Use of cellular systems to characterise plant tissue behaviour

M. Ferrando and W.E.L. Spiess

Federal Research Centre for Nutrition, Karlsruhe, Germany

Abstract

Two cellular systems have been studied to characterise the physiological and physical changes, i.e. cell viability and cellular shrinkage, which take place in plant tissue during osmotic treatments. The behaviour in hypertonic solutions of isolated protoplasts, i.e. single cells without cell wall, from cortex tissue of carrots and cortex tissue of strawberry has been evaluated at several operation conditions. CSLM (Confocal Scanning Laser Microscopy) in fluorescent mode has been used to determine cell volume changes upon osmotic treatment. With a minimum of sample preparation, on-line micro-experiments in which the osmotic treatment of the sample takes place directly on the microscope were carried out. Cellular shrinkage was evaluated for different operation conditions with respect to osmotic pressure which was established through sucrose concentrations ranging from 30 to 60%.

Introduction

By immersing biological materials into aqueous solutions of sufficiently high concentration of solutes as salts or sugars two major and simultaneous counter-current flows are initiated: a release of water from the tissue into the solution and a simultaneous transfer of the solute into the tissue. Although the mass transfer mechanisms involved are not fully understood, many authors have underlined the important role the plant tissue nature (e.g. composition, structure) may play in the mass transport following osmotic treatment. Osmosis seems to be the main phenomenon during the first phases of the process (Marcotte et al. 1991a, 1992; Saurel et al. 1994); because of its properties as a selective barrier, the plasma membrane hence is the most important cellular organ concerned.

Shrinkage has been considered an indicator of the structural changes undergone by the plant tissue and a necessary parameter to model the process. In this way, a relation between total shrinkage and physiological changes as plasmolysis and cellular collapse has been suggested (Yao et al. 1996). Cellular and extracellular shrinkage as a function of macroscopic parameters, such as solute concentration of the osmotic solution, solute gain and mass loss have been calculated to describe the microscopic process (Marcotte et al. 1991b).

Until now several microscopic techniques have been employed to estimate the qualitative effect of osmotic treatment on tissue structure (Saurel et al. 1994; Torreggiani et al. 1997). At present, the new Confocal Scanning Laser Microscopy (CSLM) allows the optical sectioning from bulk specimens with a minimum of sample preparation and to carry out on-line micro-experiments in which the osmotic treatment of the sample takes place directly on the microscope. The confocal microscope is based on the principle that the light is focused on a well-defined depth in the specimen and that information from this focal point is projected onto a pinhole in front of the detector. The light illuminates a very small region of the specimen and the point detector ensures that only light from that small region appears. In this way, the depth resolution in CSLM is much better than in the conventional microscope (Blonk et al. 1993). In CSLM various modes of microscopy can be performed: reflected light, transmitted light and fluorescent incident/emitted light. In biomedical applications and in food microscopy, the latter has been shown to be the most

powerful technique, because the various structural elements do not differ largely in reflective power. In biology CSLM is mainly used in animal physiology while in botany its use has not been so extensive. The application of CSLM to food technology has been restricted to the study of emulsions because of the difficulties to specifically stain plant tissue structures.

Plant single cells and isolated protoplasts, i.e. single cells without cell wall, have been proposed as a model system to evaluate the influence of unit operations or processes (Knorr 1994). These model systems resemble the state of a cell as it is found within the plant tissue and can be reproduced under controlled conditions. Furthermore, the use of plant single cells avoids masking of stress responses to previous handling as cutting or peeling. Protoplasts from plant tissue are obtained upon an enzymic treatment. Enzyme sources and maceration conditions, e.g. cellulase-pectinase ratio, pH, temperature, have to be empirically set up for each plant material. Moreover, small variations in the tissue composition due to physiological changes and age of the raw plant material can drastically modify the isolation process yield.

Other cellular systems, as cortex tissue of strawberry, seem to be particularly suitable to study microstructural changes. On ripening, the connections between the parenchyma cells of the strawberry cortex progressively decrease (Szczesniak et al. 1969). Therefore cells are separated from each other allowing a more efficient labelling of non fixed tissue.

Isolated protoplasts and cortex tissue, both made up of parenchyma cells, are used to study the behaviour of plant material under osmotic stress. These cellular systems allow to study separately the role of the plasma membrane and, on other hand, the effect of the solid matrix (cell wall, intercellular space) on the osmotic process.

The aim of this work is to show the possibilities of CSLM to characterize the physical and physiological changes, i.e. cellular shrinkage and cell viability, which take place during osmotic treatments in two cellular systems: isolated protoplasts from cortex tissue of carrots, and cortex tissue of strawberries.

Material and methods

Carrot protoplasts

Carrots (*Daucus carota L.*) from a local provider were peeled and cut in cylinders. Cortex tissue was separated using a corkborer and cut into approximately 2-mm rings. The segments were transferred to a digestion medium consisting of 0.6 M mannitol (a_w = 0.989), 2% (w/v) cellulase, 0.1 % (w/v) pectinase and 0.02M phosphate buffer (pH 4), all reagents from Merck (Darmstadt, Germany). Four g of tissue were added by 20 ml of digestion medium. The tissue was vacuum infiltrated for 5 min to facilitate penetration of the digestion medium into the cell wall space. Tissue was incubated in enzymic medium for 3 h at 24-26 °C. Protoplasts were separated from partially digested tissue by filtering with polyamide cloth. The filtrate was centrifugated at 250g for 5 min and protoplast pellet suspended in 0.7 M mannitol solution (pH 6.5). The protoplast sediment was suspended in 5 ml of Ficoll 12% (w/v) in 0.7 M mannitol, 0.02 M phosphate buffer (pH 6.5). A discontinuous gradient was formed by successively layering 5 ml of 8%, 5%, and 0% Ficoll in 0.7 M mannitol, 0.02 M phosphate buffer (pH 6.5) over the protoplast suspension. The gradient was centrifugated at 400g for 20 min and protoplasts were collected at the interface between the 0% and 5% Ficoll layers. The protoplasts were diluted with 0.7 M mannitol and centrifugated at 250g for 5 min. The final pellet was

suspended in 0.5 ml of 0.7 M mannitol prior to use in experiments (Gronwald et al. 1982; Langerbartels et al. 1981).

Cytoplasm of isolated protoplasts with enzymic activity was stained with fluorescein diacetate (Sigma Aldrich Chemie). For that, the protoplast suspension was treated with 100mg/ml fluorescein diacetate for 3 min and repeatedly washed with 0.7 M mannitol (250g, 5 min).

Isolated protoplasts were osmotically treated with 30, 40, and 50% sucrose solutions directly on an invert microscope. Experiments using 30 and 40% sucrose solutions were carried out twice using cells from the same isolation process. Four samples, two of them coming from the same protoplast isolation, were treated with 50% sucrose.

Strawberry tissue

Fresh strawberries (*Fragaria ananassa,* var. Elsanta) from a local provider were cut in halves. A 2-mm slice of each half was longitudinally cut and pith and epidermis tissue were removed with a razor. Cortex tissue cuts were held in isotonic solution, 0.3 M (a_w = 0.995) mannitol, for 1 hour (Flowers et al 1992). After that cytoplasm and cell wall were labelled. For this, cytoplasm was stained with fluorescein diacetate; cell walls were labelled with congo red (Sigma Aldrich Chemie) because of their affinity to cellulose and β-glucanes. Cortex tissue cuts were incubated in a 100 μg/ml solution of congo red for 1 hour at room temperature, then the sample was treated with fluorescein diacetate, 100 μg/ml for 60 minutes; finally it was gently washed.

As for carrot protoplast, the osmotic treatment was carried out directly on the microscope. Strawberry tissue was treated with 40 and 60% sucrose solutions. Each osmotic treatment was repeated eight times.

Microscopy

The micro-experiment took place in a cylindrical sample chamber placed over the microscope lenses. The chamber was made up of two cylindrical pieces to hold the coverslip. The coverslip was coated with poly-L-lysine solution, an effective compound in promoting adhesion of sections (Sigma Aldrich Chemie), in order to attach the plant specimen (carrot protoplasts or strawberry tissue) to the coverslip's surface. After the sample had been placed into the 5 ml volume chamber, osmotic sucrose solution was added by pumping at 20°C ± 2°C.

A Zeiss LSM 410, Anxiovert 135 invert microscope and a Zeiss 20x objective (NA 0.5) was used. An argon laser generates two excitation beams of 488 and 543 nm. Three dichroic mirrors and a set of filters for blue, green, red and far-red fluorescence were employed to split the different emission signals.

During osmotic processing images of the same group of cells were stored in a digital format for further processing.

Image analysis

OPTIMAS 6.2 was the image analysis software employed to measure the protoplast surface in different phases of the process.

Results and discussion

Dynamic microscopic studies of cell plants have been found to be an efficient tool not only to describe qualitative structural changes but also to estimate some parameters related to the plant structure such as cellular shrinkage. The laborious sample preparation required in classical light microscopy and also in some electronic microscopies has restricted their use to qualitative studies of structural modifications caused by osmotic treatments. CSLM allows to overcome this limitation as no chemical fixation is necessary to prepare the sample.

Protoplast suspensions are biological model systems in which a good fluorescent labelling can be obtained as the diffusional problems of on-block staining of plant tissue are negligible. Cell wall and cytoplasm of strawberry cortex tissue could be effectively stained because the rather loose connection between parenchyma cells allowed a good diffusion of the fluorescent probe. The use of fluorescent vital stains, on the other hand, provides information about the physiological state of the plant material, as the fluorescence of fluorescein diacetate, a reagent which diffuses into the cells and is colourless until its ester bonds are hydrolyzed in the cytoplasm, is related to cell viability. So the absence of a fluorescence signal indicates a loss of membrane integrity and, hence, cellular death.

Changes undergone by a sample of carrot protoplasts during the first 20 minutes of osmotic treatment with 40% sucrose solution are shown in Fig.1. During the first minute, a quick cellular shrinkage can be observed, while no important cellular volume decreasing has been found from 15 minutes of treatment. Both a breakdown of the plasma membrane and an out-of-focus image because of protoplast detachment can be the reasons of a vanishing of the signal from some protoplasts along the process.

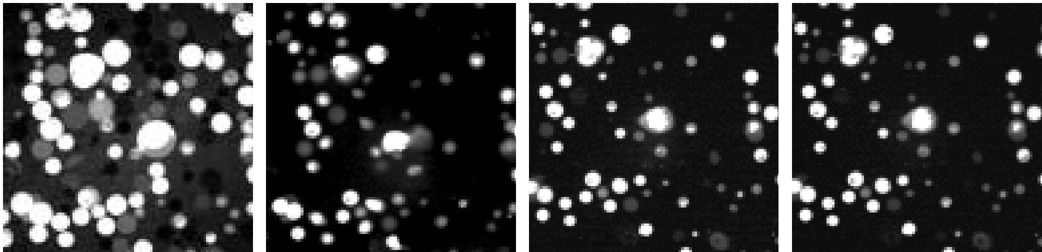

Figure 1: Carrot protoplasts from cortex tissue after 0, 1, 15 and 20 min of treatment with 40% sucrose solution at room temperature (20x magnification)

Cell wall and plasma membrane of strawberry cortex tissue are shown at different processing times in Fig. 2. It should be pointed out that, at the original colour images, protoplasts and cell wall emitted green and red fluorescence, respectively, allowing a better structure visualization. Different stages of protoplast separation from the cell wall (plasmolysis) due to the osmotic pressure of the sucrose solution can be clearly observed.

To show the possibilities of CSLM as a tool to evaluate cellular changes, protoplast shrinkage was calculated. Assuming that changes in volume are proportional to surface changes in the xy plane, cell shrinkage was calculated for every cell from the ratio of the surface at any time to the initial surface (S/S_0).

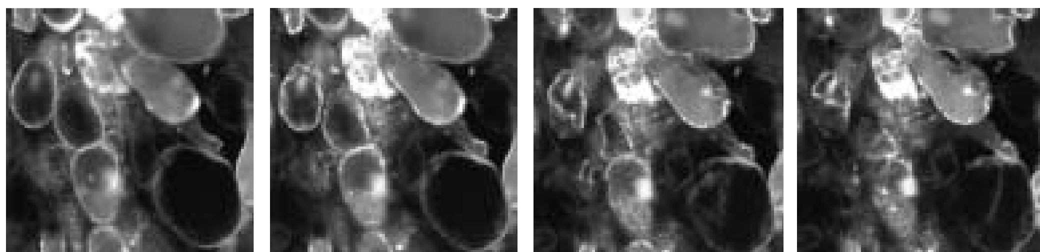

Figure 2: Strawberry cortex tissue processed with 40% sucrose solution at room temperature after 0, 15, and 30 min of treatment (20x magnification)

For carrot protoplasts the surface of approximately 100 cells corresponding to two experiments was measured for treatments with 30% and 40% sucrose solutions. Every cell could be identified and the cellular shrinkage was calculated as the average of the single cellular shrinkage of n cells ($S/S_0=\Sigma(S_i/S_{0i})$). In the case of experiments with 50% sucrose, a very fast cellular response gave rise to a high detachment rate, which hindered the identification of every protoplast along the process. Therefore cellular shrinkage was calculated from the ratio of the average surface at any time to the average initial surface ($S/S_0=\Sigma S_i/ \Sigma S_{0i}$).

A value of 0.87±0.25 was obtained for the shrinkage of carrot protoplasts after 15 minutes of 30% sucrose solution treatment; for the same processing time with 40% sucrose solution, a value of 0.56±0.15 was obtained. After 15 minutes of treatment with 50% sucrose solution 0.37±0.20 and 0.26±0.20 were the values obtained for the shrinkage of 2 different protoplast populations. The good agreement between these experimental values allows to validate the experimental procedure as an efficient tool to estimate changes at the cellular level.

Fig. 3 shows the average cellular shrinkage as a function of the processing time. For all of the operation conditions used, the cellular response was very fast and an apparent swelling in the first 5 minutes could be noticed. It could be as a consequence of a subestimation of the cell surface at the beginning of the treatment. When the cells shrink so quickly, depending on the direction of the shrinkage, the image can be out-of-focus until the operator refound the right plane. Another possible explanation is that under osmotic stress the permeability of the membrane is modified and a small gain of sucrose solution can take place (Yamaki et al. 1988).

Shrinkage of protoplasts of strawberry cortex tissue was calculated as $S/S_0=\Sigma(S_i/S_{0i})$ because cells could be easily identified during the process. Fig. 4 shows S/S_0 versus processing time of tissue treated with 40 and 60% sucrose solutions. For a high osmotic pressure (60% sucrose) the shrinkage is faster, that is, S/S_0 decreases in the first 20 minutes but with longer processing times it remains almost constant (S/S_0=0.69±0.2). On the other hand, after 25 minutes of 40% treatment S/S_0=0.82±0.2, but a more extended shrinkage could be expected because of the negative slope of the curve and a still high ratio of living cells (n/n_0=0.83). The proportion of number of living cells at any time to the initial number of living cells has been defined as living cell ratio (Fig. 5). In the first 10 minutes of 60% sucrose treatment the ratio of cells with a functional plasma membrane is still higher than 80%, but with longer processing times it decreases dramatically down to 26%.

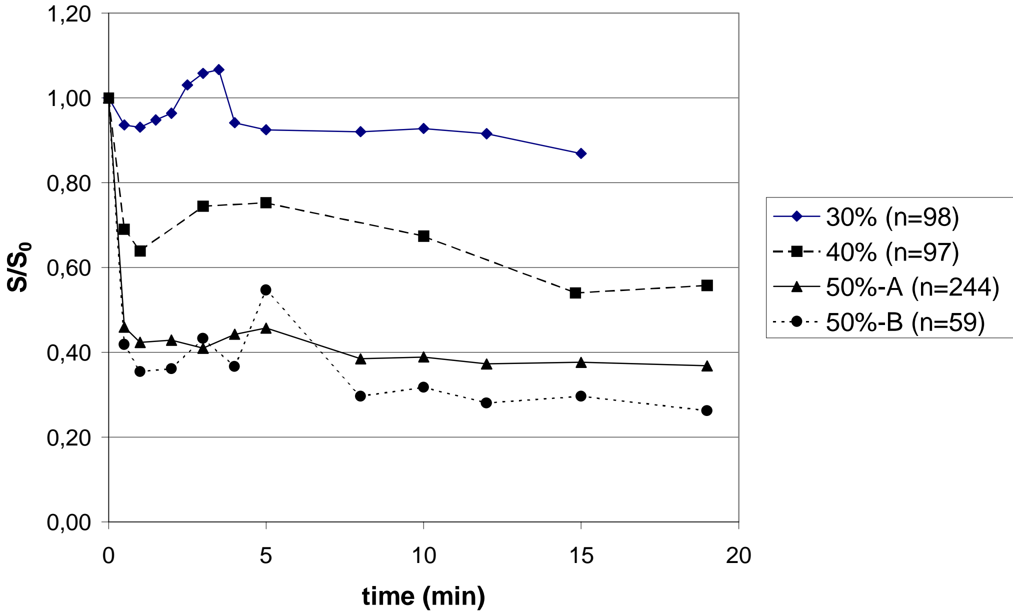

Figure 3: Shrinkage of protoplasts treated with 30, 40 and 50% sucrose solutions versus processing time

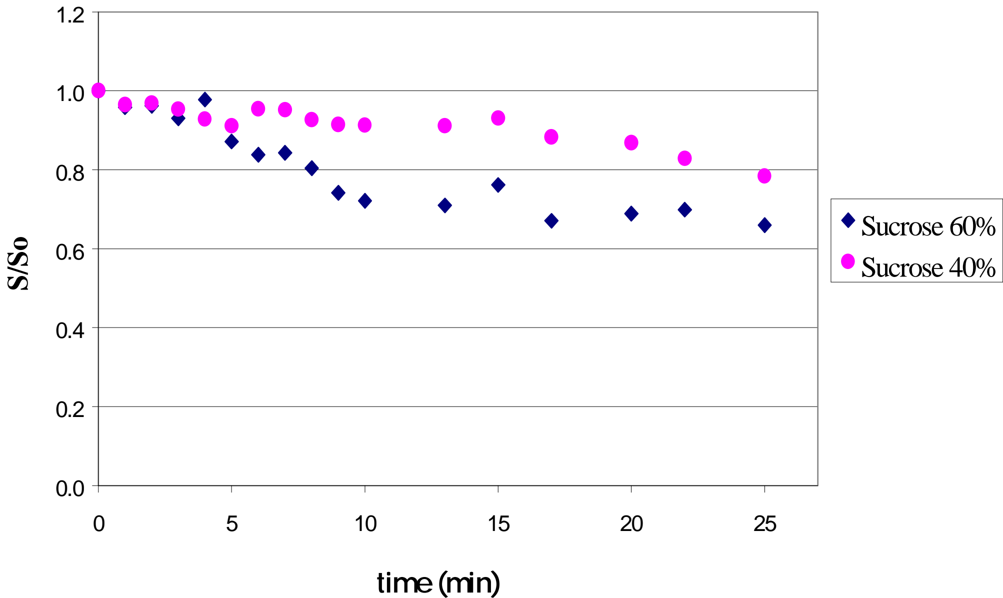

Figure 4: Cellular shrinkage (S/S_0) versus processing time of strawberry cortex tissue treated with 40% and 60% sucrose solution at room temperature

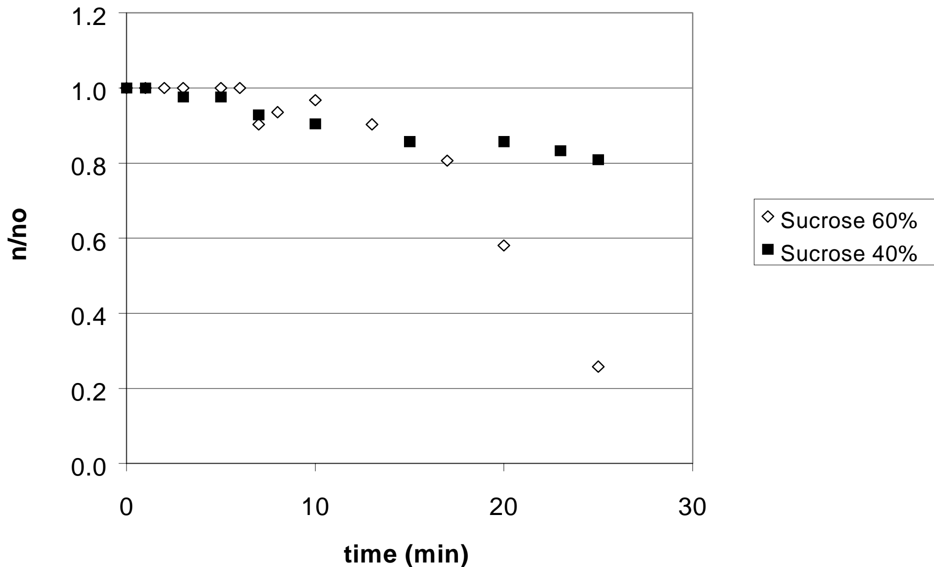

Figure 5: Living cell ratio (n/n_0) versus processing time of strawberry tissue treated with 40% and 60% sucrose solution at room temperature

In Fig.6, the protoplast shrinkage of carrot protoplasts suspension is compared with the protoplast shrinkage of strawberry cortex tissue both treated with 40% sucrose solution. A much faster cellular response is observed for protoplasts, which after 20 min of treatment seem not to shrink any more. Protoplast shrinkage is much slower in strawberry tissue, indicating that longer processing times are necessary to determine maximal protoplast shrinkage (or critical volumen) in tissue.

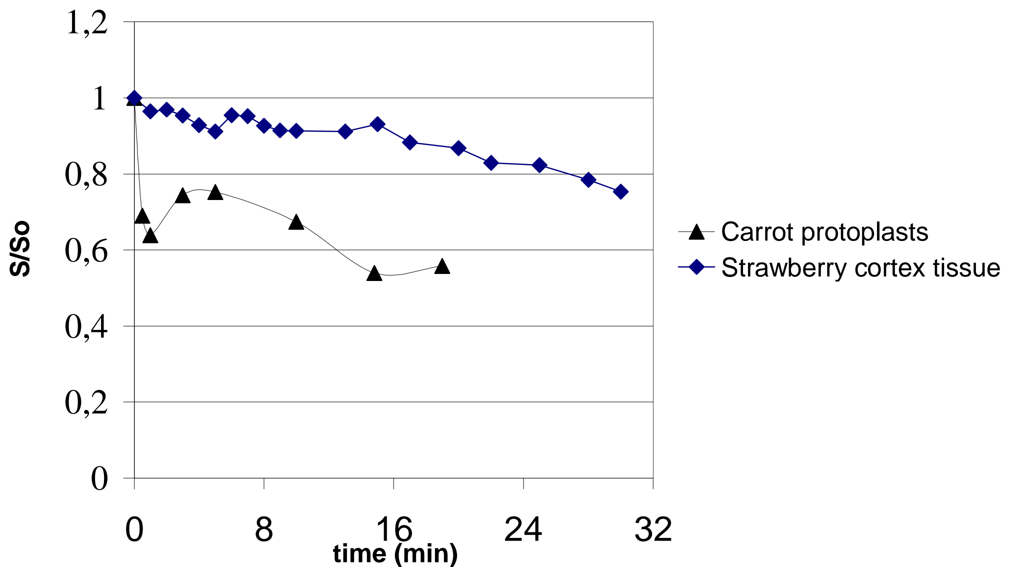

Figure 6: Cellular shrinkage (S/S0) of carrot protoplasts and strawberry cortex tissue treated with 40% sucrose solution

Although two different plant materials are used for the study, it has been shown that the combined use of isolated protoplast and some (simple) tissue structures can give information about the effect of the main cellular structures concerned - plasma membrane, cell wall, middle lamella - on the mass transfer processes.

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