Lipid content in higher plants under osmotic stress

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Abstract

In this work, we performed investigations on the lipid content of higher plants (spinach) under hyperosmotic stress, by means of thin layer chromatography (TLC) and mass spectrometry. In particular, the experiments have been performed at different plant organization levels: whole leaves, freshly prepared protoplast suspension and mesophyll cells obtained by reformation of the cell wall from protoplast suspension. The results obtained showed that hyperosmotic stress induces changes in the phospholipid content depending on the different plant organization levels studied. All phospholipids showed an increment of their content in stressed whole leaves. In particular, phosphatidylglycerol (PG) redoubles its content by 1 h of osmotic shock. Different responses to hyperosmotic stress were reported for the other systems. In the case of protoplasts, an increment of PG, phosphatidylcholine (PC) and phosphatidylinositol (PI) together with biphosphatidylglycerol (BPG) and phosphatidylethanolamine (PE) content decreasing were observed in stressed sample. For PG, identified as PG (34:4) by electrospray ionization mass spectrometry, the increment was of about 30%. In the case of cells, conversely, a decrease of PG content under osmotic stress was recorded. The results suggest an important role of phospholipids, in particular of PG, in the osmotic stress response.
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Keywords: Osmotic stress; Lipids; Higher plants; Protoplasts; Thin layer chromatography; Mass spectrometry

1. Introduction

Plants experience a wide range of environmental changes, represented both by biotic stress (bacteria, viruses, fungi and parasites in general) and by abiotic stress (salinity, drought, extreme temperature). Stress factors are numerous and, depending on their exposition time and their concentration, can reduce plant growth rate: in particular, salt stress is certainly one of the most serious abiotic factors limiting the productivity of crop plants. The understanding of the molecular elements involved in hyperosmotic stress responses can be therefore important in the study of a strategies to generate salt tolerant plants through genetic engineering [1]. Moreover, improving salt tolerance in plants might have much wider implications, because transgenic salt-tolerant plants often result tolerant also to other stresses including chilling, freezing, heat and drought [2].

In order to respond to the osmotic stress by means of survival strategies, plants need to monitor external environment, to transfer the appropriate signals inside and consequently to adapt. This response depends on different biological organization levels (cellular, tissue, organ and whole plant level) since as plant cells become specialized in tissue or organs, integration and coordination of responses are required and the adaptive mechanisms for a proper tolerance to salt stress may be different [1]. A separated study at each level of response is therefore the best way to deal with this subject.

Plants subjected to hyperosmotic stress display several complex molecular responses, which involve the modification of a large number of biochemical and developmental parameters including the synthesis of stress hormones like abscisic acid (ABA), the synthesis of proteins that prevent denaturation and

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oxidative damage [3,4], the modification of ionic fluxes and synthesis of osmolites (sugar and amino acid derivates) in order to regulate osmotic pressure and turgor [3,5].

Moreover, phospholipids signalling is an important component of the early response: some phospholipids, in fact, are not just basic constituents of membranes, but dynamic informational elements at the interface between cell and environment [6]. Plant cells contain a variety of phospholipid-based signalling pathways including phospholipase C (PLC), D (PLD), A_2 (PLA$_2$) and new pathways involving the formation of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P$_2$) [6–8]. In addition to that, numerous studies have shown that microorganisms alter their membrane lipid composition during osmotic shock. In fact, the increasing of cardiolipin levels has been observed both for microorganisms exposed to hypotonic medium (*Halobacterium salinarium*) and for microorganisms living in diluted environment exposed to high salt concentration (*Rhodobacter sphaeroides*) [9,10].

In this work, we performed investigations on the lipid content of higher plants (spinach) under hyper-osmotic stress, by means of thin layer chromatography and mass spectrometry. In particular, the experiments were performed at different plant organization levels: whole leaves, freshly prepared protoplast suspension and mesophyll cells obtained by reformation of the cell wall from protoplast suspension.

2. Materials and methods

2.1. Materials

Cellulase "Onozuka RS" (from *Trichoderma viride*) and Macerozyme R-10 (from *Rhizopus* sp. lyophil.) were obtained from Serva. All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). TLC plates (silica gel 60 A on glass 10×20 cm), obtained from Merck, were washed twice with chloroform/methanol (1:1 v/v) and activated at 120 °C before use. Lipid standards (BPG from bovine heart, PI from soybean, PG from egg yolk lecithin, and PC, PE and PS from bovine brain) were obtained from Sigma and were solubilized in chloroform (10 mg/ml). FDA, Calcofluor (Fluorescent Brightener 28) and molybdenum blue were gained from Sigma. Microscopic images were performed using AXIOPLAN 2 ZEISS Microscope equipped with AXIOCAM CCD CAMERA and AXIOVISION 3.1 PROCESSOR.

2.2. Preliminary treatment on leaves

Spinach leaves were quickly washed, deprived of their midribs and of their secondary veins, and cut into irregular pieces, which were incubated for 3 h in 5 mM MES-KOH (pH 5.5), 1 mM KCl.

2.3. Effect of increasing osmolarity on the lipid content in the whole leaves

Equal amounts (6 g) of spinach leaves were incubated at room temperature in 500 ml of MES buffer (pH 5.5) containing, respectively, no NaCl, 0.5 M NaCl, 1 M NaCl and 1.5 M NaCl . These pieces of leaves were gently stirred for 1 h in an open flask. Finally, the lipids were extracted from each sample by the standard procedure (see below).

2.4. Effect of high external osmolarity on the lipid content in the whole leaves

Equivalent aliquots (6 g) of leaf pieces were gently stirred in 500 ml of MES buffer (pH 5.5) containing, respectively, no osmolite, 1.5 M NaCl, 1.5 M KCl and 3 M sucrose. The incubation of each sample was performed at room temperature for 1 h and was followed by the standard lipid extraction.

2.5. Time course of lipids extracted from leaves under high external osmolarity

6 g of leaves were kept floating in 500 ml of MES buffer (pH 5.5) containing 1.5 M NaCl for different incubation times (15 min, 30 min, 1 h, 2 h, overnight). Lipids were extracted from each sample by the standard procedure (see below).

2.6. Effect of pH on lipid composition in leaves under hyperosmotic stress

Same quantities of leaf pieces (6 g) were incubated at room temperature in 500 ml of MES buffer (pH 5.5) both with no NaCl and with 1.5 M NaCl at different pH (5.5, 6.0, 7.0). After 1 h incubation, the lipids were extracted from each sample by the standard procedure (see below).

2.7. Mesophyll protoplast extraction

Upper and lower epidermises of 10 g of leaves were scraped off by rubbing with fine carborundum powder. Then leaves were quickly washed and cut into little pieces, which were incubated in a dish containing 100 ml of digestion medium (500 mM D-sorbitol, 1 mM CaCl$_2$, 5 mM MES-KOH pH 5.5, 2% Cellulase "Onozuka RS", 0.3% Macerozyme R-10) for 3 h at 25 °C, covered with a plastic film. It was advantageous to replace the digestion medium at intervals of 1 h, as the enzymes became inactivated by substances released from broken cells. After 3 h, the digestion medium was removed and discarded, while the plant tissues were washed three times by shaking gently for 1 h with 20 ml of wash medium (500 mM D-sorbitol, 1 mM CaCl$_2$, 5 mM MES-KOH pH 6.0). After each wash, the tissues were collected by pouring through a tea strainer (0.5 to 1 mm pore size) and the combined washes were then filtered through nylon mesh (100–200 μm pore size) to remove vascular tissue and undigested material. The protoplasts were collected by centrifugation of the combined filtered washes for 3 min at $50 \times g$ and the supernatant was aspirated and discarded. This crude protoplast preparation was finally purified by means of repeated washes with isotonic solution and centrifugation at $50 \times g$.

2.8. Protoplast viability test

The viability of protoplasts was assessed by FDA staining. A little volume of protoplasts suspension was mixed with an equal
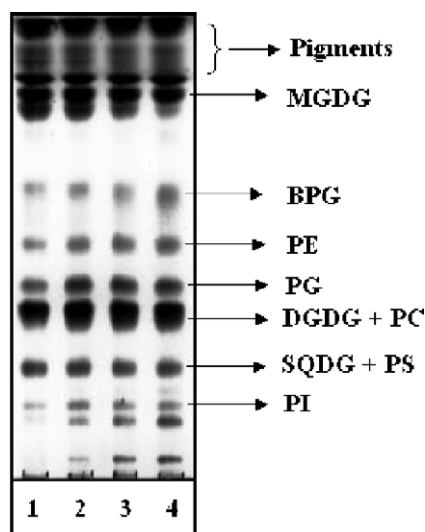
Fig. 1. Effect of external osmolarity on lipid composition of whole leaves: TLC separation of polar lipids extracted from spinach leaves (6 g) incubated for 1 h in 500 ml of MES buffer (pH 5.5) containing, respectively, no NaCl (1), 0.5 M (2), 1 M (3), 1.5 M NaCl (4). Plate was stained by 5% H_2SO_4 solution.

Fig. 2. Time course of the whole leaves lipid content under a fixed hyperosmotic stress (NaCl 1.5 M).

volume of cold FDA stock solution 0.5% w/v (in acetone). As a result of FDA staining, green fluorescence of intact viable protoplasts was observed microscopically using a 450–490-nm exciter filter, while non-viable protoplasts were not stained.

2.9. Protoplast stability test

Cell wall digestion in freshly prepared protoplasts and cell wall regeneration in protoplasts in time were monitored by the fluorescence microscope using a 350–380-nm UV light filter, after staining with Calcofluor white (Fluorescent Brightener 28). A little volume of cold stock solution 0.1% Calcofluor white was mixed 1:10 with protoplasts suspension and observed by the fluorescence microscope. Since Calcofluor white is a specific dye for polysaccharides with β-1,4 bounds, such as cellulose, only elements with cell wall fluoresced blue-white.

2.10. Effect of high osmolarity on the lipid content in protoplasts suspension

Equal amounts of freshly isolated protoplasts were incubated at room temperature in MES buffer (pH 6) containing, respectively, no NaCl and 1 M NaCl. The protoplasts suspensions were gently stirred for 1 h in an open flask. Finally, the lipids were extracted from each sample by the standard procedure (see below).

2.11. Lipid extraction

Total lipids were extracted using the Bligh and Dyer method [11]; the extracts were carefully dried under N_2 before weighting and then stored as 10 mg/ml chloroform solution.

2.12. Thin layer chromatography

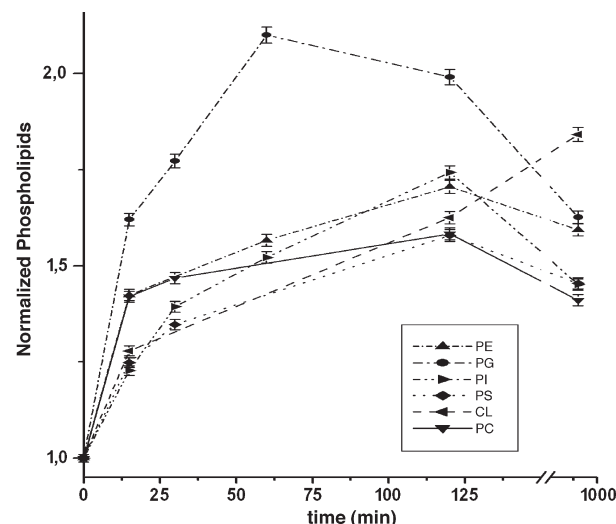Total lipid extracts were analyzed by TLC on silica gel (20 × 10 cm, layer thickness 0.2 mm). Lipids were eluted with

chloroform/methanol/acetic acid/water 75:13:9:3 mixture and detected spraying the TLC plate with 5% sulphuric acid in water, followed by charring at 120 °C for 30 min, and by spraying with 4.2 M molybdenum blue in 0.5% sulphuric acid (1:1 v/v) which is specific for phospholipids.

Quantitative analysis of phospholipid content were performed by video densitometry using the software Image J (http://rsb.info.nih.gov/ij).

2.13. Mass spectrometry

Dried lipid extract samples were dissolved in chloroform/ methanol (1:1 v/v). Electrospray ionization mass spectra (ESI– MS) were obtained with a QSTAR hybrid Qq–TOF mass spectrometer (Applied Biosystem/MSD Sciex) equipped with a turbo ion spray interface. MS–MS measurements were carried
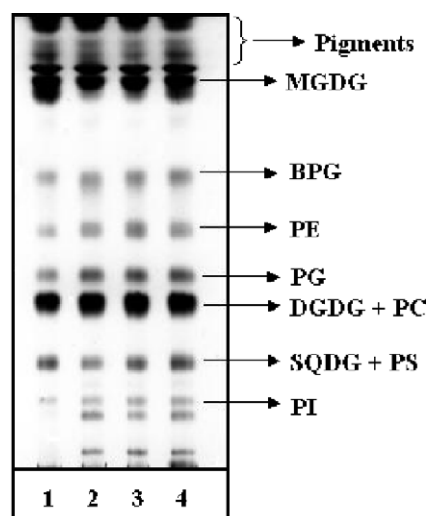

Fig. 3. Effect of different osmolites on lipid content of whole leaves. (1) Control, (2) NaCl 1.5 M, (3) KCl 1.5 M, (4) sucrose 3 M. Plate was stained by 5% H_2SO_4 solution.

out by fragmenting the target ions at proper collision energy (usually −80 eV).

2.14. Statistical analysis

All reported data represent mean values±standard deviation obtained from three replicates.

3. Results and discussion

3.1. Spinach whole leaves

Equivalent aliquots of spinach leaves (6 g) were incubated for 1 h in 500 ml of MES buffer (pH 5.5) containing, respectively, no NaCl, 0.5 M, 1 M and 1.5 M NaCl. Lipids were extracted from each sample by a standard method [4] and analyzed by TLC on silica gel (Fig. 1).

Lipid components of the extracts were identified by comparison of their Rf values with those of authentic standard markers. Besides the pigments at the solvent front and some minor lipid bands that could not be identified at this stage, the main lipid components of spinach leaves were identified as glycolipids: MGDG, DGDG, SQDG and phospolipids: BPG, PE, PG, PI. Blue molybdenum staining (data not shown) allowed to identify PC and PS, which comigrate with DGDG and SQDG, respectively. Stressed samples showed the formation of a further lipid, migrating just below PI, which could be identified as a phosphatidyl-inositol 3,5-biphosphate isomer, as just reported for several plant species exposed to hyperosmotic stress [7,12]. The increase of this band and the concomitant decrease in the PI content, observed at higher NaCl concentration, would confirm the hypothesis of an interconversion of PI in its phosphorylated form under osmotic shock and a key role of this PI isomer in osmo-signalling pathway in plant [7,12]. The analysis also evidenced an increase of all phospholipid content proportional to salt concentration. The total lipid extract yield for the samples exposed to hyperosmotic stress results almost two-fold of that relative to non-treated samples, indicating a biosynthesis of phospholipids in stimulated plant cells. In fact, it has been reported that stimulated cells with certain agonist, such as salts, induced hydrolysis and/or phosphorylation of some phospholipids with

Fig. 5. Images of protoplasts obtained by means of fluorescence microscopy. Pictures A and C show microscopy images in the visible region. Pictures B and D show fluorescence images in presence of Calcofluor and fluorescein diacetate (FDA), respectively.

consequent second messenger production, which are utilized not only in the osmo-signalling pathway but also in the phospholipid biosynthesis [6].

Time course of the lipid content under a fixed hyperosmotic stress (NaCl 1.5 M), normalized to their control values, is shown in Fig. 2. Phospholipid content increases at all incubation time explored reaching maxima values by 2 h. PG, which showed greater content than other lipids in the course of the overall experiment, redoubled its content by 1 h of osmotic shock. Only BPG continued to increase its amount during an overnight incubation, overtaking also the PG content. An increase of BPG content at PG expense under osmotic shock was observed for extreme halophilic archaeon [13]; moreover, similar increasing of BPG response to long incubation time in hyperosmotic medium were just reported for photosynthetic *R. sphaeroides* cells [9]
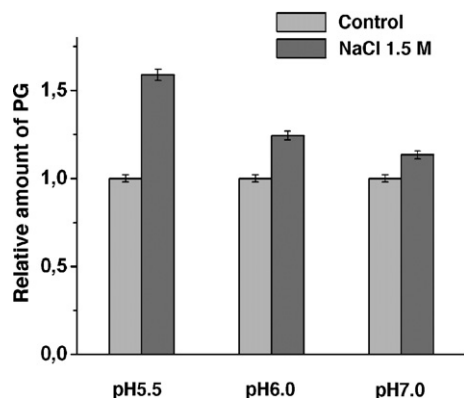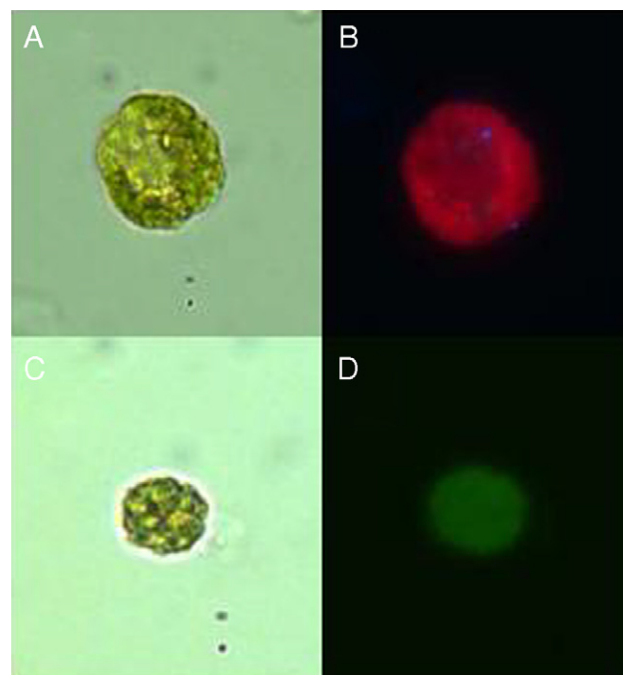

Fig. 4. Effect of pH on PG content between a control whole leaf sample (absence of NaCl) and a sample incubated for 1 h in hyperosmotic conditions (NaCl 1.5 M).
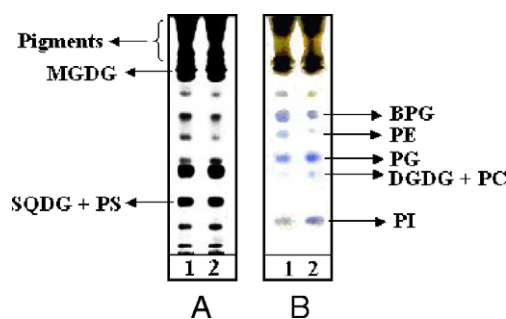

Fig. 6. Effect of external osmolarity on lipid composition of freshly prepared protoplasts: TLC separation of polar lipids extracted from protoplasts incubated for 1 h in MES buffer (pH 6) containing, respectively, no NaCl (1), 1 M NaCl (2). Plate (A) was stained by 5% H_2SO_4 solution; plate (B) was stained by molybdenum blue.
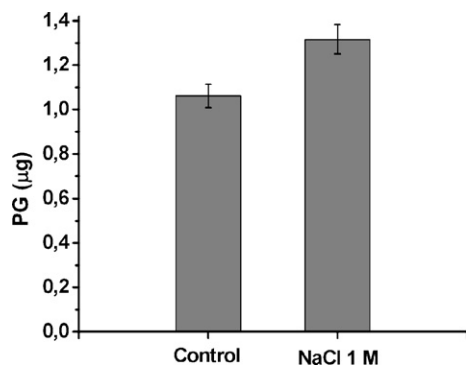
Fig. 7. Video densitometric analysis of PG content in protoplasts in absence and presence of 1 M NaCl obtained from TLC profiles.

suggesting that this lipid would help to sustain the cell bioenergetic system contributing to render the ATP synthesis more efficient.

Since the effect of NaCl could be due to Na-toxicity rather than hyperosmotic stress, KCl and sucrose, at the same osmolarity of 1.5 M NaCl, were tested (Fig. 3). All tested osmolites induced the same effect, indicating that phospholipid amount increment was due to the osmotic stress.

Moreover, the effect of pH on lipid content was investigated. Fig. 4 reports the effect of pH on PG content changes between a control sample (absence of NaCl) and a sample incubated for 1 h in hyperosmotic conditions (NaCl 1.5 M). The histogram points out an increment of PG content for stressed samples at each considered pH. In particular, the highest increment of PG (62%) was detected for the stressed samples at pH 5.5.

3.2. Protoplasts

Images of protoplasts were obtained by means of fluorescence microscopy (Fig. 5). Pictures A and B show that freshly isolated protoplasts were spherical in shape and lacked the cell wall, as confirmed by the absence of blue fluorescence of Calcofluor. Pictures C and D demonstrate that obtained protoplasts were viable, as assessed by FDA staining.

Equal amounts of freshly isolated protoplasts were incubated for 1 h in MES buffer (pH 6) containing, respectively, no NaCl (control) and 1 M NaCl. Fig. 6 shows the extracted lipids
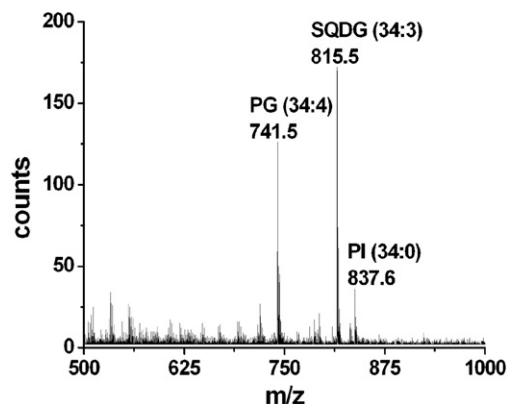

Fig. 8. ESI–MS negative ions of the lipid extract of protoplasts exposed to hyperosmotic shock with 1 M NaCl.
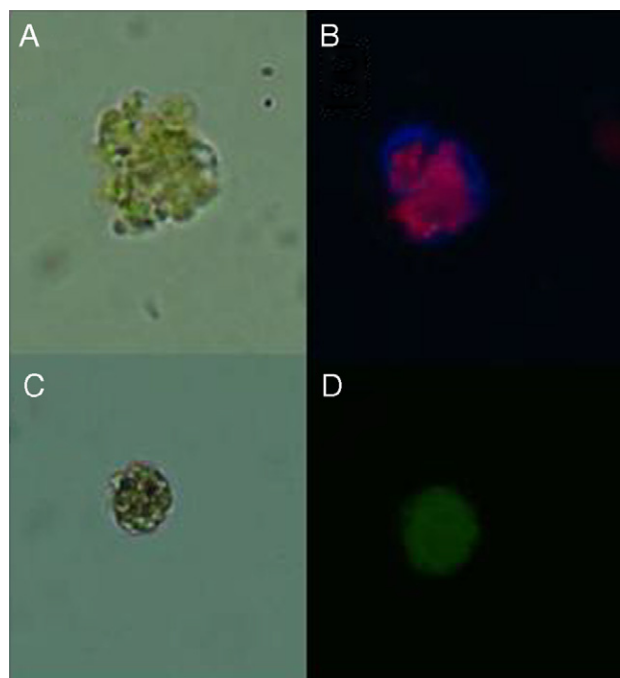

Fig. 9. Images of protoplasts after 3 days from their preparation. Pictures A and C show microscopy images in the visible region. Pictures B and D show fluorescence images in presence of Calcofluor and fluorescein diacetate (FDA), respectively.

analyzed by TLC on silica gel. Fig. 6A shows the total lipid content, while Fig. 6B evidences only phospholipids. It is evident an increase of PG, PC and PI in stressed sample and, conversely to leaves, a decrease of BPG and PE. Selective changes in lipid content and in biophysical properties of plant membranes have been reported for maize protoplast under osmotic shock [14]. In particular, a loss of phospholipids, mainly those containing unsaturated fatty acyl chains, and a concomitant increase in neutral lipids such as triacylglycerols has been documented.

Video densitometry evidenced a PG amount increment of 30% in stressed sample (Fig. 7).
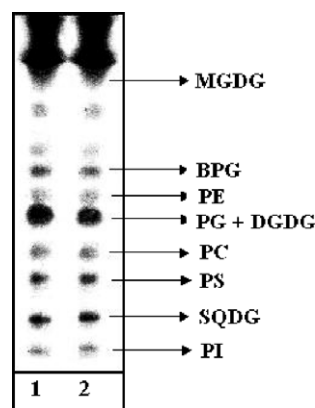

Fig. 10. Effect of external osmolarity on lipid composition of protoplasts which have reformed cell wall: TLC separation of polar lipids extracted from protoplasts incubated for 1 h in MES buffer (pH 6) containing, respectively, no NaCl (1), 1 M NaCl (2). Plate was stained by 5% H_2SO_4 solution.

Fig. 8 reports ESI–MS negative ions of the lipid extract of stressed protoplasts. In the high-mass range of the spectrum, the most intense peaks can be assigned to SQDG (34:3), PG (34:4) and PI (34:0), which represent the most abundant ionizable lipids as evidenced by TLC.

3.3. Cells

After few days, protoplasts synthetized the cell wall [15]. Images of protoplasts after 3 days from their preparation show the presence of cell wall, as confirmed by the blue fluorescence of Calcofluor (A and B) reported in Fig. 9. Pictures C and D demonstrate that obtained cells were still viable, as evidenced by the green fluorescence of FDA.

Equal amounts of cells were incubated for 1 h in MES buffer (pH 6) containing, respectively, no NaCl (control) and 1 M NaCl. Extracted lipids were analyzed by TLC on silica gel (Fig. 10). Figure shows the total lipid content. It is noteworthy a decrease in PG content under osmotic stress, as confirmed by blue molybdenum staining (data not shown). This behaviour opposite to that observed for stressed leaves and protoplasts underlines that hyperosmotic stress induces changes in the phospholipid content depending on the different plant organization levels studied.

4. Conclusion

Hyperosmotic stress induces changes in the phospholipid content depending on the different plant organization levels studied. In particular, an increment of the total phospholipid content together with the formation of a further lipid, which could be identified as a phosphatidyl-inositol 3,5-biphosphate isomer, were recorded in stressed whole leaves, suggesting a salt stimulated phospholipid biosynthesis. All phospholipids showed an increment of their content at all incubation time explored reaching maximum values by 2 h. In particular, PG presented greater content than other lipids in the course of the overall experiment, redoubling its content by 1 h of osmotic shock. BPG had a different behaviour with respect to other observed phospholipids: it continued to increase its amount during an overnight incubation, overtaking also the PG content. Different responses to osmotic shock were reported for the other systems. In the case of protoplasts, an increment of PG, PC and PI together with BPG and PE content decreasing were observed in stressed sample. For PG, the increment was of about 30%. In the case of cells, instead, a decrease of PG content under

osmotic stress was recorded, emphasizing how plant organization influences phospholipid response to hyperosmotic conditions. Modifications in lipid composition, in fact, could affect the physicochemical properties of the membrane such as bilayer thickness, membrane fluidity and transport properties as well as would help to sustain the cell bioenergetic systems under osmotic stress conditions.

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