in alterable minerals, the saturation in bases and the soil pH will go down rapidly in response to an acid deposition, until a new balance is reached.

In these conditions the drainage waters will also become more acid and will contain more aluminium and less calcium.

3 CONTAMINATION OF THE SOIL BY SALTS: SALINISATION AND ALKALINISATION. EFFECTS ON THE SOIL

The concentration of salts confers the soil certain very particular properties with very harmful effects for the crops. This may be due to natural causes or be the result of anthropic actions. Additionally two situations, with very different morphologies, properties, genesis and soil uses, according to the predominant cation, are present.

If the predominant cation is Ca^{+2} or Mg^{+2}:

-The soluble salts are very abundant in the soil.
-The soil profile shows very little difference, but its structure tends to be stable.
-The high osmotic pressure of the soil solution is responsible for the low productivity.
-They are denominated saline soils (or halomorphic soils).
-The process of accumulation of these salts is called salinisation.

If the predominant cation is Na^{+}:

-The dispersion of the clays occurs, which leads to a destruction of the soil structure.
-The hydrolysis of the sodic clays leads to the alkalinisation of the profile, and this provokes intense mineral alteration.
-The profile is well differentiated from a morphological point of view.
-These are called sodic soils (alkaline).
-This process is called alkalinisation.

When there is a high salts content and these are sodium:

-Sodium predominates in the exchange complex.
-The soils are saline-sodic.

The three cases are typical in regions with dry climates. They are therefore abundant in Egypt, Iran, India, Pakistan, China, Ecuador, Peru, Chile, Mexico, etc. However, they are also present in zones with wet climates such as Holland, Belgium or Canada, in locations influenced by sediments or waters with high salts concentrations. In Spain more than 800,000 ha are estimated to be affected by salts. At world level the figure is as much as 300/400 million ha, an important part of which are the consequence of anthropic activities.

There are two conditions necessary for the accumulation of salts to occur: input of salts and their possible elimination must be impeded.

3.1 Input of salts

Natural causes

-These may proceed directly from the parent material. Some rocks contain salts as constituent minerals, or if the parent material does not contain these salts, they can be produced in the soil by weathering of the rock minerals.
-The salts dissolved in runoff water are accumulated in the depressions and when the solution evaporates so saline accumulations are formed.

-Take the salts from the sufficiently surface phreatic levels (normally at less than 3m). The existence of the water table at the surface occurs in the depressions and low-lying lands, and thus the relationship salinity-topography.
-Contamination by wind origin salts. The wind in the arid regions drags a great quantity of particles in suspension, principally carbonates, sulphates and chlorides which can contribute to the formation of saline soils.
-This can occur in coastal zones by direct contamination from the sea, from the saline phreatic level and due to the contribution of the wind.
-The decomposition of plant waste liberates the salts which were included in their tissues and contributes to increasing the salinity; on other occasions the plants contribute to the decomposition of relatively insoluble minerals and from them salts are formed. This effect is not important at global level.

Anthropic contamination
-Agrarian activity, and especially irrigation, has provoked remote processes of salinisation of different degrees since remote times. One such example is that of Mesopotamia in which the use of saline irrigation waters led to the salinisation of the soils, and that was the cause of the fall of the Sumerian civilisation around 5000 years ago. Nowadays it is accepted that the majority of irrigated soils present some losses in yield due to problems of salinity.
Causes:
The use of waters which contain salts without due control (accumulating directly in the soils or contaminating the water table).
Drop in the regional water table and the intrusion of layers of saline waters, situated in deeper zones, as a consequence of overexploitation.
In low-lying surfaces, when transformations in irrigation are carried out in areas situated in higher zones. Directly by the action of the irrigation waters, but it can also be produced by the mobilisations of lands which can provoke the appearance of saline rocks on the surface.
Use of elevated amounts of fertilisers, especially the most soluble ones, beyond the crops needs, contaminating the aquifers and as a consequence the soils which receive these waters.
Industrial activity, by means of atmospheric pollution or by means of the waters which flow through its hydrographical basin.
All these situations are very typical in more or less arid or semiarid zones subjected to very intense agricultural activity, such as occurs for example, on the coastlines of Murcia, Granada and Almeria.

3.2 Elimination of salts impeded

It is produced by the action of the drainage and climate.
-Drainage: it is necessary for the water to circulate slowly, so that it impregnates the soil, dissolves the salts and so they are distributed in the profile without large amounts of them being eliminated.

-Climate: also exerts a fundamental action in the formation of these soils, to such an extent that it was thought that an arid climate was essential.
The majority occur in more of less arid or semiarid climates, although this is not an indispensable requirement. The brief wet periods provoke the dissolution of the salts, and thus their mobilisation, while with the intense and long droughts major evaporation occurs, which produces the rise in the groundwater and as the evaporation intensifies so the salts in the soils are concentrated, which precipitate, accumulating in determined horizons of the profile.
In wet climates the soluble salts, which are on principle present in the soil materials, are washed out and transported to lower horizons, toward the underground aquifers and finally carried to the oceans. This does not suppose a problem except in the event of agricultural or industrial contamination or in zones exposed to the influence of the sea, as occurs in deltas or marshland.

3.3 Effects on the soil

Salinity
The effects of salinity can be grouped under three different aspects:
1.-Water relations. The concentration of soluble salts increases the osmotic pressure of the soil solution.
2.-Energy balance. The plants, as the osmotic pressure of the soil solution increases, are obliged to an osmotic adaptation of their cells to be able to continue absorbing water; adaptation which requires a consumption of energy which is at the cost of lower growth. Salts may also be affecting cell division; this produces a premature swelling of the cell walls and irreversibly limits the growth.
3.-Nutrition. In the nutritional aspect, a series of important modifications occur, due, on the one hand, to the variations in pH which affect the availability of the nutrients, and on the other, to the interactions for the excessive presence of certain elements. Synergism and antagonism are present. The presence in excess of certain ions can provoke toxicity, due to the accumulation in different parts of the plants.

Alkalinity
This develops when an elevated concentration of sodium salts exist which are capable of suffering alkaline hydrolysis, of sodium carbonate and bicarbonate type. Together with these salts with a strong NaOH and weak acid (H_2CO_3) base, important quantities of neutral sodium salts lacking alkalinising properties exist (principally chlorides and sulphates).

An elevated content in Na^+, in relation with the Ca^{2+} and Mg^{2+}, provokes, given its low load density (elevated radius of hydration and low load), the increase in thickness of the diffuse double layer, the effects of repulsion between the colloids and, with them, the dispersion of the clay and the solubilisation of the organic matter.

The concentration of Na^+ versus Ca^{+2} and Mg^{+2} in the soil solution needs to be greater than 70% for the Na^+ to be able to displace the Ca^{+2} and Mg^{+2} in the exchange complex, given the lower adsorption energy of sodium.
For sodium to play an important role in the evolution of the soil, the concentration of sodium adsorbed versus the other cations must surpass the critical value of 15% (Na/S >15% (S = sum of other cations adsorbed).
The clays saturated in Na in the presence of rainwater, thus with dissolved CO_2, are hydrolysed, liberating Na^+ and OH^-:

$$2Clay\text{-}Na + H_2O + CO_2 \Leftrightarrow 2Clay\text{-}H + Na_2CO_3 \qquad \text{(equation 1)}$$
$$Na_2CO_3 + 2H_2O \Leftrightarrow 2Na^+ + 2OH^- + H_2CO_3 \qquad \text{(equation 2)}$$

As a consequence the medium is rapidly alkalinised, reaching progressively higher pH values; 9, 10 or even higher.
The alkalinisation produces a series of unfavourable consequences for the physico-chemical properties of the soil:
-Sodium clays and humus are dispersed, the structural aggregates are destroyed.
-The clays and the humic acids are illuviated, accumulating in the B horizon, forming a horizon of accumulation of sodium clays, that is to say, that they are originating a natric horizon.
-Seasonal changes produce the swelling and contraction of the sodium clays (montmorillonite type) forming a strongly developed prismatic structure. Finally, as the medium has become strongly alkaline, the crystallinity of the clays falls, they become unstable, part of them are decomposed, the upper vertices and edges of the prisms are destroyed originating a columnar structure which presents an upper face of rounded prisms.
-On occasions, the illuviated sodium humates are accumulated in these surfaces giving them very dark colours.
-This process can occur directly in the soil or can appear following the salinisation process, when the washing out of the most soluble salts occurs and the sodium carbonates and bicarbonates are accumulated.
This does not occur in the presence of other soluble salts, since their presence in the soil solution limits the formation of the sodium carbonate and bicarbonate.

$$Na_2CO_3 + CaSO_4 \Leftrightarrow CaCO_3 + Na_2SO_4 \qquad \text{(equation 3)}$$
$$Na_2CO_3 + MgSO_4 \Leftrightarrow MgCO_3 + Na_2SO_4 \qquad \text{(equation 4)}$$

In both, $CaCO_3$ and $MgCO_3$ they are not very soluble and precipitate, so the reactions are displaced to the right.
In the presence of NaCl the solubility of the sodium carbonate and bicarbonate also diminishes by common ion effect.

Sodium causes toxicity which can be centred in three different ways:
-Harmful effect of the active sodium for the plants' metabolism and nutrition.

-Toxicity due to the bicarbonates and other ions.
-Elevation of the pH to extreme values due to the action of the sodium carbonate and bicarbonate.

4 CONTAMINATION OF THE SOIL BY NUTRIENTS. DYNAMIC OF NITRATES AND PHOSPHATES IN THE SOIL. EFFECTS ON THE SOIL AND WATERS. EUTROPHICATION

Plants synthesise their food from chemical elements that they take from the air, water and soil. There are 60 chemical elements which constitute the plants, of which 16 are essential and they can be divided into macronutrients (primary and secondary) and micronutrients or trace elements.
-Primary macronutrients: N, P and K.
-Secondary macronutrients: Ca, S and Mg.
-Micronutrients: B, Cu, Cl, Fe, Mn, Mo and Zn.
Apart from this, the plants take carbon, hydrogen and oxygen from the air and water, so that CO_2 and H_2O in practice represent the only source of energy for their synthesis reactions.
A deficiency in some of the nutrients can complicate the assimilation of others. Thus, the development of the plants responds to the elements which are found in smaller amounts than the optimum.
The incorporation of fertilisers and similar residues is desirable to increment the crop yield and to recycle nutrients, but it shows the need for a regulation of the doses added, based on the knowledge of the initial situation of the soil and on the properties which have an influence in the cushioning of the effects of these substances. Many of the current problems of contamination in soils and water are caused by the contribution of fertilisers in crop soils.
This fundamentally affects the most developed agricultures based on major *inputs* of N and P, which has forced the development of different political actions and normatives.

4.1 Dynamic of nitrates and phosphates in the soil

4.1.1 Nitrogen

It is an essential nutrient for plant growth, as it is a constituent of all the proteins, and it is absorbed by the roots generally in the forms of NO_3^- and NH_4^+.
As a consequence of its cycle, in the soil we can find organic (proteinic, nucleic acids, sugars, etc.) and inorganic (NH_4^+, NO_3^-, NO_2^-, etc.) nitrogen. The organic is generally the most abundant (85 to 95%).
Basically the nitrogen cycle is composed of four types of processes: 1.-Fixation of the molecular nitrogen; 2.-Nitrification; 3.-Reduction of the ion nitrate; 4.-Denitrification.
<u>Relation with crop types:</u>
-In inundated crops: transformation of the organic nitrogen to ammonia form, not forming nitrate due to the shortage of oxygen in the medium. Thus, a large part of the

nitrate contributed as manure is transformed into ammonia, being lost rapidly through volatisation.
-In dryland farming: losses by denitrification are low, however they can be elevated by volatisation of the ammonia. Lixiviation only occurs if the period of application coincides with that of the rains.
-In irrigated crops or in wet zones: the most important losses are due to lixiviation of the nitrate, as well as by denitrification in conditions of waterlogging.
Relation with soil properties:
+Denitrification is more active in clayey soils, since it requires anaerobic conditions and a low hydraulic conductivity and also to be rich in organic matter, which stimulates the microbial activity and thus the consumption of oxygen.
Relation with the period of fertiliser application:
-If its application is carried out in rainy periods, the losses due to runoff or infiltration will be high.
-The amount of fertiliser elements utilised by the crops is different in the different growing periods.
-The type of product utilised as fertiliser also has an influence on the possibilities of contamination, depending on the ease with which it passes to the soil solution.

Nitrogen is the most frequently found element as a contaminant of the waters as it is used considerably more than the other elements (the proportion in Spain is 1N:0.5P:0.3K), coupled with its greater ease to pass to the soil solution.
It is found fundamentally in the form of nitrates (NO_3^-); it also exists as ammonium (NH_4^+) and nitrite (NO_2^-). Irrigated agriculture involves a greater ease of this element contaminating both the groundwater as well as the surface waters.
Added as fertiliser, it can be in the forms of $CO(NH_2)_2$ (urea), NH_4^+ and NO_3^-.
The urea is subjected to ammonification (formation of NH_4^+) and nitrification prior to its utilisation by the microorganisms and plants. The ammonium can be oxidised to NO_3^-, be fixed by the solid particles of the soil or be utilised without change by the microorganisms and plants. The nitrates can be absorbed directly by microorganisms and plants or can be lost by volatilisation and washout.

With regard to the side effects of nitrogenised fertilisation we have:
-Input of nutrients, apart from nitrogen, such as S, Mg, Ca, Na and B.
-Variation in the soil reaction (acidification or alkalinisation).
-Increment in the biological activity of the soil with important indirect effects on the global dynamic of the nutrients.
-Damage by salinity and the contamination of aquifers, caused by very high doses.
-Damage caused by impurities and decomposition products.
-Side effect, herbicide and fungicide, of the calcium cyanamid.

4.1.2 Phosphorus

It is, after nitrogen, the second element in importance for plant growth. The lack of this element in the soil can impede other elements from being absorbed by the plants.
With regard to the phosphorus cycle in nature we have the remains of harvests, human wastes, animal manure and phosphorus fertilisers as being the principal sources. In the soils there are two principal pools: organic which is found in the humus and in plant microbial residues, and inorganic with compounds of Ca, Fe and Al. In a normal mineral soil between 40-60% would be in each of the two forms.
It is an element with little mobility in the soil, so it appears in lower concentrations as a contaminant of water, than nitrogen. It is only washed out when it has been applied in excess over a long time and the soil's fixation capacity is saturated. The soluble forms of phosphorus correspond to phosphate ions ($PO_4H_2^-$ and PO_4H^{-2}).
Its availability is determined by the following factors:
-Soil pH.
-Soluble Fe, Al, and Mn and the presence of minerals containing Fe, Al and Mn.
-Available calcium and magnesium minerals.
-Quantity and decomposition of organic matter.
-Microorganism activity.
-All these factors are influenced by the soil pH. The maximum availability of P occurs for pH between 6 and 7. At low pH, acid soils, Fe, Al and Mn exist in solution which react with the phosphoric acid giving insoluble hydroxide phosphates. Fixation also exists for the oxides and hydroxides forming insoluble phosphate hydroxides. The fixation by silicates-clays is carried out in conditions of moderate acidity. In alkaline soils, the phosphates precipitate with the exchange Ca and with that of the $CaCO_3$. There is only one rank of pH (around 6.5) in which the phosphate remains soluble, which is the situation in which a certain risk of lixiviation can occur.
With regard to the side effects of phosphate fertilisers we have:
-Input of nutrients, as well as phosphorus, such as sulphur, calcium, magnesium, manganese and others; and also useless substances, from the point of view of fertility: sodium and silica.
-Input of substances that improve the structure: lime and gypsum.
-Variation of the soil pH.
-Immobilisation of heavy metals.

4.2 Environmental impact of the excess of n and p fertilisers

1.-Nitrate salts are very soluble, so the possibility of lixiviation occurring is elevated and more so taking into account the low adsorption power that the majority of soils present for negatively charged particles. Therefore, the most important environmental problem is the accumulation of nitrates in the subsoil which, by lixiviation, can be incorporated into the groundwaters or be dragged to the waterways and surface pools, such as lakes, reservoirs, seas, etc.
-Surface waters

In them the movement of the water is reduced giving rise to major growth in the algae. The development can be so great that it impedes the light from reaching the deepest zones, where the algae will die. Due to this, the populations of fish and other living beings will diminish and also due to the reduction in food available in the food chains. Over time the different organisms will disappear, until life disappears completely.

-Groundwaters

The lixiviation of nitrates towards the subsoil can contaminate the aquifers, creating serious health problems if water rich in nitrates is consumed, due to its transformation into nitrites by the participation of bacteria which exist in the stomach and bladder. In turn the nitrites are transformed into certain carcinogenic compounds (nitrosamines), which affect the stomach and liver. On the other hand, the habitual consumption of waters containing large amounts of nitrates in solution, leads to the development of metahaemoglobinaemia, a disease which is manifested by respiratory difficulties and dizziness.

2.-Applying fertilisers, whether they are inorganic or organic, causes changes to occur in the soil ecology. The populations of organisms increase or decrease according to the situation, additionally, changes occur in the chemical characteristics of the soil: pH, electrical conductivity, etc. The application of fertilisers with a high heavy element content originates the adsorption of these elements into the cation exchange capacity and their possible assimilation by the crops. Moreover the compost, sludge and some peats are carriers of large amounts of salts which can provoke the salinisation of the soil or toxicity in the plants.

3.-To the negative effects on local waters and organisms other far reaching considerations must be added, since in many soils part of the nitrogenised fertilisers are reduced and can be lost into the atmosphere, by denitrification processes, in the form of different compounds. Their oxidation originates a subsequent input of acid rain.

4.-Organic fertilisers can contaminate the surface waters with bacteria and other microorganisms which are harmful for the health. Nevertheless, the contamination of groundwaters by pathogenic microorganisms is almost always non-existent.

With regard to the environmental impact of the phosphate fertilisers, the problem of phosphates is, as with N, the eutrophication of the waters.

There is not such a rapid or elevated mobilisation due to the important fixation capacity of phosphates which many components of the soil have, and also due to the low general solubility of phosphates.

However, it can move as particulate material and also in organic and inorganic forms once the phosphate adsorption capacity of each soil has been exceeded.

5 CONTAMINATION OF THE SOIL BY PESTICIDES. PERSISTENCE AND EVOLUTION IN THE SOIL

These include pesticides and herbicides. With regard to the factors that regulate the evolution in the soil, both the properties of the pesticides as well as the characteristics of the soil and the medium in which it is found must be considered.

Properties

1.-Chemical structure. Compounds with a stable structure are the most persistent, specifically the organochlorines. The characteristics that are generally associated with the greatest adsorption are: high molecular mass; tendency to form ions (+); and the presence of chemical groups which increase the affinity of the molecule by the topsoil.

2.-Volatility. Represents the tendency of the pesticide to pass to the gaseous phase. Depends on the pressure of the vapour in liquid state and of the solubility in water. Represents therefore the distribution of the pesticide between the liquid phase of the soil and the atmosphere.

3.-Distribution coefficient. Relation of concentrations of any molecular species between two phases (for example, in two immiscible liquids, or a liquid and a gas) in balance. This relation is expressed using the equation:

K = C(phase 1)/C(phase 2) (equation 5)

4.-Solubility. Transcendental factor for two reasons:

-The liquid phase of the soil is an aqueous phase, which conditions the dynamic of the pesticide associated to said phase.

-The pesticides which most contaminate in soils are those which are not very soluble in water.

5.-Adsorption. Regulates the tendency of the pesticide to be retained in the soil. If the adsorption coefficient is small, it indicates a high mobility.

6.-Dose. Low doses soon disappear, but it depends on the compound.

7.-Presentation. The penetrability and persistence can be influenced depending on the presentation of the product: emulsion, powder, granulated, etc.

8.-pKa, pKb. Significant parameters for the pesticides which behave as acids or as weak bases, since they determine the pH rank in which they behave as neutral or ionised species.

Soil characteristics

1.-Soil colloids. Soils rich in colloids adsorb the pesticides more readily and within this group the organic ones do so with greater intensity than the mineral ones.

-Clay characteristics: 1.-the nature of the silicate. 2.-density of the laminar charge. 3.-nature of the exchange cations.

-Organic matter: It is the component which acts decisively in the phenomena of pesticide adsorption.

2.-Cation Exchange Capacity.

3.-pH.

4.-Structure and texture.

5.-Microorganisms.

Characteristics of the medium

1.-Temperature.

2.-Pluviometry.

3.-Vegetation cover.

6 CONTAMINATION OF THE SOIL BY HEAVY METALS. FORMS AND DYNAMIC OF HEAVY METALS IN THE SOIL. FACTORS THAT AFFECT THEIR MOBILITY

Heavy metal: element that has a density equal to or greater than 5 g cm^3 when it is in element form, or whose atomic number is greater than 20. There are two groups:

-Oligoelements or micronutrients: required in small quantities or trace amounts by plants and animals, and they are necessary for the organisms in order to complete their life cycle. Above a certain threshold they are toxic.

-Heavy metals with no known biological function: their presence in determined amounts in living beings brings dysfunctions. They are highly toxic and present the property of accumulating in living organisms. They are, principally: Cd, Hg, Pb, Cu, Ni, Zn, Sb and Bi.

In relation to the source of the heavy metals in soils we have:

Natural origin

-The content in the original material, upon weathering, is concentrated in the soils. These natural concentrations can become toxic for plant growth, such as for example nickel can appear in toxic concentrations in soils derived from ultrabasic rocks: in those derived from serpentines the Ni content can be in excess of 2000 ppm.

-Volcanic activity must also be considered; heavy metals such as: As, Hg, Se, etc. are emitted.

Anthropogenic sources. Human activities have exercised a considerable effect on the concentration and mobility of the metals in soils. Among the most important of these are the followings:

-Agricultural chemical products (fertilisers and pesticides).

-Mining and foundry activities. The mining process implies: extraction of the ores, preliminary processing, evacuation of the residues and transport of the semi-processed products. All of these operations can cause a localised contamination by metals. The dust originated can be deposited in the soils even at distances of many kilometres. Thus, in mining areas, the top horizons of mineral soils present elevated concentrations of copper, nickel, arsenic, selenium, iron and cadmium.

-Electricity generation and other industrial activities. The combustion of coal is one of the principal sources of the deposition of metals in soils. Thermal electric power stations using oil combustion can be sources of lead, nickel and vanadium. The largest industrial sources of metals include iron and steel foundries which emit metals associated with iron ores, such as nickel. Battery factories can emit considerable quantities of lead. Likewise, the metals associated with highly industrialised areas include arsenic, cadmium, chromium, iron, nickel, lead, zinc and mercury.

-Domestic wastes and residual sludges. Approximately 10% of rubbish is composed of metals. One of the most serious problems facing modern societies is how to dispose of this volume of rubbish. The two alternatives are burial or incineration. Burying it can contaminate groundwaters, while incineration can contaminate the atmosphere.

The heavy metals incorporated into the soil can follow many different routes:

1.-They can be retained in the soil by adsorption, complexation, and precipitation processes.
2.-They can pass to the atmosphere by volatilisation.
3.-Thay can be absorbed by the plants and thus enter into food chains.
4.-They can be mobilised to the surface waters or groundwaters.
We shall take particular note of point 1, since their form depends on that factor, and therefore also the dynamic of the metals in the soil, and thus their possibilities of passing to the other earth systems: atmosphere, biosphere and hydrosphere.

Factors that affect their mobility
-pH. Adsorption is strongly conditioned by the soil pH.
-Texture.
-Organic matter.
-Exchange capacity.
-Redox conditions.
-Salinity.

CHAPTER 6

Regulation of Growth in Ornamental Plants

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1 INTRODUCTION

Growth control of commercial ornamental plants became a reality many years ago. The first steps in techniques for growth regulation were based on manipulating the crops' environments (temperature, light, etc.) in order to achieve satisfactory growth. Nevertheless, in the mid 20th century it was found that plant development was controlled by phytohormones, chemical messengers that coordinate cellular activities and are produced in the plant itself. At that time it was observed that the application of plant growth regulators was very effective to control stem elongation and produce compact plants; thus, plant growth regulators replaced the former practices. Currently, phytohormones are used on a wide variety of ornamental crops, to improve both aesthetic quality (more compact plant with more branches and greener colour) and physiological status (increasing resistance to stresses during growing and postharvest).

The application of plant growth regulators is an efficient way of improving quality in a large number of floriculture crops, but the concern over how these chemicals impact on human health and the environment may limit their availability in the future. This concern leads to a plant growth control which is more respectful towards the environment. We think it can be achieved by developing three aspects: (a) introducing new less residual chemicals; (b) improving our knowledge in selecting and using plant growth regulators; and (c) combining phytoregulators with non-chemical growth regulation. The latter suggests a return to days passed. However, now there are monumental advances in greenhouses technology, such as computer-controlled temperature manipulation, photoselective plastics, more advances in nutrition and the technology of irrigation, and a more complete understanding of how we can affect stem elongation through mechanical manipulation.

2 COMMON PLANT GROWTH REGULATORS

There are numerous chemicals to control plant growth available in a variety of commercial formulations. We have divided them into two groups: a) natural and synthetic hormones, and b) plant growth retardants.

2.1 Natural and synthetic phytohormones

The natural hormones include auxins, gibberellins, cytokinins, abscisic acid and ethylene. Collectively, they regulate many facets of plant growth and development including seed germination, root growth, stem elongation, leaf expansion, flowering, seed development, fruit ripening, and dropping of leaves and fruits. The first discovery of natural plant growth regulators was with the indoleacetic acid, which promotes growth by stimulating cell enlargement. Auxins are responsible for apical dominance and tropism. They are important in root cutting because they enhance the formation of roots. The natural auxin, indoleacetic acid, is not used for commercial compounds because it is unstable; instead, synthetic auxins (naphtaleneacetic acid) are used because they are more effective. The synthetic auxin 2.4-D is often used as a herbicide.

Gibberellins promote plant growth by stimulating cell division and enlargement. In floriculture, they are mainly used to achieve longer stems, stimulate flowering, reduce crop time, promote germination and overcome seed and bulb dormancy. There are many different natural gibberellins, but commercial products contain gibberellic acid (GA_3) or a mixture of GA_4+GA_7.

Cytokinins were found in coconut milk and malt extract, promoting cell division and shoot formation when the level is high relative to the auxin level. The most commonly commercial products use benzyladenine, an active synthetic cytokinin, which promotes branching and reduces leaf yellowing in cut flower stems stored in the dark.

Ethylene is an important natural regulator produced by all plants, which has a wide range of desirable and undesirable effects, such as flower induction, preventing flowering, early flower death, leaf, bud and flower abscission, enhancing ripening and senescence, causing epinasty, decreasing internode length, increasing branching, promoting the break of dormancy in seeds and bulbs, and causing foliar yellowing. Ethylene is usually produced by plants subjected to stress. So, sleeving, boxing and shipping plants can cause ethylene build-up in the shipping box and truck, and then, affect plant growth and development. Economically it is more important to avoid the effects of ethylene than to use them, because ethylene can be responsible for severe losses during shipping and handling. To prevent the symptoms associated with ethylene, compounds which specifically block the action of ethylene (1-MCP) or inhibit the biosynthesis of ethylene (AVG) are applied.

2.2 Plant growth retardants

Plant growth regulators are chemicals that are designed to achieve crop-specific height control. Internode lengths are shorter, but the number of internodes is not usually changed. Leaves result smaller with a darker green colour too. There are six primary growth retardants commonly used in ornamental crops in Spain: chlormequat chloride, mepiquat chloride, prohexadione calcium, daminozide, trinexapac-ethyl and paclobutrazol. We can also include in this group ethephon, an ethylene-releasing

product. These growth retardants interfere with the biosynthesis of the gibberellins; thus, the appearance of plants treated with a growth retardant is the opposite to plants sprayed with gibberellins.

Chlormequat chloride was the first retardant labelled for use in the floriculture industry and it is still widely used. Its main use has been on poinsettias, azaleas, geraniums, and hibiscus, but its use on other crops is expanding. Chlormequat chloride can be applied as a spray or as a drench; however, drench applications are seldom cost-effective. It is rapidly metabolised in plant and transformed into other compounds that can cause ineffectiveness and even stimulate growth.

Daminozide was labelled after chlormequat. This chemical was initially used to improve resistance to drought, pests and diseases in bedding plants, and later to reduce the plant height in chrysanthemum and hydrangea. Its mode of action is either to inhibit translocation of gibberellins or to promote their degradation; it also affects auxin transport. Daminozide is applied only as a spray, while it is not active as a drench to the media because it is broken down rapidly in substrate. The low activity of daminozide means that it must be sprayed more frequently to maintain control over vigorous crops. This retardant is very active in chrysanthemum, sunflower, hydrangea and gardenia, while in other crops, such as impatiens, geranium and lily it has less effect.

Prohexadione calcium is a plant growth retardant that is primarily used to inhibit excessive vegetative growth in fruit trees and other crops. Prohexadione-Ca also triggers pathogen resistance. Effects on the incidence of bacterial and fungal diseases and on insect pests are often observed. Furthermore, the compound may reduce abortion of fruitlets, thereby increasing the fruit set. Its use in ornamental crops is not yet very common. Prohexadione-Ca is relatively short-lived (it degrades relatively rapidly after application, and there is no risk of carry-over or bioaccumulation) and possesses very favourable toxicological and ecotoxicological features. It inhibits gibberellins biosynthesis; as a result, less growth-active gibberellins are formed and treated plants remain compact. Treatments with prohexadione-Ca lead to reduced ethylene formation too. Lowered ethylene levels, together with elevated availability of assimilates that are no longer needed for shoot growth, explain increases in the fruit set. Prohexadione-Ca must be applied as a spray, producing effects between two and five weeks after application. As it is very little residual, repeat applications at 14-21-day intervals are required.

Since ethylene is a gas it is difficult to use directly. However, ethephon, a liquid, is converted to gaseous ethylene when it is absorbed by the plant. Unlike other retardants, ethephon does not inhibit gibberellins production. The increased ethylene causes cells to limit elongation and increase in width instead. The release of ethylene reduces apical dominance, which can increase axillary branching, thereby reducing stem elongation. However, if the application is made close to flowering, the ethylene can result in flower abortion and delayed flowering. Ethephon sprays are usually applied to many floriculture crops. The currently available ethephon products are not labelled for drench

application, but their application as a substrate drench can be effective for height control.

Mepiquat chloride is a growth regulator that has been used in cotton production for several decades as a management tool in controlling vegetative growth. Mepiquat is mainly used in cotton to increase early fruit retention, enhance earliness and promote more uniform capsules. It is also used to prevent lodging (by shortening the stem and strengthening the stem wall) in cereals, and to inhibit sprouting in onions, garlic and leeks. Mepiquat is a gibberellic acid suppressant that is absorbed by the green portions of the plant and serves to reduce cell elongation, but it is not thought to be readily transported through the plant. Mepiquat can be applied as a foliar spray or soil drench, but the former is usually more effective than the latter. All surfaces of the plant including stems and leaves have to be sprayed with the solution until they are saturated. Mepiquat has little activity, consequently, the application process has to be repeated with the same amount of active ingredient several times during the growing period, spacing each application at least 7 to 14 days apart.

Trinexapac-ethyl is a synthetic plant growth regulator that is derived from cyclohexanecarboxylate, used in turf maintenance to reduce vegetative growth. Golf course superintendents have observed that multiple applications of trinexapac-ethyl may increase the stress tolerance of grasses. Some authors observed no reduction of root length or root density and an increase in visual quality of perennial ryegrass after treatment with trinexapac-ethyl under drought conditions. This compound must be converted to its acidic form (trinexapac) for activation. This process takes place in the plant and is dependent on temperature and ultraviolet light. Trinexapac is applied as a foliar spray and is translocated to the growing shoot. It acts as an inhibitor of the action of a key enzyme in the formation of gibberellic acid, preventing the formation of this natural plant growth regulator.

Paclobutrazol is included within the triazoles, a group of chemicals that have both growth retardant activity and fungicide activity. This anti-gibberellin is much more potent than most of the previous compounds and it has a broad label for ornamentals that includes use on greenhouse or outdoor-grown and containerised crops. It affects almost all plant species. The triazoles fungicides such as triadimefon or bromuconazole have stronger fungicide activity, but also have some growth retardant effects. The primary transport is within of xylem system; it is absorbed by the roots and stems. Paclobutrazol can be applied as either a spray or drench, being more effective applied as a drench when there is not pine bark in the media. Paclobutrazol is very persistent on plastic surfaces and in soil. Some authors have verified it can last for several years in the soil, which could contaminate both people and the environment. Thus, flats, trays, pots, or soil from treated plants should not be reused, especially for plug production of sensitive crops. Paclobutrazol induces hardening in plants, stimulating their ability to counteract abiotic and biotic stresses.

3 EFFICACY OF THE PLANT GROWTH REGULATORS

3.1 Species sensitive to growth regulator

The response to a specific growth regulator varies not only among species but also among cultivars within a species. For example, it is usually apply chlormequat in poinsettia to reduce its growth, but when a greater effectiveness is required we have to spray daminozide. Daminozide is recommended for gardenia and hydrangea, because they are plants harder than poinsettia; however, it is ineffective for controlling stem elongation in *Hypoestes phyllostachya*. Paclobutrazol reduces stem height of numerous pot-grown bedding plants, except for *Zinnia elegans*; this plant required a concentration eightfold great than for pelargonium. Among lily cultivars, a paclobutrazol drench of 4-20 ppm controlled stem elongation of the cultivar Ace, but not that of Nellie White.

Applying growth regulators at the appropriate stage of development is important for all crops. Use of daminozide is restricted to early-season applications in poinsettia, since it can severely reduce bract expansion and delay flowering if applied too near the time of flower initiation. Chlormequat spray should cease at the appearance of first bract colour in warmer high-light climates so as to not affect bract size. However, paclobutrazol drenches are commonly applied in this plant during the late cultivation period to control the last stretch. Generally, growth retardants applied within a few days after a pinch are more effective than those applied latter.

3.2 Interaction with cultural and climate factors

The effect obtained from a growth retardant application varies according the to conditions under which they are growing, even by weather conditions at the application time. Growth retardants are more active when plants have a great level in growth. Plants cultivated during cooler months require less growth retardants than ones under warm conditions. Under higher temperatures retardants are often less effective due to diminished chemical activity. Chlormequat chloride is a clear example of this behavior.

Environmental conditions during the treatment may alter effectiveness too. According to the mode of action of the phytoregulator, it will be more or less effective in summer or winter. We studied the effect of high temperatures on the efficacy of ethephon at the moment of its application in geranium. Under high temperature (polycarbonate greenhouse), plants receiving ethephon grew like the control, while under lower temperature (plastic shade cloth) ethephon reduced geranium growth. In general, the most appropriate ambient conditions for chemical application on crops are low light level, high ambient humidity and cool temperatures (early morning, in the evening, or on cloudy days).

Water stress and fertiliser levels affect growth rates and alter retardant effects. Drench applications can be affected by growing media, because they tied up by organic compounds. A clear example is found from pine bark, which reduces the effect of

paclobutrazol. The more pine bark is decomposed, the less paclobutrazol has chemical action.

3.3 Amount of active ingredient applied

The dosage of the active ingredient is the most important aspect in effectiveness of a plant growth regulator. The amount of active ingredient applied to plants depends on both the concentration of the solution and the volume applied. Therefore, for soil-active growth regulators dosage will be the applied active ingredient amount (mg) per plant or pot, which is dependent on both the concentration of the solution and the volume of that solution applied. In general, the spray application rates will be sufficient to cover the plant and permit a small amount of runoff onto the medium.

The commercial products provide specific recommendations about application dosage for a large number of ornamental plant species. We must apply a higher dose of chemicals to achieve desirable control with more rapidly growing plants, while plants growing under suboptimal conditions require lower ones. On the whole, the higher active ingredient doses lead to great effects. However, a particular concentration of chemical may result in little or no reduction in stem elongation, or extreme and prolonged stunting of plant growth (phytotoxicity), depending on the plant species. Sometimes, a repeated application at lower concentration results in growth control, while a single higher dosage produces a phytotoxic response. Chlormequat often causes discoloration of leaves, especially with rates above 1500 mg/L. On the contrary, daminozide has few incidences of phytotoxicity or overstunting. Phytotoxicity by paclobutrazol includes overstunting (very short internodes) and may cause black spots on annual vinca and creased young leaves in oleander. Toxicity by ethephon excess includes blade yellowing (destruction of chlorophyll), while prohexadione-Ca excess may cause yellowing leaf margin.

Dosage depends both on the type of plant (bedding plants, annual flowers, perennial flower, shrubs, trees, flower bulbs, etc.) and commercially available phytoregulators (paclobutrazol, ethephon, etc.). Herein we provide general recommendations for spray and drench applications, which allow growers to experiment with these chemicals. Growers should do trials to determine the best chemical application procedures and rates under their production. On the whole, paclobutrazol is applied as a spray on plants between 1 and 90 mg/L, while drench applications will need from 0.1 to 50 mg per plant. However, spray concentrations need to be about 40 times greater for chlormequat and 100 times greater for daminozide and mepiquat. Chlormequat is usually sprayed between 500-3000 mg/L, and when it is applied as a drench you have to use between 0.5 g and 2 g per pot (drenching increases the cost of the treatment). Usual concentrations of daminozide are between 1500 and 5000 mg/L, while for prohexadione-Ca you need 200-1000 mg/L when it is applied on herbaceous ornamentals. For ethephon, foliar spray requires concentrations between 500 and 1500 mg/L, and for drench applications 1-1.5 g per pot. When the recommendations for

drench applications are given as concentrations, you must assume a standard delivery volume that allows the solution to be uniformly distributed throughout the entire volume of a moist potting media. A good recommendation for all compounds is about 60 mL per litre of potting media.

3.4 Methods of application

Plant growth regulators can be applied in several ways, including foliar sprays, substrate drenches (applying solutions to the growing substrate) and bulb or seed soaks. However, the majority of applications are made as a foliar spray or substrate drench. Both of these methods have pros and cons. Drenching is a popular phytoregulator application method. However, not all phytoregulators are effective as drenches. Drenching provides more uniform results and increases the duration of effectiveness compared with foliar sprays. The efficacy of growth regulators drenches can be affected by the amount of active ingredient, solution volume applied, and substrate components. Sprays are more likely to be phytotoxic. Their effectivity is greater when they are sprayed along with a surfactant. Absorption sites of paclobutrazol are stems and roots, while for daminozide, ethephon and chlormequat are leaves. An implicit assumption in the recommendations for spray application is that larger plants will receive a greater volume of spray than smaller plants because they have a larger leaf surface are and occupy a larger area of bench space. Depending on the type of phytoregulator, one method can be more or less effective. Thus, daminozide, prohexadione-Ca and mepiquat chloride have to be applied in spray, while ethephon, paclobutrazol and chlormequat can be applied in both ways. Auxins, cytokinins and gibberellins are usually applied as a spray, but can be taken up by roots too. There is a commercially prepared auxin powder for direct contact with cuttings.

3.5 Combining phytoregulators

In some case, combinations of two or more chemicals are used because this provides a synergistic effect. You can mix chemicals in tank or buy trade products containing mixtures. A typical case is a mixture of daminozide and chlormequat, most notably with poinsettia, which provides an amount of high control greater than that obtained with either retardant alone. So, lower rates of each retardant can be used, which can be important for reducing the phytotoxicity causes by chlormequat.

The BASH chemical company offers two commercial formulations containing mixture chemicals, Terpal and Canopy. The former contains 305 g/L mepiquat chloride plus 155 g/L 2-chloroethylphosphonic acid, and the latter contains mepiquat chloride (300 g/L) plus prohexadione-Ca (50 g/L). The two active ingredients in Canopy complement each other, because both have different modes of action, uptake via, translocation of active ingredient and residual effect. The addition of chlormequat provides additional activity because canopy formulation has been shown to be synergistic to the uptake and activity of chlormequat.

3.6 Water quality

Water quality, especially pH and alkalinity, can affect the efficiency of a phytoregulator. If the pH is relatively high (≥7) and the alkalinity is also relatively high (≥100 mg/L of calcium carbonate), the water is said to be higher buffered (resistant to change in pH). This type of water can decrease the chemical regulator affectivity.

Ethephon is stable in aqueous solution below pH 4; as the pH is raised above this, the compound decomposes to ethylene. Therefore, using acidic water to make spraying solution is recommended.

On the other hand, the true active ingredient of prohexadione-Ca is the free acid prohexadione, which, due to its lack of stability, is rather unsuitable for being contained in products. Instead, its stable calcium salt is used and prohexadione is generated only upon preparing an aqueous spray solution. Its efficacy is reduced if the source of spray water is high in calcium carbonate (hard water). The commercial product contains ammonium sulphate for this reason. Ammonium sulphate has been used to prevent the deactivation of prohexadione-Ca by calcium or other cations in hard water.

3.7 Uptake and movement in plant

The consensus is that growth regulators are taken up by roots or through the bark, and transported in the xylem only toward the leaves and stem apex. When they are applied to the aerial part, the active ingredient must contact with leaves and stems and then penetrate into the plant. Water solubility affects the entry into the plant of the active ingredients. There are water soluble products such as ethephon, chlormequat, daminozide, etc., and other poorly soluble ones such as paclobutrazol. More specifically, chlormequat chloride is highly water soluble while paclobutrazol has very low water solubility. The solubility of prohexadione-Ca in water is relatively low. Water soluble growth regulators move slowly through the wax layer in leaves and stems, while the less soluble move quickly. When leaves are wet, the uptake rate of the water soluble chemicals will be better, while with dried leaves very little product comes into the plant. Thus, plants take up paclobutrazol relatively quickly, but plants require more time to take up chlormequat chloride, daminozide, and ethephon. The low solubility in water of paclobutrazol promotes a quick diffusion through the wax layer of leaves and stems. Compound solubility causes daminozide activity which is adversely affected by overhead irrigation applied hours after treatment (it needs, as with ethephon, leaves to not be watered for at least 12 hours after application); while paclobutrazol activity is not affected by overhead irrigation applied 30 minutes after treatment. Prohexadione-Ca demands that a long-lasting liquid film remains on treated plants.

3.8 Commercial formulations

Commercial phytochemicals have different percentages of active ingredients that may decide the efficacy of the compound. This is more relevant when the presence of the active ingredient is relatively low, because it means the addition of a significant amount

of unknown compounds which could influence plant growth. For example, Cultar is a commercial formulation containing paclobutrazol at a concentration of 25%. In addition, it contains other products such as polyglycol, an alcohol which has other effects (fungicide, etc.). The commercial formulation available for using prohexadione-Ca (Regalis) contains 10% w/w active ingredient. It also contains primarily ammonium sulphate (round 57%) as well as acidifying adjuvants.

4 TEMPERATURE

4.1 Average daily temperature

Overall plant growth and speed of development (root growth, seedlings develop as plugs, and plants reach flowering) is dependent on the average daily temperature (ADT). Plant growth is increasingly slow as the ADT (24-hour average temperature), decreases, and increasingly fast as the ADT increases. The rate of plant growth is based on the ADT from the time plants enter a greenhouse until they leave it. It integrates temperature day by day and week by week. If crops are grown cool one week and warmer another week, then their development is a function of the average temperature for both weeks. Thus, if a crop is behind schedule, then temperature can be raised to speed growth. If crops are ahead of schedule, then the brakes can be applied by lowering the temperature to slow down plant development.

4.2 Difference between day temperature and night temperature (DIF)

DIF is a tool to help reduce stem elongation because, in many plants, stem length is influenced by the way temperature is delivered in each 24-hour period. In particular, plants are often shorter when the day is cooler than the night. A cooler day than night is commonly referred to as a negative DIF. But providing a warm night (negative DIF) can be expensive because most of the energy consumed to heat a greenhouse occurs at night. The opposite is true with a positive DIF. Stem elongation increases when the day temperature is warmer than the night (positive DIF).

Stem elongation rate is DIF dependent, with day temperature apparently being more influential than night temperature. Hence, dropping the temperature at the beginning of the morning, which is referred to as a temperature drop, can provide at least an important negative DIF response. Using a temperature drop also has little impact on heating costs. On a daily basis, the stem elongation rate is at its maximum at sunrise and shortly thereafter. DROP is the practice of lowering the temperature, typically by 5-6ºC, before sunrise for a two to three-hour period. For the best and most consistent results, the temperature drop must be achieved before plants perceive the start of the day. Therefore, a common goal is to attain the desired temperature DROP setpoint 30 minutes before sunrise. Temperature drops are generally not effective when delivered at other times of the day or night.

We do not have a clear understanding of how DIF and DROP mediate stem extension of plants. Some scientific evidence indicates that temperature influences the biosynthesis of gibberellins. So, in some way, a negative DIF and a temperature drop inhibit the biosynthesis of gibberellins. There is also likely to be some interaction with phytochrome, the pigment in plants that perceives whether light is present.

DIF can cause undesirable side effects, such as reducing ADT, which has an influence on crop time and plant development. On the other hand, growing plants with cooler day temperatures than night temperatures will present yellowish leaves because it reduces leaf chlorophyll content. Plant dry weight can be reduced when a crop is grown using negative DIF because the rate of photosynthesis is reduced during the day when day temperature is cooled. It has also been suggested that plants under negative DIF may be reducing postharvest life.

5 FERTILISATION

Nutritional manipulation to control growth is usually focused on deciding aspects such us: compactness, flowering, sepal colour, plant dry weight and postharvest quality.

5.1 Compact plant

An effective way to achieve compact plants is using low phosphorus or low nitrogen. The goal is to achieve sufficient compaction avoiding necrosis, abscission and too long a growing period. Low N is commonly used by seedling and bedding plant growers to produce compact plants with short internodes and less foliar expansion. Plants result noticeably chlorotic, yet recover normal green colour and rate of growth after one or two standard fertiliser applications. It is important for plant appearance to regulate the length of time under low N fertiliser.

Marginally low P is an equally effective alternative to low N stress for producing compact plants. However, low P stress is generally slower to produce than low N stress. The aim is to obtain the initial symptoms of P deficient that include reduced growth and green normal foliage. The latter effect is a great advantage compared with the low N stress. More advanced stages of deficiency need to be avoid because they include chlorosis of lower leaves, followed by necrosis.

5.2 Enhanced flowering

Nitrogen is the principal nutrient that interacts with flowering. To stimulate flowering low N rates have to be added. Low N enhances earliest flowering in many, but not all crops. On the contrary, high N application delays flowering. N reductions have to be done when the flower development is completed, and stress should not progress beyond the point of slightly lighter green foliage than normal. Applying high P dose you can also improve flowering. A P_2O_5 concentration two to three times as high as the N concentration should be used.

5.3 Postharvest quality

Decreasing N during the last period of the growing season reduces speed growth and improves postharvest characteristics of flowers and ornamental plants. Benefits of decreased N include longer vase life for cut flowers, better acclimation of foliage plants to the poorer consumer environment and better resistance for flowering potted plants during shipping and handling. N is usually restricted by reducing the concentration of complete fertiliser applied at the end of crop.

5.4 Flower colour control

The sepal colour of hydrangea is famous for its colour changes under different cultivation conditions. Whether a hydrangea (excluding white cultivars) develops a pink or blue inflorescence is dependent on the presence of aluminium. The absence of aluminium assures pink flowers, while high availability of aluminium leads to blue flowers. By regulating aluminium, flower colour can be controlled. Any factors that increase the amount of aluminium in the substrate solution lead to the production of blue flowers. An acid pH and a low concentration of P enhance availability of aluminium and, therefore, the blue colour. P antagonises aluminium uptake and helps assure pink flowers. To achieve pink flowers we have to avoid supplying aluminium to plants; do not use mineral soil in the substrate and use fertilisers that do not contain aluminium. Relatively high levels of phosphorus should be used in the fertiliser program too.

6 ENHANCE GROWTH BY CARBON FERTILISATION

Carbon dioxide is an important growth factor beside warmth, light, nutrients and water. Approximately 45% of dry matter in plant is carbon, and its main source is air, and to a lesser extent from respiration and soil organic matter. Most crops show that increasing the CO_2 level will increase the photosynthesis by about 50% over ambient CO_2 levels. Increased CO_2 levels will cause other effects: shorter growing period, improved crop quality and yield, as well as increased leaf size and leaf thickness.

Air generally contains approximately 350 µL/L CO_2. During the day, the carbon dioxide levels can easily drop below 350 µL/L (midday), which has a significant negative effect on the crop. Ventilation during the day can raise the CO_2 levels closer to ambient but never back to ambient levels of 350 µL/L. The level to which the CO_2 concentration should be raised depends on the crop, light intensity, temperature, ventilation, stage of the crop growth and the economics of the crop. For the majority of greenhouse crops, net photosynthesis increases as CO_2 levels increase from 350 to 1000 µL/L. Even lower levels (500-800 µL/L) are recommended for many ornamental plants; however, for other plants such as tulips and Easter lilies no response has been observed. In order to improve economic efficiency, CO_2 levels can be set depending on light levels. On sunny days when the vents are closed, supplement with 1000 µL/L CO_2 while on cloudy days when the light level is below 40 W/m^2 supplement with only 400 µL/L CO_2.

Carbon dioxide enters into the plant through the stomatal openings by the process of diffusion. Stomata open and close allowing gas exchange to occur. The concentration of CO_2 outside the leaf strongly influences the rate of CO_2 uptake by the plant. The higher the CO_2 concentration outside the leaf, the greater the uptake of CO_2 by the plant. Light levels, leaf and ambient air temperatures, relative humidity, plant water status and the CO_2 concentration in the air and the leaf, are many of the key factors that determine the opening and closing of the stomata

Carbon dioxide can be obtained by burning carbon-based fuels such as natural gas, propane, and kerosene, or directly from tanks of pure CO_2. It is very important to have an adequate distribution system. The distribution of CO_2 depends mainly on air movement within the greenhouse, as CO_2 does not travel very far through diffusion. For instance, when a single source of CO_2 is used for a large surface area or various connecting greenhouses, a distribution system must be installed. Air circulation using horizontal airflow fans or a fan-jet system provides uniform distribution by moving large volumes of air within the greenhouse when top vents are closed.

7 DEFICIT IRRIGATION

Before the days of chemical growth regulators, underwatering was practical for many crops to retard height. Plants were allowed to wilt, sometimes severely, between irrigations. Today, low water stresses are practiced, such as deficit irrigation and partial root drying. Both are tools of importance in the production of ornamental plants because they can improve ornamental plant quality, water use efficiency and tolerance to adverse environmental conditions.

Deficit irrigation is the application of water at a rate and volume lower than the evapotranspiration level. It can be applied either as sustained deficit irrigation, applying water at a constant fraction of potential evapotranspiration through the season, or as regulated deficit irrigation, in which water deficits are imposed only at certain crop developmental stages, the duration of the water stress period during each growth phase being very important. Under partial root drying half the roots are well watered while the other half are left to dry. The chemical signals produced in the drying roots reduce stomata conductance and control vegetative vigour, while the fully hydrated roots maintain a favourable water status. An additional benefit of such techniques is that they can also reduce the volume of water used in nurseries and improve commercial plant quality by reducing excessive growth (more compact plants).

7.1 Deficit irrigation to control growth and development

Different water stress levels applied induce different growth responses in the plants, suggesting that the severity of the water stress is an important aspect when deficit irrigation is used as an irrigation strategy to save water without reducing quality in ornamental species. While moderate water stress produced no significant changes in aerial development of several shrubs, a severe water deficit clearly reduced that

parameter. We verified that water deficit also had a significant effect on biomass accumulation when geranium plants were exposed to regulated deficit irrigation in different phenological stages. Aerial dry weight decreased with deficit irrigation too; however, root dry weight was not modified and the root to shoot ratio increased in the geranium plants grown under deficit irrigation conditions, regardless of the time when the reduction was applied (during the vegetative growth phase or during the flowering development phase). We also observed that the aerial dry weight of carnation plants stressed throughout the whole experiment was lower than in control plants and those exposed to deficit irrigation only during the first and third growth phases (outside blooming). These findings may be important for growers of ornamental plants because plants are often exposed to drought treatments during nursery production to reduce excessive growth. However, it goes without saying that it is first necessary to know the level of drought at which a species can maintain healthy growth and acceptable quality.

Deficit irrigation will be more effective to growth control in the plants which demand a large amount of water, such as hydrangea.

7.2 Deficit irrigation to make hardened plants

Deficit irrigation can also confer another advantage by pre-conditioning plants against drought in the field. It is well known that deficit irrigation during nursery production affects some morphological and physiological aspects (stomata closure, epinasty, reductions in leaf area, alteration in leaf water potential, osmotic adjustment) that might provide capacity to adapt to adverse conditions. These features contribute to increase relative growth and net CO_2 assimilation rates after rewatering and reduce the mortality rates after transplantation under semi-arid conditions. However, the duration of any benefits induced by deficit irrigation should be evaluated for each species.

Acclimation during exposure to deficit irrigation and low humidity was associated with smaller plant size and leaf area, and shorter, thicker, denser and less ramified roots, osmotic adjustment and more efficient stomatal regulation, which reduced the mortality rate of oleander seedlings after transplantation.

Other aspects are that the effect of drought stress is usually greater on shoot growth than on root growth. In addition, the root system morphology can be modified by water stress in an attempt to improve plant physical support. Root length is a critical factor in influencing the ability of a plant to absorb water from the soil under water stress conditions. In this sense, previous studies in some ornamental species have indicated that plants subjected to low-moisture regimes can develop more root length. Also, root distribution for each root diameter can also be modified by the irrigation. Thinner roots, compared with non-stressed controls, have been reported for drought-stressed shrubs.

8 LIGHTING

Knowing how the light quality and quantity (duration and intensity) can affect plant growth will help in managing a crop's growth.

8.1 Photosynthetic lighting (lighting at a high intensity)

The term "daily light integral" (DLI) refers to the number of light particles, or photons, received during one day in the PAR region (400-700 nm). DLI represents the amount of CO_2 that is extracted for photosynthesis. The rate of photosynthesis increases with DLI (mol/m^2 d). Under very low light intensities, plants actually have negative net photosynthesis, because the energy used for plant maintenance exceeds any gain of energy from photosynthesis. The light compensation point is the amount of light in which the energy used for plant maintenance is equal to the amount of energy generated from photosynthesis. This value varies among species and is typically lower for shade plants. As light intensity increases, net photosynthesis increases but at a decreasing rate. Finally, it reaches the light saturation point, which is the point at which further increases in light do not increase photosynthesis. Many growers also provide supplemental light to shorten schedules and reduce the growing period. In photosynthesis lighting, the light intensity (quantity) is more important than light spectral distribution (quality). In the areas above 45 degrees north latitude, the total quantity of light received per day in winter can be a limiting factor in plant production. Then, it will be justified to supplement light with artificial lighting systems. Therefore, supplemental lighting is typically not used on ornamentals grown in greenhouses in sunny winter countries.

8.2 Light for photoperiodism (control of daily light duration)

Photoperiodism is the physiological reaction of plants to the length of day or night (photoperiod), and it is frequently associated with the initiation of flowering. With many plants, the time of flowering is influenced by the photoperiod. For example, a chrysanthemum plant will only bloom when the night is long. We call them "short day plants". However, when you apply long day light to them, the flowering will be suppressed. Plants that require short days for flowering such as chrysanthemum and poinsettia will not flower if exposed to long-day lighting. On the contrary, there is a long list of crops that flower earlier when provided long days, and some crops even require long days to flower.

Lighting to stimulate flowering requires much less light intensity than photosynthetic lighting. Using light levels around 1.5 mmol m^{-2} s^{-1} are usually sufficient. In this case, light provided to stimulate plant development is absorbed by photoreceptors in chloroplast. There, conversion takes place of light energy to biochemical energy and, then, a response is produced: flowering or vegetative growth. Activation of phytochrome is caused by red light, and the effect is that flowering is delayed. This occurs with short day plants when long daylength is provided with incandescent or high pressure sodium lamps. Cyclical lighting is provided when light is delivered to plants intermittently but not constantly during the night. A common technique to deliver cyclic

lighting is to turn lamps on for 6-10 minutes every half hour during the desired lighting period.

8.3 Light for photomorphogenesis

The mix of colours in the light (spectral distribution or quality) strongly influences the appearance of the plant or morphogenesis. The control of morphogenesis by light is called photomorphogenesis. Lamps emit large amount of far red relative to red light promote stem elongation, particularly in open-air crops. Leaves and stems are effective filters of red light but reflect most far-red light. Thus, under a dense crop canopy stem elongation is promoted because the far red/red ratio dropped (it enhances the activation of phytochrome). Knowing the distribution of light and the red/ far red ratio can be useful to predict the impact of different light sources on plant development. Incandescent lamps emit a large amount of far-red light, but they are being forbidden in various countries because of their electrical inefficiency. LEDs that emit light at a low intensity are being developed to replace incandescent lamps. As LEDs advance and their costs decrease, we believe they will become the preferred light source for both low-intensity (photoperiodic) and high-intensity (photosynthetic) lighting. Far-red LEDs will be the green future of horticulture.

9 MECHANICAL CONDITIONING

Mechanical conditioning is defined as using a physical or mechanical treatment to reduce plant growth and improve plant quality. These treatments are designed to simulate plant exposure to outdoor conditions, including high wind, rain, physical, contact with surrounding plants or animals, etc. The application procedures reported have included brushing, shaking, wind, and more recently impedance (placing a physical barrier above the floor); all of which result in physical displacement of the growing points. The effects of these treatments appear to be related with the effect of stress-induced ethylene. Most of the methods are impractical for large scale or require significant engineering investments because they are difficult to automate.

Brushing plants is the most effective and efficient method of mechanical conditioning in high-density plantings, like vegetable transplant or bedding plant production. Brushing involves the movement of a PVC pipe, wooden dowel rod, or burlap bags over the top third of the plant. This treatment is a very effective way of controlling plant height (30 percent to 50 percent reductions) of many crops. These reductions in growth are similar to those obtained with plant growth retardants, but brushing provides more flexible growth control than is possible with chemicals. In general, as the frequency and duration of the brushing treatment is increased, so is the amount of growth reduction. It recommends bruising twice daily with a minimum contact time of 1.5 to 2 minutes (generally 40 strokes) with each treatment. Also, be aware that the effects of brushing on plant growth dissipate within three to four days after you stop applying the treatment. Brushing provides benefits such as: 1) plants are more resistant to stress, 2) enhances the establishment in the field, 3) leaves are darker green in colour and are thicker, 4)

canopy is denser, 5) stems and petioles are stronger, and 6) stem and flower stalk elongation is diminished. On the contrary, this treatment has some limitations: 1) there are plants with low response to brushing, 2) do not brush plants that have wet or wilted foliage (it increases leaf tearing), 3) it can cause unsightly flower damage, 4) it could spread diseases, and 5) the commercial application of brushing is limited by a lack of automation.

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CHAPTER 7

Irrigation scheduling and agronomic-physiological crop responses to water regime

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1 INTRODUCTION

This subject consists of two clearly distinguishable parts: one of a more fundamental character consisting in the first five units, and the other which is more practical for the five remaining units. In general these will cover functional crop study relating to the movement of water in the plant through the model: *the soil – plant - atmosphere continuum (SPAC).* In the more practical units and in relevant crops the correlations found between typical variables in studying water relations and specifically agronomic factors: growth, production, crop quality, etc. are analysed. The objective is to use the water relations (of plants and soils) as a tool to approach understanding of water and agronomic functioning of the plant and its application to management or irrigation scheduling.

To achieve this objective the subject starts with the topic of *Measuring and Estimation of Crop Evapotranspiration* as a prior step to the *Determination of Crop Water Requirements and Irrigation Scheduling*. This final topic is the core of the subject as a compulsory requisite in any project related with water in agriculture. The unit *the Soil-Plant-Atmosphere Continuum* provides a solid theory and functional basis as a progression from previous units and contributes to fitting the programmes in order to apply the treatments defined precisely in any research project. The crop response to water regime is studied in the following four units through data from trials carried out by the subject's teaching group. The use of salt and reutilised water as an efficient mechanism to regulate the water resources in areas with major deficits has acquired force and thus merits special attention. Finally, and given the need for the application of new information and communication technologies (ICTs) in agriculture and to innovate to face the shortage of water, these are covered in the remaining two units.

Although the subject has a strong research profile within Irrigation Agronomy, its design is such that new knowledge can be provided and skills can be gained at social,

organisational and technical level of interest for the use in professions of the sector of plant production.

2 MEASUREMENT AND ESTIMATION OF EVAPOTRANSPIRATION AND DETERMINATION OF THE CROP WATER REQUIREMENTS

2.1. Reference and crop evapotranspiration

The estimation of evapotranspiration (ET) and its components (evaporation from the soil surface, E_s and transpiration or plant evaporation, E_p) has been a key issue for improving irrigation management of agricultural crops and in hydrological studies.

The most widely adopted method in agricultural water management for calculating ET is the one originally proposed by FAO (Doorenbos and Pruitt, 1977) which uses the product of two factors, the reference ET (ET_0) that corresponds to a hypothetical grass surface and a crop coefficient (K_c) that is proportional to crop ground cover and frequency of soil wetting. The hypothetical grass (or alfalfa) is a reference crop with an assumed crop height of 0.12 m, a fixed surface resistance of 70 s m^{-1} and an albedo of 0.23. The reference surface closely resembles an extensive surface of green, well-watered grass of uniform height, actively growing and completely shading the ground. The fixed surface resistance of 70 s m^{-1} implies a moderately dry soil surface resulting from about a weekly irrigation frequency.

ET_0 is an agro-climatic parameter and can be computed from weather data and expresses the evaporating power of the atmosphere at a specific location and time of the year and does not consider the crop characteristics and soil factors (Allen et al., 1998).

2.2. Standard methods for estimating the evapotranspiration

A large number of more or less empirical methods have been developed over the last 50 years by numerous scientists and specialists worldwide to estimate evapotranspiration from different climatic variables. Relationships were subject to rigorous local calibrations and proved to have limited global validity. Testing the accuracy of the methods under a new set of conditions is laborious, time-consuming and costly, and yet evapotranspiration data are frequently needed at short notice for project planning or irrigation scheduling design. To meet this need, four main methods for calculating the reference crop evapotranspiration (ET_0) have been commonly adopted: the Blaney-Criddle, radiation, modified Penman ("Penman-Monteith") and pan evaporation methods. The Penman-Monteith method is considered to offer the best results with minimum possible error in relation to a living grass reference crop and is recommended as the sole standard method for the definition and computation of the reference evapotranspiration. It is a method with a strong likelihood of correctly predicting ET_0 in a wide range of locations and climates and has provision for application in data-short situations.

Considering the other methods, the pan method gives acceptable estimates, depending on the location of the pan. The radiation method is suggested for areas where available

climatic data include measured air temperature and sunshine, cloudiness or radiation, but not wind speed and air humidity. Finally, the use of the Blaney-Criddle method is suitable for areas where available climatic data cover air temperature data only.

The FAO Penman-Monteith method considers many meteorological parameters related to the evapotranspiration process (net radiation, air temperature, vapour pressure deficit, wind speed) and has presented very good results when compared to data from lysimeters populated with short grass (Allen et al., 2006). The analysis of the performance of the various calculation methods reveals the need for formulating a standard method for the computation of ET_o.

Allen et al. (1998) defined crop evapotranspiration through the development of a dual approach - crop coefficient, which separated K_c into a "basal" (crop) and a soil component - that *grosso modo* corresponds to separating E_p and E_s. However, in this case the basal crop coefficient includes E_p and some E_s when the soil is dry, which makes it almost impossible to check the validity of ET partitioning. The dual approach of Allen et al., was based on the original work of Wright (1982), which may be valid as an approximate engineering approach for ET estimation, but may not be precise enough in many situations. Furthermore, the precise quantification of E_p is also important as it is related to canopy performance in terms of assimilation, and thus, productivity. Also, evaluation of alternatives for water savings based on reduction/suppression of $E_{s,}$ - an important issue that demands critical evaluation and that is currently a subject of debate (Gleick et al., 2011) - depends on our ability to estimate E_p.

The ET of fruit trees presents two distinct features as compared to that of annual crops; one is the large fraction of soil evaporation due to an incomplete ground cover that changes with plantation age and may be highly variable among orchards, but that seldom reach complete cover due to traffic and orchard management needs. The second is the tighter coupling of fruit tree canopies to the atmosphere due to the large roughness. The latter implies that transpiration will be mainly modulated by canopy conductance and vapour pressure deficit as opposed to short crops (including the reference grass surface), where transpiration is mostly dependent on solar radiation (Jarvis, 1985). Transferability of standard crop coefficients for fruit trees among different environmental and orchard management conditions is thus open to some question, and alternative approaches to quantify orchard ET need to be sought.

2.3. Estimation of crop water status

To develop **new techniques of irrigation programming**, the knowledge of the water status of the crop is essential, which is suitably defined by indicators of a physiological nature. The evaluation of the water status, by means of direct measurements in the plants, constitutes a valuable tool for irrigation management given its dynamic nature, which is closely related to the climatic and soil conditions, as well as the productivity of the culture (Goldhamer et al., 2003).

In relation to the most used **methods to evaluate the crop water status**, the most prevalent is the traditional evaluation of the leaf water potential by means of a pressure chamber (Hsiao, 1990). However, many authors have demonstrated that the stem water potential is much more adequate to irrigation programming. In any case, both indicators present a relatively complex procedure of measurement, with the necessity of frequent displacements to the field, the use of manual labour, and are impossible to automate. Consequently, the discontinuous nature of these indicators tends to be of little practical use in relation to other indicators, such as the sap flow and the micrometric variations of the diameter of the trunk, which are able to provide continuous and real-time information of the crop water status.

Under water scarcity, such as in the south-eastern region of Spain, suitable indicators of crop water status are becoming ever more relevant to assist in **precision irrigation management**. Indicators suitable for tracking crop water status exist (Fernández and Cuevas, 2010) but they reflect a single plant behaviour and their representativeness is always questionable. Given that deficit irrigation increases the risks of developing excessive water deficits in some parts of the orchard (Fereres and Soriano, 2007), it is important to have access to tools that can account for the spatial variability in water status, without resorting to installing an unreasonable number of sensors that would be needed to characterise it. Nowadays, thermal imagery acquired remotely provides a means of characterising orchard variability and can assist in making decisions concerning irrigation management in precision agriculture (González-Dugo et al., 2012).

3 IRRIGATION SCHEDULING

3.1 Introduction

The rationalisation for the use of water is an objective which is becoming increasingly important to many sectors of our society and in particular to the Spanish agricultural sector, the major consumer of available water resources. One of the fundamental techniques for efficiently managed irrigation water with maximum water productivity with a minimal environmental impact is the roll out of a well-planned irrigation scheduling.

Any irrigation scheduling involves a procedure to determine irrigation frequency and gross irrigation dose to apply at each irrigation. Therefore irrigation scheduling is the process of answering two basic questions:

Do I need to irrigate?

How much water should I apply?

3.2. Methods and criteria for irrigation scheduling

The most accepted form of scheduling is to perform it according to the meteorological variables of the site. It is also usual to take into account soil water status, that is, on the basis of the water content and soil matric potential. However, methods that are

supported in the measurements of parameters related to plant water status are virtually restricted to research centres and large scale farming. Regardless of the chosen procedure for its implementation, this will depend on the purpose. Thus, irrigation scheduling can be carried out with the following purposes: i) purely technical, whose aim is to achieve maximum output per unit of surface; (ii) economic, whose aim will be to ensure the highest production per unit of water applied (maximum water use efficiency); and (iii) technical-economic whose aim will be to obtain the maximum benefit in the implementation of the water.

In order to achieve desirable agronomic results (i.e. maximum yield per unit area) soil moisture depletions below a certain critical value should be avoided in order to make sure the crop evapotranspiration (ETc -crop evapotranspiration) is not limited.

In research studies aimed at the characterisation of the crop agronomic response to different irrigation regimes, an essential treatment is the so-called treatment control or full irrigation. This treatment is usually also scheduled according to purely technical criteria.

In arid and semi-arid areas where water is a scarce and expensive resource, optimum technical output arises as the achievement of maximum yields per unit of water applied. For this reason, it is vital to be aware of the exact duration of critical periods of the crop growth cycle in order to avoid, at all times, the development of a water deficit that could dramatically reduce the yields. Finally, there is the objective of all agricultural businessmen, the realisation of economic growth, that is to say, to optimise the benefits of his agricultural management. So we can say that irrigation scheduling is the use of water management strategies both to prevent over application of water and under watering while minimising yield and crop quality loss due to water deficit and waterlogging.

This topic lists the steps to follow to make the scheduling of under drip irrigation taking a purely technical approach and from different programming methods. Equally, it addresses the calculation of doses and irrigation run times for orchards under regulated deficit irrigation (RDI) and sustained deficit irrigation (SDI) as a percentage of the ETc or the irrigation dose corresponding to the control treatment. The percentage replacement applied will depend on the type of fruit species and the phenological state of the tree. The formula commonly used to calculate irrigation run time is:

$$\text{Run time (h)} = \frac{ET_c\ (mm\ d^{-1})\ x\ row\ spacing\ (m)\ x\ tree\ spacing\ (m)\ x\ percentage\ replacement}{number\ emitters\ per\ tree\ x\ emitter\ discharge\ (L\ h^{-1})\ x\ (Ea\ x\ DU)}$$

where Ea is the estimated application efficiency, and DU is the distribution uniformity. Replacement percentages are derived from the original RDI experiments.

3.3 Results and discussion

Finally, the results obtained in different experiments are compared and discussed at the level of water relations and productive response. Additionally, the pros and cons of using one or another of the methods of programming are discussed. Likewise, the need

to rely on more than one single method for decision-making in irrigation management is presented and discussed. In subsequent themes of the subject, crop response is analysed through the study of different productive efficiencies.

4 SCHEDULING AND MANAGEMENT OF IRRIGATION WITH SALINE WATER. STUDY OF THE WATER RELATIONS, GROWTH, AND YIELD FROM FIELD TRIALS

4.1 Introduction

In arid and semi-arid regions, it is not uncommon, with regard to scarcity of water resources that this problem then goes hand in hand with low quality water. The basic principle for sustainable irrigation when saline water is being used is to maintain the soil salts concentration to a relatively constant level below a specific threshold value for each species. Fulfilling this requirement on the basis of programming techniques of irrigation is a precise practice proven profitable. However, the availability of water in these areas remains scarce, therein arises the option to grow with less water, and if possible, by minimising the losses in production and quality of the harvest.

As we know, the salts exert both general and specific effects on plants which directly affect crop growth and yields. Similarly, salts alter certain soil physico-chemical properties which at the same time alter the suitability of the soil as a medium for plant growth.

4.2 Assessing the suitability of irrigation water for crop production

To determine the availability of water for irrigation, accurate information is needed of both quantity and quality. The quantity allows us to infer the degree to which the water supplies are able to meet the needs and is data that is required in the planning of crops and irrigation. The quality usually indicates appropriateness of use, and it is therefore difficult to assess, unless it is carried out in relative terms to its specific use. The suitability of irrigation water is set in relation to the evolution of the potential danger within the soils and crops that involves its use, and management strategies of irrigation able to reduce the risks associated with their quality.

Water irrigation quality is normally assessed using four related categories:

Salinity hazard - Total soluble salt content.

Sodium hazard. -Sodicity. Infiltration/Permeability Problems.

Specific ions toxicity: chloride, sodium, boron, sulphate and nitrate.

Other problems.

For better salinity control, in the majority of cases, it will be necessary to make an appropriate choice and combination of practices in order to guarantee an adequate plant growth medium for plant growth.

4.3 Study of the water relations, growth, and production from field trials

The scarcity of water in the southeast of Spain and indeed in other parts of the world has led to the employment of highly efficient irrigation systems; but also to the use of groundwater that contain medium to high soluble salts levels.

Although several studies have pointed to the good response of several fruit trees to deficit irrigation, in most cases good quality water was used. Taking into account the salt-sensitive nature of fruit trees and the shortage and poor quality of the water available for irrigation in SE Spain, we undertook studies in order to: i) characterise the plant water relations of almond trees; ii) evaluate vegetative growth and production and the quality of the harvest; and iii) establish relationships between these and other adaptive mechanisms in the response to saline and restricted irrigation.

4.4. Experimental conditions and conclusions

An experiment was performed for a period of three years, in an orchard planted with mature almond trees (Prunus dulcis (Mill.) Webb cv. 'Colorada') grafted on 'Garrigues' rootstock (Photo 1). The trees were planted at a spacing of 6 m x 6 m and drip irrigated with highly saline well water. The orchard is located in the province of Murcia (SE Spain), where the climate is semiarid Mediterranean with 225 mm rainfall and a yearly evaporative demand, ETo (Penman-Monteith), of 1350 mm.

The soil is a silt-clay-loam with a petrocalcic horizon at 1.2 – 1.4 m depth and an available water capacity of about 0.17 m m^{-1} and bulk density ranging from 1.35 to 1.55 Mg m^{-3}. The soil is poor organic matter, and has high levels of lime and salinity ($EC_{1:5}$ = 0.8 dS m^{-1}, Photo 2).

The irrigation water used had an average electrical conductivity (EC25ºC) of 4.2 dS m^{-1}, an average chloride and sodium content of 16.2 and 19.0 meq L^{-1}, respectively. During the experiment the trees were drip irrigated using two drip irrigation lines for each row, with twelve emitters per tree, each with a flow rate of 8 L h^{-1}.

Three irrigation treatments were applied according to a randomised block statistical design, with three blocks.

CTL, control irrigated at 125% of the potential crop evapotranspiration (ETc)

RDI, regulated deficit irrigation, irrigated at 100% CTL level until the fruit reached their definitive external size (mid-late April), then 70% CTL (until late May), 30% until the fruit were harvested (mid-August) and 70% CTL during the rest of the irrigation season

DRY, dry treatment, not irrigated at any time.

The data obtained indicated that almond trees water relations were adversely affected by drought and salinity and equally their vegetative growth and yields. The mechanisms developed to confront drought periods in conditions of high salinity were based on:

- Lower leaf water potential values than in control plants, which could permit a steeper gradient in water potential between the leaf and soil, favouring water absorption.
- Early stomatal regulation from the first hours of the day, which would lead to greater efficiency in the absorption of CO_2 in relation with water losses.
- The development of osmotic adjustment during the greater part of the growing season, which allows trees to maintain turgor and compensate for the reductions in leaf water potential.
- Smaller LAI, which could have made a further contribution to turgor maintenance via a decrease in the shoot/root ratio and hence reduced transpirational demand.
- The increase in the chloride content of leaves (Photo 3) in all the irrigation treatments was correlated with lower osmotic potentials.

Photo 1. Colorada/Garrigues trees at the beginning of the experiment.

Photo 2. Accumulation of salts at the periphery of the wetted section.

Photo 3. Symptoms of chlorine by late summer.

5 FRUIT TREES IRRIGATION WITH TREATED WASTEWATER. PHYSIOLOGICAL AND AGRONOMICAL RESPONSE

5.1. Use of treated municipal wastewater in agriculture

The motivation for agricultural and landscape irrigation using treated municipal wastewater arises from a variety of reasons, including: (1) the unavailability of a freshwater supply at a competitive price; (2) potential use of plant nutrients contained in treated municipal wastewater; (3) availability of high quality treated wastewater; (4) desirability of establishing comprehensive water resources planning, including water conservation and reuse; (5) avoidance of more stringent water pollution control

requirements including needs for advanced wastewater treatment facilities; and (6) reduction in wastewater discharge to the ecologically-sensitive aquatic environment and tourist beaches such as on the Mediterranean coasts.

5.2. Water quality of treated wastewater

The water quality of treated wastewater depends to a great extent on the quality of the municipal water supply, nature of the wastes added during use, and the degree of treatment the wastewater has received. Wastewater quality data routinely measured and reported at the wastewater treatment plant are mostly for treated effluent disposal or discharge requirements in terms of gross pollution parameters [e.g., biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS)] that are of interest in water pollution control. In contrast, the water characteristics of importance in agricultural and landscape irrigation are specific chemical elements and compounds that affect plant growth or soil permeability. Consequently, when obtaining data to evaluate a treated wastewater irrigation system, it is often necessary to sample and analyse the wastewater for those constituents that define the suitability of the water for agricultural and landscape irrigation (Pettygrove and Asano, 1985).

5.3. Irrigation water analysis

Historically, the quality of irrigation water has been determined by the quantity and kind of salt present in these water supplies. As salinity increases in the treated wastewater used for irrigation, the probability for certain soil, water, and cropping problems increases. These problems are related to the total salt content, to one or more types of salt, or to excessive concentrations of one or more trace elements (National Water Research Institute, 2008). The problems, however, are no different from those caused by salinity or trace elements in freshwater supplies and are of concern only if they restrict the use of the water or require special management to maintain acceptable yields (Westcot and Ayers, 1985; Ayers and Westcot, 1989). For irrigation with treated municipal wastewater, therefore, the suitability of water is judged against the level of management needed to cope successfully with the water-related problems that are expected to develop during use. The most common water quality problems are:

Salinity. Salinity, measured by electrical conductivity, is the single most important parameter in determining the suitability of water for irrigation. Plant damage from both salinity and specific ions is usually tied closely to an increase in salinity. Establishing a net downward flux of water and salt through the root zone is the only practical way to manage a salinity problem (Westcot and Ayers, 1985; National Water Research Institute, 2008). Under such conditions, good drainage is essential in order to allow a continuous movement of water and salt below the root zone. Long-term use of reclaimed wastewater for irrigation is not generally possible without adequate drainage.

Specific ion toxicity. Toxicity due to a specific ion occurs when that ion is taken up by the plant and accumulates in the plant in amounts that result in damage or reduce yield. The ions of most concern in treated wastewater are sodium, chloride, and boron. The

most prevalent toxicity from the use of treated municipal wastewater is from boron. The source of boron is usually household detergents or discharges from industrial plants. Chloride and sodium also increase during domestic usage, especially where water softeners are used. For sensitive crops, toxicity is difficult to correct short of changing the crop or the water supply. The problem is usually accentuated by severe (hot) climatic conditions (Westcot and Ayers, 1985).

Soil permeability. In addition to their effects on the plant, sodium in irrigation water may affect soil structure and reduce the rate at which water moves into the soil as well as reduce soil aeration. If the infiltration rate is greatly reduced, it may be impossible to supply the crop or landscape plant with enough water for good growth. In addition, reclaimed wastewater irrigation systems are frequently located on less desirable soils or those already having soil permeability and management problems, which increases the probability of a problem. A permeability problem usually occurs in the surface few centimetres of the soil and is mainly related to a relatively high sodium or very low calcium content in this zone or in the applied water (Westcot and Ayers, 1985). At a given SAR, the infiltration rate increases as salinity increases or decreases as salinity decreases. Therefore, SAR and ECw should be used in combination to evaluate the potential permeability problem. Treated municipal wastewaters are normally high enough in both salt and calcium, and there is little concern for the water dissolving and leaching too much calcium from the surface soil. On occasions, treated wastewaters are relatively high in sodium; the resulting high SAR is a major concern in planning wastewater reuse projects.

Nutrients. The nutrients in treated municipal wastewater provide fertiliser value to crop or landscape production but in certain instances are in excess of plant needs and cause problems related to excessive vegetative growth, delayed or uneven maturity, or reduced quality. Nutrients occurring in quantities important to agriculture and landscape management include nitrogen and phosphorus and occasionally potassium, zinc, boron, and sulphur (Westcot and Ayers, 1985). The most beneficial and the most frequently excessive nutrient in treated municipal wastewater is nitrogen unless nutrients (e.g., N and P) are removed in the wastewater treatment process.

Miscellaneous problems. Clogging problems with sprinkler and drip irrigation systems have been reported. Growth (slimes and biomass) in the sprinkler head, emitter orifice, or supply line cause plugging, as do heavy concentrations of algae and suspended solids. The most frequent clogging problems occur with drip irrigation systems. From the standpoint of public health such systems are, however, often considered ideal, as they are totally closed systems and avoid the problems of worker safety and spray drift. Excessive residual chlorine in treated effluent causes plant damage when sprinklers are used if the high chlorine residual exists at the time the effluent is sprinkled on plant foliage. Residual chlorine less than 1 mg/L should not affect plant foliage, but when chlorine residual is in excess of 5 mg/L, severe plant damage can occur.

5.4. Case study: influence of two different treated wastewaters on citrus trees.

Pedrero et al. (2007) evaluated the effects of applying treated wastewater on citrus trees. Two experimental plots irrigated with two different treated wastewater effluents were compared. The experimental sites were located in Murcia, in the south-east of Spain. The first experimental plot was placed on a silty-loam soil in Cartagena, south-east of Murcia, where the treated wastewater originated from a secondary treatment plant. The second experimental plot was placed on a silty-clay soil located in Campotejar, north-east of Murcia, where the treated wastewater originated from a tertiary treatment plant. Soil, leaf, and water chemical and microbiological analyses were performed in order to detect these effects. The physical parameters pH, EC, turbidity, and total dissolved solids (TDS) were higher in Cartagena's treated wastewater than in Campotejar's, which has higher levels of nitrates, sulphates and suspended solids (SS). High accumulation of K, Fe, P and some heavy metals like Ni, Cr and Pb in Cartagena's soil were incorporated in the leaf, whereas high accumulation of chlorides in Campotejar's soil were reflected in this site's leaf dry matter. The microbiological analysis revealed an absence of E.coli and helminth eggs in the treated wastewaters and soils, but in Cartagena's treated wastewater faecal coliforms exceeded health standards (WHO, 2006).

It was concluded that: (1) the possibility of using reclaimed wastewater mixed with well water is a good solution for improving the agronomic quality of treated wastewater; (2) the high salinity and boron concentrations are the main problems associated with treated wastewater use in the Region of Murcia; (3) treated municipal wastewater seems to be an alternative water resource for citrus trees irrigation with a correct salts management.

6 IRRIGATION WATER MANAGEMENT BASED ON INDICATORS OF SOIL AND PLANT WATER STATUS. ANALYSIS AND DISCUSSION OF FIELD TRIALS RESULTS

One way to optimise the use of irrigation water through a more efficient control of irrigation scheduling is through the use of indicators of plant water status, which quickly detect water stress in the plant and thus the need for watering. Among these indicators, the stem water potential has proven to be a robust and sensitive indicator (Shackel et al., 1997), but its use has the disadvantage of requiring a great deal of work to properly monitor the water status of the plant. But from the knowledge that plant growth is an excellent indicator of water stress, continuous information provided by the LVDT sensors placed in the trunk of the tree has mitigated this problem.

Maximum daily trunk shrinkage (MDS) is considered by many researchers as an indicator of plant water status more sensitive to water deficit. Advances in computing and electronics made it possible to continuously record the trunk diameter fluctuations (TDF) discovered by Kozlowski and Winget (1964). From such data, Goldhamer and Fereres (2001) proposed a number of parameters such as maximum (MXTD), minimum

trunk diameter (MMTD), maximum daily trunk shrinkage (MDS) and trunk growth rate (TGR) to characterise daily plant water status.

The sensitivity of these variables to water stress has been repeatedly tested in different crops like almond (Nortes et al. 2005); apple (De Swaef et al. 2009); cherry (Livellara et al. 2011); citrus (Ortuño et al. 2009); grapevines (Intrigliolo and Castel 2007); olive (Moriana and Fereres 2004); peach (Conejero et al. 2011); plum (Intrigliolo and Castel 2004); pomegranate (Intrigliolo et al. 2011); and table grape (Selles et al. 2004).

This high sensitivity combined with the ability to continuously record its measurements (Ortuño et al., 2010) has opened up great expectations for future automation of irrigation based on its measurements.

These variables may be useful in irrigation scheduling, by comparing the ratio of signal intensity (the ratio of values obtained from trees exposed to water deficit, DI, and those from control, well watered trees, CTL) and the coefficient of variation (CV - noise) as proposed by Goldhamer and Fereres (2001). The signal intensity (SI) would be a direct measurement of the sensitivity to water stress of these plant water status indicators.

The early detection of plant water stress is also important, and MDS was considered to show the greatest sensitivity to water stress (Fernández and Cuevas 2010). In contrast, TGR was generally more influenced by crop phenology, and only responded to water stress in two-year-old trees (Nortes et al. 2005) in which growth rates were fractionally higher than trunk shrinkage. Effectively, in young almond trees there was a constant trunk growth throughout the growing season, and it tested that a water deficit during the stage IV of the crop promoted a slow-down of that growth, decreasing MXTD and TGR, meanwhile MDS values increased. In this way TGR presented a sensitivity of water stress higher than MDS, with values of 2.93 and 1.44, respectively, during the first week of water deficit application. Nevertheless, TGR of adult mandarin trees was highly sensitive to water stress when trunk growth rates were high (Pagán et al. 2012).

Although signal intensity is always similar or slightly higher for MDS compared to Ψstem, the very high coefficient of variation (CV) of MDS (>25%) compared to Ψstem (< 10%) suggests that trunk diameter sensors should be installed on multiple trees to achieve robust results.

MDS depends on both the soil water available to plants and the evaporative demand of the atmosphere, so, reference values are required for plant-based irrigation scheduling and to detect water stress (Ortuño et al., 2010). Values of MDS from non-limiting soil water conditions trees reflect the effect of weather demand on trunk shrinkage; so by relating the actual values of a certain plant indicator (e.g. MDS) with several meteorological variables (e.g. air vapour pressure deficit (VPD); reference crop evapotranspiration (ETo); temperature (T); etc.) reference baselines are obtained.

These reference baselines depend on weather and water in soil but are also affected by other factors such as tree age (Moriana and Fereres 2004), the phenological period

(Egea et al. 2009; Pagán et al. 2012) and crop load (Intrigliolo and Castel 2007), so there are limitations to the use of MDS in irrigation scheduling, so it is necessary to check the degree of interaction with these factors. In addition, there are others such as salinity; the effect on them is unknown.

The irrigation scheduling consisted in maintaining different SI (MDSDI/MDSCTL) values according to the sensitivity to water stress of the crop phenology. Then, the SI values could be higher than 1 to increase the water stress, during the non-critical period, when a deficit irrigation could not reduce the yield and quality of the harvest.

At experimental level, Goldhamer and Fereres (2001 and 2004) were the first to verify that the irrigation scheduling in almond from MDS was both possible and advantageous over other programming methodologies. These authors consider two threshold values of SI of MDS, 1.75 and 2.75, promoting a light and medium plant water stress, respectively. The lower SI values treatment did not decrease the fruit size. The irrigation rate was decreased or increased by 10% to maintain those threshold values of SI.

Subsequently, other authors and other crops reached similar conclusions, such as Ortuño et al. (2009) in lemon, Velez et al. (2007) in clementine and Bonet et al. (2010) in plum.

In almond trees, Pérez-Pastor et al., (2009) maintained SI values around 1.1 throughout the growing season (from March to October), during the first year, the irrigation was scheduled every week, obtaining a CV in the SI values around 22% (Fig. 1A), meanwhile, when the frequency of the irrigation scheduling was increased the CV was reduced to 8% (Fig. 1B), with a higher water use efficiency. The average of water saved was 21%.

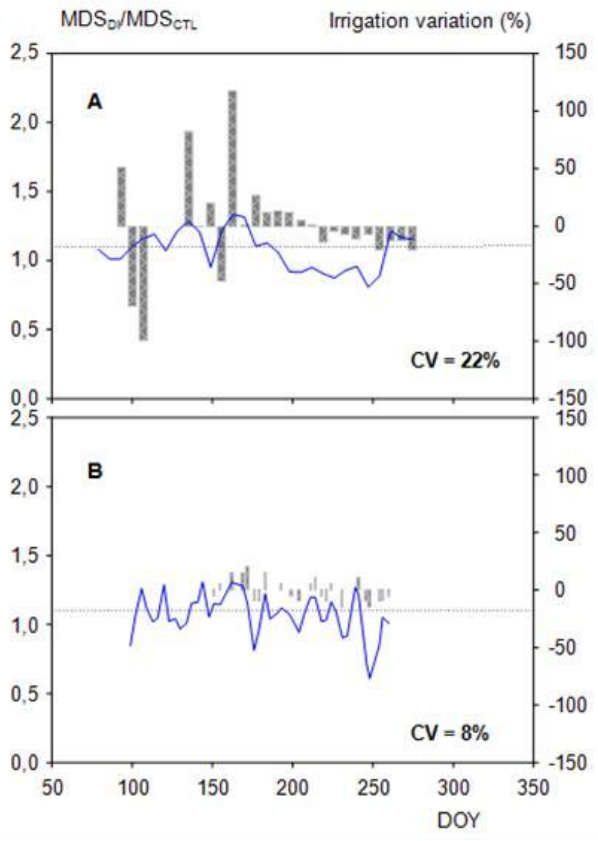

Figure 1. Signal intensity values of MDS (MCDDI /MCDCTL) and the variation of water applied in DI treatment (bars) in almond trees during two years when the frequency of irrigation scheduling was weekly (A) and every 3-4 days (B).

Similarly, Ortuño et al. (2009) after three years scheduling the irrigation of adult lemon trees based MDS found some difficulty in autumn and winter to maintain the SI value of about 1.0 from trees subjected to deficit irrigation continued, so they proposed to increase SI or decrease the irrigation frequency. The irrigation rate was decreased by 10% when the MDS signal intensity did not exceed the threshold value on at least two of three consecutive days. The MDS signal intensity threshold values of 1.15, 1.25 and 1.35 were adopted to induce different water stress levels.

Conejero et al. (2011) by daily adjusting of irrigation doses achieved substantially reduced SI deviations with respect to SI. However, we must take into account the multidependent nature of MDS, as it has been shown by several authors.

In mandarin trees, Velez et al., (2007) scheduled the irrigation during two years, maintaining SI of MDS around 1.25 from June to October. In this way, the water saved in the DI treatment amounted to nearly 15% of the amount used in the control treatment, without negatively affecting the yield and fruit weight.

In a study of deficit irrigation scheduling in plum, Bonet et al. (2010) had difficulty in maintaining SI about to default. These authors estimated the MDS for trees without limiting soil water obtained in the same plot years ago, so they attributed the difficulty in adjusting SI to the desired value to the various factors that may have modified MDS

values for certain climate conditions. Other authors have obtained good results combining MDS absolute threshold values with measures of matric potential of soil water. Bonany and collaborators, cited by Ortuño et al. (2010), managed water savings over traditional control of 38% from trials in apple trees when they were watered to maintain soil matric potential (Ψm) and MDS values around -15 kPa and 200 μm, respectively.

7 INSTRUMENTATION AND REMOTE CONTROL FOR IRRIGATION SCHEDULING. WIRED AND WIRELESS SENSORS NETWORK

7.1. Introduction

For irrigation scheduling, you must have very precise information on the variables of soil, water and plant affecting the calculation of crop water requirements. Variables such as MCD, water content in the soil or sap flow rate are very important to estimate the volume of water required.

Furthermore, the implementation of these variables requires the use of information and communications technologies to measure, record, interpret and communicate properly the values acquired by the sensors.

The aim of this subject is to describe the diverse technologies that can be used to deploy the necessary instrumentation to allow irrigation scheduling; from traditional wired instrumentation, to modern wireless sensor networks.

7.2 Instrumentation

The basic instruments for on-line water scheduling are divided in two groups:

- Soil measurement (Potential soil water, FDR, etc...)
- Plant measurement. (Dendrometers-LVDT, sap flow meters, etc...)
- Air temperatures.

The potential of the soil water refers to its energy, and only indirectly reflects the water status of the plant, as it is affected also by weather conditions and the plant itself. FDR sensors reflect the soil water content. The Dendrometers measure the maximum daily (MXDT) and minimum (MNDT) trunk diameter, these data are a powerful tool for growth and tree water running while recording the wet and dry temperatures to determine the relative humidity and the deficit steam pressure air (DPV). From this information relationships can be established vs. MCD type DPV; non-limiting under water in the ground (reference lines) are of great interest for scheduling irrigation dendrometers.

7.3. Data Collecting-Wired

Centralised data registration is the most common way to deploy sensors in a cultivation plot with different irrigation treatments. It requires all sensors to be wired to a recording or storage unit which can work in harsh environments.

In most cases, the data storage unit consists of a Campbell CR1000 datalogger (see Figure) with multiplexers, capable of storing more than 100 signals.

The Campbell CR1000 datalogger captures the signals of the differential voltage LVDT format with a resolution of + / - 2,500 mV and Watermark signals in Wheatstone half bridge format; the sensors of soil water content measurement are captured in SDI12 digital format. The same unit manages the power supply for the LVDTs, the Wheatstone half-bridges and SDI12 sensors. The system is powered by a battery which is continuously maintained by a charger.

The recorded data extraction can be done in several ways, the manufacturer (Campbell Scientific) provides connection via serial port (RS-232 or RS-485) or by wireless connections GPRS.

7.4. Data Collecting - Wireless

The use of these systems for centralised data-logging requires the installation of wires from the sensor location to where the datalogger is. This installation is a major drawback due to the difficulty and high cost involved in the installation of a large number of wires.

As an alternative to wired solution, the technology of wireless sensor networks, WSN, has been used. The WSN are an emerging technology that has experienced widespread growth in recent years and are formed by sensor nodes or motes, which are composed of a computing device such as a microcontroller, storage elements, a power supply system, one radio transceiver and the interface for sensors and/or actuators. Processing algorithms are simple because these devices require a high autonomy. The wireless communication system is usually based on a communications standard such as IEEE 802.15.4 or ZigBee. See Figure 1.

The great advantage of installing a wireless sensor network is that it requires no cables. The data measured by the sensors are recorded and transmitted via radio by one mote or sensor node. The data are received by another node called a coordinator. If the distance between the sensor node and the coordinator node is high (more than 300-400 metres) it is necessary to introduce other nodes called routers.

All nodes are autonomous, i.e. their power supply comes from batteries whose duration is very high (2-3 months). These batteries can be recharged, if necessary, with small solar panels. The installation of the different nodes that performs the WSN is very flexible because sensor nodes allow “multi-hop” connections, i.e. if the node does not

have enough coverage; it seeks another node that does have coverage close to the router or coordinator node, and sends the information from the sensors or actuators to this node.

In addition, each sensor node has local storage, that allows to not lose acquired data if there is any fault in the transmission of the communication.

The data from all the sensor nodes are received at the coordinator node directly or through router nodes. This coordinator node classifies and transmits the data to a database that can be local (in the same node or in some computer connected to it) or remotely via Internet.

Figure 1. Campbell Scientific CR1000 Datalogger vs Wireless sensor node

In Figure 2, on the left, we can see a WSN deployment in an irrigation schedule wine crop. This deployment is composed by 21 sensor nodes (seven irrigation treatments with three repetitions), two routers and a coordinator. Each sensor node is equipped with two LVDT dendrometers, four watermark soil moisture and one SDI12 FDR water content sensor. The other figure shows the data collected by the coordinator node.

Figure 2.- WSN deployment in a wine crop. Data collected by the coordinator node.

8 SOURCES OF INNOVATION AND TECHNOLOGY TO DEAL WITH WATER SHORTAGES AND DROUGHT

8.1. Water in agriculture (More food for every drop of water).

Irrigated agriculture is highly productive compared to rainfed, providing over 40% of current food production in a relatively small land area (<20%). In this sense, the influences of irrigated agriculture on economic and social development are clearly positive. However, the irrigation may also cause adverse environmental conditions such as the pollution of surface water, groundwater and soil salinisation. The water distribution networks and modern pressurised irrigation systems are characterised by an advanced level in terms of efficiency and uniformity, although the amount of applied water and fertilisers often exceeds the real needs of the crops.

8.2. Vision and objectives

The main aim of our research should be the development of a sustainable irrigated agriculture to reduce the use of inputs (water, fertiliser, energy). This requires expanding our knowledge in relation to the water-soil-plant-atmosphere. We should be able to enhance the implementation of new irrigation systems and programming strategies. We need to know which varieties are able to withstand water shortages and drought, enhancing their breeding and their incorporation into the production system. The new technological alternatives in water resources, based on desalination, water reuse and optimisation of scheduling systems and irrigation management, will provide a clear opportunity for technological development.

The results of all these actions should allow a reduction of the environmental impact of irrigation with respect to the current level, and the development of a reference technology industry to lead the "Water and Agriculture" sector worldwide.

8.3. Priorities for water research and technological innovation.

Line 1. Irrigation Engineering (programmers, filters, pumps, droppers, etc...).

Coverage eco-industrial opportunities in the sector, aimed at efficient and sustainable use of water, energy and fertiliser.

Line 2. Irrigation management strategies at farm level.

Development and application of deficit irrigation strategies, partial drying irrigation system, etc...

Line 3. Irrigation water management models at district and basin level.

Development and application of models to aid decision making.

Line 4. Precision irrigation based on environmental, soil and plant sensors.

Development and application of irrigation self-programming systems with feedback from environmental, soil and plant sensors.

Line 5. Precision irrigation based on remote sensing.

Using satellites and manned flight devices / unmanned sensors equipped with remote sensing.

Line 6. ICTs at the service of water management and governance.

Development and application of remote sensing techniques to estimate irrigation needs and water stress.

Line 7. Agricultural use of alternative water resources.

Reuse of treated wastewater, reuse of drainage water, use of desalinated water.

Line 8. Biotech industry in improving the efficiency of water use.

Development and commercialisation of new varieties resistant to water stress, and use of microorganisms in sustainable production systems

8.4. Case study: SIRRIMED project

The SIRRIMED project is a European Project financed by FP7 and coordinated from Spain by CEBAS-CSIC (www.sirrimed.eu). This project is addressing issues related to sustainable use of water in Mediterranean irrigated agricultural systems, with the overall aim of optimising irrigation water use. The approach proposed in SIRRIMED for reaching this goal will be based in an Integrated Water Irrigation Management (IWIM) where the improved water use efficiency will be considered at farm, irrigation district and watershed scales. These strategies include innovative and more efficient irrigation techniques for improving water productivity and allow savings in water consumption. SIRRIMED considers the development, test and validation of new deficit irrigation strategies, the sustainable and safe use of poor quality waters and the improvement of precise irrigation scheduling using plant sensors. These new techniques are integrated with suitable husbandry irrigation practices. At district scale, efforts are directed towards an integrated policy of water allocation which accounts for the characteristics and specificity of each farm, requiring the availability of data bases and efficient management tools (decision support systems) specifically designed to fulfil the objectives of maximising water use efficiency. At watershed scale, priority is devoted to the assessment of new models of water governance, and the definition of strategies and policies aimed at promoting a more responsible use of irrigation water. Finally, SIRRIMED is establishing a sound dissemination strategy for transfer of knowledge towards the end users, with a real participatory approach to facilitate an adequate involvement of stakeholders (farmers, association of irrigation users, water authorities and SMEs).

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CHAPTER 8

Irrigation Districts and their Planning and Management

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PRESENTATION

The maintenance and improvement of irrigated land is essential for facing the increasing food demand that is prospected under future scenarios with a growing population as well as important water resources constraints associated to weather change. The subject "Irrigation Districts and their Planning and Management" presents the fundamentals for the planning and management of irrigation areas, the main agents and infrastructures implied in these irrigation schemes, and the available technological tools for tackling the planning and management issues (Geographical Information Systems (GIS), automation and remote control systems, etc.).

The subject is organised in five teaching units, which are succinctly treated in this chapter, following this syllabus:

1. Introduction.
2. Institutions and policies for agricultural water governance in Spain.
3. The role of Irrigation Water Users Associations in agricultural water management.
4. The planning and modernisation of irrigation districts and their collective irrigation infrastructure.
5. Automation and remote control systems in irrigation districts.
6. GIS basis and functioning for irrigation districts management.
7. References and bibliography.

1 INTRODUCTION

The world's population is rapidly increasing and the greatest potential for the needed food production is often to be found in the development of new irrigated areas. These areas are usually organised as irrigation districts, which are a cooperative, self-governing public corporation, with definite geographic boundaries, organised, and having the power to obtain and distribute water for the irrigation of lands within the district.

The world community is also facing a situation of increasing competition for the water resource among its many sectors. Specifically, water resources are severely constrained in Spain. The National Hydrologic Plan indicates that about 3.5 Mha of land are

currently being irrigated for food production in Spain, with an important water deficit in many places. Improved performance of irrigation districts must be part of the solution to regional or national water deficits since the sector of agriculture is the main user of water resources in Spain (about 75%).The maintenance and improvement of this irrigated land is essential for the country's development.

Many recent studies of irrigation projects have indicated that for various reasons (some of them connected with the technical skills of operating staff, some with obsolete water distribution infrastructures and some with social factors) collective systems for water distribution are frequently very unsatisfactory. Where this is the case, it follows that proposals to improve water management practices at field and watercourse levels, in the absence of simultaneous or prior measures to improve practices or the irrigation system at project level, will lead to disappointing results.

Unsatisfactory performance in irrigation districts is not only a consequence of technical deficiencies in the design of irrigation systems or in the infrastructure preservation, but many of the problems stem from weaknesses in the organisation and management of the district. It cannot be assumed that the most important problems in irrigation water management can be solved by concentrating attention exclusively on improving water management capabilities at field level. Only a comprehensive analysis of all the factors that may be contributing to poor performance at the lower levels of the system can indicate the correct mixture of remedies required, in the correct sequence. In other words, organisation and management at the irrigation district need to be fully reviewed, as well as the constraints at farm level.

It is necessary for government planners to understand the importance of an appropriate organisational structure, the application of suitable management methods and the provision of technically adequate services as factors which determine good irrigation district performance. The gap between actual and expected performances has led governments to undertake different types of actions. In this context, the concept of planning in irrigated agriculture and "modernisation" in irrigated districts is broadly applied.

The modernisation of irrigated districts requires the combination of technical improvements and infrastructure investment, together with institutional and organizational changes in the way irrigation water is managed. Water distribution is clearly of central importance in any irrigation project, but many other aspects of project organisation and management also have a profound influence on performance. These include the project's organisational structure, its overall direction and coordination, and the provision of other services such as operation, maintenance, irrigation assistance to farmers, finance, and administration. Therefore, Irrigation Water Users' Associations (IWUAs) play a major role in decision-making and operate and maintain the collective irrigation infrastructures by themselves.

Specific guidelines for the planning and management of irrigation districts cannot be given since local conditions will bear strongly on decisions regarding the structure of the organisations. However, the subject provides some general criteria and guidelines to help students to identify a suitable organisation. Technical activities related to operation, maintenance, irrigation assistance to farmers, administration and finance are covered in the subject in considerable detail. Organisational aspects related to the management of these services, such as IWUAs description and analysis, and available technological options for possible alternatives in the technical organisation, are also presented.

There is a great need to train professionals and technicians in matters related to irrigation districts organisation and management. Unfortunately, with very few exceptions, the subject is largely neglected in the curricula of universities. The present subject deals with the topics in a rather comprehensive manner, in order to introduce the subject topics to a wider audience. Efforts have been made to reduce the use of mathematical formulae or detailed technical discussions. For those interested in the detailed discussions of some of the technical issues, the bibliography and references will provide further information

2 INSTITUTIONS AND POLICIES FOR AGRICULTURAL WATER GOVERNANCE IN SPAIN

Spain is a southern European country with a large irrigated agricultural sector, which covers 3.5 Mha and consumes 24,100 hm^3 of the total 30,400 hm^3 water demand. The policy issue has been addressed by both national as well as European Union legislation. While the Spanish government has enacted the National Hydrological Plan (NHP) and the National Irrigation Plan (PNR), the European Union (EU) has enacted the Water Framework Directive.

The National Hydrological Plan (NHP) involves significant investments aimed at increasing the water supply for irrigation, urban, and industrial users. The key project of the NHP was the Ebro interbasin transfer, from north-eastern to south-eastern Spain, designed to solve the severe degradation of water resources in the receiving basins, although this supply increasing approach has been criticized by some experts.

The Spanish National Irrigation Plan (PNR) subsidises the modernisation of the largely outdated irrigation facilities, in order to save water resources and improve the competitiveness of agricultural production. Up to the 2008 horizon, planned investments were 19 billion euro under the National Hydrological Plan, and 5 billion euro under the National Irrigation Plan.

The PNR aims to help the irrigation-based agriculture to face modernisation and efficient water management, developing five programmes and building a new financing

system tailored to the different irrigation systems and reinforcing the relationship between administrations and stakeholders, mainly the IWUAs.

The most important programme of the PNR was "Improvement and modernisation of traditional irrigation systems" in which the four State owned companies for agricultural infrastructures called SEIASAS (in Spanish, Sociedad Estatal de Infraestructuras Agrarias. Sociedad Anónima) played a key role. There were three more action programmes less financed and affecting a more reduced area: "Works in irrigation zones with infrastructures into execution"; "New infrastructures for social purposes"; and "New infrastructures in private irrigation communities". The fifth programme included supporting actions such as an Environmental Monitoring Programme, training and educational programmes for farmers using irrigation, complementary studies, knowledge and dissemination of new technologies, etc.

The expected outcomes were: water savings; increased efficiency in water management; control of inputs use and consequent improvement of water quality; extended implementation of automated irrigation technologies; increased capacity to diversify crops for a market-oriented production; increased competitiveness facing global markets; improvement of irrigating farmers work conditions; and a more balanced territorial distribution of the population. In short, the PNR had to contribute to an economically, socially and environmentally sustainable development of many Spanish rural zones.

Over the last 20 years, water management policy in Spain has shown signs of a gradual transition. Traditionally, Spain's problem-solving approach to water policy has consisted of the regulation of the water supply by means of state-subsidised construction of large-scale infrastructure. While not completely abandoned, the supply-based approach to water policy has taken tentative steps towards a concept based more on sustainability of the resource.

The Actions for Water Use and Management Programme (Programa AGUA) was proposed in 2004 as a replacement for the PNR and represented a fundamental policy shift in national water management from large inter-basin water transfers to a commitment to desalination. The PNR adopted by the Popular Party administration in 2001, which rested heavily on traditional management and decision making principles and actors, was revoked three months after the Socialist Party won the national elections in March 2004. The newly-elected government adopted the Programa AGUA shortly afterwards, introducing desalinisation as a policy aim intended as part of a shift to alternative supply-based regulation methods. Twenty-one desalination facilities were planned for six provinces on the Spanish Mediterranean coast to supplement their water needs. The programme was expected to represent a fundamental shift in water management policy, although the outlooks for change are as yet uncertain.

The Programa AGUA was forthright in stating its compliance with EU environmental legislation and made specific reference to the EU Water Framework Directive in its stated aim of promoting water savings through full-cost recovery by 2010. However, recognising that water savings alone will not be sufficient to meet changing demands for water in the Mediterranean regions, it emphasised desalination as the means to 'better guarantee its availability and its quality'.

The Programa AGUA put new order in water policy through the explanation and extension of the specific actions designed to guarantee the availability and quality of water in each territory. The programme also included actions to improve the management and supply of water of quality to optimise the existing storage and distribution infrastructures (both irrigation and urban supply ones), as well as treatment and re-use. Other specific plans and programmes are being executed to face up to the main water challenges in a sustainable way, particularly with regards to environmental management, improving water use guarantee, management of risks like drought, floods, climate change, water planning, management of water in an international context, as well as research, development and innovation.

The European Water Framework Directive protects all continental, coastal and subsurface waters, and is aimed at improving water quality and ecosystems conditions, promoting the sustainable use of water, and reducing emissions and discharges to water media. The directive is aimed at securing a good quality water supply while reducing water pollution, and is based on the principles of river basin management and public participation. Water pricing should approximate full recovery costs to increase water use efficiency, and costs should include extraction, distribution and treatment costs, environmental costs, and resource value costs. The directive introduces a combination of emission limits and water quality standards, with deadlines to achieve good status for all waters. The new Water Framework Directive introduces drastic changes in the control, evaluation and management of water resources, and requires the achievement of good ecological status and good ecological potential of water bodies across the European Union by 2015.

The approval of the Royal Decree on the Irrigated Lands Sustainable Modernisation National Strategy-Horizon 2015 was foreseen in 2011-2012, but the new change in the national government has halted its development. Its aims will include increasing efficiency in water management, promoting saving this resource, improving environment sustainability, harmonising irrigation lands modernisation with the maintenance of good agricultural practices and use of the most advanced technologies to avoid pollution of surface water and groundwater as well as improving conditions of flora and fauna, soils and landscape in irrigated areas.

3 THE ROLE OF IRRIGATION WATER USERS ASSOCIATIONS IN AGRICULTURAL WATER MANAGEMENT

In Spain, about 75% of water resources are used by the Irrigation sector. By legal force, water users, as well as users for any other public purpose that share the same outlet or concession shall organize themselves into "Users Communities". When the water is used only for irrigation these communities are named "Irrigation Water Users Associations" (IWUAs) or "Irrigators Communities". Nowadays in Spain there are around 6500 IWUAs that manage about 2/3 of the total national water resources.

The IWUAs could be defined as the grouping of all the owners who own an irrigating area forced by law to join together, for the autonomous and common administration of the public waters, without profit making intentions (i.e. an irrigation district). It should be noted that the water concession is linked to the land and not a grant to the landowner. Accordingly, when the landowner sells his field he transfers, along with the land, the right to the water that cannot be sold separately, for it does not belong to him.

The main objective of the IWUAs is to distribute the limited water resources available for irrigation with the maximum strictness and equity. Then they have the obligation to administrate collectively the public, surface and underground waters they share. The reason why irrigation water users shall gather in IWUAs is the existence of common properties and related equipment, such as water, transport and distribution hydraulic networks, rural roads, valve boxes and the right-on-ways caused by this works.

The article 73.1 of the waters Low establishes that the IWUAs will be regulated by statutes and ordinances which, once they have been redacted and approved by the users, should be submitted for administrative approval to the Basin Water Agency. These statutes and ordinances have to include the aim and the territorial scope where the public property hydraulic goods can be used, regulating the participation of the water users.

IWUAs management includes all what is related to the internal running and to its relations with third parties. For this reason they will have a General Assembly composed of all the irrigation water users, a Board of Governors composed of users elected by vote of the General Assembly, and the Irrigation Juries required.

The regulations and ordinances will also force all the holders to contribute to pay, in the same proportion, the common expenses of the exploitation of the collective irrigation infrastructures, their maintenance and their improvement.

Of all tasks that an IWUA has to assume, the most important one is the fair and equitable distribution of water to each of the irrigated plots. Hence the importance of a good and permanent agricultural management, made by experts on overall aspects of cropping techniques and, in particular related to a better knowledge of the relationship

between soil water and plants. It should be known which stages of the vegetative development of the crops are more affected by temporary water deficit conditions because of its effects on agricultural yield loses, with the final object to improve available water resources management, especially in regions as is the case of south and east Spain and many other parts of the world, where water resources are limited, both because of unfavourable climatic conditions and the increasing demand of water for other uses that might be priority, as happens with the population supplies.

In the light of the highly competitive and non aggressive for the environment situation in worldwide irrigation agriculture, IWUAs are challenged to channel their members on the best way to make use of the available water volumes and teach them how to do so. This participation will be completed by a control on the water consumed by the farmers. This task will be successfully assumed provided that it is complemented by the appropriate flow measuring instruments and a good water pricing policy.

The main purpose of this subject unit is to introduce the objectives, legal basis and managing operation in the IWUAs.

4 THE PLANNING AND MODERNISATION OF AN IRRIGATION DISTRICT AND ITS COLLECTIVE IRRIGATION INFRASTRUCTURE

Irrigated districts involve the transport of relatively large volumes of water over extensive areas. Their infrastructure is generally complex with long distribution networks. Careful engineering design and construction are needed to keep costs down and to maximise water and energy use efficiency.

The planning of an irrigation district is also a complex process. A preliminary step in the planning of an irrigation project or its rehabilitation is the definition of project objectives that depend on the country's policy for irrigated agriculture. The objective could be to maximise the value of production by unit of water or unit of land. Project designs vary whether the project objective is to develop export-oriented commercial farming or to support rural population, alleviate poverty in rural areas and limit rural migration to urban centres. The original design of a project may no longer be compatible with modern irrigation practices, which are affecting irrigated agriculture in many countries.

The second step in the planning of an irrigation district is the decision about water delivery. This can be described as the frequency, rate and duration of water deliveries at all levels of an irrigation system. The various systems of water delivery are described in the literature (FAO, 1996; FAO 1986): rotation, arranged schedule, limited rate demand and centralised scheduling. Most traditional delivery systems have no or little flexibility built into them. They do not attempt to match water deliveries to crop needs. The stated objective is to obtain equity through simplicity of design, although poor design, maintenance and operational problems may prevent the objective from be achieved.

Modern irrigation projects are designed with the stated objective to deliver water according to crop requirements. At a minimum, the frequency and sometimes the duration of irrigation should be adjustable.

The configuration of an irrigation system is obviously determined by the relation of land and water resources, the topography and economic considerations. The design should incorporate as much as possible features that facilitate operation and provide flexible irrigation service, such as buffer reservoirs and on-farm reservoirs (Stephens, 2010) and use low-pressure pipes instead of canals for distribution. Buffer reservoirs can be located alongside main systems or at the interface between two levels of management. The incorporation of reservoirs reduces to some extent the need for sophisticated water control methods.

The designer has the choice between many control strategies for the operation of the system:

- Upstream, downstream control or controlled volume.
- Local versus remote monitoring and control.
- Proportional versus adjustable control.

The irrigation system proper consists of transport structures to convey, regulate and deliver the water to the users. Two basic types of irrigation systems exist: open canal systems and pressured piped systems. This subject unit concentrates on the latter system. Experience gained from many countries in arid and semi-arid zones has shown that pressure piped irrigation techniques are replacing successfully the traditional open canal surface methods at irrigation district and farm levels.

A pressure piped irrigation system is a network installation consisting of pipes, fittings and other devices properly designed and installed to supply water under pressure from the source of the water to the irrigable area. The pipelines that convey and distribute the irrigation water to the individual plots are usually buried, and are so protected from low temperatures, farming operations and traffic hazards. Off take hydrants, rising on the surface, are located at various spots according to the planned layout. In all piped systems the main component parts are:

- The control centre or station (head control unit, operation and automation equipment).
- The mains and submains (pipelines and connector fittings).
- Valve boxes and hydrants (valves and other flow control devices).

Any device installed in a fluid supply system, in order to ensure that the fluid reaches the desired destination, at the proper time, in the required amount (the flow rate), and under the right pressure, is called a control appliance. As such an appliance controls proper operation of a fluid system, selecting its type, size and placement is of utmost

importance and ought to be done with the full knowledge of the various features of the device and with complete understanding of the way it performs. Equally important is proper maintenance in order to ensure faultless and sound performance of the appliance.

Fluid control devices can be divided into three main classes:

- Directional devices or valves. These serve to directly regulate the fluid flow. Installed in the pipeline, they enable starting or stopping the flow, and setting its rate, pressure and direction. Examples of such devices are the stop valves, the check valves and the regulating valves.
- Measuring devices or valves. In order to ensure the appropriate flow regime, just regulating the flow is not enough. It is also necessary to obtain accurate information about flow parameters, so that adjustments can be made, as required, to achieve the desired flow conditions. Water and flow meters and pressure gauges belong to this group.
- Auxiliary devices. These do not directly influence fluid flow, but ensure an undisturbed functioning of a system. To this group belong air valves and safety valves.

The overriding principle of modern irrigation districts is that irrigation is a service to farmers which should be as convenient and efficient as possible. Farmers ultimately have to generate the benefits which keep the system functioning.

Several definitions of modern design have been proposed. The following definition was adopted during an FAO seminar on modernisation held in Bangkok: *"Modernization is a process of improving resource (labour, water, economic and/or environmental) utilization by upgrading (as opposed to merely rehabilitating) the hardware and software in irrigation projects, while maintaining or improving water delivery service to farms".* Another definition was proposed during another FAO-supported workshop on the valorisation of irrigation water in the large-scale irrigation schemes of North Africa: *"Modernization is a process of rehabilitation of irrigation systems during which substantial modifications of the concept and design are made to take into consideration the changes in techniques and technology and to adapt the irrigation systems to the future requirements of operation and maintenance. Delivery of water should be as flexible as possible, with demand irrigation being the ideal solution."*

These two definitions slightly diverge on the technical aspects. The first one focuses on the shift from supply to service-oriented and the second one on future requirements of operation and maintenance. The first one suggests the use of advanced concepts of hydraulic engineering, the second of new techniques and technology, which may include modern equipment for remote control. The main difference is that institutional and organizational changes, including more active farmer participation, are attached to the first definition.

Summarising, in this subject unit it will be studied that a modern design is the result of a thought process that selects the configuration and physical components in light of a well-defined and realistic operational plan that is based on the service and energy efficiency concepts. A modern design is not defined by specific hardware components and control logic, but advanced concepts of hydraulic engineering, irrigation, agronomy and social science should be used to arrive at the simplest and most workable solution.

5 AUTOMATION AND REMOTE CONTROL SYSTEMS IN IRRIGATION DISTRICTS

Irrigation districts face many challenges in their daily operations. Automation, remote monitoring (telemetry) and control show promise in addressing such issues and can result in more efficient water management, provide real-time flow control and measurement, reduce operational and maintenance costs, and provide alarming capabilities. This subject unit is focused on the knowledge of different aspects of telemetry, remote control, and automation.

The selection of a control strategy is not limited to a simple choice between different systems. There are a number of options available for each level of a piped network system and they can be combined to define the most desirable global solution in order to provide ease of operation and a higher level of service. The designer of an irrigation district project or its modernisation also has the choice between various configurations for the automation of the pressured piped network:

- Distributed control in which control is achieved through independent automatic units.
- Centralised control in which control is achieved through a master station.
- Supervisory control combining distributed automation under master supervisory control. This configuration is known as SCADA (Supervisory Control And Data Acquisition).

The advantages and disadvantages of these different configurations are as follows. Under distributed control, the system manager is not in a position to supervise or control the entire network system. Centralised automatic control makes it possible to use highly efficient control logics but the operation depends on the reliability of a communication system. Under supervisory control, the central station makes decisions on the lower-level strategy based on the data received from the local controllers, also known as remote terminal units or programmable logics controllers. These local controllers make changes to the control devices according to the target instructions received from the master station, such as maintaining a target flow rate or water level. This system is less susceptible to communication system failure. The centralised and supervisory control methods can involve varying levels of participation of the master station personnel in making decisions from manual to computer-directed control, which uses specially developed computer programs using data from the entire canal system and modelling

studies. Computer-directed control is applicable to the most complex systems involving a number of pipes, reservoirs, pumping and/or power stations.

Additional physical, social and institutional factors should be considered in the selection of control strategy and equipment. Questions such as the possibility of crop diversification or conversion to crops with higher irrigation requirements; the capabilities of the field staff to operate and maintain electronic equipment; the acceptance of the operating rules by the farmers, and their understanding of how the system function should all be considered. The answers to some of these questions are beyond the scope of responsibilities of a design engineer. However, it is his/her responsibility to select control devices that are robust and easy to operate.

An emerging approach commonly adopted in the project of new irrigation districts or in the modernisation of existing irrigation districts is the SCADA. The term SCADA usually refers to centralised systems which monitor and control entire sites, or complexes of systems spread out over large areas (anything from an industrial plant to a nation). A SCADA system usually consists of the following subsystems:

- A human–machine interface (HMI) which presents process data to a human operator, and through this, the human operator monitors and controls the process.
- A supervisory (computer) system, gathering (acquiring) data on the process and sending commands (control) to the process.
- Remote terminal units (RTUs) connecting to sensors in the process, converting sensor signals to digital data and sending digital data to the supervisory system.
- Programmable logic controller (PLCs) used as field devices because they are more economical, versatile, flexible, and configurable than special-purpose RTUs.
- Communication infrastructure connecting the supervisory system to the remote terminal units.
- Various process and analytical instrumentation

Data acquisition begins at the RTU or PLC level and includes meter readings and equipment status reports that are communicated to SCADA as required. Data are then compiled and formatted in such a way that a control room operator using the HMI can make supervisory decisions to adjust or override normal RTU (PLC) controls. Data may also be fed to a Historian, often built on a commodity Database Management System, to allow trending and other analytical auditing.

6 GIS BASIS AND FUNCTIONING FOR IRRIGATION DISTRICTS MANAGEMENT

A Geographic Information System (GIS) is a system designed to capture, store, manipulate, analyse, manage, and present all types of geographically referenced data. GIS technology enables community planners, economists, agronomists, and farmers to research and devise practices that will enable the sustainability of food production.

The information systems flourished from the developments of computer technology. Most of the information systems in the early stage, such as the Management Information System (MIS), were mostly for the manipulation of numeric and alphanumeric data. The closed linkage of spatial distributed data and image data with the management of natural resources and regional planning creates the needs for a technology to incorporate these spatial data and imageries with the traditional MIS. Therefore the GIS integrates the MIS, CAD, Topology and Statistics into an effective tool for the acquisition, storage, retrieval, analysis and display for the spatial data. The GIS is not necessarily an independent discipline but is an integration or application of information science with other disciplines. GIS is a product integrating the technologies of digital computing, computer graphics, databases, remote sensing and spatial statistics and analysis. GIS can be effectively applied to tasks such as natural resource planning and management. Spatial databases such as for soil, rainfall, geology, land use, transportation, topography, demography and socioeconomics can be implemented for better decisions in resource or facilities planning and management.

The choice of the GIS software is an important issue for successful GIS implementation. There are many considerations for software choice, including: market share, the capacity of data exchange with other software, future expansion, application development support and ease of use.

The main data format is another important concern. There are two major formats in GIS: vector and raster. In the raster system, the area is uniformly divided into grids with attributes such as elevation, rainfall or land use categories assigned to each grid cells to represent the phenomena in the real world. Data resolution is represented by the size of the grids. More information of the real world can be preserved as the grid size becomes smaller. In vector data system, the spatial information is expressed by discrete spatial features such as points, lines and polygons. A spatial relationship description scheme called Topology is used to record the locations of these spatial objects and the relationships among them.

These two data formats have their own advantages as well as drawbacks. Raster data are good for description of continuously varied geographic phenomena such as terrain, vegetation and precipitation. The vector data format is more suitable for data with a definite position and boundaries such as land plot or administration boundaries, land use zoning, buildings and traffic networks.

Management of irrigation districts demands the handling of large amounts of data. As most of these data are spatially distributed, one can expect that the quality of decisions made by irrigation managers could be promisingly improved by applying GIS. Most of the spatial information in irrigation management, such farmland plots, irrigation management unit, canal network or distribution and regulation structures are appropriate to be handled using vector data format.

In irrigation district management, GIS is very useful to partition the irrigated area into homogeneous units based on hydrological properties relevant to the managers. In particular, it is possible to map the sensitivity of the infrastructure with respect to operation by overlaying several layers of information.

GIS can be used also as a data visualisation tool, 'a dashboard,' by connecting the existing database with all the water releases to the GIS interface. The pictorial presentation of the water status of the main reservoir and intermediate storages, the releases and the deliveries to each sub-command area in the dashboard give a very comprehensive knowledge of the whole project area in an attractive manner. The colour codes used are very easily understood and interpreted by the manager at a single glance. Therefore, in a minimum time he is in a position to understand what is happening and to manage every situation.

The main objective of this subject section is to present the main concepts related with basic GIS applications, and to develop an appropriate information system to improve decision making in the operation and management of an irrigation district.

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BLOCK IV:

FOOD TECHNOLOGY AND ENGINEERING

CHAPTER 9

Modelling of food processing systems

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The modelling procedures for food processing systems and auxiliary systems are presented in this chapter, as a summary of chapter 2 of the book by López-Gómez and Barbosa-Cánovas (2005). These tools are useful because they assist the design engineer in the screening and evaluation of different design and operation alternatives for the above systems.

1 TRANSFER PHENOMENA AND PROPERTY BALANCES

The physical state of a body is absolutely defined when the following characteristics are defined: (i) Quantity of matter and composition; (ii) Total energy (internal, electrical, magnetic, potential, kinetic, etc.); and (iii) Components of the velocity at which the body is circulating. And, in this way, changes that take place during the realisation of a unit operation, on raw matter or an intermediate product, are:

- Changes in the mass or composition (phase separation, blending, transformation by biochemical reactions, etc.)
- Changes in level or quality of the energy of the product (cooling, vaporisation, heating, pressure increasing, etc.)
- Changes in motion conditions (increasing velocity or changing direction, etc.)

In addition, changes taking place in a system must follow conservation laws: (i) Law of conservation of mass; (ii) Law of conservation of energy; and (iii) Law of conservation of momentum. Generally, these changes that occur in a system, body, or product during the realisation of a unit operation can be carried out by means of transfer phenomena of mass, energy, and momentum. In effect, when a system is not at a state of equilibrium, it is inclined to achieve it, in such a way that, when the system is going to this equilibrium, the property transfer phenomena take place. These transfer phenomena of mass, energy, and momentum can be designated property transfer phenomena, considering mass or matter, energy, and momentum as properties.

So, when there is a temperature difference between two points of a system, a heat transfer phenomenon takes place, from a point of greater temperature to a point of lower temperature. This transfer phenomenon takes place until the state of equilibrium, where all points are at the same temperature, is reached.

For example, a case of heat transfer phenomenon is presented during the cooling of fruits in cold storage. Fruit coming from the field is at 25ºC, while the air of the cold chamber is at 0.5ºC. The heat transfer phenomenon takes place within the fruit (in this case, the fruit is the system) because the skin, the exterior part of the system, is in contact with the air temperature of 0.5ºC and the internal points of the fruit are at 25ºC. The heat transfer takes place from the internal points at greater temperature (Ti) to the skin points at lower temperature (Te) until all points, external and internal, are at the cold chamber air temperature (0.5ºC). In this situation, the fruit –the system– will be at a state of equilibrium with respect to temperature. This equilibrium situation will change, however, because the cold chamber air temperature will increase in time due to heat entering through the walls. Then, as the air temperature is greater than 0.5ºC, heat transfer occurs again from the air to the fruit, which is colder than the air of the chamber. When the air temperature is too high (for example, 1ºC), the evaporator of the refrigeration system is switched on to cool the chamber air until an air temperature of 0.5ºC is reached. Now, as the air temperature will be lower than the fruit temperature (close to 1ºC), heat transfer takes place again from the internal to the external points of the fruit, and from here to the air, until the new equilibrium state is reached.

Heat transfer density within a solid body is given by Fourier's equation:

$$\vec{q} = -k \cdot \vec{\nabla} T \qquad \text{[eq. 1]}$$

where q is the heat transfer density (in J/s.m2), k the conductivity coefficient (in J/s.m.ºC), and ∇T the gradient of temperature between the different points of the solid (in ºC/m). From this equation, heat transfer takes place from the points of the solid at greater temperature to the points at lower temperatures.

When there is a velocity difference between two points of a fluid, a momentum transfer phenomenon takes place, from points at greater velocity to points at lower velocity. This transfer phenomenon takes place until the equilibrium state is reached, where all points are at the same velocity.

In this case, Newton's equation for a Newtonian liquid circulating in laminar flow between two parallel planes is given by:

$$\tau_{yx} = -\mu \cdot \frac{dv_x}{dy} \qquad \text{[eq. 2]}$$

where τ is the shear stress (in Pa), μ the viscosity of the liquid (in Pa.s), and dv/dy the shear rate or velocity gradient (in s-1) between the different points of the fluid. This expression shows the relationship between the deformation of the fluid (given by the velocity gradient) and the cause of this deformation: the shear stress.

In the same manner, when there is a difference in concentration between the points of a system, the mass transfer phenomenon takes place from points at greater concentration

to points at lower concentration, until all points are at the same concentration. This is the equilibrium state of the system.

For example, during brine salting of cheese curd, salt penetration takes place from the brine to the internal parts of the cheese. Salt transfer phenomenon occurs because the concentration of salt in the brine (and in the external points of the cheese in contact with the brine) is greater than in the internal points of the cheese. There is a situation of non-equilibrium with respect to the salt concentration within the system of the cheese. This salt transfer will take place until all points of the system have the same salt concentration, which will be approximately the salt concentration of the brine.

Mass transfer density is given by Fick's equation:

$$\vec{\eta} = -D \cdot \vec{\nabla} \rho \qquad \text{[eq. 3]}$$

where η is the mass transfer density (in kg/s.m^2), D the coefficient of diffusion (in m2/s), and ∇ρ the gradient of concentration (in kg/m^3.m).

From the above laws of Fourier, Newton, and Fick, it is deduced that the property transfer rate is directly proportional to the property gradient. The greater the property gradient is, the greater the transfer rate will be. That is, the greater the temperature difference, the greater the heat transfer rate; the higher the velocity gradient in a Newtonian fluid, the higher the momentum transfer rate; and the greater concentration difference, the greater the mass transfer rate.

On the other hand, property (mass, energy, and momentum) transfer can take place by means of two mechanisms: molecular and turbulent transport. Molecular transport is based on interaction between or motion of individual molecules. This is the case, for example, of heat transfer through a stationary solid material (the wall of a tank, the insulated wall of a cold chamber, etc.). Turbulent transport is based on the motion of large groups, or clusters, of molecules, which transport mass, energy, and momentum at the same time. There is also interaction between groups or clusters of molecules. The mechanism of turbulent transport is evidenced only in fluids, while molecular transport takes place in solids and fluids.

1.1 Macroscopic balances and physical properties

1.1.1 Mass balance

Given an open system where there are T mass inlet and outlet flow streams, with S mass components in each stream, the mass macroscopic balance applied on this system is given by the relationship:

$$\left\{\begin{array}{c}\text{Rate of mass}\\ \text{accumulation in}\\ \text{the system}\end{array}\right\}=\left\{\begin{array}{c}\text{Net rate of}\\ \text{mass entering}\\ \text{system}\end{array}\right\}+\left\{\begin{array}{c}\text{Rate of mass}\\ \text{generation in}\\ \text{the system}\end{array}\right\}$$

When this balance is applied for only one component j, as indicated above, this relationship takes the following form:

$$\frac{dn_j}{dt}=\sum_{m=1}^{T}\dot{m}_{m,j}+R_j \qquad \left(j=1,2,...,S\right) \qquad \text{[eq. 4]}$$

where dn_j/dt represents the variation with time of the mass quantity of component j in the system (n_j), that is, the accumulation term; represents the rate of component j which moves in or out of the system via stream m, considering T streams in or out. Finally, Rj is the quantity of component j generated per unit of time in the system.

In the case of steady-state systems, like process equipment working continuously (heat exchangers, freezers, refrigeration systems, etc.), this mass balance can be simplified, as in this type of system there is only one inlet of mass flow corresponding to the product (or refrigerant in the case of a refrigeration system). When the inlet mass flow is equal to the outlet mass flow, there is no accumulation of refrigerant in the element of the refrigeration system. The equipment will be in steady state, without a mass generation term, the equation for which (with only one component) is given as:

$$\sum_{m=1}^{T}\dot{m}_m=0 \qquad \text{[eq. 5]}$$

1.1.2 Energy balance

The knowledge of mass macroscopic balance is not enough to know, for example, the system thermal behaviour, and it will be interesting to know the temperature of each stream, the amount of thermal energy which is exchanged in a particular equipment, etc. That is to say that it will be necessary to apply an energy macroscopic balance to the system.

The different kinds of energy that take part in the energy macroscopic balance are (Costa et al., 1994): Internal energy (Ui), Potential energy (ϕ), Kinetic energy (K), Heat (Q), and Work (W).

The three first kinds of energy are state functions, while heat and work depend on thermodynamic processes that occurs as mass flows along the system. Heat and work are types of energy exchanged between the system and the surroundings through the walls of the system, and these energies are not associated with mass flows. Usually, internal energy, heat, and work are considered in the energy macroscopic balance around the different food processing equipment and elements of the refrigeration system, while variations of the potential and kinetic energies are negligible.

Internal energy (Ui) is the sum of the energies of the particles that constitute a substance. These particles (atoms, molecules, ions, etc.) are in continuous motion (rotation, vibration, and translation), and the total internal energy of the system is the sum of energies of these particles due to their motions. The value of the internal energy is a function of the amount of matter, specific heat, and temperature of the system. Thus, for a mass n of substance, the internal energy is given by the equation:

$$U_i = \int_n \hat{c}_v \cdot T \cdot dn \qquad \text{[eq. 6]}$$

where $\hat{c}_v$ is the specific heat at constant volume, and T is the temperature.

The heat term (Q) is the heat exchanged between the system and the surroundings, and depends on the difference of temperatures between both sides of the exchange surface. The heat transfer per unit of time between the system and the surroundings is given by the equation:

$$\dot{Q} = A \cdot U \cdot \Delta T \qquad \text{[eq. 7]}$$

where U is the overall coefficient of heat transfer (in $J/m^2.^oC.s$), A the exchange surface (in m^2), and ΔT the difference of temperatures between the system and the surroundings (in ^oC).

Work (W) can be mechanical, electrical, etc. The mechanical work of compression is the integral of the product of a force F and distance. For fluids compressed within a closed system, work is given by the equation:

$$W = \int_x F \cdot dx = \int_x p \cdot S \cdot dx = \int_V p \cdot dV \qquad \text{[eq. 8]}$$

When we have an open system, with mass flows taken in and out, the energy macroscopic balance is given by the expression:

$$\left\{\begin{array}{c}\text{Rate of accumulation}\\ \text{of}\\ \text{energy}\end{array}\right\} = \left\{\begin{array}{c}\text{Net rate of}\\ \text{energy entering}\\ \text{system}\end{array}\right\}$$

Since only energy forms that are state functions can be accumulated in the system (internal, kinetic, and potential energy), the above accumulation term is given by:

$$\left\{\begin{array}{c}\text{Rate of accumulation}\\ \text{of energy}\end{array}\right\} = \frac{d}{dt}\left(U_i + K + \Phi\right) \qquad \text{[eq. 9]}$$

Within the energy net rate term, there are two kinds of energy: energy associated with the in and out mass rates, and the energy exchanged with the surroundings of the system:

$$\left\{\begin{array}{c}\text{Net rate of}\\ \text{energy entering}\\ \text{system}\end{array}\right\}=\begin{array}{l}\sum_m \hat{U}_{i,m}\cdot\dot{m}_m+\sum_m \hat{K}_m\cdot\dot{m}_m+\\ \sum_m \hat{\Phi}_m\cdot\dot{m}_m+\sum_m p_m\cdot S_m\cdot v_m\end{array} \qquad \text{[eq. 10]}$$

where: $\hat{U}_{i,m}$ is the Internal energy per unit of mass of the stream m; $\hat{K}_m$ is the Kinetic energy per unit of mass of the stream m; $\hat{\phi}_m$ is the Potential energy per unit of mass of the stream m; p_m is the Pressure of the stream m, at inlet of the system; *Sm* is the Crossing section of the stream m, at inlet of the system; and v_m is the Average velocity in the stream m. The product $S_m.v_m$ is the volume rate, which is the same as $m_m\cdot\hat{V}_m$. The enthalpy per unit of mass is given by:

$$\hat{H}_m=\hat{U}_m+p_m\cdot\hat{v}_m \qquad \text{[eq. 11]}$$

Thus, the overall energy balance is the following:

$$\frac{d}{dt}(U_i+K+\Phi)=\sum_m\left(\hat{H}+\hat{K}+\hat{\Phi}\right)_m\cdot\dot{m}_m+\dot{Q}+\dot{W} \qquad \text{[eq. 12]}$$

This expression of the energy macroscopic balance is difficult to use, however, because absolute values of enthalpy and kinetic, internal, and potential energies are used. It is necessary, therefore, to transform this expression into another written in relative values. The definition of these reference values can be done by means of temperature for the enthalpy *H**, a distance above the ground, or another reference point, for the potential energy *Φ**, and a coordinates system for the kinetic energy *K**.

The expression of the overall energy balance with these reference values will be the following:

$$\frac{d}{dt}\left(U_i-H^*+K-K^*+\Phi-\Phi^*\right)=$$

$$\sum_m\left(\hat{H}-\hat{H}^*+\hat{K}-\hat{K}^*+\hat{\Phi}-\hat{\Phi}^*\right)_m\cdot\dot{m}_m+\dot{Q}+\dot{W}+\frac{d(\rho\cdot V)}{dt} \qquad \text{[eq.13]}$$

The variation of the internal energy can be written as following:

$$\frac{dU_i}{dt}=\frac{dH}{dt}-\frac{d(p\cdot V)}{dt} \qquad \text{[eq.14]}$$

Then the expression of the macroscopic energy balance will be:

$$\frac{d}{dt}\left(U_i - H^* + K - K^* + \Phi - \Phi^*\right) = \sum_m \left(\hat{H} - \hat{H}^* + \hat{K} - \hat{K}^* + \hat{\Phi} - \hat{\Phi}^*\right)_m \cdot \dot{m}_m + \dot{Q} + \dot{W}$$

[eq.15]

This general equation can be simplified. For example, in macroscopic energy balances applied to food processing systems, the possible variations in kinetic and potential energies are negligible. In this case, the above equation will be transformed as follows:

$$\frac{d}{dt}(H - H^*) = \sum_m (\hat{H} - \hat{H}^*)_m \cdot \dot{m}_m + \dot{Q} + \dot{W} + \frac{d(p \cdot V)}{dt} \qquad \text{[eq.16]}$$

When there is heat generation in the system, and the system works at constant pressure and volume, the energy balance is given by the equation:

$$\frac{d}{dt}(H - H^*) = \sum_m (\hat{H} - \hat{H}^*)_m \cdot \dot{m}_m + \dot{Q} + \sum_j R_j \Delta \hat{H}_j^* \qquad \text{[eq.17]}$$

where ΔH is the reaction enthalpy of food processing operations like fermentation (in the making of alcoholic drinks such as wine, beer, and cider), barley germination (in malting plants), and cold storage of fruits and vegetables (where the respiration of fruits and vegetables is a process that generates heat).

If the system is in steady state and there is no heat generation because it is working continuously and the biochemical reactions are negligible (for example, as occurs in continuous heat exchangers, or in concentration systems by evaporation), the above equation is transformed to:

$$0 = \sum_m (\hat{H} - \hat{H}^*)_m \cdot m_m + \dot{Q} \qquad \text{[eq.18]}$$

where Q is the heat exchanged with the surroundings through the walls of the system. Generally, if the system is insulated, the Q value is negligible with respect to the enthalpy entering and leaving the system associated with the mass flow of the different mass streams. In this case, the above equation is reduced to:

$$0 = \sum_m (\hat{H} - \hat{H}^*)_m \cdot \dot{m}_m \qquad \text{[eq.19]}$$

1.1.3 Momentum balance

In operations with changes in momentum, as occurs in fluid transport through tubes or in other unit operations, there are problems that are not solved only by means of

applying mass and energy conservation laws. For example, the calculation of the falling velocity of a solid spherical particle within a fluid (sedimentation operation) cannot be completed using only mass and energy balances. It is necessary to apply the momentum balance.

A system with mass n increases the velocity and, in this manner, the momentum is given by the equation:

$$n \cdot \vec{v} = \vec{P} \qquad \text{[eq.20]}$$

if, and only if, a force acts on this system. In this case, the momentum change and the force acting on the system are related by means of the following expression:

$$\vec{F} = \frac{d(n \cdot \vec{v})}{dt} \qquad \text{[eq.21]}$$

In an open system, the momentum balance or the expression of the momentum conservation law will be a force balance, and it is given by:

$$\left\{\begin{matrix}\text{Rate of accumulation}\\ \text{of momentum in the}\\ \text{system}\end{matrix}\right\} = \left\{\begin{matrix}\text{Net rate of}\\ \text{momentum entering}\\ \text{system}\end{matrix}\right\} + \left\{\begin{matrix}\text{Sum of forces}\\ \text{acting on system}\end{matrix}\right\}$$

It is interesting to say that in fluids circulation (fluid dynamics), the system is the fluid portion without considering the container (the tube within which the fluid circulates, for example). On the contrary, in problems of particles falling within a fluid, the system is the particle and the fluid is the surroundings.

The momentum accumulation term is:

$$\frac{d(n \cdot \vec{v})}{dt} = \frac{d(\vec{P})}{dt} \qquad \text{[eq.22]}$$

The mass flow entering through each stream m is mm (a quantity of mass per unit of time, in kg/s). Then, if v_m is the mean velocity of the mass stream m, and there are T streams (crossing T inlet and outlet sections), the net rate of momentum entering the system is given by the equation:

$$\sum_{m=1}^{T} \dot{m}_m \cdot \vec{v}_m \qquad \text{[eq.23]}$$

where the momentum given out is negative and the momentum taken in is positive.

The force acting on cross section m (of stream m) due to pressure p_m is: $-p_m \cdot \vec{S}_m$ and the force acting on cross section m' (of stream m' at the outlet) due to pressure pm' will be:

$$- p_{m'} \cdot \vec{S}_{m'} \qquad \text{[eq.24]}$$

In this manner, the net force due to pressure acting on the system is given by:

$$-(p_m \cdot \vec{S}_m - p_{m'} \cdot \vec{S}_{m'}) \qquad \text{[eq.25]}$$

The negative sign means that the force taken into the system is negative while the force given out is positive, because the surface vector is oriented out of the system. In the system with T streams (with T inlet/outlet cross sections), the forces acting on the inlet/outlet cross sections are:

$$-\sum_{m=1}^{T} p_m \cdot \vec{S}_m \qquad \text{[eq.26]}$$

When the system has a mass n, the gravity force will be:

$$n \cdot \vec{g} \qquad \text{[eq.27]}$$

The force from the system acting on the surroundings will have a negative sign, and it will be the force resulting from the momentum balance. This force is manifested as pressure of the fluid on the tube and friction on the tube walls. In this manner, the momentum macroscopic balance is given by the equation:

$$\frac{d(n \cdot \vec{v})}{dt} = \sum_{m=1}^{T} \dot{m}_m \cdot \vec{v}_m - \sum_{m=1}^{T} p_m \cdot \vec{S}_m + n \cdot \vec{g} - \vec{F} \qquad \text{[eq.28]}$$

As this balance has a vectorial character, it must be solved with respect to a system of coordinates. As an example, this momentum balance can be applied to study the gravity sedimentation operation of solid particles within a food fluid (like the clarification of grape juice in white-wine making). In this case, the solid particle, with mass n and density ρ, is considered as a sphere suspended within a fluid with density ρ_f, and the macroscopic balance of forces is given by the equation [eq.29].

$$\frac{d(\vec{P})}{dt} = n \cdot \vec{g} - \vec{F} \qquad \text{[eq.29]}$$

where the force F is the sum of friction force Fr and the reaction force of the system (the particle) to exterior pressure Fp (the particle is considered to be a solid that cannot be deformed). This force due to exterior pressure is given by Archimedes' principle (the ascendant force executed by the fluid on a solid immerged in it is equal to the weight of the fluid displaced by the solid), which is given by the expression:

$$\vec{E} = \int_S p \cdot d\vec{S} = -\rho_f \cdot V \cdot \vec{g} = -\vec{F}_p$$
$$\vec{E} = -\vec{F}_p \qquad \text{[eq.30]}$$

where V is the particle volume and p the exterior pressure. In this manner, the momentum balance is:

$$\frac{d\vec{P}}{dt} = n\vec{g} - (\vec{F}_p + \vec{F}_r)$$
$$\frac{d\vec{P}}{dt} = \rho_s V\vec{g} - \rho_f V\vec{g} - \vec{F}_r = (\rho_s - \rho_f)V\vec{g} - \vec{F}_r \qquad \text{[eq.31]}$$

and, as all forces have the same direction (on the Y axis), the above equation can be expressed as the following:

$$\frac{dP}{dt} = (\rho_s - \rho_f)Vg - F_r \qquad \text{[eq.32]}$$

Experimentally, it is stated that the friction force is a function of kinetic energy of the particle per unit of mass ($v^2/2$), on the area A (projected by the particle on a plane perpendicular to the direction of the motion of the falling particle), and the density of the fluid:

$$F_r = C_D A\rho_f \frac{v^2}{2} \qquad \text{[eq.33]}$$

where C_D is a friction coefficient, which is a function of the motion conditions of the fluid. If it is considered that $A/V = 3/(2d_p)$ and $P = nv = \rho_s Vv$, the above equation can be written as follows:

$$\frac{d(\rho_s Vv)}{dt} = (\rho_s - \rho_f)Vg - C_D A\rho_f \frac{v^2}{2}$$
$$\frac{dv}{dt} = \frac{(\rho_s - \rho_f)}{\rho_s} g - \frac{3}{4} C_D \left(\frac{\rho_f}{\rho_s}\right)\frac{v^2}{d_p} \qquad \text{[eq.34]}$$

where v is the falling velocity of the particle and dp the particle diameter. From this equation, at the initial instant v = 0 and Fr = 0, and from this moment, the particle will start to ascend or to descend (depending on whether $\rho_s<\rho_f$ or $\rho_s>\rho_f$). In this manner, the greater the particle velocity, the greater the force Fr, until the value of Fr is equal to $(\rho_s-\rho_f)Vg$, at the moment at which the particle momentum is not altered, and dP/dt = 0, when steady state is reached.

The limit velocity is reached, and is given by the equation:

$$0 = \left(\frac{\rho_s - \rho_f}{\rho_s} \right) g - \frac{3}{4} C_D \left(\frac{\rho_f}{\rho_s} \right) \frac{v^2}{d_p}$$

$$v_s = \left[\frac{4}{3} \frac{g}{C_D} \left(\frac{\rho_s - \rho_f}{\rho_f} \right) d_p \right]^{\frac{1}{2}} \qquad \text{[eq.35]}$$

1.1.4 Physical properties

To solve the mass, energy, and momentum balances in a food processing plant, it is necessary to know the physical properties of each substance acting in the system being studied:

- Air (acting in food processing operations such as drying, cold storage, freezing by cold air, germination ventilated with cold air, etc.; and in auxiliary systems such as refrigeration systems, pneumatic transport systems, etc.)
- Water, as liquid or steam (in food-processing operations such as cooling by water, heating by hot water, sterilisation with steam, blanching with hot water, washing, etc.; and in auxiliary systems such as refrigeration systems, steam generation and distribution installations, etc.)
- Refrigerant fluids, such as those used in refrigeration systems (R-22, NH3, R-134a, etc.), working in most cooling and freezing processes in food factories
- Packaging materials (in cold storage, in thermal treatments with liquid or solid packed food, in packages such as glass bottles, plastic bottles or boxes, wood boxes, plastic film applied to the product, etc.)
- Food (liquid or solid, such as fruits, vegetables, liquid milk and milk products, meat and meat products, juices, etc.)
- Cleaning and sanitation chemicals to be used in CIP systems
- Construction materials of food processing equipment (such as stainless steel, rubbers, elastomers, and thermal insulation materials), floors, walls, and ceilings

It will be necessary to know physical properties:

- Density and specific gravity of solids: solid density, bulk density, liquid density, gases and vapour density, density of aerated products (over-run)
- Surface properties: surface tension, surface activity, interfacial tension, detergency, foaming, wettability
- Thermodynamic and thermal properties: specific heat, specific enthalpy, specific enthalpy of reaction, and latent heat for solids, liquids, gases, and vapours.

There are good books dealing with the physical properties of foods as well as those physical properties and principles involved in food processing operations, from which

these physical properties can be obtained (Lewis, 1990; Jowitt et al., 1983; Mohsenin, 1970; Heldman and Lund, 1992; Heldman and Singh, 1981; Hayes, 1987; Peleg and Bagley, 1983; Perry et al., 1992; Rao and Rizvi, 1986; Singh and Medina, 1988; Toledo, 1991).

2 MICROSCOPIC BALANCES AND TRANSFER PHENOMENA

The macroscopic balances described above result from the application of conservation laws to systems considered as black boxes. In this way, the relationships between entering and exiting property flows are obtained, also explaining the generation and accumulation of property taking place within the system. Really by means of these macroscopic balances, it is not possible to know what occurs at each point in the system. For example, the system can be a batch dryer for cereals. In this case, the cereal deep bed is located in a silo with a perforated floor, through which the hot air enters to dry the cereal. Here, by means of macroscopic balances the temperature and relative humidity of hot air at the inlet and outlet can be calculated. It is also possible to calculate the average moisture content of the dried cereal once the mass flow of dry air is known, as well as its moisture content and temperature at the dryer inlet and outlet, and the time interval the hot air has been passed through. However, it is not possible to calculate the cereal's moisture content and temperature at each point within the dryer, or similarly, the relative humidity and temperature of hot air within the deep cereal bed in the silo dryer. In fact, the temperature of the hot air and the cereal changes as the cereal layer rises in the silo dryer: the air temperature decreases and the moisture content and relative humidity of the air increases. At the same time, the higher the cereal layer becomes, the higher the moisture content.
To calculate the moisture content of the cereal at each point in the bed, it is necessary to apply microscopic balances of mass and energy to the system. As a result, the system is considered as a box filled with mechanisms and not as a black box.

2.1 Microscopic balances.

2.1.1 Mass microscopic balance. Fick's Laws

To obtain microscopic balances, microscopic description parameters are used:

- Partial mass density ρ_j for the component *j* (with concentration units kg/m^3), which is a function at locations (x, y, z) within the system and for time (t).
- Mass flow density n_j for the component *j* (with units kg/m^2.s), which is the mass quantity (in kg) crossing over a unit of surface (m^2) in the system per unit of time (s), calculated as follows:

$$n_j = \rho_j \cdot v_j \qquad \text{[eq.36]}$$

- Velocity of the component *j* at each point, v_j (in m/s), in the system.

It is considered a system immobile in space, without displacements, with volume V and surface S. Thus, the mass balance for component *j* can be written as follows:

$$\begin{Bmatrix}\text{Rate of accumulation}\\ \text{of mass component j}\\ \text{in the system}\end{Bmatrix}=\begin{Bmatrix}\text{Net flow rate of mass}\\ \text{component j entering}\\ \text{system}\end{Bmatrix}+\begin{Bmatrix}\text{Generation of mass}\\ \text{component j per unit of}\\ \text{time in system}\end{Bmatrix}$$

The mass quantity of component *j* within the system is expressed as

$$\int_V \rho_j dV \qquad \text{[eq.37]}$$

where dV is a differential of volume for the system (in m^3). Thus, the accumulation term in the above mass balance expression is

$$\frac{d}{dt}\int_V \rho_j dV \qquad \text{[eq.38]}$$

which represents the change in the time of mass of *j* component in the system; this is an accumulation term for mass balance expressed microscopically.

If the mass enters through the system's surface, and it is considered a surface element *dS*, then the mass quantity entering the *dS* is

$$-\vec{n}_j \cdot d\vec{S} \qquad \text{[eq.39]}$$

When the mass flow leaves the system, the above scalar product must be positive. Therefore, the net mass flow rate of component *j* entering the system is

$$-\int_S \vec{n} d\vec{S} \qquad \text{[eq.40]}$$

On the other hand, if the mass generation of component *j* per unit of time and unit of volume is r_j, then the total mass of component *j* in the system is

$$R_j = \int_V r_j dV \qquad \text{[eq.41]}$$

Thus, mass balance for component *j* can be written as follows:

$$\frac{d}{dt}\int_V \rho_j dV = -\int_S \vec{n}_j d\vec{S} + \int_V r_j dV \qquad \text{[eq.42]}$$

This expression can be converted to systems without biochemical reactions, by applying the Gauss-Ostrogradskii and Leibnitz principles:

$$\int_V \frac{\partial}{\partial t}\rho_j dV = -\int_V \left(\vec{\nabla}\vec{n}_j\right) dV \qquad \text{[eq.43]}$$

From this expression the continuity equation for the j component becomes

$$\frac{\partial \rho_j}{\partial t} + \vec{\nabla}\vec{n}_j = 0 \qquad \text{[eq.44]}$$

which is the microscopic mass balance for the *j* component. The overall microscopic mass balance is the sum of S microscopic balances for S components.

$$\sum_{j=1}^{S} \frac{\partial \rho_j}{\partial t} + \sum_{j=1}^{S} \vec{\nabla}\vec{n}_j = 0 \qquad \text{[eq.45]}$$

If it is taken into account that

$$\sum_{j=1}^{S} \rho_j = \rho \qquad \text{[eq.46]}$$

and

$$\sum_{j=1}^{S} \vec{n}_j = \rho \cdot \vec{v} \qquad \text{[eq.47]}$$

then

$$\frac{\partial \rho}{\partial t} + \vec{\nabla}\rho\vec{v} = 0 \qquad \text{[eq.48]}$$

where ρ is the overall density and v the average velocity for all mass points entering the system. In the above expression, if the gradient vector is in rectangular coordinates:

$$\vec{\nabla} = \frac{\partial}{\partial x}\vec{i} + \frac{\partial}{\partial y}\vec{j} + \frac{\partial}{\partial z}\vec{k} \qquad \text{[eq.49]}$$

then the equation of continuity is

$$\frac{\partial \rho}{\partial t} + \frac{\partial}{\partial x}(\rho v_x) + \frac{\partial}{\partial y}(\rho v_y) + \frac{\partial}{\partial z}(\rho v_z) = 0 \qquad \text{[eq.50]}$$

In an immobile material, the heat and mass transfer phenomena mainly occur through the molecular transport mechanism; the turbulent transport mechanism is negligible. For this

kind of material the partial mass density or mass concentration of component *j* at a determined point and moment is

$$\rho_j = \rho_j(x, y, z, t) \qquad \text{[eq.51]}$$

The constant concentration surfaces in immobile material are given by the equation:

$$\rho_j(x, y, z, t) = constant \qquad \text{[eq.52]}$$

such that the concentration difference between two immediate surfaces is $d\rho_j$, a value infinitely small. The First Law of Fick is given as follows:

$$\vec{n}_j = -D_j \vec{\nabla} \rho_j \qquad \text{[eq.53]}$$

where D_j is the coefficient of diffusion. From this equation, the mass flow density vector is a function of the gradient vector of concentration. Thus, when a concentration gradient in the system exists, there is a mass flow density from points with greater concentration to zones with lower concentration, in contrast to the gradient vector (oriented from lower to greater concentration). Diffusion of mass occurs in a direction normal to constant concentration surfaces. With rectangular coordinates, and for cases in which the coefficient of diffusion is constant and equal at all points in the system, the mass flow density vector is given by

$$\vec{n}_j = -D_j \left(\frac{\partial \rho_j}{\partial x} \vec{i} + \frac{\partial \rho_j}{\partial y} \vec{j} + \frac{\partial \rho_j}{\partial z} \vec{k} \right) \qquad \text{[eq.54]}$$

From the continuity equation or microscopic mass balance, plus the above expression, the Second Law of Fick is obtained:

$$\frac{\partial \rho_j}{\partial t} = -\vec{\nabla} \vec{n}_j = -\vec{\nabla} \left(-D_j \vec{\nabla} \rho_j \right) = D_j \nabla^2 \rho_j \qquad \text{[eq.55]}$$

which for rectangular coordinates and cases in which the coefficient of diffusion is constant and equal for all points of the system:

$$\frac{\partial \rho}{\partial t} = D_j \left(\frac{\partial^2 \rho_j}{\partial x^2} + \frac{\partial^2 \rho_j}{\partial y^2} + \frac{\partial^2 \rho_j}{\partial z^2} \right) \qquad \text{[eq.56]}$$

2.1.2 Momentum Microscopic balance. Newton's Law

By means of momentum microscopic balance, it is possible to know the velocity profile for the different points of a fluid circulating through a tube or within a particles bed. The procedure used to obtain the momentum microscopic balance is similar to that used for

mass microscopic balance. It is considered a system with volume V and is enclosed in surface S. Therefore, the momentum is given by:

$$\left\{\begin{array}{l}\text{Rate of accumulation}\\ \text{of momentum}\\ \text{in the system}\end{array}\right\}=\left\{\begin{array}{l}\text{Net flow rate of}\\ \text{momentum}\\ \text{entering the system}\\ \text{through mass flow}\end{array}\right\}+\left\{\begin{array}{l}\text{Net flow rate of}\\ \text{momentum entering}\\ \text{the system by}\\ \text{molecular flow}\end{array}\right\}+\left\{\begin{array}{l}\text{Sum of}\\ \text{exterior}\\ \text{forces}\end{array}\right\}$$

where each representative term in brackets has a unit of force (N). The momentum per volume unit is given by the expression:

$$\rho \cdot \vec{v} \qquad \text{[eq.57]}$$

and the momentum of volume differential element *dV* by

$$\rho \vec{v} dV \qquad \text{[eq.58]}$$

From here, the momentum of a system with volume V is given by

$$\int_V \rho \vec{v} dV \qquad \text{[eq.59]}$$

And the momentum change in time for this system is

$$\frac{d}{dt}\int_V \rho \vec{v} dV \qquad \text{[eq.60]}$$

Given the mass flow density $\rho\vec{v}$, if it is considered as surface element $d\vec{S}$, the mass flow crossing this will be

$$\rho \vec{v} \cdot d\vec{S} \qquad \text{[eq.61]}$$

and the momentum flow rate crossing the surface element $d\vec{S}$ will be

$$(\rho \vec{v} \cdot d\vec{S}) \cdot \vec{v} \qquad \text{[eq.62]}$$

and crossing all of surface S in the system results in

$$-\int_S (\rho \vec{v}\vec{v}) \cdot d\vec{S} = -\int_V \vec{\nabla} \cdot (\rho \vec{v}\vec{v}) \cdot dV \qquad \text{[eq.63]}$$

If the molecular flow of momentum through the unit surface is $\vec{T}_m$, the molecular flow of momentum through all of surface *S* is expressed as

$$-\int_S \vec{T}_m dS \qquad \text{[eq.64]}$$

Regarding external forces, if $\rho\vec{g}$ is the force per volume unit due to gravity, the force acting on volume *V* is expressed as

$$\int_V \rho\vec{g}dV \qquad \text{[eq.65]}$$

On the other hand, the force due to pressure acting on a differential element of surface is $-pd\vec{S}$, and the total pressure force acting on surface *S* of the system is

$$-\int_S pd\vec{S} = -\int_V \vec{\nabla}pdV \qquad \text{[eq.66]}$$

Thus, the expression for microscopic momentum balance becomes

$$\frac{d}{dt}\int_V \rho\vec{v}dV = -\int_V \vec{\nabla}\cdot(\rho\vec{v}\vec{v})dV - \int_S \vec{T}_m dS + \int_V \rho\vec{g}dV - \int_V \vec{\nabla}pdV \qquad \text{[eq.67]}$$

Consider an experimental situation in which a fluid is located between two parallel and horizontal planes, with area *A*, and at a distance with very little *y**. If the inferior plane begins moving with a constant velocity *v**, and the other superior plane remains immobile, it is observed that the fluid layer in contact with the moving plane is moved (gains momentum), and the remaining layers (ones in contact with others) are put into motion at lower velocities since the layers are at a greater distance from the moving inferior plane. After a few minutes, a steady state is reached with a linear distribution of velocities. This is manifested as fluid deformation with displacement of the different fluid layers, one on top of the other.

The velocity at direction X, v_x, changes in a linear manner with distance *y*, given by

$$v_x = v^* - by \qquad \text{[eq.68]}$$

where b is the slope of the linear distribution of velocities. To maintain the above condition in a steady state, it is necessary to apply continual force on the plane in direction X. This force F (in N) is called shear force, and τ_{yx} (shear stress, in N/m^2) is given as follows:

$$\tau_{yx} = \frac{F}{A} \qquad \text{[eq.69]}$$

where A is the area (in m^2) on which force F is applied. In this example, the shear stress is the agent causing the velocity's gradient along axis Y. Experimentally, it is found for Newtonian fluids that slope *b* is directly proportional to the shear stress, and it is expressed as follows:

$$b = \left(\frac{dv_x}{dy} \right) = -\frac{1}{\mu} \cdot \tau_{yx} \qquad \text{[eq.70]}$$

that is,

$$\tau_{yx} = -\mu \cdot \left(\frac{dv_x}{dy} \right) \qquad \text{[eq.71]}$$

This is Newton's Law, where the proportionality constant μ (in kg/m.s, or Pa.s) is equal to the viscosity of the fluid and $\left(\frac{dv_x}{dy} \right)$ is the velocity's gradient or shear rate (s^{-1}). This shear rate is also given by parameter $\dot{\gamma}$.

Referring back to the concept of molecular flow density, of momentum or viscous flow, it is known that molecular flow is related to shear stress via the following equation:

$$\vec{T}_m = \left[u_x \; u_y \; \mathrm{u}_z \right] \cdot \begin{bmatrix} \tau_{xx} & \tau_{xy} & \tau_{xz} \\ \tau_{yx} & \tau_{yy} & \tau_{yz} \\ \tau_{zx} & \tau_{zy} & \tau_{zz} \end{bmatrix} = \vec{u} \cdot \vec{\vec{\tau}} \qquad \text{[eq.72]}$$

where the nine components of shear stress tensor are the shear stress coming from the shear forces actuating in x, y, z directions and forming velocity gradients along directions x, y, z.

In this manner, the motion equation or general expression for momentum microscopic balance is given:

$$\frac{\partial}{\partial t}(\rho \vec{v}) = -\vec{\nabla} \cdot (\rho \vec{v} \vec{v}) - \vec{\nabla} \cdot \vec{\vec{\tau}} - \vec{\nabla} p + \rho \vec{g} \qquad \text{[eq.73]}$$

If conservation laws and transfer rate equations are applied to the study of fluid flow through cylindrical tubes, it is possible to determine the relationship between flow and pressure drop of a fluid circulating within a tube, taking into account the viscosity of the fluid and tube diameter. Consider a Newtonian and incompressible fluid circulating in a laminar regime that is in a steady state within a cylindrical tube. If the total pressure at the ends (1) and (2), of the tube, is $P = p + \rho g h$, the shear stress is given by the equation:

$$\tau_{rz} = \left(\frac{P_1 - P_2}{2L} \right) \cdot r \qquad \text{[eq.74]}$$

where it is manifested that the shear stress increases linearly from the tube centre to the tube wall. In the above equation, a close relationship can be seen between the pressure

drop of a fluid circulating within a tube and the shear stress. The velocities profile within the tube is calculated as follows:

$$v_z = \frac{(P_1 - P_2)\cdot R^2}{4\mu L}\left[1-\left(\frac{r}{R}\right)^2\right] \qquad \text{[eq.75]}$$

which is a parabola. The maximum velocity is at the tube centre and its value is

$$(v_z)_{max} = \frac{(P_1 - P_2)\cdot R^2}{4\mu L} \qquad \text{[eq.76]}$$

And the mean velocity is given by the equation:

$$(v_z)_{mean} = \frac{\int_S v_z dS}{\int_S dS} = \frac{(P_1 - P_2)\cdot R^2}{8\mu L} \qquad \text{[eq.77]}$$

which is half of the maximum velocity. The velocity is at maximum when the shear stress is zero (at the tube centre), and this velocity is zero at the wall when the shear stress is at maximum. From the above [eq. 77], the volumetric flow of fluid circulating within the tube is obtained:

$$\dot{V} = (v_z)_{max}\cdot S = \frac{\pi(P_1 - P_2)\cdot R^4}{8\mu L} \qquad \text{[eq.78]}$$

which is the Hagen-Poiseuille equation for Newtonian liquid fluids.

On the other hand, the shear force at the tube wall is given by the following equation:

$$F_z = (2\pi RL)\cdot\left(\frac{P_1 - P_2}{2L}\right)\cdot R \qquad \text{[eq.79]}$$

2.1.3 Energy Microscopic balance. Fourier's Law

By means of an energy microscopic balance, it is possible to know, at any given moment, the temperature evolution during thermal processing of every point within a food product mass. This is interesting because a food product can lose its quality if the processing temperature is excessive, as can occur during the blanching and sterilisation processes. When it is considered an open system with volume *V* and is enclosed in surface *S*, the conservation law of energy establishes that

$$\left\{\begin{matrix}\text{Rate of energy}\\ \text{accumulation}\\ \text{in the system}\end{matrix}\right\}=\left\{\begin{matrix}\text{Net flow rate}\\ \text{of energy entering}\\ \text{the system}\end{matrix}\right\}$$

If only internal energy, kinetic energy, and potential energy are accumulated in the system, and the energy contained in volume V is

$$\int_V \rho\cdot\left(\hat{U}+\hat{K}+\hat{\Phi}\right)\cdot dV \qquad \text{[eq.80]}$$

then, the accumulation can be expressed as

$$\frac{d}{dt}\int_V \rho\cdot\left(\hat{U}+\hat{K}+\hat{\Phi}\right)\cdot dV=\frac{d}{dt}\int_V \rho\cdot\hat{E}\cdot dV \qquad \text{[eq.81]}$$

where $\hat{E}$ is the total energy per mass unit in volume dV. The above expression can also be written as

$$\int_V \frac{\partial}{\partial t}\rho\hat{E}\cdot dV \qquad \text{[eq.82]}$$

Concerning the net flow rate of energy entering the system, there are three types of entry procedures: energy entering via mass flow, energy due to surface forces, and energy entering via molecular flow.

Through the differential surface $d\vec{S}$ of the system, if $\vec{v}$ is the velocity of the fluid taken in, the mass flow entering is given as follows:

$$-\rho\cdot\vec{v}\cdot d\vec{S} \qquad \text{[eq.83]}$$

and the mass flow has energy $\hat{E}$. Then, the net flow rate of internal, kinetic and potential energy taken in is

$$\int_S \rho\cdot\vec{v}\cdot d\vec{S}\cdot\hat{E}=\int_V -\vec{\nabla}\cdot\left(\rho\cdot\hat{E}\cdot\vec{v}\right)\cdot dV \qquad \text{[eq.84]}$$

Through differential surface $d\vec{S}$, the intake of energy due to pressure is $-p\cdot\vec{v}\cdot d\vec{S}$, and the energy due to viscous forces is $-\left(\vec{\vec{\tau}}\cdot\vec{v}\right)\cdot d\vec{S}$. Then, the total energy taken into the system due to the action of viscous forces is

$$-\int_S\left(p\cdot\vec{v}+\vec{\vec{\tau}}\cdot\vec{v}\right)\cdot d\vec{S}=-\int_V\vec{\nabla}\left(p\cdot\vec{v}+\vec{\vec{\tau}}\cdot\vec{v}\right)\cdot dV \qquad \text{[eq.85]}$$

If $\vec{q}$ is the energy flow density by conduction (molecular transport of energy) on the differential surface $d\vec{S}$, the net flow rate of energy taken in via molecular transport is obtained as follows:

$$-\int_S \vec{q}\cdot d\vec{S} = -\int_V \left(\vec{\nabla}\vec{q}\right)\cdot dV \qquad \text{[eq.86]}$$

In sum, the net flow rate is given by the expression:

$$\int_V -\vec{\nabla}\cdot\left(\rho\cdot\hat{E}\cdot\vec{v} + p\cdot\vec{v} + \vec{\vec{\tau}}\cdot\vec{v} + \vec{q}\right)\cdot dV \qquad \text{[eq.87]}$$

which must be equal to the accumulation term:

$$\int_V \frac{\partial}{\partial t}\rho\cdot\hat{E}\cdot dV \qquad \text{[eq.88]}$$

In this manner, the energy microscopic balance is calculated:

$$\frac{\partial}{\partial t}\left(\rho\hat{E}\right) = -\vec{\nabla}\left(\rho\hat{E}\vec{v} + p\vec{v} + \vec{\vec{\tau}}\vec{v} + \vec{q}\right) \qquad \text{[eq.89]}$$

Similar to the study of mass transfer (Fick's Law), if a solid is heated by means of a flame, surfaces at constant temperature are formed, described as follows:

$$T = T(x, y, z, t) \qquad \text{[eq.90]}$$

Thus, the gradient vector of *T*, $\vec{\nabla}T$, will be perpendicular to these constant temperature surfaces and will have the way from lower to higher temperatures. Fourier's Law states that heat flow density $\vec{q}$ (by molecular transport or conduction) is directly proportional to the temperature gradient:

$$\vec{q} = -k\cdot\vec{\nabla}T \qquad \text{[eq.91]}$$

But, heat flows from higher temperature points to lower temperature points.

The coefficient *k* in Fourier's Law (Eq. 91) is called thermal conductivity, and its units are W/(m.K). Its value depends on the material type and the physical state.

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CHAPTER 10

Chilling and coadjutants for optimising the postharvest quality and safety of fruit and vegetables

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1 FACTORS AFFECTING THE SHELF-LIFE OF FRUIT AND VEGETABLES UNDER CHILLING

Fruit and vegetables are unique foods very often consumed raw or after minimal preparation. Consumers around the world perceive fruits and vegetables as being fresh, healthy, tasty, convenient, and suitable among the most popular foods at a reasonable price. In recent years the handling and shipping fresh plant foods industry has shown a continuous growth in the world with increasing consumption, sales and space dedicated to them in markets and restaurants. This growth trend requires accurate techniques and food quality and safety systems for growing, harvesting, handling, packaging, and distribution of fresh plant produce from the field to the consumers. Although effective strategies for assuring safety have been developed, they cannot fully eliminate microbial hazards linked with raw produce consumption. Thus, preventing contamination of horticultural products with microbial pathogens, risky levels of chemical residues, or physical contaminants is the most effective strategy to assure that such produce is wholesome and safe for human consumption (USFDA, 2001).

After picking, fruits and vegetables as living organs, gradually lose water, firmness, nutritive value and sensory quality (visual appearance, texture, taste and aroma), while the safety risks increase. The postharvest treatments basically determine their final overall quality and safety. As living organs, fruits and vegetables respire and transpire and their consequences are crucial for keeping quality and increasing their shelf-life. Factors that slow respiration can retard senescence and keep quality; however, some respiration must continue or the products will rapidly senesce and die. Cooling the produce slows down respiration and transpiration as well as many adverse changes in horticultural products, but many of them are intolerant to low temperatures above freezing point, causing chilling injuries (CI). Thus, knowing the fresh produce physiology is fundamental to understanding their stability and likely shelf-life (Aked, 2002). The expression shelf-life of a plant product refers to the period of time during which it retains a certain level of quality under specific storage conditions. When trying to reach optimal shelf life of non-climacteric fruit and vegetables, the main aim is to

minimise decline (by lowering metabolism), while in the climacteric ones maturation must be optimised depending on the final use (consumed fresh or processed). The optimal maturity stage at harvest is the main factor influencing its final quality and shelf life, usually established by sensory properties and microbial safety (Shewfelt, 1986, Artés, 2004).

Maturity is the stage of development of a plant organ at which, after harvesting and handling, its quality will at least be at the minimum level acceptable to the final consumers. Ripening starts in the last stage of maturity and is the start of senescence. During ripening, the intracellular organisation deteriorates, the gas permeability of cell membranes is reduced, inducing anaerobic respiration, and toxic compounds (like ethanol, acetaldehyde and ethyl acetate) accrue causing cell death. During senescence, plant organs become increasingly liable to microbial attacks, not only because biochemical changes induce promote microbial growth, but also through loss of natural immunity (Reid, 2002; Artés, 2004).

The degree of excellence or quality of fresh plant products is the result of the combination of a mixture of many biophysical and biochemical parameters that give value to each product as a food. Several characteristics, attributes and properties determine the product value for food or its enjoyment when consumed, and the relative importance of each quality factor depends on the type of product and its final use. The main quality factors are visual appearance (absence of defects, including CI), colour, texture, flavour, nutritional value and safety. For each of these factors, there is a matching series of attributes useful to evaluate the produce according to the demanded quality standards, selection in breeding programmes, and evaluation of responses to ecological factors and postharvest treatments (Kader, 2002b, Artés, 2004).

Fruits and vegetables are essential in human nutrition, being major sources of macro and micronutrients. Among the latter, vitamins (C, A, B6, thiamine, niacin, E), essential fatty acids, riboflavin, minerals (Mg, Fe, Ca, K, P and Zn), and dietary fibre are found. In addition, diets rich in fruits and vegetables have shown health benefits by decreasing chronic diseases, and reducing the risk of many forms of cancer. But delays between harvest and consumption as well as the methods of processing and cooking can induce nutritional losses (USDA, 2004).

2 MAIN POSTHARVEST DISORDERS OF FRUIT AND VEGETABLES

Although estimates of postharvest losses of fruit and vegetables are hard to judge, the average in industrialised countries is between 5 and 25%, and in developing ones between 20 and 50%. Losses are mainly due to metabolic changes (respiration, ethylene production, and compositional changes), growth and development, bruising and other mechanical injuries, weight loss (transpiration), physiological breakdown (CI, browning and others), pathological breakdown (decay), accidental freezing, those due to insects, pest, and rodents, and others. Factors affecting post-harvest losses vary widely from place to place and become increasingly complex as marketing systems become more

complex. However, small changes in attitudes toward the prevention of losses may profit more than changes in the techniques of the marketing chain. In order to satisfy the growing global food demand around the world more efforts must be made in reducing these losses rather than in increasing yield production (FAO, 1989, Artés, 1997, Kader, 2002a).

3 ENVIRONMENTAL FACTORS AFFECTING DETERIORATION

Maturity at harvest and the harvesting method influence the produce's quality and extent of physical injuries. Delays between harvest and final use can result in losses of overall quality. The magnitude of these losses increases with exposure to temperature, relative humidity (RH), and/or levels of O_2, CO_2, and C_2H_4 outside the optimum ranges for each product throughout the postharvest handling system (Lee and Kader, 2000).

Chilling is the most effective means of keeping quality and extending the shelf-life of fresh fruit and vegetables, because lowering the temperature reduces the growth rate of even cold-adapted microorganisms, mainly if the produce has been earlier damaged. In order to optimise the efficacy of combined preservation techniques and to provide a synergistic extension of the safe shelf-life of fruit and vegetables, a better knowledge of the molecular basis of the physical treatments lethal action is needed (Dinçer Baysal and Baysal, 2007). Chilling helps to keep quality by preserving freshness and avoiding spoilage, mainly by slowing microbial growth and that of pathogens, such as *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes* or *Clostridium* spp., among others. However, although the correct low temperature (depending on the product) should be kept throughout shelf-life, harvested plant organs can still spoil, as shown by fungal attacks or even bacterial growth and negative quality changes (Artés, 2004).

For assuring food safety and quality, the implementation of good agricultural and good manufacturing practices as well as hazard analysis critical control points system, in fruit and vegetable production and postharvest handling and treatments is crucial.

The main environmental factors influencing deterioration of fruit and vegetables are temperature, RH, atmospheric composition including C_2H_4, and light. All of them must be well adapted to each product, and in particular, temperature must be selected to avoid CI and accidental freezing. Some socio-economic factors also cause postharvest losses of fruit and vegetables, such as: inadequate marketing systems and facilities; inefficient methods of transportation; regulations set by government; absence of quality grades; preferences of the consumers; unavailability of needed tools and equipment; lack of proper maintenance; and inadequate extension of information.

4 INNOVATIONS IN CHILLING STORAGE TO AVOID POSTHARVEST DISORDERS

The most important goal of postharvest handling is keeping the product cool, to reduce moisture loss and adverse changes, and to avoid mechanical damage to delay visual appearance loss and spoilage. Precooling and cooling reduce qualitative and quantitative losses due to physiological disorders and decay, retard ripening and senescence, extend the shelf-life with right quality, and regulate supplying to industrial factories. The distribution chain is generally composed of many different steps in storage and transportation up until consumption, and traceability is today a key concept (Artés, 2004). However there are certain coadjutants that could effectively improve the postharvest quality of horticultural products during cold storage.

Consumers are progressively more concerned about chemical residues that are potentially harmful to humans and/or cause ecological problems. Due to this, there is renewed interest in the use of physical methods to control postharvest quality attributes losses, diseases and disorders in plant products. This kind of treatments can favourably replace, or at least minimise, the regular use of agro-chemicals in postharvest treatments (Artés, 1995a). Such coadjutants treatments are subsequently briefly reviewed.

4.1 Thermal treatments

Heat has fungicidal and insecticidal action, but heat regimes optimal for insect control may not be adequate for disease control; in some cases they may even be detrimental. A thermal treatment that is developed for fungal or insect control should not damage the product and may even have beneficial effects. These benefits include slower ripening of climacteric products, enhanced sweetness by increasing the amount of sugars or decreasing acidity, and preventing some storage disorders. Thermal pre-treatments could be used for pathogen, insect, or CI control, although most of them are still experimental (Ben-Yehoshua, 2003, Irtwange, 2006).

Heat treatments for controlling postharvest decay have been employed in the past, before the advance of systemic fungicides such as thiabendazol (TBZ) and imazalil. However, the current increasing consumer demand towards reducing the use of synthetic fungicides together with the development of resistance against these fungicides and the growing cost of developing new fungicides have raised the option of restoring the use of heat treatments. In fact they could be considered as an environmentally friendly method for decay control. Some of these methods offer real practical interest for storage of horticultural products sensitive to chilling. But there are no fully effective means to fight against CI, except perhaps those which minimise water stress caused secondarily by low temperature. As a way forward, work must be done on achieving transgenic species resistant to CI (Marcellin, 1992)

Storage at low temperature could prolong shelf-life and maintain fruit and vegetables quality by decreasing the rate of metabolism, delaying ripening and controlling

microbial growth, although CI may appear. When chilling stress is prolonged, these alterations and dysfunctions will lead to the development of a variety of CI symptoms such as surface lesions, internal discoloration, water soaking of the tissue and failure to normally ripen. Several postharvest heat treatments have been reported to induce fruit tolerance to cold temperatures and to inhibit, or at least to reduce CI development during cold storage. Such treatments include pre-storage curing in air or in water and intermittent warming (IW), combined or not with wrapping or seal-packaging in plastic film, and the application of benzimidazole-type fungicides. However, occasional contradictory results related to decay and off-flavours developed in some particular applications of these techniques have been shown (Artés, 1995ab, Artés et al., 1993, Martínez, et al., 1987, Lurie, 1998, Schirra and Mulas, 1995).

IW consists of exposing fruits during chilling storage to several periods of warm temperature. IW increases fruit resistance to low temperatures, enabling the commodity to be stored at temperatures below the ones usually recommended, keeping quality for longer periods. IW has been shown to be effective to reduce CI in several produces, like citrus fruits, peaches, pomegranates (Artés et al., 2000) and plums. The greatest difficulty in creating optimum condition with IW lies in the need to operate with temperatures, duration and frequency that may greatly change among cultivars, maturity stage and growing conditions. It has been hypothesized that IW reduces CI because it accelerates fruit ripening and increase the exchange between internal and external fruit atmosphere thus eliminating harmful metabolites (Marcellin, 1992).

Air renewal and keeping 90-95% RH are crucial factors for the successful application of IW. Moreover, the first temperature rise during an IW treatment must be applied during the induction period of the physiological disorder to be controlled. This stage, when any damage is still not visible, is defined as the latency period. If the temperature increase is achieved too early it results in vain, and if it is performed too late it will hasten the onset of the disorder (Marcellin, 1992).

Several procedures of commercial heat application of long and short duration have been successfully implemented like conditioning or curing citrus fruits for 72 hours at 36ºC, or immersing or drenching them in hot water. The combination of brushing and drenching with hot water has been shown as positive, especially for bell peppers (Ben-Yehoshua, 2003, Fallik, 2004). Air curing at 38 ºC for 12 h combined with 1μmol L^{-1} of methyl jasmonate vapour was an effective treatment to alleviate CI in peaches after storage for 5 weeks at 0 ºC. This was very probably due to the fact that the combination induced the antioxidant superoxide dismutase enzyme activity, thus protecting the cellular membrane against the chilling stress (Jin et al., 2009).

An experimental example of results reached heat treatments was as follows (Artés et al., 1998). In ‘Navelate’ oranges to be stored for 7 weeks at 2 ºC and 90-95 % RH and then for 1 week at 20 ºC, air curing for 3 days at 36 ºC or dipping in water at 53 ºC for 2 min, combined or not with 1 g L-1 TBZ were applied. Also, during storage at 2 ºC, an IW for 1 day at 20 ºC after every week was applied. After shelf-life weight losses and CI

(pitting and rind scald) increased while decay and cracking developed. In control fruit 26.3% decayed by Penicillium spp and Alternaria citri and 34.5% suffered CI. Despite the low dose, TBZ was highly effective in preventing decay and reducing CI (16.5%), but no synergistic effect was shown between hot water or curing with TBZ in preventing decay. A high effect on reducing CI of curing (5.6%) was found, although it was the only treatment that increased cracking (6.9%) and decay (5.5%). Dipping in hot water reduced decay (5.5%) and prevented CI. Hot water plus TBZ caused the lowest weight loss, prevented both decay and CI and resulted in the lowest total losses, being the best treatment. IW also avoided fungal attacks and CI and resulted in a quite similar level in total losses, being the second best treatment. No deleterious effect on quality attributes on treated oranges was found.

For optimising CI sensitive peaches and nectarines eating quality and extending fruit market life an air preconditioning treatment at commercial scale has been implemented. It aimed to reduce CI symptoms and consists in keeping fruits immediately after harvest at 20 ºC for 1 to 2 days in special chambers prior to cold storage at about 0ºC up to 40 to 50 days. Compared to control this treatment increased minimum market life by up to 14 days in the cvs. tested. Weight loss and softening occurred during the preconditioning (cooling delay), but did not reduce fruit quality after storage. Proper use of fungicides is essential for a successful result. Rapid cooling after preconditioning is needed to stop further flesh softening, senescence, decay and weight loss (Crisosto et al., 2004, Infante et al., 2009).

4.2 Gaseous treatments

The conventional cold storage in air can be optimised acting on the gases involved in the respiration, since it is possible to control the metabolism, within certain limits, by controlling the O_2, CO_2 and/or C_2H_4 surrounding the stored produce to levels different from those in ambient air. This change is based on the effects of low O_2 and/ or moderate CO_2 to restrain respiration and other vital processes. Both O_2 and CO_2 play a decisive role in primary and secondary metabolism in plant organs. Their global influence has led to their use with the aim of extending both storage duration and shelf-life.

Therefore, the principle of the storage of fruits and vegetables under a modified atmosphere consists in the change of the quantitative relation of the normal ambient air components by eliminating or adding gases in a refrigerated and gastight area. Depending on how the modification of the atmosphere is reached and applied this technique is known as controlled atmosphere (CA) or modified atmosphere (MA). CA storage consists in a permanent stable control of the gas composition during the chilling storage or transport at optimal temperature and RH in an airtight cold room or container (Artés et al., 2006).

When the generation and stabilisation of favourable atmospheres are obtained by packaging the refrigerated produce in hermetically closed polymeric films (bags, boxes,

pallets) the technique is known as modified atmosphere packaging (MAP). The intended gas composition is generated by the interaction between the respiration of the product and the gas permeability of the polymer (passive modification) or is prepared externally and injected into the package (active modification) in order to accelerate the stabilisation of this atmosphere. In passive MAP, depending on the respiratory quotient, the O_2 consumed by the respiration of the produce is replaced by the CO_2 emitted within the sealed package. If the combination of package-produce has been properly designed and the product is stored at the right temperature, the desired gas composition will be stabilised throughout cold storage or transportation. Early advances in MAP are centred on matching the right films to specific products. As an optimal equilibrium MA within a package depends on the reported parameters, a diversity of produce requires a diversity of packaging films (Artés et al., 2006).

The beneficial or detrimental effects of MAP depend on numerous factors. Among them it should be emphasized the species, cv., growing conditions, harvesting system, physiological stage, postharvest handling, gas composition, storage temperature and time. Efficacy of MAP requires that the recommended steady atmosphere must be reached quickly, without anoxia or generating excessive CO_2 levels. A slight decrease in O_2 is usually not effective and must be lowered under 10-12 kPa. On the other hand, O_2 levels of 1-2 kPa (the lowest O_2 level at which fermentation is completely displaced by aerobic respiration or compensation anaerobic point) could derive in anaerobic conditions within the package and thus will negatively affect the produce quality. The efficacy of MAP depends on a series of factors, including the sensitivity of the products to O_2 and CO_2 levels. Beneficial effects of CA and MAP are commonly pronounced in increased safety and a reduction of qualitative or quantitative losses during handling, storage, transport, distribution and retail sale. However, abnormal ripening as well as physiological disorders can occur. Off-flavours and off-odours might develop due to the organic volatile emission under anaerobic conditions. A decrease in some bioactive compounds levels could also occur (Artés et al., 2012).

4.3 Variable conditions during chilling storage

Technical advances in the development of storage equipment and automation have made possible the implementation of new and industrial CA compositions, including O2 levels of 2-3 kPa (Low Oxygen -LO-) and even below 1 kPa (Ultra Low Oxygen -ULO-) with between 0.5 kPa and 1.5 kPa CO2. In addition, this CA may be achieved using static or dynamic (DCA) systems. In the static CA, the O2 level is maintained at a pre-determined rank throughout the storage period. However, in DCA, O2 levels are defined by a low O2 stress (<1-2 kPa) response from the fruit, which may change during storage. At the start of DCA storage, the O2 level is decreased until low O2 stress is detected, and the O2 level is then increased enough to alleviate the stress. The cycle of stress and recovery is repeated at intervals through the storage period. A sensor is required to detect low O2 stress in the fruit prior to irreversible damage occurs. Two physiological responses to low O2 stress have been tested during DCA storage: the

ethanol production and chlorophyll fluorescence. The use of DCA in apples and pears has been suggested to control superficial scald, and therefore avoids the use of the postharvest antioxidants diphenylamine or ethoxyquin (Watkins and Nock, 2007).

The gas 1-methylcyclopropene (1-MCP), which binds to C_2H_4 receptors with 10 times more affinity than C_2H_4 itself, is one such C_2H_4 antagonising compound, and a useful tool at commercial scale. 1-MCP treatment of fruit has been shown to keep flesh firmness and acidity, but it has also been reported to decrease juiciness and to increase the incidence of some physiological disorders. In addition, 1-MPC could control scald in some apple varieties, although this control can be variable. Loss of scald control is associated with release of fruit from inhibition of C_2H_4 production. The combination of DCA and 1-MCP techniques slow softening, including during shelf life, resulting in better quality produce for the consumer, inhibiting superficial scald development, but their effects on softening and scald are affected by variety **(**Watkins and Nock, 2007**).**

5 EFFECTS OF HIGH CARBON DIOXIDE LEVELS ON HORTICULTURAL METABOLISM

The postharvest tolerances of most commercially important fruit and vegetables to high CO_2 levels are well known in conjunction with the associated O_2 level, which will result in optimal shelf-life without any injury. When a fruit or vegetable is subjected to atmospheres outside safe limits at any temperature/time combination, damage may be manifested as irregular ripening, initiation and/or aggravation of certain physiological disorders, development of off-flavours, and increased susceptibility to decay. Responses of horticultural commodities to CO_2 vary radically between species, cultivars, organ types, and developmental stages, and can be either unwanted or highly desirable, depending on the product. Generally it is assumed that CO_2 directly affects respiration and associated primary and secondary metabolism, including production of C_2H_4, volatiles and pigments. A very wide range of CO_2 levels beyond which injury occurs exists for different products. Whereas 2 kPa CO_2 is the upper level for long-term storage of lettuce, strawberry, figs or cherimoya can tolerate 25 kPa or even higher (Artés et al., 2006).

At the metabolic level, more information is available about the effects of low O_2 than for CO_2 levels. In general, there are many similarities between the effects of low O_2 and high CO_2 on metabolism, with most effects being suppression of various metabolic processes. But, while respiration is usually inhibited by low O_2, it can be inhibited, unaffected or stimulated by high CO_2. When stimulation happens, it may be due to stress responses by the tissue. Moreover, it is known that high CO_2 is a competitive inhibitor of C_2H_4 action (Watkins, 2000).

High CO_2 affects the primary metabolic pathways, such as glycolysis, fermentation, Krebs cycle, and the mitochondrial respiratory chain. CO_2 may affect enzyme activities by changing the rates of degradation and/or synthesis, activation and/or inactivation, substrate and cofactor availability, or a combination of these processes. In addition, CO_2

may cause interactions in tissues, including changes in the kinetics of allosteric proteins. Above 5 kPa CO_2 usually generates HCO_3 decreasing intracellular pH, and affecting the activity of several key enzymes responsible of deterioration. However, the natural buffer capacity of the tissues must be considered (Kader and Watkins, 2000).

Tolerance of plant products to CO_2 depends on its level, that of O_2, and the storage temperature and length of exposure to the gas. Some products are able to resist very high CO_2 levels for a short period (up to 60 kPa for asparagus). Also, tolerance of high CO_2 levels is higher at lower temperatures than at elevated temperatures. As examples, beneficial CO_2 levels for asparagus are 10 to 14 kPa at 0 to 3ºC and 5 to 9 kPa at 3 to 6ºC, and for chilli pepper are 15 to 20 kPa at 5ºC and 0 to 5 kPa at 10ºC (Saltveit, 1997).

Minimal processing or fresh-cutting has direct effects on the tolerances of products to CO_2 by disrupting physical barriers between the external and internal environments, and removing the protective effect of the skin and the outer tissue layers. In that way, tolerances to CO_2 differ greatly between whole and minimally processed products. For example, higher CO_2 levels are suggested for fresh-cut lettuce than for the whole product (about 2 kPa). Whole lettuce can be damaged by higher CO_2 levels causing disorders known as brown stain, and heart leaf injury, and/or accumulation of fermentation products. These differences can occur because storage periods are typically much shorter for fresh-cut than for whole products. In addition, a fresh-cut product has greater surface area, and is potentially subject to greater water loss, more damaged sites, higher respiration and C_2H_4 production rates, and higher microbial growth. Usually, for fresh-cut produces a higher CO_2 level than for original whole produce is more beneficial (Artés et al., 2006).

Some persimmon cvs. (‘Rojo Brillante’, ‘Triumph’, etc.) are astringent when harvested. This makes it necessary to apply a postharvest deastringency treatment of large commercial impact before the fruit can be marketed. The current method used to remove astringency, while keeping the right firmness, involves holding the fruit in air-tight chambers for 24 h under 95–98% CO_2 at 20 ºC and 90% RH. The effectiveness of this method lies in the fact that it triggers anaerobic respiration in the fruit, which gives rise to an accumulation of acetaldehyde and then a reaction with the soluble tannins that are responsible for the astringency. The tannins then become insoluble and the astringency disappears (Salvador et al., 2007). A particular case is the gas treatment used during postharvest persimmons storage, since 1-MCP needs to be used for prolonged storage under chilling conditions and 80 kPa CO_2 during 24 h at 20ºC are needed for deastringency of persimmon (Besada et al., 2008).

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CHAPTER 11

Predictive Microbiology and Improvement of Food Safety

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1 INTRODUCTION

Within the past decade, there has been an increased demand for fresh and minimally processed fruits and vegetables, mainly because of the health benefits associated with their consumption. However, foodborne illnesses associated with the consumption of these produces has also increased. Concerning muscle-based foods, given the significant market trends toward fully-cooked and ready-to-eat food products, there is a growing technical need for ensuring food safety from the start of processing through to distribution to the consumer. Food safety is therefore an important concern in the food industry and in the entire food supply chain.

2 PREDICTIVE MICROBIOLOGY

Predictive microbiology is the part of microbiology devoted to analysing the response of microorganisms in particular conditions and to predict their behaviour. It is based upon the premise that the responses of microorganisms to environmental factors are reproducible, and that it is possible to predict their responses, just taking into account past observations. In this regard it is important to predict the possibilities for growth of microorganisms, as affected by different factors, and for the survival of a microbial population exposed to a preservative treatment. These microbial responses are summarised in the form of predictive models, which quantify the effects of interactions between two or more factors, and by interpolation can be used to predict responses to conditions that have not been tested explicitly. With this purpose, mathematical or probabilistic models with which it is possible to describe the growth or inactivation of foodborne microbes in specific environmental conditions are built and validated.

Predictive microbiology is gaining interest as a tool to guarantee the production of safe foodstuffs, and it has also been recognised as a versatile and helpful tool for risk assessment. Mathematical and probabilistic models are very useful not only for hazard analysis and critical control points, but also for making decisions in scenarios where there is uncertainty. The successful application of predictive microbiology depends on the development and validation of appropriate models. Many manufacturers have used

predictive microbiology to reduce consumer risk associated with pathogen growth, and even to prevent the growth of spoilage microorganisms.

2.1 Inactivation models

The former predictive inactivation models can be attributed to Esty and Meyer in 1922, who found that the inactivation kinetics of *Clostridium botulinum* spores exposed to wet heat followed a logarithmic linear relationship between the number of microorganism survivors and time after heat treatment (first order kinetics). From the survival curve, a D value can be derived, as the time to inactivate 90% of the population. In such models, a deterministic nature of the microbial inactivation is assumed, each microorganism having the same probability of dying and death being due to one single event. D values have been successfully used in the food-canning industry for almost a century, in order to avoid the risk of botulism. Nevertheless, in most cases, this assumption is not valid because of the presence of shoulders and tails in the survival curve. In this context, classical deterministic models, based on first order kinetics, can no longer be used.

Hence, lately, some authors have considered the survival curve as the cumulative form of lethality event distribution, considering the death of each microorganism as a probabilistic case. In these probabilistic models, it is assumed that each microorganism of the population has a different sensitivity to an inactivating agent and dies at a specific time. Hence, the inactivation time is different for each microorganism and the form of the survival curve depends on the resistance distribution of the microbial population. One stochastic alternative that has been widely used by different authors to describe non linear survival curves is based on the Weibull distribution function. The Weibull distribution has been successfully applied for modelling inactivation kinetics of microorganisms exposed to lethal agents, such as natural antimicrobial substances, high hydrostatic pressures, or pulsed electric fields.

2.2 Growth models

There are a number of sigmoid equations and several models that have been used as growth functions. They all differ in their ‘ease of use’ and the number of parameters in the equation. After Monod, who in 1949 successfully applied a kinetic exponential model to describe the growth of fermentative microorganisms, several growth models have been created or adapted to describe microbial growth. Probably, three-phase linear, Gompertz, logistic, Richards and Baranyi are the most preferred models among researchers.

Gibson et al. were the first to use the Gompertz equation to describe the growth curve in 1988. The Gompertz function describes an asymmetric sigmoid curve, which fits most of the growth curves reasonably well. However, it also shows some disadvantages, derived from its own nature.

Some years later, in 1993, Baranyi created his own model, to be used specifically for microbial growth curves. The Baranyi model considers lag phase as an adaptation stage

of the microbial cells to the new environmental conditions. This model, although complex, was provided with a software to facilitate its use.

2.3 Databases

Linked to the development of powerful computers, a great advance in predictive microbiology took place in the 1980s. The huge amount of data generated afterwards has allowed for the development of large databases and computer programs, which facilitate its usage. These data sets are available for food microbiologists, manufacturers, risk assessors, and legislative officers. Some of these programs are free while others are commercial and need to be registered before use. As an example of a free model package, the Pathogen Modeling Program, was created by the United States Department of Agriculture. This predictive microbiology application was designed as a research and an instructional tool for estimating the effects of multiple variables on the growth, inactivation or survival of foodborne pathogens. The first validated, commercialised predictive package, the Food MicroModel, predicts the growth, survival, and thermal death of major foodborne pathogen and spoilage microorganisms in a wide range of foods. These two independent data sets constitute thousands of microbial growth and survival curves that are the basis for numerous microbial models used by the industry, academia, and government regulatory agencies.

In 2003, these two data sets were unified in a common database, the ComBase, and are now publicly and freely available. Data have also been compiled from scientific literature. At present, ComBase contains up to 40,000 records containing full bacterial growth and survival curves.

In ComBase, factors such as pH, NaCl or water activity can be entered as independent variables to obtain the variation in the dependant variable, usually the inactivation or growth kinetic parameter. The system has become a vital tool to ensure the safety of foods in international trade. The use of ComBase avoids unnecessary repetition of experiments, increases the efficiency of research efforts, standardises the data sources for microbial risk assessors, and helps to improve food safety and quality. Nevertheless, many of these models have been built with a focus on the inactivation of microorganisms exposed to heat or on the growth under usual storage conditions.

As stated at the second research summit of the Institute of Food Technologists, held in Orlando in January 2003, “the paramount purpose of microbial inactivation data and calculations deriving from whatever model is applied is the development of a calculated process that is safe, robust in its design, and flexible in approach such that deviations can be evaluated for safety impact”.

3 NON-THERMAL PROCESSING TECHNOLOGIES

Increasing consumer demand for “fresh-like” foods has led to much research effort in the last years in the development of new mild methods for food preservation. Microorganisms are the main agents responsible for food spoilage and food poisoning

and therefore food preservation procedures are targeted towards them. Thermal treatment is the most widely used procedure for microbial inactivation in foods. Thermal treatments generally, however, cause undesirable changes in food flavour, colour, texture, and nutritional attributes such as protein and vitamin destruction.

On the other hand, non-thermal processing/preservation methods are of interest to food scientists, manufacturers and consumers because of the minimal impact these methods have on the nutritional and sensory properties of foods, while they achieve a shelf life extension because of the inactivation of microorganisms at sublethal temperatures. Non-thermal food processing methods are also considered more energy efficient and environmentally-friendly than conventional thermal-based treatments. Together these reasons have promoted the development of non-thermal processing methods for microbial inactivation, among which high hydrostatic pressure, ionising radiation, pulsed electric fields, pulsed light, ultrasound, magnetic fields, and dense phase carbon dioxide are attracting much interest. However, the high resistance of certain enzymes and microorganisms to non-thermal processes, especially bacterial spores, limit their application. To expand their use in the food industry, combinations of non-thermal technologies with traditional or emerging food preservation techniques are being studied (as hurdle technologies), and could present a number of potential benefits to food preservation. There are foods for which thermal technologies are, and will continue to be, the best process alternative. However, some market niches have appeared for non-thermal technologies to produce food products, which are healthy, retain a greater proportion of their fresh properties and most importantly are safe from a microbiological point of view. Thus, there are cases where these are the most appropriate technologies to meet consumer demand.

3.1 High Hydrostatic Pressure (HHP)

High Hydrostatic Pressure involves the application of pressures ranging from 100 to 1000 MPa. The process is based on Pascal's law and Le Chatelier's principle, according to which the pressure applied at one point is transmitted immediately and uniformly throughout the liquid and will provoke reactions which favour a volume reduction.

It inactivates vegetative microbial cells by breaking non-covalent bonds and causing damage to the cell membrane. HHP disrupts secondary and tertiary structures of macro-molecules, such as proteins and polysaccharides, and alters their structural and functional integrity in a pressure-dependent way. So far, little or no inactivation has been observed in spores when only pressure is applied at levels up to 900-1,000 MPa. However, a germination effect is observed at lower pressures, which is understood to be one of the inactivation paths for spores.

Nowadays, there are already approximately 100 industrial applications of HHP in active use worldwide. The most widely used commercial applications of HHP are for products such as refrigerated guacamole, salsas, entrées, and delicatessen meats, which are all processed within packages. This technology can also be used for non-thermal

processing of avocado halves, apple sauce, cured ham and chopped onions. It is further employed to pasteurise oysters while maintaining a raw designation. HHP can be used as a post-packaging lethality step for the inactivation of *Listeria monocytogenes* as well on ready-to-eat meats such as sliced ham and deli meat. The integration of high pressure with other developed processing operations such as blanching, dehydration, rehydration, frying, freezing/thawing and solid-liquid extraction has been shown to open up new processing options. Overall, HHP has demonstrated sufficient benefits with minimal alterations to the product, leading to the conclusion HHP could be a more significant food preservation force in the future.

3.2 Ionising Radiation (IR)

Food irradiation is a non-thermal food pasteurisation process that involves the application of ionising radiation to food. Radiation sources can be gamma rays from cobalt-60, electron beams, or X-rays. It has been found that at low doses, irradiation has little effect on food's nutritional and sensorial qualities. More than 40 countries have approved irradiation for over 100 food items. Further, the World Health Organization has declared that irradiation of any food commodity up to 10 kGy is not a toxicological hazard.

Ionising radiation reduces or eliminates spoilage and pathogenic microorganisms by fragmenting DNA. Irradiation processes minimise post-harvest loss and inhibit sprout formation in products such as potatoes. Post-packaging potential for irradiation includes the disinfection of grains, legumes, spices, fruits, vegetables and tubers, and microbiological control in eggs, poultry, pork and other meats.

3.3 Pulsed Electric Fields (PEF)

Pulsed Electric Fields technology is based on the application of short duration (1-100 µs), high electric field pulses (10-50 kV/cm). Although PEF is a non-thermal process, an increase in temperature occurs in the processing chamber. This process attains a 5 log-reduction of most pathogenic bacteria in liquid media. It causes only minimal detrimental changes to the physical and sensory properties of foods, helps retain food's "fresh" quality, and assists in nutrient retention.

The most accepted hypothesis to interpret the inactivation mechanism for PEF is based on the theory of dielectric breakdown, whereby an external electric field applied to a cell disrupts the stability of the electric charges on the cell membrane. When the electric field applied exceeds a critical threshold value, it causes pore formation that may be either reversible or irreversible, in which case the cell loses membrane integrity and cell material leading to cell death.

PEF can be applied to the pasteurisation of liquid products in continuous systems, such as milk, yogurt, juices, liquid eggs, soups, brines and other products that can withstand high electric fields. PEF has limited effects on microbial spores, cannot be used on products that contain or could form air bubbles and cannot be used on foods having

higher or variable electrical conductivity. PEF has mainly been applied to improve the quality of foods and a lot of research is ongoing today.

3.4 Ultrasound

Ultrasound is defined as sound waves with frequencies above the threshold for human hearing (> 16 kHz). Ultrasound utilises at least 20,000 vibrations per second to achieve a bactericidal effect by cell lysis and causes enzyme inactivation. In liquid media, ultrasounds are transmitted as waves, creating compression and decompression cycles. These pressure changes lead to the formation and growth of microscopic bubbles in the liquid. When these bubbles reach a critical size they implode, in a phenomenon called cavitation. At the cavitation spot, very high temperatures and pressures are reached, which are thought to have the microbicidal effect.

Ultrasound is one of the simplest and most versatile methods used for cellular disruption and food extract production. This technology works best when used in conjunction with heat and pressure, but it can be used alone for fruit juices, sauces, purees and dairy products. Foods with particulates and other interfering substances do not react well to ultrasound. Ultrasound has not yet demonstrated nor achieved any major beneficial effects that warrant serious consideration for processing or packaging.

3.5 Natural Antimicrobials

The essential oils of plants are complex mixtures of different compounds. Many exhibit antimicrobial properties against bacteria, moulds and yeasts. Essential oils and their components are used as raw materials for the food industries, where their main use is as food flavouring agents. The European Union has recorded various components of essential oils, considering that at the recommended dose, they do not present risks to the health of consumers. Among the most important essential oil components, carvacrol, carvone, cinnamaldehyde, citral, p-cymene, eugenol, limonene, menthol, and thymol can be cited.

Natural antimicrobials from animal origin can also be found. Eggs and milk are good sources for antimicrobial substances. The lysozyme, present in the egg yolk or in milk, is an example in this group.

Among the natural antimicrobial substances from microbial origin, nisin, produced by *Lactococcus lactis* is probably the most used.

4 NON-THERMAL TECHNOLOGIES AND FOOD SAFETY

In the last years several new non-thermal technologies have been developed, and some have been successfully applied to the food industry. When these technologies are used for food preservation, it is important to know the mechanism through which the new technology is inactivating microorganisms, which microorganisms are being inactivated, whether these microorganisms can develop resistance or cross resistance

(and to which level), as well as the effect of physical and/or chemical factors on its effectiveness and to which point these effects can be predicted. Here is where predictive microbiology has a new field of application.

Mild preservation technologies have in common that they are often limited in the type and number of microorganisms killed. For example, pulsed electrical field treatment does not inactivate bacterial spores, and high pressure inactivates spores only if applied at elevated temperatures. The implementation of mild preservation technologies requires special attention to the quality of raw materials, optimisation of process conditions, hygiene of process lines, high quality packaging (pack integrity, packing under modified atmosphere), and specific storage conditions.

In this context, Food Safety Authorities can play an important role in assessing the safety of the new technologies applied in the food industry. Recently, the European Commission has requested an initial scientific opinion from EFSA relating to the risks arising from nanoscience and nanotechnologies on food and feed safety and the environment. The request also asks to identify the nature of the possible hazards associated with actual and foreseen applications in the food and feed area, and to provide general guidance on data needed for the risk assessment of such nanotechnologies and applications. In this case, EFSA's mission is: (i) revision of scientific advice and scientific and technical support in all fields that have a direct or indirect impact on food and feed safety, to provide to European Commission, Parliament, and Member States independent information on all matters within these fields, and (ii) risk communication within its remit networking and collaboration with Member States. Other opinions have been prepared in response to questions raised from the Commission in relation to the use of different technologies for preservation and food preparation.

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CHAPTER 12

Control Engineering of Food Recontamination

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1 FOOD SAFETY ENGINEERING: A NEW CONCEPT

Within the past decade, food safety has been an increasing concern for consumers, retailers, and all production and processing areas of the food industry. Food safety is also of crucial importance to a nation's economy and health systems. Calculation of annual cases of salmonellosis and campylobacteriosis shows that the yearly number of cases in Europe is likely to exceed five million (Jevsnik et al. 2008). An estimated 25-81 million cases of foodborne illness and an estimated 9000 deaths are associated with consumption of contaminated foods each year in the USA (Center for Food Safety Engineering - CFSE 2007; Gerner-Smidt and Whichard 2007). Even though an economic value can never be assigned to lost lives, the USDA's Economic Research Service (ERS 2004) estimated that each year in the United States, five foodborne illnesses—*Camploybacter* spp., *Salmonella* spp., *E. coli* O157:H7, *Listeria monocytogenes*, and *Toxoplasma gondii*—cause $6.9 billion in medical costs, lost productivity and premature deaths (Kowalcyk 2004). In 2006, it was estimated that foodborne diseases cost the economies of England and Wales slightly less than £1.5 billion (FSA 2008).

Nevertheless, the incidence of foodborne diseases is rising in developing countries, as well as in the developed world (Redmond and Griffith 2003). For example, in the UK while cases of *Campylobacter* and *Salmonella* have fallen (due to regulation, training and public information campaigns), *E. coli* and *Listeria* are on the upswing; the number of *Listeria* cases has almost doubled since 2000, a troubling indication that more work needs to be done in combating these pathogens (ElAmin 2006).

There has been an increased demand for fresh and minimally processed fruits and vegetables mainly because of the health benefits associated with their consumption, but foodborne illnesses associated with consumption of these produces have also increased in the last two decades. In fact, the proportion of foodborne disease outbreaks associated with these fruit and vegetable products doubled from 1988 to 1991 (Mahmoud et al. 2008). Also, concerning muscle-based foods, given the significant market trends toward fully-cooked and ready-to-eat food products, there is a growing technical need for

ensuring food safety from the start of processing to distribution to the consumer (Marks 2001).

Food safety is therefore an important concern in food engineering. In fact, the safety of the entire food supply chain depends on food engineering innovations and designs that apply the latest technologies to real-world problems in production and processing (Marks 2001; López-Gómez and Barbosa-Cánovas 2005; López-Gómez et al. 2009, 2013; Ramaswamy et al. 2007; Barron 2007).

After CFSE in 2007 it was clear that engineering should be an integral component of food safety research. Engineering is necessary in the development of physical and chemical mechanisms and devices for detection of microbial and chemical hazards in the food supply. In fact, if we define engineering as the hardware that makes it possible to carry out a technology (using software or "know how"), food safety engineering could be considered as a type of food engineering hardware (e.g., the physical solutions for processing; packaging and storage equipment; facilities–including control systems; rooms in food factory; other facilities used in food supply chain) that could be used to achieve the required levels of food safety and security in the food supply chain (Raspor 2008). Marks (2001) stated that while epidemiologists, food microbiologists, and chemists advance the understanding of foodborne microorganisms (e.g., explaining how microorganisms cause diseases, react to environmental influences, and can be isolated and identified), this knowledge must ultimately be scaled-up by food engineers to design and implement technical solutions to the real problems facing the food industry (Fig. 1).

Figure 1 Bulk storage aseptic tanks (Photo by A. López-Gómez).

Food safety engineering is an emerging specialisation that involves the application of engineering principles to address microbial and chemical safety challenges (Balasubramaniam 2006). According to Ramaswamy et al. (2007), food safety engineering study must include: (i) intervention technologies (traditional and novel non-

thermal intervention technologies, chemical interventions, and hurdle approach); (ii) control/monitoring/identification techniques (biosensors); (iii) packaging applications in food safety (active packaging, intelligent or smart packaging, tamper evident packaging); and (iv) tracking and traceability systems. At Cornell University a graduate training program offered in Food Safety Engineering (Moraru 2007) included: (i) novel processing methods for food pathogen inactivation; (ii) modelling of microbial inactivation kinetics; (iii) development of antimicrobial techniques; and (iv) development of novel microbial detection methods.

At Pennsylvania State University, a Food Safety Engineering course introduces diverse topics in microbial food safety from an engineering perspective, including: plant layout; construction materials; equipment design; predictive microbiology and modelling; conventional and novel detection and enumeration methods; conventional and novel processing methods; emergency contingency plans; and current responsibilities and regulations of federal agencies for food safety (PSU 2008).

Another important reference to food safety engineering is the Center for Food Safety Engineering at Purdue University, originally a "food safety engineering project" involving a five-year cooperative agreement with USDA-ARS to develop better methods of detection and prevention of biological and chemical foodborne contaminants. Today, the mission of that Center is to develop new knowledge, technologies, and systems for detection, and prevention of chemical and microbial contamination of foods, with a multi-disciplinary approach, including a strong engineering component (CFSE 2007).

This chapter demonstrates the need for the food safety engineering perspective. This perspective is needed in order to produce high quality food products (minimally processed) that are both safe and secure, and involves a multi-disciplinary approach. The main components of this engineering are: (i) predictive microbiology as a tool to evaluate and improve food safety in traditional and new processing technologies; (ii) advanced food contaminants detection methods; (iii) advanced processing technologies and food safety; (iv) advanced systems for food re-contamination control; and (v) advanced systems for active and intelligent packaging (López-Gómez et al. 2009).

2 CONTROL ENGINEERING OF FOOD RECONTAMINATION

2.1 Barriers technology for food safety and security

Early on, the main concern of food security was that the rights of people to have a steady diet be assured. Later, food security referred to measures that could be taken to obtain healthy food that was beneficial and not harmful to health, and food not contaminated accidentally. In 2001, the concept of food security was also extended to all measures designed to prevent the occurrence of intentional contamination of food harmful to people's health. NFPA (2001), after the terrorist attacks of September 11th, elaborated a Food Security Manual, for processors, distributors and retailers, including a

security checklist with the objective of providing companies with a document to facilitate self-assessment of food security measures by identifying a wide range of factors that should be considered (independent of specific processing steps). The focus of this document is distinct from GMPs and HACCP, and is on the prevention of intentional product contamination.

Safety and security are a concern in virtually all engineering processes and systems. In engineering practices, there are many principles and methods recommended for the engineer as means to ensure safety. Möller and Hansson (2008) state that safety considerations can be divided into three different types: (i) adherence to good practice, (ii) safety analysis, and (iii) inherently safe design. A recommended first step in safety engineering is to minimise the inherent dangers in the process as far as possible. This means that potential hazards are excluded rather than just enclosed or otherwise coped with. Also, the concept of fail-safe is in practice mainly used for specific methods and principles for keeping the system safe in case of failure, such as shutting down the components or the entire system. An application of the fail-safe principle is the usage of several safety barriers (Möller and Hansson 2008). The NFPA (2001) document considers a kind of barriers technology, as applied to protect the food product from intentional contamination but which is equally valid to protect it from accidental contamination.

The first barrier refers to outside premises, such as the fencing or other barriers, to prevent unauthorised access within the boundaries of the facility (monitoring and controlling these areas, with access control to transport vehicles, personnel, and domestic and non-domestic animals). The basic idea is that the first barrier must be normally closed, with a single access (a door or several doors) for entry personnel and transport vehicles with raw matters and end products. The second barrier concerns the inside premises, involving the closing of the buildings of the food factory, which must be closed in a normal manner (windows and doors). All entrances/exits must be controlled, in the same building and between buildings, as well as connections to other areas through openings for vents, air circulation lines, pipes, electrical lines, drains, etc. In this way, the entry of insects, rodents, and personnel is controlled within the processing and packaging rooms (through doors, windows, floor drains, and ceilings). The third barrier is the segregation of restricted areas (zones) within the plant, which have different hygienic requirements and controlled access (Van Donk and Gaalman 2004). The fourth barrier involves the clothes worn to protect the product from human contamination, mainly in the clean room areas dedicated to ultra-clean and aseptic processing and packaging (Burfoot et al. 2000) (Figs. 2 and 3). The fifth barrier is the processing equipment (including storage and conveying systems), which must have an adequate hygienic design and must be closed normally to protect the food product from external contamination (López-Gómez and Barbosa-Cánovas 2005; López-Gómez et al. 2009, 2013; Jensen 2007). The food safety engineering must solve the detail engineering (drawings and specifications) to implement each one of these barriers to protect the food product from intentional and accidental contamination.

2.2 Hygienic design in the food factory

As cleaning in place (CIP) procedures are used throughout the food industry as the only practical way to clean closed process equipment (Friis and Hensen 2002), some design details are reported as being hygienically risky because of the undesired fluid flows, such as those found in up-stands, dead-ends, heat exchangers, expansions or contractions, etc. (EHEDG 1992; Hauser et al. 1989). Therefore, to produce safe and wholesome foods the food safety engineer should carefully check the hygienic design, installation, handling and maintenance of production or the processing "hardware" (Jensen 2007; López-Gómez et al. 2013).

Figure 2 Ultra-clean packaging facility for fruit juices (Photo by A. López-Gómez).

Figure 3 Class 10.000 clean room as a closing of a Class 100 clean room, for ultra-clean packaging of fruit juices (Photo by A. López-Gómez).

Food engineering provides a number of rules and knowledge to design and run food processing plants. Substantial knowledge is available with respect to equipment design, plant design (stressing all aspects of civil engineering such as walls, floors, heating, piping), air control, control of personnel, use of materials (Lelieveld et al. 2003; EHEDG 2003; López-Gómez et al. 2013), paying special attention to specific technical aspects of hygienic design at the detailed level of machines, materials used, floors, piping, etc. However, one aspect of hygienic design must not be neglected, as distinguished by Holah (2000): the systematic analysis and evaluation of an overall factory with the aim of segregating work areas to control hazards. Segregation of work areas (or hygienic zoning, EHEDG 2003) is important for food processing industries. Van Donk and Gaalman (2004) have developed a decision aid that can be used to evaluate the design or redesign of the layout of food processing plants, explicitly taking into account hygiene of the product and process and aiming to find the appropriate segregation of work areas or different hygienic zones. This usually must be considered as one of the aspects of hygienic design (Holah 2000). The approach of Van Donk and Gaalman (2004) is inspired by principles of layout planning and design, as developed in the field of production engineering on the one hand, and the specific characteristics of food processing industries and its products on the other hand.

2.3 Ultra-clean and aseptic processing, storage, and packaging

The Gram-positive *Listeria monocytogenes* is the pathogen of concern in ready-to-eat meat and poultry products that allow growth of the organism during storage, if exposed to recontamination during slicing and packaging, without posterior treatment (Sofos 2008). In fact, *L. monocytogenes* continues to be the number one target for control in ready-to-eat meat and poultry products, considering its ubiquitous presence, potential to contaminate products after processing, and the ability to multiply even under cold temperatures (Tompkin 2002).

Types of antimicrobial interventions or hurdles used to control pathogens in further processed meat and other food products are of a physical, physicochemical, or biological nature (ILSI 2005). A recent regulation in the United States (FSIS 2003) was established for the control of *L. monocytogenes* in ready-to-eat meat and poultry products that may be contaminated after processing (during handling for slicing and packaging). After this new regulation the industry must choose one of three next alternatives: (i) application of a post-lethality treatment (maybe an antimicrobial agent) to reduce or eliminate microorganisms on the product and an antimicrobial agent or process to suppress or limit growth of *L. Monocytogenes*; (ii) application of a post-lethality treatment (maybe an antimicrobial agent) to reduce or eliminate microorganisms on the product or an antimicrobial agent or process to suppress or limit growth of *L. Monocytogenes*; and (iii) a combination of sanitation and microbiological testing programs for food contact surfaces and holding of the product when results of testing are positive (FSIS 2003; Sofos 2008).

An advanced alternative for prevention of contamination and control of *L. monocytogenes* (and other damage and pathogen microorganisms) is ultra-clean and aseptic processing (e.g., slicing), storage (Fig. 1), and packaging technology (Figs. 2 and 3), involving a combination of sanitation and microbiological testing programs for food contact surfaces (Ros-Chumillas et al. 2007a,b; López-Gómez et al. 2013). But, it is very important to understand the difference between non-hygienic, hygienic, ultra-clean, and aseptic processing and packing and their consequences for processing and food safety (Jensen 2007; López-Gómez et al. 2013).

2.4 Food Safety and Quality Standards

Quality standards have contributed to food safety (Escriche et al. 2006). During the last decade there was a strong trend towards quality certification by large Western retailers. Private safety control systems, standards, and certification programs are used to respond to higher consumer expectations, because quality is no longer related to the product alone, but also to the characteristics of the production and distribution processes, including the hygienic design (Trienekens and Zuurbier 2008).

Contrary to more general quality systems like HACCP and ISO, systems used by retailers often cover more parties in the chain (Korel et al. 2003). ISO standards are international standards, enacted to achieve uniformity and to prevent technical barriers against trade throughout the world. The standard extends the ISO 9001:2000 quality management system standard, which is widely implemented in all sectors, but does not specifically address food safety (www.ISO.org; Trienekens and Zuurbier 2008). Demands regarding private food safety and quality standards are best represented by three examples of systems used world-wide: Eurep-GAP, British Retail Consortium (BRC), and Safe Quality Food (SQF). Eurep-GAP is an organisation of more than 20 large European retailers and purchase organisations (e.g., AHOLD, TESCO). GAP stands for Good Agricultural Practice. In 1998, the BRC, with participants such as TESCO and Sainsbury, took the initiative to define common criteria for the inspection of suppliers of food products. The norms of the BRC are converging with HACCP norms, although more attention is being paid to a documented quality management system, factory environments and facilities (including hygienic design), product and process control, and personnel. SQF aims at quality assurance from a total supply chain perspective, including food safety (Trienekens and Zuurbier 2008).

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CHAPTER 13

Technology and engineering of minimally processed products

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1 INTRODUCTION

The current high demand for minimally processed or fresh-cut fruit and vegetables is a result of the consumer's desire for healthy, convenient, fresh and ready-to-eat plant-derived commodities. The fresh-cut industry is increasing in many EU countries with UK, France and Italy as the main leaders. These kinds of products are fruits/vegetables prepared with single and slight processing operations (peeling, cutting, shredding, washing, drying, etc.), and packed in suitable polymeric films under active or passive modified atmosphere packaging (MAP) that are marketed under refrigerated conditions (5ºC) as a ready-to-eat produce. For that reason, they show similar characteristics to the whole original product, contain exclusively natural ingredients, are of good quality with relatively low price and do not need time for preparation. Since a well-balanced diet, rich in fruits and vegetables, promotes good health and may reduce the risk of certain diseases, fresh-cut products are an important source of antioxidants and other phytochemicals, which play important roles in human nutrition due to free radical scavenging activities. As consumers increasingly perceive fresh food as healthier than heat-treated food, it motivates a general search for food production methods with reduced technological input, with natural fresh-like appearance, and reduced time to prepare into minimal processing operations. The expected attributes of these products by consumers are freshness, optimum overall quality (general appearance, sensory quality –texture/firmness, aroma and taste– and nutritional quality) and safety. However, during fresh-cut processing and the retail period some physiological, physical and nutritional changes may occur, reducing the expected quality attributes of the fresh-cut products. Furthermore, some pathological disorders can appear which can highly limit the shelf-life and safety of such products.

Several factors affect the shelf-life and microbial quality of fresh-cut vegetables, such as agricultural practices at the farm; hygienic practices during harvesting and handling; quality of washing water; processing technologies; packaging methods and materials; and transportation, processing and storage temperatures. In order to reach the best results in this industrial activity the plant raw materials must be carefully chosen considering their ability to support the different processing steps. In addition to this, to ensure the safety and quality of all incoming raw materials, implementation of a quality

management standard such as ISO 9000 has been recommended. A hazard analysis of critical control points (HACCP) should also be included to identify what could go wrong with incoming produce. Proper storage conditions of fruits and vegetables before processing are vital for good quality production. Finally, it is necessary to evaluate rapidly and non-destructively the quality of plant raw materials upon reception in the factory, for safety aspects such as pesticide residues, microbial load, toxic metals, naturally present undesirable compounds, and plant growth regulators.

2 PHYSIOLOGY AND BIOCHEMISTRY

After wounding, living tissue begins a flow of metabolic reactions that starts with increased respiration rate and can result in texture changes, accelerated ripening and/or senescence, off flavours, discolouration and other undesirable events. Handling and processing also result in an increased ethylene production which promotes ripening and senescence. Microbiologically, removing the protective peel of fresh produce leaves a cut surface that is covered with water from the cell contents, which makes it convenient for microbial development. Since the minimal processing damages plant tissues, leading to additional quality losses, the derived fresh-cut commodities are in fact more sensitive to disorders than the original ones. Therefore, deterioration of fresh-cut fruits and vegetables is mainly due to further physiological aging, biochemical changes and microbial spoilage which turn the product unmarketable. The adverse changes affecting such products are off-flavours, discolouration or chlorophyll loss, softening and water loss. The most important characteristic of fresh fruit and vegetables is that they continue living after harvesting. The respiratory activity is a metabolic process that provides the energy for plant cells to stay alive and to develop physiological and biochemical processes. Furthermore, wounding increases the respiration rate of the plant tissue, probably as a consequence of induced C_2H_4 biosynthesis, which stimulates respiration. During minimal processing, products are subjected to mechanical damages that stimulate fast physiological and biochemical pathways, which are recognised by an increase in their metabolism.

Browning of fresh fruits and vegetables is a frequent problem during postharvest handling, processing and storage, and is one of the major causes of quality loss and spoilage, which reduces produce quality and is very often the factor limiting shelf-life and marketability of minimally processed products. This phenomenon can be due to enzymatic and non-enzymatic reactions. Enzymatic browning or oxidative browning requires different components: enzymes such as polyphenol oxidase (PPO), and peroxidase (POD), a substrate and co-substrates such as O_2. Browning takes place at the cut surface of fruits and vegetables because of decompartimentation that occurs when cells are broken, allowing substrates and oxidisers to be in touch. The brown colour development is related to oxidation of phenolic compounds (monophenols, triphenols, and o- and p-diphenols) to o-quinones, catalysed by PPO and POD.

3 PREHARVEST FACTORS AFFECTING POSTHARVEST QUALITY

The effects of preharvest factors on postharvest quality are often overlooked and underestimated. However, many of the decisions made during production can greatly influence the postharvest quality. A range of biotic and abiotic elements can alter their appearance determining postharvest life. The first factor is the plant itself: large genotypic variation given by many different botanical species and genetic type or variety has been described. Secondly, there are external factors. The factors that have been most studied are the environmental (climatic conditions), the agronomic (cultural practices) and the physiological conditions. The first one includes temperature, RH, rainfall, wind, soil, etc. In the second one fertilisation, watering, pruning, etc. should be mentioned, and in the third the maturity stage at harvest.

Genetic aspects. It is considered that genome is responsible of plant behaviour in relation to environmental conditions and that the achievement of a certain product depends on the expression of a variety in a given external environment. The cultivar genetic variability within the same species is relatively large, so selection of the most appropriate is of vital importance for the quality of the final product.

Environmental aspects. Although in the field most of the environmental factors are hardly modulated, they have a strong influence on the quality and nutritional value of many products. Light Intensity and quality, temperatures to which the plants are exposed, CO_2 content in the atmosphere, etc. are the most relevant. In general, the lower the light intensity is, the lower the ascorbic acid content is. Light quality can also affect the physiology of the product. UV-B decreases the synthesis of β-carotene and ascorbic acid, while the red light increases the synthesis of lycopene.

Agronomic Aspects. Soil type, mulching, irrigation and fertilisation influence the water and nutrient supply, which can affect the nutritional quality of the product. Nutrient content as well as the balance between two or more of them can affect growth and induce both disorders due to deficiency and to overdose. Those that have aroused greater interest are nitrogen and calcium. The nitrogen content is directly related to protein and carotenoids synthesis (influencing colour). Excess soil nitrogen can be problematic (accumulation in leaves, increased leaves production, decrease of texture, vitamin C and essential amino acids content, etc.). The chlorophyll content is usually higher and sugar content and acidity lower. On the other hand, soil nitrogen deficiencies may lead to lower protein and pigments concentrations. Deficiencies in soil calcium have been associated with a great number of physiological disorders. High soil calcium concentrations reduce such disorders and are associated with increased vitamin C content; extended storage life; delayed ripening; increased firmness; and reduced respiration and C_2H_4 production. Water stress during the growing season can affect the size. However, a controlled stress would induce slight negative effects on production, but with some improvements in quality. Water amount also affects the development of postharvest physiological disorders such as pithiness.

Physiological aspects. Maturity at harvest has an essential role in biochemical and physiological activity and, therefore, influences quality attributes. The ripeness also affects the fruit susceptibility to certain physiological disorders (i.e. chilling injury).

4 SAFETY OF MINIMALLY PROCESSED PRODUCTS. LEGISLATION

From 1996 to 2006, 72 foodborne illness outbreaks were associated with the consumption of fresh produce, and in 25% of them a fresh-cut produce was implicated. Foodborne microbial pathogens associated with the consumption of fresh fruits and vegetables include *Cyclospora cayetanensis*, *Escherichia coli* O157:H7, hepatitis A virus, *Listeria monocytogenes*, Norovirus, *Salmonella* spp. and *Shigella* spp. Potential sources of microbial contamination are the ingredients (fruits and vegetables), packaging materials, processing aids, facility environment, food contact surfaces, nonfood-contact surfaces, etc. Table 1 shows the limits on microbiological criteria for fresh-cut products (Regulation EC 1441/2007, 2007). Fresh-cut processing increases the risk of bacterial growth and contamination. The release of plant cellular fluids when produce is chopped or shredded provides a nutritive medium in which pathogens, if present, can survive or grow. Such processing has the capability to reduce the risk of contamination by placing the fresh-cut produce in a controlled, sanitary facility, including screening materials entering the processing chain, suppressing microbial growth, reducing the microbial load during processing and preventing post-processing contamination. The main sustainable sanitation strategies which can be used for keeping quality and safety of fresh-cut commodities are chemical antimicrobial solutions and physical treatments.

Sodium (NaOCl) or calcium hypochlorite ($Ca(OCl)_2$), are potent disinfectants with powerful oxidising properties, being the most commonly used by the food industry for sanitising products, lines, surfaces and processing equipments. They are generally effective and comparatively inexpensive. Their effectiveness against microorganisms depends on pH, temperature, concentration, organic matter present in the washing water and plant product, time of exposure, and the initial microbial load. Their efficacy increases with increasing concentration of available chlorine, but high levels may cause product tainting and residue on the product and equipment.

Table 1. EU Commission regulation on microbiological criteria for fresh-cut products. (Regulation EC 1441/2007, 2007).

Food category	Micro-organism	Sampling plan ([1])		Limits ([2])		Stage where the criterion applies
		n	c	m	M	
HYGIENIC PROCESSING CRITERIA						
Pre-cut fruit and vegetables (ready-to-eat).	*E. coli*	5	2	100 CFU g^{-1}	1000 CFU g^{-1}	Manufacturing process.
FOOD SAFETY CRITERIA						
Pre-cut fruit and vegetables (ready–to–eat).	*Salmonella*	5	0	Absence in 25 g		Products placed on the market during their shelf-life.
Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes.	*Listeria monocytogenes*	5	0	100 CFU g^{-1} ([3])		Products placed on the market during their shelf-life.
				Absence in 25 g		Before the food has left the immediate control of the food business operator, who has produced it.

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M // (2) For points 1.1–1.25 m = M. // (3) This criterion shall apply if the manufacturer is able to demonstrate that the product will not exceed the limit of 100 CFU/g throughout the shelf-life. The operator may fix intermediate limits during the process that must be low enough to guarantee that the limit of 100 CFU/g is not exceeded at the end of shelf-life.

5 TECHNOLOGY OF MINIMALLY PROCESSED PRODUCTS: UNIT OPERATIONS AND EQUIPMENTS

The fresh-cut processing usually consists of a sequence of minimal /simple operations and generally, the extension of the shelf-life depends on a combination of a proper temperature management throughout the entire production chain, optimum sanitising, dips in anti-browning solutions, use of edible coatings, optimal packaging conditions (usually under MAP) and good manufacturing and handling practices in well-designed factories. Figure 1 illustrates the general unit operations and the maximum recommended temperatures to each processing step in the production line of a general fresh-cut product factory. The main objective of the fresh fruit and vegetable processors throughout all operations involved in the production of a fresh-cut produce is food safety, quality optimisation and reduction of losses. The first step is generally sanitation of whole products (with NaOCl) to eliminate dirt, pesticide residues, plant debris, soil, insects and foreign matter, and retardation of the enzymatic discolouration reactions. Cutting and peeling steps constitute a critical hygienic point in the processing line, and the equipment used in this operation needs to be cleaned, disinfected and sharpened at

regular intervals every working day. Attention must be paid to the physical damage, physiological stress, and increase of microbial growth caused in this step. Such changes are mainly due to the increase of wound respiration and C_2H_4 production due to mechanical injuries which results in the release of intracellular oxidising enzymes and substrates and leads to various biochemical deteriorations such as browning, and increased availability of cell juice and nutrients that will decrease the shelf life of fresh-cut produce. Therefore, cutting appears to have a dramatic effect on nutritional value, overall quality and shelf life of minimally processed fruits and vegetables. Many different peeling machines are commercially available but peeling is normally accomplished manually, mechanically, chemically or in high-pressure steam peelers. At the same time, several industrial machines are able for cutting, grating, chopping, shredding or slicing fresh produce into pieces of several shapes and sizes.

Washing and disinfection, mainly with NaOCl, after peeling and/or cutting is the only step where a reduction in the microbial load can be obtained, thus minimising populations of potential pathogens. It is remarkable that chlorinated washing produce can effectively remove sand, soil and other debris from fruits and vegetables but should not be relied upon to completely remove organisms. However, sanitisers are primarily used to maintain bacteriological quality of the water rather than the produce. Washing can be applied with a spraying of potable water, or by showers, although it generally involves the immersion of the product during 1-5 minutes in refrigerated (1-10 ºC) sanitised water in a bath or wash-tanks usually containing between 50 to 150 ppm of NaClO solution, and acidified with about 150 to 200 ppm of citric acid to manage pH values between 6.5 and 7.5 for optimising the chlorine efficacy.

The next critical processing operation is dewatering. Drying / dewatering wet surfaces must be carried out carefully to avoid unnecessary damage to the plant tissues, reducing the product moisture content and removal of cell leakage that can support microbial growth. Dewatering systems include draining systems, gentle removal with cheesecloth, centrifugal spin dryers, vibrating racks, rotating conveyors, hydro-sieves, forced air and spinless drying tunnels. The high centrifugal force not only removes water, but also cracks and crushes the tissues, which is a great inconvenient that should be avoided. Additionally, between the drying and packaging steps it could be interesting to introduce techniques already applied in clean room technology by installing a filtered air system that is able to assure a reduced number of particles with a low diameter, which include microorganisms.

The correct quantity of product may be weighed and disposed in bags or trays before the packaging step, which is usually achieved under MAP to reach the needed commercial shelf-life. The aim of MAP is to create an atmosphere around the packaged produce, which retards its respiration and deterioration rates in such way that the tolerated minimal O_2 or maximum CO_2 concentrations are not exceeded, in order to avoid a shift towards fermentation or other metabolic or biochemical disorders. The design and selection of the appropriate polymeric film for trays or bowls as well as for sealing is crucial. In this way, when temperature abuse (over 8ºC) occurs during transport,

distribution and, particularly, retail sale could commonly occur, the use of little perforations or microperforations in MAP polymeric films should be suggested. It is known that on one hand, atmospheres of high CO_2 and low O_2 levels could control microbial growth but, on the other hand, the risk of recontamination of commodities within packages increases. Before the expedition of the product from the factory, it has to pass an effective quality control system to guarantee the safety, suitability and compliance of specifications. In addition, it needs a procedure of product recall when the specifications are not met.

The equipment has to be frequently revised, adjusted and calibrated. It is well-known that temperature is the most important environmental factor that influences the deterioration rate of harvested commodities. Knowledge about the time-temperature conditions in the cold chain is necessary to determine its influence on the quality loss and the shelf-life of these products. Although throughout the distribution chain commodities must be kept at 1-5ºC to ensure quality, it is almost impossible to guarantee such temperature during transit, distribution and retail display.

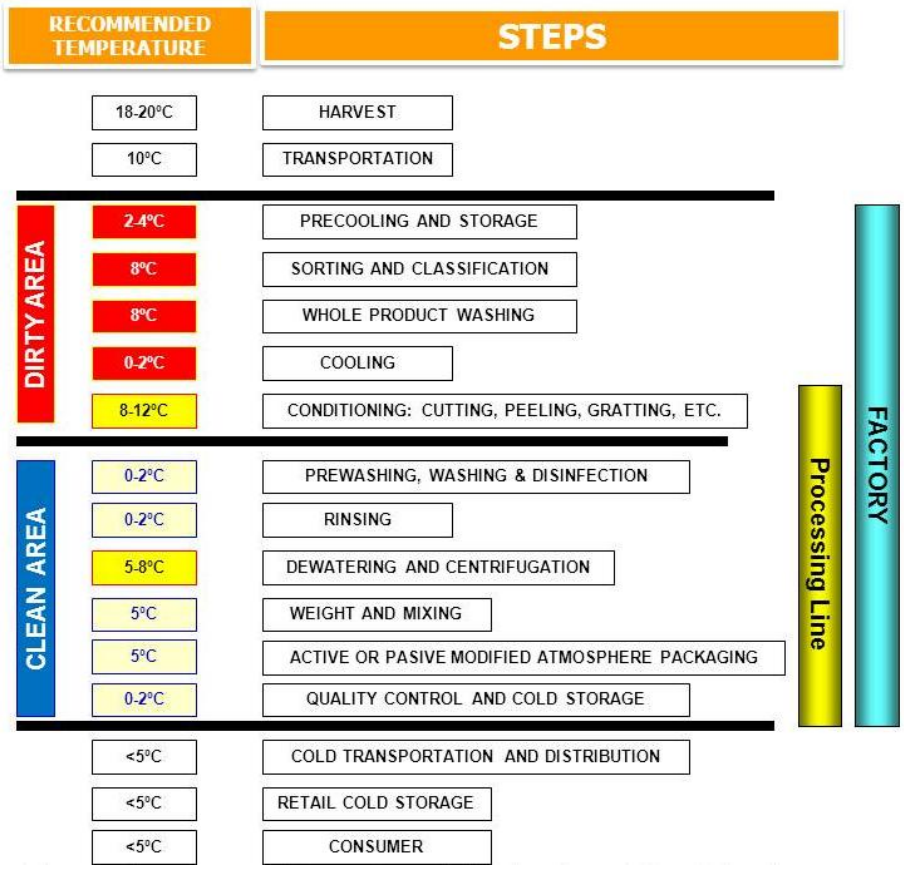

Figure 1.- General unit operations and maximum recommended temperatures for each processing step in the production line of a general fresh-cut product factory.

6 TECHNOLOGICAL INNOVATIONS TO PRESERVE QUALITY AND SAFETY OF FRESH-CUT PRODUCTS

NaClO is commonly an efficient sanitation agent but there is a risk of undesirable by-products upon reaction with organic matter such as chloroform ($CHCl_3$), haloacetic acids or other trihalomethanes (THM) that have known carcinogenic effects and may lead to new regulatory restrictions in the future. Moreover, its efficacy is poor for some products. Consequently the fresh-cut industry wants alternatives.

Improved ecofriendly strategies are promissory alternatives to preserve the high quality and safety demanded by consumers, and many papers in the scientific literature deal with this fact. During the washing and disinfection step with water, some GRAS compounds with antimicrobial and/or antibrowning properties, ozonated, ionised or electrolysed water can be used. Before packaging, UV-C illumination or intense light pulses seem to be promising tools to ensure safety, enhancing quality of fresh-cut produce. Meanwhile, in the packaging step, non-conventional gas mixtures, including noble gases, to generate an alternative active MAP can reduce the initial microbial load and its development, thus 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.

Peroxyacetic acid is a combination of peracetic acid (CH_3CO_3H) and H_2O_2. It is not particularly harmful for the ecosystem and it has tolerance to several factors such as temperature, pH (from 1 to 8), hardness and soil contamination. **Chlorine dioxide** (ClO_2) is a stable dissolved gas that is environmentally friendly, having a higher oxidation and penetration power than NaClO. ClO_2 is a strong bactericide and virucide at levels as low as 0.1 ppm. It attacks bacteria, fungi and viruses, disinfects surfaces, and prevents and rapidly removes biofilms, avoiding bacterial re-growth. With minimal contact time, it is highly effective against many pathogenic organisms. **Acidified sodium chlorite** is obtained by reducing the pH to values between 2.5 to 3.2, a solution of sodium chlorite ($NaClO_2$) with an acid as citric, phosphoric, malic or acid sodium sulphate. It is commercially obtained through the reaction of citric acid and $NaClO_2$ in equal proportions. **Organic acids** (citric, ascorbic, etc) and **calcium salts** have been largely applied for the prevention of enzymatic and non-enzymatic browning, texture deterioration and microbial growth at levels that did not adversely affect taste and flavour of plant commodities. They are more effective for bacteria than for moulds and yeasts due to the low pH (between 2.1 and 2.7) at which they are applied. **Ozone** (O_3) is a highly unstable tri-atomic oxygen molecule. O_3 is commercially generated by passing O_2 through an electrical charge. The decomposition of O_3 is so rapid in the water phase of food that its antimicrobial action may take place mainly at the surface, leaving no residues. The bactericidal effects of O_3 have been shown on a wide variety of bacteria, spores and vegetative cells. O_3 destroys microorganisms by the progressive oxidation of vital cell components, preventing microbial growth and extending the shelf-life of many fruit and vegetables. The application systems include gaseous or as ozonated dips. **Electrolysed water** (EW) is formed by adding a very small amount of NaCl (≅0.1%) to

pure water, and conducting a current across an anode and cathode. The cathode area produces alkaline reducing water while the anode area produces acidic oxidising water. EW has a strong bactericidal effect against pathogens and spoilage microorganisms, more effective than NaClO due to its high redox potential. The use of hot water, steam and forced air can be used as **heat treatment**. Immersion in hot water or dips has traditionally been used to control fungi and insects and is developed for fresh-cut fruits. Temperatures generally used are in the range of 50 to 60ºC during 1 to 5 min.

Among other non-watered physical methods, the use of **UV–C radiation** before packaging is remarkable. The use of non-ionising, germicidal and artificial UV at a wavelength of 190–280nm (UV–C) is effective for surface decontamination of fresh-cut products. This treatment does not leave any residue; it is easy to use and lethal to most types of microorganisms. UV–C damages microbial DNA, therefore, cells which are unable to repair radiation damaged DNA die and sub-lethally injured cells are often subject to mutations. The more common UV–C radiation used in fresh-cut products is from 0.5 to 20 kJm^{-2}.

7 BYPRODUCTS REVALORISATION

Fresh-cut products generate high amounts of wastes (peels, seeds, stones and unused flesh that are generated by different steps of the industrial process) which constitute an important environmental problem. A beneficial impact on the environment, leading to a greater diversity of products directed to human usage for their biological compounds such as natural antioxidants or functional ingredients that could be further processed into functional food and could represent a solution to the environmental problem. Several studies have shown that the content of phytochemicals is higher in peel and seeds than in the edible tissue. Peels from apples, peaches, pears as well as yellow and white flesh nectarines were found to contain twice the amount of total phenolic compounds as that contained in fruit pulp. Pomegranate peels contain about 10-folds higher amount of phenolic compounds as compared to the arils, and mango peel contains a number of valuable compounds such as polyphenols, carotenoids, enzymes, dietary fibre and pectin. In the asparagus industry there is more than 30% by-product produced, mostly peel stem and crown. These by-products are a rich source of rutin and protodioscin which is the active ingredient in the dietary supplement. Artichoke processing also generates high by-products volumes. Extract from the edible part and other artichoke parts are rich in polyphenols, and show a high antioxidant activity. Isorhoifolin (apigenin-7-O-rutinoside), narirutin, cynarin (1,5-dicaffeoylquinic acid and 1,3-dicaffeoylquinic acid), chlorogenic acid, caffeic acid and cynaroside are identified in the different parts of the plant. Broccoli by-products, consisting of leaves and stalks, are rich in nitrogen-sulphur compounds (glucosinolates and isothiocyanates) and phenolics (chlorogenic and sinapic acid derivatives, and flavonoids), as well as essential nutrients (minerals and vitamins). Broccoli by-products are a source of bioactive ingredients to design novel beverages, using organic green tea as a food matrix. The incorporation of cauliflower by-products into ready-to-eat snacks has also been studied.

Onion has also shown a variety of pharmacological effects such as growth inhibition of tumour and microbial cells, reduction of cancer risk, scavenging of free radicals, and protection against cardiovascular disease, which are attributed to specific sulphur-containing compounds and flavonoids.

A number of researchers have used fruits and vegetable by-products from apple, pear, orange, peach, blackcurrant, cherry, artichoke, asparagus, carrot pomace as sources of dietary fibre supplements in refined food. Fruits and vegetables by-products can also provide lipids and amino acids. Seeds from a melon hybrid contained high percentages of lipids (35% dw) and proteins (29.9 g 100 g^{-1} dw), and the presence of 25 fatty acids, with the principal fatty acids linoleic, oleic, palmitic and stearic acids. Seed proteins were rich in arginine, aspartic and glutamic acids. It has also been reported that the watermelon seeds can be utilised successfully as sources of good quality oil and protein for human consumption. In addition, watermelon skin, a typical by-product from the fresh-cut industry, is a rich source of biological amino acids, such as citrulline. This amino acid is used in the nitric oxide system in humans and is an efficient hydroxyl radical scavenger, a strong antioxidant and it has vasolidatador roles. There is increasing worldwide interest in the importance of dietary minerals in the prevention of several diseases such as cancer, diabetes, and osteoporosis.

8 FIFTH RANGE PROCESSING TECHNOLOGY

The ready-to-eat vegetable industry has recently developed new products in order to diversify its production and increase the offer for a consumer who is constantly demanding new healthy and natural products with high quality but low preparation time. The fifth range industry has found in the cooked vegetables sector a good market opportunity. Some of this demand comes also from the catering services and fast food chains, which require larger volumes of these products.

The application of heat treatments is the most widespread food preservation technique. However, this treatment is very aggressive with the nutritional and sensory properties, and especially with vegetables, causing consumers rejection. In order to solve these technological problems, mild cooking techniques with a subsequent refrigerated storage have appeared to configure the fifth range industry of vegetables. The technology used to preserve the fifth range vegetables includes mild heat treatment, cooling and packaging and can be described by the following characteristics: heat-treated products, ready-to-eat and marketed under refrigeration; heated prior to consumption, usually in microwave or conventional oven, usually packaged in plastic materials; keeping the cold chain until consumption (processing, packaging, storage and distribution). The name of fifth range vegetables has been attributed primarily to vacuum-packaged boiled vegetables ready-to-heat and eat. Lately, the fifth range vegetables have been described as plant based products which have been heat-treated to guarantee a conservation period of minimum six weeks. In the Anglo-Saxon term the fifth range products are sometimes referred to as ‘Refrigerated Pasteurized Foods of Extended Durability’ (REPFEDs). These products are characterised by a processing at temperatures of 65-95 °C. The

higher temperature range (> 70 ºC) is usually applied to vegetables. After heat treatment food is rapidly cooled and stored <5ºC until consumption. The fifth range products market has considerably grown in the recent years. Europe is largely the most active market in this sector in 2010, with a 50% worldwide market share, followed by the USA with 23%.

The increasing demand of low and easy-preparation foods has attached a considerably long expiration date, usually of several weeks, expected by the consumers. Throughout the recent and emerging trajectory of the fifth range vegetable industry, conventional cooking techniques, such as boiling, steaming, deep-frying and grilling have been applied. Together with the latter cooking methods, new techniques like vacuum cooking (cook vide), sous vide ('under vacuum' from French) and microwaving are being adapted to the fifth range vegetable industry in order to: diversify the product offer; reduce the nutritional losses during thermal treatments; maximise the sensory properties of the product; and reduce the production costs (versatile and effective equipment, less energetic cost, etc). Furthermore, when this kind of food is destined to catering services, it is also necessary to implement effective packaging techniques which guarantee its safety and long commercial distribution. 'Cook and chill' is a system where food is prepared and subjected to a heat treatment, packaging (prior to or after cooking), fast cooling (blast chilling) and chilling storage until consumption.

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